The role of augurin in the hypothalamo-pituitary-adrenal axis and the regulation of food intake

A thesis submitted for the degree of Doctor of Philosophy

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ABSTRACT

Novel secreted peptides represent an important field of research because delineation of their function affords new insights into the pathophysiological processes causing disease, and provides opportunities for the development of pharmacological therapies. Augurin is a recently identified secreted peptide of unknown function expressed in the central nervous system (CNS), pituitary, and several endocrine tissues. Because of its expression pattern I hypothesised that augurin may be involved in the neuroendocrine system. I therefore investigated the effects of central injection of augurin on the hypothalamo-pituitary axes and food intake in male Wistar rats.

Intracerebroventricular (ICV) or intraparaventricular nucleus (iPVN) injection of augurin increased plasma adrenocorticotrophic hormone (ACTH) and corticosterone levels compared with vehicle-injected controls. Augurin also increased the release of corticotrophin releasing hormone (CRH) from hypothalamic explants, and pre-treatment with a CRH receptor antagonist in vivo prevented the rise in ACTH and corticosterone caused by ICV augurin, suggesting activation the hypothalamo-pituitary-adrenal (HPA) axis via the release of CRH from neurons in the PVN. In addition to stimulating the HPA axis, iPVN injection of augurin at the start of either the light or dark phase caused a robust increase in food intake.

To assess whether augurin might have a physiological role in the regulation of the HPA axis or food intake, I investigated its endogenous distribution in the rat CNS using immunohistochemistry and in situ hybridisation histochemistry. Augurin positive neurons were present in several regions of the CNS including the hypothalamus and the dorsal vagal complex of the brainstem, a distribution consistent with a possible role in neuroendocrine function. Augurin is therefore anatomically positioned to play a role in the hypothalamic regulation of the HPA axis and food intake.

While the data presented suggest a novel role for augurin, these are preliminary studies. Currently, little is known about the physiology of the augurin system. Further characterisation of the neuroanatomy, identification of receptors, and the development of receptor agonists and antagonists would provide further data on the physiological role of augurin, and on the potential therapeutic benefits of manipulating the augurin system.
DECLARATION OF CONTRIBUTORS

The author performed the majority of the work described in thesis. Any collaboration or assistance is described below.

Chapters 2 and 3 - *In vivo* studies were carried out with the assistance of the 'hypothalamic group'. All radioimmunoassays were carried out under the supervision of Professor Mohammad Ghatei and Dr Michael Patterson (Section of Investigative Medicine). All in house radioimmunoassays were established and maintained by Professor Mohammad Ghatei.

Chapter 4 – Immunohistochemistry and *in situ* hybridisation histochemistry were carried out with the guidance of Dr Bradley Spencer-Dene (Experimental Pathology Laboratory, Cancer Research UK London Research Institute).
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And finally, James, Gav, and Kev, for teaching me how to do science.
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<tbody>
<tr>
<td>3V</td>
<td>Third cerebral ventricle</td>
</tr>
<tr>
<td>ABC</td>
<td>Avidin-biotin complex</td>
</tr>
<tr>
<td>aCSF</td>
<td>Artificial cerebrospinal fluid</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotrophic hormone</td>
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<tr>
<td>AgRP</td>
<td>Agouti-related protein</td>
</tr>
<tr>
<td>AII</td>
<td>Angiotensin II</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ANS</td>
<td>Autonomic nervous system</td>
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<td>AP</td>
<td>Area postrema</td>
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<td>ARC</td>
<td>Arcuate nucleus of the hypothalamus</td>
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<td>AVP</td>
<td>Arginine vasopressin</td>
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<tr>
<td>BBB</td>
<td>Blood brain barrier</td>
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<tr>
<td>BCIP/NBT</td>
<td>4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate,</td>
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<tr>
<td>bp</td>
<td>Base pairs</td>
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<td>BSA</td>
<td>Bovine serum albumin</td>
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<td>C2orf40</td>
<td>Chromosome 2 open reading frame 40</td>
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<td>CART</td>
<td>Cocaine-and amphetamine-regulated transcript protein</td>
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<td>CCK</td>
<td>Cholecystokinin</td>
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<td>cDNA</td>
<td>Complimentary deoxyribonucleic acid</td>
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<td>Central nervous system</td>
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<td>Corticosterone</td>
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<td>CRH</td>
<td>Corticotrophin-releasing hormone</td>
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<td>CVO</td>
<td>Circumventricular organ</td>
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<td>DA</td>
<td>Dopamine</td>
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<td>DMX</td>
<td>Dorsal motor nucleus of vagus</td>
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<td>DNA</td>
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<td>dNTP</td>
<td>Deoxynucleotide triphosphate</td>
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<td>DTT</td>
<td>Dithiothreitol</td>
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<td>Esophageal cancer related gene 4</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic acid</td>
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<td>eGFP</td>
<td>Enhanced green fluorescent protein</td>
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<td>ER</td>
<td>Endoplasmic reticulum</td>
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<td>FI</td>
<td>Food intake</td>
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<td>GABA</td>
<td>γ-amino butyric acid</td>
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<td>GH</td>
<td>Growth hormone</td>
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<td>GHRH</td>
<td>Growth hormone releasing hormone</td>
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<td>GHSR</td>
<td>Growth hormone secretagogue receptor</td>
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<td>GLP-1</td>
<td>Glucagon-like peptide-1</td>
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<td>GLP-2</td>
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<td>GnRH</td>
<td>Gonadotrophin releasing hormone</td>
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<td>GPCR</td>
<td>G-protein coupled receptor</td>
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<td>Glucocorticoid</td>
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<td>HCRT</td>
<td>Hypocretin</td>
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<td>HMM</td>
<td>Hidden Markov model</td>
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<td>HPA</td>
<td>Hypothalamo-pituitary-adrenal</td>
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<td>HFP</td>
<td>Hippocampal formation</td>
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<td>HPT</td>
<td>Hypothalamo-pituitary-thyroid</td>
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<td>HRP</td>
<td>Horseradish peroxidase</td>
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<td>ICV</td>
<td>Intracerebroventricular</td>
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<td>IGF</td>
<td>Insulin like growth factor</td>
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<td>IHC</td>
<td>Immunohistochemistry</td>
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<td>ip</td>
<td>Intraperitoneal</td>
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<td>iPVN</td>
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<td>In situ hybridisation histochemistry</td>
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<td>Kilodalton</td>
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<td>Knockout</td>
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<td>LH</td>
<td>Luteinising hormone</td>
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<td>LHA</td>
<td>Lateral hypothalamic area of the hypothalamus</td>
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<tr>
<td>LI</td>
<td>Like immunoreactivity</td>
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<td>MCH</td>
<td>Melanocortin-concentrating hormone</td>
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<tr>
<td>ME</td>
<td>Median eminence</td>
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<tr>
<td>MFB</td>
<td>Medial forebrain bundle</td>
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<tr>
<td>MOPS</td>
<td>3 (N Morpholino)propanesulphonic acid</td>
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<td>MPOA</td>
<td>Medial preoptic area of the hypothalamus</td>
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<td>MR</td>
<td>Mineralocorticoid</td>
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<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
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<td>MSH</td>
<td>Melanocyte-stimulating hormone</td>
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<tr>
<td>NEP</td>
<td>Neuroendocrine peptide</td>
</tr>
<tr>
<td>NPC</td>
<td>Neural precursor cell</td>
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<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
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<tr>
<td>NSC</td>
<td>Neural stem cell</td>
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<td>Nucleus of the tractus solitarius</td>
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<td>Olfactory bulb</td>
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<td>ORF</td>
<td>Open reading frame</td>
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<td>OSCC</td>
<td>Oesophageal squamous cell carcinoma</td>
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<td>OXT</td>
<td>Oxytocin</td>
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<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PC</td>
<td>Prohormone convertase</td>
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<td>PCN</td>
<td>Parvocellular neurosecretory neurons</td>
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<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>POMC</td>
<td>Proopiomelanocortin</td>
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<td>Pancreatic polypeptide</td>
</tr>
<tr>
<td>PRL</td>
<td>Prolactin</td>
</tr>
<tr>
<td>PrRP</td>
<td>Prolactin-releasing peptide</td>
</tr>
<tr>
<td>PVN</td>
<td>Paraventricular nucleus of the hypothalamus</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
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<tr>
<td>PYY</td>
<td>Peptide YY</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RMS</td>
<td>Rostral migratory stream</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse transcription</td>
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<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus of the hypothalamus</td>
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<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
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<tr>
<td>SON</td>
<td>Supraoptic nucleus of the hypothalamus</td>
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<td>SP</td>
<td>Secretory signal peptide</td>
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<tr>
<td>SS</td>
<td>Somatostatin</td>
</tr>
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<td>SVZ</td>
<td>Subventricular zone</td>
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<tr>
<td>T₃</td>
<td>3,5,3’-triiodo-L-thyronine</td>
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<td>T₄</td>
<td>3,5,3’,5’-tetraiodo-L-thyronine</td>
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<tr>
<td>TAE</td>
<td>Tris-acetate-ethylenediaminetetraacetic acid</td>
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<tr>
<td>TE</td>
<td>Tris-ethylenediaminetetraacetic acid</td>
</tr>
<tr>
<td>TH</td>
<td>Tyrosine hydroxylase</td>
</tr>
<tr>
<td>TRH</td>
<td>Thyrotrophin-releasing hormone</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid-stimulating hormone</td>
</tr>
<tr>
<td>VMN</td>
<td>Ventromedial nucleus of the hypothalamus</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral tegmental area</td>
</tr>
<tr>
<td>WT</td>
<td>Wild-type</td>
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1 General Introduction
1.1 NEUROENDOCRINE PEPTIDES

This thesis aims to examine the biological and physiological role of augurin, a novel neuroendocrine peptide (NEP), in the hypothalamic regulation of feeding and endocrine function. The characterisation of novel human proteins furthers our understanding of normal physiology, and provides new opportunities for the treatment of diseases. Uncharacterised NEPs are particularly good targets for therapeutic intervention as both peptide and cellular target are readily accessible via the circulation. In cases where deficiency causes disease, such as the lack of insulin in type 1 diabetes mellitus, purified peptide can be administered directly to an individual (Banting et al., 1922). Alternatively, specific antibodies or receptor antagonists can readily suppress biological activity (Elliott et al., 1994, Hokfelt et al., 2003). In some cases a NEP system may have therapeutic potential even if it does not play a direct role in the pathophysiology of a disease (Izzo and Zion, 2011).

The term NEP encompasses both neuropeptides and peptide hormones; a critically important and diverse set of regulatory molecules whose functions are to convey specific information amongst cells and organs. Many arose early in the evolution of life and are highly conserved throughout lower vertebrates, insects, yeasts, and bacteria (Roth et al., 1982). NEPs are a subset of secreted proteins loosely defined by their shared characteristics. They are typically short polypeptides produced by the proteolytic cleavage of preprohormone precursors (Hook et al., 2008).

Mature peptides then localise to the secretory pathway where particular stimuli regulate their secretion (Burbach, 2010). Once released into the extracellular space they can bind to specific cell surface receptors through which they modulate cellular functions. The majority of NEPs are ligands for G-protein-coupled receptors (GPCRs), all of which share common structural features (Lagerström and Schiöth, 2008). The high degree of homology between GPCRs makes them particularly amenable to bioinformatic analysis which has suggested there may be some 25-30 orphan GPCRs which have as yet undiscovered peptide ligands (Vassilatis et al., 2003).
NEPs function both as hormones whose actions on distant organs are mediated by way of their transport through the bloodstream, and as local cell-to-cell communicators. Many are pleiotropic and in addition to both endocrine and paracrine activity, function as neurotransmitters in the central, autonomic, and peripheral nervous systems (Hokfelt, 1991). Irrespective of the mechanism, the primary action of NEPs is to coordinate the behaviour of receptive cells to ultimately advantage the organism as a whole. They modulate processes as diverse as neurogenesis, blood pressure regulation, glucose homeostasis, arousal, feeding behaviour, fear, anxiety and stress, addiction, and pleasure (Hokfelt et al., 2000, Stanic et al., 2008, Ogren et al., 2010). Given their diverse functions and their potential as therapeutic targets, considerable efforts have been made to identify and characterise the complement of human NEPs and their receptors (Clark et al., 2003).

1.1.1 METHODS OF IDENTIFYING NEPS

The discovery of thyrotrophin-releasing hormone (TRH) by Guillemin and Schally exemplifies the classical peptide discovery method; bioactivity assays guiding repeated fractionation of peptides extracted from specific tissues (Guillemin et al., 1965, Schally et al., 1966). Since 1986, homology screening and bioinformatics analyses have identified hundreds of GPCRs, and those whose ligand was unknown were termed ‘orphan’ GPCRs (Dixon et al., 1986). Concomitant improvements in molecular cloning technology enabled the ‘reverse pharmacology’ strategy for deorphanising receptors, whereby ligands were identified based on their activity at GPCRs coupled to an in vitro reporter assay (Civelli et al., 1998). Reverse pharmacology has led to the discovery of several notable NEPs within the last two decades, including the orexins, ghrelin, and kisspeptin (Sakurai et al., 1998, Kojima et al., 1999, Ohtaki et al., 2001). Nevertheless, although the identification of putative GPCRs can be accomplished relatively easily, the discovery of the endogenous ligands that activate these receptors is far more difficult. This has fuelled the increasing use of bioinformatics approaches to try to identify potential NEPs (Gustincich et al., 2003, Sonmez et al., 2009).
1.1.2 BIOINFORMATICS APPROACHES TO IDENTIFYING NEPs

The successful sequencing of entire genomes of many different organisms has yielded a large number of deoxyribonucleic acid (DNA) sequences that potentially encode novel candidate NEPs, as well as hundreds of orphan GPCRs with no known cognate ligands (Chung et al., 2008). In 2007, Mirabeau and colleagues developed an algorithm based on the hidden Markov model (HMM) to quantify the likelihood that a given protein contained a NEP (Mirabeau et al., 2007). Taking advantage of the fact that the primary sequences of NEPs contain several common features, they identified esophageal cancer related gene 4 protein (ECRG4) as likely to contain a NEP which the authors named augurin (Mirabeau et al., 2007).

Although a formal definition of an HMM is beyond the scope of this thesis, I will outline the principles of HMMs, and their strengths and weaknesses when applied to protein prediction. HMMs are a mathematical foundation for making probabilistic models of linear sequence ‘labelling’ problems (Eddy, 1998). HMMs are ‘learning’ algorithms; they adjust their free parameters gradually by repeated presentation of a training data set, and thereby learn to generalise from the examples on which they have been trained. As a result, an HMM can learn the probability that an amino acid sequence corresponds to a particular state defined by a training set. A state might be a signal peptide or a transmembrane domain, while the training set would be a list of amino acid sequences known to correspond to that state. An HMM can incorporate multiple features as separate states, and calculate the probabilities that a sequence corresponds to a particular state, and that one state will transition into the next (Winters-Hilt, 2006). For example, NEPs have an identifiable secretory signal peptide (SP) cleavage site following the SP. An HMM should therefore ‘learn’ that, for NEPs, the probability of a transition from SP to SP cleavage site is equal to 1. It follows that if a sequence does not transition from SP to SP cleavage site, it is unlikely to belong to the NEP family.

The HMM’s strength lies in the simplicity with which individual HMMs can be combined to model complex sequences. In the case of protein profiling, individual HMM states, for example the existence of an SP or peptidase cleavage site, can be incorporated into a single HMM by modelling the transition from the former state to the latter (Eddy, 1996). The major drawback of
HMMs is their short ‘memory’ span. The HMM assumes that the probability of transitioning to the subsequent state is entirely dependant on the current state (Eddy, 2004). In practise, this makes it difficult for an HMM to model correlations between distant states because it assumes the probability of each state depends only on the previous one.

As a group, NEPs share several common amino acid motifs and features that are highly conserved relative to other regions of the primary sequence, illustrated in Figure 1.1A. These include a relatively short length, an N-terminal SP, basic residue prohormone cleavage sites, and an absence of transmembrane domains (Sonmez et al., 2009).

1.1.2.1 N-terminal secretory signal peptide

Much of the information about the subcellular localisation of a protein is localised at the N-terminus of a protein (Devillers-Thiery et al., 1975, Wiedmann et al., 1987). As it starts to be translated in the cytoplasm, a number of RNA/protein complexes binds to the nascent protein and target it into specific organelles (Wiedmann et al., 1987). The prototypical N-terminal signal is the SP, which targets a protein for translocation across the endoplasmic reticulum (ER) membrane in eukaryotes (Von Heijne, 1990). It is typically 15–30 amino acids long and is cleaved during translocation of the protein across the membrane (Nagai et al., 2003). There is no simple consensus sequence for SPs, but they typically show three distinct compositional zones: an N-terminal region which often contains positively charged residues, a hydrophobic region of at least six residues, and a C-terminal region of polar uncharged residues (Perlman and Halvorson, 1983).

It should be noted that not all proteins with SPs are actually secreted to the outside of the cell. In eukaryotes, proteins translocated across the ER membrane are by default transported through the Golgi apparatus and exported by secretory vesicles, but many have specific retention signals that hold them back in the ER or the Golgi, or divert them to the lysosomes (Van Vliet et al., 2003). In addition, several proteins such as the fibroblast growth factors are secreted without an SP through the non-classical pathway, which does not follow the classical route through the ER and Golgi (Nickel and Rabouille, 2009).
Figure 1.1: Alignment of preproaugurin orthologues and potential peptidase cleavage sites. Orthologues were identified using a Markov Clustering algorithm (Kim et al., 2008). The multiple sequence alignment was generated using the T-Coffee algorithm and annotated using Jalview alignment editor (Notredame, 2007, Waterhouse et al., 2009). Amino acid residues are coloured with the Clustal X colour scheme (Procter et al., 2010). The SignalP 3.0 algorithm predicted amino acid residues 1-31 comprised the signal peptide sequence (Dyrlv Bendtsen et al., 2004, Emanuelsson et al., 2007). Cleavage sites, marked with red arrows, were compiled from the following studies (Mirabeau et al., 2007, Gotze et al., 2009, Kujuro et al., 2010, Gonzalez et al., 2011, Ozawa et al., 2011). The consensus sequence is shown below the multiple species alignment, amino acid conservation is represented by relative bar height on the histogram.

Ailuropoda melanoleuca (giant panda), Bos taurus (cow), Callithrix jacchus (marmoset), Canis lupus familiaris (dog), Danio rerio (zebrafish), Equus caballus (horse), Gallus gallus (chicken), Homo sapiens (human), Loxodonta africana (elephant), Mus musculus (mouse), Oryctolagus cuniculus (rabbit), Osmerus mordax (rainbow smelt) Pan troglodytes (chimpanzee), Pongo abelii (sumatran orangutan), Rattus norvegicus (rat), Salmo salar (atlantic salmon), Sus scrofa (wild boar), Taeniopygia guttata (zebra finch), Xenopus laevis (african clawed frog), Xenopus silurana tropicalis (western clawed frog), Macaca mulatta (macaque)
1.1.2.2 **Absence of transmembrane domains**

By default, SP-containing proteins are trafficked into the secretory pathway, however, if in addition to an SP a protein contains stretches of 15-25 hydrophobic residues, it is incorporated into the plasma membrane of the cell (Krogh *et al.*, 2001). GPCRs are an example of transmembrane proteins that have SPs.

1.1.2.3 **Preprohormone cleavage sites**

NEPs are initially synthesised as relatively large preprohormone precursors that gradually mature into specific bioactive forms via the sequential action of post-translational processing enzymes (Hook *et al.*, 2008). The SP is removed by signal peptidases, forming the prohormone, which is commonly further cleaved by prohormone convertase (PC) enzymes. The PCs are a family of at least seven enzymes that typically cleave C-terminally to basic residue (Arg/Lys) motifs (Fricker, 2005, Hook *et al.*, 2008). PC1/3 and PC2 are primarily responsible for prohormone maturation; they are specifically expressed in neuroendocrine tissues where they localise to secretory vesicles (Azaryan *et al.*, 1995, Zhou *et al.*, 1999). Other enzymes including carboxypeptidase E/H, cathepsin L, and Arg/Lys aminopeptidase may be necessary for cleavage and maturation of a prohormone (Fricker, 1988, Hook *et al.*, 2004, Biswas *et al.*, 2009). In many cases processed peptides undergo further post-translational modifications which are necessary for biological activity such as C-terminal amidation (Eipper *et al.*, 1992).
1.2 Augurin is a Candidate NEP

Mirabeau et al. constructed an HMM based on the aforementioned features to predict the probability a peptide belonged to the NEP family. When applied to the mammalian proteome it identified ECRG4, now named preproaugurin (RefSeq NP_115787.1), as likely to contain a NEP (Mirabeau et al., 2007). Since then, several studies using similar methodology have identified homologues of preproaugurin in cattle, zebrafish, and chicken, as likely to contain a NEP (Southey et al., 2009, Delfino et al., 2010, Xie et al., 2010).

1.2.1 The Chromosome 2 Open Reading Frame 40 Gene

Preproaugurin is encoded by the Chromosome 2 open reading frame 40 (C2orf40) gene. C2orf40 was first identified in 1998 as a potential tumour suppressor gene in human oesophageal epithelium (Su et al., 1998). It is located on human chromosome 2q14.1-14.3, spans approximately 13kb, and is highly conserved amongst vertebrate animals (Figure 1.1). C2orf40 has 4 exons, all of which are coding (Su et al., 1998). No alternative splicing has been reported yet in mammals, however there are two paralogous genes in the zebrafish and chimpanzee (Figure 1.1). C2orf40 does not appear to be a member of any known gene family.

1.2.2 Preproaugurin

Preproaugurin is a 17kDa peptide that has several, highly conserved, characteristic features of a preprohormone; it is short at 148 residues (in most species), contains a canonical SP, and several potential PC cleavage sites (Figure 1.1). Based on its distribution, subcellular localisation, post-translational processing, and secretion from cells, there is now considerable evidence to support the hypothesis that preproaugurin contains one or more secreted peptides.

1.2.2.1 Post-translational processing

Post-translational processing of preproaugurin is poorly understood. In particular, the sites of prohormone cleavage, and the identity and nature of the resultant peptides are not known. Six potential cleavage sites have been suggested based predominantly on data from in vitro studies (Figure 1.1). Prior to beginning work for this thesis, only the canonical dibasic PC cleavage site corresponding to residues Lys69 and Arg70 was supported by direct experimental evidence.
Cleavage at this site has been demonstrated in vitro following transfection of two different rat pancreatic β-cell lines with preproaugurin cDNA, in which the FLAG-tag was inserted adjacent to Arg70 (Mirabeau et al., 2007). Western blotting of culture medium from FLAG-preproaugurin transfected cells demonstrated a 10kDa immunoreactive band consistent with secretion of an Arg70 cleaved peptide (Mirabeau et al., 2007). Furthermore, the 10kDa peptide is immunoreactive when probed with an antibody specific for FLAG at the N-terminus, suggesting the C-terminal sequence from residues 71-148 is secreted.

Since then, several studies have investigated the post-translational processing of preproaugurin in vitro and various other cleavage sites have been inferred based on the presence of multiple bands on western blots. Of the six studies that have investigated the post-translational processing of preproaugurin, identification of a 14kDa, a 10kDa, and an 8kDa immunoreactive band is almost universal (Mirabeau et al., 2007, Gotze et al., 2009, Kujuro et al., 2010, Gonzalez et al., 2011, Ozawa et al., 2011). In one study, no 8kDa band was observed transfection into chondrocytes (Huh et al., 2009). These bands most likely correspond to preproaugurin (31-148) following removal of the signal peptide sequence, preproaugurin (71-148) following cleavage at Arg70, and preproaugurin (71-132) following further cleavage at Arg132. The 14kDa, 10kDa, and 8kDa bands are consistently identified in the culture medium of preproaugurin transfected cells, suggesting predominant secretion of these forms (Mirabeau et al., 2007, Gotze et al., 2009, Gonzalez et al., 2011).

Despite the reasonable consistency between studies, extrapolation of these results to endogenous processing of preproaugurin is potentially misleading. Because the preproaugurin cDNA used in each of the aforementioned studies lacks intron-exon boundaries, alternative mRNA splicing cannot occur (Johnson et al., 2003, Adamson et al., 2011). Furthermore, post-translational processing depends on the coexistence of both prohormone and protease enzyme in the same cell, and varies depending on which proteases are expressed (Ozawa et al., 2010). For example, co-expression of preproglucagon and PC2 predominantly generates glucagon in pancreatic α-cells, whereas in enteroendocrine L-cells glucagon like peptide-1 (GLP-1) and glucagon like peptide-2 (GLP-2) are the predominant products due to
co-expression with PC1/3 (Rothenberg et al., 1995, Furuta et al., 1997, Rouille et al., 1997). Therefore, although several preproaugurin products have been identified in vitro, their existence in vivo will depend on the co-expression of both preproaugurin and the appropriate cleavage enzymes. To date, only one study has investigated preproaugurin processing in vivo. Gonzalez et al. demonstrated that in the choroid plexus, a tissue expressing high levels of preproaugurin mRNA, the vast majority of preproaugurin-like immunoreactivity (LI) is a 14kDa band, while an 8kDa band is also present at much lower levels (Gonzalez et al., 2011).

The data above support the hypothesis that post-translational processing of preproaugurin generates one or more secreted peptides, however their identity is very much unclear. I began the work presented in this thesis soon after the identification of augurin by Mirabeau et al., at which time the primary sequence of endogenous augurin was unknown (Mirabeau et al., 2007). I hypothesised preproaugurin (71-148) was a likely endogenous form because it appeared to be the predominant peptide secreted from preproaugurin transfected β-cells, and its primary sequence is highly conserved between species (Figure 1.1) (Mirabeau et al., 2007). I therefore chose to investigate the biological effects of residues 71-148 of preproaugurin, a likely endogenous form, which I refer to as augurin throughout this thesis. Although further cleavage at Arg132 into an 8kDa product had been suggested, the identity of the C-terminal cleavage site had not been confirmed (Mirabeau et al., 2007). PC cleavage at a single Arg residue is atypical, and compared to the canonical dibasic cleave site residues Lys69/Arg70, Arg132 is less well conserved (Figure 1.1) (Fricker, 2005).

1.2.2.2 Subcellular localisation

Several studies have investigated the subcellular localisation of preproaugurin, and the majority demonstrate trafficking into the secretory pathway. FLAG-preproaugurin and insulin co-localise to punctate intracellular bodies in transfected β-cells, while in the HCT116 colorectal carcinoma cell line, eGFP-tagged preproaugurin predominantly localises to the Golgi apparatus (Mirabeau et al., 2007, Gotze et al., 2009). Although these studies suggest preproaugurin is trafficked into the secretory pathway, they should be interpreted with caution as these cells may not express augurin endogenously, and the presence of a tag can alter the post-translational processing of
peptides (Hanson and Ziegler, 2004). To date, no studies have examined the subcellular localisation of endogenous preproaugurin in situ.

1.2.3 Function

Despite being identified in 1998 very little is known about the physiological functions of preproaugurin, although it is recognised as down-regulated in many, if not most cancers (Su et al., 1998, Yue et al., 2003, Mori et al., 2007, Gotze et al., 2009, Li et al., 2009, Vanaja et al., 2009, Li et al., 2010a, Li et al., 2010b, Gonzalez et al., 2011). Very recently, a role for preproaugurin in the central nervous system (CNS) has been proposed as a secreted inducer of neural precursor cell (NPC) senescence (Kujuro et al., 2010). RNA interference-mediated knockdown prevented the onset of senescence in NPCs, suggesting preproaugurin is a necessary for NPC senescence (Kujuro et al., 2010).

1.2.4 Distribution

Preproaugurin is broadly expressed in rodents and in humans. Using reverse transcription polymerase chain reaction, in situ hybridisation histochemistry (ISH), or northern blotting, preproaugurin mRNA has been demonstrated in the heart, placenta, lung, liver, lung, skeletal muscle, kidney, and skin (Steck et al., 2002, Mirabeau et al., 2007, Huh et al., 2009, Gonzalez et al., 2011). High levels of preproaugurin mRNA are consistently found in the choroid plexus, cartilage, adrenal gland, pancreas, and in areas of the CNS (Mirabeau et al., 2007, Huh et al., 2009, Gonzalez et al., 2011). Preproaugurin-LI in neurons has been reported in an abstract submitted for a recent scientific meeting (Roberton et al., 2009). In addition to the choroid plexus, preproaugurin-LI was detected in the paraventricular nucleus (PVN) and supraoptic nucleus (SON) of the hypothalamus, where it co-localised with arginine vasopressin (AVP) and oxytocin (OXT). This pattern of immunoreactivity is characteristic of a NEP and suggests that preproaugurin or a product thereof may be a novel hypothalamic neuropeptide. Prominent preproaugurin expression in the CNS and endocrine organs is consistent with a physiological role in the neuroendocrine system.
1.3 THE NEUROENDOCRINE SYSTEM

The primary biological imperatives are to first ensure an organism’s survival and second to propagate its species. To satisfy these necessities, multicellular organisms must coordinate complex physiological processes with diverse environmental conditions, and elaborate appropriate adaptive responses to specific sensory cues. Thus, the maintenance of homeostasis represents a key component of biological adaptation, and depends upon the successful integration of behavioural, somatomotor, and visceral function by the autonomic nervous and endocrine systems.

There are considerable similarities between autonomic nervous system (ANS) and endocrine system. Endocrine cells and neurons are prototypical secretory cells; both have electrically excitable plasma membranes and specific ion conductances that regulate exocytosis of their signalling molecules from storage vesicles (De Camilli and Jahn, 1990). NEPs often have a role in both systems, acting as hormones in the periphery and as neurotransmitters in the nervous system (Hokfelt et al., 2000). The ANS and the endocrine system can therefore be considered as a single neuroendocrine regulatory system.
1.4 THE HYPOTHALAMUS

Normal cellular function is exquisitely sensitive to the local environment. In order to maintain the cellular environment within the narrow optimum range, mammals have evolved an array of regulatory mechanisms. Commonly these regulatory mechanisms operate on the principle of negative feedback, whereby, deviations from a given set point are detected by a sensor, and signals from the sensor trigger appropriate antagonistic responses that continue until the set point is re-established (Fleming, 1984). Many of the complex mechanisms that maintain the internal environment are mediated via the hypothalamus (Swanson and Mogenson, 1981).

The hypothalamus is considered the critical integratory relay between afferent and efferent homeostatic signals (Figure 1.2). In mammals for example, the hypothalamus has been shown to regulate multiple hormonal axis, circadian rhythms, body temperature, as well as appetitive, sexual, and affective behaviours (Paxinos, 2004). It is also a site at which the majority of NEPs have some biological activity (Williams and Bloom, 1989, Hokfelt et al., 2000, Burbach, 2010). Sensory information from both the internal and external environment is relayed directly to the hypothalamus, which also monitors and responds to changes in numerous plasma hormone and metabolite levels (Kastin and Pan, 2008, Stefater and Seeley, 2010). The hypothalamus processes sensory and hormonal inputs and triggers appropriate responses through outputs to key regulatory sites. These include the pituitary gland, cerebral cortex, premotor and motor neurons in the brain stem and spinal cord, and parasympathetic and sympathetic preganglionic neurons (Swanson and Mogenson, 1981, Thompson and Swanson, 2003). The orchestrated hypothalamic output to these effectors ultimately results in a coordinated endocrine, behavioural, and autonomic response designed to maintain homeostasis (Figure 1.2).
Figure 1.2: The pivotal position of the hypothalamus in maintaining homeostasis. Central nervous system signals including physiological and processive stress, afferent neuronal activation, neuropeptide, and hormone signals are processed in the hypothalamus, affecting the synthesis and secretion of hypothalamic hormones and neuropeptides, and regulating patterned neuronal outputs. Both afferent and efferent signals converge at the hypothalamus. Multiple levels of negative feedback are also shown.

Abbreviations: CNS, central nervous system; ANS, autonomic nervous system.
1.4.1 ANATOMY

The hypothalamus is located in the ventral diencephalon, bound anteriorly by the optic chiasm, laterally by the sulci formed with the temporal lobes, and posteriorly by the mamillary bodies. Dorsally, the hypothalamus is delineated from the thalamus by the hypothalamic sulcus. The smooth, rounded base of the hypothalamus encircled by the circle of Willis is the tuber cinereum. The pituitary stalk descends from the median eminence (ME), found in the central part of the tuber cinereum (Figure 1.3). The hypothalamus can be functionally and anatomically subdivided into three longitudinally orientated zones: the periventricular, medial, and lateral zones (Figure 1.4) (Crosby and Woodburne, 1940). Each zone can be further subdivided into discrete regions or nuclei (Figure 1.4). An overview of the structure and function of the hypothalamus is presented below, and the roles of the hypothalamus in the regulation of endocrine function and feeding are discussed in detail in chapters 2 and 3 respectively.

1.4.2 DEVELOPMENT AND BIOLOGICAL FUNCTION

The development of the hypothalamus reflects its longitudinal subdivisions. The neurons of the lateral zone are generated first during gestation (Altman and Bayer, 1986). The lateral zone structures are generally associated with arousal and autonomic responses in feeding, defensive behaviour, and reproduction, whereas their connections are dominated by the medial forebrain bundle and the fornix (Hahn and Swanson, 2010). The second wave of neurogenesis provides neurons of the nuclei of the medial zone, characterised by limbic afferents from the amygdala and septum, and major intrahypothalamic connections (Altman and Bayer, 1986). Functionally, these cell groups are thought to generate motivated behaviours such as feeding and social interaction (Swanson, 2005). The last to be generated are the neurons of the periventricular zone (Altman and Bayer, 1986). The periventricular zone contains the classical magnocellular and parvocellular neurosecretory systems, and is critical for the regulation of biological rhythms and neuroendocrine output (Swanson, 2005).
Figure 1.3: Diagrammatic representation of the hypothalamus showing individual nuclei and adjacent structures. Taken from (Berthoud, 2002).

Abbreviations: AHA, anterior hypothalamic area; ARC, arcuate nucleus; AV3V, anteroventral area of third ventricle; CI, capsula interna; DP, dorsal parvocellular subnucleus of paraventricular nucleus; DMH, dorsomedial nucleus; F, fornix; LHA, lateral hypothalamic area; LM, lateral magnocellular subnucleus of paraventricular nucleus; LPOA, lateral preoptic area; ME, median eminence; MP, medial parvocellular subnucleus of paraventricular nucleus; MPO, medial preoptic area; OT, optic tract; Sch, suprachiasmatic nucleus; SON, supranoic nucleus; SI, substantia inomminata; ST, subthalamic nucleus; VMH, ventromedial nucleus; VP, ventral parvocellular subnucleus of paraventricular nucleus.
1.4.3

1.4.4
Figure 1.4: Organisation of the hypothalamus. A highly schematic representation of mediolateral and rostrocaudal subdivisions of the hypothalamus and their functions. Adapted from (Paxinos, 2004).
1.4.4.1 The periventricular hypothalamic zone

The periventricular zone is considered the ‘neuroendocrine motor zone’. It contains two histochemically well-defined neuronal systems, the magnocellular and the parvocellular, which convey the neuroendocrine output from the hypothalamus to the pituitary (Markakis and Swanson, 1997). The pituitary gland is intimately linked to the hypothalamus; the two form a unit that exerts control over the function of several endocrine glands, and therefore a wide range of physiological functions. The pituitary has two distinct subdivisions, the adenohypophysis and the neurohypophysis. The neurohypophysis is embryologically and anatomically continuous with the hypothalamus; it contains axon terminals arising from magnocellular secretory neurons located in the paraventricular and supraoptic nucleus of the hypothalamus.

Parvocellular neurons project to the median eminence, and other CNS regions. Secretion from the pituitary adenohypophysis is predominantly regulated by subpopulations of parvocellular hypothalamic neurons whose axons terminate in the median eminence (ME). The majority are located in the periventricular zone, including those expressing growth hormone releasing hormone (GHRH), somatostatin (SS), gonadotrophin releasing hormone (GnRH), corticotrophin releasing hormone (CRH), thyrotrophin releasing hormone (TRH), and other factors known to regulate pituitary functions such as dopamine (DA), vasoactive intestinal peptide (VIP), and γ-amino butyric acid (GABA) (Witkin et al., 1982, Bloch et al., 1983, Makara et al., 1983, Meister et al., 1988, Ceccatelli et al., 1989, Fliers et al., 1994, Simmons and Swanson, 2009).

The periventricular zone also contains the suprachiasmatic nucleus that regulates the circadian cycle, and a large group of autonomic premotor neurons that project to preganglionic autonomic neurons in the brainstem and the spinal cord (Saper et al., 1976, Thompson and Swanson, 2003, Geerling et al., 2010). The hypothalamo-pituitary axes are discussed in more detail in chapter 2.

1.4.4.2 The medial hypothalamic zone

The medial zone of the hypothalamus contains a series of large nuclei that collectively play key roles in the initiation of motivated behaviours (Swanson et al., 1987b, Swanson, 2000). Recently it has been suggested that the medial hypothalamic zone and ventral midbrain nuclei constitute a ‘motivational’ control column (Risold et al., 1997, Swanson, 2005). The motivation
control column can be divided on structural and functional grounds into rostral and caudal segments (Thompson and Swanson, 2003, Swanson, 2005). The caudal segment includes the dorsal and ventral regions of the paraventricular, median preoptic, and dorsomedial nuclei, and is primarily implicated in consummatory behaviours such as hyperphagia and polydipsia (Weiss and Leibowitz, 1985). The rostral segment is further subdivided; the anterior hypothalamic, dorsomedial parts of the ventromedial, and dorsal premammillary nuclei, and primarily mediates the expression of defensive or antagonistic behaviours (Risold et al., 1997, Cezario et al., 2008, Lin et al., 2011). In contrast, the medial preoptic, ventrolateral parts of the ventromedial, and ventral premammillary nuclei are implicated in the expression of reproductive behaviours (Thompson et al., 1996, Thompson and Swanson, 1998, Swanson, 2000, Lin et al., 2011).

1.4.4.3 Lateral hypothalamic zone

The lateral hypothalamic area (LHA) extends caudally from the lateral preoptic area to the rostral aspect of the mammillary body (Geeraedts et al., 1990). It consists of a group of medium- and large-sized neurons which are distributed along the course of the medial forebrain bundle (MFB; Figure 1.3) (Nieuwenhuys et al., 1982). Despite being the largest region of the hypothalamus, and in stark contrast to the periventricular and medial zone, the cytoarchitectural differentiations and functions of the LHA are poorly understood.

The functional importance of the LHA has been appreciated for several decades. Classic studies carried out in the 1940s and 1950s described syndromes of ravenous overeating and obesity as a consequence of lesioning the ventromedial nucleus of the hypothalamus (VMN) (Hetherington and Ranson, 1940), and of a failure to eat and drink following damage to the LHA (Anand and Brobeck, 1951). These observations fostered the ‘dual centre’ hypothesis, whereby food intake was regulated by the LHA feeding and VMN satiety centres (Stellar, 1954). However, it was soon recognised that lesions of the LHA encompassed the nigrostriatal dopaminergic pathway, which travels in the MFB, and resulted in not only in hypophagia but a near complete inability to respond to acute stimuli of any kind (Ungerstedt, 1970, Marshall et al., 1974). Evidence from recent studies suggests the LHA is critical for behavioural arousal and motivation, and thereby
modulates diverse functions including feeding and drinking, foraging, sleeping, and social behaviours (Watts et al., 1999, Taheri et al., 2002, Goto et al., 2005, Adamantidis et al., 2007). Much of the progress identifying the physiological functions of the LHA has been facilitated by the recent discovery the melanin-concentrating hormone (MCH) and the hypocretin (HCRT) neuropeptide systems.

Neurons within the LHA do not form well-defined nuclei; rather, there is subtle heterogeneity in which regional borders are not easily discernable in common Nissl preparations (Sawchenko, 1998). The discovery of MCH and HCRT has considerably advanced our understanding of its cytoarchitecture. HCRT A and HCRT B are 33 and 28 amino acid neuropeptides generated from the same precursor, and are predominately expressed by neurons whose soma lie within the LHA (De Lecea et al., 1998, Sakurai et al., 1998, Swanson et al., 2005, Hahn, 2010). HCRT-LI fibres project widely throughout the brain to areas including the cerebral cortex, thalamus, hypothalamus, ventral tegmental area, and brainstem (Elias et al., 1998, Peyron et al., 1998). MCH is a 19 amino acid neuropeptide isolated first from teleost pituitary (Kawauchi et al., 1983). It is produced by neurons of the LHA and the adjacent zona incerta that project extensively throughout the CNS (Bittencourt and Elias, 1998, Swanson et al., 2005, Hahn, 2010). Both neuropeptide systems are heavily implicated in the regulation of arousal and motivation (Chemelli et al., 1999, Lin et al., 1999, Adamantidis et al., 2007, Hassani et al., 2009).
1.5 **HYPOTHESIS**

Augurin is a recently identified secreted peptide of unknown function expressed in several endocrine tissues and in the central nervous system (CNS). Because of its prominent expression in the hypothalamus, pituitary, and endocrine organs, I hypothesised augurin may have a physiological role in the neuroendocrine system. My aims were therefore to investigate the potential roles of augurin in the neuroendocrine system.
2 The effects of augurin on the hypothalamo-pituitary axes
2.1 **INTRODUCTION**

One of the major efferent pathways through which the hypothalamus maintains homeostasis is via the pituitary gland. The two form a unit that controls the function of numerous endocrine glands, and therefore a wide range of physiological functions. The pituitary is situated directly below the hypothalamus and lies in the hypophyseal fossa of the sphenoid bone. It is a composite organ comprised of two distinct subdivisions, the adenohypophysis and the neurohypophysis (Figure 2.1). Hormone secretion from both the adenohypophysis and neurohypophysis is regulated primarily by output from the hypothalamic neuroendocrine motor zone. In the case of the neurohypophysis, axons from hypothalamic neurons project to and release hormones directly into the circulation (Figure 2.2A). In contrast, hormone secretion from the adenohypophysis is controlled by populations of hypothalamic neurons that secrete hypophysiotrophic hormones into the hypophyseal portal circulation (Figure 2.2B).

With the exception of the supraoptic nucleus (SON) and gonadotrophin-releasing hormone expressing cells, the vast majority of neurons that regulate pituitary function lie in the periventricular region of the hypothalamus, referred to as the neuroendocrine motor zone (Swanson *et al.*, 1987a). Within the neuroendocrine motor zone are two histochemically well-defined neuronal systems, the magnocellular and the parvocellular, which convey the neuroendocrine output from the hypothalamus to the pituitary (Swanson and Sawchenko, 1983, Markakis and Swanson, 1997).
Figure 2.1: Schematic representation of the human pituitary and the hypophyseal portal vasculature. Taken from (Larsen, 2003).
Figure 2.2: Diagrammatic representation of the regulation of hormone secretion from the adenohypophysis (A) and the neurohypophysis (B) of the pituitary.

Abbreviations: ACTH, adrenocorticotrophic hormone; AVP, arginine vasopressin; CRH, corticotrophin-releasing hormone; FSH, follicle-stimulating hormone; GH, growth hormone; GHRH, growth hormone–releasing hormone; GnRH, gonadotrophin-releasing hormone; LH, luteinising hormone; OXY, oxytocin; TRH, thyrotrophin-releasing hormone; TSH, thyroid-stimulating hormone. Adapted from (Larsen, 2003).
2.1.1 Magnocellular neurosecretory system

Magnocellular neurons are located in the SON, ventrolateral paraventricular nucleus (PVN), and accessory supraoptic cell groups (Swanson and Kuypers, 1980, Cunningham and Sawchenko, 1991). They are large relative to neighbouring hypothalamic neurons and synthesise the neuropeptides arginine vasopressin (AVP) or oxytocin (OXT) at high levels (Mezey and Kiss, 1991). Magnocellular neurons typically have a single axon that project along the infundibular stalk (Sofroniew and Glasmann, 1981). Magnocellular neuronal axons collectively form the posterior lobe of the neurohypophysis where these hormones are released into the circulation (Sofroniew and Glasmann, 1981).

2.1.1.1 The neurohypophysis

The neurohypophysis is embryologically and anatomically continuous with the hypothalamus. It is composed of the posterior lobe, the infundibular stalk, and the median eminence (ME). The infundibular stalk is surrounded by the pars tuberalis, and together they constitute the hypophyseal stalk (Figure 2.1). Axon terminals from magnocellular neurons are the major constituent of the neurohypophysis (Swanson and Sawchenko, 1983). Neurohypophyseal morphology is characterised by the terminal arborisations of these axons, a rich, fenestrated vasculature, and glial cells (Hatton, 1990). These axon terminals are in close association with a capillary plexus, and they secrete substances including AVP and OXT into the general circulation via the hypophyseal veins. OXT release during parturition induces uterine contractions that facilitate birth, and release in response to suckling causes milk let-down from the mammary glands (Renaud and Bourque, 1991). AVP is released in response to a rise in plasma osmotic pressure or a fall in blood volume; it acts at the kidneys to promote water reabsorption, and causes vasoconstriction in some vascular beds (Renaud and Bourque, 1991).

2.1.2 Parvocellular neurosecretory system

Secretion from the adenohypophysis is predominantly regulated by subpopulations of hypothalamic neurons known collectively as parvocellular neurosecretory neurons (PCNs). Hypophysiotrophic hormones are released into the ME, and then travel via the hypophyseal-portal circulation to the anterior pituitary, where they regulate adenohypophyseal
hormone synthesis and release. The six classical hypophysiotrophic hormones are growth hormone releasing hormone (GHRH), somatostatin (SS), gonadotrophin releasing hormone (GnRH), corticotrophin releasing hormone (CRH), thyrotrophin releasing hormone (TRH), and dopamine (DA).

2.1.2.1 The distribution of PCNs in the rat hypothalamus

All of the hypophysiotrophic hormones are synthesised by non-neuroendocrine neurons within and outside the hypothalamus (Hokfelt et al., 2000). Projection to the ME distinguishes true PCNs from the larger number of neurons that use hypophysiotrophic hormones as a neurotransmitter. CRH, TRH, GHRH, SS, and DA PCNs are distributed along a region of the periventricular hypothalamus bound rostrally by the vascular organ of the lamina terminalis (OVLT), and caudally by the posterior periventricular nucleus (PeP), with the majority clustered in the PVN and ARC (Markakis and Swanson, 1997). SS PCNs are commonest in the anterior periventricular nucleus (aPV), and the anterior and medial parvocellular portions of the PVN (Simmons and Swanson, 2009). Almost all TRH and CRH PCNs are located in the dorsal medial parvocellular (PaDM) part of the PVN (Simmons and Swanson, 2009). From medial to lateral, these PCNs are distributed SS, TRH, and then CRH. Scattered DA PCNs, identified by their expression of tyrosine hydroxylase, and GHRH PCNs are also seen in the PVN, but they are predominantly located in the ARC (Simmons and Swanson, 2009). The majority of GHRH and DA PCNs are located in the ventrolateral and dorsomedial ARC respectively (Markakis and Swanson, 1997). Thus, SS, TRH, CRH, DA, and GHRH PCNs are distributed along a continuous periventricular zone that includes the aPV, parvocellular PVN, and ARC.

As a result of their unusual embryonic origins and in contrast to the other five, GnRH PCNs are displaced rostrally rostral, clustered in the medial preoptic area of the hypothalamus (MPOA) and in the medial septal-diagonal band complex of the telencephalic septal region (Barry et al., 1973, Markakis and Swanson, 1997).

2.1.2.2 The median eminence

Anatomically the ME lies immediately below the third cerebral ventricle, posterior to the optic chiasm and rostral to the infundibulum. It is composed of an extensive array of blood vessels
and nerve endings. Its blood supply arises from the superior hypophyseal artery, which sends off many small branches that form capillary loops (Figure 2.1). The small capillary loops anastomose and drain into the pituitary portal veins that enter the vascular pool of the adenohypophysis (Knigge and Scott, 1970). The vascular arrangement connecting the hypothalamus and the adenohypophysis is termed the hypophyseal portal circulation.

There are two distinct compartments within the ME, the internal zone and the external zone (Evans et al., 1994). The internal zone contains the axons of magnocellular and parvocellular destined for the neurohypophysis and external zone of the ME respectively. The external zone contains PCN terminals and the blood vessels of the hypophyseal portal system (Evans et al., 1994). The ME is a circumventricular organ (CVO); its ventral surface is exposed to the perivascular space of capillaries allowing the passage of hypophysiotrophic hormones into the hypophyseal portal circulation (Knigge and Scott, 1970, Norsted et al., 2008).

2.1.2.3 The adenohypophysis

The adenohypophysis consists of the anterior lobe (pars distalis), the intermediate lobe (pars intermedia), and the pars tuberalis. Both the anterior and intermediate lobes of the pituitary derive from a dorsal invagination of the pharyngeal epithelium, called Rathke’s pouch, in response to inductive signals from the overlying neuroepithelium of the ventral diencephalon (Zhu et al., 2005). The main function of the anterior pituitary is the regulation of peripheral endocrine organ function through the secretion of trophic hormones.

2.1.2.3.1 The intermediate lobe

A well-developed intermediate lobe is found in most mammals, however, only rudimentary vestiges are detectable in adult humans, with the bulk of intermediate lobe cells being dispersed in the anterior and posterior lobes (Wingstrand, 1966). The intermediate lobe contains melanotroph cells that process the prohormone proopiomelanocortin (POMC) into β-endorphin and α-melanocyte-stimulating hormone (α-MSH) (Mains and Eipper, 1979, Chang and Loh, 1984). The physiological function of the intermediate lobe is poorly understood.
2.1.2.3.2 The anterior lobe

The anterior lobe is the largest part of the adenohypophysis. In the rat it surrounds all but the dorsal aspect of the posterior lobe of the neurohypophysis. The anterior lobe secretes ‘trophic’ hormones, under the regulation of the hypothalamus. Five distinct hormone-secreting cell types are present in the mature anterior pituitary gland. Corticotrophs express POMC peptides including adrenocorticotropic hormone (ACTH); somatotrophs express growth hormone (GH); lactotrophs express prolactin (PRL); thyrotrophs express the common glycoprotein α-subunit and the specific thyroid-stimulating hormone (TSH) β-subunit, while gonadotrophs express the same α-subunit but β subunits for follicle-stimulating hormone (FSH) and luteinising hormone (LH).

The distinct endocrine cell types are heterogeneously distributed in the anterior lobe (Larsen, 2003). Somatotrophs account for 35-45% of pituitary cells and are concentrated laterally. Thyrotrophs are also concentrated laterally and these make up roughly 5% of anterior pituitary cells. Corticotrophs are found more medially and comprise 15-20% cells (Moriarty and Garner, 1977). Lactotrophs and gonadotrophs are both randomly distributed and make up 25 and 10% respectively (Larsen, 2003).

2.1.3 The anterior hypothalamo-pituitary axes

The five classical anterior hypothalamo-pituitary axes are the hypothalamo-pituitary-adrenal (HPA) axis, hypothalamo-pituitary-thyroid (HPT) axis, hypothalamo-pituitary-gonadal (HPG) axis, hypothalamo-pituitary-prolactin axis, and the hypothalamo-pituitary-somatotrophic axis (Figure 2.3). They all share a common schematic whereby neural and humoral signals converge on specific PCNs and modulate their secretion of hypophysiotrophic hormones into the ME. Hypophysiotrophic hormones travel via the hypophyseal portal circulation to the anterior pituitary lobe, where they regulate trophic hormone secretion. Anterior lobe trophic hormones in turn regulate the secretion of circulating hormones by target endocrine organs, with the exception of prolactin. Control of secretion is exerted through negative feedback at multiple levels by circulating, trophic, and hypophysiotrophic hormones.
Figure 2.3: Diagrammatic representation of five classical anterior hypothalamo-pituitary axes. Although sex steroids act predominantly via negative feedback as shown, in females a transient positive feedback loop occurs during the late follicular phase (discussed in 2.1.3.4).

Abbreviations: ACTH, adrenocorticotrophic hormone; CRH, corticotrophin-releasing hormone; FSH, follicle-stimulating hormone; GH, growth hormone; GHRH, growth hormone-releasing hormone; GnRH, gonadotrophin-releasing hormone; IGF, insulin-like growth factor; LH, luteinising hormone; T₄, 3,5,3',5'-tetraiodo-L-thyronine; T₃, 3,5,3'-triiodo-L-thyronine; TRH, thyrotrophin-releasing hormone; TSH, thyroid-stimulating hormone. Taken from (Melmed, 2003)
2.1.3.1 The hypothalamo-pituitary-adrenal axis

Internal or external stimuli that are perceived to challenge homeostasis are termed stressors (De Kloet et al., 2005). The HPA axis is the humoral component of an integrated neural and endocrine system that functions to orchestrate the response to stressors and maintain homeostasis. Activation of the HPA axis culminates in the secretion of glucocorticoids which act at multiple levels, temporarily redirecting resources to restorative processes (Selye, 1936). Much of the data presented in this chapter describes the effects of augurin on the HPA axis.

The CRH system in the hypothalamus is vitally important for the integrated behavioural, endocrine, and autonomic response to stressors. In addition to CRH, there are three CRH-like peptides (urocortin I, urocortin II, and urocortin III), at least two cognate receptors (CRHR1 and CRHR2), and a high-affinity CRH-binding protein, each with distinct distribution in the CNS (Behan et al., 1989, Chen et al., 1993, Chalmers et al., 1995).

At the hypothalamic level, CRH PCNs within the PVN control glucocorticoid secretion, and are the focal point for stress neurocircuitry (Whitnall, 1993). Under basal conditions, a circadian pulsatility of glucocorticoid secretion is maintained by direct projections from the SCN (Reppert and Weaver, 2002). In response to stressors however, a complex neural network that converges on the PVN initiates a surge in glucocorticoid levels (Ulrich-Lai and Herman, 2009). The PVN receives direct and indirect afferents from multiple sources including hypothalamic, brainstem, and limbic forebrain structures which modulate the activity of CRH PCNs (Herman et al., 2003).

CRH is a 41-amino-acid peptide that stimulates the release of ACTH from pituitary corticotrophs (Vale et al., 1981). Two CRH G-protein-coupled receptors (GPCRs) have been identified; CRHR1 and CRHR2 (Bale and Vale, 2004). CRHR1 is expressed on pituitary corticotrophs and widely throughout the brain, while CRHR2 is less broadly expressed and predominantly found in the CNS (Chalmers et al., 1995). The nonapeptide AVP is co-expressed by a by a subset of CRH PCNs and it synergistically enhances the stimulation of ACTH release by CRH (Gillies and Lowry, 1979, Gillies et al., 1982).
ACTH is a 39-amino-acid peptide produced by cleavage of POMC (Lee et al., 1961). Circulating ACTH acts via the melanocortin 2 receptor (MC2R) to stimulate glucocorticoid synthesis and secretion from the adrenal gland (Ramachandran, 1984). The adrenal gland contains two endocrine components. The adrenal medulla is located at the centre of the adrenal gland and secretes catecholamines into the circulation. Surrounding the adrenal medulla is the cortex of the adrenal gland, which synthesises and secretes steroid hormones. The adrenal cortex is functionally and histologically comprised of three subdivisions. From exterior to interior they are the zona glomerulosa, zona fasciculate, and the zona reticularis (Mitani et al., 1999). Glucocorticoids (predominantly cortisol in humans, corticosterone in rats) are secreted from the zona fasciculata under the control of ACTH, whereas mineralocorticoids (predominantly aldosterone) are secreted from the zona glomerulosa under the principal control of angiotensin II (Brecher et al., 1974). Cells of the zona reticularis secrete adrenal androgens that exert minor effects on reproductive function under physiological conditions (Migeon, 1972).

Glucocorticoids and mineralocorticoids exert their effects predominantly via the glucocorticoid and mineralocorticoid receptors (GR and MR) (Lu et al., 2006). Both receptors are members of the nuclear receptor superfamily (Chambon, 2005). They reside in the cytoplasm complexed with molecular chaperone proteins (Pratt and Toft, 1997). On ligand binding, the receptors translocate into the nucleus where they interact with specific responsive elements within the DNA, thus modulating the transcription of hormone responsive genes (Lu et al., 2006). The MR and GR share considerable homology and as a result, there is substantial promiscuity of ligand binding. While the GR is specifically activated by glucocorticoids, data from in vitro studies suggest aldosterone, corticosterone, and cortisol all bind to and activate the MR with equal affinity (Arriza et al., 1987, Lu et al., 2006). The average plasma glucocorticoid concentration is several orders of magnitude higher than plasma mineralocorticoid levels, however, tissue specificity at the MR and GR is conferred through the “pre-receptor” metabolism of steroid hormones via the 11β-hydroxysteroid dehydrogenase enzyme family (Edwards et al., 1988, Funder et al., 1988). Although the GR is encoded by single a gene, splice variants have been
described which, together with tissue specific post-translational modification are thought to account for many of the diverse actions of glucocorticoids (Pascual-Le Tallec and Lombes, 2005, Zhou and Cidlowski, 2005). Like many of the steroid hormones, there is evidence glucocorticoids also exert rapid nongenomic effects, not mediated by the GR or MR (Evanson et al., 2010).

Glucocorticoids regulate a wide range of physiological functions including intermediate metabolism, growth, immune function and bone and calcium metabolism (Buckingham, 2006). Circulating glucocorticoids negatively feedback on the HPA axis, acting on both the pituitary corticotrophs and the hypothalamic neurons that secrete CRH and AVP (Ulrich-Lai and Herman, 2009).

2.1.3.2 The hypothalamo-pituitary-somatotrophic axis

At the hypothalamic level GHRH and SS PCNs regulate the secretion of GH from pituitary somatotrophs. Post-translational processing of a single preproGHRH precursor yields several molecular forms of GHRH in the rat and human hypothalamus, and bioactivity lies in the N-terminal 29-amino-acids (Rivier et al., 1982, Mayo et al., 1983, Frohman et al., 1989). Somatostatin occurs in two major forms; a 14-amino-acid cyclic peptide (SS-14), and a 28-amino-acid N-terminally extended form (SS-28). SS-14 is the predominant form in the CNS, whereas SS-28 is the major form in the gastrointestinal tract (Bloom and Polak, 1987).

Pituitary somatotrophs expresses the GHRH receptor, GH secretagogue receptor (GHSR), and SS receptor subtypes 2 and 5, all of which regulate GH secretion (Shimon et al., 1997a, Mayo et al., 2000). GHRH selectively induces GH gene transcription and hormone release (Barinaga et al., 1983, Mayo et al., 2000), while SS suppresses basal and GHRH-stimulated GH release, and also supresses the release of TSH, ACTH, and PRL (Shimon et al., 1997a, Shimon et al., 1997b). The hypophyseal release of SS and GHRH oscillate 180 degrees out of phase, resulting in pulsatile hormone secretion characteristic of GH (Plotsky and Vale, 1985, Vance et al., 1985). Acyl-ghrelin, a 28-amino-acid peptide, is the only known endogenous ligand for the GHSR (Kojima et al., 1999). Acyl-ghrelin dose-dependently evokes GH release from somatotrophic cells (Kojima et al., 1999).
GH is an anabolic hormone that stimulates increased protein synthesis, promotes lipolysis and gluconeogenesis and increases bone mineralisation (Davidson, 1987, Moller and Jorgensen, 2009). The principal form of GH is a 191-amino-acid single-chain polypeptide, accounting for 90% of pituitary and 75% of plasma GH (Baumann et al., 1983). GH mediates its pleiotropic effects through the GH receptor (GHR), both directly by agonising GHRs on target organs, and indirectly through stimulation of insulin-like growth factor-1 (IGF-1) secretion by the liver (Lupu et al., 2001).

2.1.3.3 The hypothalamo-pituitary-thyroid axis

The HPT axis directly regulates energy expenditure and metabolic rate, and has a much broader permissive role in development, growth, reproduction, and metabolism. TRH PCNs determine the set-point of feedback control, and therefore HPT axis tone (Nikrodhanond et al., 2006). TRH, a tripeptide, is the smallest known NEP (Boler et al., 1969). Rat preproTRH contains five TRH peptides (six in humans) flanked by dibasic residue cleavage sites (Lee et al., 1988). Two TRH GPCRs have been identified in rodents, TRHR1 and TRHR2, however humans appear to lack a functional TRHR2 gene (Straub et al., 1990, Heuer et al., 2000, Sun et al., 2003). TRH stimulates the secretion of TSH via TRHR1 on thyrotrophs, while DA and SS have modest inhibitory effects (Arimura and Schally, 1976, Morley, 1981, Jackson, 1982).

TSH is a glycoprotein heterodimer of two non-covalently linked α and β subunits (Pierce and Parsons, 1981). TSH, LH, FSH and human chorionic gonadotrophin share a common α-subunit, and each has a unique β-subunit that confers specificity of action (Fiddes and Goodman, 1981, Grossmann et al., 1997). TSH acts via a single TSH receptor expressed by thyroid follicular cells, stimulating the synthesis and release of 3,5,3′,5′-tetraiodo-L-thyronine (T₄) and 3,5,3′-triiodo-L-thyronine (T₃) (Vassart et al., 1995, Rapoport et al., 1998). Normal plasma T₄ levels are roughly 40 fold higher than plasma T₃, and the vast majority of both are bound and sequestered in the plasma by thyroxine-binding globule, transthyretin, or albumin (Robbins, 1992, Schussler, 2000). Only free T₄ and T₃ are bioactive (fT₄ and fT₃ respectively), the latter 15-fold more active than the former (Brent, 1994). Both fT₄ and fT₃ circulate in equilibrium with the bound fraction so that roughly 0.02% and 0.3% of total plasma T₄ and T₃ are in the free
form (Robbins and Rall, 1957, Schussler, 2000). Negative feedback on thyrotrophs and TRH PCNs maintains plasma fT₄ and fT₃ within a remarkably narrow range (Reichlin and Utiger, 1967, Segerson et al., 1987, Dyess et al., 1988).

Intracellular transport of T₄ and T₃ is near universal but tissue specificity is conferred by expression of particular membrane transporter, iodothyronine deiodinase, and thyroid hormone receptor subtypes (Brent, 1994, Gereben et al., 2008, Van Der Deure et al., 2010). The iodothyronine deiodinases are a family of selenoprotein enzymes that catalyse the dehalogenation of iodothyronines (Gereben et al., 2008). Intracellular T₄ is metabolised into T₃ by type 1 and type 2 deiodinase, or inactive rT₃ by type 3 deiodinase (St Germain and Galton, 1997). T₃ is the principal ligand for the ubiquitously expressed thyroid hormone receptors (TRs), which are members of the nuclear receptor superfamily. There are four TRs, TRα1 and TRβ1, TRβ2, and TRβ3 (Lazar, 2003). Ligand binding alters the affinity of the TR complex for thyroid hormone response elements in the DNA, thereby regulating transcription (Lazar, 2003).

2.1.3.4 The hypothalamo-pituitary-gonadal axis

The physiology of the mammalian HPG axis is a highly complex, sexually dimorphic, and changes dramatically depending on reproductive status. Plasma reproductive hormone levels, and thus fertility and reproductive behaviour, are regulated by GnRH PCNs (Seeburg and Adelman, 1984). There are two mammalian GnRH genes; Gnrh1 and Gnrh2 (Sherwood et al., 1993). GnRH-I, encoded by the Gnrh1 gene, is expressed by GnRH PCNs, and this form regulates gonadotroph function (Fernald and White, 1999). GnRH-I is a decapeptide produced from a 92-amino-acid preproGnRH1 precursor (Seeburg and Adelman, 1984). Following cleavage by PC enzymes, the N-terminal Gln is cyclised, while the C-terminal Gly is amidated (Amoss et al., 1971, Baba et al., 1971, Matsuo et al., 1971, Schally et al., 1971). The N-terminal pGlu-His-Trp-Ser and C-terminal Pro-Gly-NH₂ are critical for biological activity, and are highly conserved throughout vertebrates (Fernald and White, 1999, Millar, 2005). GnRH-I stimulates the release of LH and FSH via the GnRHR1, a GPCR expressed by pituitary gonadotrophs (Cheng and Leung, 2005).
GnRH PCNs fire in coordinated and episodic manner, producing distinct pulses of GnRH-I in the hypophyseal circulation (Carmel et al., 1976). The regulation of GnRH-I pulses is highly complex; their amplitude and frequency determine the pattern of LH and FSH secretion, and vary depending on pubertal development, circadian rhythms, and the stage of the oestrus cycle in females (Crowley et al., 1991, Krsmanovic et al., 2009). The pulsatile release of GnRH is critical for fertility because continuous GnRH administration down-regulates pituitary gonadotroph activity, leading to suppression of gonadotrophin secretion and subsequent infertility (Belchetz et al., 1978). The determinants of GnRH-I secretion are collectively termed the ‘GnRH pulse generator’, and their identity is a fundamental, unsolved question in reproductive biology. There is evidence GnRH PCNs are inherently pulsatile (Wetsel et al., 1992, Suter et al., 2000), but they also receive peptidergic inputs from neurons expressing kisspeptin (KISS) (Cravo et al., 2011), nesfatin (Garcia-Galiano et al., 2010), neurokinin B (NKB) (Goodman et al., 2007), dynorphin A (DYN) (Wakabayashi et al., 2010), gonadotrophin inhibitory hormone (GnIH) (Kriegsfeld et al., 2006), neuropeptide Y (Klenke et al., 2010), galanin (Rajendren and Gibson, 2001), galanin-like peptide (Matsumoto et al., 2001), and POMC (Hill et al., 2010), as well as classical neurotransmitters such as glutamate and GABA (Chen and Moenter, 2009, Khan et al., 2010), all of which regulate hypophysiotrophic GnRH release.

Of the aforementioned neuronal projections those expressing KISS and those expressing GnIH have received considerable attention recently (Clarke, 2011). KISS neurons reside within the preoptic region and ARC (Mikkelsen and Simonneaux, 2009). ARC KISS neurons co-express NKB and DYN (KNdy neurons) and project directly to GnRH PCNs (Goodman et al., 2007, Lehman et al., 2010). Although there is some variability between species considerable evidence suggests KNdy neurons are important regulators of the mammalian HPG axis. Hypothalamic injection of KISS or NKB stimulates the release of GnRH into the hypophyseal circulation (Thompson et al., 2004b, Navarro et al., 2011). Furthermore loss-of-function mutations in the genes encoding KISS (Kiss1), NKB (Tac3), or their cognate receptors (Gpr54 and Tacr3 respectively), cause congenital isolated hypogonatrophic hypogonadism (Seminara et al., 2003,
D'anglemont De Tassigny et al., 2007, Topaloglu et al., 2009. GnIH was identified as a hypothalamic neuropeptide that inhibits HPG axis activity in the quail and a large body of work has established this as a bone fide regulatory peptide in avian species (Tsutsui et al., 2000, Tsutsui et al., 2009). Nerveless, although an analogous effect has been described in mammals, discrepancies in the literature regarding the magnitude, sites, and relevance of its biological effects mean its physiological role is unclear (Pineda et al., 2010).

Both LH and FSH are synthesised and intermittently secreted from gonadotrophs in response to the pulsatile release of GnRH (Evans, 1999). They act via their respective GPCRs on the gonads where they regulate gametogenesis and the production of sex steroids (Misrahi et al., 1996, Themmen and Huhtaniemi, 2000). In males, LH promotes the secretion of testosterone and other androgens from Leydig cells in the testes (Bhalla et al., 1992), while in females, LH and FSH act together to regulate the production of oestrogens and progesterones in the ovaries (Themmen and Huhtaniemi, 2000). Sex steroids act predominantly via negative feedback at the hypothalamic and pituitary levels to supress HPG axis tone (Damassa et al., 1976, Tilbrook and Clarke, 2001). In females however, prolonged exposure to oestrogens during the late follicular phase of the oestrous cycle results in a transient positive feedback loop that triggers ovulation (Moenter et al., 1990).

### 2.1.3.5 Hypothalamo-pituitary-prolactin axis

Human PRL is a 199-amino-acid polypeptide necessary for lactation in mammals (Li et al., 1969, Ormandy et al., 1997). In contrast to the other trophic hormones, the hypothalamic regulation of PRL is predominantly inhibitory, mediated by DA PCNs (Ben-Jonathan, 1985). DA inhibits PRL secretion via type 2 dopamine receptors on pituitary lactotrophs (Ben-Jonathan, 1985). No primary PRL releasing hormone has been identified, but numerous hypothalamic and systemic factors have been shown to stimulate PRL secretion, including vasoactive intestinal polypeptide (VIP), OXT, PRL-releasing peptide (PrRP), TRH, and oestrogen (Lloyd et al., 1975, Bjoro et al., 1984, Liu and Ben-Jonathan, 1994, Hinuma et al., 1998).
Circulating PRL is a mixture of monomeric and polymeric forms with varying bioactivity (Suh and Frantz, 1974, Farkouh et al., 1979). All forms of PRL act via a single PRL receptor (PRLR) that is similar in structure and function to the GHR (Arden et al., 1990, Bazan, 1990, Goffin and Kelly, 1997). PRLRs are broadly expressed, and in addition to stimulating lactation, prolactin has been implicated in the regulation of fluid and electrolyte balance, growth and development, behaviour, and immune function (Bole-Feysot et al., 1998).
2.2 HYPOTHESIS AND AIMS

Augurin is a putative neuroendocrine peptide of unknown function, and is expressed in the CNS, pituitary, and endocrine organs. I hypothesise that augurin may play a role in the neuroendocrine system. As the hypothalamus and pituitary are sites at which many NEPs have biological activity, and are pivotal components of the neuroendocrine system, I began by investigating the effects of augurin on the hypothalamo-pituitary axes in male Wistar rats.

2.2.1 AIMS

1. Investigate the effects of augurin on plasma hormone levels following central or peripheral injection in vivo.
2. Investigate the direct effect of augurin on the release of neurohypophyseal hormones from the hypothalamus.
3. Investigate the direct effect of augurin on trophic hormone release from the pituitary.
2.3 METHODS

2.3.1 Peptides

A synthetic fragment of human augurin corresponding to amino acids 71 to 148 of preproaugurin protein was synthesised by Bachem UK Ltd. (Merseyside, UK). The synthesis was performed using a Symphony automated peptide synthesiser (Protein Technology, Inc., USA). The product was initially purified by flash chromatography with a reversed phase resin (Daisogel, Daiso Ltd. Japan), followed by reversed phase high-pressure liquid chromatography (HPLC). Analyses of the purified peptide by matrix-assisted laser desorption/ionisation mass spectroscopy, HPLC, and amino acid analysis showed above 91% purity (average molecular weight 9689). Rat neuromedin U (NMU) 23, angiotensin II (AII), and astrostin (cyc30,33[D-Phe12,Nle21,38,Glu30,Lys33]CRH-(12-41)) were purchased from Bachem UK Ltd.

Augurin is poorly soluble in water; therefore, for all studies lyophilised augurin was first dissolved in a small amount of 0.05M HCl, and then diluted in saline (in vivo studies) or artificial cerebrospinal fluid (aCSF; hypothalamic and pituitary explant studies) containing sufficient NaOH to neutralise the HCl. Vehicle was prepared by mixing the same amounts of 0.05M HCl, 0.05M NaOH, and saline/aCSF. The pH of vehicle and peptide solutions was checked prior to injection by pipetting a small amount onto pH indicator paper. AII, astrostin, and NMU were dissolved into a minimal quantity of sterile water, and then diluted into sterile saline (AII and astrostin) or vehicle (NMU 23) prior to injection.

2.3.2 Animals

Male Wistar rats (specific pathogen free; Charles River, Margate, UK), weighing 250-300g were maintained in individual cages under controlled temperature (21-23 C) and light (12 hour light, 12 h dark cycle; lights on at 0700 h) conditions with ad libitum access to food (RM1 diet, SDS Ltd., Witham, UK) and water. Animal procedures were approved under the British Home Office Animals Scientific Procedures Act 1986 (Project Licence 70/6402).
2.3.3 **INTRACEREBROVENTRICULAR CANNULATION AND INJECTIONS**

Intracerebroventricular (ICV) cannulation was carried out using an established protocol (Rossi et al., 1997). Prophylactic antibiotics were administered to prevent post-operative infection. An ip injection of a mixture of amoxycillin (37.5mg/kg) and flucloxacillin (37.5mg/kg) was given prior to surgery. Animals were anaesthetized by ip injection of a mixture of xylazine (10mg/kg) (Rompun, Bayer UK Ltd.) and ketamine (100mg/kg) (Ketalar, Parke-Davis, Pontypool, UK) in a 2:5 ratio. The head was cleaned with 10% w/v povidone/iodine solution (Betadine, Seton Healthcare) and the animal placed in a stereotaxic frame (David Kopf instruments, supplied by Clark Electromedical Instruments) with the incisor bar set at 3mm below the interaural line. Prior to commencement of surgery, each rat received a single sub-cutaneous injection of buprenorphine (45µg/kg; Schering-Plough, Welwyn Garden City, UK) for analgesia. A 1.5cm incision was made in the scalp and the skull surface exposed by removal of the periosteum. A hole was drilled 0.8mm posterior to the bregma on the midline. A permanent 22-gauge intracerebroventricular (ICV) stainless steel cannula (Plastics One Inc. USA) was stereotaxically implanted 6.5mm below the outer surface of the skull projecting to the third cerebral ventricle. Co-ordinates were calculated from the Rat Brain Atlas (Paxinos, 2007). Three stainless steel screws were secured in the skull in three additional drill holes to anchor the dental cement (Associated Dental Products Ltd) used to hold the cannula in position. A plastic-topped obturator (Plastic One Inc.) was then inserted into the cannula to prevent blockage. Following surgery 5ml 0.9% sodium chloride was injected ip to prevent hypovolemia.

Following surgery, rats were allowed a seven-day recovery period during which time they were checked daily. Prior to the studies, each rat received two sham injections (one of AII and one of saline) to acclimatise them to the procedure. Only animals with correct cannula placement, as confirmed by a sustained drinking response to ICV AII (50ng/rat), were included in the studies. Peptide/vehicle was administered in 5µl volume to conscious, freely moving rats using a stainless steel injector (Plastics One Inc.) placed into, and projecting 1mm below, the cannula tip. The injector was connected by polythene tubing to a Hamilton gas tight syringe (Fischer Scientific, UK) in a Harvard infusion pump (Harvard Apparatus, USA). The tubing was filled
with water and a 1µl bubble drawn up into it to separate the experimental compound from the water. Injections were given over 1 minute, and the injector left in place for a further 30 seconds to allow diffusion of the compound and to prevent retrograde flow. Rats were returned to their own cages following the injection procedure.

2.3.4 INTRAPARAVENTRICULAR CANNULATION AND INJECTIONS

Unilateral intrahypothalamic cannulation directed at the PVN (iPVN) was carried out using an established protocol (Abbott et al., 2003). Animals were maintained and handled for iPVN cannulation as in 2.3.3. Anaesthesia, surgical equipment, and post cannulation recovery were the same as in 2.3.3. Only the site and cannula gauge differed. Permanent 26-gauge stainless steel guide cannulae (Plastics One Inc.) were stereotaxically implanted using established coordinates obtained from the Rat Brain Atlas (1.8mm posterior to the bregma, 0.3mm laterally and implanted 7.5mm below the outer surface of the skull) (Paxinos, 2007).

For iPVN injections, peptides were dissolved as described in 2.3.1 and administered in 1µl volume via a stainless steel injector projecting 1mm beyond the tip of the cannula. Correct cannula placement was confirmed histologically at the end of the study period by injection of India ink using an established protocol (Abbott et al., 2003). Following decapitation 1µl of India ink was injected into the cannula using the same protocol as iPVN injection of peptide. The ink was allowed to diffuse for 5 minutes, following which the guide cannula was removed. The brain was rapidly dissected out, fixed overnight in 4% formaldehyde, cryoprotected in 40% sucrose, and frozen in an isopentane/dry ice bath. Brains were stored at -70 C until sectioning. A freezing sled microtome (Shandon Southern Products Ltd, Cheshire, UK) was used to take 40µm coronal sections. Sections were stained with cresyl violet and compared with the corresponding section from the rat brain atlas (Nissl, 1894, Paxinos, 2007).

Data from rats were excluded if the injection site extended more than 0.4mm outside the intended injection site, or if any ink was detected in the cerebroventricular system. In total 71% of cannulae were correctly positioned. Figure 2.4 shows a representative section of the PVN containing India ink.
Figure 2.4: Photomicrograph of the spread of 1μl India ink injected into the PVN. The section was not stained so that the ink could be clearly seen.

Abbreviations: 3V, 3rd ventricle; ot, optic tract.
2.3.5 The effect of ICV augurin on plasma hormone levels

Each rat received a single ICV injection of vehicle or 5 nmol augurin (n = 9-10 per group) in the early light phase (0900-1200 h). Rats were killed by decapitation 60 minutes after injection. Trunk blood was collected in plastic lithium heparin tubes containing 4200 kallidinogenase inactivator units of aprotinin (Bayer Corp., Haywards Heath, UK) for LH, FSH, GH, TSH, PRL, testosterone, fT3, fT4, and corticosterone assays, and in plastic ethylenediaminetetraacetic acid (EDTA) tubes for ACTH assays. Plasma was immediately separated by centrifugation at 1600g for 10 minutes, frozen on dry ice, and stored at -20°C (lithium heparin tubes) or -80°C (EDTA tubes).

2.3.6 The effect of ICV augurin on plasma corticosterone and ACTH

Each rat received a single ICV injection of vehicle, 0.6, 1.7, or 5 nmol augurin in the early light phase (0900-1200 h). Rats were killed by decapitation 20 or 60 minutes post injection (n = 8-10 per group per time point). Trunk blood was collected and stored as in 2.3.5.

2.3.7 The effect of iPVN augurin on plasma corticosterone and ACTH

Each rat received a single iPVN injection of vehicle, 0.1, 0.3, or 1 nmol augurin in the early light phase (0900-1200 h). Rats were killed by decapitation 20 or 60 minutes post injection (n = 5-8 per group per time point). Trunk blood was collected and stored as in 2.3.5.

2.3.8 The effect of iPVN augurin on behaviour

The acute effects of iPVN augurin on behaviour were formally assessed by blinded observers using an established protocol (Smith et al., 2006). Each rat received a single iPVN injection of vehicle, 1 nmol augurin, or 0.3 nmol NMU 23 (n = 6-8 per group) in the early light phase (0900-1200 h). Behavioural patterns were monitored for 120 minutes following injection by observers blinded to the experimental treatment. Behaviour was classified into eight different categories; feeding, drinking, grooming, burrowing, rearing, locomotion, head down, and sleeping, adapted from Fray et al (Fray et al., 1980). These methods have previously been used to demonstrate abnormal behaviour following iPVN injection of peptides (Wren et al., 2002).
During the analysis, each rat was observed for 15 seconds every 5 minutes. Each 15-second period was further subdivided into three and the predominant behaviour of the rat during each 5-second episode was noted. NMU was used as a positive control as it is known to increase grooming behaviour and decrease sleeping (Wren et al., 2002).

2.3.9 The effect of augurin on CRH and AVP release from hypothalamic explants

Static incubation of hypothalamic explants was carried out using an established technique (Dhillo et al., 2002). Ad libitum fed male Wistar rats were killed by decapitation and the whole brain immediately removed. The brain was mounted with the ventral surface uppermost and placed in a vibrating microtome (Microfield Scientific Ltd., Dartmouth, UK). A 1.9mm slice was taken from the basal hypothalamus and blocked lateral to the circle of Willis. The hypothalamic slices were incubated in individual chambers containing 1ml aCSF (20mM NaHCO_3, 126mM NaCl, 0.09mM Na_2HPO_4, 6mM KCl, 1.4mM CaCl_2, 0.09mM MgSO_4, 5mM glucose, 0.18mg/ml ascorbic acid, and 100µg/ml aproatin) equilibrated with 95% O_2 and 5% CO_2.

The tubes were placed on a platform in a water bath maintained at 37°C. After an initial 2 h equilibration period, hypothalami were incubated for 45 min in 600µl aCSF (basal period), before being treated with 1, 10, or 100nM augurin in 600µl aCSF for 45 min (n = 13-15 hypothalamic slices per group). Finally, tissue viability was assessed by incubation for 45 min in aCSF containing 56mM KCl. Isotonicity was maintained by substituting K⁺ for Na⁺. Explanted hypothalami that failed to show peptide release above the basal level in response to aCSF containing 56mM KCl were excluded from the data analysis. At the end of each period, aCSF was collected and stored at -20°C until measurement of CRH and AVP by radioimmunoassay (RIA).

2.3.10 The effect of peripheral CRHR antagonism on augurin induced ACTH and corticosterone secretion

ICV cannulated rats were injected ip with 100µg/kg astressin or vehicle 15 minutes before receiving an ICV injection of 5 nmol augurin or vehicle. Astressin is a CRHR₁ receptor
antagonist (Gulyas et al., 1995), not known to cross the blood-brain barrier. This dose of
dose of astressin has previously been used to block the increase in plasma ACTH and corticosterone
caused by ICV injection of GLP-1 (Kinzig et al., 2003). Rats were decapitated 30 minutes after
the ICV injection (n = 7-9 per group). Trunk blood was collected and stored as in 2.3.5.

2.3.11 THE EFFECT OF PERIPHERALLY ADMINISTERED AUGURIN ON
PLASMA HORMONE LEVELS

Rats were handled daily and received twice-weekly ip injections to acclimatise them to the
procedure. On the day of the study, rats were injected ip with 100 nmol/kg augurin or vehicle
during the early light phase (0900-1200 h). Rats were decapitated 30 minutes after the ip
injection (n = 7-9 per group). Trunk blood was collected and stored as in 2.3.5.

2.3.12 THE EFFECT OF AUGURIN ON ACTH RELEASE FROM PITUITARY
SEGMENTS

Static pituitary explants were performed using an established technique (Smith et al., 2006).
The anterior pituitaries of ad libitum fed rats were harvested immediately following
decapitation. The posterior pituitary was removed from the anterior pituitary which was bisected
along the mid-sagittal line and then divided into four pieces of approximately equal size. After
an acclimatisation period, the segments were then incubated in one of aCSF alone, or aCSF
containing 10nM augurin, 100nM augurin, 1000nM augurin or 100nM CRH for 4 h (n = 17-19
per group). At the end of this period the incubation medium was collected and stored at -20°C
until assayed for ACTH by RIA.

2.3.13 RADIOIMMUNOASSAYS

RIAs for CRH and AVP were performed using established protocols (Dhilllo et al., 2003). The
intra and interassay coefficient of variation (CV) were <10% for the CRH RIA, and 11% and
20% respectively for the AVP RIA. Plasma corticosterone was measured using an RIA kit from
MP Biomedicals, Inc. (Orangeburg, NY, USA), for which the intra and interassay CV were less
than 10% and 7% respectively. For studies 2.3.5, 2.3.6, and 2.3.7, plasma ACTH was measured
by immunoradiometric assay purchased from Euro-Diagnostica B.V. (Arnhem, The
Netherlands). The intra and interassay CV were both less than 4%. For study 2.3.10 plasma
ACTH was measured by immunoradiometric assay purchased from BioSource Europe S.A. (Nivelles, Belgium) because the previously used kit was no longer available. The intra and interassay \( C_v \) were 6.4% and 6.2% respectively. Plasma TSH, LH, FSH, PRL, GH, were measured, using methods and reagents provided by the National Hormone and Pituitary program, as was and ACTH release from pituitary segments. Total plasma testosterone, \( fT_3 \) and \( fT_4 \) were measured using commercial Coat-a-Count RIA kits (Euro/DPC Limited, Caenarfon, UK). The relevant theoretical and practical aspects of RIAs are described in more detail in appendix I.

### 2.3.14 Statistics

Data from hypothalamic explant, pituitary explant, and terminal studies are presented as mean ± SEM. Data from behavioural analyses are presented as median frequency and interquartile range for each behaviour. Data from hypothalamic explant release were analysed using paired Student \( t \) test between the basal period and the test period. Data from pituitary explant release were analysed using one-way ANOVA followed by post hoc Holm-Sidak test (SigmaStat 3.5, San Jose, CA, USA). Data from the behavioural study were analysed using Kruskal-Wallis one-way ANOVA on ranks (Systat 11, San Jose, CA, USA).

The normal distributions of plasma ACTH and corticosterone levels are positively-skewed (Ganong and Systems, 1995). Therefore plasma ACTH and corticosterone values were log transformed to homogenise variances among groups, and to improve the normality of residuals (Bland and Altman, 1996). Transformed values were then analysed by Student \( t \) test, or one- or two-way ANOVA followed by post hoc Holm-Sidak test (SigmaStat 3.5, San Jose, CA).
2.4 RESULTS

2.4.1 THE EFFECT OF ICV AUGURIN ON PLASMA HORMONE LEVELS

Augurin significantly increased plasma ACTH 60 minutes post injection (ACTH pg/ml; vehicle 21.3 ± 3.2, 5 nmol augurin 66.7 ± 16.3, p<0.001). Plasma corticosterone was raised but failed to reach statistical significance (corticosterone ng/ml; vehicle 152.8 ± 25.9, augurin 5 nmol 300.0 ± 65.1). There were no significant changes in plasma LH, FSH, GH, TSH, prolactin, testosterone, fT3, or fT4 (Table 2.1).

2.4.2 THE EFFECT OF ICV AUGURIN ON PLASMA CORTICOSTERONE AND ACTH

Plasma ACTH and corticosterone levels were significantly elevated following ICV injection of augurin compared with vehicle injected controls (Figure 2.5). In the case of ACTH, two-way ANOVA revealed that there was a significant main effect of dose ($F_{(3,60)} = 11.789; \ p<0.001$), a significant main effect of time ($F_{(1,60)} = 7.956; \ p<0.01$), and a significant interaction ($F_{(3,60)} = 3.181; \ p<0.05$). Post hoc analysis demonstrated that ICV injection of 5 nmol augurin significantly increased plasma ACTH 20 minutes post injection (p<0.001). All doses of augurin significantly increased plasma ACTH 60 minutes post injection (p<0.01 for all doses).

In the case of corticosterone, two-way ANOVA revealed that there was a significant main effect of dose ($F_{(3,68)} = 8.510; \ p<0.001$), a significant main effect of time ($F_{(1,68)} = 3.799; \ p<0.05$), and no significant interaction ($F_{(3,68)} = 0.954; \ p=0.420$). Post hoc analysis demonstrated that ICV injection of 5 nmol augurin significantly increased plasma corticosterone 20 minutes post injection (p<0.001).
Table 2.1: Effect of a single ICV injection of augurin (5 nmol) or vehicle in *ad libitum* fed rats on plasma hormone concentrations at 60 minutes post injection. *** p<0.001 vs. vehicle, n = 9-10 per group. Results are mean ± SEM

<table>
<thead>
<tr>
<th></th>
<th>Vehicle Mean</th>
<th>Vehicle SEM</th>
<th>Augurin (5 nmol) Mean</th>
<th>Augurin (5 nmol) SEM</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTH (pg/ml)</td>
<td>21.3</td>
<td>3.2</td>
<td>66.7***</td>
<td>16.3</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>152.8</td>
<td>25.9</td>
<td>300.0</td>
<td>65.1</td>
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<td>LH (ng/ml)</td>
<td>0.60</td>
<td>0.09</td>
<td>0.54</td>
<td>0.12</td>
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<tr>
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<td>7.18</td>
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<tr>
<td>Testosterone (ng/dL)</td>
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<td>87.2</td>
<td>495.7</td>
<td>139.2</td>
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<td>TSH (ng/ml)</td>
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<td>0.14</td>
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<td>Free T&lt;sub&gt;4&lt;/sub&gt; (ng/dL)</td>
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<td>0.11</td>
<td>2.27</td>
<td>0.12</td>
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<tr>
<td>Free T&lt;sub&gt;3&lt;/sub&gt; (pg/dL)</td>
<td>1.28</td>
<td>0.11</td>
<td>1.21</td>
<td>0.13</td>
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<tr>
<td>PRL (ng/ml)</td>
<td>5.28</td>
<td>0.79</td>
<td>5.15</td>
<td>0.81</td>
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<tr>
<td>GH (ng/ml)</td>
<td>20.27</td>
<td>4.38</td>
<td>22.39</td>
<td>11.54</td>
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</table>
Figure 2.5: Effect of a single ICV injection of augurin (0.6, 1.7, or 5 nmol) or vehicle in *ad libitum* fed male Wistar rats on plasma ACTH and corticosterone at 20 (A and C) and 60 (B and D) minutes post injection. **p<0.01, ***p<0.001 vs. vehicle, n = 8-10 per group per time point. Results are mean ± SEM.
2.4.3 The effect of iPVN augurin on plasma corticosterone and ACTH

Plasma ACTH and corticosterone levels were significantly elevated following iPVN microinjection of augurin compared with vehicle-injected controls (Figure 2.6). In the case of ACTH, two-way ANOVA revealed that there was a significant main effect of dose ($F_{(3,45)} = 9.891; p<0.001$), a significant main effect of time ($F_{(1,45)} = 5.363; p<0.05$), and no significant interaction ($F_{(3,45)} = 0.600; p=0.618$). Post hoc analysis demonstrated that iPVN microinjection of 1 nmol augurin significantly increased plasma ACTH 20 and 60 minutes post injection ($p<0.01$ for both time points).

In the case of corticosterone, two-way ANOVA revealed that there was a significant main effect of dose ($F_{(3,45)} = 6.247; p<0.001$), no significant main effect of time ($F_{(1,45)} = 2.275; p=0.138$), and no significant interaction ($F_{(3,45)} = 1.129; p=0.347$). Post hoc analysis demonstrated that iPVN microinjection of 1 nmol augurin significantly increased plasma corticosterone 20 minutes post injection ($p<0.01$).

2.4.4 The effect of iPVN augurin on behaviour

There were no significant behavioural differences between iPVN augurin or vehicle injected rats. Injection of 0.3 nmol NMU significantly increased time spent grooming, and decreased time spent sleeping or feeding (Table 2.2).

2.4.5 The effect of augurin on CRH and AVP release from hypothalamic explants

Augurin caused a significant increase in CRH and AVP release from hypothalamic explants. Neither assay demonstrated cross-reactivity with aCSF, aCSF containing 1, 10, or 100 nM augurin, nor the high potassium aCSF. CRH and AVP release is presented in Table 2.3 and graphically as a percent of basal in Figure 2.7.

2.4.6 The effect of peripheral CRHR antagonism on augurin induced ACTH and corticosterone secretion

Pre-treatment with astressin completely blocked the increase in plasma ACTH and corticosterone 30 minutes post ICV injection of 5nmol augurin (Figure 2.8).
Figure 2.6: Effect of a single iPVN injection of augurin (0.1, 0.3, or 1 nmol) or vehicle in *ad libitum* fed male Wistar rats on plasma ACTH and corticosterone at 20 (A and C) and 60 (B and D) minutes post injection. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle, n = 5-8 per group per time point. Results are mean ± SEM.
Table 2.2: Effect of a single iPVN injection of augurin (1 nmol), NMU (0.3 nmol), or vehicle in *ad libitum* fed male rats on behaviour. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle, n = 6-8 per group. Data presented are median frequency and interquartile range.

<table>
<thead>
<tr>
<th>Time Period</th>
<th>Feeding</th>
<th>Rearing</th>
<th>Drinking</th>
<th>Locomotion</th>
<th>Grooming</th>
<th>Burrowing</th>
<th>Head down</th>
<th>Sleeping</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0-60 minutes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>NMU 0.3 nmol</td>
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<td>0 [0:0]</td>
<td>5 [3:6]</td>
<td>21 [18:24]**</td>
<td>1 [0:4]</td>
<td>1 [0:3]</td>
<td>0 [0:0]**</td>
</tr>
<tr>
<td><strong>61-120 minutes</strong></td>
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<td></td>
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<td></td>
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</tr>
<tr>
<td>Vehicle</td>
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<td>0 [0:2]</td>
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<tr>
<td>Augurin 1 nmol</td>
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<td>0 [0:0]</td>
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<td>0 [0:0]</td>
<td>2 [0:4]</td>
<td>32 [30:36]</td>
</tr>
<tr>
<td>NMU 0.3 nmol</td>
<td>0 [0:0]</td>
<td>0 [0:0]</td>
<td>0 [0:0]</td>
<td>0 [0:1]</td>
<td>10 [2:18]*</td>
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<td><strong>0-120 minutes</strong></td>
<td></td>
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</table>
Table 2.3: Effect of augurin (1, 10 or 100nM) on CRH and AVP release from hypothalamic explants. *p<0.05 vs. basal release. Results are mean ± SEM

<table>
<thead>
<tr>
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<th>Concentration of augurin in aCSF</th>
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<tbody>
<tr>
<td></td>
<td>1nM</td>
</tr>
<tr>
<td></td>
<td>Basal</td>
</tr>
<tr>
<td>CRH (pmol/explant)</td>
<td>1.3 ± 0.35</td>
</tr>
<tr>
<td>AVP (fmol/explant)</td>
<td>700.3 ± 151.82</td>
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</table>
Figure 2.7: Effect of augurin (1, 10, or 100nM) on CRH (A) and AVP (B) release from hypothalamic explants. Data presented as percent of basal release. *p< 0.05 vs. basal release, n = 13-15 hypothalami per group. Results are mean ± SEM.
Figure 2.8: Effect of a single ICV injection of augurin (5 nmol) or vehicle in ad libitum fed male Wistar rats pre-treated with 100nmol/kg astressin on plasma ACTH (A) and corticosterone (B) 30 minutes post ICV injection. **p<0.01, ***p<0.001 vs. vehicle, n = 7-9 per group. Results are mean ± SEM.
2.4.7 THE EFFECT OF PERIPHERALLY ADMINISTERED AUGURIN ON PLASMA HORMONE LEVELS

There were no significant differences in plasma corticosterone, LH, FSH, GH, TSH, PRL, fT₃, or fT₄ at 30 minutes post ip injection of 100nmol/kg augurin (Table 2.4).

2.4.8 THE EFFECT OF AUGURIN ON ACTH RELEASE FROM PITUITARY SEGMENTS

No significant change in ACTH release was seen following incubation of pituitary segments with 10, 100, or 1000nM augurin, compared with aCSF alone. Incubation with 100nM CRH resulted in a significant increase in ACTH release (ACTH ng/explant; vehicle 192.9 ± 23.1; CRH 100nM 296.7 ± 36.2, p<0.01) (Figure 2.9).
Table 2.4 Effect of a single ip injection of augurin (100 nmol/kg) or vehicle in *ad libitum* fed rats on plasma corticosterone, LH, FSH, GH, TSH, prolactin, free T3, and free T4 at 30 minutes post injection. Results are mean ± SEM, n = 7-9 per group.

<table>
<thead>
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<th>Vehicle</th>
<th>Augurin (100 nmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>Corticosterone (ng/ml)</td>
<td>34.5</td>
<td>12.6</td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>0.84</td>
<td>0.11</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
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</tr>
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<td>TSH (ng/ml)</td>
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</tr>
<tr>
<td>Free T₄ (ng/L)</td>
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<td>1.1</td>
</tr>
<tr>
<td>Free T₃ (pg/L)</td>
<td>10.8</td>
<td>1.5</td>
</tr>
<tr>
<td>PRL (ng/ml)</td>
<td>3.87</td>
<td>0.66</td>
</tr>
<tr>
<td>GH (ng/ml)</td>
<td>105.9</td>
<td>33.1</td>
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</table>
Figure 2.9: Effect of augurin (10, 100, or 1000nM) or CRH (100nM) on ACTH release from pituitary segments. **p<0.01, n = 17-19 per group. Results are mean ± SEM.
2.5 DISCUSSION

Augurin is a recently identified secreted peptide of unknown function. It is expressed in the CNS and endocrine organs. I therefore examined the effects of augurin on the hypothalamo-pituitary axes. Both ICV and iPVN injection of augurin elevated plasma ACTH and corticosterone compared with vehicle-injected controls, and augurin increased the release of CRH and AVP from hypothalamic explants. The rise in plasma ACTH and corticosterone following ICV injection of augurin was blocked by pre-treatment with a CRH receptor antagonist.

ICV injection of 5 nmol augurin increased plasma ACTH three fold 20 minutes after injection, versus vehicle injected controls. Plasma corticosterone also increased three fold over the same period. Interestingly, ICV injection of 0.6 or 1.7 nmol augurin caused a delayed rise in plasma ACTH, evident only at 60 minutes (Figure 2.5). The reason for this delay is unclear; it is possible that it represents the time taken for the peptide to diffuse to its site or sites of action. Alternatively, small differences in plasma ACTH at 20 minutes may have been masked by the stress of the experimental procedure. ICV injection of 5 nmol augurin had no effect on plasma LH, FSH, testosterone, TSH, fT₃, fT₄, PRL, or GH at 60 minutes post injection, compared with vehicle injected controls, suggesting the neuroendocrine effects of augurin are specific to the HPA axis.

CRH and AVP are the main regulators of ACTH levels; they act synergistically on the pituitary to stimulate ACTH secretion (Whitnall, 1993). Augurin increased the release of CRH and AVP from explanted hypothalami, and pre-treatment with the non-selective CRHR antagonist astressin blocked the rise in plasma ACTH and corticosterone caused by ICV injection of augurin. This suggests augurin stimulates the secretion of ACTH by stimulating the release of CRH and AVP from hypothalamic PCNs, although the relative contribution of AVP cannot be determined from the antagonist study as CRH is necessary for AVP stimulated ACTH release (Whitnall, 1993).
CRH neurons in the PVN are the major conduit for endocrine, autonomic, and behavioural responses to stress (Herman, 1997). The PVN contains a subpopulation of hypophysiotrophic neurons that constitute the primary source of CRH and AVP (Hashimoto et al., 1982, Sawchenko et al., 1993). I therefore examined the effects of injecting augurin directly into the PVN on the HPA axis. Intranuclear injection of lower doses minimises any nonspecific side effects of peptide administration that may occur following ICV injection (Dhillo et al., 2002).

Microinjection of 1 nmol augurin iPVN significantly increased plasma ACTH and corticosterone; a much lower dose than required for a similar effect ICV. This could indicate receptors for augurin are found within or close to the PVN. It is, however, possible that lower concentrations of augurin could diffuse into adjacent nuclei, and I cannot therefore discount administration of augurin to other hypothalamic areas having a similar effect. There were no significant effects on ACTH or corticosterone following iPVN injection of 0.1 or 0.3 nmol augurin. There were no significant differences in behaviour in the two hours following iPVN microinjection of 1 nmol augurin, compared with vehicle injected controls. In particular, no adverse behaviours such as hunched posture or tremors were observed in the augurin group, indicating that nonspecific activation of the HPA axis secondary to a noxious effect is unlikely (Deacon, 2006, Kalueff et al., 2007).

Basal and augurin-stimulated levels of ACTH and corticosterone varied somewhat between studies. In study 2.4.2 basal ACTH is 41.1 ± 9.6pg/ml at 20 min and 14.9 ± 2.1pg/ml at 60 min, in study 2.4.3 the values are 72.8 ± 18.1pg/ml and 56.7 ± 16.6pg/ml, while in study 2.4.6 basal ACTH is 16.3 ± 2.6pg/ml at 30 min. Unfortunately it was necessary to measure ACTH levels using a different assay kit for study 2.4.6 because the previously used assay kit was no longer being manufactured. For studies 2.4.2 and 2.4.3 plasma ACTH was measured by immunoradiometric assay manufactured by Euro-Diagnostica B.V. whereas for study 2.4.6, plasma ACTH was measured by immunoradiometric assay manufactured by BioSource Europe S.A. It is common to see variations in levels measured by different assay kits and this may account for the differences between studies (Bieling et al., 1988).
Peripheral administration of augurin at doses of 100nmol/kg had no effect on plasma corticosterone at 30 minutes post injection. This dose is relatively high; ip injection of 4.2 nmol/kg of CRH causes a significant rise in plasma ACTH (Watanabe et al., 1991). Furthermore, incubation of pituitary segments with augurin at concentrations of up to 1000nM had no effect on ACTH release. It therefore appears unlikely that peripheral augurin acts directly on the pituitary to release ACTH, unless it is released into the circulation at very high concentrations. To date, the presence of augurin in the circulation has not been investigated.

The data presented in this chapter indicate hypothalamic injection of augurin (preproaugurin 71-148) stimulates the HPA axis. It is also possible shorter fragments of augurin will be biologically active. The N-terminal segment of augurin adjacent to the Lys69/Arg70 dibasic cleavage site has substantially higher interspecies conservation than the rest of the peptide (Figure 1.1). I hypothesise that the increase in conservation implies the N-terminal segment is important for biological activity. Identifying the endogenous forms of augurin and determining the minimum biologically active sequence would provide a basis for developing structural analogues acting as antagonists or agonists of the augurin system.

Hypophysiotrophic CRH neurons within the PVN control glucocorticoid secretion and are the focal point for neurocircuitry projecting from hypothalamic, brainstem and forebrain nuclei conveying information regarding stressors (Ulrich-Lai and Herman, 2009). In response to stressors, a surge in plasma glucocorticoids is initiated by the activity of stress responsive neurocircuitry directly or indirect on PVN CRH neurons (Ulrich-Lai and Herman, 2009). The studies in this chapter suggest that hypothalamic injection of augurin stimulates the HPA axis in rats, and that this effect is likely mediated by the release of CRH from neurons in the PVN. Nevertheless, these studies show a pharmacological rather than physiological effect. Two studies published during the preparation of this thesis indicate that endogenously preproaugurin-like immunoreactivity (LI) is present within neurons of the CNS, suggesting augurin could play a physiological role in the HPA axis (Roberton et al., 2009, Kujuro et al., 2010).
Early studies of hypocretin (HCRT) illustrate the accuracy with which neuropeptide function can be inferred from its neuroanatomy. Based on the distribution of HCRT neurons, a role in the regulation of food intake, blood pressure, body temperature, and the sleep-waking cycle were hypothesised, all of which have since been confirmed (Peyron et al., 1998, Tsujino and Sakurai, 2009, Scammell and Winrow, 2011). A similarly detailed analysis of the distribution of augurin neurons, their projections, and their neurochemical identity might indicate possible physiological roles within the CNS. Furthermore, differences in the distribution or intensity of preproaugurin-LI or mRNA in response to a stressor, or following suppression or elevation of circulating glucocorticoids would suggest a physiological role for augurin in the regulation of the HPA axis.

Multiple neuropeptides including HCRT, neuropeptide Y, cocaine-and amphetamine-regulated transcript protein, glucagon-like peptide-1, melanocortin-concentrating hormone, and PrRP are all released into the PVN and peri-PVN area where they contribute to the PVN peptidergic microenvironment (Rinaman, 1999, Stanley et al., 2001, Lin et al., 2002, Mihaly et al., 2002, Sarkar and Lechan, 2003, Swaab, 2004, Pissios et al., 2006). Neuropeptides released in this manner regulate HPA axis activation, and particularly the intra-hypothalamic processing of stressful stimuli (Herman et al., 2002). The extent of intra-hypothalamic processing depends on the precise nature of the ‘stressful’ stimulus and ensures that stimuli are interpreted in the context of cardiovascular, nutritional, immune, and reproductive status, in order that the appropriate response ensues (Herman et al., 2002, Swaab, 2004). It is possible augurin my have a similar role to the aforementioned neuropeptides. Further characterisation of the neuroanatomy, identification of receptors, and the development of specific antagonists are important to establish whether augurin has a physiological role in the regulation of the HPA axis and whether it may represent a novel target for the treatment of stress related diseases.
3 The effects of Augurin on food intake
3.1 INTRODUCTION

The rise in obesity and its comorbid associations is one of the most pressing issues facing the medical and scientific community. The prevalence of obesity amongst adults worldwide has risen by 75% since 1980, and has been rising even faster in children (Bray, 1998, Flegal, 2005). The threat to public health services is unparalleled and obesity has become a socioeconomic time bomb because of its well known cardiovascular, metabolic, reproductive, neoplastic, musculoskeletal, and psychosocial complications (Ogden et al., 2006, Welborn and Dhaliwal, 2011).

Despite the alarming figures, the increasing incidence of obesity is not reflected by a proportionate increase in body weight (Friedman, 2003). Body weight is kept remarkably constant during most of adult human life, in spite of large variations in daily energy intake and expenditure (Jéquier and Tappy, 1999). The regulation of body weight is incredibly precise, typically food intake matches expenditure to within 0.17% over a decade (Harris, 1990). This degree of precision implies the active regulation or defence of energy intake and expenditure. Indeed, evidence suggests humans and rodents have a complex “adipostatic” system that defends body weight in the face of positive or negative energy balance (Bandini et al., 1989, Keesey and Corbett, 1990).

3.1.1 THE HYPOTHALAMUS AND THE REGULATION OF FOOD INTAKE

Food intake is controlled by a complex and diverse neural network, within which the hypothalamus plays a critical and largely non-redundant role. The hypothalamus integrates both long- and short-term signals about energy flux, and orchestrates the compensatory responses to positive or negative energy balance (Morton et al., 2006). Although simplistic, the melanocortin model, in which orexigenic agouti-related protein (AgRP) and anorexigenic proopiomelanocortin (POMC) neurons within the hypothalamic arcuate nucleus (ARC) take centre stage, is the most widely accepted framework for explaining hypothalamic regulation of food intake.
Figure 3.1: The melanocortin model. Activation of AgRP neurons promotes food intake and reduces energy expenditure, whereas activation of POMC neurons reduces food intake and increases energy expenditure. Insulin and leptin inhibit AgRP neurons and stimulate POMC neurons, thereby reducing food intake, whereas ghrelin activates AgRP neurons and thereby increases food intake.

Abbreviations: AgRP, agouti-related protein; CART, cocaine-and amphetamine-regulated transcript protein; Ghsr, growth hormone secretagogue receptor; Lepr, leptin receptor; Mc3r/Mc4r, melanocortin 3/4 receptor; NPY, neuropeptide Y; POMC, proopiomelanocortin; Y1r, Y1 receptor. Adapted from (Barsh and Schwartz, 2002).
3.1.1.1 The melanocortin model

The hypothalamic melanocortin system comprises two opposing populations of ‘first order’ peptidergic neurons in the ARC (Figure 3.1) (Cone, 2005). One population express POMC and their activity suppresses food intake (POMC neurons), while the other subset co-express AgRP and neuropeptide Y (NPY), and their activity stimulates food intake (AgRP neurons) (Cowley et al., 2001, Aponte et al., 2011). Within the ARC, POMC neurons are located lateral to AgRP neurons, and in rodents roughly 50% of POMC neurons co-express cocaine-and amphetamine-regulated transcript protein (CART) (Elias et al., 1998, Hahn et al., 1998). In humans, however, CART co-localises with AgRP, and is absent from POMC neurons (Menyhert et al., 2007).

3.1.1.1.1 POMC neurons

The majority of POMC neurons in the CNS are located in the hypothalamus and nucleus of the tractus solitarius (Civelli et al., 1982, Joseph et al., 1983). Within the hypothalamus, POMC is expressed exclusively in the ARC (Jacobowitz and O'donohue, 1978, Gee et al., 1983). The melanocortins α- and γ-melanocyte-stimulating hormone (MSH) are the major products of POMC processing in the rodent central nervous system (CNS), and both are potently anorexigenic when injected ICV (Panskepp et al., 1976, Jacobowitz and O'donohue, 1978, Emeson and Eipper, 1986). Subsets of ARC POMC neurons also synthesise and release the neurotransmitters γ-amino butyric acid (GABA) or glutamate (Horvath et al., 1997, Hentges et al., 2004, Hentges et al., 2009).

Of the five melanocortin receptors, subtype 4 (MC4R), and to a lesser extent subtype 3 (MC3R), are important for the regulation of feeding (Cone, 2006). The CNS distribution of MC4Rs is broader than that of MC3Rs, but both receptors highly expressed in hypothalamic nuclei, the paraventricular nucleus (PVN), lateral hypothalamic area (LHA), and ventromedial nucleus (VMN) (Roselli-Rehfuss et al., 1993, Mountjoy et al., 1994). The orexigenic peptide AgRP acts as a competitive antagonist at both receptors, and has also been shown to inhibit constitutive activity in the MC4R (Ollmann et al., 1997, Nijenhuis et al., 2001). In the rodent
hypothalamus, α-MSH is believed to be the main endogenous agonist of the MC3R and MC4R (Panskepp et al., 1976, Adan et al., 1994, Oosterom et al., 1998).

Studies of genetic defects that impair α-MSH signalling clearly demonstrate the critical role of the melanocortin system in energy homeostasis. In both humans and mice, homozygous loss-of-function mutations in the gene encoding the α-MSH precursor (Pomc), the enzymes required for post translational processing into α-MSH (Pcsk1, Cpe), it’s receptor (Mc4r), or several downstream targets of the melanocortin system (Sim1, Bdnf, and Ntrk2), each cause marked hyperphagia and adiposity (Cool et al., 1997, Huszar et al., 1997, Jackson et al., 1997, Krude et al., 1998, Vaisse et al., 1998, Yeo et al., 1998, Farooqi et al., 2000, Holder et al., 2000, Xu et al., 2003, Yeo et al., 2004, Han et al., 2008). It is noteworthy that heterozygous mutations result in an intermediate phenotype, suggesting there is little redundancy in anorectic tone from the melanocortin system (Huszar et al., 1997, Jackson et al., 1997, Farooqi et al., 2000, Holder et al., 2004, Farooqi et al., 2006).

3.1.1.1.2 AgRP neurons

AgRP neurons in the ARC co-express NPY, a potent orexigen (Stanley and Leibowitz, 1985, Hahn et al., 1998). NPY is a member of the PP-fold peptide family, all of which bind and activate members of the Y-receptor family (Lin et al., 2004). NPY is abundant throughout the CNS, and particularly within the hypothalamus (Adrian et al., 1983, Allen et al., 1983). Of the five known Y-receptors, evidence suggests Y1R and Y5R mediate the orexigenic effect of NPY, and both receptors are expressed throughout the hypothalamus (Gerald et al., 1996, Kanatani et al., 1996, Parker and Herzog, 1999).

Although hypothalamic injection of NPY stimulates feeding, blockade of NPY signalling has minor effects, if any, on energy homeostasis, and its precise physiological role in regulating feeding is therefore unclear. Mice with homozygous loss-of-function mutations in Npy, Npy1r, or Npy5r have normal food intake and body weight, or, paradoxically, a slight increase in adiposity and mild hyperphagia (Erickson et al., 1996a, Marsh et al., 1998, Pedrazzini et al., 1998, Ishihara et al., 2006). Genetic or pharmacological blockade of NPY signalling in adults has a similarly innocuous effect, suggesting the mild phenotype in Npy-, Npy1r-, or
Npy5r- knockout (KO) mice is not a result of developmental compensation (Ste Marie et al., 2005, Erondu et al., 2006). Nevertheless, ARC NPY expression is elevated during negative energy balance and in most genetic models of obesity (Sanacora et al., 1990, Kalra et al., 1991, Wilding et al., 1993, Kesterson et al., 1997, Schwartz et al., 1998). Furthermore, loss of Npy attenuates the hyperphagia caused by leptin deficiency and fasting, suggesting a specific role for NPY in the acute response to starvation (Erickson et al., 1996b, Segal-Lieberman et al., 2003).

A series of elegant experiments over the last decade have shed considerable light on the role of AgRP neurons in energy homeostasis, and demonstrate that, in contrast to POMC neurons, the AgRP neurons themselves, rather than their characteristic neuropeptides, play an essential role in feeding behaviour. Firstly, transgenic mouse models enabling the ablation or depolarisation of AgRP neurons demonstrate their activity is both necessary and sufficient for feeding (Gropp et al., 2005, Luquet et al., 2005, Aponte et al., 2011, Krashes et al., 2011). Secondly, although AgRP neurons project to and hyperpolarise POMC neurons, and AgRP antagonises MC3/4Rs, the acute stimulation of feeding by AgRP neurons appears to be independent of melanocortinergic signalling (Horvath et al., 1992, Cowley et al., 2001, Cowley et al., 2003, Tong et al., 2008, Wu et al., 2008, Aponte et al., 2011). And thirdly, that the release of GABA by AgRP neurons is necessary for energy homeostasis, while null mutations in Npy, Agrp, or both have little effect on appetite (Qian et al., 2002, Wortley et al., 2005, Phillips and Palmiter, 2008, Tong et al., 2008, Wu et al., 2009).

3.1.1.2 The regulation of the melanocortin system by the periphery

The ARC integrates a number of peripheral signals as part of its role in regulating energy homeostasis. Peripheral signals can be classified into a short-term feedback signals that regulate the initiation and termination of meals depending on the contents of the gastrointestinal tract, and long-term feedback signals of energy stores such as leptin and insulin (Morton et al., 2006, Suzuki et al., 2010).

3.1.1.2.1 Long-term feedback signals

Leptin is the archetypal adipostatic hormone; mathematical modelling of both food intake and energy expenditure over time as a function of plasma leptin alone matches experimental data
from numerous in vivo studies with remarkable accuracy (Tam et al., 2009). Leptin is secreted by primarily by white adipocytes and circulates in approximately direct proportion to adipose mass (Frederich et al., 1995, Considine et al., 1996). During acute starvation, however, plasma leptin levels decrease excessively relative to the mass of adipose lost (Weigle et al., 1997). Leptin insufficiency profoundly affects whole body physiology to promote positive energy balance at the expense of reproductive, immune, and anabolic processes (Friedman and Halaas, 1998). Loss-of-function mutations in the gene encoding leptin or the gene encoding the leptin receptor cause extreme hyperphagia and adiposity in both rodents (Lepob/ob and Leprdb/db mice respectively) and humans (Zhang et al., 1994, Lee et al., 1996, Phillips et al., 1996, Montague et al., 1997, Clement et al., 1998). Circulating leptin acts in the CNS to promote negative energy balance by suppressing food intake and increasing energy expenditure (Cohen et al., 2001). Leptin traverses the blood brain barrier (BBB) via a specific transport system, and has direct effects on disparate neuronal targets within the hypothalamus, brainstem, hippocampus, cortex, and limbic system (Balthasar et al., 2004, Dhillon et al., 2006, Leinninger et al., 2009, Yadav et al., 2009, Hayes et al., 2010). Administration of leptin to fasted mice reverses many of the neuroendocrine consequences of starvation, suggesting that the physiological role of leptin is to inhibit the starvation response (Ahima et al., 1996).

Insulin also meets the criteria for an adipostatic hormone. Secreted by pancreatic β-cells, plasma insulin correlates positively with adiposity (Polonsky et al., 1988). Insulin crosses the BBB via receptor-mediated transport process, and acts in the hypothalamus to promote negative energy balance (Bagdade et al., 1967, Schwartz et al., 1991, Baura et al., 1993). Blockade of hypothalamic insulin signalling by injection of anti-insulin antibodies, by neuron-specific insulin receptors (IRS) knockout, or by antisense mediated IRS knockdown, results in hyperphagia and increased adiposity (Strubbe and Mein, 1977, Brüning et al., 2000, Obici et al., 2002). These data support a physiological role for hypothalamic insulin signalling in the regulation of energy homeostasis.
3.1.1.2.2  Short-term feedback signals from the gut

The ingestion of food gives rise to mechanical and chemical stimulation of the gastrointestinal tract (GIT) and, in turn, to the secretion of numerous gut hormones. These neurohormonal signals are primarily implicated in the regulation of meal initiation and termination. Gut hormones are produced from several different regions of the GIT, and include cholecystokinin (CCK), peptide YY$_{3-36}$ (PYY$_{3-36}$), glucagon-like peptide-1, oxyntomodulin, and pancreatic polypeptide (PP) (Suzuki et al., 2010). Plasma gut hormone levels rise rapidly after food is ingested, and in general they act to inhibit further food intake (Suzuki et al., 2010). Gut hormones signal to the CNS either indirectly via the vagus nerve and circumventricular organs, or cross the BBB and act directly on brainstem, hypothalamic, and limbic areas (Cummings and Overduin, 2007, Kastin and Pan, 2008, Price et al., 2008).

Ghrelin is the only known gut hormone which is orexigenic (Kojima et al., 1999). It is secreted primarily by the stomach and has a role in both long- and short-term energy homeostasis. Circulating levels of the ghrelin increase before each meal and decline rapidly afterwards, implicating it in meal initiation (Cummings et al., 2001, Tschop et al., 2001a). In addition, plasma ghrelin is inversely proportional to body mass index, and is altered by changes in body weight (Tschop et al., 2001b, Purnell et al., 2007). In rodents, central or peripheral injection of ghrelin increases food intake and adiposity, while peripheral injection of a ghrelin receptor antagonist has the opposite effect (Tschop et al., 2000, Esler et al., 2007).

3.1.1.3  The regulation of the melanocortin system by the CNS

In addition to neurohormonal signals from the periphery, the ARC reciprocally innervates structures throughout the CNS that play a role in feeding. Efferent projections from the brainstem, LHA, and VMN to the melanocortin system are amongst the most important and best-characterised CNS pathways.

3.1.1.3.1  Brainstem

Within the caudal brainstem, the dorsal vagal complex (DVC) is crucial in the interpretation and relaying of peripheral signals of energy balance to the hypothalamus. Vagal nerve endings are found throughout the oesophagus, stomach, liver, and both small and large intestines, the
majority of which are afferent sensory fibres (Powley et al., 2005). The vagus conveys information from mechano- and chemoreceptors activated by the presence of nutrients in the stomach and the intestine to the DVC (Gao and Horvath, 2008). In addition, vagal nerves express receptors for several gut hormones, and the effects of CCK, ghrelin, GLP-1, and PYY$_{3-36}$ on food intake appear to be mediated in part via the vagus nerve (Abbott et al., 2005, Le Roux et al., 2005, Berthoud, 2008).

The DVC consists of the dorsal motor nucleus of vagus (DMX), area postrema (AP), and the nucleus of the tractus solitarius (NTS). Specific subnuclei of the NTS are the first central neurons to process ingestion-related vagal afferent signals (Sawchenko, 1983). The NTS receives and integrates information about energy status from numerous sources as well as the vagus. NTS neurons also express receptors for, and alter their firing in response to, peripheral hormones such as ghrelin and leptin (Mercer et al., 1998, Grill et al., 2002, Ellacott et al., 2006, Hisadome et al., 2010, Cui et al., 2011). Additional blood-borne signals related to nutrient status are relayed via dense projections from the AP, a sensory circumventricular organ (CVO) immediately adjacent to the NTS (Berthoud et al., 2006, Price et al., 2008, Wang et al., 2008). Efferents from the NTS project caudally to the ascending visceral afferent pathway, which includes neurons located in the parabrachial nucleus, ARC, PVN, ventral forebrain, thalamus, and insular cortex (Berthoud et al., 2006, Grill, 2010).

3.1.1.3.2 The VMN

Classic lesioning studies in the 1940s and 1950s fostered the ‘dual centre’ model, whereby appetite was regulated by the LHA feeding and VMN satiety centres (Hetherington and Ranson, 1940, Anand and Brobeck, 1951, Stellar, 1954). However, the role of the VMN was subsequently questioned because the process of lesioning also damaged nearby nuclei and traversing neuronal fibres (Gold, 1973, Parkinson and Weingarten, 1990). More recent studies of the molecular biology, neurochemistry, and neurophysiology of VMN neurons have in large part validated the conclusions drawn from the early lesions studies, and suggest the VMN is indeed a satiety centre.
Within the CNS, the transcription factor steroidogenic factor-1 (SF1) is expressed exclusively by VMN neurons (Ikeda et al., 1995). Deletion of SF1 in mice results in agenesis of the VMN and leads to hyperphagia and increased adiposity (Dellovade et al., 2000, Majdic et al., 2002). Leptin has been shown to directly activate SF1 neurons in the VMN, and deletion of Lepr from SF1 lineage neurons causes hyperphagia and increased adiposity (Dhillon et al., 2006). Although these studies demonstrate an important role for the VMN in suppressing food intake, how the VMN regulates output from the ARC is poorly understood. The recent identification of neuronal projections from the VMN to the ARC implies the VMN might promote negative energy balance by directly regulating the activity of melanocortin system neurons (Sternson et al., 2005). Using a combination of laser scanning photo-stimulation and slice electrophysiology, Sternson et al. demonstrated neurons within the lateral and medial VMN project to the ARC where they predominantly synapse POMC neurons. Furthermore, while VMN-to-ARC inhibitory projections were essentially absent, VMN-to-POMC neuron projections were almost universally excitatory, and were markedly reduced by fasting (Sternson et al., 2005). These data suggest VMN neurons regulate energy homeostasis via direct excitatory stimulation of POMC neurons.

3.1.1.3.3 The LHA

Within the LHA, two intermingled but distinct cell populations express the orexigenic neuropeptides melanin-concentrating hormone (MCH) and the hypocretins (HCRT) (Qu et al., 1996, Broberger et al., 1998, Sakurai et al., 1998). Although primarily implicated in arousal, MCH and HCRT neurons play a prominent role in the coordinated behavioural responses to negative energy balance (Van Den Pol et al., 2004, Williams et al., 2008, Silva et al., 2009). MCH- and HCRT-LI fibres directly synapse neurons throughout the hypothalamus and CNS, including the majority of structures involved in energy homeostasis (Bittencourt et al., 1992, Peyron et al., 1998, Yamanaka et al., 2003). Despite their similarities MCH and HCRT neurons interact with the melanocortin system essentially independent of each other. HCRT neurons are activated rapidly by fasting, and project heavily to AgRP, but not POMC, neurons within the ARC (Horvath et al., 1999, Diano et al., 2003). Furthermore, their activity and synaptic profiles
on AgRP neurons is regulated by peripheral feedback signals such as ghrelin, leptin, and insulin (Toshinai et al., 2003, Horvath and Gao, 2005, Louis et al., 2010). Feeding in response to ICV HCRT is dependant on Y1 and Y5 receptors, suggesting the orexigenic effects of HCRT are mediated by direct stimulation of AgRP neurons (Jain et al., 2000, Yamanaka et al., 2000). In contrast, MCH-LI projections in the ARC are relatively sparse, but MCH increases food intake by functionally antagonising the effects of α-MSH at ‘second order’ neurons (Abbott et al., 2003, Crozier et al., 2010). The difference in phenotype between Hcrt\(^{-/-}\) and Mch\(^{-/-}\) mice illustrates the distinct roles of MCH and HCRT neurons in energy homeostasis. Hcrt\(^{-/-}\) mice are overweight and hypophagic, while Mch\(^{-/-}\) mice are lean and hypophagic (Shimada et al., 1998, Chemelli et al., 1999, Hara et al., 2001, Willie et al., 2003).

3.1.1.4 Downstream targets of the melanocortin system

Neuronal and hormonal signals converge on POMC and AgRP neurons in the ARC, which in turn project to ‘second order’ neurons throughout the CNS (Figure 3.1). Within the prosencephalon, the distribution of γ-MSH and AgRP fibres overlap, however, with the exception of the parabrachial and trigeminal nuclei, γ-MSH fibres innervate the mesencephalon and rhombencephalon in the absence of AgRP (Bagnol et al., 1999, Haskell-Luevano et al., 1999). POMC and AgRP neurons also project extensively within the hypothalamus. Most nuclei contain some POMC and AgRP fibres, but the PVN receives by far the densest innervation and is recognised as a key downstream target of the melanocortin system (Elias et al., 1998, Bagnol et al., 1999, Haskell-Luevano et al., 1999, Atasoy et al., 2008).

3.1.1.5 The PVN

Evidence indicates the PVN is a critical node in neural circuitry that coordinates neuroendocrine, autonomic, and behavioural responses underlying energy balance. Firstly, the PVN receives peptidergic and non-peptidergic afferents from diverse regions of the CNS that regulate feeding, including prominent projections from hypothalamic and brainstem nuclei (Swanson and Sawchenko, 1983, Sawchenko et al., 1985, Elmquist et al., 1998, Patterson et al., 2011). Secondly, PVN neurons express receptors for the majority of neurotransmitters, neuropeptides, and hormones that regulate feeding, including GABA, glutamate, leptin, insulin,

MC4R expressing PVN neurons are synapsed by both POMC and AgRP neurons, which stimulate and inhibit their activity respectively (Cowley et al., 1999, Cowley et al., 2003, Ghamari-Langroudi et al., 2011). Rodent models in which ARC-PVN signalling is disrupted demonstrate the importance of the PVN in for the regulation of energy homeostasis by the melanocortin system. Discrete lesions of the PVN cause marked hyperphagia and adiposity, as do loss-of-function mutations in the Sim1 gene, a haploinsufficient transcription factor necessary for the normal development of the PVN (Leibowitz et al., 1981, King et al., 1989, Holder et al., 2000, Michaud et al., 2001, Kublaoui et al., 2006). Furthermore, overexpression of AgRP in the PVN causes hyperphagia and adiposity, while restoration of MC4R expression in Sim1 lineage neurons is sufficient to normalise food intake in MC4R−/− mice (Balthasar et al., 2005, De Backer et al., 2010). These data demonstrate the importance of the PVN in the regulation of energy homeostasis, and imply that net autonomous output from the PVN is anorexigenic and maintained under normal conditions by direct melanocortinergic tone.

3.1.1.5.1 The regulation of food intake by the PVN

There are three predominant efferent pathways from the PVN; endocrine via the pituitary; autonomic via direct projections to preganglionic neurons in the spinal cord (sympathetic) and the vagus (parasympathetic); and behavioural via projections to the brainstem and LHA (Saper et al., 1976, Thompson and Swanson, 2003, Geerling et al., 2010). Evidence suggests a subset of non-hypophysiotrophic CRH and OXT neurons, which form part of the behavioural output from the PVN, are important second order targets for both melanocortinergic and non-melanocortinergic pathways regulating appetite.
3.1.1.5.2 CRH

A significant number of MC4R expressing neurons in the medial parvocellular PVN also express functional leptin receptors and the anorexigenic neuropeptides CRH and/or OXT (Hakansson et al., 1998, Lu et al., 2003, Ghamari-Langroudi et al., 2011). Hypothalamic CRHR agonism consistently causes a reduction of food intake and body weight, with greater attribution to the CRHR2 subtype (Morley and Levine, 1982, Krahn et al., 1988, Gardner et al., 1998, Cullen et al., 2001, Stengel et al., 2009). A physiological role for the CRH system is supported by the apposite variation of CRH and CRHR2 mRNA, and of CRH peptide, in response to exogenous leptin, food deprivation, or overfeeding (Schwartz et al., 1996, Gardner et al., 1998, Makino et al., 1998, Cullen et al., 2001). Furthermore, ICV co-injection of a CRHR antagonist attenuates the anorexigenic effects of α-MSH and leptin (Gardner et al., 1998, Uehara et al., 1998, Lu et al., 2003).

3.1.1.5.3 OXT

OXT neurons in the PVN are necessary for energy homeostasis, and are important downstream targets of leptin’s effects on food intake. Firstly, mouse models in which CNS oxytocin signalling is disrupted, such as or loss-of-function mutation of the gene encoding oxytocin (Oxt), its receptor (Oxtr), or Sim1 or Syt4 which regulate the expression and release of OXT respectively, all result in hyperphagia (Kublaoui et al., 2008, Takayanagi et al., 2008, Camerino, 2009, Tolson et al., 2010, Zhang et al., 2011). Secondly, PVN OXT neurons express c-fos, a marker of neuronal activation, following ICV injection of leptin, and leptin’s effects on food intake are attenuated by ICV co-injection of an OXTR antagonist (Blevins et al., 2004). PVN OXT neurons project heavily to hindbrain nuclei that regulate satiety, the NTS and parabrachial nucleus in particular, where it is suggested OXT suppresses feed intake by enhancing the sensitivity to satiety signals from the GIT (Blevins et al., 2003, Blevins et al., 2004, Geerling et al., 2010, Olszewski et al., 2010).
3.2 **HYPOTHESIS AND AIMS**

Studies in chapter 2 suggest that ICV or iPVN injection of augurin stimulates the hypothalamo-pituitary-adrenal (HPA) axis in rats, and that this effect is likely mediated by the release of CRH from neurons in the PVN. As CRH is potently anorexigenic, I hypothesise that hypothalamic injection of augurin will acutely suppress food intake in male Wistar rats by stimulating the central release of CRH.

3.2.1 **AIMS**

1. Investigate the effects of augurin on food intake following ICV injection.
2. Investigate the effects of augurin on food intake following iPVN injection.
3. Investigate whether the effects of augurin on food intake are mediated by CRH.
3.3 METHODS

3.3.1 PEPTIDES

A synthetic fragment of human augurin corresponding to amino acids 71 to 148 of preproaugurin protein was synthesised and purified by Bachem UK Ltd. as described in 2.3.1. Angiotensin II (AII) and the non-specific CRHR antagonist α-helical CRH-(9-41) (α-hCRH) were purchased from Bachem UK Ltd. Augurin was prepared for injection as described in 2.3.1. Prior to injection, α-hCRH was dissolved into a minimal quantity of sterile water, and then diluted into vehicle.

3.3.2 ANIMALS

Male Wistar rats (specific pathogen free; Charles River), weighing 250-300 g were maintained in individual cages under controlled temperature (21-23 C) and light (12 h light, 12 h dark cycle; lights on at 0700 h) conditions with ad libitum access to food (RM1 diet, SDS Ltd.) and water. Animal procedures were approved under the British Home Office Animals Scientific Procedures Act 1986 (Project Licence 70/6402).

3.3.3 INTRACEREBROVENTRICULAR CANNULATION AND INJECTIONS

ICV cannulation and injections were carried out as described in 2.3.3.

3.3.4 INTRAPARAVENTRICULAR CANNULATION AND INJECTIONS

Unilateral iPVN cannulation, iPVN injections, and histological verification of cannula placement were carried out as described in 2.3.4.

3.3.5 THE EFFECT OF ICV AUGURIN INJECTION DURING THE EARLY DARK PHASE ON FOOD INTAKE

The effect of augurin on dark phase food intake was investigated first. Ad libitum fed rats received a single ICV injection of vehicle, 0.3, 1 or 3 nmol augurin in the early dark phase (1900-2000 hours, n = 8-10 per group). Animals were returned to their own cages with a preweighed quantity of chow. Food intake was measured 1, 2, 4, 8 and 24 hours after injection. Body-weight was measured directly before and 24 hours after injection.
3.3.6 THE EFFECT OF ICV AUGURIN INJECTION DURING THE EARLY LIGHT PHASE ON FOOD INTAKE FOLLOWING A FAST

As the first study showed a mild reduction in food intake the effect of augurin on food intake following a fast was investigated. Rats had their chow removed 24 hours prior to the study. Each rat received a single ICV injection of vehicle, 1, 3 or 10 nmol augurin in the early light phase (0900-1000 hours, n = 5-10 per group), and was immediately returned to its own cage with a preweighed quantity of chow. Food intake was measured 1, 2, 4, 8 and 24 hours after injection. Body-weight was measured directly before and 24 hours after injection.

3.3.7 THE EFFECT OF iPVN AUGURIN INJECTION DURING THE EARLY DARK PHASE ON FOOD INTAKE

This study was performed twice, each time in a different batch of iPVN cannulated rats. During study one, *ad libitum* fed rats received a single injection of vehicle or augurin (0.1, 0.3 or 1 nmol) in the early dark phase (1900-2000 hours, n = 5-6 per group). Rats were returned to their own cages with a preweighed quantity of chow. Food intake was measured 1, 2, 4, 8 and 24 hours after injection. Body-weight was measured directly before and 24 hours after injection.

The second study was carried out in order to confirm the findings of the first study, and to investigate a higher range of doses. During study two, rats received a single injection of vehicle or augurin (0.25, 0.5, 1 or 2 nmol, n = 8-15 per group). Rats were returned to their own cages with a preweighed quantity of chow. Food intake was measured at 1, 2, 4, 8 and 24 hours after injection. Body-weight was measured directly before and 24 hours after injection.

3.3.8 THE EFFECT OF iPVN AUGURIN INJECTION DURING THE EARLY LIGHT PHASE ON FOOD INTAKE

This study was performed twice, each time in a different batch of iPVN cannulated rats. Both studies were performed as described in 3.3.7, except that rats were injected during the early light phase (0900-1000 hours) rather than the early dark phase.
3.3.9 The effect of iPVN injection of augurin and α-hCRH during the early dark phase on food intake

Ad libitum fed rats received a single iPVN injection of vehicle, 1 nmol augurin, α-hCRH (0.3µg or 1µg), or 1 nmol augurin combined with α-hCRH (0.3µg or 1µg), in the early light phase (1900-2000 hours, n = 8-13 per group). These doses of α-hCRH have previously been shown to potentiate the orexigenic effect of iPVN injection on NPY (Heinrichs et al., 1993). Animals were returned to their own cages with a preweighed quantity of chow. Food intake was measured 1, 2, 4, 8 and 24 hours after injection. Body-weight was measured directly before and 24 hours after injection.

3.3.10 Statistics

All food intake data are presented as mean ± SEM. All data were analysed using One Way ANOVA followed by post hoc Dunnett’s test, with the exception of the antagonist study, which was analysed using One Way ANOVA followed by post hoc Tukey’s Multiple Comparison Test, to allow for comparison of all groups (Prism Graph Pad 5).
3.4 RESULTS

3.4.1 The effect of ICV Augurin injection on food intake

Augurin had no significant effects on food intake (FI) between any time points following ICV injection at the start of the dark phase. It appeared to have a mild anorexigenic effect 1 hour post injection (Figure 3.2A) (FI/g; vehicle 2.6 ± 0.5; augurin 0.3 nmol 1.8 ± 0.3, p=0.37; 1 nmol 1.7 ± 0.3, p=0.25; 3 nmol 2.3 ± 0.4, p=0.87).

The effect of higher doses of augurin on food intake was then investigated in fasted rats to clarify the robustness and potency of any anorexigenic effect. Again, only a mild, non-significant inhibition of food intake over the first hour was seen (Figure 3.2B) (FI/g; vehicle 6.7 ± 0.6; augurin 1 nmol 6.2 ± 0.8, p=0.93; 3 nmol 5.5 ± 0.6, p=0.54; 10 nmol 6.0 ± 0.8, p=0.91). There were no significant differences in food intake between any time points. There were no significant differences in body weight change between groups over the 24 hour duration of either study.

Figure 3.2: The effect of ICV augurin on food intake A) The effect of vehicle or augurin (0.3, 1 or 3 nmol) injected at the start of the dark phase ICV into male ad libitum fed Wistar rats on food intake over the first hour. B) The effect of ICV injection of vehicle or augurin (1, 3 or 10 nmol) at the start of the light phase into 24 h fasted male Wistar rats on food intake over the first hour.
3.4.2 The effect of iPVN augurin injection during the early dark phase on food intake

3.4.2.1 Dark phase study one

Microinjection of 1 nmol augurin into the PVN significantly increased food intake over the first hour post injection (FI/g; vehicle 2.7 ± 0.8; augurin 1 nmol 6.5 ± 0.7, p<0.01) (Figure 3.3A). Between the first and second hour following injection of 0.3 or 1 nmol augurin food intake was significantly decreased (FI/g; vehicle 3.7 ± 0.5; augurin 0.3 nmol 1.2 ± 0.5, p<0.01; 1 nmol 1.0 ± 0.5 g, p<0.01) (Figure 3.3B). There were no significant differences in cumulative food intake at any time points (Figure 3.4). There were no significant differences in body weight change between groups over the 24 hour duration of the study.

3.4.2.2 Dark phase study two

Injection of 1 or 2 nmol augurin into the PVN significantly increased food intake 1 hour following injection (FI/g; vehicle 2.4 ± 0.3; augurin 1 nmol 4.6 ± 0.6, p<0.001; 2 nmol 4.0 ± 0.3, p<0.01) (Figure 3.5A). Food intake was also significantly increased between the first and second hour following injection of 2 nmol augurin (FI/g; vehicle 2.4 ± 0.4; augurin 2 nmol 3.8 ± 0.3, p<0.05) (Figure 3.5B).

Injection of 1 or 2 nmol augurin significantly increased cumulative food intake over the first two hours post injection (FI/g; vehicle 4.8 ± 0.3; augurin 1 nmol 6.8 ± 1.0, p<0.05; 2 nmol 7.3 ± 0.4, p<0.01) (Figure 3.6A). The increase in cumulative food intake following injection of 2 nmol augurin remained significant over 4, 8 and 24 hours post injection (0-4 h cumulative FI/g; vehicle 8.1 ± 0.6; augurin 2 nmol 11.4 ± 0.6, p<0.01; 0-8 h cumulative FI/g; vehicle 13.9 ± 0.9; augurin 2 nmol 19.5 ± 0.8, p<0.01; 0-24 h cumulative FI/g; vehicle 26.1 ± 0.6; augurin 2 nmol 29.5 ± 0.9, p<0.05) (Figure 3.6A, B and C). There were no significant differences in food intake between any other time points. There were no significant differences in body weight change between groups over the 24 hour duration of the study.
Figure 3.3: Dark phase study one. The effect of microinjection of vehicle or augurin (0.1, 0.3 or 1 nmol) at the start of the dark phase into the paraventricular nucleus of male *ad libitum* fed Wistar rats on (A) 0-1h food intake, (B) 1-2h, (C) 2-4h, (D) 4-8h, (E) 8-24h. *p<0.05, **p<0.01, vs. vehicle.
Figure 3.4: Dark phase study one. The effect of microinjection of vehicle or augurin (0.1, 0.3 or 1 nmol) at the start of the dark phase into the paraventricular nucleus of male *ad libitum* fed Wistar rats on (A) 0-2h cumulative food intake, (B) 0-4h, (C) 0-8h, (D) 0-24h.
Figure 3.5: Dark phase study two. The effect of microinjection of vehicle or augurin (0.25, 0.5, 1 or 2 nmol) at the start of the dark phase into the paraventricular nucleus of male ad libitum fed Wistar rats on (A) 0-1h food intake, (B) 1-2h, (C) 2-4h, (D) 4-8h, (E) 8-24h. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle.
Figure 3.6: Dark phase study two. The effect of microinjection of vehicle or augurin (0.25, 0.5, 1 or 2 nmol) at the start of the dark phase into the paraventricular nucleus of male ad libitum fed Wistar rats on (A) 0-2h cumulative food intake, (B) 0-4h, (C) 0-8h, (D) 0-24h. *p<0.05, **p<0.01, ***p<0.001 vs. vehicle.
3.4.3 THE EFFECT OF iPVN AUGURIN INJECTION DURING THE EARLY LIGHT PHASE ON FOOD INTAKE

3.4.3.1 Light phase study one
Microinjection of 1 nmol augurin into the PVN at the start of the light phase significantly increased food intake over the first hour post injection (FI/g; vehicle 1.2 ± 0.4; augurin 1 nmol 2.9 ± 0.5, p<0.05) (Figure 3.7A). Injection of 0.3 or 1 nmol augurin caused a significant increased cumulative food intake over the first 2 h (FI/g; vehicle 1.5 ± 0.4; augurin 0.3 nmol 2.7 ± 0.3, p<0.05; 1 nmol 3.4 ± 0.3, p<0.01) (Figure 3.8A). There were no significant differences in 24 hour cumulative food intake (Figure 3.8D). There were no significant differences in body weight change between groups over the 24 hour duration of the study.

3.4.3.2 Light phase study two
Injection of 0.5, 1 or 2 nmol augurin into the PVN significantly increased food intake 1 h post injection (Figure 3.9A) (FI/g; vehicle 0.7 ± 0.1; augurin 0.5 nmol 1.8 ± 0.4, p<0.05; 1 nmol 2.1 ± 0.4, p<0.05; 2 nmol 2.1 ± 0.3, p<0.01). There were no significant differences in food intake between any other time points. Cumulative food intake remained significantly elevated up to 2 hours post injection of 0.5 or 1 nmol augurin, and up to 4 hours post injection of 2 nmol augurin (Figure 3.10A and B) (0-2 h cumulative FI/g; vehicle 0.9 ± 0.2; augurin 0.5 nmol 2.1 ± 0.4, p<0.05; 1 nmol 2.3 ± 0.4, p<0.05; 2 nmol 2.3 ± 0.3, p<0.01; 0-4 h cumulative FI/g; vehicle 1.4 ± 0.2; augurin 2 nmol 2.9 ± 0.5, p<0.01). There were no significant differences in 24 h cumulative food intake (Figure 3.10D). There were no significant differences in body weight change between groups over the 24 hour duration of the study.
Figure 3.7: Light phase study one. The effect of microinjection of vehicle or augurin (0.1, 0.3 or 1 nmol) at the start of the light phase into the paraventricular nucleus of male ad libitum fed Wistar rats on (A) 0-1h food intake, (B) 1-2h, (C) 2-4h, (D) 4-8h, (E) 8-24h. *p<0.05, vs. vehicle.
Figure 3.8: Light phase study one. The effect of microinjection of vehicle or augurin (0.1, 0.3 or 1 nmol) at the start of the light phase into the paraventricular nucleus of male *ad libitum* fed Wistar rats on (A) 0-2h cumulative food intake, (B) 0-4h, (C) 0-8h, (D) 0-24h, *p*<0.05, **p**<0.01, vs. vehicle.
Figure 3.9: Light phase study two. The effect of microinjection of vehicle or augurin (0.25, 0.5, 1 or 2 nmol) at the start of the light phase into the paraventricular nucleus of male *ad libitum* fed Wistar rats on (A) 0-1h food intake, (B) 1-2h, (C) 2-4h, (D) 4-8h, (E) 8-24h. *p<0.05, **p<0.01, vs. vehicle.
Figure 3.10: Light phase study two. The effect of microinjection of vehicle or augurin (0.25, 0.5, 1 or 2 nmol) at the start of the light phase into the paraventricular nucleus of male ad libitum fed Wistar rats on (A) 0-2h cumulative food intake, (B) 0-4h, (C) 0-8h, (D) 0-24h, *p<0.05, **p<0.01, vs. vehicle.
3.4.4 The Effect of iPVN Injection of Augurin and α-hCRH During the Early Dark Phase on Food Intake

Microinjection of 1 nmol augurin, with or without α-hCRH, into the PVN at the start of the light phase significantly increased food intake over the first hour post injection compared to vehicle or α-hCRH injected controls (FI/g; vehicle 2.5 ± 0.3; augurin 1 nmol 4.1 ± 0.5; augurin 1 nmol and α-hCRH 1µg 4.8 ± 0.4; augurin 1 nmol and α-hCRH 0.3µg 4.3 ± 0.4; α-hCRH 1µg 3.0 ± 0.3; α-hCRH 0.3µg 2.7 ± 0.3) (Figure 3.11A). There were no significant differences in food intake at any time point when augurin was co-injected with 0.3µg or 1µg α-hCRH, compared with injection of augurin alone (Figure 3.11). There were no significant differences in food intake between vehicle and either dose of α-hCRH at any time point, and there were no significant differences in body weight change between any groups over the 24 hour duration of the study.
Figure 3.11: The effect of microinjection of vehicle, augurin (1 nmol), \( \alpha \)-hCRH (0.3 or 1 \( \mu \)g), or augurin and \( \alpha \)-hCRH at the start of the dark phase into the paraventricular nucleus of male ad libitum fed Wistar rats on (A) 0-1h, (B) 1-2h, (C) 0-2h, (D) 0-4h. Columns without any letters in common are significantly different (p<0.05).
3.5 **DISCUSSION**

Augurin is a recently identified secreted peptide of unknown function. The studies discussed in chapter 2 suggest that ICV or iPVN injection of augurin stimulates the HPA axis in rats, and that this effect is likely mediated by the release of CRH from neurons in the PVN. As CRH is potently anorexigenic when injected ICV or iPVN I hypothesised that hypothalamic injection of augurin would reduce food intake by stimulating the central release of CRH. ICV injection of augurin either at the start of the dark phase or in 24 hour fasted rats caused a mild, non-significant decrease in food intake over the first hour post injection compared with vehicle injected controls. In contrast with the ICV studies, iPVN injection of lower doses of augurin caused a robust orexigenic effect when injected at the start of either the dark or light phase.

Initially the effect of iPVN augurin on dark phase food intake was investigated because I hypothesised that augurin would be anorexigenic. During dark phase study one food intake increased two fold over the first hour following injection of 1 nmol augurin, compared with vehicle injected controls. Between the first and second hours post injection, doses of 1 and 0.3 nmol augurin caused a significant decrease in food intake. The pattern of alternating orexigenic and anorexigenic effects following administration of appetite regulatory peptides is not uncommon, and it is possible that the delayed decrease in food intake following iPVN augurin occurred to compensate for the preceding increase (Parkinson *et al.*, 2008). Alternatively it could have been an erroneous finding resulting from the inherent variability of the experiment. In order to confirm the results from this study and assess whether higher doses of augurin further increased food intake, the study was repeated.

During dark phase study two doses of 1 or 2 nmol augurin increased food intake two fold over the first hour compared with vehicle injected controls. Food intake following iPVN injection of 2 nmol was also significantly increased between the first and second hours post injection. In contrast to night time study one augurin did not cause a delayed anorexigenic effect at any dose. Interestingly, cumulative food intake after injection of 2 nmol augurin was significantly increased at 2, 4, 8, and 24 hours post injection. The data from light phase feeding study two shows that 24 hours post injection of 2 nmol augurin, food intake remains higher that in the
vehicle group but this effect is not statistically significant. Of the known orexigenic peptides very few have been shown to significantly increase cumulative food intake 24 hours after central administration (Rossi et al., 1998, Tang-Christensen et al., 2004, Semjonous et al., 2009), therefore further feeding studies need to be carried out to clarify the duration of augurin’s orexigenic effect.

The effect of iPVN augurin injected during the early light phase was also investigated. During Light phase study one injection of 1 nmol augurin increased food intake twofold over the first hour, compared with vehicle-injected controls. No significant differences in food intake were seen between any other time points. During light phase study two injection of 0.5, 1 or 2 nmol augurin significantly increased food intake over the first hour by two, three, and three fold respectively. Again, no significant differences in food intake were seen between any other time points.

The dark phase feeding study is considered a more physiologically relevant experimental setting in which to investigate food intake, compared with the light phase feeding study. Light phase food intake can be increased by non-specific or stressful stimuli such as the ‘tail pinch’ (Rowland and Antelman, 1976). That augurin should stimulate food intake when injected at the start of the dark phase suggests a relatively potent effect, as rats will eat predominantly during this period anyway.

The reasons for the different effect on food intake depending on the site of injection are currently unknown. It is possible that augurin, like other neuropeptides, has different actions in different hypothalamic nuclei (Williams et al., 2001). Following ICV injection augurin is likely to diffuse into many of the hypothalamic nuclei around the 3rd ventricle, and possibly into the brainstem (Bittencourt and Sawchenko, 2000). The net effect of simultaneous activation of augurin responsive signalling pathways at multiple sites following ICV injection could mask the orexigenic effect seen following iPVN injection. Similar observations have been described for other neuropeptides such as CART.
CART is a neuropeptide implicated in the regulation of the HPA axis and energy homeostasis (Murphy, 2005). ICV injection of recombinant CART (55-102) was shown to inhibit feeding in both satiated and 24 hour fasted rats in a dose dependent manner, and suppress food intake when co-administered with NPY (Kristensen et al., 1998, Vrang et al., 1999). The role of CART peptides in appetite has become less clear, since the discovery that ICV injection of CART (55-102) causes behaviour reminiscent of elevated anxiety in a number of tests (Kask et al., 2000, Aja et al., 2001). It is therefore possible that the anorexigenic effects of ICV CART may be secondary to behavioural changes. It was subsequently shown that microinjection of CART (55-102) into the individual hypothalamic nuclei, including the ARC and PVN, significantly increases food intake compared to controls (Abbott et al., 2001, Hou et al., 2010). In addition, chronic up regulation of CART mRNA in the ARC or PVN of male Wistar rats significantly increases cumulative food intake and body mass (Kong et al., 2003, Smith et al., 2008). In light of this, it is possible that, like CART, non-specific effects following injection of augurin ICV could mask the orexigenic effect seen after iPVN microinjection. Investigation of the behavioural effects caused by ICV injection may suggest reasons that ICV augurin is not orexigenic.

In addition to increasing food intake, my studies in chapter 2 suggest iPVN injection of augurin stimulates the HPA axis via the release of CRH. It is possible that augurin has two or more separate functions within the PVN, and that microinjection of augurin indiscriminately activates augurin responsive neurons resulting in activation of the HPA axis and increased food intake via separate or overlapping pathways. Under physiological conditions, neuropeptide release can be highly regulated at the synaptic level, allowing neuropeptidergic neurons to independently regulate anatomically adjacent targets (Mihaly et al., 2002, Landry et al., 2003, Horvath, 2006). In contrast, the iPVN microinjection technique I used in this chapter and chapter 2 is likely to cause an unphysiologically rapid and widespread increase in extracellular augurin (Levin, 2010).

The HPA axis and energy metabolism are closely linked, and many peptides that regulate food intake play a role in the control of the HPA axis (Ludwig et al., 1998, Spinedi and Gaillard,
A role for augurin in the regulation of both the HPA axis and energy homeostasis is therefore reasonable. The relationship between HPA axis activity and food intake is complex, and it appears that CRH and glucocorticoids have opposing effects on food intake. Obesity in humans and rodents is associated with chronically increased circulating glucocorticoid levels (Leibel et al., 1989, Guillaume-Gentil, 1990). Suppression of endogenous glucocorticoids by adrenalectomy has an anorexic effect in rats which can be corrected by corticosterone replacement (Cohn et al., 1955). Furthermore, adrenalectomy normalises body weight in lep(ob) mice (Solomon and Mayer, 1973). The limited data available from human studies supports the orexigenic effect of glucocorticoids seen in rodents (Walker and Seckl, 2003). While chronically increased glucocorticoid levels promote caloric intake, glucocorticoid and mineralocorticoid receptor agonists have no acute effect on food intake in rats with an intact HPA axis (Tempel et al., 1992). The increase in circulating glucocorticoids caused by iPVN augurin is therefore unlikely to cause the orexigenic effect seen.

In addition to stimulating the release of ACTH from the adenohypophysis, CRH is potently anorexigenic (Morley and Levine, 1982, Krahn et al., 1988). That iPVN augurin should stimulate the HPA axis and acutely increase food intake therefore seems counterintuitive given that PVN CRH neuron project within the PVN to other parvocellular neurons, however a similar relationship has been observed with NPY (Liposits et al., 1985, Silverman et al., 1989). The PVN is the main site of accumulation of nerve terminals of NPY-containing neurons, the perikarya of which are located primarily in the ARC and the brainstem (Cowley et al., 1999, Fuzesi et al., 2007). Electron microscopy has revealed synapses between NPY containing neurons and the dendrites and cell bodies of CRH synthesising PVN neurons, suggesting a physiological role for NPY in the regulation of both the HPA axis and energy homeostasis (Liposits et al., 1988). ICV or iPVN injection of NPY potently increases food intake and reduces energy expenditure (Stanley et al., 1985, Stanley et al., 1986, Zarjevski, 1993). ICV or iPVN injection of NPY also stimulates the HPA axis (Haas and George, 1989, Albers et al., 1990, Brooks et al., 1994). Interestingly, iPVN co-injection of NPY with CRH or α-hCRH
attenuate and potentiate its orexigenic effect respectively, suggesting CRH and NPY interact within the PVN to regulate both food intake and the HPA axis (Heinrichs et al., 1993, Menzaghi et al., 1993).

I therefore investigated whether a similar effect is seen when augurin is co-administered with α-hCRH. Co-injection of α-hCRH at a dose shown to potentiate the orexigenic effect of NPY did not alter the orexigenic effect of augurin, suggesting in contrast to NPY, augurin does not stimulate the local release of CRH within the PVN (Heinrichs et al., 1993). This hypothesis is consistent with the findings from my behavioural study in chapter 2. ICV or iPVN Injection of CRH causes a reliable behavioural response, characterised by an increase in grooming and a decrease in time spent sleeping (Krahn et al., 1988). ICV injection of NPY has also been shown to increase time spent grooming (Clark et al., 1984). My results from chapter 2 demonstrate that augurin, however, had no effects on grooming or sleeping behaviour when injected iPVN (Table 2.2). Measurement of neuropeptide release using in vivo microdialysis is a well-established technique, and could be used to investigate the effects of augurin on the release of CRH within the PVN (Cook, 2001).
4 THE DISTRIBUTION OF AUGURIN IN THE CNS
4.1 INTRODUCTION

The studies discussed in chapters 2 and 3 suggest injection of augurin into the paraventricular nucleus (PVN) of the hypothalamus stimulates the hypothalamo-pituitary-adrenal (HPA) axis and increases food intake in male Wistar rats. These data, however, demonstrate a pharmacological effect of augurin. As described in chapter 1, relatively little known about distribution of augurin in the central nervous system (CNS). In order to investigate the potential physiological functions of augurin I aimed to characterise the distribution of preproaugurin-like immunoreactivity (LI) and messenger ribonucleic acid (mRNA) in the rat CNS. An overview of the relevant techniques, their strengths, and limitations is provided below.

4.1.1 IMMUNOHISTOCHEMISTRY

Immunohistochemistry (IHC) is a powerful technique that can be used to localise specific antigens within a tissue or cell (Coons et al., 1942). The principles underlying IHC are simple; an antibody is raised against the epitope of interest, applied to the appropriately fixed and pre-treated tissue, and the site of reaction revealed by a suitable detection system (Montero, 2003). Nevertheless, there are many variables that can affect the sensitivity and specificity of staining such as the type and quality of antibody, method of tissue fixation and processing, and detection system (Saper, 2005, Elias et al., 2008).

4.1.1.1 Antibodies

IHC relies on the ability of the immune system to produce antibodies directed against foreign antigenic substances. Antibodies are raised by injecting appropriate hosts (commonly rabbits) with purified or synthetic antigen, usually dissolved in an adjuvant solution (Ward et al., 1959, Montero, 2003). Large antigens are often sufficiently immunogenic that they can be used neat to immunise an animal, however, small antigens such as neuropeptides are weakly immunogenic and must therefore be chemically coupled to a larger carrier protein such as bovine serum albumin (Sutcliffe et al., 1983, Polak et al., 2003). The resultant antiserum contains a heterogeneous mix of polyclonal antibodies against several regions of the immunogen produced by activated B-lymphocytes (Polak et al., 2003).
Polyclonal antisera have certain drawbacks, such as possible cross-reactivity with related antigens, or the presence of native immunoreactive antibodies (Larsson, 1988). In addition, a limited quantity of serum can be obtained from the host, and it may be difficult to reproduce antibodies for some antigens (Polak et al., 2003). These drawbacks fuelled the development of monoclonal antibodies (Kohler and Milstein, 1975). The production of monoclonal antibodies is possible because individual B-lymphocytes produce only one type of antibody. Following immunisation, activated B-lymphocytes from the host spleen isolated and fused with non-secreting myeloma cells in vitro, to form an immortalised hybridoma cell line which produces a single antibody specific for a single epitope (Kohler and Milstein, 1975). Although highly specific, the specificity of monoclonal antibodies can occasionally result in falsely negative staining. Where polyclonal antibodies will generally bind to multiple epitopes on the same molecule, a monoclonal will bind to only one, resulting in weaker staining (Ramos-Vara, 2005). In addition, if the epitope is altered by fixation the antibody may not bind at all (Ramos-Vara, 2005).

4.1.1.2 Preparation of tissues for IHC

Tissues must be fixed and sectioned before they are suitable for IHC. Ideally prepared tissues should render antigens insoluble in situ, without hindering the availability of antigenic sites to antibodies (Slaoui and Fiette, 2011). Processing should also preserve tissue architecture and cellular morphology, allowing the identification of immunoreactivity in context (Yan et al., 2010). Formaldehyde fixation followed by embedding in paraffin has been the preparation method of choice for decades because this treatment maintains tissue morphology and immunoreactivity particularly well (Bancroft and Gamble, 2008, Shi et al., 2008).

4.1.1.2.1 Fixation

The process of fixation aims to preserve antigens in their endogenous loci within a tissue, while protecting the tissue during the subsequent staining process. Perfusion fixation with buffered formaldehyde is the gold standard for fixing the CNS prior to IHC (Lamberts and Goldsmith, 1986, Fix and Garman, 2000). Cross-linking fixatives such as formaldehyde fix tissues by chemically linking reactive moieties on adjacent proteins (Fox et al., 1985). Although the
chemical crosslinking during formaldehyde fixation can adversely affect protein antigenicity, immunoreactivity can be restored by heating sections in acidic or alkaline buffer prior to IHC (Shi et al., 1993, D'amico et al., 2009).

4.1.1.2.2 Paraffin embedding

Once fixation is complete, tissues can be embedded in a support material such as paraffin wax. The aim of paraffin embedding is to replace water within the tissue with a support medium that provides sufficient strength to allow sectioning of the tissue without damage (Bancroft and Gamble, 2008). The tissue is first immersed in increasing concentrations of ethanol until all the water has been removed from the tissue. The ethanol is then replaced with xylene, which is in turn replaced with molten paraffin wax. The tissue is then embedded in wax on a tissue cassette for sectioning. The result is a tissue block which can be stored indefinitely, and from which thin sections can be obtained without compromising morphology (Schrell and Sofroniew, 1982). After mounting on to slides, sections can be rapidly de-waxed, rehydrated, and are then ready for use.

4.1.1.3 Detection of the antibody-antigen reaction

Labelling of the antibody-antigen binding sites is required for it to been seen with the light microscope (Montero, 2003). The most commonly used labels are enzymes such as horseradish peroxidase (HRP), and while fluorescent labels are gaining popularity, their use requires special equipment in order to detect fluorescent light (Polak et al., 2003). The primary antibody can directly conjugated to the label, but this method lacks the sensitivity required for the detection of most antigens so the three step avidin-biotin complex (ABC) method is more commonly used (Coons et al., 1942, Bayer et al., 1976). Avidin is a large glycoprotein that has four high affinity binding sites for a low molecular weight vitamin called biotin (Guesdon et al., 1979). Each biotin molecule has a single binding site for avidin, and can be attached through other sites to an antibody (Guesdon et al., 1979). Following application of the unlabelled primary antibody or antiserum, a biotin-conjugated secondary antibody is applied (Hsu et al., 1981). The third layer consists of large complexes of HRP-conjugated biotin with avidin, which binds to the biotin-conjugated secondary antibody (Lloyd et al., 1985).
4.1.1.4 Specificity controls for IHC

Because of the numerous technical pitfalls that can produce spurious results, a crucial part of IHC is selecting the appropriate controls (Petrusz et al., 1976, Swaab et al., 1977). When evaluating a new antiserum, demonstrating an absence of immunoreactivity in an identically processed section from the relevant knockout (KO) mouse is gold standard test of specificity (Saper and Sawchenko, 2003). Nevertheless, KO mice do not exist for the vast majority of proteins, and therefore the adsorption specificity control is most commonly employed (Larsson, 1988, Saper, 2005). An absence of immunoreactivity following incubation of the antiserum with an excess of synthetic antigen suggests the immunoreactive component of the antiserum is indeed raised against that antigen (Larsson, 1988). One caveat is that certain antisera have been shown to avidly bind basic amino acids (Scopsi et al., 1986). This can result in non-specific staining within sections, and, in cases where the immunogen contains many basic amino acids, can also cause false-negative results from the adsorption control (Scopsi et al., 1986).

4.1.2 In situ hybridisation histochemistry

In situ hybridisation histochemistry (ISH) is a technique that allows localisation of a messenger ribonucleic acid (mRNA) or deoxyribonucleic acid (DNA) sequence within a tissue, whilst maintaining morphological integrity (Pardue and Gall, 1969). ISH takes advantage of the specific annealing of complementary nucleic acids through hydrogen bonds formed between bases attached to a sugar-phosphate backbone (Watson and Crick, 1953). This base pairing underlies the formation of a double stranded complex. Any nucleic acid sequence can therefore be specifically detected by use of a labelled nucleotide probe that is the ‘antisense’ reverse complementary sequence (Wilcox, 1993). The two main considerations for ISH are specificity and sensitivity, which are determined in large part by tissue preparation, probe type, and detection method (Wilkinson and Nieto, 1993).

4.1.2.1 Tissue preparation

The preparation of tissues for ISH is critical for preserving mRNA in situ (Yan et al., 2010). In unstabilised tissue, ubiquitously expressed RNase enzymes quickly degrade mRNAs, and it is therefore imperative to block their activity as quickly as possible (Yang et al., 1999).
Transcardial perfusion with buffered formaldehyde is the preferred method for preparation of tissues for ISH as formaldehyde rapidly inhibits RNAse enzymes, and effectively preserves tissue morphology during the hybridisation procedure (Koopman, 2001, Palop et al., 2011).

4.1.2.2 Riboprobe preparation

With the continued development of RNA polymerase methodologies, the use of complimentary RNA (cRNA) riboprobes is preferred over cDNA or oligonucleotide probes for several reasons (Karr et al., 1995). First, cRNA probes can easily be synthesised easily and rapidly by in vitro transcription of a DNA template generated by polymerase chain reaction (PCR) (Young et al., 1993, Karr et al., 1995). Second, cRNA-mRNA hybridisation complexes are more stable than comparable cDNA-mRNA complexes, permitting the use of stringent hybridisation conditions specifically conducive to the formation of riboprobe-mRNA complexes (Meltzer et al., 1998). Finally, non-specific hybridisation can be drastically reduced by digestion of single stranded probe using RNAse (Wilkinson and Nieto, 1993).

4.1.2.3 Hybridisation

The main goal of hybridisation is for the probe to anneal to complementary mRNA strands under the optimal condition (Kadkol et al., 1999). Factors that affect how well a riboprobe will bind to the target mRNA include the pH and ionic concentration of the hybridisation buffer, and the temperature and duration of hybridisation (Moens, 2008). Changing these parameters will influence the probe’s affinity for its target sequence. High ionic concentration and low temperatures and pH enhance hybridisation, but the specificity of hybridisation is also reduced (Schaeren-Wiemers and Gerfin-Moser, 1993). Following hybridisation, the slides are washed under high stringency conditions to remove unbound probes or probes loosely bound to improperly matched sequences (Kelsey et al., 1986, Wilkinson and Nieto, 1993).

4.1.2.4 Detection

There are two approaches for localisation of hybridised probes, non-radioactive and radioactive (Komminoth et al., 1992). Although historically associated with lower sensitivity, technical advances mean that non-radioactive haptens such as digoxigenin (DIG) have largely superseded the use of radioisotopes for ISH (Komminoth et al., 1992, De Block and Debrouwer, 1993).
DIG labelled probes are detected using an antibody raised against DIG and conjugated to a chromogenic enzyme (Wilkinson, 1995). Advantages of DIG labelling include subcellular resolution of hybridisation, and the short time required to yield a signal; minutes to hours in the case of DIG probes, rather than days to weeks for autoradiographic detection of radioactive isotopes (Knoll et al., 2007, Pinaud et al., 2008).

4.1.2.5 Specificity controls for ISH

It is essential to establish that a probe has hybridised selectively to the target mRNA sequence, and that the resultant pattern of hybridisation is therefore specific (Larsson, 1989). As with IHC, the absence of hybridisation in a section from the relevant KO mouse is the gold standard specificity control (Larsson and Hougaard, 1993). Nevertheless, as this is often unfeasible hybridisation of matched sections with a labelled sense probe is commonplace (Wilkinson and Nieto, 1993). An absence of staining in sections hybridised with sense probe indicates that the pattern of anti-sense hybridisation is sequence dependant, and therefore likely to be specific to the target mRNA (Larsson and Hougaard, 1990).
4.2 **HYPOTHESIS AND AIMS**

Augurin is a recently identified secreted peptide of unknown function. Preproaugurin mRNA has been detected in the brain, but relatively little known about its distribution within the CNS. Studies in chapters 2 and 3 suggest injection of augurin into the PVN of male Wistar rats stimulates the hypothalamo-pituitary-adrenal (HPA) axis and increases food intake. These data, however, demonstrate a pharmacological effect of augurin. In order to investigate the potential physiological role of augurin, I aimed to characterise the distribution of preproaugurin -LI and mRNA in the rat CNS.

### 4.2.1 AIM

1. Characterise the distribution of preproaugurin-LI in the CNS of male Wistar rats.

2. Characterise the distribution of preproaugurin mRNA in the CNS of male Wistar rats.
4.3 METHODS

4.3.1 PEPTIDES

A synthetic fragment of human augurin corresponding to amino acids 71 to 148 of preproaugurin was synthesised and purified by Bachem UK Ltd. as described in 2.3.1. Synthetic fragments of human augurin corresponding to amino acids 71 to 107, 108 to 132, or 133 to 148 of preproaugurin were purchased from Phoenix Pharmaceuticals, (Belmont, CA, USA).

4.3.2 WATER

Water used to make solutions was ultra pure (18.2mΩ) quality obtained through an ELGA system (ELGA Labwater, Marlow, UK). RNase-free water or TE (Sigma-Aldrich, Poole, UK) was used for all experiments involving RNA, with the exception of pre- and post-hybridisation ISH washes for which ultra pure water was used.

4.3.3 ANIMALS

Male Wistar rats (specific pathogen free; Charles River), weighing 250-300 g were maintained in individual cages under controlled temperature (21-23°C) and light (12 h light, 12 h dark cycle; lights on at 0700 h) conditions with ad libitum access to food (RM1 diet, SDS Ltd.) and water. Animal procedures were approved under the British Home Office Animals Scientific Procedures Act 1986 (Project Licence 70/6402).

4.3.4 TISSUE PREPARATION

Sections for both IHC and ISH were prepared from tissues fixed by transcardial perfusion and embedded into paraffin wax. Tissues preparation was identical for both ISH and IHC.

4.3.4.1 Perfusion fixation

4.3.4.1.1 Materials

Flush solution (appendix)

Fixative solution (appendix)

Pentobarbitone (Merial)
4.3.4.1.2 Method

Fixation by transcardial perfusion was carried out using an established protocol (Fix and Garman, 2000). Each rat was injected ip with a lethal dose of pentobarbitone (Merial Animal Health Ltd, UK). The pedal reflex test was used to ensure that a surgical plane of anaesthesia had been reached. The rat was placed in a dorsal recumbent position and a transverse incision was made just caudal to the xiphoid process. The diaphragm was incised, followed by the left and right lateral ribcage. The xiphoid process was clamped and held rostrally to expose the chest cavity. An 18-gauge needle, connected to a two-way valve was then inserted into the left ventricle and clamped into position. A small nick was made in the right atrium and the animal was perfused with from a 1m height under gravitational pressure. Transcardial perfusion was done in two stages. Firstly, exsanguination was accomplished using 40-50mls flush solution, and then the brain was fixed using fixative solution (1ml/g body weight fixative was perfused over 10-15 minutes). Perfused brains were left in situ for two hours, sectioned coronally into 5mm thick blocks and then post-fixed is the same fixative for 16 hours at 4°C.

4.3.4.2 Paraffin-embedding

Following fixation, tissue blocks were placed in plastic cassettes and transferred to 70% ethanol for paraffin embedding. Tissues were paraffin-infiltrated in a V.I.P. Tissue Tek Processor (Miles/Sakura, Torrance, CA) and then embedded in Paraplast X-Tra Tissue Embedding Medium (Fisher Scientific, Pittsburgh, PA) at a Tissue-Tek embedding station (Miles/Sakura, Torrance, CA). Four µm sections were sectioned using a rotary microtome (Leitz, Wetzlar, Germany), floated onto ultra pure water, and mounted on poly-L-lysine-coated slides.

4.3.5 ANTISERA

Details for each antiserum used in this chapter are shown in Table 4.1.
Table 4.1: Antisera used in this chapter.

Abbreviations: AP, alkaline phosphatase; DIG, digoxigenin.

<table>
<thead>
<tr>
<th>Name</th>
<th>Immunogen</th>
<th>Type</th>
<th>Host species</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-(71-107)</td>
<td>Amino acids 71-107 of human preproaugurin protein</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>Phoenix Pharmaceuticals, Belmont, CA, USA</td>
</tr>
<tr>
<td>Anti-(108-132)</td>
<td>Amino acids 108-132 of human preproaugurin protein</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>Phoenix Pharmaceuticals, Belmont, CA, USA</td>
</tr>
<tr>
<td>Anti-(133-148)</td>
<td>Amino acids 133-148 of human preproaugurin protein</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>Phoenix Pharmaceuticals, Belmont, CA, USA</td>
</tr>
<tr>
<td>Anti-(71-148)</td>
<td>Amino acids 71-148 of human preproaugurin protein</td>
<td>Polyclonal</td>
<td>Rabbit</td>
<td>Peninsula Laboratories, Belmont, CA, USA</td>
</tr>
<tr>
<td>Anti-DIG-AP</td>
<td>Digoxigenin</td>
<td>Polyclonal</td>
<td>Sheep</td>
<td>Roche Diagnostics, Mannheim, Germany</td>
</tr>
<tr>
<td>Biotinylated anti-rabbit</td>
<td>Rabbit immunoglobulin G (heavy and light chain)</td>
<td>Polyclonal</td>
<td>Goat</td>
<td>Vector Laboratories, Peterborough, UK</td>
</tr>
</tbody>
</table>

Table 4.2: Primer sequences used to amplify preproaugurin cDNA by PCR. The expected size of the product is in base pairs. Accession number refers to that published in the Nucleotide Sequence Database (Sayers et al., 2011).

Abbreviations: T3, T3 RNA polymerase; T7, T7 RNA polymerase; bp, base pairs.

<table>
<thead>
<tr>
<th>Name</th>
<th>Promoter sequence</th>
<th>Complimentary sequence</th>
<th>bp</th>
<th>Accession Number</th>
</tr>
</thead>
<tbody>
<tr>
<td>T3 Forward</td>
<td>ATTAACCCTCACTAAAGGGA</td>
<td>GACCTGTTCATCAAAGACG</td>
<td>350</td>
<td>NM_032411</td>
</tr>
<tr>
<td>T7 Reverse</td>
<td>TAATACGACTCACTATAGGG</td>
<td>TCATAGTGTGACTGGCTCC</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
4.3.6 IHC

All IHC protocols were adapted from (Polak et al., 2003).

4.3.6.1 IHC staining

4.3.6.1.1 Materials

Ethanol (VWR)

Xylene (VWR)

0.01M PBS (appendix)

0.01M citrate buffer pH6 (appendix)

Normal goat serum (NGS) (Invitrogen)

Antibody diluent (appendix)

Biotinylated goat anti-rabbit (Jackson Labs)

Avidin Biotin Complex (ABC) (Vector Labs)

3’3’-diaminobenzidine tetrahydrochloride hydrate (DAB) (Sigma-Aldrich)

Copper sulphate solution (appendix)

DPX mountant (Thermo Fisher Scientific)

Wax pen (Vector Labs)

Haematoxylin (Vector Labs)

4.3.6.1.2 Method

Paraffin sections were dewaxed in two changes of xylene (5 minutes each) and rehydrated through graduated ethanol washes (2 x 100%, 1 x 70%, 1 x 50%) for 3 minutes each before rinsing in ultrapure water. Antigen retrieval was carried out on the sections by boiling for 20 minutes in a microwave oven in pre-boiled 0.01M citrate buffer pH6, after which, sections were allowed to cool for a further 15 minutes before rinsing in 0.01M PBS. Endogenous peroxidase activity was blocked by incubation in 0.01M PBS containing 1.6% v/v hydrogen peroxide for 10 minutes. Following a 5 minute wash in water, sections were incubated for 30 minutes at room temperature with 10% v/v NGS in antibody diluent. The primary antiserum was diluted to the appropriate concentration in antibody diluent and incubated on the sections overnight at room temperature. Negative control sections were routinely processed by omitting the primary
antiserum to detect any non-specific staining caused by unwanted binding of the secondary antibody, ABC, or any endogenous enzyme activity. Sections were then washed in 0.01M PBS, incubated in biotinylated goat anti-rabbit for 30 minute at room temperature, before being washed in PBS and incubated with avidin-biotin complex horseradish peroxidase at room temperature for a further 30 minutes. After a final wash in 0.01M PBS, the antigen-antibody complex was visualised by incubation in 0.01M PBS containing 0.05% w/v DAB and 0.03% v/v hydrogen peroxide. After 10 minutes sections were rinsed in PBS and then water. DAB staining was enhanced by incubation in copper sulphate for 4 minutes. Sections were then rinsed in water before being dehydrated though ascending ethanol concentrations (50-100%) for 3 minutes each, incubated in xylene for 2 x 5 minutes, and then mounted using DPX. In some instances sections were lightly counterstained using Haematoxylin before dehydration and mounting.

4.3.6.2 Titre test
A titre test was used initially to evaluate each primary antiserum. IHC was performed as described in section 4.3.6.1, and each primary antiserum was serially diluted in antibody diluent to a final concentration of 1:30, 1:100, 1:300, 1:900, or 1:2700.

4.3.6.3 Adsorption control

4.3.6.3.1 Materials
Synthetic peptide used to raise antiserum (Table 4.1)
Poly-L-lysine MW 3000-6000 (Sigma)
Antibody diluent (appendix)

4.3.6.3.2 Method
 Appropriately diluted antiserum was incubated at 4°C overnight with 10 nmol/ml of the corresponding synthetic peptide used to raise the antiserum (Polak et al., 2003). To control for antibody binding to bind basic amino acids, synthetic peptide was replaced with the equivalent concentration of poly-L-lysine (Polak et al., 2003). The following day IHC staining was performed as described in section 4.3.6.1.
4.3.7 **ISH**

4.3.7.1 **RNA extraction**

4.3.7.1.1 *Materials*

Tri-Reagent (Helena Biosciences, Sunderland UK)

1-Bromo-Chloro-Propane (Sigma-Aldrich)

Propan-2-ol (VWR)

Ethanol (VWR)

4.3.7.1.2 *Method*

Total RNA was extracted using Tri-reagent according to the manufacturer’s protocol. Frozen hypothalami were homogenised using an Ultra Turrax T25 homogeniser (IKA Labortechnik, Staufen, Germany) in Tri-reagent (10x v/w Tri-reagent solution per hypothalamus). Following 2 minutes incubation at room temperature the mixture was transferred to silanised Corex tubes and incubated for a further 5 minutes at room temperature. To separate the mixture into aqueous and organic phases, 0.1x v/v of 1-bromo-chloro-propane was added and mixed vigorously. The mixture was incubated for 5 minutes at room temperature before being centrifuged at 12000g for 15 minutes at 4°C (centrifuge 5417 C/R, Eppendorf, Hamburg, Germany). The upper, aqueous phase containing the RNA was then transferred to a new Corex tube, precipitated by addition of 0.5x v/v propan-2-ol for 10 minutes at room temperature and then centrifuged at 12000g for 10 minutes at 4°C. The supernatant was removed and the pellet washed twice with 70% ethanol before being centrifuged at 12000g for 10 minutes at 4°C. The supernatant was then removed and the pellet allowed to air dry before being re-suspended in 400µl BPC water. RNA concentration and purity was determined spectrophotometrically, and the integrity was then confirmed by denaturing agarose gel electrophoresis.

4.3.7.2 **Verification of RNA integrity**

4.3.7.2.1 *Materials*

Agarose, type II-A medium EEO (Sigma-Aldrich)

Formaldehyde (VWR)

20 x MOPS pH7 (appendix)
Ethidium bromide (VWR)
DENAT (appendix)
Gel loading buffer (appendix)
TE pH7.5 (appendix)
2M Sodium Acetate (NaAc) pH5.2 (appendix)
Ethanol (VWR)

4.3.7.2.2  Method
A 1% agarose denaturing gel was prepared in 1 x MOPS buffer with 7.5% formaldehyde (v/v). 5 µg of RNA was added to 12µl DENAT then the samples were denatured at 65°C for 5 minutes and 3µl gel loading buffer was added. The samples were then loaded onto the gel and run in 1 x MOPS buffer with 7.5% formaldehyde v/v at 140 volts for approximately 40 minutes until the gel front had run 10cm. The gel was then stained in TE with 0.01% v/v ethidium bromide on a shaking platform for 30 minutes. The gel was then de-stained overnight in 1 x TE to allow visualisation under ultraviolet light of the 18s and 28s ribosomal bands and to ensure that the RNA had not degraded.

4.3.7.3  Reverse transcription reaction

4.3.7.3.1  Materials
RNA samples
10mM dNTPs (Amersham Biosciences, Little Chalfont, UK)
5 x reverse transcriptase buffer (Promega, Madison WI)
Avian myoblastoma virus reverse transcriptase (RT) 10 U/µl (Promega)
Oligo dT (12-18) 200 ng/µl (Amersham Biosciences)

4.3.7.3.2  Method
The reaction was set up in a final volume of 20µl containing the following: 1 µg RNA, 1 x reverse transcriptase buffer, 1mM dNTPs and 10 mg/ml oligo dT. The solution was heated to 65°C for 5 minutes and allowed to cool to room temperature for 30 minutes. 10 U RT was added and the reaction incubated at 42°C for 60 minutes. The reaction was used directly as the PCR template.
4.3.7.4 Riboprobe template design

A riboprobe template was generated by polymerase chain reaction (PCR) (Sitzmann and Lemotte, 1993). Primer-BLAST was used to generate 20 pairs of primers to which T3 or T7 polymerase sequences were added to the 5’ ends (Sayers et al., 2011). Each pair of primers was checked for specificity, self-complementarity, and the propensity to form primer dimers until a suitable pair was identified (Rozen and Skaletsky, 2000). The primers selected to amplify the DNA template are detailed in Table 4.2.

4.3.7.5 Preparation of DNA template by PCR

4.3.7.5.1 Materials

- 10 x Taq buffer (Sigma-Aldrich)
- 20 µM oligonucleotide primers (Eurogentec)
- Taq DNA polymerase (5U/µl) (Sigma-Aldrich)
- 10mM dNTPs (Amersham Biosciences)

4.3.7.5.2 Method

A 2µl aliquot of the reverse transcription reaction generated in 4.3.7.3 was added to a tube containing 1 x Taq buffer, 0.2mM dNTPs, 200 nM forward and reverse primers. The reaction was heated to 95°C for 5 minutes and then 5 U Taq DNA polymerase added. The reaction was cycled 25 times through the following temperatures: 95°C for 30s, 55°C for 30s and 72°C for 30s. After completion of the reaction, correct size of the PCR was confirmed by agarose gel electrophoresis.

4.3.7.6 Agarose gel electrophoresis of DNA template

4.3.7.6.1 Materials

- Agarose, type II-A medium EEO (Sigma-Aldrich)
- 50x Tris-acetate-EDTA buffer (TAE) (appendix)
- Ethidium bromide (10mg/ml) (VWR)
- DNA marker (1Kb ladder, Invitrogen)
- Gel loading buffer (appendix)
4.3.7.6.2 Method

A 1% w/v agarose gel was prepared by dissolving the agarose in 1 x TAE using a microwave oven (Panasonic, NNE205W). The gel was cooled to 45°C and ethidium bromide added to a final concentration of 0.5 µg/ml. Once set, the gel was placed into an electrophoresis tank containing 0.5x TAE, run for 150 volts for approximately 40 minutes and visualised under ultraviolet light.

4.3.7.7 Phenol-chloroform extraction of DNA template

4.3.7.7.1 Materials

Phenol chloroform (VWR)

Ethanol (VWR)

2M sodium acetate (NaAc) pH5.2 (appendix)

4.3.7.7.2 Method

Once size had been verified the remaining PCR reaction was phenol chloroform extracted by addition of an equal volume of phenol chloroform. Solutions were thoroughly mixed before centrifugation at 12000g for 3 minutes. The supernatant was carefully removed and precipitated with the addition of 0.1x v/v of NaAc and 2.5x v/v of ethanol for one hour at -20°C. Following precipitation, suspensions were centrifuged at 12300g for 7 minutes, the supernatant was removed and the pellet washed twice with 70% ethanol before being centrifuged at 12000g for 10 minutes at 4°C. Pellets were re-suspended in 100µl TE and the concentration and purity of the DNA template was determined spectrophotometrically.

4.3.7.8 Riboprobe preparation

4.3.7.8.1 Materials

DNA riboprobe template

100mM dithiothreitol (DTT) (Promega)

30U/µl RNAse inhibitor (Invitrogen)

T3 or T7 RNA polymerase (Promega)

10x DIG RNA labelling mix (Roche)

5x polymerase buffer (Promega)
DNAse I (Invitrogen)

10x DNAse buffer (appendix)

8M lithium chloride (LiCl) (Sigma-Aldrich)

Ethanol (VWR)

TE (Sigma-Aldrich)

4.3.7.8.2 Methods

The DIG-labelled riboprobe was produced by in vitro transcription of the DNA template. Two hundred nanograms of DNA template was added to a reaction containing 5mM DTT, 1x polymerase buffer, 30 U RNAse inhibitor and 1x DIG labelling mix in a total volume of 19µl. Finally, 20 U RNA polymerase was added (T3 to the anti-sense reaction and T7 to the sense reaction) and this was incubated at 37°C for 2 hours. After incubation, 3µl DNAse buffer and 7.5 U DNAseI were added and the reaction incubated for a further 15 minutes at 37°C. The riboprobe was precipitated by addition of 0.1x v/v 8M LiCl and 2.5x v/v ethanol. The precipitate were centrifuged at 12300g for 7 minutes, the supernatant was removed and the pellet washed twice with 70% ethanol before being centrifuged at 12000g for 10 minutes at 4°C. The pellet was then resuspended in 100µl TE. The concentration and purity of the DIG-labelled probe was determined spectrophotometrically, and the integrity was then confirmed by denaturing agarose gel electrophoresis as described in 4.3.7.2.

4.3.7.9 Hybridisation and detection

4.3.7.9.1 Materials

0.01M PBS (Gibco)

0.01M citrate buffer (appendix)

Ethanol (VWR)

Hybridisation buffer (appendix)

20x SSC (appendix)

1x RNAse buffer (Promega)

RNAse A (Promega) (appendix)

Maleic buffer (Roche)
Blocking solution (appendix)

Anti-DIG-AP, Fab fragments (Roche)

DIG wash (Roche)

Detection buffer (appendix)

4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indoyl-phosphate, 4-toluidine salt kit (BCIP/NBT alkaline phosphatase substrate kit) (Vector Laboratories)

Xylene (VWR)

DPX mountant (Thermo Fisher Scientific)

Wax pen (Vector Laboratories)

10% w/v Poly-vinyl alcohol solution

Formamide (VWR)

4.3.7.9.2 Methods

Paraffin sections were dewaxed in two changes of xylene (5 minutes each) and rehydrated through graduated ethanol washes (2 x 100%, 1 x 70%, 1 x 50%) for 3 minutes each before rinsing in ultrapure water. Antigen retrieval was carried out on the sections by boiling for 20 minutes in a microwave oven in pre-boiled 0.01M citrate buffer pH6, after which, sections were allowed to cool for a further 15 minutes before rinsing in ultra pure water (Sperry et al., 1996). Slides were then dehydrated through 50%, 70%, and 100% ethanol for 3 minutes each, and individual sections were circumscribed using a hydrophobic wax pen and allowed to air dry. Pre-hybridisation of sections was carried out using hybridisation buffer not containing any DIG-labelled probe. Slides were incubated in this solution for 1 hour at 57°C in a humidified chamber. Fifteen minutes before hybridisation, probes were added to hybridisation buffer at a final concentration of 1ng/ml and denatured at 75°C for 15 minutes, before cooling on ice for 5 minutes. Hybridisation buffer containing the denatured probe was applied to the sections which were then hybridised overnight at 57°C. Negative control sections were routinely hybridised with the same concentration of sense rather than antisense riboprobe to identify non-specific staining.
Slides were then washed once in 5x SSC at 60°C for 5 minutes, before RNAse treatment by incubation in 1x RNAse buffer containing 50µg/ml RNAse A for 30 minutes at 37°C (Sitzmann and Lemotte, 1993). Slides were again washed in 5x SSC at 60°C for 10 minutes, before a series of increasing stringency washes at 60°C in 50% formamide/2 x SSC for 30 minutes, 2 x SSC for 30 minutes, and twice in 0.2x SSC for 30 minutes each. Slides were then incubated in maleic buffer at room temperature for 5 minutes before undergoing blocking in blocking solution for 30 minutes at room temperature. Sections were then subjected to two hours incubation in a humidified chamber with blocking solution containing anti-DIG-AP, Fab fragments at a 1:2000 concentration (Weiszmann et al., 2009).

Slides were then washed in DIG wash twice for 15 minutes at room temperature. Sections were briefly rinsed in detection buffer for 5 minutes before incubation in detection buffer, an equal volume of 10% poly-vinyl alcohol, and 2 drops of reagents 1, 2 and 3 (BCIP-NBT kit) per 5 ml detection buffer (De Block and Debrouwer, 1993). Slides were incubated in this solution in the dark and development was monitored microscopically over the following 1-2 hours. Following sufficient development, slides were washed in water three times for 5 minutes each, before dehydration through 50%, 70% and 100% ethanol each for 15 seconds. Finally slides were immersed in xylene for 30 seconds and mounted using DPX.

4.3.8 PHOTOMICROGRAPHY

Representative images rat brain sections were photographed using a GXCAM-3 camera (GX Optical, Suffolk, UK) attached to a Nikon Eclipse 50i microscope (Nikon, Melville, NY). 1000x images were photographed using a Leica DFC320 camera attached to a Leica DM LB2 microscope (Leica Microsystems Inc., Bannockburn, IL). Only linear adjustments to the contrast, brightness, or colour of photomicrographs were made using Adobe Photoshop Elements 7 (Adobe, San Jose, CA).
4.4 RESULTS

4.4.1 EVALUATION OF AUGURIN ANTISERA

4.4.1.1 Antibody titre test

The choroid plexus (CP) was used as the positive control for antibody titration as high levels of mRNA have been reported there previously (Mirabeau et al., 2007). Anti-(71-148) showed high levels of uniformly distributed non-specific staining. The estimated optimal dilution for the three remaining antisera tested was 1:300 (Table 4.3).

Table 4.3: Antisera titration using serial dilutions. Relative intensities were estimated by visual comparison of stained sections: -, absent; +, weak; ++, moderate; ++++, strong; ++++, very strong.

<table>
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<tbody>
<tr>
<td>1:30</td>
<td>+++</td>
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<td>+++</td>
<td>+++</td>
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<td>1:100</td>
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<td>1:300</td>
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<td>1:900</td>
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<td>1:2700</td>
<td>-</td>
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Abbreviations: CP, choroid plexus.

4.4.1.2 Adsorption specificity control

Overnight incubation of anti-(133-148) at the estimated optimal dilution with 10 nmol/ml of the corresponding synthetic peptide used to raise the antiserum resulted in the absence of staining (Figure 4.1). Substitution of synthetic peptide for an equal mass of poly-L-lysine had no effect on the pattern of staining (Figure 4.1). Some staining remained following incubation of anti-(108-132) or anti-(71-107) at the estimated optimal dilution with 10 nmol/ml of the corresponding synthetic peptide used to raise the antiserum (Figure 4.1). The specificity of augurin (71-148) was not tested because of its high background staining. Anti-(133-148) was used for mapping the distribution of preproaugurin-LI because the adsorption control was successful, and it demonstrated high signal to noise ratio.
Figure 4.1: Representative light-field photomicrographs of rat CP showing adsorption specificity controls. Anti-(133-148) showed strong immunoreactivity in the CP (Brown; A), which was absent following pre-adsorption of the primary antibody with 10nmol/ml synthetic preproaugurin-(133-148) (B). Pre-adsorption of the primary antibody with 10nmol/ml poly-L-lysine had no effect (C). Anti-(108-132) and anti-(71-107) also showed immunoreactivity in the CP (D and F respectively), however some staining remained following pre-adsorption of the primary antibody with 10nmol/ml of the corresponding synthetic peptide (E and F respectively). All sections were counterstained with Haematoxylin (blue nuclei).

Figure 4.2: Representative light-field photomicrographs of male Wistar rat CP showing preproaugurin antisense (A [200x]) and sense (B [200x]) riboprobe hybridisation. Prominent antisense riboprobe hybridisation (blue) is observed, while sense riboprobe hybridisation is negligible (B).
4.4.2 Evaluation of Augurin Riboprobe

4.4.2.1 Riboprobe production

Rat hypothalamus tissue expressed preproaugurin mRNA as shown by RT-PCR (Figure 4.3). Sequencing of the purified PCR reaction confirmed it matched the PCR product predicted by Primer-BLAST. The size of both the sense and antisense riboprobe generated by \textit{in vitro} transcription matched that of the DNA template.

4.4.2.2 Hybridisation specificity

Pronounced hybridisation of the antisense probe was observed in the choroid plexus (Figure 4.2). No hybridisation was detected when the antisense probe was omitted or replaced with the same concentration of sense probe (Figure 4.2). The observed pattern of staining is in line with the published distribution of preproaugurin, suggesting the antisense riboprobe hybridised preproaugurin mRNA (Mirabeau \textit{et al.}, 2007, Gonzalez \textit{et al.}, 2011).

![Image of RT-PCR gel showing preproaugurin mRNA expression](image-url)

Figure 4.3: Preproaugurin mRNA expression in rat hypothalamus as determined by RT-PCR. The size of the expected PCR product for preproaugurin was 350 base pairs. L, 1Kb ladder.
4.4.3 DISTRIBUTION OF AUGURIN IN THE CNS

4.4.3.1 General expression pattern

The distributions of preproaugurin-LI and mRNA in the CNS are summarised in Table 4.4. The distribution of preproaugurin-LI matched that of augurin-mRNA throughout the CNS and discordance was not observed at any location. In line with previously published data, preproaugurin-LI and mRNA were found in the choroid plexus (Figure 4.1) (Mirabeau et al., 2007, Gonzalez et al., 2011). Preproaugurin-LI and mRNA were also observed in other CNS regions including the telencephalon, diencephalon, rhombencephalon, and cerebellum.

Table 4.4: Distribution and intensity of preproaugurin-LI and mRNA in the rat CNS. Relative intensities were estimated by visual comparison: -, absent; +, weak; ++, moderate; +++, strong; ++++, very strong.

<table>
<thead>
<tr>
<th>CNS region</th>
<th>IHC</th>
<th>ISH</th>
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<tbody>
<tr>
<td>1. Telencephalon</td>
<td></td>
<td></td>
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<tr>
<td>1.1. Olfactory bulb</td>
<td>++++</td>
<td>++++</td>
</tr>
<tr>
<td>1.2. Cerebral cortex</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>1.3. Hippocampal formation</td>
<td>++</td>
<td>++</td>
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<tr>
<td>1.4. Amygdala</td>
<td>-</td>
<td>-</td>
</tr>
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<td>1.5. Basal ganglia</td>
<td>-</td>
<td>-</td>
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<tr>
<td>2. Diencephalon</td>
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<td></td>
</tr>
<tr>
<td>2.1. Thalamus</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2.2. Hypothalamus</td>
<td>++</td>
<td>++</td>
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<tr>
<td>3. Mesencephalon</td>
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<tr>
<td>3.1. Sensory</td>
<td>-</td>
<td>-</td>
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<td>3.2. Motor</td>
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<tr>
<td>3.3. Behavioural</td>
<td>-</td>
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</tr>
<tr>
<td>4. Rhombencephalon</td>
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<tr>
<td>4.1. Pons</td>
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<td>4.2. Medulla</td>
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<td>5. Cerebellum</td>
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<tr>
<td>5.1. Cerebellar cortex</td>
<td>+++</td>
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<tr>
<td>5.2. Cerebellar nuclei</td>
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</table>
4.4.3.2 Telencephalon

Within the rat telencephalon preproaugurin-LI and mRNA were observed in the olfactory bulb (OB), subventricular zone (SVZ), and hippocampal formation (HPF). Within the OB, high levels of preproaugurin-LI and mRNA were observed in the mitral layer of the OB (Figure 4.4). More caudally, preproaugurin-LI and mRNA were prominent in the rostral migratory stream (RMS) and the SVZ (Figure 4.4). Within the HPF, preproaugurin-LI and mRNA were observed in neuron-like cells throughout the CA1-3 regions of the HPF, and the subgranular zone (SGZ) of the hippocampal dentate gyrus (Figure 4.4).

4.4.3.3 Diencephalon

Within the rat diencephalon preproaugurin-LI and mRNA were only observed in the hypothalamus. Within the hypothalamus, high levels of preproaugurin were observed in the ependymal cell layer lining the 3rd cerebral ventricle (3V) (Figure 4.5). Preproaugurin-LI and mRNA were also found in several areas of the hypothalamic parenchyma. Immunoreactive cells with neuronal appearance and punctate immunoreactivity were most prominent in the SON and PVN (Figure 4.5). Some immunoreactive neurons in the supraoptic nucleus (SON) displayed morphological features of magnocellular cells (Figure 4.5), with staining of the somata, sparing of the nuclei, and labelling of processes with dendritic appearance.

4.4.3.4 Mesencephalon

No preproaugurin-LI or mRNA was observed in any part of the mesencephalon.

4.4.3.5 Rhombencephalon

Scattered cells within nucleus of the tractus solitarius (NTS) of the dorsal vagal complex (DVC) showed strong preproaugurin-LI and mRNA expression (Figure 4.6).

4.4.3.6 Cerebellum

Within the cerebellum, the cell soma of Purkinje cells showed strong preproaugurin-LI and mRNA expression, and preproaugurin-LI was also observed in Purkinje cell dendritic processes (Figure 4.6). Both preproaugurin-LI and mRNA were absent from granular and molecular cell layers (Figure 4.6).
Figure 4.4: Representative light-field photomicrographs of preproaugurin-LI and mRNA in the telencephalon of male Wistar rats. Preproaugurin-LI was seen in the MCL of the OB (A [40x] and B [200x]), SVZ of the LV (D [100x]), migratory chains within the RMS (F [200x]), and in the HPF (H [40x]). Preproaugurin mRNA was also present in the MCL (C [40x]), SVZ (E [200x]), and HPF (G [10x] and I [40x]). A, B, D, and F, were counterstained with Haematoxylin.

Abbreviations: CP, choroid plexus; DG, dentate gyrus; GL, granule layer; GLL, glomerular layer; LV, lateral ventricle; MCL, mitral cell layer; OPL, outer plexiform layer; SGZ, subgranular zone; SVZ, subventricular zone.
Figure 4.5: Representative light-field photomicrographs of preproaugurin-LI and mRNA in the hypothalamus of male Wistar rats. Preproaugurin-LI was observed in the ependymal cell layer adjacent to the 3V (A [200x]), the PVN (B [40x]), the SON (D [100x] and E [200x]), and ARC (I [100x]). Preproaugurin immunoreactive cell within the SON with the morphological appearance of a magnocellular neuron (F [1000x]), immunoreactivity is restricted the cell cytoplasm where it is diffuse and punctate. Preproaugurin mRNA was also present in the PVN (C [200x]), SON (G [200x]), and ARC (H [100x]). A, B, D, and E, were counterstained with Haematoxylin.

Abbreviations: 3V, 3rd cerebral ventricle; ARC, arcuate nucleus; PVN, paraventricular nucleus; SON, supraoptic nucleus; ot, optic tract.
Figure 4.6: Representative light-field photomicrographs of preproaugurin-LI and mRNA in the cerebellum and brainstem of male Wistar rats. Within the cerebellum, preproaugurin-LI was restricted to Purkinje cell soma and their dendrites (A [40x] and B [200x]). Within the brainstem, preproaugurin-LI was observed in the NTS (D [100x]), in what appeared to be neurons (E [200x]). Preproaugurin mRNA was also present in Purkinje cells within the cerebellum (C [100x]), and the brainstem NTS (F [200x]).

Abbreviations: 4V, 4th cerebral ventricle; GL, granule cell layer; ML, molecular layer; NTS, nucleus of the tractus solitarius; PU, Purkinje cell layer; WM, white matter.
4.5 DISCUSSION

The studies discussed in chapters 2 and 3 suggest injection of augurin into the hypothalamic PVN stimulates the HPA axis and increases food intake in male Wistar rats. To investigate whether augurin could have a physiological role in the regulation of the HPA axis or food intake, I aimed to characterise its endogenous distribution in the rat CNS using IHC and ISH. My results suggest discrete populations of cells positive for preproaugurin-LI and mRNA are present in several regions of the CNS including the hypothalamus and brainstem.

The primary consideration when evaluating a new antiserum or riboprobe is the specificity of staining (Kadkol et al., 1999, Saper and Sawchenko, 2003). Taken together, the studies in this chapter indicate that the observed pattern of both preproaugurin-LI and preproaugurin anti-sense riboprobe hybridisation represent the distribution of endogenous preproaugurin in the CNS. With regards to the IHC, the absence of staining following incubation of the antiserum with an excess of synthetic antigen, but not poly-L-lysine, suggests the immunoreactive component of the antiserum recognises that antigen specifically (Scopsi et al., 1986, Larsson, 1988). With regards to the ISH, no staining was observed following hybridisation with the same concentration of sense riboprobe as antisense, indicating that the pattern of anti-sense riboprobe hybridisation is sequence dependent, and therefore likely to be specific to the target mRNA (Larsson and Hougaard, 1990). Most importantly, the patterns of immunoreactivity and hybridisation in my studies showed strict concordance, strongly supporting the specificity of my results (Saper and Sawchenko, 2003). Furthermore, my results are in agreement with several studies published during preparation of this thesis (Roberton et al., 2009, Kujuro et al., 2010, Gonzalez et al., 2011).

Prior to beginning work for this thesis, the only study investigating the distribution of augurin within the CNS was carried out by Mirabeau et al. who reported high levels of preproaugurin mRNA in the CP (Mirabeau et al., 2007). In addition to the CP, I observed the highest levels of preproaugurin-LI and mRNA in the SVZ, RMS, and SGZ; regions of the CNS primarily associated with neural stem cells (NSCs) (Merkle et al., 2007, Conti and Cattaneo, 2010). Although neurogenesis primarily occurs during embryonic development, it continues into adult
life in rodents and humans largely confined to the SVZ and SGZ (Lledo et al., 2006). In the SVZ, for example, slowly dividing astrocyte-like NSCs give rise to neural precursor cells (NPCs) that migrate toward the OB in chains, forming the RMS (Mirzadeh et al., 2008, Zhao et al., 2008). After reaching the OB the NPCs differentiate into functional local interneurons (Imayoshi et al., 2008, Lledo et al., 2008). Although adult neurogenesis is now a well-established occurrence, much is still unknown about the signalling mechanisms that regulate adult neurogenesis and cell fate (Suh et al., 2009). Given the high levels of preproaugurin in regions of the CNS associated with NSCs, it is possible augurin regulates NSC/NPC proliferation, migration, or both.

A role for augurin as in neurogenesis would be consistent with the distribution of preproaugurin, and its reported anti-proliferative effects. The gene encoding preproaugurin, Chromosome 2 open reading frame 40 (C2orf40), was originally suggested to be a tumour suppressor gene in human oesophageal squamous cell carcinoma (OSCC), and decreased expression has since been associated with multiple forms of cancer (Su et al., 1998, Mori et al., 2007, Gotze et al., 2009, Li et al., 2009, Vanaja et al., 2009). Preproaugurin mRNA expression correlates negatively with OSCC disease stage, and may be an independent prognostic factor for poor survival (Mori et al., 2007, Li et al., 2009). In vitro studies indicate that preproaugurin regulates numerous aspects of tumour progression and metastasis. Transfection with preproaugurin cDNA reduces the rate of proliferation, invasion, and migration of several cancer cell lines (Li et al., 2009, Li et al., 2010b). Based on the evidence indicating C2orf40 serves as a tumour suppressor gene, and the studies of Kujuro et al. suggesting preproaugurin is a secreted inducer of cell NPC senescence, I hypothesise that augurin is an endogenous inhibitor of adult neurogenesis in the SVZ and SGZ (Gotze et al., 2009, Li et al., 2009, Kujuro et al., 2010). If augurin is a physiological inhibitor of neurogenesis, antagonists of its receptor could be candidates for neurorestorative therapeutics (Geraerts et al., 2007, Taupin, 2009, Lindvall and Kokaia, 2010).

Two studies published during the preparation of this thesis have reported preproaugurin-LI in cells with neuronal morphology in the rodent CNS (Roberton et al., 2009, Kujuro et al., 2010). In accord with their observations, my studies indicate that preproaugurin-LI is present in
neurons within the rat CNS. Furthermore, the results discussed in this chapter are the first to report preproaugurin mRNA in neurons. Preproaugurin-LI cells in the OB, hypothalamus, and cerebellum exhibited quintessential neuronal morphology (Paxinos, 2004, Su et al., 2004, Young et al., 2008). Within neurons, preproaugurin-LI was diffuse, punctate, and present throughout the cytoplasm and in what appeared to be dendritic and axonal processes. This pattern of staining is consistent with storage in cytoplasmic secretory granules, and is characteristic of a neuropeptide (Polak and Bloom, 1987).

The data presented in this chapter are in agreement with a recent study by Roberton et al. describing the distribution of preproaugurin-LI in the hypothalamus (Roberton et al., 2009). They observed preproaugurin-LI in magnocellular neurons within the PVN and SON, synaptic-capillary interfaces in the median eminence, and in Herring bodies in the posterior pituitary (Roberton et al., 2009). Furthermore, preproaugurin-LI co-localised with arginine vasopressin (AVP)- and oxytocin (OXT)-LI to secretory granules (Roberton et al., 2009). The majority of magnocellular neurons in the PVN and SON project to the posterior pituitary where they release neuropeptides such as AVP and OXT into the circulation (Bourque, 2008). Augurin may therefore also be released in the circulation, and exert a regulatory effect on target organs.

My studies in chapters 2 and 3 suggest iPVN injection of augurin is biologically active, stimulating the HPA axis and increasing food intake in male Wistar rats. Multiple neuropeptides including neuropeptide Y, cocaine-and amphetamine-regulated transcript protein, glucagon-like peptide-1, melanocortin-concentrating hormone, and prolactin releasing peptide, are all released into the PVN and peri-PVN area where they modulate neuroendocrine function (Rinaman, 1999, Stanley et al., 2001, Lin et al., 2002, Mihaly et al., 2002, Pissios et al., 2006). The data presented in this chapter suggest preproaugurin positive neurons are present within the PVN itself, and, in areas that project directly to the PVN such as the ARC and NTS (as discussed in chapter 3). Furthermore, my data are in agreement with several studies published during preparation of this thesis (Roberton et al., 2009, Kujuro et al., 2010, Gonzalez et al., 2011). The distribution of preproaugurin neurons in the CNS is therefore consistent with a role in the
regulation of food intake and the HPA axis via the PVN (Larsen and Mikkelsen, 1995, Ulrich-Lai and Herman, 2009).

Considerable work remains to delineate the neuroanatomy of the CNS augurin system, and to establish whether endogenous augurin has a physiological role within the hypothalamic PVN. Neuropeptides usually do not exist alone as neurotransmitters but colocalise with classical neurotransmitters, with other neuropeptides, or both (Hokfelt et al., 2000). Identification of coexisting transmitters in augurin-producing neurons would help to develop new hypotheses about anatomical connections of augurin neurons and possible regulatory roles of the augurin system. Induction of the immediate-early gene cfos is a well-established marker of neuronal activation (Sagar et al., 1988, Hoffman and Lyo, 2002). Retrograde neuronal tracing combined with IHC for Fos-LI would indicate which augurin neurons, if any, projected directly to the PVN, and whether those neurons were activated in response to altered energy balance or stressors (Li and Sawchenko, 1998, Vinuela and Larsen, 2001).
5  **FINAL DISCUSSION**
5.1 **FINAL DISCUSSION**

Work presented in this thesis has attempted to investigate whether the putative neuroendocrine peptide augurin has a role in the regulation of neuroendocrine function. Considerable efforts have been made to identify and characterise novel neuroendocrine peptides (NEPs) because of their capacity to modulate physiological functions and the opportunities for development of drug therapies resulting from their characterisation (Clark *et al.*, 2003, Hokfelt *et al.*, 2003). NEPs function as both hormones and neurotransmitters, and as a result frequently play important roles in the orchestration of both somatic and behavioural responses to relevant environmental stimuli (Hokfelt, 1991). Augurin is a recently identified putative NEP of unknown function (Mirabeau *et al.*, 2007). Because of its prominent expression in the central nervous system (CNS), pituitary, and endocrine organs, I hypothesised augurin may have a physiological role in the neuroendocrine system (Steck *et al.*, 2002, Mirabeau *et al.*, 2007).

As the hypothalamus and pituitary are sites at which many NEPs have some biological activity, and are pivotal components of the neuroendocrine system, I began by investigating the effects of augurin on the hypothalamo-pituitary axes in male Wistar rats. Studies in chapter 2 suggest that intracerebroventricular (ICV) or intraparaventricular nucleus (iPVN) injection of augurin selectively activates the hypothalamo-pituitary-adrenal (HPA) axis in rats, and that this effect is likely mediated by the release of corticotrophin releasing hormone (CRH) from neurons in the PVN. The HPA axis is the final common pathway in the stress response. It is the humoral component of an integrated neural and endocrine system that maintains homeostasis in response to stressors (Ulrich-Lai and Herman, 2009). Activation of the HPA axis culminates in the secretion of glucocorticoids (Herman *et al.*, 2003). Hypophysiotrophic CRH and arginine vasopressin (AVP) parvocellular neurosecretory neurons (PCNs) within the PVN control glucocorticoid secretion, and are the focal point for neurocircuitry projecting from hypothalamic, brainstem and forebrain nuclei conveying information regarding stressors (Ulrich-Lai and Herman, 2009). Glucocorticoids act at multiple levels, temporarily redirecting resources to restorative processes and promoting allostasis.
Nevertheless, inappropriate or sustained glucocorticoid secretion is highly detrimental to an organism. Dysregulation of the HPA axis and of plasma glucocorticoid levels has been implicated in the pathogenesis of hypertension, obesity, type II diabetes, and depression (Buckingham, 2006). Various pharmacotherapies have been developed based on components of the HPA axis. Glucocorticoid receptor agonists are widely used as anti-inflammatory and immunosuppressive agents (Rhen and Cidlowski, 2005). More recently, CRH receptor (CRHR) antagonists have shown promise in the treatment of anxiety, depression, sleep disorders, addictive behaviour, and preterm labour (Habib et al., 2000, Zoumakis et al., 2006). Based on my results it is conceivable that the augurin system may be a viable pharmacological target for manipulation of the HPA axis.

Neuropeptides commonly have several biological functions within the hypothalamus (Williams et al., 2001). The proopiomelanocortin derived peptide α-melanocyte-stimulating hormone (α-MSH), for example, has been implicated in the regulation of energy homeostasis, HPA axis activity, reproduction and fertility, blood pressure, and arousal (Wardlaw and Ferin, 1990, Van Der Ploeg et al., 2002, Leibel, 2006, Belgardt et al., 2008, Greenfield et al., 2009). Results from chapter 2 suggest that augurin stimulates the release of CRH from neurons in the PVN. The PVN has an important role in the regulation of food intake as well as HPA axis activity, and in addition to stimulating the secretion of glucocorticoids, hypothalamic CRH is a potent physiological inhibitor of food intake (Grill and Kaplan, 2002, Morton et al., 2006). Studies have shown that CRH is a downstream mediator of α-MSH’s effects on both food intake and the HPA axis (Gardner et al., 1998, Uehara et al., 1998, Lu et al., 2003). I therefore hypothesised that hypothalamic injection of augurin would reduce food intake by stimulating the central release of CRH.

Unexpectedly, my results from chapter 3 indicate that augurin had a robust orexigenic effect when injected into the PVN at the start of either the dark or light phase. My results from chapter 2 suggest that augurin stimulates the release of CRH in vivo following hypothalamic injection, and from ex vivo hypothalamic explants. That iPVN augurin should stimulate the HPA axis and increase food intake therefore seems counterintuitive, however, many peptides that regulate
food intake play a role in the control of the HPA axis, and role for augurin in the regulation of both is therefore possible (Ludwig et al., 1998, Spinedi and Gaillard, 1998, Krysiak et al., 1999, Pecoraro et al., 2004, Thompson et al., 2004a).

The data presented in chapters 2 and 3 demonstrate pharmacological effects of augurin following injection into the PVN. To explore whether augurin might have a physiological role in the regulation of the HPA axis or food intake, I investigated its endogenous distribution in the rat CNS using immunohistochemistry (IHC) and in situ hybridisation histochemistry (ISH). The studies in chapter 4 suggest preproaugurin positive neurons are present within the PVN itself, and, in areas that project directly to the PVN such as the arcuate nucleus and dorsal vagal complex (as discussed in chapter 3). The distribution of preproaugurin neurons in the CNS is therefore consistent with a role in the regulation of food intake, the HPA axis, or both, via the PVN (Larsen and Mikkelsen, 1995, Ulrich-Lai and Herman, 2009).

The data presented in chapter 4 are in agreement with several studies published during preparation of this thesis describing preproaugurin neurons within the PVN itself, and, in areas that project directly to the PVN (Roberton et al., 2009, Kujuro et al., 2010, Gonzalez et al., 2011). These data indicate that preproaugurin derived peptides are present endogenously in the PVN. Results discussed in chapter 3 suggest augurin has an orexigenic effect in the PVN, and it is therefore possible augurin may have a physiological role promoting food intake. If augurin is a physiological orexigen, antagonists at its receptor could be a potential treatment for obesity.

The prevalence of obesity is reaching epidemic proportions (Abelson and Kennedy, 2004). Obese people, defined as having a body-mass index of 30.0 or higher, have increased all cause mortality, and higher death rates from heart disease, stroke, and cancer (Whitlock et al., 2009, Welborn and Dhaliwal, 2011, Zheng et al., 2011). Treatment options for obesity are limited. Studies of lifestyle advice and dietary intervention show that while moderate, clinically significant weight loss is achievable and results in concomitant improvements in comorbidities, it is invariably transient and recidivism is almost universal (Yanovski and Yanovski, 2002, Padwal and Majumdar, 2006). Although pharmacotherapeutic options have shown promise, they are currently inadequate to address many obesity-associated comorbidities (Padwal and
Majumdar, 2007, Field et al., 2009). Further understanding of augurin’s role in the neuroendocrine circuits that regulate energy homeostasis would indicate whether it could provide a bases for novel therapies for the treatment of obesity.

Neuropeptides are a critically important and diverse set of regulatory molecules whose functions are to convey specific information among cells and organs. Their diversity makes defining a neuropeptide challenging, and in general they are best characterised by their shared characteristics. Hallmarks of neuropeptides are 1) preprohormone gene expression and posttranslational processing by neurons, 2) regulated release, and 3) the ability to modulate or mediate neural functioning by acting on specific receptors (Burbach, 2010). Although no receptor for augurin has been identified, augurin appears to fulfil the first and possibly second criteria.

Firstly, the studies presented in this thesis, and those recently published by Roberton et al. and Kujuro et al., suggest preproaugurin is endogenously expressed in several neuronal populations within the CNS, and that posttranslational cleavage produces several candidate neuropeptides (Roberton et al., 2009, Kujuro et al., 2010). Secondly, experimental evidence suggests that secretion of augurin by neurons is likely to be regulated rather than constitutive. Neurons expressing classical neuropeptides use the regulated secretory pathway, allowing storage of neuropeptides in large dense-cored vesicles (LDCVs) and controlled release upon a stimulus (Brownstein et al., 1980). Several studies have demonstrated that the preproaugurin-LI co-localises with peptides known to be stored in LDCVs, such as insulin, AVP, and OXT, strongly suggesting stimulus dependant release (Watson et al., 1982, Leng et al., 1999, Mirabeau et al., 2007, Roberton et al., 2009).
5.2 final conclusion

Taken together, the results discussed in this thesis, and those of several recently published studies, support the hypothesis that augurin is a novel neuropeptide with a role in the neuroendocrine system (Mirabeau et al., 2007, Roberton et al., 2009, Kujuro et al., 2010, Gonzalez et al., 2011). Further work is required to determine if pharmacological manipulation of the augurin system holds therapeutic potential for the treatment of diseases. Identifying the endogenous forms of augurin, their receptor(s), and structural analogues acting as antagonists or agonists would provide valuable tools for investigating the physiological role of augurin, and its potential as a therapeutic target.
REFERENCES


hormone releasing factor (LRF) of ovine origin. *Biochemical and biophysical research communications*, 44, 205-10.


Hahn, J. D. & Swanson, L. W. 2010. Distinct patterns of neuronal inputs and outputs of the juxtaparaventricular and suprafornical regions of the lateral hypothalamic area in the male rat. *Brain research reviews*, 64, 14-103.


Hetherington, A. & Ranson, S. 1940. Hypothalamic lesions and adiposity in the rat. *The Anatomical Record*, 78, 149-172.


Ishihara, A., Kanatani, A., Mashiko, S., Tanaka, T., Hidaka, M., Gomori, A., Iwaasa, H., Murai, N., Egashira, S., Murai, T., Mitobe, Y., Matsushita, H., Okamoto, O., Sato, N., Jitsuoka,


Kujuro, Y., Suzuki, N. & Kondo, T. 2010. Esophageal cancer-related gene 4 is a secreted inducer of cell senescence expressed by aged CNS precursor cells. *Proceedings of the National Academy of Sciences*, 107, 8259-64.


Shimon, I., Yan, X., Taylor, J. E., Weiss, M. H., Culler, M. D. & Melmed, S. 1997b. Somatostatin receptor (SSTR) subtype-selective analogues differentially suppress in


APPENDICES
APPENDIX I: RADIOIMMUNOASSAY

RIA is a technique for measuring low levels of biological substances in small volume tissue and plasma samples. The principle of RIA is competition between unlabelled antigen (in the sample) and radiolabelled antigen to bind to a fixed concentration of antibody. The most commonly used radiolabel is $^{125}$I, and antibodies are often obtained from rabbit immunisation with antigen/peptide plus adjuvant (though polyclonal antibodies from other species and monoclonal antibodies can be used). The concentrations of radiolabelled antigen and antibody remain constant. Increasing the concentration of unlabelled antigen will therefore decrease the binding sites available for binding to the radiolabelled antigen. Thus the amount of radiolabelled antigen bound to the antibody is inversely proportional to the amount of unlabelled antigen in the sample being examined. A series of reference standards or a ‘standard curve’ is constructed of known concentrations of the peptide.

Each RIA has optimum conditions in terms of buffer, volume, antibody titre, incubation time, and separation method. Phosphate and sodium barbitone buffers are used for a wide range of RIAs. Assays are often incubated at 4°C for 3-5 days. Once equilibrium has been achieved, the amount of antibody bound to antigen, and the amount of free antibody and antigen are constant. Separation of the unbound radiolabelled antigen from the radiolabelled antigen-antibody complex and counting the proportion of radiolabel present in the two fractions enables direct measurement of the amount of unlabelled antigen that has bound to the antibody. By reference to the standard curve, the concentration of unknown samples can be calculated.

The simplest method of separation of free radiolabelled antigen from the antibody complex is adsorption, for example with charcoal. Dextran is added to a charcoal suspension to block the larger holes in the porous charcoal. The suspension is then added to the RIA tubes, where it traps the free radiolabelled antigen. The tubes are then centrifuged and the supernatant (bound antibody complex) and carbon pellet (free radiolabelled antigen) separated by aspiration, and counted in a gamma counter.
Appropriate quality control is must for obtaining robust and reproducible data. The precision of an assay is commonly estimated by calculating the intra and interassay coefficients of variation ($C_v$). The $C_v$ is a measure of the dispersion of a probability distribution. It is defined as the ratio of the standard deviation ($\sigma$) to the mean ($\mu$), and reported as a percentage by multiplying by 100 (Equation 1). The $C_v$ is appropriate when describing the distribution of positive valued variables that approximate a normal distribution. Inter-assay variation is calculated by assaying aliquots of the same sample in each assay performed and comparing the concentrations obtained in each, while intra-assay variation is calculated by assaying aliquots of the same sample in separate assays.

Equation 1 The coefficient of variation

$$C_v = \frac{\sigma}{\mu} \times 100$$
## APPENDIX II: AMINO ACID THREE AND ONE LETTER CODES

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<th>Amino Acid</th>
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<td>A</td>
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<td>Isoleucine</td>
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<tr>
<td>Theronine</td>
<td>Thr</td>
<td>T</td>
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</tbody>
</table>
APPENDIX III: SUPPLIERS

Associated Dental Products Ltd, Kemdent Works, Purton, Swindon, UK
Bachem St. Helens, UK
Bayer UK, Bury St. Edmonds, UK
BioXtal, Mundelsohn, France
Bright Instruments, Huntingdon, UK
Campden Instruments, Loughborough, UK
Charles River, Bicester, UK.
Clark Electromedical Instruments, Pangbourne, Reading, UK
Fisher Scientific, Leicestershire, UK
Gibco BRL (Life Technologies Ltd.), Paisley, Renfrewshire, UK.
GraphPad, La Jolla, CA, USA
Harvard Apparatus, Massachusetts, USA
Helena Biosciences, Sunderland, UK
Invitrogen, Paisley, UK
Life Sciences Technology, Eggenstein, Germany
Millipore S.A., France
New England Bioloabs, Hitchin, Herts, UK
Parke-Davis, Pontypool, Gwent, UK.
Peptide Institute, Kyoto, Japan.
Perkin Elmer, Massachusetts, USA
Pharmacia Biotechnology Ltd., St. Albans, Hertsfordshire, UK.
Phoenix Pharmaceuticals Belmont, CA, USA
Plastics One Inc, Roanoke, VA, USA.
Promega, Southampton, UK
Research Diets Inc, New Brunswick, NJ, USA
Schering-Plough Corporation, Welwyn Garden City, Herts, UK
Sigma-Aldrich Company Ltd., Poole, Dorset, UK.
Systat Ltd., Evanston, Illinois, USA.
VWR, Poole, UK
APPENDIX IV: SOLUTIONS

Fixative
15g of NaCl, 28.2g Na₂HPO₄·2H₂O and 5.44g KH₂PO₄ were dissolved in 800mls H₂O. 100mls of 40% formaldehyde were added and the solution made up to 1L. Adjust to pH 7.4 with NaOH.

Flush
15g of NaCl, 28.2g Na₂HPO₄·2H₂O and 5.44g KH₂PO₄ were dissolved in 900mls H₂O and the solution made up to 1L. Adjust to pH 7.4 with NaOH.

Artificial cerebrospinal fluid (aCSF)
Make aCSF immediately before use. To make 500 ml aCSF mix 100ml 0.1M NaHCO₃, 100ml 0.63M NaCl, 5ml 0.09M Na₂HPO₄·2H₂O, 5ml 0.59M KCl, 5ml 0.09M MgSO₄·7H₂O, 450 mg glucose, 88 mg ascorbic acid, 5ml aprotinin (Trasylol, Bayer Corp) and 270ml ultra pure water. Saturate with 95% O₂ and 5% CO₂. Add 10ml 0.07M CaCl₂·2H₂O after gassing.

56mM KCl (stimulatory) aCSF
Make aCSF immediately before use. To make 100ml 56mM KCl aCSF, mix 20ml 0.1M NaHCO₃, 11.6ml 0.63M NaCl, 1ml 0.09M Na₂HPO₄·2H₂O, 10ml 0.59M KCl, 1ml 0.09M MgSO₄·7H₂O, 90 mg glucose, 17.6mg ascorbic acid, 1ml aprotinin (Bayer Corp) and 53.4ml ultra pure water. Saturate with 95% O₂ and 5% CO₂. Add 2ml 0.07M CaCl₂·2H₂O after gassing.

aCSF stock solutions
0.09M disodium hydrogen orthophosphate dehydrate: Dissolve 1.56g Na₂HPO₄·2H₂O in 100ml ultra pure water
0.07M calcium chloride dehydrate: Dissolve 1.04g CaCl₂·2H₂O in 100ml ultra pure water
0.09M magnesium sulphate heptahydrate: Dissolve 2.2g MgSO₄·7H₂O in 100ml ultra pure water
0.59M potassium chloride: Dissolve 4.42g KCl in 100ml ultra pure water
0.1M sodium bicarbonate: Dissolve 4.62g NaHCO₃ in 500ml ultra pure water
0.63M sodium chloride: Dissolve 18.43g NaCl in 500ml ultra pure water

0.01M citrate buffer
Dissolve 19.2g citric acid in 900ml ultra pure water. pH to 6 and make up to 1L with ultra pure water.

1% Copper sulphate solution
Dissolve 1g of copper sulphate in 100ml ultra pure water.

Deionised formamide
Add 1g of amberlite MB6113 (indicator) mixed resin per 10ml formamide. Incubate for 1 hour in the 37°C shaker until indicator turns yellow. Pass through a sterile filter before use.

Denhart’s solution
Dissolve 10g of each of ficoll, polyvinyl-pyrrolidone and BSA in 500ml ultra pure water. Store in aliquots at -20°C.

**Detection buffer**
Mix 0.8ml 5M NaCl, 1.6ml MgCl₂, 8ml 0.5M Tris-HCl pH9.8 and add 20mg levamisol. Make up to 20ml with ultra pure water and add 20ml of 10% polyvinyl acetate. For development, omit the MgCl₂ and add 2 drops of reagent 1, 2, and 3 per 5 ml detection buffer.

**Dextran coated charcoal**
Add 2.4g charcoal and 0.24g dextran to 100ml phosphate buffer with gelatine and mix for 20 mins at 20°C

**DNA loading buffer**
Mix 3.125ml 80% glycerol, 50µl 0.5M CH₁₄H₂O₈Na₂.2H₂O (EDTA) and 6.075ml ultra pure water, add 10mg orange G.

**dNTP mix for PCR and reverse transcription**
Mix 20µl each of 100mM dGTP, dCTP, dATP and dTTP in 120µl filtered ultra pure water.

**0.5M ethylenediaminetetra-acetic acid (EDTA)**
Dissolve 186.1g CH₁₄H₂O₈Na₂.2H₂O (EDTA) in 800ml ultra pure water and adjust pH to 8.0. Make up to 1L with ultra pure water.

**Hybridisation buffer**
Add 5ml deionised formamide, 2.5ml 20x SSC, 200µl 100 x Denhardts Solution, 100µl 1M Tris pH7.5, 250µl 10% SDS, 20µl 0.5M EDTA pH8, 1.25ml 10mg/ml tRNA, 20µl 0.5M EDTA pH8, 1.25ml 10mg/ml tRNA, 680µl BPC water, 1g dextran sulphate together and mix well.

**Kemteck buffer (0.05M) (RIA buffer)**
Dissolve 22.4g NaH₂PO₄.2H₂O, 9.96g Na₂HPO₄.2H₂O, 14.8g C₁₀H₁₄H₂O₈Na₂.2H₂O, 1g C₃H₆HgNaO₂S, 26.6ml of 30% BSA in 5L of ultra pure water that has been boiled and allowed to cool. Adjust to pH 7.4 with sodium hydroxide. Store at 4°C.

**1M magnesium chloride**
Dissolve 203.3g MgCl₂ in 1L ultra pure water.

**Maleic buffer**
Dissolve 116g maleic acid and 88g NaCl into 800ml ultra pure water. pH to 7.5 with NaOH and make up to 1L with ultra pure water

**20x MOPS**
Dissolve 83.6g 3-(N-Morpholino)propanesulphonic acid (MOPS), 8.1g sodium acetate, 7.4g C₁₀H₁₄H₂O₈Na₂.2H₂O and 2.5ml formaldehyde to 800ml ultra pure water. Adjust to pH 7.0 with 10M sodium hydroxide and make up to 1L.

**Phosphate buffer (0.06M) (RIA buffer)**
Dissolve 48 g of Na₂HPO₄.2H₂O, 4.13 g KHPO₄, 18.61 g C₁₀H₁₄H₂O₈Na₂.2H₂O, 2.5 g NaN₃ in 5 L of ultra pure water that has been boiled and allowed to cool. Measure the pH to confirm it is 7.4±0.1. Store at 4°C.
Phosphate buffer (0.06M) with gelatin
The buffer is produced as above with 12.5 g of gelatine dissolved in the boiling ultra pure water then cooled before the addition of the other ingredients.

10x Phosphate buffered saline (PBS)
Add 80g of NaCl, 2g KCl, 14.4g Na2HPO4, 2.4g KH2PO4 to 800ml ultra pure water. Adjust volume to 1L with ultra pure water, adjust pH to 7.4 and sterilise by autoclaving.

1x PBS
Add 100ml 10x PBS to 900ml ultra pure water. Sterilise by autoclaving.

10% Polyethylene glycol (RIA)
Dissolve 100g of polyethylene glycol into 1L ultra pure water.

10mg/ml RNAse A
Add 100mg RNAse A, 50µl 2M Tris and 30µl 5M NaCl to 9.92ml ultra pure water, and boil for 15 minutes. Allow to cool and aliquot.

20 x Standard sodium citrate (SSC)
Add 1753g NaCl and 882g Na3C6H5O7.2H2O to 8L ultra pure water, adjust to pH 7.0 and make up to 10L.

5M sodium chloride
Dissolve 292.2g NaCl in 1L ultra pure water.

10M sodium hydroxide
Dissolve 400g of NaOH in 500ml ultra pure water. Once dissolved, make up to 1L with ultra pure water.

30% sucrose solution
Dissolve 300g of sucrose in 1L 1xPBS.

50 x Tris-acetate-EDTA (TAE) buffer
Dissolve 242g Trizma base in 843ml ultra pure water and mix in 57ml glacial acetic acid and 100ml 0.5M C10H14H2O8Na2.2H2O.

TAE running buffer
To 1L ultra pure water, add 75µl ethidium bromide and 10ml 50 x TAE.

100 x TE
Dissolve 121.1g Trizma base and 3.7g C10H14H2O8Na2.2H2O in 800ml ultra pure water and adjust to pH 7.5 with HCl. Make up to 1L.

1M Tris-HCl pH 7.5
Dissolve 121.1g Trizma base in 800ml ultra pure water and adjust to pH 7.5 with HCl. Make up to 1L.

2M Tris-HCl pH 8.0
Dissolve 121.1g Trizma base in 450ml ultra pure water and adjust to pH 8.0 with HCl. Make up to 500ml.
APPENDIX V: PUBLICATIONS RELATING TO THIS THESIS

Augurin stimulates the hypothalamo-pituitary-adrenal axis via the release of corticotrophin-releasing factor in rats

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1Department of Investigative Medicine, Imperial College London, London, UK, and 2Bachem UK Ltd., Merseyside, UK

Background and purpose: The functional characterization of secreted peptides can provide the basis for the development of novel therapeutic agents. Augurin is a recently identified secreted peptide of unknown function expressed in multiple endocrine tissues, and in regions of the brain including the hypothalamus. We therefore investigated the effect of hypothalamic injection of augurin on the hypothalamo-pituitary-adrenal (HPA) axis in male Wistar rats.

Experimental approach: Augurin was given as a single injection into the third cerebral ventricle (i.c.v.) or into the paraventricular nucleus (iPVN) of the hypothalamus. Circulating hormone levels were then measured by radioimmunoassay. The effect of augurin on the release of hypothalamic neuropeptides was investigated ex vivo using hypothalamic explants. The acute effects of iPVN augurin on behaviour were also assessed.

Key results: i.c.v. injection of augurin significantly increased plasma ACTH and corticosterone, compared with vehicle-injected controls, but had no effect on other hypothalamo-pituitary axes hormones. Microinjection of lower doses of augurin into the PVN caused a similar increase in plasma ACTH and corticosterone, without significant alteration in behavioural patterns. Incubation of hypothalamic explants with increasing doses of augurin significantly elevated corticotrophin-releasing factor (CRF) and arginine vasopressin release. In vivo, peripheral injection of a CRF1/2 receptor antagonist prevented the rise in ACTH and corticosterone caused by i.c.v. augurin injection.

Conclusions and implications: These data suggest that augurin stimulates the release of ACTH via the release of hypothalamic CRF. Pharmacological manipulation of the augurin system may therefore be a novel target for regulation of the HPA axis.

Keywords: augurin; hypothalamo-pituitary-adrenal axis; paraventricular nucleus; CRF

Abbreviations: aCSF, artificial cerebrospinal fluid; AVP, arginine vasopressin; CRF, corticotrophin-releasing factor; ECRG4, oesophageal cancer-related gene 4; HPA, hypothalamo-pituitary-adrenal; i.c.v., intracerebroventricular; PVN, paraventricular nucleus

Introduction

Novel secreted peptides represent an important field of research because delineation of their function affords new insights into the pathophysiological processes causing disease, and provides opportunities for the development of pharmacological therapies. Secreted peptide systems are particularly amenable to pharmacological modulation as their receptors are generally found on the cell surface and accessible to circulating factors. Synthetic secreted peptides or structural analogues can therefore be used directly as therapeutic agents for various diseases.

Augurin is a recently identified secreted peptide of unknown function encoded by the gene c2orf40 (Mirabeau et al., 2007), a potential tumour suppressor gene in human oesophageal epithelium (Su et al., 1998). Down-regulation of c2orf40 expression due to hypermethylation can occur in human oesophageal squamous cell carcinoma and may be an independent prognostic factor for poor survival (Yue et al., 2003). The product of c2orf40 is the highly conserved 148-amino-acid oesophageal cancer-related gene 4 protein (ECRG4 protein). ECRG4 protein has characteristic features of a preprohormone; it contains a canonical signal peptide...
sequence and at least one basic residue, prohormone cleavage, site. Augurin is produced by post-translational processing of ECRG4 protein (Mirabeau et al., 2007).

The primary sequence of endogenous augurin has not been determined. ECRG4 protein contains one canonical dibasic prohormone cleavage site corresponding to residues Lys69 and Arg70. Cleavage at this site has been demonstrated in vitro following transfection of a pancreatic cell line with the c2orf40 gene in which the Flag antigen sequence was inserted between Arg70 and Gin71 (Mirabeau et al., 2004). Western blotting of supernatant from Flag augurin-transfected cells revealed two immunoreactive bands consistent with secretion of an Arg70 cleaved 10 kDa peptide (augurin 71–148) and a smaller 8 kDa peptide. It is suggested the 8 kDa peptide results from a second, unknown, C-terminal non-canonical cleavage site (Mirabeau et al., 2007).

c2orf40 expression has been quantified in various tissues. Microarray data suggest that, in humans, the highest expression is found in the thyroid, pituitary, testis and adrenal. In mice, the highest expression is found in the adrenals, ovaries, digits, retina and trachea [GNF Symatlas: http://symatlas.gnf.org/SymAtlas/ (last accessed 12 April 2009)] (Su et al., 2004). In the human brain c2orf40 expression is highest in the olfactory bulb, cerebellum, hypothalamus and amygdala, an expression pattern mirrored in mice (Su et al., 2004). In situ hybridization in mouse embryos has detected c2orf40 mRNA in the intermediate lobe of the pituitary, glomerular layer of the adrenal cortex, choroid plexus and atrioventricular node of the heart (Mirabeau et al., 2007).

The distribution of ECRG4-like immunoreactivity in hypothalamus has been reported in an abstract submitted for a recent scientific meeting (Roberton et al., 2009). In addition to the choroid plexus, ECRG4-like immunoreactivity was detected in the paraventricular nucleus (PVN) and supraoptic nucleus of the hypothalamus, where it colocalized with vasopressin (AVP) and oxytocin. Immunoreactivity was localized to synaptic-capillary interfaces in the median eminence, and Herring bodies in the posterior pituitary. This pattern of immunoreactivity is characteristic of a secreted peptide and suggests that ECRG4 or a product thereof may be a novel hypothalamic neuropeptide.

As c2orf40 is expressed in the brain and endocrine organs, we hypothesized that its product may be involved in the neuroendocrine system. Substantial expression in the hypothalamus could be consistent with a physiological role in the hypothalamic-pituitary axis. We therefore investigated the effects of hypothalamic injection of augurin 71–148, a probable endogenous form, on the hypothalamic-pituitary axis.

The present study found that injection of augurin into the third cerebral ventricle (i.c.v.) or into the PVN (iPVN) elevated plasma adrenocorticotrophin (ACTH) and corticosterone compared with vehicle-injected controls, and that incubation with augurin increased the release of corticotrophin-releasing factor (CRF) and AVP from hypothalamic explants. The rise in plasma ACTH and corticosterone following i.c.v. injection of augurin was blocked by pretreatment with a CRF receptor antagonist. Dysregulation of the hypothalamic-pituitary-adrenal (HPA) axis and of plasma glucocorticoid levels have been implicated in the pathogenesis of hypertension, obesity, type II diabetes and depression (Buckingham, 2006). These studies suggest that the augurin system may be an attractive pharmacological target for manipulation of the HPA axis.

Methods

Animals

All animal care and experimental procedures complied with the British Home Office Animals (Scientific Procedures) Act 1986 (Project Licence 70/6402). Male Wistar rats (specific pathogen free; Charles River, Margate, UK) weighing 250–300 g were maintained in individual cages under controlled temperature (21–23°C) and light conditions (12 h light, 12 h dark cycle; lights on at 0700 h), with ad libitum access to food (RM1 diet, SDS Ltd., Witham, UK) and water.

Peptides

A synthetic fragment of human augurin corresponding to amino acids 71–148 of ECRG4 protein was synthesized by Bachem UK Ltd. (Merseyside, UK). The synthesis was performed using a Symphony automated peptide synthesizer (Protein Technology, Inc., Woburn, MA, USA). The product was initially purified by flash chromatography with a reversed phase resin (Daisogel), followed by reversed phase high pressure liquid chromatography (HPLC). Analyses of the purified peptide by matrix-assisted laser desorption/ionization mass spectrometry, HPLC and amino acid analysis showed above 91% purity (average molecular weight 9689).

Rat neuromedin U (NMU) 23 and astressin were purchased from Bachem UK Ltd.

For all studies lyophilized augurin was first dissolved in a small amount of 0.05 M HCl, and then diluted in saline for in vivo studies or in artificial cerebrospinal fluid [(aCSF; see below for composition) for hypothalamic or pituitary explant studies] containing sufficient NaOH to neutralize the HCl. For all studies the vehicle control was prepared using identical amounts of HCl and NaOH.

Intracerebroventricular cannulation and injections

Intracerebroventricular cannulation was carried out using an established protocol (Rossi et al., 1997). A 22-gauge stainless steel guide cannula (Plastics One, Roanoake, VA, USA) projecting into the third cerebral ventricle was stereotactically implanted into each rat using coordinates calculated from the rat brain atlas of Paxinos and Watson (0.8 mm caudal to bregma in the midline and implanted 6.5 mm below the outer surface of the skull) (Paxinos and Watson, 2007), as previously described (Rossi et al., 1997). Prior to commencement of surgery, each rat received a single s.c. injection of buprenorphine (45 µg·kg⁻¹; Schering-Plough, Welwyn Garden City, UK) for analgesia. Following surgery, rats were allowed a 7 day recovery period during which time they were checked daily. Prior to the studies, each rat received two sham injections (one of angiotensin II and one of saline) to acclimatize them to the procedure. Only animals with correct cannula placement, as confirmed by a sustained drinking response to i.c.v. angiotensin II (50 ng per rat), were included in the studies.

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For i.c.v. injections, peptides were dissolved as described above and administered in 5 µL volume via a stainless steel injector projecting 1 mm beyond the tip of the cannula. Rats were returned to their own cages following the injection procedure.

Intraparaventricular cannulation and injection
Unilateral intrahypothalamic cannulation directed at the PVN (iPVN) was carried out using an established protocol (Abbott et al., 2003). Rats were maintained as for i.c.v. cannulation. A 26-gauge stainless steel guide cannula (Plastics One) was stereotactically implanted using established coordinates obtained from the Paxinos and Watson rat brain atlas (Paxinos and Watson, 2007).

For iPVN injections, peptides were dissolved as described above and administered in 1 µL volume via a stainless steel injector projecting 1 mm beyond the tip of the cannula. Correct cannula placement was confirmed histologically at the end of the study period by injection of India ink as previously described (Abbott et al., 2003). Data from rats were excluded if the injection site extended more than 0.4 mm outside the intended injection site, or if any ink was detected in the cerebral ventricular system. In total 71% of cannulae were correctly positioned. Figure S1 shows a representative section of the PVN containing India ink.

Study 1: The effect of i.c.v. augurin on plasma hormone levels
Each rat received a single i.c.v. injection of vehicle or 5 nmol augurin (n = 9–10 per group) in the early light phase (0900–1200 h). Rats were killed by decapitation 60 min after injection. Trunk blood was collected in plastic EDTA containing tubes containing 4200 kallidinogenase inactivator units of aprotinin (Bayer Corp., Haywards Heath, UK) for luteinizing hormone (LH), follicle stimulating hormone (FSH), growth hormone (GH), thyroid stimulating hormone (TSH), prolactin, testosterone, free tri-iodothyronine (T3), free thyroxine (T4) and corticosterone assays, and in plastic EDTA containing tubes for ACTH assays. Serum was immediately separated by centrifugation, frozen on dry ice and stored at −80°C (EDTA tubes).

Study 2: The effect of i.c.v. augurin on plasma corticosterone and ACTH
Each rat received a single i.c.v. injection of vehicle, 0.6, 1.7 or 5 nmol augurin in the early light phase (0900–1200 h). Rats were killed by decapitation 20 or 60 min post injection (n = 8–10 per group per time point). Trunk blood was collected and stored as above.

Study 3A: The effect of iPVN augurin on plasma corticosterone and ACTH
Each rat received a single iPVN injection of vehicle, 0.1, 0.3 or 1 nmol augurin in the early light phase (0900–1200 h). Rats were killed by decapitation 20 or 60 min post injection (n = 5–8 per group per time point). Trunk blood was collected and stored as above.

Study 3B: The effect of iPVN augurin on behaviour
Each rat received a single iPVN injection of vehicle, 1 nmol augurin or 0.3 nmol NMU 23 (n = 6–8 per group) in the early light phase (0900–1200 h). Behavioural patterns were monitored for 120 min following injection by observers unaware of the experimental treatments. Behaviour was classified into eight different categories: feeding, drinking, grooming, burrowing, rearing, locomotion, head down and sleeping, adapted from Fray et al. (1980). These methods have previously been used to demonstrate abnormal behaviour following iPVN injection of peptides (Wren et al., 2002). During the analysis, each rat was observed for 15 s every 5 min. Each 15 s period was further subdivided into three and the predominant behaviour of the rat during each 5 s episode was noted. NMU was used as a positive control as it is known to increase grooming behaviour and decrease sleeping (Wren et al., 2002).

Study 4: The effect of augurin on the release of CRF and AVP from ex vivo hypothalamic explants
A static incubation system was used as described previously (Bewick et al., 2005). Briefly, a 1.9 mm slice was taken from the basal hypothalamus and incubated in individual tubes containing 1 mL aCSF (20 mM NaHCO3, 126 mM NaCl, 0.09 mM Na2HPO4, 6 mM KCl, 1.4 mM CaCl2, 0.09 mM MgSO4, 5 mM glucose, 0.18 mg·mL−1 ascorbic acid and 100 µg·mL−1 aprotinin) saturated with 95% O2 and 5% CO2 at 37°C. After an initial 2 h equilibration period, hypothalamic slices were incubated for 45 min in 600 µL aCSF (basal period), before being treated with 1, 10 or 100 nM augurin in 600 µL aCSF for 45 min (n = 13–15 hypothalami per group). Finally, tissue viability was assessed by 45 min incubation in isotonic aCSF containing 56 mM KCl. Explants that failed to show peptide release above the basal level in response to aCSF containing 56 mM KCl were excluded from the data analysis. At the end of each period, aCSF was collected and stored at −20°C until measurement of CRF and AVP by radioimmunoassay (RIA).

Study 5: The effect of peripheral CRF receptor antagonism on augurin-stimulated ACTH and corticosterone secretion
Intracerebroventricular cannulated rats were injected i.p. with 100 µg·kg−1 astressin or vehicle 15 min before receiving an i.c.v. injection of 5 nmol augurin or vehicle. Astressin (cyclopentyl[D-Phe213,Leu215,Glu125,Lys512]CRF-(12-41)) is a CRF1 receptor antagonist (Gulyas et al., 1995), not known to cross the blood–brain barrier. This dose of astressin has previously been used to block the increase in plasma ACTH and corticosterone caused by i.c.v. injection of GLP-1 (Kinzig et al., 2003). Rats were decapitated 30 min after the i.c.v. injection (n = 7–9 per group). Trunk blood was collected and stored as above.

Study 6A: The effect of peripherally administered augurin on plasma hormone levels
Rats were handled daily and received twice weekly i.p. injections to acclimatize them to the injection procedure. On the
day of the study, rats were injected i.p. with 100 nmol·kg⁻¹ augurin or vehicle during the early light phase (0900–1200 h).

Study 6B: The effect of augurin on ACTH release from pituitary segments

Static pituitary explants were performed as previously described (Smith et al., 2006). Briefly, the pituitaries of ad libitum fed rats were harvested immediately following decapitation. The posterior pituitary was removed and discarded, and the remaining anterior pituitary was bisected along the mid-saggital line and then divided into four pieces of approximately equal size. After an acclimatization period, the segments were then incubated in one of aCSF alone, or aCSF containing 10 nM augurin, 100 nM augurin, 1000 nM augurin or 100 nM CRF for 4 h (n = 17–19 per group). At the end of this period the incubation medium was collected and stored at –20°C until assayed for ACTH.

Radioimmunoassays

RIAs for CRF immunoreactivity and AVP immunoreactivity were performed using established methods (Dhillon et al., 2003). The intra- and interassay coefficients of variation were <10% for the CRF RIA, and 11% and 20%, respectively, for the AVP RIA. Plasma corticosterone was measured using an RIA kit from MP Biomedicals, Inc. (Orangeburg, NY, USA), for which the intra- and interassay coefficients of variation were less than 10% and 7% respectively. For studies 1, 2 and 3A, plasma ACTH was measured by immunoradiometric assay purchased from GuC Diagnostica B.V. (Arnhem, the Netherlands). The intra- and interassay coefficients of variation were both less than 4%. For study 5, plasma ACTH was measured by immunoradiometric assay purchased from BioSource Europe S.A. (Nivelles, Belgium) because the previously used kit was no longer available. The intra- and interassay coefficients of variation were 6.4% and 6.2% respectively. Plasma TSH, LH, FSH, prolactin, GH and ACTH release from pituitary segments were measured, using methods and reagents provided by the National Hormone and Pituitary programme. Total plasma testosterone, free T3 and free T4 were measured using commercial Coat-a-Count assay kits (Euro/DPC Limited, Caernarfon, UK).

Statistics

Data from hypothalamic explant, pituitary explant and terminal studies are presented as mean ± SEM. Data from behavioural analyses are presented as median frequency and interquartile range for each behaviour. For plasma ACTH and corticosterone studies, values were log-transformed to homogenize variances among groups, and to improve the normality of residuals (Bland and Altman, 1996). Transformed values were then analysed by Student’s t-test, or one- or two-way ANOVA followed by post hoc Holm-Sidak test (SigmaStat 3.5, San Jose, CA, USA). Data from the behavioural study were analysed using Kruskal-Wallis one-way ANOVA on ranks (Systat 11, San Jose, CA, USA).

Nomenclature

The nomenclature of receptors and peptides described in this manuscript conform to BJP’s Guide to Receptors and Channels (Alexander et al., 2009).

Results

Study 1: The effect of i.c.v. augurin on plasma hormone levels

Augurin significantly increased plasma ACTH 60 min after injection (Table 1). Plasma corticosterone was raised but failed to reach statistical significance. There were no significant changes in plasma LH, FSH, GH, prolactin, testosterone, free T3 or free T4 (Figure 1).

<table>
<thead>
<tr>
<th>Study</th>
<th>Hormone</th>
<th>Vehicle (pg·mL⁻¹)</th>
<th>Augurin (5 nmol) (pg·mL⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
<td>Mean</td>
</tr>
<tr>
<td>ACTH</td>
<td>21.3</td>
<td>3.2</td>
<td>66.7***</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>152.8</td>
<td>25.9</td>
<td>300.0</td>
</tr>
<tr>
<td>LH</td>
<td>0.60</td>
<td>0.09</td>
<td>0.54</td>
</tr>
<tr>
<td>FSH</td>
<td>8.97</td>
<td>0.65</td>
<td>7.18</td>
</tr>
<tr>
<td>Testosterone</td>
<td>4.25</td>
<td>0.87</td>
<td>4.96</td>
</tr>
<tr>
<td>TSH</td>
<td>2.38</td>
<td>0.14</td>
<td>2.21</td>
</tr>
<tr>
<td>Free T4 (ng·L⁻¹)</td>
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<td>1.1</td>
<td>22.7</td>
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<tr>
<td>Free T3 (pg·L⁻¹)</td>
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<tr>
<td>PRL</td>
<td>5.28</td>
<td>0.79</td>
<td>5.15</td>
</tr>
<tr>
<td>GH</td>
<td>20.27</td>
<td>4.38</td>
<td>22.39</td>
</tr>
</tbody>
</table>

Results are mean ± SEM. **P < 0.001 versus vehicle, n = 9–10 per group.

FSH, follicle stimulating hormone; GH, growth hormone; LH, luteinizing hormone; PRL, prolactin; T3, tri-iodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone.
Post hoc analysis demonstrated that i.c.v. injection of 5 nmol augurin significantly increased plasma corticosterone 20 min post injection (P < 0.001).

Study 3A: The effect of iPVN augurin on plasma corticosterone and ACTH
Plasma ACTH and corticosterone levels were significantly elevated following iPVN microinjection of augurin compared with vehicle-injected controls (Figure 2). In the case of ACTH, two-way ANOVA revealed that there was a significant main effect of dose (F(3,45) = 9.891; P < 0.001), a significant main effect of time (F(1,45) = 5.363; P < 0.05) and no significant interaction (F(3,45) = 0.600; P = 0.618). Post hoc analysis demonstrated that iPVN microinjection of 1 nmol augurin significantly increased plasma ACTH 20 and 60 min post injection (P < 0.001 and P < 0.01 respectively).

In the case of corticosterone, two-way ANOVA revealed that there was a significant main effect of dose (F(3,45) = 6.247; P < 0.001), no significant main effect of time (F(1,45) = 2.275; P = 0.138) and no significant interaction (F(3,45) = 1.129; P = 0.347). Post hoc analysis demonstrated that iPVN microinjection of 1 nmol augurin significantly increased plasma corticosterone 20 min post injection (P < 0.01).

Study 3B: The effect of iPVN augurin on behaviour
There were no significant behavioural differences between iPVN augurin- or vehicle-injected rats. Injection of 0.3 nmol NMU significantly increased time spent grooming and decreased time spent sleeping or feeding (Table 2).

Study 4: The effect of augurin on the release of CRF and AVP from ex vivo hypothalamic explants.
Incubation of hypothalamic explants with augurin significantly increased the release of CRF and AVP (Figure 3).

Study 5: The effect of peripheral CRF receptor antagonism on augurin-induced ACTH and corticosterone secretion
Pretreatment with astressin completely blocked the increase in plasma ACTH and corticosterone 30 min post i.c.v. injection of 5 nmol augurin (Figure 4).

Study 6A: The effect of peripherally administered augurin on plasma hormone levels
There were no significant differences in plasma corticosterone, LH, FSH, GH, TSH, prolactin, free T3 or free T4, 30 minutes following i.p. injection of 100 nmol·kg⁻¹ augurin (Table S1).

Study 6B: The effect of augurin on ACTH release from pituitary segments
No significant change in ACTH release was seen following incubation of pituitary segments with 10, 100, or 1000 nM augurin, compared with aCSF alone. Incubation with 100 nM CRF resulted in a significant increase in ACTH release (ACTH ng/explant; vehicle 192.9 ± 23.1; CRF 100 nM 296.7 ± 36.2, P < 0.01) (Figure S2).

Discussion
Augurin is a recently identified secreted peptide of unknown function. It is expressed in the CNS and endocrine organs (Su...
et al., 2004; Mirabeau et al., 2007). We therefore examined the effects of augurin on the hypothalamo-pituitary axes. Both i.c.v. and iPVN injection of augurin elevated plasma ACTH and corticosterone compared with vehicle-injected controls, and augurin increased the release of CRF and AVP from hypothalamic explants. The rise in plasma ACTH and corticosterone following i.c.v. injection of augurin was blocked by pretreatment with a CRF receptor antagonist.

Intracerebroventricular injection of 5 nmol augurin significantly increased plasma ACTH and corticosterone 20 min after injection, compared with vehicle-injected controls. Injection of 0.6 or 1.7 nmol augurin appeared to cause a delayed rise in plasma ACTH, evident only at 60 min. The reason for this delay is unclear; it is possible that it represents the time taken for the peptide to diffuse to its site(s) of action. Alternatively, small differences in plasma ACTH at 20 min may have been masked by the stress of the experimental procedure.

Corticotrophin-releasing factor and AVP are the main regulators of ACTH levels; they act synergistically on the pituitary to stimulate ACTH secretion (Whitnall, 1993). Augurin increased the release of CRF and AVP from explanted hypothalami, and pretreatment with a CRF\(_{12}\) receptor antagonist blocked the rise in plasma ACTH and corticosterone caused by i.c.v. injection of augurin. This suggests augurin stimulates the secretion of ACTH via the release of hypothalamic CRF and AVP, although the relative contribution of AVP cannot be determined from the antagonist study as CRF is necessary for AVP stimulated ACTH release (Whitnall, 1993).

![Figure 2](image-url)  
**Figure 2** Effect of a single iPVN (paraventricular nucleus) injection of augurin (0.1, 0.3 or 1 nmol) or vehicle in *ad libitum* fed male Wistar rats on plasma ACTH and corticosterone (CORT) at 20 (A and C) and 60 (B and D) min post injection. *P* < 0.05, **P* < 0.01, ***P* < 0.001 versus vehicle, *n* = 5–8 per group per time point. Results are mean ± SEM.

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Effect of a single iPVN injection of augurin (1 nmol), NMU (0.3 nmol) or vehicle in <em>ad libitum</em> fed male rats on behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Feeding</td>
</tr>
<tr>
<td>0–60 min</td>
<td></td>
</tr>
<tr>
<td>NMU 0.3 nmol</td>
<td>0 [0:0]</td>
</tr>
<tr>
<td>61–120 min</td>
<td></td>
</tr>
<tr>
<td>Vehicle</td>
<td>0 [0:0]</td>
</tr>
<tr>
<td>Augurin 1 nmol</td>
<td>0 [0:0]</td>
</tr>
<tr>
<td>NMU 0.3 nmol</td>
<td>0 [0:0]</td>
</tr>
<tr>
<td>0–120 min</td>
<td></td>
</tr>
<tr>
<td>NMU 0.3 nmol</td>
<td>0 [0:0]*</td>
</tr>
</tbody>
</table>

Data presented are median frequency and interquartile range.  
*P* < 0.05, **P* < 0.01, ***P* < 0.001 versus vehicle, *n* = 6–8 per group.  
NMU, neuromedin U; PVN, paraventricular nucleus.
Corticotrophin-releasing factor neurons in the PVN are the major conduit for endocrine, autonomic and behavioural responses to stress (Herman, 1997). The PVN contains a subpopulation of hypophysiotropic neurons that constitute the primary source of CRF and AVP (Whitnall, 1993). We therefore examined the effects of injecting augurin directly into the PVN. Intranuclear injection of lower doses minimizes any non-specific side effects of peptide administration that may occur following i.c.v. injection (Dhillo et al., 2003).

Microinjection of 1 nmol augurin iPVN significantly increased plasma ACTH and corticosterone, a much lower dose than required for a similar effect when given i.c.v. This could indicate that the receptors for augurin are found within or close to the PVN. It is, however, possible that lower concentrations of augurin could diffuse into adjacent nuclei, and we cannot discount administration of augurin to other hypothalamic areas having a similar effect. We did not observe any significant effects on ACTH or corticosterone following iPVN injection of 0.1 or 0.3 nmol augurin. However, we cannot rule out slightly elevated ACTH levels in the vehicle group masking minor effects at the 20 min time point. There were no significant differences in behaviour in the 2 h following iPVN microinjection of 1 nmol augurin, compared with vehicle-injected controls. In particular, no adverse behaviour was observed in the augurin group, indicating that non-specific activation of the HPA axis as a result of a noxious effect is unlikely.

Intracerebroventricular injection of 5 nmol augurin had no effect on plasma LH, FSH, testosterone, TSH, free T3, free T4, prolactin or GH at 60 min post injection, suggesting the neuroendocrine effects of augurin are specific to the HPA axis. Peripheral administration of augurin at doses of 100 nmol·kg−1 had no effect on plasma corticosterone at 30 min (Table S1). This dose is relatively high; i.p. injection of 4.2 nmol·kg−1 of CRF causes a significant rise in ACTH (Watanabe et al., 1991). In addition, we have shown that incubation of pituitary segments with augurin at concentrations of up to 1000 nM has no effect on ACTH release. It therefore appears unlikely that peripheral augurin acts directly on the pituitary to release ACTH unless it is released into the circulation at very high concentrations. To date, the presence of augurin in the circulation has not been investigated.

The primary sequence or sequences of endogenous augurin are unknown. While these data show residues 71–148 have a biological effect, it is also possible shorter fragments of augurin are active.
Augurin may be biologically active. The N-terminal segment of augurin adjacent to the dibasic cleavage site has substantially higher interspecies conservation than the rest of the peptide (Mirabeau et al., 2007). We hypothesize that the increase in conservation implies the N-terminal segment is important for biological activity. Identifying the endogenous forms of augurin and determining the minimum biologically active sequence would provide a basis for developing structural analogues acting as agonists or antagonists of the augurin system.

Data from our studies demonstrate injection of augurin into the hypothalamus stimulates the HPA axis, culminating in the release of glucocorticoids. Various pharmacotherapies have been developed based on components of the HPA axis. Glucocorticoid receptor agonists are widely used as anti-inflammatory and immunosuppressive agents (Rhen and Cidlowski, 2005). More recently, antagonists of the CRF receptors have shown promise in the treatment of anxiety and depression, sleep disorders, addictive behaviour and preterm labour (Habib et al., 2000; Zoumakis et al., 2006). While the data presented suggest a novel role for augurin, these are preliminary studies. Currently, little is known about the physiology of the augurin system. Further characterization of the neuroanatomy, identification of receptors and the development of functional analogues would aid us in establishing whether the augurin system is a viable target for manipulating the HPA axis.

Acknowledgements

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Statement of conflict of interest

SM is an employee of Bachem UK Ltd. JAT, MP, KS, KEB, CKB, KLS, MAG and SRB have nothing to disclose.

References


Bewick GA, Dhillon WS, Darch SJ, Murphy KG, Gardiner JV, Jethwa PH et al. (2005). Hypothalamic cocaine- and amphetamine-regulated transcript (CART) and agouti-related protein (AgRP) neurons coexpress the NOP1 receptor and nociceptin alters CART and AgRP release. Endocrinology 146: 3526–3534.


Yue CM, Deng DJ, Bi MX, Guo LP, Lu SH (2003). Expression of ECRG4,


**Supporting information**

Additional Supporting Information may be found in the online version of this article:

**Figure S1** Photomicrograph of the spread of 1 μL India ink injected into the paraventricular nucleus. The section was not stained so that the ink could be clearly seen. 3V, third ventricle; ot, optic tract.

**Figure S2** Effect of augurin (10, 100 or 1000 nM) or corticotrophin-releasing factor (CRF) (100 nM) on ACTH release from pituitary segments. **P < 0.01, n = 17–19 per group. Results are mean ± SEM.

**Table S1** Effect of a single i.p. injection of augurin (100 nmol·kg⁻¹) or vehicle in *ad libitum* fed rats on plasma corticosterone, LH, FSH, GH, TSH, prolactin, free T3 and free T4 at 30 min post injection

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Supplemental Figure list

**Figure S1**: Photomicrograph of the spread of 1µl India ink injected into the PVN. The section was not stained so that the ink could be clearly seen. 3V, 3rd ventricle; ot, optic tract.

**Figure S2**: Effect of augurin (10, 100, or 1000nM) or CRH (100nM) on ACTH release from pituitary segments. **p<0.01, n = 17-19 per group. Results are mean ± SEM.**
Figure S2

The bar chart shows the effect of different concentrations of Augurin (in nM) on the secretion of ACTH (ng/explant). The x-axis represents the concentrations of Augurin (10, 100, 1000, and CRH), and the y-axis represents the secretion of ACTH. The data points are presented as mean ± SEM. The CRH group shows a significantly higher secretion of ACTH compared to the other groups, indicated by the ** symbol.
Supplementary Table 1

<table>
<thead>
<tr>
<th></th>
<th>Vehicle</th>
<th>Augurin (100 nmol/kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
<td>SEM</td>
</tr>
<tr>
<td>CORT (ng/ml)</td>
<td>34.5</td>
<td>12.6</td>
</tr>
<tr>
<td>LH (ng/ml)</td>
<td>0.84</td>
<td>0.11</td>
</tr>
<tr>
<td>FSH (ng/ml)</td>
<td>9.40</td>
<td>0.67</td>
</tr>
<tr>
<td>TSH (ng/ml)</td>
<td>2.21</td>
<td>0.19</td>
</tr>
<tr>
<td>Free T4 (ng/L)</td>
<td>22.5</td>
<td>1.1</td>
</tr>
<tr>
<td>Free T3 (pg/L)</td>
<td>10.8</td>
<td>1.5</td>
</tr>
<tr>
<td>PRL (ng/ml)</td>
<td>3.87</td>
<td>0.66</td>
</tr>
<tr>
<td>GH (ng/ml)</td>
<td>105.9</td>
<td>33.1</td>
</tr>
</tbody>
</table>

Effect of a single ip injection of augurin (100 nmol/kg) or vehicle in *ad libitum* fed rats on plasma corticosterone, LH, FSH, GH, TSH, prolactin, free T3, and free T4 at 30 minutes post injection. Results are mean ± SEM, n = 7-9 per group.