Modulation of gut-brain signalling: CCK effects on vagal afferent neurons

Thesis sumitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy by

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Je voudrais dédier cette thèse à mes parents. Sans leur amour, éducation, et encouragements aucun de ceci aurait été possible.

CONTENTS

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ABSTRACT

The neuropeptide transmitter cocaine and amphetamine regulated transcript (CART) inhibits food intake. CART could act as a mediator of the effects of cholecystokinin (CCK), since like CCK it can inhibit feeding when administered centrally, and co-localises with CCK1 receptors in vagal afferent neurons. This thesis focuses on the regulation of CART expression by CCK in these neurons.

Immunohistochemistry of nodose ganglia and cultured vagal afferent neurons were used to visualise changes in CART peptide expression. A CART-promoter luciferase-reporter construct was used to study transcriptional mechanisms.

In rat vagal afferent neurons, CART was virtually undetectable after energy restriction for 24 hour; administration of CCK to fasted rats increased CART, and refeeding of fasted animals promptly increased CART-immunoreactivity in vagal afferent neurons by a mechanism sensitive to a CCK-1 receptor antagonist. In vagal afferent neurons cultured in serum-free medium, CART was virtually undetectable but was rapidly induced by addition of CCK. Conversely, in serum free medium, the orexigenic peptide MCH was expressed in vagal afferent neurons and was rapidly down-regulated by CCK. Using a CART-promoter luciferase-reporter construct transfected into cultured vagal afferent neurons CCK was found to stimulate CART transcription by

activation of protein kinase C and CREB and were down-regulated by orexigenic hormones such as orexin-A and ghrelin.

The effects of CCK on vagal afferent neurons include rapid up-regulation of the putative satiety factor CART through a mechanism involving PKC and CREB, as well as down-regulation of orexigenic compounds. Conversely orexigenic hormones were found to down-regulate CART expression by inhibiting phosphoCREB nuclear localisation, while up-regulating molecules associated with stimulation of food intake.

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LIST OF ABBREVIATIONS

AgRP	Agouti related protein
Arc	Arcuate nucleus
BMI	Body mass index
Bp	Base pair
bZIP	Basic leucine zipper domain
CART	Cocaine and amphetamine related protein
CART LUC	CART-promoter luciferase-reporter construct
CB1R	Cannabinoid 1 receptor
CBP	CREB binding protein
CCK	Cholecystokinin
CCK1R	Cholechystokinin-1 receptor
CCK2R	Cholecystokinin-2 receptor
CNS	Central nervous system
CRE	cyclic AMP response element
CREB	cyclic AMP response element binding protein
DMN	Dorsal motor neuron
DTT	Dithiothreitol
E1A	Adenovirus early region 1
EGR-1	Early growth factor 1
ER	Endoplasmic reticulum
ERK	Extracellular signal-regulated kinase
FBS	Foetal bovine serum

FITC	Fluorescein
FM	Full media
GFAP	Glial fibrillary acidic protein
GFP	Green fluorescent protein
Gh	Ghrelin
GHS1R	Growth hormone secretagogue receptor 1
GI	Gastrointestinal
GPCR	G-protein coupled receptor
HBSS	Hank's buffered salt solution
HDMEM	HEPES-buffered dulbecco's modified eagle's medium
Hr	Hour
i.c.v.	intracerebroventricular
i.p.	Intraperitoneal
JAK	Janus kinase
kD	Kilodalton
LB	Luria-Bertani broth
LH	Lateral hypothalamus
MAPK	MAP kinase
MCH	Melanin concentrating hormone
Min	Minutes
mTOR	Mammalian target of rapamycin
NLS	Nuclear localisation signal
NPY	Neuropeptide Y

NSE	Neuron specific enolase
NTS	Nucleus of the tractus solitarius
OxA	Orexin-A
OxR1	Orexin receptor 1
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PI3K	Phosphoinositide kinase-3
PIKK	Phosphoinositide kinase-related kinase
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PKC-I	Protein kinase C inhibitor
PLC	Phospholipase C
PMA	Phorbol 12-myristate 13-acetate
POMC	Pro-opiomelanocortin
PVN	Paraventricular nucleus
RIA	Radioimmunoassay
rlCART	Long form of rat CART
rsCART	Short form of rat CART
sec	Seconds
SF	Serum free
STAT	Signal transducers and activators of transcription
TGN38	Trans-golgi network 38

TRH	Thyrotropin-releasing hormone
TxR	Texas Red
VAN	Vagal afferent neurons
VMN	Ventromedial nucleus
WT	Wild type

CHAPTER ONE

Introduction

The role of the vagus nerve in the control of the digestive tract, the heart and respiratory tract, and in the regulation of appetite, has attracted intense attention since the 19th century. At the start of the 20th century Pavlov received a Nobel Prize for his work on the role of the vagus in the control of acid secretion. In addition, Pavlov observed that in dogs with pancreatic fistulas, electrical stimulation of the vagus nerve elicited a strong secretory response of the exocrine pancreas. This and subsequent findings prompted him to conclude that the pancreatic secretory response to nutrients is mediated by enteropancreatic reflexes involving the vagus nerves (Pavlov, 1910). However, with the discovery of the first hormone, secretin, by Bayliss and Starling in 1902, the idea that control of pancreatic secretory responses to nutrients was exclusively neural was overturned (Bayliss and Starling, 1902). In recent decades, the idea has emerged that there are interactions at many levels between the gut hormones and other regulatory mechanisms including those involving the vagus nerve.

It was already clear early in the 20th century that the vagus nerve subserves both afferent and efferent functions (Hertz, 1911). Work on other afferent systems (e.g., vision, audition, olfaction, taste) involving the isolation and characterisation of receptor cells, the site of stimulation, specification of the energetic event that is transduced to a neural signal, the cloning of relevant membrane-bound receptors, and that identification of the second-messenger pathways has transformed

our understanding of these systems in recent years. In the case of gastrointestinal vagal afferent mechanisms, it is clear that these are initiated when vagal afferent terminals are activated by chemical, physical or heat stimuli. Importantly it is now also clear that vagal afferent nerve fibres respond to gut hormones, thereby linking the original work of Bayliss & Starling, and of Pavlov.

In recent years there has been growing interest in the role of the vagus in the control of food intake (Schwartz, 2000). The decision to begin or to stop eating is derived from complex processes. Many factors have been identified as contributors to the control of eating. Several peptides and hormones from the gut have been shown to act either peripherally or in the central nervous system (CNS) to influence the relevant systems. Interest in these mechanisms has grown considerably with the recognition that an obesity epidemic is sweeping the developed, and parts of the developing, world. Current drug treatments for obesity lead to modest weight loss, and may be poorly tolerated. Continuing research in the physiology of appetite regulation may therefore provide the basis for further drug treatments of obesity.

1.1 THE GUT

The primary function of the gut is the uptake of water and nutrients. Most of the nutrient molecules present in food are large polymeric molecules that must be mechanically and enzymatically broken down into smaller molecules before they can be absorbed. Digestion, absorption, and secretion take place in different organs of the gastrointestinal (GI) tract and specific functions tend to be localized to the main regions of the gut, namely the stomach, the small intestine, and the distal and proximal colon.

There is a continuous back and forth passage of information between the GI tract, and the brain and spinal cord, known collectively as the brain-gut axis. These interrelated circuits can influence both brain processes and bowel functions, affecting pain perception, gut sensitivity, secretion, inflammatory responses and motility. Peptide hormones from the GI tract play an important role in the short-term regulation of food intake and satiety by influencing the initiation and termination of meals. Both the vagus nerve and non-vagal splanchnic mesenteric nerves supply pre-absorptive upper gastrointestinal sites that handle ingested nutrients, and together they comprise the peripheral extrinsic neural component of the gut–brain axis.

1.2 THE VAGUS NERVE

Vesalius originally referred to the vagus as the 'wandering nerve', in recognition of its wide distribution to thoracic and abdominal organs (Skandalakis *et al.*, 1986). The vagus nerve is the tenth of twelve paired cranial nerves, and is the only one that starts in the brainstem (within the medulla oblongata) and extends, through the jugular foramen, down below the head, to the abdomen (see figure 1.1).

The earliest studies of the effect of the vagus on gut function centred on its efferent activities. In 1814, Brodie noticed that the mucosa of the empty stomach was dry following vagal section (Brodie, 1814), whilst later, Bernard observed that the same procedure led to a reduction in gastric contractions (Bernard, 1858). In addition to motor functions, it is now apparent that vagal trunks contain both afferent and efferent fibres in a ratio of about 9:1 (Grundy, 1989; Sengupta, 1994).

1.2.1 Vagal afferent neurons (VAN)

Vagal afferent nerve fibres carry information about the physiological status of the gut directly to brainstem circuits regulating digestive function (see figure 1.1). They innervate the entire gut except the distal third of the colon (Grundy, 1989; Sengupta, 1994). Most of the cell bodies of VAN are contained within the nodose ganglion

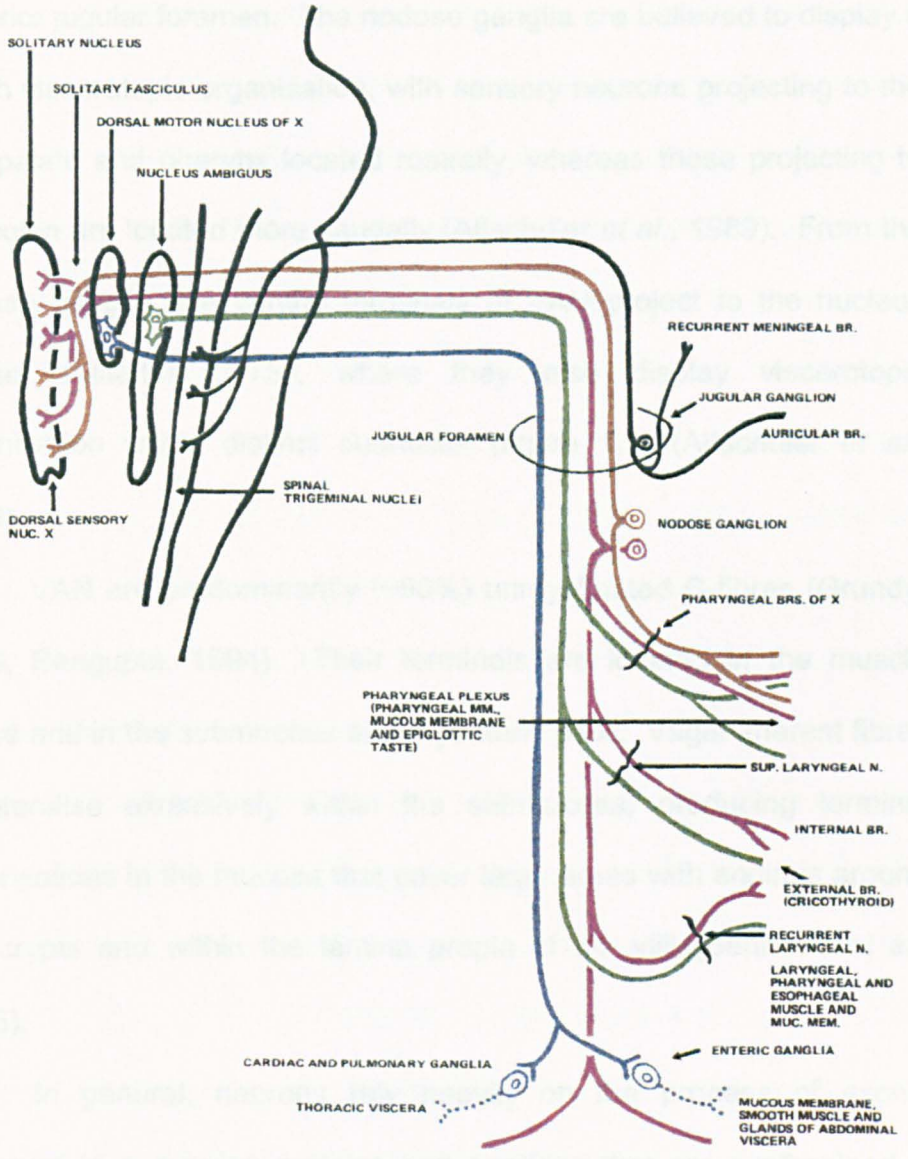


Figure 1.1. The vagus nerve. The origin and site of action of the different fibres of the vagus nerve. The orange line indicates vagal afferent fibres. The blue line indicates vagal efferent fibres.

located just below the jugular foramen, with a minority residing in the superior jugular foramen. The nodose ganglia are believed to display a rough viscerotopic organisation, with sensory neurons projecting to the soft palate and pharynx located rostrally, whereas those projecting to the colon are located more caudally (Altschuler *et al.*, 1989). From the nodose ganglia, the central terminals of VAN project to the nucleus tractus solitarius (NTS), where they also display viscerotopic organisation within distinct subnuclei (figure 1.1) (Altschuler *et al.*, 1989).

VAN are predominantly (~90%) unmyelinated C-fibres (Grundy, 1989; Sengupta, 1994). Their terminals are located in the muscle layers and in the submucous and myenteric plexi. Vagal afferent fibres collateralise extensively within the submucosa, producing terminal arborisations in the mucosa that cover large areas with endings around the crypts and within the lamina propia of the villi (Berthoud *et al.*, 1995).

In general, neurons rely heavily on the process of axonal transport to conveying proteins and peptides that are synthesised in nerve cell body to the sites at which they function within axons, dendrites or nerve terminals. The vagus nerve provides an excellent model for the study of transport phenomena since both cell body and nerve trunk are easily accessible by surgery, and its length permits experiments to be undertaken over a sufficient period of time for the

various phases of transported materials to be separated. As in other nerves, proteins are axonally transported in a number of distinct ways classified according to the rates of transport and the particular protein concerned. Radiolabeling techniques, in which radiolabeled amino acids are injected into the nodose ganglia have demonstrated that a major group of proteins is transported at a fast rate of around 400 mm/day (Sjostrand, 1970; McLean *et al.*, 1976; Tashiro, 1980). The polypeptide composition of the fast transported proteins has been determined in guinea pig and rabbit vagus down to a limit of molecular weight around 15kDa (Black and Lasek, 1978; Tashiro, 1980; McLean *et al.*, 1983), but our knowledge of the functions of the fast transported proteins is extremely limited.

Rather better characterised are the slowly transported proteins in the vagus nerve. Two main groups of proteins are transported in the anterograde direction in both motor and sensory vagal fibres with transport rates of 12 to 15 and 25 to 30mm/day respectively in the rabbit (Sjostrand, 1970; McLean *et al.*, 1976; Bajada *et al.*, 1980; Sato *et al.*, 1986) and similar rates in the rat and guinea pig (Tashiro, 1980; Yokokoyama, 1980; Varro *et al.*, 1988). The polypeptide composition of transported proteins in the vagus is similar to that in other neurons, comprising a group of proteins in the faster moving phase, and a slower phase (McLean *et al.*, 1983; McLean, 1985).

1.2.2 Vagal efferent nerves

Subsequent to the work of Brodie and Bernard, Pavlov and his pupils established, that the vagus was the main secretomotor nerve of the gut (Pavlov, 1910). Efferent fibres of the vagus originate in the dorsal motor nucleus (DMN) (see figure 1.1). Vagal efferent neurons that innervate smooth muscle may be excitatory or inhibitory, whilst those that innervate gastric or duodenal glands are principally stimulatory secretomotor neurons (Wood, 1999).

1.3 THE SPLANCHNIC NERVE

The lower gut is supplied by the thoracic and lumber spinal nerves. These arise from vertebrae T7-T10. Axons of thoracic and lumbar spinal primary afferent nerves pass through sympathetic ganglia to reach the gut via splanchnic and mesenteric nerves; whilst axons of spinal primary afferent neurons, with cell bodies in sacral ganglia, follow the pelvic nerves to reach the large intestine. Approximately 10-20% of fibres in the major splanchnic nerves are afferent in nature and are unmyelinated C and A-delta fibres, with terminals mainly in the muscle layer, serosal layer and mesentery of the gut (Grundy, 1989; Sengupta, 1994). These fibres are believed to transmit painful sensations: in general, low-threshold afferent endings are vagal in origin and high-threshold endings are of spinal origin. However, some

spinal primary afferent neurons can also be activated by low-threshold stimuli, implying that these fibres also transmit non-nociceptive signals (Sengupta, 1994). In contrast to VAN, there is much less evidence that spinal afferent terminals reach the mucosa of the GI tract. However, the existence of spinal afferent fibres supplying the gastric mucosa of the rat has been proposed, and these may play a role in cytoprotection (Holzer *et al.*, 1991; Raybould *et al.*, 1992).

Spinal afferent neurons have their cell bodies in dorsal root ganglia and their central processes terminate in the dorsal horn of the spinal cord. Spinal afferent neurons are organised segmentally, albeit somewhat diffusely over several spinal segments, which probably explains the poor viscerotopic localisation of nociceptive sensation in the GI tract (Grundy, 1989; Sengupta, 1994). Second order neurons ascend in the spinoreticular and spinothalamic tracts, although the dorsal columns have also been recognised to convey information from the gut (Aziz and Thompson, 1998). These pathways project to the thalamus via relays in the brainstem and the midbrain. From the thalamus, sensory information passes to the insular cortex, the primary somatosensory cortex and the prelimbic, limbic and infralimbic areas of the medial prefrontal cortex (Aziz and Thompson, 1998). Sympathetic efferent nerves from cervical, thoracic and lumbar spinal cord segments project to intrinsic myenteric ganglia and mainly exert an inhibitory effect on GI function (Aziz and Thompson, 1998).

1.4 GUT ENDOCRINE CELLS

1.4.1 History

While investigating the innervation of the pancreas and duodenum, Bayliss and Starling were repeating Pavlov's experiments on the nervous control of the gut (Bayliss and Starling, 1901; Bayliss and Starling, 1902). Pavlov believed that pancreatic secretion was solely controlled by the vagus; when acid gastric contents passed into the duodenum, vagal afferent fibres in the duodenal wall passed to the brain, and vagal efferent fibres returned to the pancreas, thus stimulating the secretion of pancreatic juice into the duodenum. Bayliss and Starling carefully dissected away all the nerves supplying the jejunum. They then put acid into the jejunum and pancreatic secretion occurred in the normal way. So it was possible that some other mechanism was responsible for pancreatic secretion in response to acid in the duodenum. Working on the hypothesis that acid caused the release of something from the small intestine into the blood, they scraped some jejunal mucosa, added acid to it, ground it up with sand, filtered it and injected it intravenously (i.v.) into an anaesthetised dog and found that pancreatic secretion followed. They called the active factor "secretin" and then investigated the nature of secretin. The working hypothesis was that it existed in the wall of the small intestine as a precursor ('pro-secretin'), which was released under the influence of acid. Hence the first GI hormone was discovered.

1.4.2 Structure

Basal-granulated cells dispersed in the epithelium comprise the endocrine elements of the gut. Early investigators mostly believed that the gut basal-granulated cells consisted of a single cell type, the enterochromaffin cell secreting seretonin (Fujita and Kobayashi, 1977). However, electron microscope studies and immunocytochemical evidence from about 1970 onwards made it clear that numerous cell types, besides the enterochromaffin cells, represented the sources of different hormones.

Gut endocrine cells are distributed from the cardia nearly down to the anus. The cells are pyramidal or spindle-shaped and generally extend a cytoplasmic process to the gut lumen (i.e. "open" cells). However, in the oxyntic area of the stomach the cells lie flat on the basement membrane and do not reach the epithelial surface (i.e. "closed" cells) (Fujita, 1973).

The secretory granules vary between cell types in their immunochemical properties and electron microscopic morphology. Peptides secreted by gut endocrine cells, as in other secretory cells, are synthesised as precursors in rough endoplasmic reticulum (ER) and transported to the Golgi apparatus to be packed in secretory granules (Kobayashi, 1975; Kobayashi, 1976).

The apical process of the open type endocrine cell is covered by microvilli whose shape is characteristic of the cell type. These microvilli

are believed to constitute a sensory apparatus that detects changes in the chemical content of the lumen and modulates the endocrine secretion of the cell appropriately (Fujita, 1973; Fujita and Kobayashi, 1977). Many different types of enteroendocrine cells have been identified and are categorised by the regulatory peptides that they synthesise and secrete (Table 1). Different enteroendocrine cells are sensitive to different classes of nutrients such as carbohydrates, fats, or proteins (Roberge and Brubaker, 1991; Pironi *et al.*, 1993; Layer *et al.*, 1995; Tso *et al.*, 1995; Raybould, 1999; Xiao *et al.*, 1999). The secretions of enteroendocrine cells are mainly peptides, and they either enter the bloodstream and act as hormones, or diffuse through the extracellular fluid to act in a paracrine fashion on nearby cells (Reidelberger, 1994; Holzer *et al.*, 2001; Lundgren, 2004). The hormonal actions of these peptides are relatively well-known and involve, for example, actions at the gallbladder (Liddle *et al.*, 1985), pancreas (Chey and Chang, 2001), CNS, vagus, immune cells, gastric epithelium, and smooth muscle (Brubaker *et al.*, 1997). These are the actions that enable the proper mix of digestive enzymes to be added to the chyme to ensure optimal digestion, and this subsequently regulates movement of the gut contents from one part to the next.

Table 1. Gastrointestinal Hormones. The cells that produce GI hormones and their main location.

Hormones	Cell type	Gut region
Amylin	B	Pancreas
CCK	I	Small intestine
Gastric leptin	Chief	Stomach
Gastrin	G	Stomach
Ghrelin	X/A-like	Stomach
GIP	K	Small intestine
GLP-1	L	Ileum and colon
Glucagon	L	Ileum and Colon
Insulin	B	Pancreas
Motilin	Mo	Small intestine
Neurotensin	N	Ileum and colon
Oxyntomodulin	L	Ileum and colon
PYY (3–36)	L	Colon
Secretin	S	Small intestine
Serotonin	EC	Small intestine
Somatostatin	D	Stomach

1.5 CHOLECYSTOKININ

1.5.1 History

In 1902, Bayliss and Starling first proposed the concept of hormonal signalling (Bayliss and Starling, 1902), but it was not until 1928, that Ivy and Oldberg (Ivy, 1928) demonstrated that the intestinal mucosa could release a substance, in response to lipids in the duodenum, which caused contraction of the gallbladder. They showed that a purified fraction of duodenal extracts lead to the emptying of the gallbladder in dogs (Ivy 1928). They named the substance "cholecystokinin": i.e. that which "excites or moves the gallbladder". Ivy later confirmed that injecting cholecystokinin (CCK) into human subjects caused evacuation of the gallbladder (Ivy, 1930).

In 1943, Harper and Raper (Harper, 1943) purified a substance from the duodenal mucosa of the cat which induced pancreatic enzyme secretion. They named the active substance "pancreozymin" (Harper, 1943). It took 25 years before Jorpes and Mutt (Mutt and Jorpes, 1968) were able to show that CCK could induce both effects, thus confirming that CCK and pancreozymin were the same substance (Jorpes and Mutt, 1966). For a short period thereafter, the peptide was known as 'cholecystokinin-pancreozymin', but more recently, it has been referred to simply as 'cholecystokinin'.

1.5.2 CCK peptides and structure

CCK was originally purified from hog intestine as a 33-amino acid peptide. The carboxyl terminal pentapeptide sequence, Gly-Trp-Asp-Met-Phe-NH_2, is identical to that of gastrin. The carboxyl terminus of CCK is the biologically active portion of the hormone, and because of sequence similarity between CCK and gastrin, each hormone can interact with the receptor of the other. Therefore, gastrin has slight CCK-like bioactivity and CCK possesses gastrin-like activity.

The carboxyl terminal octapeptide of CCK (CCK-8) is the most biologically potent small peptide of CCK that has been isolated. However, amino-terminal extended forms have been extracted from brain and intestine of the pig, dog, rat, and human. Using techniques to minimise protein degradation, recent studies have demonstrated that the most abundant molecular form of CCK in these species is the NH2-terminally extended peptide of 58 residue, i.e. CCK-58. Intermediate-sized peptides of 39, 33, 25, 22, 18, 7, 5, and 4 amino acids have been isolated from several species (Mutt, 1976; Eng *et al.*, 1982; Eysselein *et al.*, 1982; Tatemoto *et al.*, 1984; Eberlein *et al.*, 1988; Rehfeld, 1998).

Deschenes et al starting with a thyroid cell line of rat medullary carcinoma, isolated sufficient amounts of CCK mRNA to clone and sequence the cDNA. Hence the structure of preproCCK was deduced and the rat CCK gene was charaterised (Deschenes *et al.*, 1985).

Since then porcine (Gubler *et al.*, 1984), human, and mouse CCK cDNA (Takahashi *et al.*, 1985) have been sequenced.

1.5.3 Genetics

The transcription unit of the CCK gene is 7 kilobases. It is made up of three exons broken up by two introns. The first of the three exons is the smallest and is completely non-coding. The structure of preproCCK deduced from the cDNA sequence of the human and rat consists of 116 amino acid residues, whereas porcine preproCCK consists of one less residue. PreproCCK is made up of 4 domains. The first is an NH_2-terminal signal peptide of 20 residues. The second, varying considerably between species, probably acts as a spacer. The third sequence of 58 amino acids contains the bioactive region. The final domain of preproCCK is a COOH-terminal dodecapeptide, of which the first 3 amino acid residues, Gly-Arg-Arg, constitute the characteristic α-amidation signal for the post-translational maturation of active CCK peptides.

1.5.4 Translational and post-translational modifications

Extensive post-translational processing of preproCCK is required for production of bioactive peptides. These modifications are highly tissue and cell specific (Rehfeld *et al.*, 1985; Rehfeld and Hansen, 1986). Cleavage of peptide bonds and sulphation are the two

predominant post-translational processes. Within the CCK-58 sequence there is proteolysis at mono-basic sites, i.e., at Arg-64 (to release CCK-39), Arg-70 (to release CCK-33), Arg-91 (to release CCK-12) and Arg-95 (to release CCK-8). In addition, naturally occurring CCK-5 has been described implying that further cleavage can occur at Met-98. The Tyr-97 of preproCCK (96 of porcine preproCCK) is nearly always sulphated in secreted peptides (Dockray and Gregory, 1980). To function as a CCK molecule, a peptide must have the sequence Trp-Asp-Met-Phe-NH_2 (See figure 1.2). However, because this sequence is identical to the carboxyl terminus of gastrin, this peptide does not confer CCK-like specificity and a sulphated tyrosine at position 7 from the COOH terminus is required for full biological activity.

Molecular forms ranging in size from CCK-7 to CCK-33 appear to be of similar biological potency on pancreatic acinar cells (Otsuki *et al.*, 1986). However, CCK-58 is somewhat less potent on a molar basis than CCK-8 and is less immunoreactive with CCK antibodies (Reeve *et al.*, 1994). Although CCK-58 is the major molecular form in intestine, brain, and the circulation of many species, identification of multiple smaller forms of CCK in circulation, even under conditions that preserve CCK-58, suggests that intracellular processing of CCK occurs to produce small forms of CCK that are secreted into the blood. Recently Reeve has suggested that CCK-58 is not only the major molecular form, but using radioimmunoassay (RIA) has shown that it

can bind and activate both the CCK1 receptor (CCK1R) and the CCK2 receptor (CCK2R). More recently, he suggested that the sulphated form of CCK-58 binds to the CCK1R, while the non-sulphated form binds predominantly the CCK2R, and with very low affinity to the CCK1R (Reeve *et al.*, 2006).

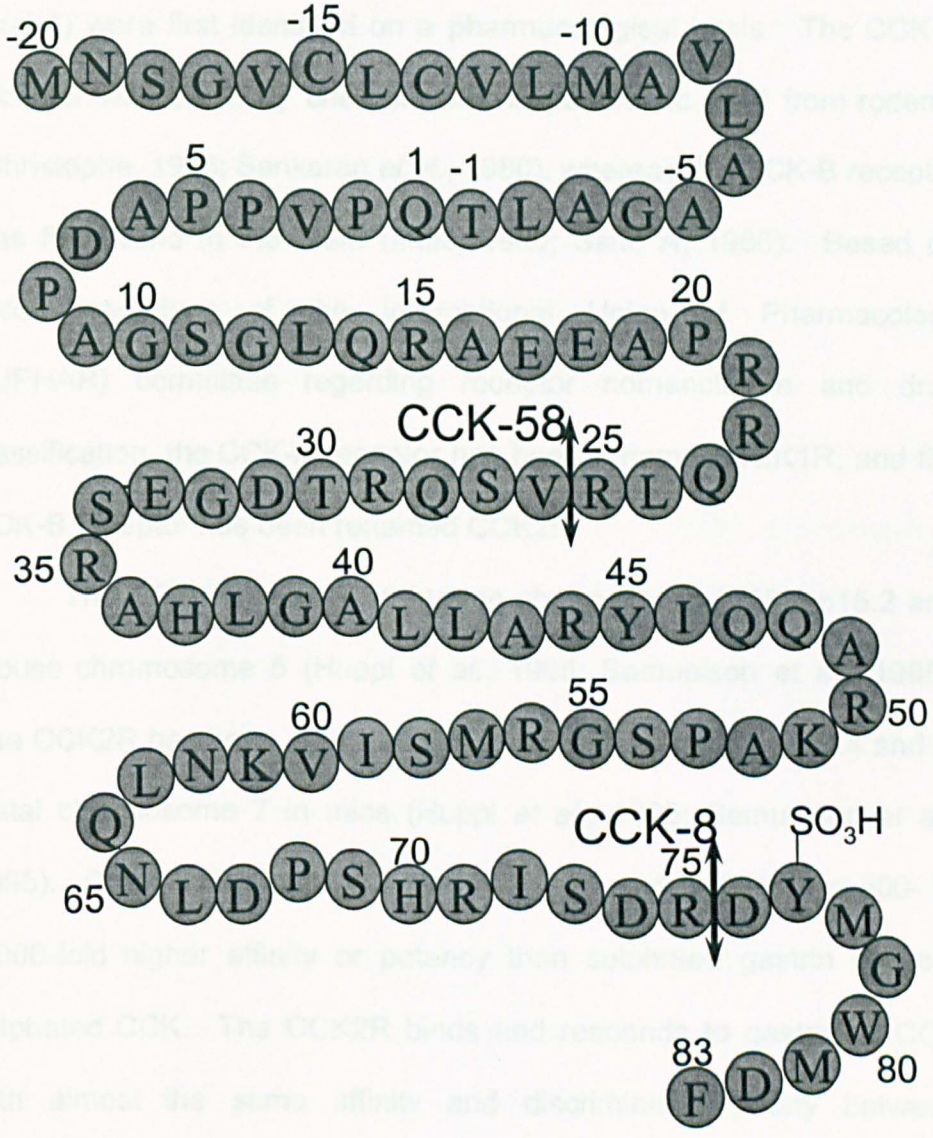


Figure 1.3. Predicted sturcture of human preprocholecystokinin. The largest characterised peptide from brain and intestine, CCK-58, consists of residues 26 to 83. Other active molecular forms are derived from this precursor, such as CCK-8. (Adapted from (Noble *et al.*, 1999)).

1.5.5 CCK receptor

Two types of CCK receptors (type A, "alimentary," and type B, "brain") were first identified on a pharmacological basis. The CCK-A receptor was originally characterised in pancreatic acini from rodents (Christophe, 1978; Sankaran *et al.*, 1980), whereas the CCK-B receptor was first found in the brain (Innis, 1980; Saito A, 1980). Based on recommendations of the International Union of Pharmacology (IUPHAR) committee regarding receptor nomenclature and drug classification, the CCK-A receptor has been renamed CCK1R, and the CCK-B receptor has been renamed CCK2R.

The CCK1R gene is on human chromosome 4p15.1-p15.2 and mouse chromosome 5 (Huppi *et al.*, 1995; Samuelson *et al.*, 1995). The CCK2R has been assigned to human chromosome 11p15.4 and to distal chromosome 7 in mice (Huppi *et al.*, 1995; Samuelson *et al.*, 1995). CCK1R binds and responds to sulphated CCK with a 500- to 1,000-fold higher affinity or potency than sulphated gastrin or non-sulphated CCK. The CCK2R binds and responds to gastrin or CCK with almost the same affinity and discriminates poorly between sulphated and non-sulphated peptides. In the periphery, the CCK2R can be considered as the "gastrin receptor". The CCK1R and CCK2R exhibit a relatively low degree of sequence homology (50%) but present seven hydrophobic segments, likely corresponding to transmembrane domains, with extracellular NH_2-terminal and intracellular COOH-

terminal ends. Such structures are characteristic of G protein-coupled receptors (GPCRs) (Palczewski *et al.*, 2000).

The natural ligand with the highest affinity for CCK1R is CCK-8 (Solomon *et al.*, 1984; Reeve *et al.*, 2002). Gastrin, at physiological concentrations, is likely a poor activator of CCK1R, its affinity being 100- to 500-fold lower than that of sulphated CCK-8. Structure-activity relationship studies with synthetic CCK analogs have indicated that sulphation of the position 2 tyrosine in CCK-8 is critical for binding to CCK1R, since its removal causes a 500-fold drop in affinity. The two natural ligands with the highest affinities for CCK2R are sulphated gastrin-17 and sulphated CCK-8 (Huang *et al.*, 1989; Dufresne *et al.*, 1996). Non-sulphated gastrin-17 exhibits a 3- to 10-fold lower affinity than sulphated gastrin-17. This affinity order and the fact that postprandial blood levels of gastrins are 5- to 10-fold more elevated than those of CCK lead one to consider that sulphated gastrin-17 is the preferred ligand and probably the physiological ligand of most of the peripheral CCK2R. On the other hand, due to the abundance of sulphated CCK-8 in the CNS, it is likely the ligand that naturally activates brain CCK2R. Compared with sulphated gastrin-17, the COOH-terminal tetrapeptide common to gastrin and CCK and non-sulphated CCK-8 interacts with the CCK2R with only a 10- to 50-fold decreased affinity.

1.5.6 Gut endocrine I cells

According to the Wiesbaden classification CCK cells, by their ultra-structural characteristics, have been officially named I cells (Solcia *et al.*, 1973). Both in animals and humans, I cells have not been shown to contain other gut hormones. A gradient of cell density exists such that I cell abundance is greatest in the proximal small intestine and less in the distal intestine. The I-type cell is flask-shaped with an apical microvilli contacting the luminal contents (Polak *et al.*, 1975; Buchan *et al.*, 1978) (see figure 1.3). Secretory granules, which are 250 nm in size and contain CCK, are concentrated around the basolateral surface of the cell. It is this orientation that allows food or other factors, within the intestinal lumen to interact with the apical surface of the I cell, and initiate a series of as yet unknown intracellular signalling events that ultimately result in secretion of CCK from the basal surface of the cell into the blood.

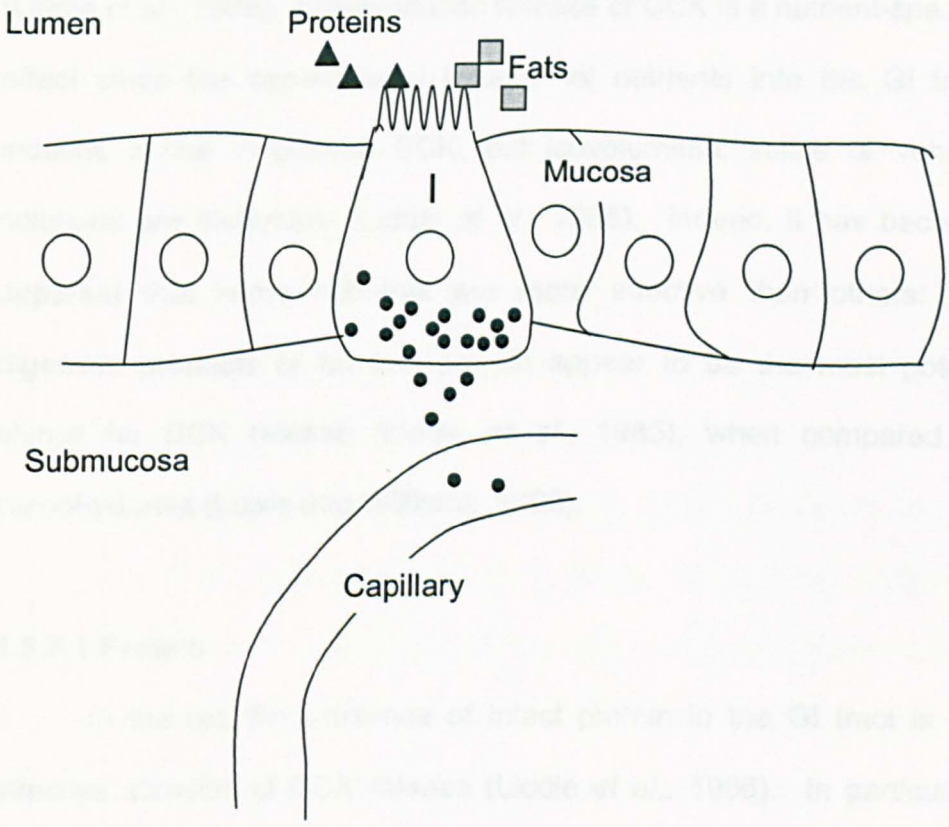


Figure 1.3. Secretion of CCK. Schematic diagram of a CCK (I) cell in the upper small intestine. Note that its apical surface is exposed to the lumen of the gut where it may sample luminal contents.

1.5.7 Effect of nutrients on CCK

As mentioned earlier, CCK is released from the small intestine in response to ingestion of food. Basal plasma CCK concentrations are ~1 pM in most species and rise to about 5 pM following meal ingestion (Liddle *et al.*, 1985). Food-induced release of CCK is a nutrient-specific effect since the experimental infusion of nutrients into the GI tract induces a rise in plasma CCK, but isovolumetric saline or vehicle solutions are ineffective (Liddle *et al.*, 1985). Indeed, it has become apparent that some nutrients are more effective than others: the digestion products of fat and protein appear to be the most potent stimuli for CCK release (Liddle *et al.*, 1985), when compared to carbohydrates (Lewis and Williams, 1990).

1.5.7.1 Protein

In the rat, the presence of intact protein in the GI tract is an effective stimulus of CCK release (Liddle *et al.*, 1986). In particular, tryptophan and phenylalanine each produce marked elevation in circulating CCK levels when they are administered into the stomach or duodenum. However, it is not known how amino acids actually affect the CCK producing cells to stimulate secretion. It is thought that protein-induced CCK release is likely to depend on the inhibition of proteases, preventing the breakdown of a trypsin-sensitive CCK releasing factor, which is believed to interact with the I cell to stimulate

CCK secretion (Liddle *et al.*, 1984). *In vitro*, phenylalanine exposure to STC-1 cells causes hormone release (Colombel *et al.*, 1988). This effect is concentration-dependent and the secretory response is associated with a parallel increase in cytoplasmic calcium concentration. Whether an extracellular site is involved or co-transport of phenylalanine into the cell is required for calcium channel activation will be important areas of future investigation.

1.5.7.2 Lipid

Triglycerides are broken down into glycerol and fatty acids before the release of CCK from the small intestine. It is now fairly well established that the acyl chain length is critically important to the modulation CCK release (French *et al.*, 2000). McLaughlin controlled the physicochemical differences of free fatty acids of different chain lengths to demonstrate that dodecanoic acid (C12) was more effective than decanoic acid (C10) in inhibiting antral contractions in human subjects (McLaughlin *et al.*, 1999).

Some light has recently been shed on the cellular mechanisms that may mediate the effect of free fatty acids on the enteroendocrine cell. The chain length dependency of fatty acid-induced CCK release has been reproduced in two murine enteroendocrine cell lines: one of colonic origin, GLUTag cells (McLaughlin *et al.*, 1999), and one of duodenal origin, STC-1 cells (McLaughlin *et al.*, 1998). In these cells,

fatty acid-evoked CCK secretion is dependent on the presence of a free carboxyl group, but not on cellular metabolism of the fatty acid (McLaughlin *et al.*, 1998). Additionally, CCK release is accompanied by a rise in intracellular calcium concentration, which is largely mediated by the influx of extracellular calcium, probably through L-type calcium channels (McLaughlin *et al.*, 1998; McLaughlin *et al.*, 1999). The mechanism by which the enteroendocrine cell recognises fatty acid chain length to induce CCK release is, as yet unknown, although the recent discovery that a lipid sensitive Apolipoprotein A-IV activates a CCK1R-dependent vagal afferent pathway suggests that apolipoprotein A-IV could stimulates CCK release (Glatzle *et al.*, 2004). Furthermore a recently discovered member from the G-protein coupled receptor family of receptors, GPR40, may provide some interesting insight in years to come.

1.5.7 Cellular mechanisms of CCK action

Much work on the cellular mechanisms of action of CCK has come from studies on pancreatic acinar cells. Work by Williams suggests that binding of CCK to its receptors will act via the $G\alpha_{q/11}$ protein which leads to the activation of phospholipase C (PLC) (Williams, 2001). PLC can activate the breakdown of phosphatidylinositol-4, 5-bisphosphate into diacylglycerol and inositol-1, 4,5-trisphosphate, which will lead to the increased release of Ca^{2+} from internal stores (Wakui *et al.*, 1991).

1.5.8 CCK functions within the gut

CCK acts to control the digestion of fat and protein by regulating both the capacity for digestion by stimulating pancreatic and gallbladder secretions and inhibiting gastric emptying and food intake (see appetite section).

1.5.8.1 Pancreatic secretion

Following the finding that rodent pancreatic acinar cells express CCK1R and that CCK stimulates enzyme secretion *in vitro*, it was believed that CCK acts directly on these cells. Interestingly however, there is evidence that in human studies CCK1R are not expressed on pancreatic acinar cells. Since CCK1R antagonists inhibit postprandial pancreatic enzyme secretion in both rodents and humans it was suggested that CCK activates vago-vagal reflexes to stimulate enzyme secretion (Li and Owyang, 1994). There is also cholinergic stimulation of pancreatic acinar cells by acetylcholine released from postganglionic vagal efferent fibres.

CCK has also been found to stimulate the synthesis of pancreatic enzymes and cellular proliferation (Petersen *et al.*, 1978; Solomon *et al.*, 1983). Diets thought to induce CCK secretion in rats, such as diets containing trypsin inhibitors or protein rich diets, have been found to stimulate pancreatic growth, and these effects were reversed by administration of a CCK1R antagonist (Wisner *et al.*, 1988;

Green *et al.*, 1992). Although interestingly pancreatic hypertrophy persists in response to a high-protein diet in mice lacking CCK (Lacourse *et al.*, 1999).

1.5.8.2 Gall bladder

Both endogenous and exogenous CCK stimulate contraction of the gall bladder and relaxation of the sphincter of Oddi, which regulate the delivery of bile (Liddle *et al.*, 1985). CCK1R antagonists block postprandial gall bladder contraction indicating that this is a physiological action of the hormone (Liddle *et al.*, 1989; Hildebrand *et al.*, 1990). In CCK1R null mice, there is evidence of biliary stasis (Wang *et al.*, 2004). Since gall bladder smooth muscle expresses the CCK1R it is likely that CCK acts directly on the smooth muscle (Schjoldager *et al.*, 1988). Nonetheless, there is evidence for a pre-synaptic site of action to facilitate gall bladder contraction in guinea pigs (Mawe, 1991; Shaffer, 2000).

1.5.8.3 Gastric emptying

The gastric emptying of test meals is reduced by administration of CCK1R antagonists, suggesting this is a physiological effect of the hormone (Forster *et al.*, 1990; Fried *et al.*, 1991). To reduce nutrient flow across the pylorus one site of action of CCK could be the smooth muscle in the distal stomach (Morgan *et al.*, 1978). In humans,

ultrasound studies indicate that endogenous CCK plays a role in regulating contractions of the antrum (McLaughlin *et al.*, 1999). In addition, however, there is considerable evidence that CCK activates a vago-vagal reflex leading to relaxation of the proximal stomach (Raybould *et al.*, 1987; Raybould and Tache, 1988; Forster *et al.*, 1990).

1.6 APPETITE

Appetite is the desire to eat food, felt as hunger. Appetite exists in all higher lifeforms, and serves to regulate adequate energy intake to maintain metabolic needs. Food intake is regulated by the brain, which monitors the close interplay between inhibitory (anorectic) and stimulatory (orexigenic) function. Hormones released from the GI tract (eg. ghrelin) or the adipose tissue (eg. leptin) can act directly at the brain by crossing leaky regions of the blood brain barrier or by acting through the vagus nerve (eg. CCK).

1.6.1 Signalling of appetite by circulating hormones

Kennedy proposed in 1953 that inhibitory signals generated in proportion to body fat stores act in the brain to reduce food intake (Kennedy, 1953). He hypothesised that weight loss induced by caloric restriction reduced the level of these inhibitory signals, and that an energy deficit is corrected by increasing consumption of food. This model, however, does not explain how energy intake is controlled during individual meals. Twenty years later, Gibbs and Smith proposed that signals generated during a meal (Gibbs *et al.*, 1973a), including peptides secreted from the GI tract, provide information to the brain that inhibits feeding and leads to meal termination.

The pancreatic hormone, insulin, which enters the brain from the circulation (Baura *et al.*, 1993) and acts there to reduce food intake

(Woods *et al.*, 1979), was the first hormonal signal to be implicated in the control of body weight by the CNS. Later the profound hyperphagia and obesity of *ob/ob* mice was found to result from autosomal recessive mutation of the gene encoding leptin, a hormone secreted by adipocytes (Zhang *et al.*, 1994). This provided compelling evidence of a second adiposity signal. Subsequent studies demonstrated that both insulin and leptin fulfil the criteria that need to be met by any candidate adiposity signal. Both hormones circulate at levels proportional to body fat content (Bagdade *et al.*, 1967; Considine *et al.*, 1996) and enter the CNS in proportion to their plasma level (Baura *et al.*, 1993; Schwartz *et al.*, 1996). Leptin receptors and insulin receptors are expressed by brain neurons involved in energy intake (Baskin *et al.*, 1988; Cheung *et al.*, 1997; Baskin *et al.*, 1999), and administration of either peptide directly into the brain reduces food intake (Woods *et al.*, 1979; Campfield *et al.*, 1995; Weigle *et al.*, 1995), whereas deficiency of either hormone does the opposite (Zhang *et al.*, 1994; Sipols *et al.*, 1995).

Ghrelin is a recently discovered orexigenic hormone synthesised in X cells of the gut. Plasma ghrelin levels increase immediately before each meal and fall to minimum levels within 1hr after eating (Cummings *et al.*, 2001; Tschop M, 2001). The clear pre-prandial rise and postprandial fall in plasma ghrelin levels support the hypothesis that ghrelin is an initiation signal for meal consumption. When ghrelin is

injected into the cerebral ventricles of rats, their food intake is potently stimulated (Tschop *et al.*, 2000; Nakazato *et al.*, 2001; Kaiya *et al.*, 2003). Among all the known orexigenic peptides, ghrelin has been found to be the most powerful. Chronic intracerebroventricular (i.c.v.) injection of ghrelin increases cumulative food intake and decreases energy expenditure, resulting in body weight gain. Ghrelin-treated mice also increase their fat mass, both absolutely and as a percentage of total body weight. Not only i.c.v. injection, but also i.v. and subcutaneous injection of ghrelin have been shown to increase food intake (Tschop *et al.*, 2000; Nakazato *et al.*, 2001).

1.6.2 Neuropeptide signalling pathways in the hypothalamus

Brain lesioning and stimulation studies implicate the hypothalamus as a major centre controlling food intake and body weight. These studies identify the ventromedial hypothalamic nucleus (VMN) as a 'satiety centre', while the lateral hypothalamic area (LHA) has been termed the 'hunger centre' (Kennedy, 1953). These designations reflect the ability of electrical stimulation of the VMN to suppress food intake, and of bilateral VMN lesions to induce hyperphagia and obesity. Conversely, stimulation or lesioning of the LHA induced the opposite set of responses. More recently the notion of specific 'centres' of the brain that control food intake and body weight has been replaced by that of discrete neuronal pathways that generate

integrated responses to afferent input related to changing body fuel stores (Woods *et al.*, 1998).

Situated adjacent to the floor of the third ventricle, the arcuate nucleus is an elongate collection of neuronal cell bodies occupying approximately one-half of the length of the hypothalamus. Neuropeptide Y (NPY) and agouti related protein (AgRP) are co-localised in some arcuate nucleus neurons (Broberger *et al.*, 1998; Hahn *et al.*, 1998), demonstrating that a single neuronal cell type can contain multiple anabolic effector molecules. The subsequent finding that proopiomelanocortin (POMC) and cocaine and amphetamine regulated transcript (CART) are co-localised in a distinct, but adjacent, subset of arcuate nucleus neurons (Elias *et al.*, 1998) indicates that circuits originating in this brain area have highly specialised roles in energy homeostasis (see figure 1.4).

The hypothesis that the arcuate nucleus transduces information related to signalling by leptin, into a neuronal response, is supported by the anorexic response to local microinjection of leptin into this area (Satoh *et al.*, 1997), and the inability of i.c.v. leptin to reduce food intake after the arcuate nucleus has been destroyed (Dawson *et al.*, 1997; Tang-Christensen *et al.*, 1999). A majority of both NPY/AgRP and POMC/CART neurons have been found to co-express leptin receptors (Cheung *et al.*, 1997; Baskin *et al.*, 1999) and both types of neurons are regulated by leptin (as judged by changes in neuropeptide

gene expression), but in an opposing manner. Thus, NPY/AgRP neurons are inhibited by leptin, and consequently are activated in conditions where leptin levels are low (Stephens *et al.*, 1995; Schwartz *et al.*, 1996; Broberger *et al.*, 1998; Hahn *et al.*, 1998). Although less well studied, a deficiency of insulin also seems to activate these neurons (Williams *et al.*, 1989; Sipols *et al.*, 1995), and insulin receptors are highly concentrated in the arcuate nucleus (Baskin *et al.*, 1988). Conversely, conditions characterised by reduced insulin or leptin inhibit POMC (Schwartz *et al.*, 1997; Thornton *et al.*, 1997) and CART (Kristensen *et al.*, 1998) expression in the arcuate nucleus, and administration of these hormones can prevent or attenuate these neuropeptide responses. Moreover, involuntary overfeeding in rats, which potently inhibits food intake once body weight has increased by more than 5%, also elicits a threefold increase of POMC messenger RNA levels in the arcuate nucleus (Hagan *et al.*, 1999). The demonstration that anorexia induced either by leptin (Seeley *et al.*, 1997) or by involuntary overfeeding (Hagan *et al.*, 1999) is reversed by central administration of a melanocortin-receptor antagonist, indicates that melanocortin signalling is a mediator of the anorexic response induced by increased adiposity signalling to the brain. Taken together, these findings indicate that the arcuate nucleus is a major site for transducing afferent input from circulating leptin and insulin into a neuronal response.

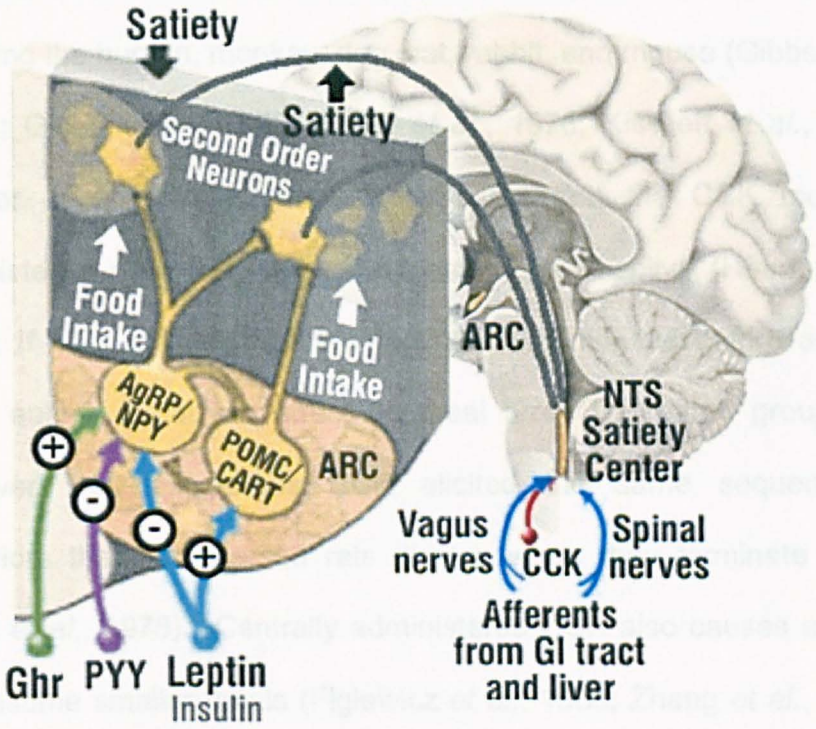


Figure 1.4. Hormonal regulation of appetite. Image of the arcuate nucleus and the effects of hormones on NPY/AgRP and POMC/CART cells. The role of CCK in regulating appetite is also detailed. (Image adapted from (Schwartz and Morton, 2002))

1.6.3 CCK signalling of appetite through vagus nerve

In 1973 Gibbs, Young and Smith discovered that peripheral administration of CCK inhibits food intake (Gibbs *et al.*, 1973b). The satiety effect was dose dependent and specific in that it mimicked the satiety induced by ingested food and was not seen with other gut peptides. The effect could be demonstrated in several mammals, including the human, monkey, dog, cat, rabbit, and mouse (Gibbs *et al.*, 1973a; Gibbs *et al.*, 1976; Houpt *et al.*, 1978; Kissileff *et al.*, 1981; Stacher *et al.*, 1982). It was observed that the CCK must be administered near the start of a meal to be effective (Houpt *et al.*, 1978). If CCK is administered more than 15 minutes before animals begin eating, it had no effect on meal size. The same group also observed that exogenous CCK elicited the same sequence of behaviors that non-injected rats display when they terminate meals (Antin *et al.*, 1975). Centrally administered CCK also causes animals to consume smaller meals (Figlewicz *et al.*, 1986; Zhang *et al.*, 1986). Chronically decerebrate animals do not initiate meals but will swallow liquid food that is slowly infused into their mouth and in this way consume normal-sized meals (Seeley *et al.*, 1994; Grill and Kaplan, 2002). These same animals eat smaller meals when CCK is administered systemically (Grill and Smith, 1988).

CCK secreted from intestinal I cells interacts with CCK1R on endings of sensory fibres of the vagus nerve, eliciting increased activity

in the form of action potentials (Moran *et al.*, 1990; Moran and Kinzig, 2004). The activated vagus in turn stimulates cells in the brainstem, eliciting reflexes that control GI function and sending signals to other brain areas that stop eating (Rinaman *et al.*, 1995; Moran *et al.*, 2001; Moran and Kinzig, 2004).

The putative factor that mediates the satiety effects of CCK acting through the vagus nerve is currently unknown. The action potentials elicited by CCK are thought to release either glutamate (Perrone, 1981) or glycine (Sved and Curtis, 1993). More recently Hokfelt's group demonstrated that CART is present in VAN sensitive to CCK, hence they suggested that CART is the putative mediator of the effects of CCK (Broberger *et al.*, 1999).

1.7 COCAINE AND AMPHETAMINE REGULATED TRANSCRIPT

1.7.1 History

CART peptides have been implicated in a range of important physiological processes such as feeding, reward and reinforcement (Hunter and Kuhar, 2003). They were discovered in two stages. The first was described in a report by Spiess *et al.* (Spiess *et al.*, 1981), who found and sequenced a hypothalamic peptide with unknown function. The second followed work of Douglass *et al.* (Douglass *et al.*, 1995), who showed that an mRNA whose levels in the nucleus accumbens changed after psychostimulant administration encoded a propeptide neurotransmitter. The protein product of this mRNA contained the sequence noted by Spiess *et al.* (Spiess *et al.*, 1981).

1.7.2 Structure and post-transcriptional modification

Several studies have since examined the processing and sequences of these peptides (Thim *et al.*, 1998; Kuhar and Yoho, 1999; Thim *et al.*, 1999). In the rat and mouse, but not in human, CART mRNA was found in two alternatively spliced forms, differing by 39 nucleotides in the coding region (see figure 1.5) (Kuhar and Yoho, 1999). Thus, in rodents, two proteins could be produced, and there is some evidence that both peptide products are made in the rat and mouse, while only one form is produced in the human (Kuhar and Yoho, 1999). Western blotting has identified a CART peptide at about

6.1 kD that is found in the rat and mouse hypothalamus and is not found in the human hypothalamus. This CART peptide fragment may therefore be derived from the long form of CART mRNA which is found in the rodent but not in the human. CART mRNA also uses alternate polyadenylation sites resulting in two mRNA species of 700 or 900 bases in length (Kuhar and Yoho, 1999).

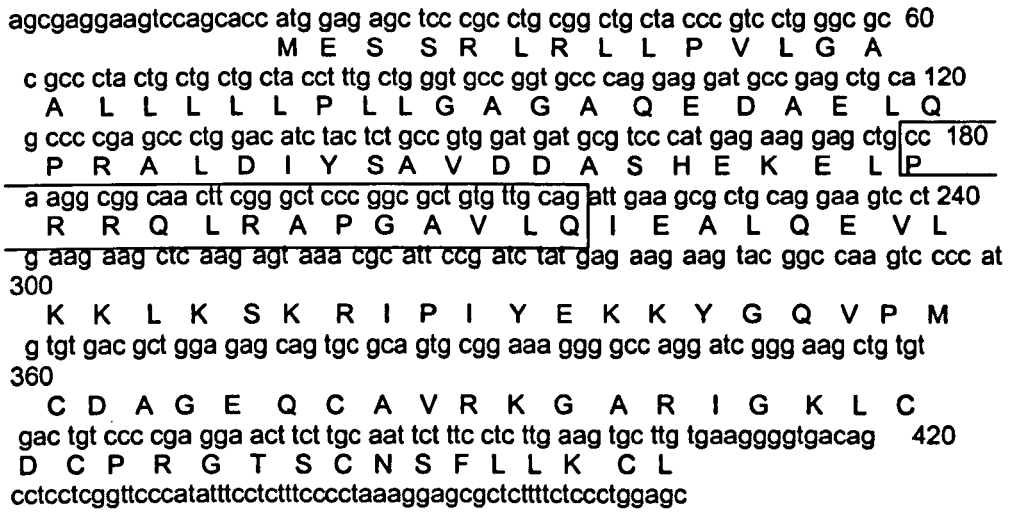


Figure 1.5 Rat CART transcript. The small letters represent the mRNA sequence, with the spacing representing the codons, the capital letters represent the amino acid sequence. The boxed area indicates the region that undergoes alternative splicing.

The human propeptide is 89 amino acids in length and is processed into two fragments of known biological activity, residues 42–89 and 49–89, which correspond to residues 55–102 and 62–102, respectively, in the long form expressed in the rat (Dylag *et al.*, 2006). The amino acid homology between the rat and human forms of the peptide is 95%. Interestingly, the two active forms of the peptide seem to have different effects on several behaviours, including feeding (Thim *et al.*, 1998; Bannon *et al.*, 2001; Kimmel *et al.*, 2002). CART protein and smaller derived peptides are found in the brain. The processing presumably occurs by enzymes such as the prohormone convertases as most of the peptide fragments can be accounted for by cleavage at dibasic amino acid sequences. For example, the long form of rat (rl)CART 55–102 is produced by cleaving at Lys^{53}–Arg^{54} by prohormone convertase 5/6 (Stein *et al.*, 2006). Thim *et al.* extracted and identified by sequencing the short form of rat (rs)CART 1–89 (adrenal gland), rsCART 42–89 (hypothalamus, nucleus accumbens and anterior pituitary), rsCART 49–89 (hypothalamus, nucleus accumbens, and posterior pituitary), and rsCART 10–89 (adrenal gland) from rat tissues (Thim *et al.*, 1998; Thim *et al.*, 1999). RsCART 10–89 could result from a proline-directed arginyl cleavage at the Pro^{8}–Arg^{9} sequence. Still, additional CART fragments appear to be produced in brain as indicated by Western blotting studies (Kuhar and Yoho, 1999).

1.7.3 Function

Koylu *et al.* (Koylu *et al.*, 1997) suggested that CART might be involved in feeding after noting that CART immunoreactivity was localised to brain regions associated with feeding behaviour (Koylu *et al.*, 1997; Koylu *et al.*, 1998), notably the ventromedial nucleus (VMN), DMN, lateral hypothalamus (LH), arcuate nucleus, paraventricular nucleus of the hypothalamus (PVN) and the nucleus accumbens. It has since been demonstrated that CART can be found in several locations, both centrally and peripherally, that are involved in feeding (Couceyro *et al.*, 1998; Jensen *et al.*, 1999; Dun *et al.*, 2000a; Dun *et al.*, 2000b; Cowles *et al.*, 2001; Ekblad *et al.*, 2003; Ellis and Mawe, 2003; Wierup *et al.*, 2004).

Within the CNS, CART peptides are co-localised with a range of other neurotransmitters and peptides that are involved in the regulation of feeding, such as melanin-concentrating hormone (MCH) in the DMN and LH (Broberger *et al.*, 1999), corticotrophin-releasing factor (CRF) in the PVN (Vrang *et al.*, 1999a; Vrang *et al.*, 1999b; Li *et al.*, 2002) and α-melanocyte-stimulating hormone from pro-opiomelanocortin in the arcuate nucleus (Elias *et al.*, 1998). MCH is an orexigenic peptide that stimulates feeding, whereas α-melanocyte-stimulating hormone inhibits feeding. Co-localisation of CART with both orexigenic and anorexigenic (or anorexin producing) neuropeptides suggests a potential modulatory role of CART in feeding behavior.

Since CART peptides are localised to many sites where they could influence feeding and body weight, interest quickly turned to testing the effects on feeding behaviour of i.c.v. CART peptides and antibodies to them. Two groups (Kristensen *et al.*, 1998; Lambert *et al.*, 1998) showed that feeding in rats was increased by i.c.v. administration of antibodies against CART peptides, and Kristensen *et al.* (Kristensen *et al.*, 1998) showed that CART peptides antagonised feeding, including feeding induced by the infusion of NPY, which is one of the most significant central stimulators of feeding (Clark *et al.*, 1984). Subsequent work in rodents has both confirmed the anorectic effects of i.c.v. CART peptides and expanded our understanding of how they might affect metabolism. Vrang *et al.* (Vrang *et al.*, 2000) showed that centrally administered CART induced c-fos expression in hypothalamic neuroendocrine neurons, pointing to one likely site of action for CART. Larsen *et al.* (Larsen *et al.*, 2000) have shown that continuous i.c.v. infusion of moderate to high doses of CART over 7 days or 10 days decreased feeding and body weight in a dose-dependent fashion, although the effects were restricted largely to the first 4–5 days of treatment. Rohner-Jeanrenaud *et al.* (Rohner-Jeanrenaud *et al.*, 2002) observed similar effects, as well as decreases in plasma insulin and leptin. Kong *et al.* (Kong *et al.*, 2003) have shown that twice-daily injection of CART peptide for 7 days reduces daytime food intake but does not effect overall food intake or body weight.

CART null mice consume more food than their wild type (WT) counterparts when fed on a high fat diet, and body weight differences were seen (Moffett *et al.*, 2006). The CART-/- mice also had higher fat mass. Wierup *et al.* found that CART-/- mice weighed significantly more than WT mice after 40 weeks of being fed a regular diet. CART-/- mice have elevated basal insulin levels with normal baseline glucose compared to WT mice (Wierup and Sundler, 2006).

1.7.4 CART and obesity

Several genetic findings link the *CART* gene to human obesity. The human *CART* gene is a positional candidate for obesity because it maps to human chromosome 5q13–14 (Douglass and Daoud, 1996; Echwald *et al.*, 1999), which has been shown to be a susceptibility locus for obesity (Hager *et al.*, 1998). Animal models also support a role for CART in obesity. As noted above mice that have a targeted deletion of the *CART* gene become more obese when fed a high-fat diet from weaning than WT littermates (Asnicar *et al.*, 2001). Interestingly, CART-deficient animals did not show an increase in body weight until after the 14th week of the high-fat diet, suggesting that CART's role in obesity requires interactions with the environment or with developmental events.

Several studies have identified polymorphisms in the *CART* gene of obese individuals (Echwald *et al.*, 1999; Challis *et al.*, 2000;

Walder *et al.*, 2000; Asnicar *et al.*, 2001; del Giudice *et al.*, 2001). Two of these studies failed to link the described polymorphisms to an obese phenotype (Echwald *et al.*, 1999; Walder *et al.*, 2000) and a third found a linkage to reduced waist-to-hip size ratio in men with an A1475G substitution in the 3' untranslated region of the *CART* gene (Challis *et al.*, 2000). The most convincing human data suggesting that the *CART* gene plays a role in human obesity come from two studies (del Giudice *et al.*, 2001; Yamada *et al.*, 2002). One study identified an Italian family with a history of early-onset obesity. Members who were heterozygous for a mis-sense mutation at codon 34 of proCART that changed Leucine to Phenylalanine were severely obese from childhood (del Giudice *et al.*, 2001) and had an average adult body-mass index (BMI) of 39 (the minimum BMI for obesity is 30). This mutation was not found in the unaffected family members, whose average adult BMI was 27. Interestingly, in *in vitro* cell culture, it was shown that transfecting with a CART cDNA that expressed the WT amino acid or a cDNA that expressed the mutant amino acid resulted in differences in CART peptide levels (Dominguez and Kuhar, 2004). Together, these data support the hypothesis that this mutation plays a role in the obesity phenotype by altering CART peptide levels.

Another study supporting a role of CART in human obesity examined polymorphisms in the promoter region. The promoter regions (−1072bp from the transcription initiation site) of the *CART*

gene from 30 obese Japanese individuals as determined by BMI (obese individuals had BMI values ≥30) and 30 lean individuals (with BMI values ≤25) were sequenced (Yamada *et al.*, 2002) and six common polymorphisms were identified in these individuals. Of the six polymorphic sites, only the transposition of A to G at −156 was associated with increased BMI. Both studies demonstrate that CART might play a significant role in human obesity. Further work should help establish whether altered CART levels in blood or brain are associated with obesity or anorexia in humans.

1.7.5 CART receptor

To date, no receptors for CART peptides have been identified, a fact that limits our understanding of the function of CART. Binding studies using radiolabeled CART were unsuccessful due to high background, probably caused by the disruption of the active ligand binding site. However a green fluorescent protein (GFP)-tagged CART has been used to identify a putative CART receptor. Keller *et al.* demonstrated that CART mainly binds subpopulations of cell bodies localised through the PVN, the DMN, and the VMN (Keller *et al.*, 2006).

There is evidence that there may in fact be multiple CART receptors. For example, differences in relative potencies for CART 55–102 and CART 62–102 have been shown in food consumption (Thim *et al.*, 1998; Bannon *et al.*, 2001), elevated plus maze activity (Chaki *et*

al., 2003), and hot plate anti-nociception, acoustic startle response, and prepulse inhibition (Bannon *et al.*, 2001). These differences in potencies were not always of the same magnitude or in the same direction. Kuhar's group suggest that the CART receptor could be a GPCR that couples to Gi/Go and is capable of activating ERK (Lakatos *et al.*, 2005). Finding the receptor will enable us to study the role of CART in more detail.

1.7.6 Regulation of CART

The CART mouse promoter has recently been described and its sequence provides insights into which transcription factors may be involved in CART mRNA regulation (Dominguez *et al.*, 2002). The CART proximal promoter contains several transcription factor binding sites, including a TATA box, STAT/cyclic AMP responsive element (CRE)/API, SP1, aAP2, and E box sites. The presence of these sites suggests that transcription factors such as CRE-binding protein (CREB), c-Jun, SP1, and AP2 may play a role in CART gene expression. The high sequence similarity in this region (>80%) between the mouse and human sequence provides additional support that these transcription factors are involved in CART mRNA regulation (Dominguez *et al.*, 2002).

GH3 cells, a rat pituitary adenoma cell line, express high level of CART mRNA and thus serve as a useful *in vitro* system to study CART

gene regulation (Barrett *et al.*, 2001; Dominguez *et al.*, 2002). In GH3 cells, CART mRNA levels increase in response to activators of adenyl cyclase and protein kinase A (PKA). Forskolin, an activator of adenyl cyclase, and dibutryl-cyclic AMP, an analogue of cyclic AMP produce a significant increase in CART mRNA abundance (Barrett *et al.*, 2001; Dominguez *et al.*, 2002). Additionally, the presence of an inhibitor of PKA (H89) results in a 50% reduction in CART mRNA levels after forskolin treatment. In contrast, treatment of GH3 cells with phorbol 12-myristate 13-acetate (PMA), an activator of the protein kinase C (PKC) signal transduction pathway, does not increase CART mRNA levels, although, PMA treatment of ovine *pars tuberalis* cell cultures resulted in an up-regulation of CART mRNA (Barrett *et al.*, 2001; Dominguez *et al.*, 2002). These data suggests that like other neuropeptides CART mRNA expression is differentially regulated in different cell types.

Transient transfection of GH3 cells with luciferase expressing constructs that contained varying lengths of CART upstream sequence displayed strong promoter activity (Dominguez *et al.*, 2002). In addition, constructs containing the STAT/CRE/AP1 site were responsive to PKA induction by forskolin, while a construct lacking the composite site was not, providing additional evidence that the STAT/CREAP1 site plays an important role. Studies aimed at identifying which transcription factors may be involved in the forskolin-induced up-regulation of CART mRNA identified CREB as a putative

mediator of the transcriptional activation (Lakatos *et al.*, 2002). To date the exact role that CREB plays in CART expression in VAN requires further clarification.

1.8 Cyclic AMP RESPONSE ELEMENT BINDING PROTEIN

CREB is a transcription factor which binds to 8-bp CRE site, 5'-TGACGTCA-3' (Montminy *et al.*, 1986). Identification and characterisation of CREB stemmed from studies of cyclic AMP regulation of peptide hormone biosynthesis, in particular from somatostatin expression in response to other hormonal stimuli. CREB was purified from nuclear extracts of the phaeochromocytoma cell line PC12 by using CRE affinity chromatography and shown to be a 43-kDA phosphoprotein (Montminy and Bilezikjian, 1987).

CREB binds to its DNA target sequence as a dimer (Yamamoto *et al.*, 1988). Dimerisation occurs through a conserved structural motif at the C-terminus of the protein formed by a heptad repeat of leucine residues, referred to as the leucine zipper (See figure 1.6) (Dwarki *et al.*, 1990; Yun *et al.*, 1990). DNA binding is mediated by a basic domain, a Lysine- and Arginine-rich stretch of amino acids just amino-terminal to the leucine zipper (Dwarki *et al.*, 1990). Presence of both the basic and leucine zipper (bZIP) domains places CREB within a larger family of bZIP transcription factors, including mammalian c-fos, c-Jun, c-Myc, and C/EBP, as well as yeast Gcn4 (Struhl, 1989; Vinson *et al.*, 1989).

Stimulus-induced activation of CREB is mediated by phosphorylation. Exposure of cells to forskolin (Seamon *et al.*, 1981), leads to CREB phosphorylation at a specific residue, serine 133

(Ser133) on the KID domain (see figure 1.6). Phosphorylation of Ser133 is required for signal-induced transcription *in vivo*, as mutation of Ser133 to a non-phosphorylatable residue (Alanine) abolishes transcriptional response to elevated cyclic AMP (Gonzalez and Montminy, 1989).

It is not clear how phosphorylation of CREB stimulates the ability to activate transcription (Karin and Hunter, 1995). Phosphorylation at Ser133 could affect the stability of CREB, so that it is less labile when phosphorylated. Phosphorylation has been shown to affect stability of c-Jun by decreasing their ubiquitin-dependent degradation (Musti *et al.*, 1997). Since CREB functions in the nucleus, phosphorylation at Ser133 might induce translocation of cytoplasmic CREB to the nucleus. Within the nucleus, phosphorylation at Ser133 might affect the ability of CREB to dimerise with different bZIP partners, or might promote CREB binding to CRE. One report suggests that PKC phosphorylation of CREB regulates its dimerisation (Yamamoto *et al.*, 1988).

However the most recent hypothesis is that the phosphorylation of CREB allows binding to CREB-binding protein (CBP), a transcriptional co-activator of CREB, and that this leads to the activation of transcription (Wood *et al.*, 2006). CBP and its homologue p300 interact with the basal transcription factors TFIID and TFIIB, as well as with the RNA polymerase II holoenzyme component, RNA helicase A (Swope *et al.*, 1996; Nakajima *et al.*, 1997; Felzien *et al.*,

1999). These results suggest that one function of this co-activator is to stabilise the preinitiation complex and therefore initiate transcription (Wood *et al.*, 2006).

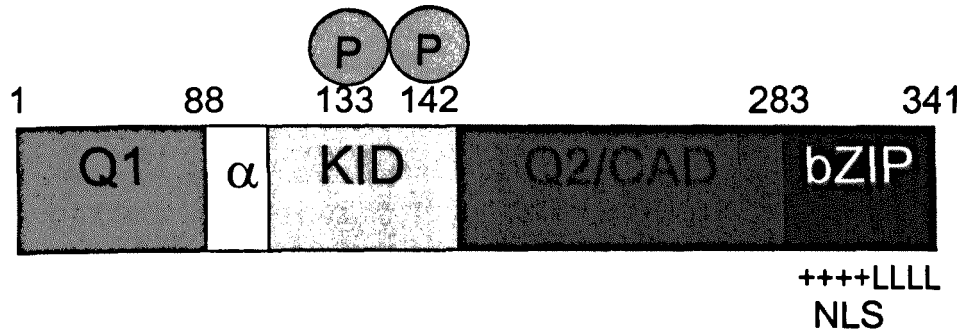


Figure 1.6 CREB domains. Domain Q1 is required for binding elements of basal transcription. KID is a kinase inducible domain. Q2 is a constitutive activation domain. bZIP is the DNA binding domain (contains leucine zipper).

1.9 AIMS

The work in this thesis set out to answer the following questions:

1. How is CART regulated in nodose ganglia of rats *in vivo*?

2. Can *in vivo* data be reproduced in a culture system?

3. What are the mechanisms by which CART is up-regulated in VAN?

4. What are the mechanisms by which CART is down-regulated in VAN?

5. Are orexigenic receptors and peptides regulated in VAN? What are the mechanisms?

CHAPTER TWO

Materials and Methods

2.1 MATERIALS

2.1.1 Chemicals and reagents

Routine chemicals were obtained from BDH Laboratory Supplies (Lutterworth, Leicestershire, UK) or Sigma (Poole, Dorset, UK). Routine tissue culture vessels were from Nunclon (Nottingham, UK). Routin lab equipment was obtained from Appleton Woods (Birmingham, UK). For immunohistochemistry, tissue culture chamber slides were form Becton Dickinson (Bedford, UK) as well as polysine-coated slides (Polysine TM, Germany). Culture media and routine tissue culture chemicals were from Life Technologies (Paisley, Glasgow, UK) or Sigma. Foetal bovine serum (FBS) was obtained from Peninsula (S. Helens, Merseyside, UK). Penicillin/strepcillin and antibiotic/actinomycotic were obtained from Sigma.

CCK-8 was obtained from Bachem (St Helens, Merseyside, UK), CCK1 receptor (CCK1R) antagonist, Lorglumide from Axxora Platform (Nottingham, UK). PMA, Actinomycin D, and ghrelin were all obtained from Sigma. Orexin-A was obtained from Bachem. Protein Kinase C inhibitor (Ro-32-0432) was obtained from Calbiochem (San Jose, CA). Dithiothreitol (DTT) was obtained from BDH and collagenase type Ia from Sigma. TRI RNA, DNA and Protein Isolation Reagent were obtained from Sigma, avian myeloblastosis virus reverse transcriptase (Promega, Southampton, UK) and oligo(dT) primer (see table 2.1), Bioline *Thermus aquaticus*

(BIOTAQ) DNA Polymerase (Bioline, Randolph, MA, USA). Triton X-100 reagent was obtained from BDH.

2.1.2 Antibodies and antisera

Phosphorylation of CREB was detected by affinity purified rabbit polyclonal phosphoCREB (Ser133) antibody (New England BioLabs, Beverly, MA). Affinity purified rabbit polyclonal antibody raised against a peptide mapping at the carboxyl terminus of early growth response factor 1 (EGR-1) p82 of human origin was obtained from Santa Cruz Biotechnology Inc. (Calne, Wiltshire, UK). Affinity purified goat polyclonal anti-Melanin concentrating hormone (MCH) (E-16) was obtained from Santa Cruz Biotechnology Inc. Affinity purified rabbit polyclonal anti-cannabinoid-1 receptor (CB1R) (H158) was obtained from Santa Cruz Biotechnology Inc. Affinity purified mouse anti-Flag monoclonal antibody (DYKDDDDK) was obtained from Upstate Biotechnology (Hampshire, UK). Affinity purified rabbit anti-CCK1R polyclonal antibody was obtained from Santa Cruz Biotechnology Inc. Affinity purified rabbit polyclonal anti-Luciferase was obtained from Promega. Affinity purified mouse anti-transgolgi network 38 (TGN38) monoclonal antibody was obtained from Abcam plc (Cambridge, Cambrigeshire, UK). Affinity purified mouse anti-neuron specific enolase (NSE) monoclonal antibody raised against human gamma enolase was obtained from Abcam plc. Affinity-purified rabbit polyclonal antibody to CART was obtained from Phoenix peptides (San Antonio, TX). Affinity purified goat

polyclonal anti-glial fibrillary acidic protein (GFAP) (c-19):sc6170 was obtained from Santa Cruz Biotechnology Inc. Donkey serum was aquired from Santa Cruz Biotechnology Inc. Secondary antibodies were used as appropriate and included goat or donkey anti-rabbit IgG conjugated to fluorescein (FITC) or Texas Red (TxR) and were obtained from Jackson ImmunoResearch (West Grove, PA). Vectashield with DAPi was obtained from Vector Laboratories, Peterborough, UK. Specificity of immunostaining was determined by preincubation with an excess of appropriate peptide and omitting of primary antibody.

2.2 METHODS

2.2.1 Animals

Studies were made using adult male Wistar rats (250-400 grams; Biomedical Services Unit, University of Liverpool) that were housed in polycarbonate-bottomed cages at 22°C under a 12hr light/dark cycle with *ad libitum* access to food and water (unless otherwise stated). Rats were killed by increasing CO_2 concentrations followed by permanent cessation of the circulation according to Schedule 1 methods as directed by the Home Office.

Fasting–refeeding. Rats were fasted for up to 48hr (water *ad libitium*). Fasted rats were subsequently either refed for up to 2hr or killed. Some fasted animals received injections (10nmol intraperitoneal (i.p.)) of CCK-8 (referred to as CCK from now on) (Bachem) or saline and were killed after 1hr. In addition, some rats that were fasted for 24hr received the CCK1R antagonist Lorglumide (Axxora Platform) (10 mg/kg, i.p.) 15 minutes (min) before refeeding and were killed 1hr after refeeding.

2.2.1.1 Dissection of Nodose ganglia

Nodose ganglia were dissected from rats (Burdyga *et al.*, 2003). Nodose ganglia were either placed in 100ml of 0.14M sodium chloride, followed by 500-1000ml of 4% (Agar Scientific, Stansted, UK) in 0.1M phosphate-buffered saline (PBS), pH 7.4 for immunohistochemistry and immunocytochemistry.

2.2.2 Reverse Trascriptase-Polymerase Chain Reaction

2.2.2.1 RNA extraction

Eight to 14 rat nodose ganglia and vagal trunks were pooled, homogenised in 1ml TRIzol (Total RNA Isolation; Gibco BRL, Paisley, UK) and total RNA extracted according to the manufacturer's protocol. In order to eliminate residual contamination of genomic DNA, samples of total RNA were treated for 30min at 37°C with RNAse-free DNAse (1unit/μg RNA; Promega). RNA was extracted by acid phenol (Ambion, Huntington, UK) and chloroform (VWR International, Lutterworth, UK) and the aqueous phase (containing the RNA) was ethanol precipitated with an equal volume of isopropanol. RNA pellets were washed with 75% ethanol, air-dried and reconstituted in 20μl nuclease free water (Ambion). The concentration of RNA was determined using a 1:100 dilution (in diethyl pyrocarbonate treated water; DEPC) of total RNA, added to a cuvette and absorbance at 260nm (A_{260}) measured in a spectrophotometer (Uvikon 922; Kontron Instruments ltd., Bletchly, UK). The RNA concentration of each sample (μg/μl) was calculated using the formula: A_{260} x [(total volume/DNA volume)xRNA constant]/1000. Five micrograms of each RNA sample was reverse transcribed using oligo(dT) primers and 15 units of avian myeloblastosis virus reverse transcriptase (Promega) in a reaction volume of 30μl.

2.2.2.2 Polymerase chain reaction

Polymerase chain reaction (PCR) was performed using Bio-TaQ DNA polymerase in a Perkin Elmer Geneamp 2400 thermal cycler (GeneTool Inc., Milpitas, CA). The thermal cycle conditions were as follows: hot start at 94°C for 5min. Followed by 25-30 cycles with the following steps 95°C for 30sec, then annealing step for 30sec (optimal annealing temperature was used for each set of primers see table 2.1), the elongation stage was held at 72°C for 1min, and finally 7min hold at 72°C, and samples kept at 4°. The concentration of template in each sample was standardised to relevant GAPDH concentration.

Table 2.1 Primers used for polymerase chain reaction

Sample	Sense primers	Antisense primers	Annealing temp
CART	TCTACTCTGCCGTGGATGATGC	GGGGAAAGAGGAAATATGGGAACC	58.4°C
GAPDH	GACCCCTTCATTGACCTCAACT	CTCAGTGTAGCCCAGGATGCC	65.0°C

2.2.3 Immunohistochemistry

Anaesthetised rats were perfused with 100 ml of 0.14M sodium chloride, followed by 500-1000 ml of 4% paraformaldehyde in 0.1M PBS, pH 7.4. Nodose ganglia were dissected, incubated with 4% paraformaldehyde for 1hr at 22^{0}C, and then in 25% sucrose in 0.1M PBS overnight at 4^{0}C. Samples were mounted on cork discs using cryospray (Bright Instrument Co Ltd., Huntingdon, UK) and frozen in isopentanol. The samples were cut on a cryostat at a thickness of 5-12 μm and mounted onto poly-lysine coated microscope slides. Cryostat sections were rinsed briefly using distilled water and permeabilised using increasing concentrations of 70-90% ethanol for 20min. Prior to incubating with the primary antibody, non-specific binding sites were blocked by a 20min incubation phase in 10% donkey serum (species in which the secondary antibody was raised). The excess serum was then removed, and the tissue was incubated in a 1:100 dilution of primary antibody (diluted in PBS) overnight at 4°C in a humidified atmosphere. Tissue was prepared for incubation with secondary antibody by a series of PBS/NaCl washes: 5min incubation in 0.14M NaCl, 5min incubation in 0.5M NaCl and one final 5min wash in PBS. Tissue was then incubated in the appropriate secondary antibody (diluted at 1:200 in Hepes/NaCl) for 2hr in the dark. A summary of the primary and secondary antibodies used is given in table 2.2. Tissue was then washed (3 times 10min in PBS) and mounted under Vectashield to preserve fluorescence. Slides were examined using a Zeiss

Axioplan-2 Universal microscope (Zeiss Vision, Welwyn Garden City, UK) and images captured using a JVC-3 charge-coupled device camera using 10 and 40X magnification air immersion and KS300 software (Zeiss Vision). The sections were processed using the Axio Vision 3.0 Imaging system (Zeiss Vision).

Table 2.2 Primary and secondary antibodies used for immunohistochemisty
The source and additional information on specificity for each antibody is given in section 2.1.2

Primary antibody	Titre	Secondary antibody	Titre
Anti-CART	1:500	Anti-Rabbit IgG	1:400
Anti-CB1R	1:400	Anti-Rabbit IgG	1:400
Anti-CCK1R	1:80	Anti-Rabbit IgG	1:400
Anti-EGR-1	1:800	Anti-Rabbit IgG	1:400
Anti-Flag	1.200	Anti-Mouse IgG	1:200
Anti-GFAP	1:1000	Anti-Goat IgG	1:200
Anti-MCH	1:100	Anti-Goat IgG	1:200
Anti-NSE	1:1000	Anti-Mouse IgG	1.200
Anti-phosphoCREB	1:500	Anti-Rabbit IgG	1:400

2.2.4 Constructs

VAN were transfected with various constructs. Joseph Nevins (Duke University Medical Center, Durham, NC) kindly gifted plasmids for the adenovirus product early region 1A (E1A) and the inactive mutant Δ2-36 E1A (Yaciuk *et al.*, 1991). David Ginty (Department of Neuroscience, The John Hopkins University School of Medicine, Baltimore, Maryland) kindly gifted the CREB dominant negative mutant, ACREB plasmid along with the empty vector (Ahn *et al.*, 1998). Micheal Kuhar (Yerkes National Primate Research Center, Emory university, Atlanta, Georgia) gifted 3 CART promoter luciferase reporter constructs (120CART LUC, 620CART LUC, and 3451CART LUC) along with the empty vector (Dominguez *et al.*, 2002). Perry Barrett (Rowett research Institute, Aberdeen Center for Energy Regulation and Obesity, Bucksburn, UK) provided the 3.1kb CART promoter (Barrett *et al.*, 2001). The renilla construct used as an internal construct for luciferase assay experiments was bought from Promega (see table 2.3)

Table 2.3 Constructs used in transfecting VAN

Construct	Vector	Promoter	Bought/Gift
ACREB	pRc/CMV500	pSVα-1	Dr Ginty
ACREB Empty	pRc/CMV500	N/A	Dr Ginty
CART- 3100 LUC	PGL3	3.1 kb CART	Dr Barrett
CART Empty	PGL3 basic	N/A	Dr Kuhar
CART-120 LUC	PGL3	120 base pairs (bp) CART	Dr Kuhar
CART-3451 LUC	PGL3	3.45 kb CART	Dr Kuhar
CART-620 LUC	PGL3	620bp CART	Dr Kuhar
E1A Δ2-26	pGEX-3X	CMV	Dr Nevins
E1A Wt	pGEX-3X	CMV	Dr Nevins
Renilla	PGL3	SV40-PRL	Promega

2.2.4.1 Bacterial re-transformation with plasmid DNA

Subcloning efficiency bacterial DH5α competent cells (50μl), (GIBCO, BRL) were incubated with plasmid DNA (0.5μl) on ice for 30min. Cells were then subjected to heat shock (42°C, 40sec) and returned to the ice for 2min and 950ml SOC media (2% Tryptone, 0.5% Yeast extract, 10mM NaCl, 2.5mM KCl, autoclave pH7 and add further 10mM $MgCl_2.6H_2O$, 20mM Glucose) added. Bacteria were incubated with shaking (37°C, 1.5hr) then streaked on Luria Bertani-agar plates containing ampicillin (100μg/ml) for re-transformation of other plasmids. Plates were incubated over night at 37°C.

2.2.4.2 Preparation of plasmid DNA

Single colonies were selected and cultured in 3ml Luria Bertani medium containing ampicilin (50μg/ml) at 37°C for 6-8hr with shaking (225 rpm). The 3ml culture was then transferred into 100ml of LB medium and further incubated overnight at 37°C with shaking (225 rpm). Plasmid DNA was recovered using Plasmix Midipreps (Talent srl, Trieste, Italy) according to the manufacturers' protocol. Briefly, bacterial cells were pelleted by centrifugation (4200g, 10min), the supernatant was discarded and the cells were re-suspended and lysed followed by neutralisation and precipitation. The precipitated material was centrifuged (11,500g, 5min) and the cleared supernatant was transferred to a clean tube to which isopropanol (0.6 volumes) was added. The tube was subsequently

mixed by inversion and incubated on ice for 5min followed by centrifugation (11,500g, 15min). The supernatant was discarded and the pellet was resuspended. This mixture was transferred to a filter and a vacuum was applied, the resin with the bound DNA was retained on the filter. This was then washed with 80% ethanol and eluted with 500ml exonuclease free water (2500g, 2min).

2.2.5 Culturing VAN

Afferent neurons were isolated using a modified protocol of that first described by Lancaster *et al.* (Lancaster *et al.*, 2001). The nodose ganglion was dissected under aseptic conditions as described above and kept in ice-cold hanks balanced salt solution (HBSS; Sigma). The neurons were then digested for 90min in HBSS containing 1mg/ml collagenase A (Roche molecular Biochemicals, East Sussex, UK) and dispase II (BD Biosciences, Oxford, Oxfordshire), in waterbath at 37°C in an atmosphere of 95% O_2/ 5% CO_2, whilst shaking at 100 cycles/min. Once sufficiently digested, the tissue was triturated for 45 seconds (sec) using a sterile Pasteur pipette and then transferred to a clean universal tube. Isolated neuronal cells were then incubated full medium (FM) which consists of Hepes buffered Dulbecco's modified Eagle's medium (HDMEM) supplemented with 10% FBS, 1% penicillin/streptomycin, and 2% antibiotic-antimycotic solution (Sigma) and centrifuged for 5min at 800g (centrifuge 4-22, Juan). The supernatant was discarded and the process was repeated a further two times. The pellet was re-suspended in 1ml HDMEM. The neurons were allowed to adhere overnight and the medium was changed the following day. In routine experiments, neurons were cultured for 3-4 days and the medium changed every 48hr.

2.2.5.1 Immunocytochemistry of adherent VAN

Isolated neurons were cultured on 4-well chamber slides for 72hr, stimulated, and fixed for 30min at 22°C in 4% w/v paraformaldehyde. Fixed cells were washed 3 times in PBS and permeabilised for 30min using PBS containing 0.8% w/v saponin (Sigma). Permeabilised neurons were washed again (3 times PBS) in a humidified atmosphere for 1hr at 22°C, in the dark. Before adding the primary antibody, non-specific binding sites were blocked by a 30min incubation phase in 10% serum from the species in which the secondary antibody was raised. The excess serum was then removed, and cells were incubated with primary antibody (diluted in PBS) overnight at 4°C in a humidified atmosphere. A summary of the primary and secondary antibodies used is given in table 2.2. Cells were prepared for incubation with secondary antibody by a series of PBS/NaCl washes: 5min wash in PBS, 5min wash in 0.14M NaCl in PBS, 5min wash in 0.5M NaCl in PBS, and one final 5min wash in PBS. Neurons were then incubated in the appropriate secondary antibody (diluted in Hepes/NaCl) for 1hr in the dark. Neurons were then washed (3 times 10min in PBS) and mounted under Vectashield containing DAPI to preserve fluorescence and stain cell nuclei, respectively. Slides were examined using a Zeiss Axioplan-2 Universal microscope (Zeiss Vision) and images captured using a JVC-3 charge-coupled device camera using 10 and 40x magnification air immersion) and KS300 software (Zeiss Vision). The sections were processed using the Axio Vision 3.0 Imaging system (Zeiss Vision).

The sections were processed using the Axio Vision 3.0 Imaging system (Zeiss Vision). In experiments where dual immunohistochemistry was required, all secondary antibodies were raised in donkey serum (Jackson Laboratories). All non-specific binding was blocked using 10% goat and donkey serum. Primary antibodies were added to each well at the same time and the corresponding secondary antibodies added sequentially. For all immunohistochemistry, specificity of immunostaining was determined by omitting primary antibodies.

2.2.5.2 Transfection of VAN

1μg of DNA per well vortexed with 210μl SF DMEM per well. A summary of the constructs used is given in table 2.3. One milliliter of vortexed combimag (Oz Biosciences, Marseille, France) per μg DNA added to tube and mixture was vortexed. A further 6μl transfast (Promega) added to tube per μg DNA and vortexed. Mixture was then left for 15min. The wells to be transfected were then washed with PBS before adding 200μl solution per well. The plates were then incubated on magnetic plate for 15min at room temperature. Finally 800μl full serum medium added and incubated at 37°C for 24hr before being examined.

2.2.5.3 *In situ* hybridisation of VAN

VAN were prepared as described above, and fixed for 20min in 4% paraformaldehyde made up in 10x PBS diluted ten fold with

DEPC-treated water. The slides were then washed twice in 1x PBS for 1min each. Following this, the samples were left 5min to dehydrate in 70% ethanol made up in DEPC-treated water, and stored in 95% ethanol made up in DEPC-treated water. The slides were removed from the 95% ethanol and allowed to air dry.

During this time 150µl hybridisation fluid was paced in a tube per slide to be used. Oligonucleotide probes complementary to bases CTGTGTGCAGGAGACGGGTAGGTAGAGGAGCCCGGAG AGGAGTAA and ATCGGAATGCGTTTACTCTTGACCTTCTTCAG GACTTCCTGCAGCGCT of the CART and EGR-1 peptides, respectively, were 3' end labeled with [^{35}S]dATP (10mCi/ml; Amersham Biosciences, Buckinghamshire, UK), purified using QIA quick nucleotide removal kit (Qiagen, Crawley, UK). One microlitre of labeled probe was measured and from this 3000cpm of probe was added to the tube per µl of hybridisation fluid. 0.02µl (1M) DTT, per µl of hybridisation fluid, was also added to make the hybridisation buffer.

Once the slides were completely dry, 150µl hybridisation buffer was added to each slide, making sure to cover the whole sample. A piece of parafilm was used to cover the section making sure not to leave any air bubbles. The slides were then placed in a moist flat plastic container, sealed with parafilm, and incubated overnight at 42°C.

One litre 1x SSC buffer (Ambion) was heated to 55°C. Using slide forceps the parafilm was floated off the slides in the heated 1x

SSC buffer containing 20mM DTT. The slides were soaked for 30min at 55°C in 1X SSC buffer on a belly dancer, and then allowed to cool for up to 40min. The slides were then washed for 30 sec in 1X SSC buffer, followed by 30sec in 0.1X SSC buffer. The slides were then dehydrated for 30sec in 70% ethanol, and then placeinto 95% ethanol for a further 30sec. The slides were air dried very thoroughly.

During this time the Kodak NTB2 emulsion (Sigma) was melted in a water bath at 43°C, and then poured into a dipping vessel also maintained at 43°C. Each slide was dipped into the emulsion for seconds, and the back of the slide was cleaned with cotton bud. The slides were then placed horizontally on a paper towel to dry for a minimum of 2hr. Once dry the slides were placed in a box with dehydrated silica gel. The box was closed, sealed and wrapped with tin foil. The slides were left to develop for 1-2 months at 4°C.

Slides were allowed to equilibrate to room temperature in a sealed, light tight box for 30-60min. Working stocks of Kodak developer (Sigma), Kodak stop solution (Sigma) and Kodak fix solution (Sigma) were prepared. Under darkroom conditions, exposed slides were placed in developer and left 5min and were gently agitated every minute. Under darkroom conditions, the slides were transferred to stop solution for 30sec. The slides were then fixed in fixing solution for 10min and were gently agitated. The slides were placed in water and washed for 15min with a constant,

gentle flow of water. The excess water was shaken off slides and hematoxylin was added. The excess stain was washed off with water. The slides were drained, and coverslips were added. The slides were viewed using a light microscope (Axiovert 25, Zeiss Vision).

2.2.5.4 Luciferase assay

VAN were cultured in full media for 2 days in 12-welled plates before transfection (protocol above: section 2.2.5.2). The cells were incubated 24hr in full media before being stimulated. The cells were then lysed using passive lysis buffer (Promega).

Luciferase activity was measured with dual-luciferase reporter assay system (Promega) using a LumiCount Platereader (Packard BioScience) according to the manufacturer's protocol. Luciferase assay substrate was used to react with luciferase reporter produced by VAN. Stop and Glo buffer (Promega) was used to react with internal control construct, renilla. Results are presented, as fold increase over unstimulated control with a value of 1.0 signifying no change in luciferase activity.

2.2.5 Statistics

Results are represented as mean +/- standard error of the mean.; comparisons were made using a student t-test where appropriate, and were considered significant at $p<0.05\%$. Excel statiscal package was used.

CHAPTER THREE

In vivo regulation of nodose ganglion

3.1 INTRODUCTION

As described in detail earlier in chapter 1, appetite and energy balance are regulated by opposing orexigenic and anorectic pathways. CART is a component of the anorectic arm of the appetite and energy balance regulation system. Importantly, Hokfelt's laboratory have identified CART expression in nodose ganglion neurons. They show that CART co-localised with the CCK1R in the nodose ganglion which contains the cell bodies of VAN (Broberger *et al.*, 1999).

The aim of this chapter was to study the expression of CART in VAN, and to identify changes in CART expression that are dependent on the feeding state of animals. Emphasis was also placed on finding transcription factors activated during different feeding states. Following the experiments from Hokfelt's group I wanted to determine the role of CCK in regards to the regulation of CART expression and transcription factors.

3.2 METHODS

Rats were housed as described in chapter 2.2.1. Rats were fed *ad libitum* or fasted for 24hr. Fasted rats were also re-fed for 1hr, or injected with CCK (i.p., 10nmol) after 23hr fasting. Rats were also injected with Lorglumide (i.p. 15pmol) 15min before re-feeding a fasted rat for 1hr. The nodose ganglions of the rats were then dissected as described in chapter 2.2.1.1.

Reverse transcriptase PCR was performed as described in chapter 2.2.2. The following primers were used: CART (Forward: TCTACTCTGCCGTGGATGATGC Reverse: GGGGAAAGAGGAAATATGGGAACC), GAPDH (Forward: GACCCCTTCATTGACCTCAACT Reverse: CTCAGTGTAGCCCAGGATGCC)

Immunohistochemistry was performed as described in chapter 2.2.3, using the following primary antibodies: rabbit anti-CART, rabbit anti-phosphoCREB, and rabbit anti-EGR-1; and the following secondary antibodies: donkey anti-Rabbit conjugated to TxR or FITC as appropriate.

3.3 RESULTS

3.3.1 CART abundance *in vivo*

Immunohistochemical analysis of CART in nodose ganglion of fed and fasted rats revealed a dramatic difference in expression. CART was identified in the cell bodies of 37±2.1% of neurons found in the nodose ganglion of rats fed *ad libitum* (figure 3.1.A and 3.2). The expression of CART was characteristically vesicular as would be expected of a secretory protein (figure 3.1.B). Withdrawal of food for 24hr caused a significant decrease in the number of cells expressing CART compared to fed rats (figure 3.1 C). In the mid- and caudal-regions of the nodose ganglion from rats fasted for 24hr 8.0±1.6% of all neurons expressed CART immunoreactivity (figure 3.2). It can be seen that after a 24hr fast, although some neurons still expressed CART, the abundance of CART expression decreased compared to those in fed rats (figure 3.1.D).

Re-feeding a rat for one hour after fasting for 24hr (figure 3.1.E), restored CART expression to a similar level to that of the rat fed *ad libitum, i.e.* 37.7±1.8% of neurons expressed the protein (figure 3.2). After one hour of re-feeding the pattern of expression of CART was perinuclear presumably in the ER and Golgi complex (figure 3.1.F).

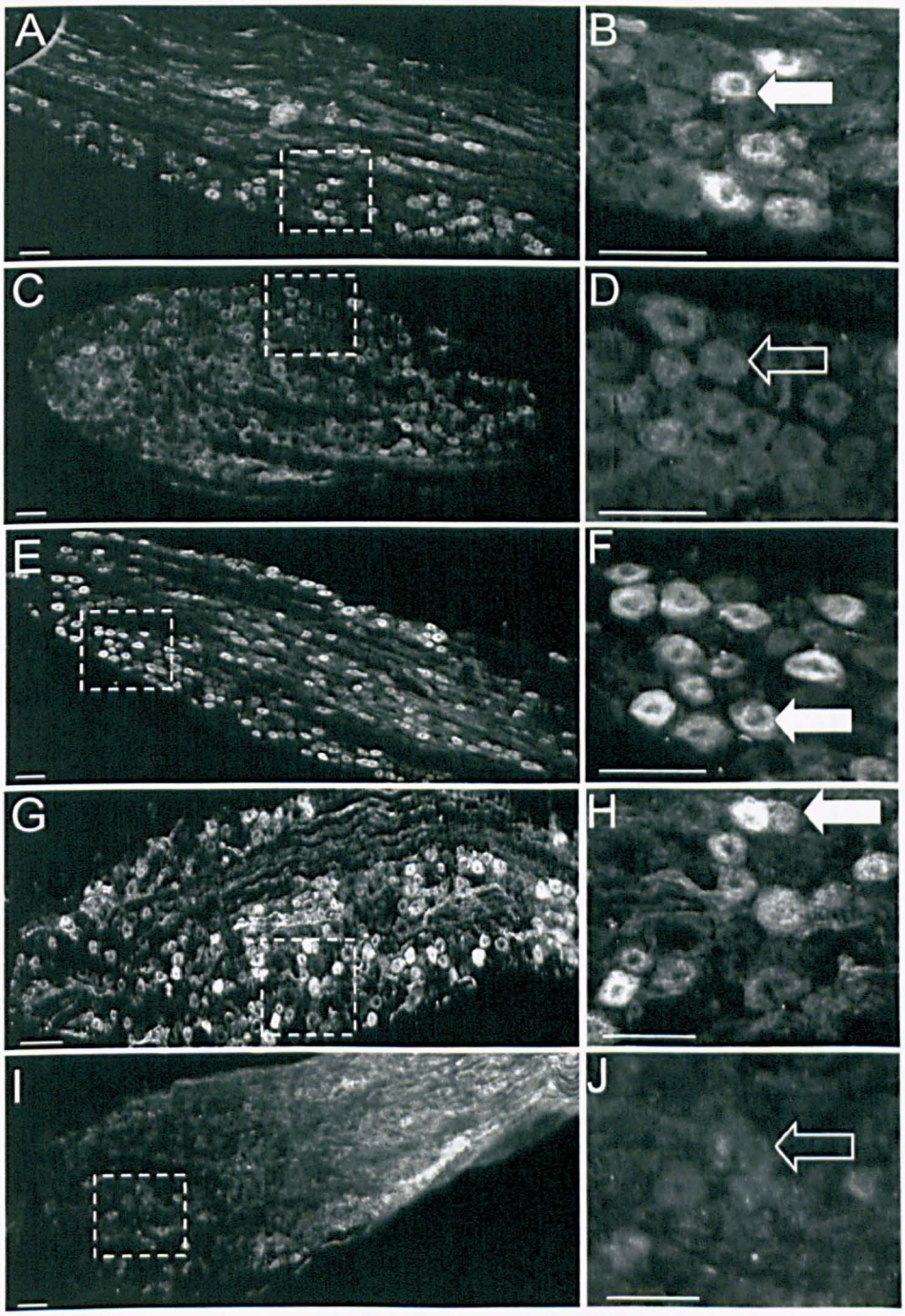


Figure 3.1. CART expression increases with either feeding or CCK in nodose ganglion of rat. Immunohistochemistry of nodose ganglia stained with CART antibodies from rats A) and B) fed ad lib C) and D) fasted 24hr E) and F) fasted 24hr and re-fed 1hr G) and H) fasted 24hr and i.p. CCK, I) and J) fasted 24hr i.p lorglumide and re-fed 1hr. Images A), C), E), G), and I) taken at x20 magnification. Images B) ,D), F), H), and J) taken at x40 magnification. Full arrows indicate positive CART staining, empty arrows indicate negative CART neurons. Scale bar = 50µm. Representative images from 10 independent experiments.

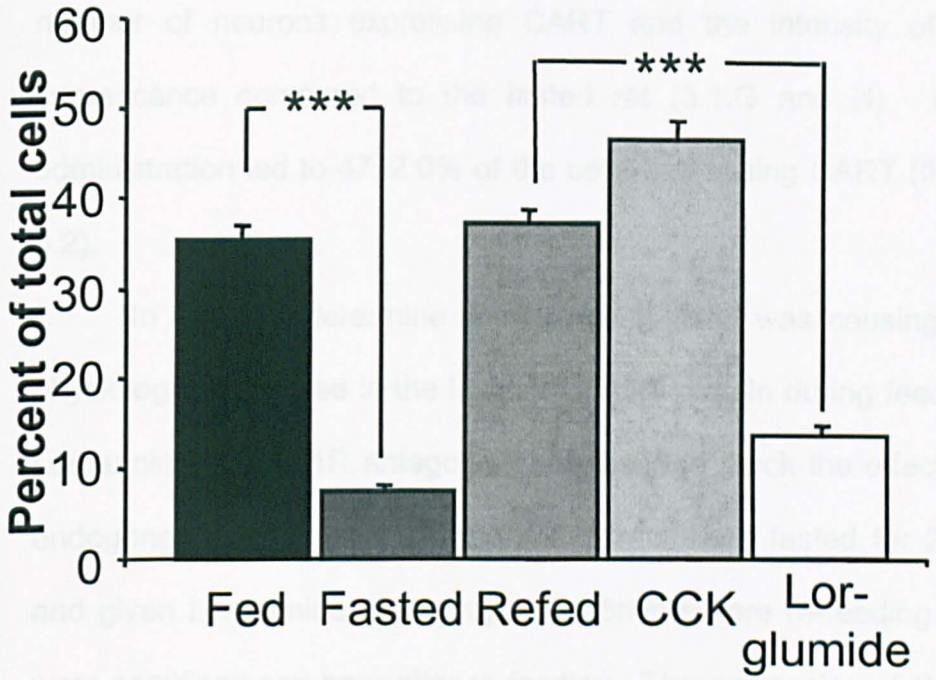


Figure 3.2. Fasting reduces the number of CART-immunoreactive cells, while re-feeding and CCK increase the number of CART-immunoreactive cells *in vivo*. CART immunoreactive cells in nodose ganglia of rats fed (36.0±1.3%), fasted (8.0±0.7%), fasted and re-fed (37.7±1.2%), fasted and receiving 10nmol i.p. injection of CCK (47±1.8), and injected with Lorglumide before 1hr re-feeding after 24hr fast (14.1±0.9%). *** represents $p<0.0001$ t-test, (N=5)

Previous findings have shown co-localisation between the CCK1R and CART in neurons of the nodose ganglion (Broberger *et al.*, 1999). For this reason I examined whether injection of CCK could induce CART expression. In this experiment, CCK (10nmol, i.p.) was administered to rats fasted for 24hr. CCK increased the number of neurons expressing CART and the intensity of the fluorescence compared to the fasted rat (3.1.G and H). CCK administration led to 47±2.0% of the cells expressing CART (figure 3.2).

In order to determine whether CCK itself was causing the physiological increase in the levels of CART protein during feeding, Lorglumide, a CCK1R antagonist, was used to block the effects of endogenous CCK. In this experiment, rats were fasted for 24hr, and given Lorglumide (10mg/kg, i.p.) 15min before re-feeding and were sacrificed one hour after re-feeding. The expression of CART was greatly diminished compared to those injected with CCK (10nmol, i.p.) (figure 3.1.I) and the pattern of expression was similar to the fasted rat. The intensity of fluorescence decreased with Lorglumide treatment in rats (figure 3.1.J). CART expression was significantly ($p<0.001$, t-test) reduced, to 14.1±0.9% of cells compared to CCK treated rats (figure 3.2).

3.3.2 CART mRNA *in vivo*

The mRNA expression of CART was also examined in both rats that were fed *ad libitum*, and fasted for 24hr. Reverse transcriptase PCR analysis of nodose ganglion revealed the expression of CART mRNA in both the fed and fasted states of rats. As described previously in chapter 1, an alternatively spliced transcript can be generated during transcription of the CART gene, resulting in the presence or absence of 39bp (Dominguez *et al.*, 2002). The transcripts encode a protein of either 116 or 129 amino acids. The primer sequence chosen encompasses the region of alternative splicing; hence both mRNA sequences are found on the RT-PCR. The top band of the RT-PCR (129 amino acids) is the transcript of interest as it forms the active $CART_{55\text{-}102}$. Analysis of the nodose ganglion showed that CART mRNA was highly expressed in the nodose ganglion of the fed rat compared to that of the fasted rat (figure 3.3). Both the top and lower bands were decreased in the fasted rats in the RT-PCR. The intensity of the band is approximately 3 times higher in fed rats compared to fasted rat as determined by densitometry, which is broadly consistent with the immunohistochemical data.

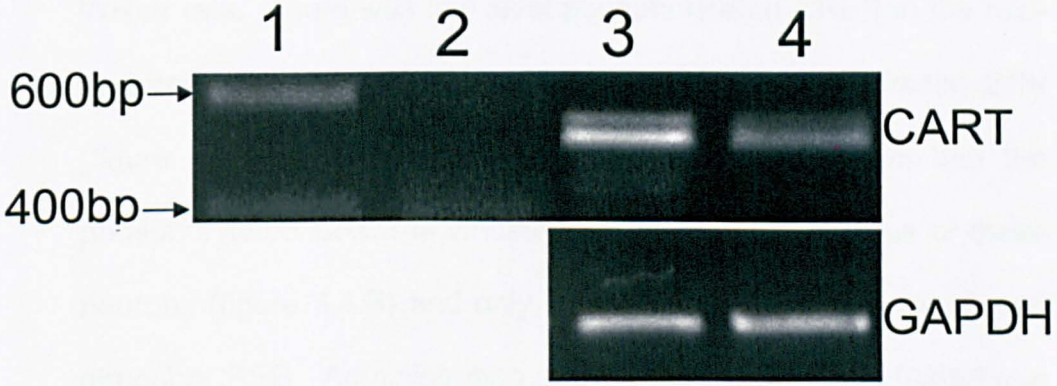


Figure 3.3. RT-PCR of CART mRNA in nodose ganglia. Agarose gel from RT-PCR samples. Lane 1 loaded with hyperladder 1, lane 2 loaded with water blank (negative control), lane 3 loaded with cDNA generated from 12 nodose ganglia from six fed rats, lane 4 loaded with cDNA generated from 12 nodose ganglia from six fasted rats. Equal loading shown by GAPDH bands. Representative sample from 8 independent experiments.

3.3.3 CCK regulation of transcription factors

Having demonstrated that feeding regulated CART transcriptionally, and that this is mediated by CCK, I explored the hypothesis that CCK regulates CART expression via CREB activation. Immunohistochemical analysis of nodose ganglion with an antibody raised against CREB, phosphorylated on serine 133, revealed sharp changes between the fed and fasted states of male Wistar rats. There was low level phosphorylated CREB in the mid- and caudal-regions of the nodose ganglion from rats fasted 24hr (figure 3.4.A). It was clear under higher magnification that the phosphorylated CREB is virtually absent from the nucleus of these neurons (figure 3.4.B) and only 2.1±0.6% of these cells expressed phosphoCREB. Administration of CCK (10 nmol. i.p.) to fasted rats increased phosphoCREB within 1hr (figure 3.4.C). Nuclear localisation of phosphoCREB after CCK administration was found in 58.8±3.5% of neurons (figure 3.4.D), suggesting the activated CREB may be binding to DNA to induce CART expression.

To identify neuronal activity in VAN, immunohistochemistry was used to detect the expression of EGR-1, an immediate early gene product, in nodose ganglion preparations. EGR-1 was expressed in the cell body of 38.0±1.2% of neurons in the nodose ganglion of rats fed *ad libitum* (figure 3.5.A). EGR-1 seemed to be present in the perinuclear area of the cell (magnification x20), but under higher magnification (Magnification x40) it seemed to be

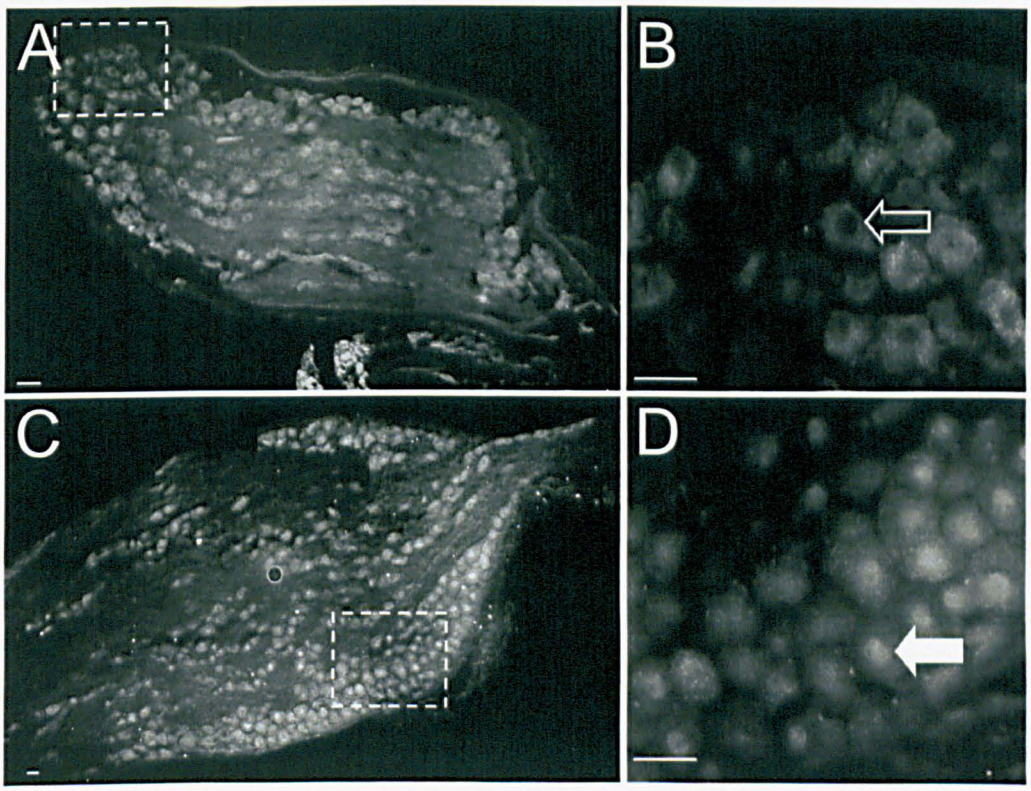


Figure 3.4. CCK increases CREB phosphorylation *in vivo*. Immunohistochemistry of nodose ganglia stained with phosphoCREB antibodies from A) and B) rat fasted 24hr, C) and D) rat fasted 24hr with a 10nmol i.p. injection of CCK (1hr). Images A, and C taken at x20 magnification. Images B and D show x40 magnification of region marked by square of corresponding x20 picture. Full arrow indicates positive phosphoCREB staining, empty arrow indicates negative phosphoCREB nuclei. Representative images from 6 independent experiments.

predominantly found in the cytoplasm (figure 3.5.B). The withdrawal of food for 24hr abolished EGR-1 expression in neurons from the middle and caudal region of the nodose ganglion (figure 3.5.C and D). This effect could be reversed with the administration of 10nmol CCK i.p. (figure 3.5.E). It was clear under high magnification that the localisation of EGR-1 in these neurons is cytoplasmic as opposed to nuclear (figure 3.5.F).

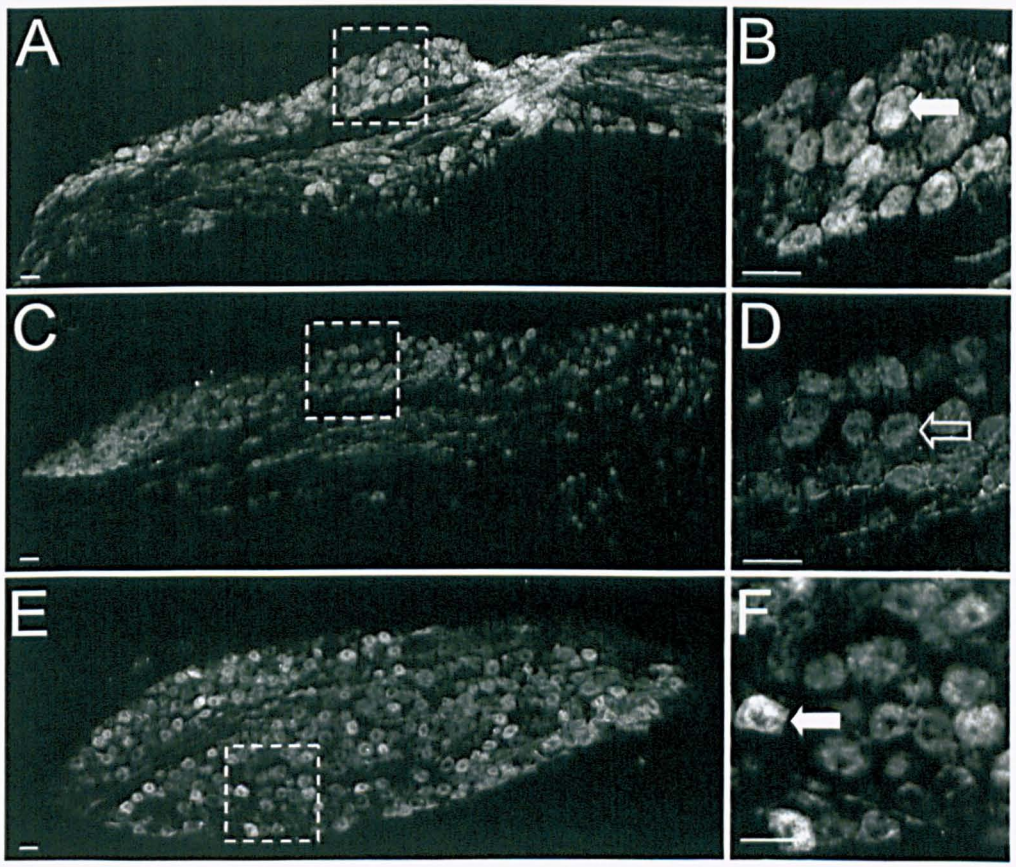


Figure 3.5. CCK regulates EGR-1 expression *in vivo*. Immunohistochemistry of nodose ganglion stained with EGR-1 antibodies from A) and B) rat fed *ad libitum*. C) and D) rats fasted 24hr. E) and F) rat fasted 24hr with a 10nmol i.p. injection of CCK (1hr). Images A), C), and E) taken at x20 magnification. Images B), D), and F) show x40 magnification of region marked by square of corresponding x20 picture. Full arrows indicate positive EGR-1 staining, Empty arrows indicates negative EGR-1 neurons. Representative images from 6 independent experiments.

3.4. DISCUSSION

The results presented here show that, in the presence of CCK, the number of VAN expressing CART is increased, confirming the findings of Broberger *et al.* that CART is localised in VAN and can co-localise with CCK1R (Broberger *et al.*, 1999). It had not been suggested until now that CART levels fluctuate with feeding in the nodose ganglion. Furthermore, these results indicate that CCK is necessary and sufficient to mediate the increased CART levels. These findings are evidence for the role of CCK in increasing the levels of CART peptide, since during feeding, the levels of CCK in the blood stream are greatly increased and can act on CCK1R at VAN endings terminating in the gut. This is an important finding since it implicates the vagus nerve not just as a means of communication between the brain and the gut, but as a potential mediator of the effects of CCK.

It is interesting to note that the effects of feeding on CART occur relatively rapidly as one hour was sufficient to induce CART synthesis. However, the singular importance of CCK in mediating CART levels was demonstrated by the Lorglumide experiment. In this experiment, the levels of CART were reduced through fasting a rat for 24hr, coupled with an injection with Lorglumide to abolish the effect of CCK at the CCK1R, prior to re-feeding for one hour. The fact that a specific antagonist of the CCK1R could abolish the effects of feeding on CART indicates that endogenous CCK is vital for production of CART.

There are a number of ways by which CCK could potentially regulate CART abundance. One possible method is that of post-translational regulation. CCK may play a role in controlling proteases that cleave CART into its final active form. CART can be cleaved by a plethora of proteases at numerous sites (Kuhar and Yoho, 1999). This would fit in with the finding that CART can be synthesised within an hour of CCK administration or feeding.

Another possibility is that CCK does not regulate the production of CART protein, but rather reduces its degradation. Ubiquitination has proven to be a very important regulator of proteins, however, there have not currently been any secretory proteins found to be regulated by ubiquitination. This is because secretory proteins never come in contact with ubiquitin, a cytosolic protein, as they are always encapsulated either by vesicle or an organelle (Golgi/ER).

There is also the possibility that CCK controls the translational regulation of CART. CCK stimulates protein synthesis in pancreatic acini at the translational level (Sans *et al.*, 2002; Sans *et al.*, 2004a; Sans *et al.*, 2004b). CCK acting through its GPCR is now known to activate a variety of intracellular signalling mechanisms and thereby regulate a complex array of cellular functions in pancreatic acinar cells. In pancreatic acinar cells CCK stimulates the rapamycin-sensitive kinase, mTOR, which independently signals to two different pathways, one leading to p70S6 kinase activation and subsequent phosphorylation of

ribosomal S6 protein, and another leading to PHAS-1 phosphorylation and subsequent regulation of eIF4E action on cap-dependent translation. Hence, the phosphoinositide-3 kinase-protein kinase B-mTOR pathway is activated by CCK in pancreatic acinar cells and plays a major role in regulating protein synthesis at the translational level (Bragado *et al.*, 1998; Bragado *et al.*, 2000).

However, with the finding that CCK regulates the phosphorylation of CREB and the synthesis of EGR-1, two transcription factors, coupled with the reverse transcriptase-PCR and *in situ* hybridization results, I have three lines of evidence to suggest that CCK regulation of CART occurs via transcriptional route. EGR-1 is an interesting finding since it is an immediate early gene, which are often referred to as third messengers, and could therefore explain the observation that CCK can induce CART expression within an hour. It remains unlikely however that EGR-1 has a major role to play in CART regulation, as there are no predictive EGR-1 binding sites on the CART promoter. There is, however, a predictive CRE site on the CART promoter, indicating that CREB could be an important regulator of CART synthesis.

In conclusion, CART expression is elevated during feeding and inhibited by fasting. CCK seems to play a crucial role in the regulation of CART expression in fed animals. In addition, CCK plays a key role in the phosphorylation of CREB and the up-regulation of EGR-1. It is unclear however, how CCK regulates the

phosphorylation of CREB and if it is involved in the mechanism of CART up-regulation.

CHAPTER FOUR

Characterisation of culture system

4.1 INTRODUCTION

In the previous chapter, I demonstrated that CART abundance in the nodose ganglia fluctuated *in vivo* depending on the feeding state of the animal. Evidence was presented that implicated a role for CCK. In order to study the cellular mechanisms by which CCK increases CART abundance the need for an appropriate experimental system was identified.

Over the last three decades, cell and tissue culture methods have been refined and have now become an essential tool in biomedical research. Animal welfare concerns have made a major contribution to the development of these models, but an essential driver has been the development of cell systems that allow the study of high resolution functions under strictly controlled environmental conditions. One advantage of these systems is that they exclude the paracrine and endocrine influence of other cells and organs, thus providing the possibility to study direct effects of exogenous factors on a defined cell population.

Commonly used cell culture models, employ either cancer cell lines or primary cells from the tissue of interest. Cell lines are commercially available or can be made in house, and provide the advantage of a homogenous cell type; however, they do not faithfully represent the behavior of primary cells. Since tissue culture systems are based on mechanical and/or enzymatic dissociation of the tissue to

single cells, they have the added advantage of maintaining the mixed cell population, however lose the three dimensional design of the tissue. Given the nature of neurons, in that they do not replicate, and the unavailability of cell lines, I decided a tissue model would be better, especially as tissue could be easily obtained.

Other groups have successfully used cultures of nodose ganglia (Lancaster *et al.*, 2001; Zhang *et al.*, 2004; Wacker *et al.*, 2005). As a result, methods for the culture of primary VAN were developed with the aim to study the role of CCK and other regulatory peptides on a single cell. In order to validate the properties of VAN initial studies sought to characterise the different cell types after dissociated nodose ganglia were cultured for 2-3 days, this included identification of the proportion of glial and neuronal cells. The environmental conditions that mimicked the *in vivo* phenotypes associated with feeding and fasting states were then identified. Finally attention was given to the question of whether cultured neurons exhibited responses to CCK that replicate *in vivo* work.

4.2 METHODS

Nodose ganglia were dissected as described in chapter 2.2.1.1, and cells were dissociated as described in chapter 2.2.5.

Immunohistochemistry was performed as described in chapter 2.2.5.1, using the following primary antibodies: rabbit anti-CART, rabbit anti-phosphoCREB, rabbit anti-EGR-1, goat anti-GFAP, mouse anti-NSE, rabbit anti-CCK1R and mouse anti-TGN38; and the following secondary antibodies: donkey anti-rabbit conjugated to TxR or FITC as appropriate, donkey anti-goat conjugated to FITC, and donkey anti-mouse conjugated to FITC. VAN were cultured in FM for 3-4 days, and then transferred to SF medium with or without CCK, and Actinomycin D as appropriate for 30-120min.

In situ hybridisation was performed as described in chapter 2.2.5.3, using oligonucleotide probes complementary to bases for CART: CTGTGTGCAGGAGACGGGTAGGTAGAGGAGCCCGGAGA GGAGTAA and for EGR-1: ATCGGAATGCGTTTACTCTTGACCTTC TTCAGGACTTCCTGCAGCGCT.

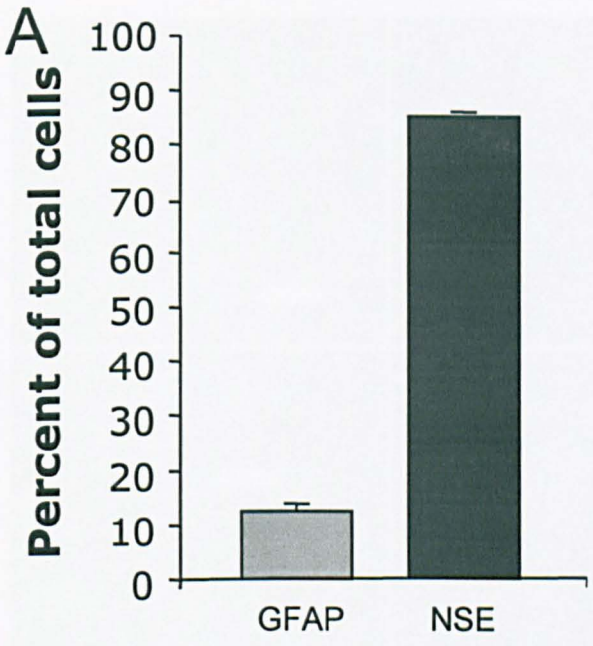
4.3 RESULTS

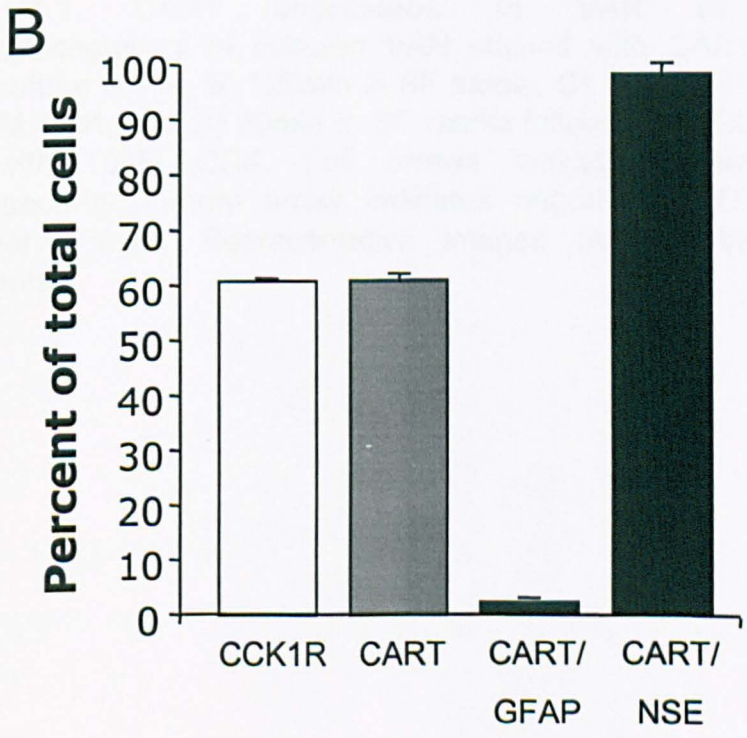
4.3.1 Characterisation of cultured VAN

After culture of dissociated rat nodose ganglia for 96hr 79.1±1.8% of cells were identified as neurons on the basis of NSE expression (figure 4.1.A) while the majority of the remaining cells (12.7±1.3%) were identified as glial cells on the basis of GFAP staining. Co-expression of CART and CCK1R was observed in 60.8% of the total cell population (figure 4.1.B). CART co-localised with 98.2±1.2% of cells stained with an anti-NSE antibody. As expected both CART and CCK1R were expressed by <1% of GFAP-positive cells.

In VAN cultured in FM, CART was relatively abundant (figure 4.2.A). However, serum withdrawal for 2hr led to virtually a complete loss of CART immunoreactivity (figure 4.2.B). Moreover, addition of CCK (10nM) during culture of serum-deprived VAN was associated with CART abundance comparable to that in the control (figure 4.2.C). In order to test whether CCK induced CART synthesis rather than prevented CART depletion, cells were pre-incubated in SF medium (30min) in order to allow for depletion to occur before the addition of 10nM CCK for 2hr (figure 4.2.D). These experiments indicated that CCK is not simply preventing degradation of CART, since CART abundance is comparable in neurons pre-incubated with medium

Figure 4.1. Composition of cell types in VAN cultures. A) VAN stained with antibodies to GFAP and NSE. B) VAN stained with antibodies to CCK1R, CART, CART and GFAP, CART and NSE shown as a percent of the total cell population. (N=6)


A
Percent of total cells
100
90
80
70
60
50
40
30
20
10
0
GFAP
NSE


B
Percent of total cells
100
90
80
70
60
50
40
30
20
10
0
CCK1R
CART
CART/ GFAP
CART/ NSE

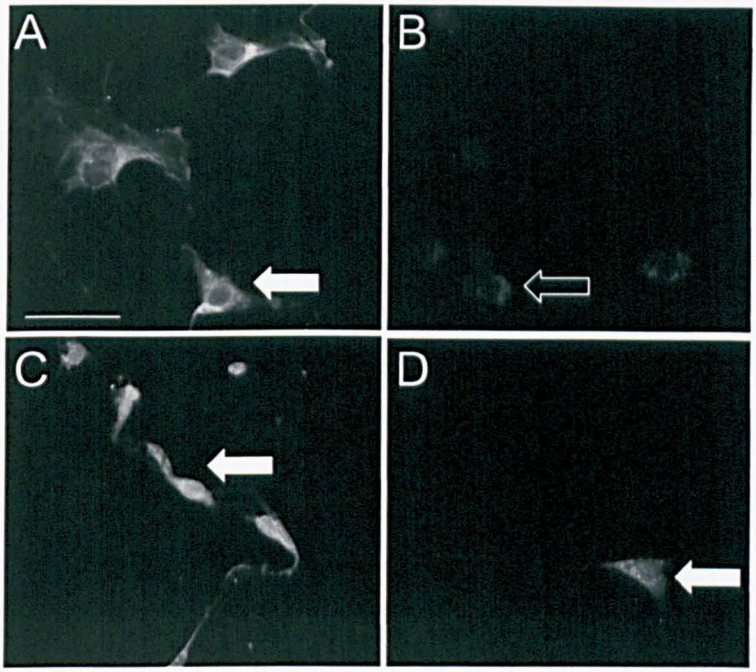


Figure 4.2. CART expression in VAN in culture. Immunocytochemistry of cultured VAN stained with CART antibody after A) culture in FM, B) 120min in SF media, C) 120min in SF media with 10nM CCK, and D) 30min in SF media followed by 120min in SF media with 10nM CCK. Full arrows indicate positive CART-immunoreactivity. Empty arrow indicates negative CART neurons. Scale bar= 50µm. Representative images from 6 independent experiments.

before addition of CCK as the positive controls.

4.3.2 CCK increases CART abundance and localisation

CCK was found to regulate CART expression in a dose-dependent manner (figure 4.3). CART was absent from neurons cultured in 2hr SF medium (figure 4.3.A). The abundance of CART increased after incubation for 2hr with 1nM CCK (figure 4.3.B). CART abundance was optimal when neurons were incubated for 2hr with 10nM CCK (figure 4.3.C). CART immunoreactivity was saturated when neurons were cultured 2hr with 100nM CCK (figure 4.3.D).

CCK was shown to regulate CART expression in a time-dependent manner (figure 4.4). CART was absent from neurons cultured in SF medium (figure 4.4.A). The abundance of CART increased after 30min cultured with 10nM CCK (figure 4.4.B). CART immunoreactivity was increased further when neurons were incubated for 1hr with 10nM CCK (figure 4.4.C). Optimal CART abundance was found after culture with 10nM CCK for 2hr (figure 4.4.D).

In order to determine CART localisation after time in the neurons a transgolgi marker, TGN38, was used. TGN38 is one of the few known resident integral membrane proteins of the TGN. Since it cycles constitutively between the TGN and the plasma membrane,

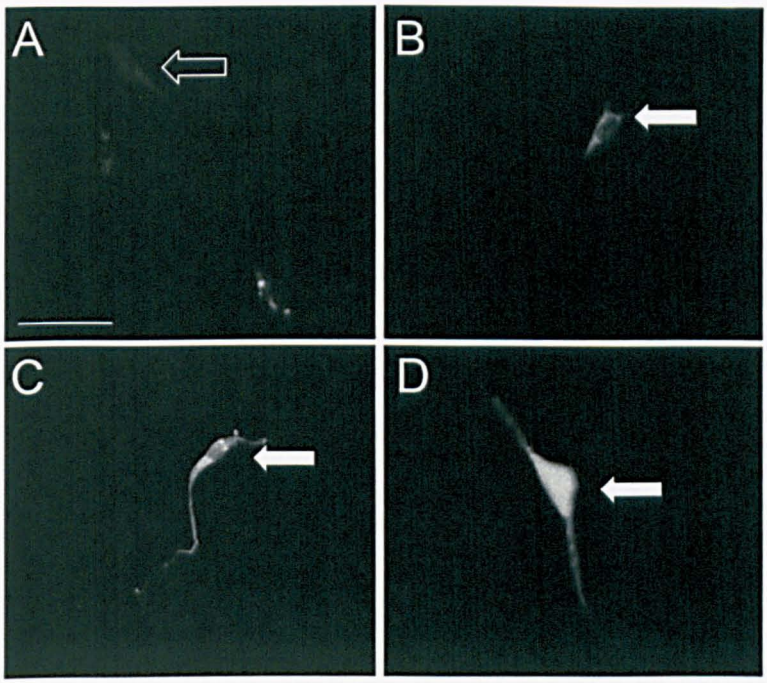


Figure 4.3. Rising CCK concentrations increase CART abundance. Immunocytochemistry of cultured VAN stained using CART antibody after A) 120min SF media, B) 120min SF media with 1nM CCK, C) 120min SF media with 10nM CCK, and D) 120min SF media with 100nM CCK. Full arrows indicate positive CART-immunoreactivity. Empty arrow indicates negative CART neurons. Scale bar=50µm. Representative images from 6 independent experiments.

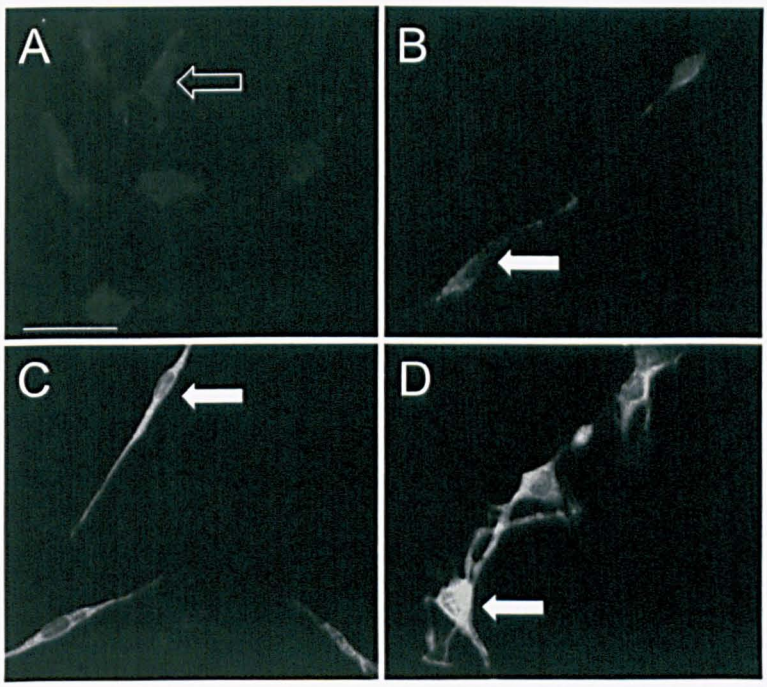


Figure 4.4. Increased exposure to CCK increases CART abundance. Immunocytochemistry of cultured VAN stained with CART antibodies after A) 120min SF media, B) 30min SF media with 10nM CCK, C) 60min SF media with 10nM CCK, and D) 120min SF media with 10nM CCK. Full arrows indicate positive CART-immunoreactivity. Empty arrow indicates negative CART neurons. Scale bar=50µm. Representative images from 6 independent experiments.

TGN38 is ideally suited as a model protein for the identification of post-Golgi trafficking. In VAN cultured 2hr in SF medium no CART-immunoreactivity was detectable, but TGN38 was localised on either side of the nucleus in globular structures. CART-immunoreactivity was detected after 30min of treatment with 10nM CCK predominantly in a perinuclear compartment with no overlap with the TGN marker (figure 4.5.D). After 60min, CART exhibited co-localisation with TGN38, although CART was also found in a perinuclear compartment (figure 4.5.I). By 120min CART was detected in secretory vesicles throughout the cell with many vesicle structures exhibiting co-localisation with TGN38 (figure 4.5.J).

4.3.3 CCK transcriptionally regulates CART expression

CART was previously shown to be transcriptionally regulated by CCK, and this was confirmed by immunocytochemistry using a transcriptional inhibitor, Actinomycin D (figure 4.6). Actinomycin D is an antibiotic that binds to DNA duplexes, thereby interfering with the action of enzymes engaged in replication and transcription. As seen previously, administration of CCK for 2hr induced CART expression in VAN (figure 4.6.A). Pre-incubating VAN with Actinomycin D for 30min before stimulating with CCK (2hr) inhibited CART transcription. Actinomycin D however inhibits all transcription in the neurons, perhaps explaining the morphological change. In the presence of

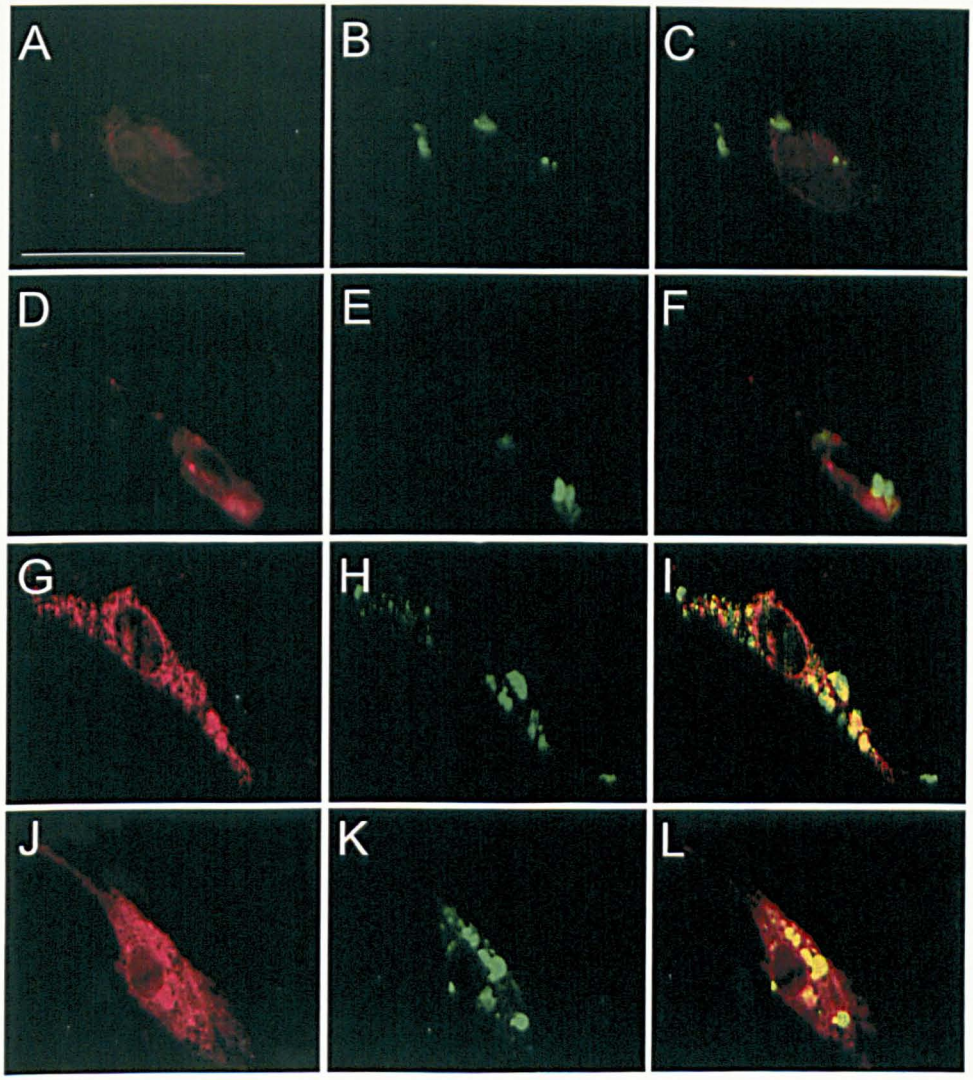


Figure 4.5. CART translocates through the secretory pathway. Immunocytochemistry of cultured VAN after A), B) and C) 120min SF medium, D), E) and F) 30min SF medium with 10nM CCK. G), H), and I) 60min SF medium with 10nM CCK. J), K), and L) 120min SF medium with 10nM CCK. Images A, D, G, J stained with CART antibodies. Images B, E, H, and K stained with TGN38 antibodies. Images C, F, I, L represent overlap of both CART and TGN38 antibodies. Scale bar=50μm. Representative images from 8 independent experiments.

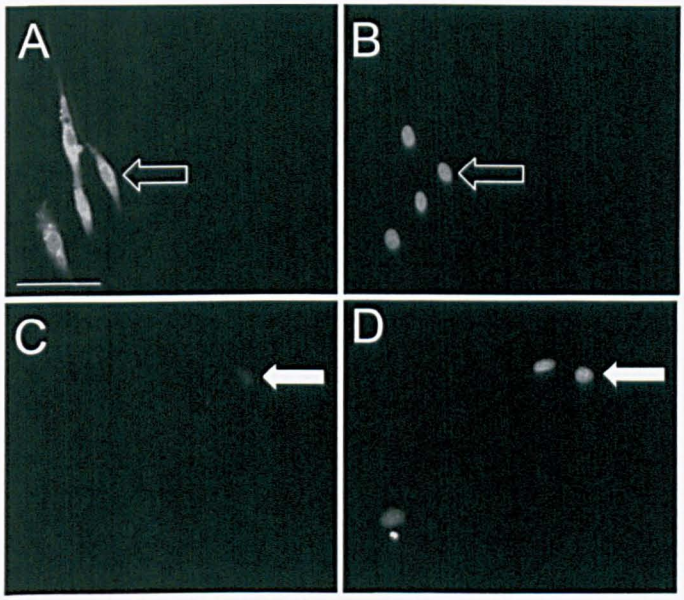


Figure 4.6. CCK transcriptionally regulates CART expression. Immunocytochemistry of cultured VAN after A) and B) SF media with 10nM CCK, C) and D) SF media with Actinomycin D and 10nM CCK. Images A) and C) stained with CART antibodies. Images B) and D) stained with DAPI. Full arrows indicate positive CART-immunoreactivity. Empty arrow indicates negative CART neurons. Scale bar=50µm. Representative images from 6 independent experiments.

SF medium with CCK the cultured neurons were elongated. Conversely, in the presence of the inhibitor the cells were rounded.

Additional evidence that CCK transcriptionally regulates CART was demonstrated by *in situ* hybridisation (figure 4.7). In the absence of CCK, there was no CART mRNA, indicated by the lack of positive silver stain (figure 4.7.B). In the presence of 10nM CCK for 2hr, CART mRNA was increased in cultured VAN indicated by positive silver stain (figure 4.7.A).

4.3.4 CCK regulates CREB and EGR-1

In the previous chapter, it was demonstrated that CCK induced CREB phosphorylation and EGR-1 expression when injected *in vivo* in rats fasted 24hr. Dapi staining indicated the presence of 10 cells (figure 4.8.B), however these cells exhibited no immunoreactivity with phosphoCREB antibodies when cultured in SF condition (figure 4.8.A). CCK increased the abundance of phosphoCREB to 53.8±0.5% of nuclei of cultured neurons within 30min (figure 4.8). It is important to note that the total CREB population does not change between SF or in the presence of CCK (figure 4.9). This indicates that the phosphoCREB antibody is reliable. EGR-1 was also absent from VAN cultured in SF medium (figure 4.10.A). CCK (2hr) also increased the abundance of EGR-1 in the nuclei of cultured neurons (figure 4.10.D). Furthermore, cell counts demonstrated that 6.2±0.7% of the total cell

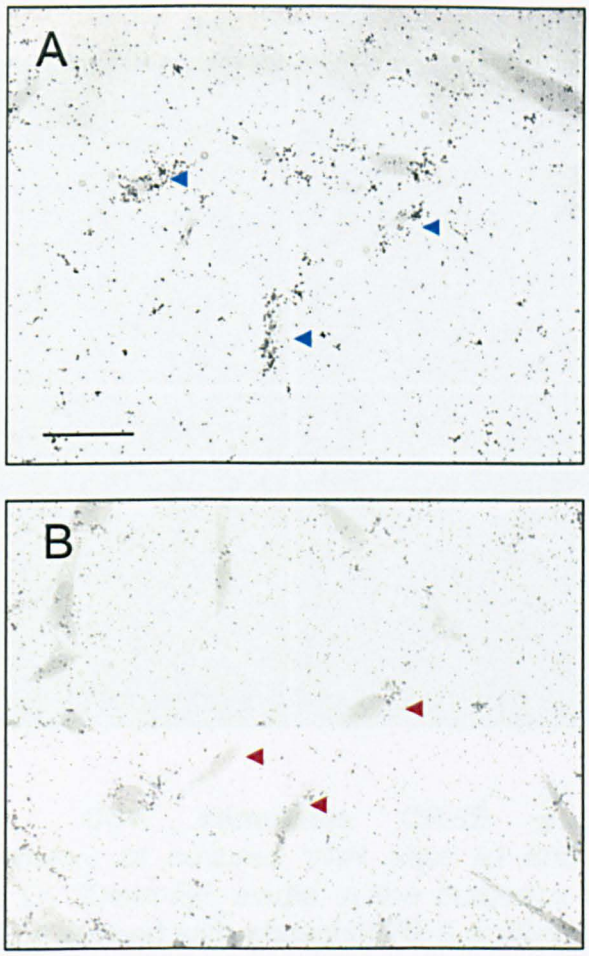


Figure 4.7. CCK regulates CART mRNA. *In situ* hybridisation of cultured VAN after A) 120min SF medium with 10nM CCK, B) 120min SF medium. Blue arrows indicate positive cells. Red arrows indicate negative cells. Scale bar= 50µm. Representative images from 4 independent experiments.

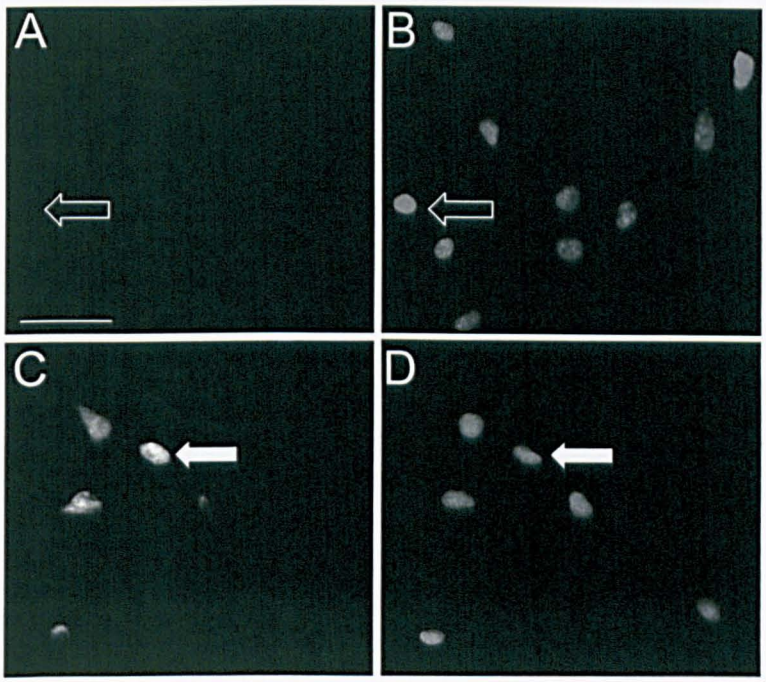


Figure 4.8. CCK stimulates CREB phosphorylation. Immunocytochemistry of cultured VAN after A) and B) 30min SF media, C) and D) 30min SF media in the presence of 10nM CCK. Images A) and C) stained with phosphoCREB antibodies. Images B) and D) stained with DAPI. Full arrows indicate positive phosphoCREB-immunoreactivity. Empty arrows indicate negative phosphoCREB neurons. Scale bar=50μm. Representative images from 6 independent experiments.

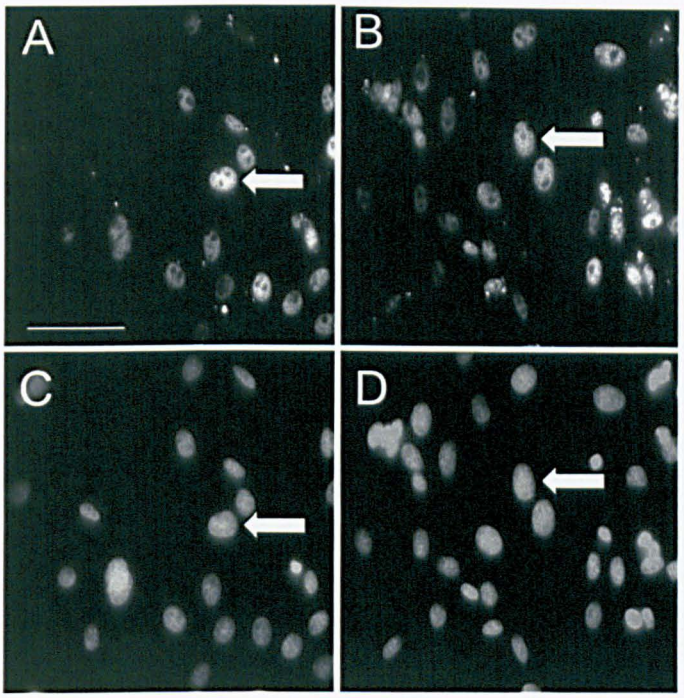


Figure 4.9. Total CREB does not change with CCK. Immunocytochemistry of cultured VAN after A) and B) 120min SF media. C) and D) 120min SF media in the presence of 10nM CCK. Images A) and B) stained with total CREB antibodies. Images C) and D) stained with DAPI. Arrows indicate total CREB-immunoreactivity. Scale bar=50µm. Representative images from 5 independent experiments.

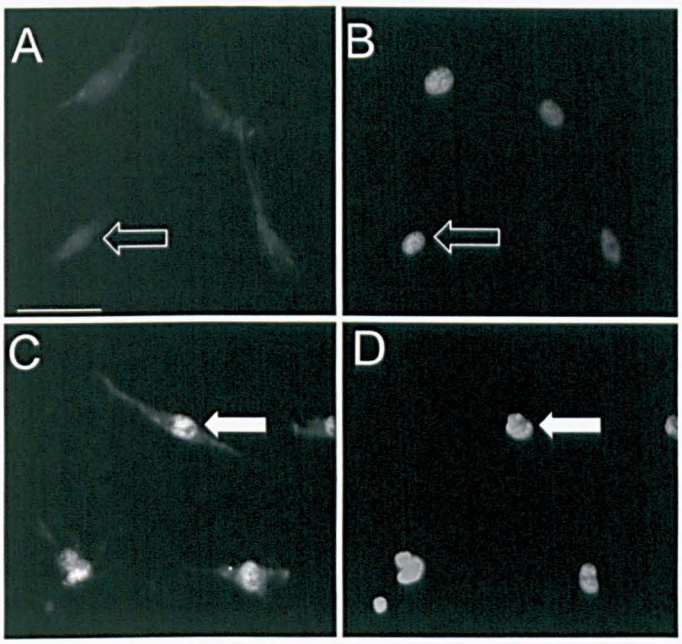


Figure 4.10. CCK regulates EGR-1 expression in culture. Immunocytochemistry of cultured VAN after treatment with A) and C) 120min SF media, B) and D) 120min SF media in the presence of 10nM CCK. Images A) and D) stained with EGR-1 antibodies. Images B) and D) stained with DAPI. Full arrow indicates positive EGR-1 immunoreactivity. Empty arrow indcates negative EGR-1 neurons. Scale bar=50μm. Representative samples from 6 independent experiments.

population express EGR-1 when cultured in SF medium. This expression increased to 16.6±2.4% within 10min exposure to 10nM CCK, and peaked at 40.4±3.9% after 2hr exposure of 10nM CCK. EGR-1 expression was regulated in a dose-dependent manner by CCK (figure 4.11).

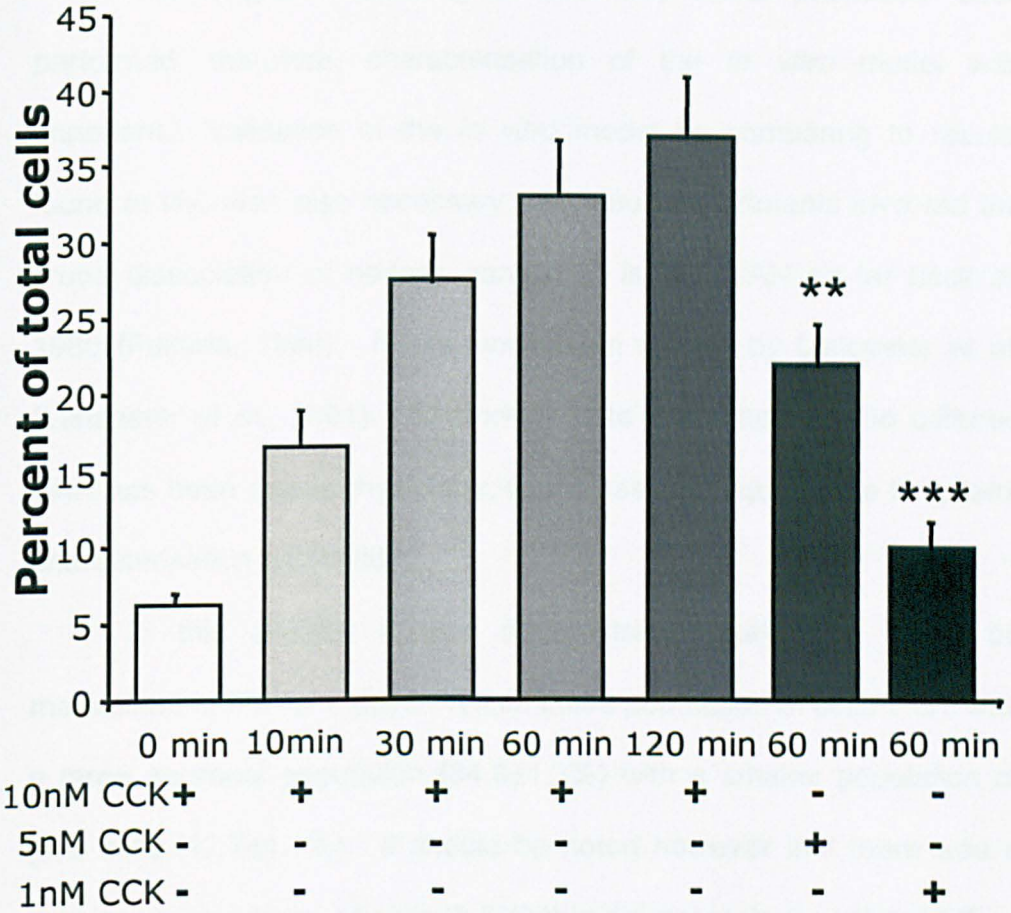


Figure 4.11. CCK regulates EGR-1 expression in a time dependent manner. Cultured VAN stained with antibodies to EGR-1 after treatment with 120min SF medium (6.16±0.7%), 10min SF medium with 10nM CCK (16.6±2.4%), 30min SF medium with 10nM CCK (22.4±3.1%), 60min SF medium with 10nM CCK (33.0±3.5%), 120min SF medium with 10nM CCK (40.4±3.9%), 60min SF medium with 5nM CCK (22.0±2.8%), and 60min SF medium with 1nM CCK (10.0±1.7%). Results are expressed as a percentage of total cells. ** represents $p<0.01$, t-test, *** represents $p<0.001$, t-test. (N=6).

4.4 DISCUSSION

The long-term culturing of VAN has never previously been performed, therefore, characterisation of the *in vitro* model was important. Validation of the *in vitro* model by comparing to results found *in vivo* was also necessary. Previous experiments involved the crude dissociation of nodose ganglia to isolate VAN as far back as 1980 (Fukuda, 1980). It has since been refined by Lancaster et al. (Lancaster *et al.*, 2001). All work to date preformed on the cultured VAN has been electrophysiology, which has not required the long-term characterisation and validity.

In this chapter it was demonstrated that VAN could be maintained in FM for 4 days. Within these population of cells there was a large neuronal population (84.8±1.3%) with a smaller population of glial cells (12.7±1.1%). It should be noted however that there was a very small population of cells (2.5%) that did not stain for either NSE or GFAP antibody. These cells are probably contamination occurring during the dissection of the tissue and are most likely fat or muscle cells, which are unlikely to have any effect on the results.

It is accepted that glial cells *in vivo* out-number neurons by about 5 to 1 (McClintic, 1985). Historically, they were seen as mere protective material holding the neuronal structure intact (with the word glia deriving from the greek work for glue). Only recently has this concept been challenged. It was found that in genetically modified

mice, an ablation of enteric glial cells, resulted in neuronal degeneration (Bush *et al.*, 1998). This suggests that glial cells are crucial for neuronal maintenance. However, the mechanisms by which they support neurons are largely unknown. They could maintain a protective microenvironment, or maintain neuronal integrity by the release of neurotrophic factors, or simply by providing structural support.

Glial cells are therefore thought to play a role in maintaining the neuronal population. However, dissociated neurons would not require glial cells to regulate the ionic microenvironment or to help them to anchor to the glass plates, but their presence in the culture may be required for their ability to release neurotrophic factors. Even though glial cells are able to proliferate after four days, the culture was only made of 12.7% glia; it was therefore decided not to prevent their proliferation in the culture. The large number of glial cells found *in vivo* was no longer a prerequisite for cell culture. In work not shown I found that a seven-day culture of nodose ganglia could provide an equal distribution of neurons to glial cells, but to the detriment of the culture, as the wells became over populated. It was decided that a four-day culture would provide the optimal confluency. The neurons did not demonstrate abnormal phenotypes at outnumbering the glial cells 7:1, since all the results found *in vivo* were reproducible in the culture.

There was high CART abundance in neurons cultured in FM, however CART was virtually absent after 2hr serum withdrawal. The abundance of CART was also elevated *in vivo* in rats fed *ad libitum*, while CART abundance was greatly reduced after fasting for 24hr. This would suggest serum withdrawal is comparable to the fasted state found *in vivo*. However it is unclear if the timing *in vivo* could happen as quickly as 2hr. Other groups have shown that serum starving G6 glioblastoma cells could modulate other regulators of appetite, providing further evidence that this system can be used as a model for fasting. Significant decreases in *leptin* gene expression were observed after 24hr in SF medium and this was fully reversible by addition of serum within 6hr (Morash *et al.*, 2000).

The mechanisms by which FM mimics the fed state, and removal of serum mimics the fasted state are unclear. The simplest explanation is that foetal bovine serum (FBS) may contain CCK (Huang *et al.*, 1995). The SF medium lacks CCK present in FBS, so CART would no longer be synthesised. FM containing FBS or administration of CCK to serum deprived VAN would reverse this effect. Another possible explanation could be the requirement of the essential amino acid L-Leucine. It has been recently demonstrated that L-Leucine had the ability to activate mTOR in the hypothalamus of rats and to inhibit food intake (Cota *et al.*, 2006). In the absence of FBS, DMEM contains 0.45mM L-Leucine. Cota *et al* found no effect of L-Leucine at

concentrations less than 16.8mM (Cota *et al.*, 2006). It is possible therefore that the cellular fuel status is too low under SF condition, but administration of CCK, or L-Leucine in FBS, is sufficient to redress the low fuel status.

Administration of CCK for 1hr to serum-deprived VAN was sufficient to increase CART abundance. CART immunoreactivity in VAN reached maximal fluorescence after 2hr, with 12-fold increase compared to serum withdrawal. This is similar to the *in vivo* results whereby 1hr administration of CCK was sufficient to reverse the lowered CART abundance seen after fasting. Increasing the concentration of CCK also increased CART expression in VAN.

In live animals, the presence of fats or proteins induces the release of CCK locally at a concentration of 1-5nM. This suggests that VAN terminating in the upper intestine could come into contact with these high CCK doses. VAN terminating in the stomach, however, would only come into contact with CCK at a maximal concentration of 15pM since CCK would have to circulate through the blood stream in order to bind to these receptors. The doses used *in vivo*, after an i.p. injection of 10nmol CCK, equates to approximately 2nM CCK, immediately after injection, reducing to 500pM after 20min (Forster *et al.*, 1990; Forster and Dockray, 1992). Administration of 10nM CCK to the cells is 2 times higher than would be found in the animal. However, CART was elevated after 1nM CCK in the dose-dependent experiment.

It is possible therefore, that increasing the exposure of lower doses of CCK may also increase CART abundance since CCK-induced CART abundance was found to be both time- and dose-dependent. In addition, increases in CART abundance could possibly be occurring at far lower concentrations of CCK but not be detectable by the relatively insensitive technique of immunocytochemistry.

After 2hr CCK in SF medium, or when cultured in FM, CART was virtually exclusively expressed in neurons. The CCK1R was found in 60% of the total cell population. Another group also reported that a similar number of cells (55%) respond to CCK in VAN (Peters *et al.*, 2006). Separately, both CART and CCK1R were present in a similar percentage of cells. It is possible therefore that they are localised to the same population of cells. However, due to species overlap with antibodies, dual immunocytochemical staining with CART and CCK1R was not possible. Another group however, has demonstrated that all CCK1R mRNA expressing profiles also contained CART mRNA in VAN (Broberger *et al.*, 1999).

Evidence from cultured VAN indicated that CCK alone was sufficient to up-regulate CART abundance. TGN38 is one of the few known resident integral membrane proteins of the trans-Golgi network (TGN). Since it cycles constitutively between the TGN and the plasma membrane, TGN38 is ideally suited as a model protein for the identification of post-Golgi trafficking motifs (Roquemore and Banting,

1998). TGN38 is widely used in the literature as a late marker for the secretory pathway (Matas *et al.*, 2004; Giau *et al.*, 2005). Within 30min of CCK administration, CART was localised to the endoplasmic reticulum and early golgi. After 2hr CCK was localised throughout the cell including in the late golgi network, and vesicles. CART has a target sequence suggesting that it is a secretory protein (Stein *et al.*, 2006; Yanik *et al.*, 2006). These new data provide further evidence that CART is a secretory protein.

In the previous chapter it was demonstrated that feeding transcriptionally regulated CART abundance. Reverse transcriptase PCR of nodose ganglia from fed animals had elevated CART mRNA levels compared to nodose ganglia from fasted rats. The experiments in this chapter in cultured VAN now provide clear evidence that CCK induces CART synthesis via a transcriptional mechanism. If CCK was preventing CART degradation as suggested earlier, incubating VAN in SF would deplete CART and administration CCK would prevent the further depletion of CART. However, this was not found to be the case, there was no difference between cells incubated with SF or FM for 30min, before the addition of 10nM CCK 2hr. Further evidence that CART is transcriptionally regulated was shown in the experiment in which the transcriptional inhibitor, Actinomycin D, prevented CCK inducing CART abundance. Finally, *in situ* hybridisation indicated that

CART mRNA was elevated in VAN treated with CCK compared to unstimulated cells.

There is a CRE site on the CART promoter, and it was shown that CCK led to the phosphorylation of the transcription factor CREB in rats fasted for 24hr. This phosphorylation of CREB in response to CCK was repeated in the culture system. Transcriptional activation of cellular genes by a CREB-mediated response usually peaks after 30min of stimulation and decreases after 2–4hr (Mayr and Montminy, 2001). In VAN, CCK was found to phosphorylate CREB within 30min. PhosphoCREB was found in the nucleus of over half the total cell population after 2hr stimulation with 10nM CCK. CCK was also shown *in vivo* to induce EGR-1 expression. EGR-1 expression was found in the nucleus of activated neurons. Within 10min of 10nM CCK 16.6±2.4% of neurons expressed EGR-1. EGR-1 expression was optimal after 2hr (40.4±3.9%). It was not possible to co-localise CART and EGR-1 immunoreactivity as both antibodies were raised in rabbit. However, a greater proportion of cells expressed CART compared to EGR-1 cells. Since there is no EGR-1 consensus binding site on the CART promoter it is unlikely that EGR-1 plays a role in regulating CART abundance. Instead EGR-1 is more useful as a neuronal marker of activation.

In this chapter it was demonstrated that VAN could be cultured for 4 days. The population of neurons in culture express CART when

cultured in FM. Withdrawal of serum in these cells down-regulated CART expression similarly to fasted animals *in vivo*, while addition of CCK up-regulated CART expression similarly to fed animals *in vivo*. It was confirmed that CCK transcriptionally regulates CART, and that CCK up-regulates EGR-1 expression and phosphorylation of CREB. I now have a successful model of appetite by which to study the cellular mechanisms of VAN.

CHAPTER FIVE

Mechanisms of CART up-regulation

5.1 INTRODUCTION

Work in previous chapters indicated that CCK regulated CART transcription and that CCK activated CREB in VAN. It was hypothesised that CCK induced CART expression via the transcription factor CREB.

A number of putative cis-regulated elements have been identified in both the human and mouse CART promoter sequences, and transcriptional regulation has been studied in cell lines. Little work has been done in primary cells or *in vivo*. In rat pituitary GH3 cells, CART mRNA levels were up-regulated by increased cyclic AMP (Barrett *et al.*, 2001) and a signalling pathway involving PKA has been identified (Dominguez *et al.*, 2002) implicating CREB in the regulation of CART transcription (Lakatos *et al.*, 2002). Regulation of CART synthesis has also been found in CATH.a cells which are derived from the locus coeruleus, a region of the brain involved in opiate dependence and withdrawal (Dominguez and Kuhar, 2004). *In vivo* work has further confirmed that CART is up-regulated by a cyclic AMP/PKA messenger system (Elefteriou *et al.*, 2005; Jones and Kuhar, 2006).

In this chapter, the activity of the CART promoter in VAN is described. Therefore the aims of this chapter were to elucidate the mechanisms by which CCK regulates CART, and phosphorylates CREB.

5.2 METHODS

Nodose ganglia were dissected as described in chapter 2.2.1.1, and cells were dissociated as described in chapter 2.2.5.

Immunohistochemistry was performed as described in chapter 2.2.5.1, using the following primary antibodies: rabbit anti-CART, mouse anti-Flag, rabbit anti-phosphoCREB; and the following secondary antibodies: donkey anti-rabbit conjugated to TxR or FITC and donkey anti-mouse conjugated to FITC as appropriate. VAN were cultured in FM for 3-4 days, and then transferred to SF medium with or without CCK, PMA, or Ro-32-0432 for 30-120min. Cells were also treated with Ro-32-0432 for 30min followed by CCK for 120min.

Luciferase assays were performed as described in chapter 2.2.5.4. VAN were cultured in FM for 3-4 days, transfected and left for 24hr as described in chapter 2.2.5.2 with the following constructs: ACREB, empty vector, 3451bp of the CART-promoter luciferase-reporter construct (3451CART-LUC), 3100CART-LUC, 620CART-LUC, 120CART-LUC, E1A, E1A mutant, and PKC-alpha GFP. VAN were then transferred to SF medium with or without CCK or Ro-32-0432 for 120min. Cells were also treated with Ro-32-0432 for 30min followed by CCK for 120min.

5.3 RESULTS

5.3.1 The role of CREB

5.3.1.1 CCK regulates CART abundance via CREB in VAN

To establish a link between CREB and CART expression I examined the consequences of transfecting cultured VAN with a dominant negative mutant A-CREB. Using a flag epitope flanking A-CREB, the vector was transfected in 8.2±0.5% of neurons. In these neurons, CCK had little effect in increasing CART expression, whereas in the same cultures untransfected cells demonstrated increased CART abundance in response to CCK (figure 5.1). In control experiments with a GFP-encoding vector, transfected cells exhibited an increase in CART abundance in response to CCK similar to controls ($p<0.001$; figure 5.2).

5.3.1.2 The effect of CCK on CART promoter luciferase reporter activity

To assess the importance of the regulatory elements within the CART upstream sequence, the activity of various 5'-proximal regions of the CART gene were tested for their ability to drive gene expression when cloned upstream of the luciferase gene. Four luciferase-expressing constructs were tested: 3451CART-LUC, 3100CART-LUC, 641CART-LUC, and 102CART-LUC. The luciferase activity of each construct was compared to that of pGL3-BASIC, the parent vector that

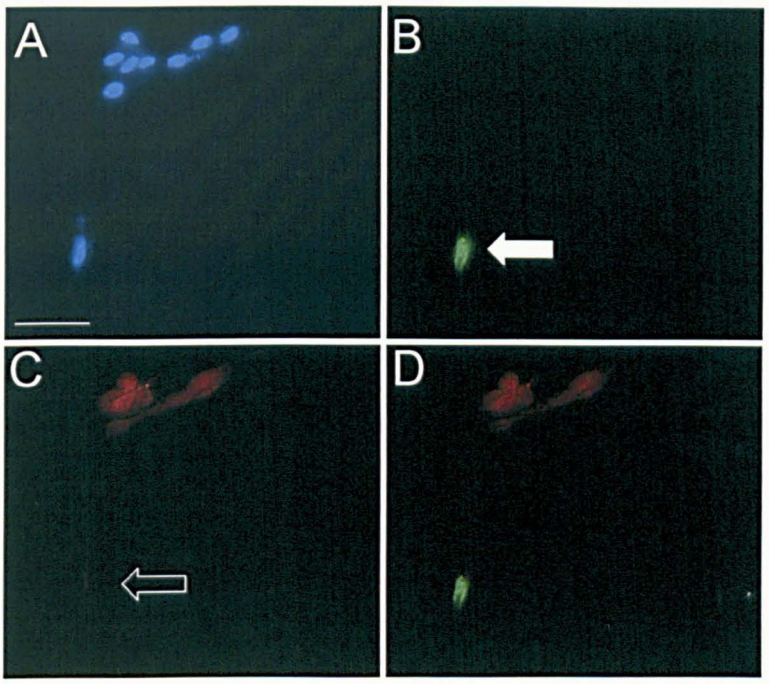


Figure 5.1. ACREB inhibits CCK induced CART abundance. Immunocytochemistry of cultured VAN after 120min SF medium with 10nM CCK stained with A) DAPI, B) Flag antibodies (indicating presence of ACREB), C) CART antibodies, and D) overlay of Flag and CART staining. Full arrow indicates ACREB positive cell. Empty arrow indicates negative CART neuron. Scale bar= 50µm. Representative images from 10 independent experiments.

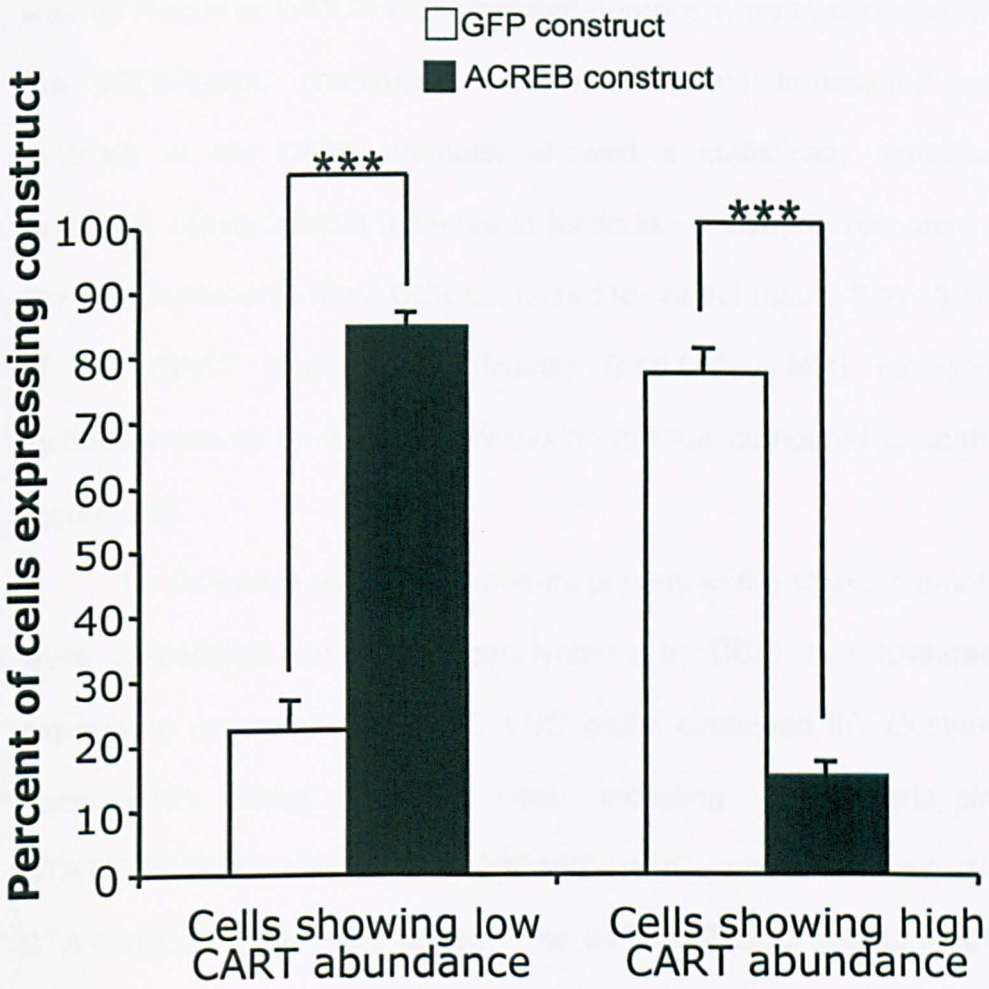


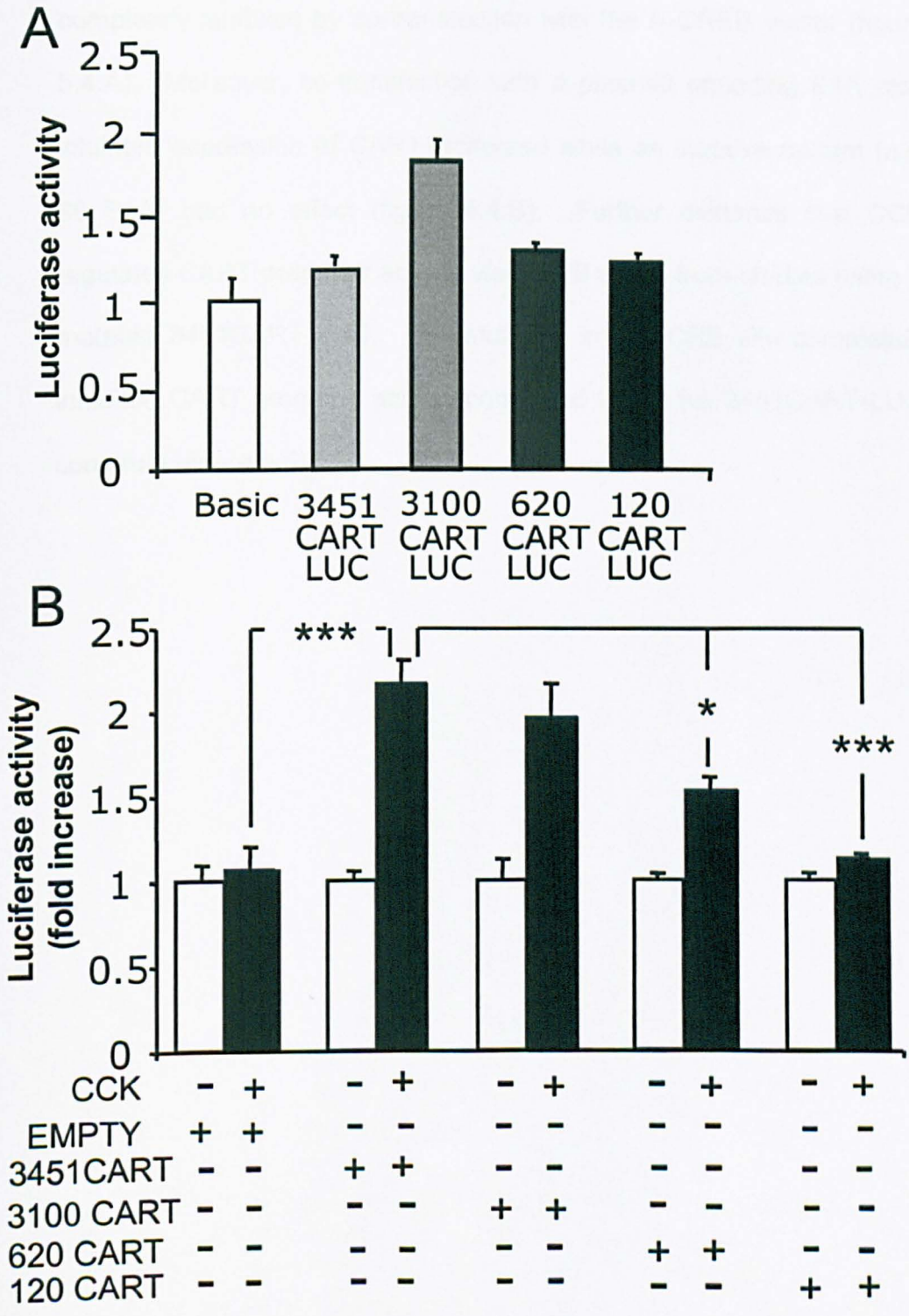
Figure 5.2. ACREB inhibits CCK induced CART expression. VAN cultured 120min in SF medium with CCK transfected with GFP construct showing high CART abundance, ACREB construct showing low CART abundance. *** represents P<0.001, t-test. (N=10)

lacks the promoter region (background activity). In figure 5.3, there was no response to CCK when cultured neurons were transfected with the pGL3-BASIC construct. However neurons transfected with 3.451Kb of the CART promoter showed a statistically significant ($p<0.001$, t-test) 2.2-fold increase in luciferase activity in response to 2hr stimulation with 10nM CCK compared to control (figure 5.3). 3.1Kb of the CART promoter significantly ($p<0.001$, t-test) increased luciferase activity by 2.0-fold in response to CCK compared to control (figure 5.3).

To determine which cis-elements present in the CART promoter were responsible for the responsiveness to CCK, two luciferase expressing constructs, 641CART LUC which contained the clustered transcription factor binding sites including the overlapping STAT/CRE/AP1 site, and 120CART LUC which lacked the STAT/CRE/AP1 site were tested. The 641CART LUC and 120CART LUC constructs were transfected into VAN and 18hr after transfection, medium was changed and cells were treated with 10nM CCK for 2hr. As illustrated in figure 5.3, a significant ($P<0.05$, t-test) decrease in luciferase activity after CCK treatment was produced with 641CART LUC compared to the largest 3451CART LUC vector (figure 5.3). There was a large statistical significant difference in luciferase activity between 120CART LUC and 3451CART LUC. There was a significant increase ($p<0.05$, t-test) in luciferase activity between 120CART LUC

and 620CART LUC, which suggests that the region between 120 and 620 contains cis-elements that are responsive to changes in cyclic AMP levels.

Figure 5.3. CCK regulates CART promoter activity. Luciferase assay of VAN transfected with various CART promoters. A) VAN cultured 2hr in SF medium. Empty CART promoter (basic), 3451CART LUC promoter, 3100CART LUC promoter, 620CART LUC promoter, 120CART LUC promoter. B) Cultured VAN transfected with empty vectors, 3451CART LUC vector, 3100CART LUC vector, 620CART LUC vector, 120CART LUC vector. Bars 1, 3, 5, 7, and 9 represent VAN cultured in SF medium 120min. Bars 2, 4, 6, 8, and 10 represent VAN cultured in SF medium 120min with 10nM CCK. *** represents $p<0.001$, t-test; * represents $p<0.05$, t-test. (N=15).


A
Luciferase activity
2.5
2
1.5
1
0.5
0
Basic
3451 CART LUC
3100 CART LUC
620 CART LUC
120 CART LUC
B
Luciferase activity (fold increase)
2.5
2
1.5
1
0.5
0
***
*
***
CCK - + - + - + - + - +
EMPTY + + - - - - - - - -
3451CART - - + + - - - - - -
3100 CART - - - - + + - - - -
620 CART - - - - - - + + - -
120 CART - - - - - - - - + +

The increase in CART promoter activity of vector 3451CART LUC was completely inhibited by co-transfection with the A-CREB vector (figure 5.4.A). Moreover, co-transfection with a plasmid encoding E1A also inhibited expression of CART-luciferase while an inactive mutant (Δ2-36 E1A) had no effect (figure 5.4.B). Further evidence that CCK regulates CART promoter activity via CREB came from studies using a mutated 3451CART LUC. The mutation in the CRE site completely inhibited CART promoter activity compared to the full 3451CART-LUC construct (figure 5.5).

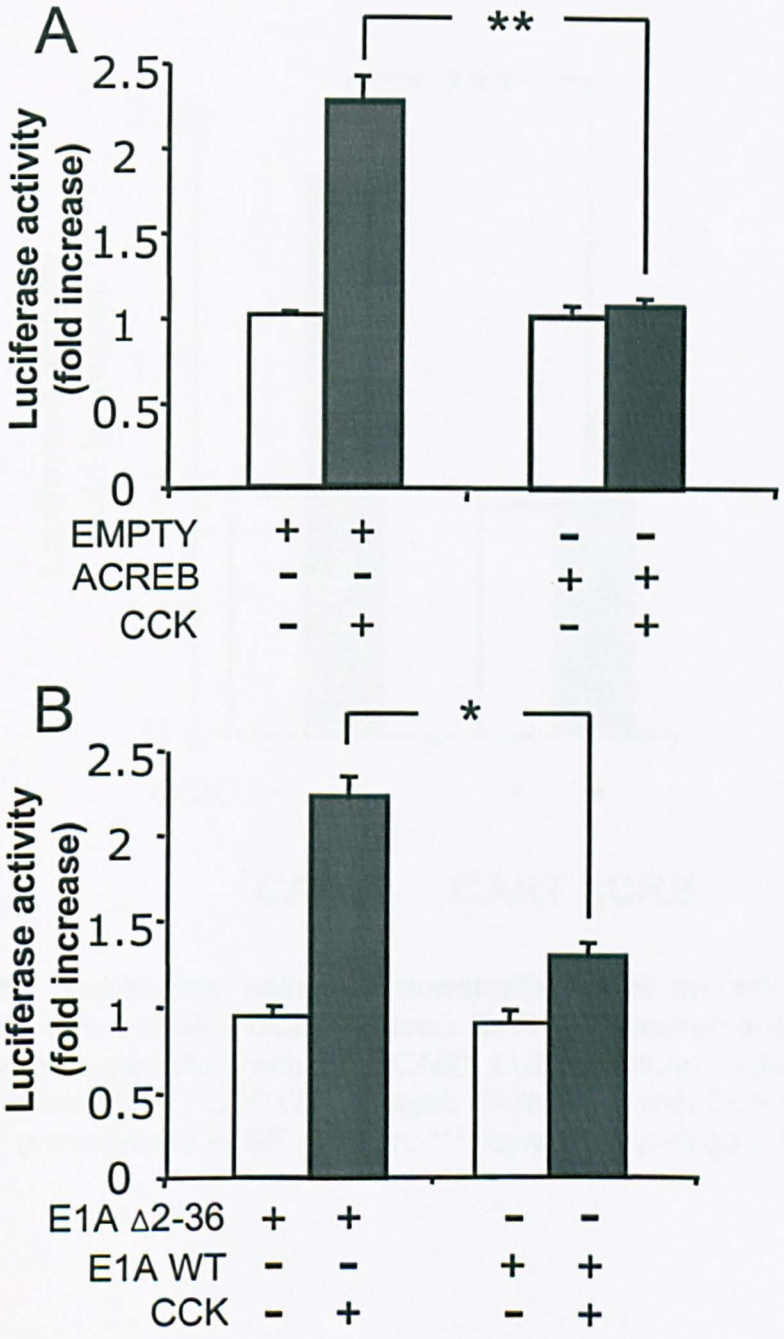


Figure 5.4. Luciferase assay demonstrating that CCK acts via CREB to regulate CART promoter activity. Cultured VAN transfected with 3451CART LUC vector and further transfected A) with ACREB or empty vector. VAN cultured with or without CCK and with in SF medium, B) E1A or E1A mutant. VAN cultured with or without CCK in SF medium. * represents $p<0.05$, t-test; ** represents $p<0.01$, t-test. (N=6)

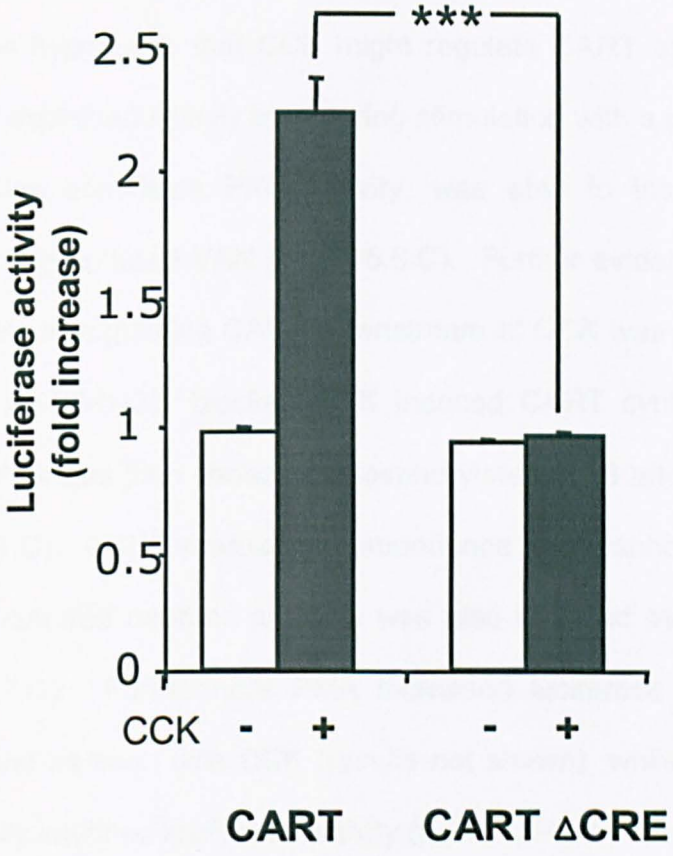


Figure 5.5. Luciferase assay demonstrating that mutation of CART CRE site inhibits CCK induced CART promoter activity. Cultured VAN transfected with 3451CART LUC promoter luciferase reporter or a 3451CART LUC CRE mutant. Stimulated with CCK in SF medium or unstimulated in SF medium. *** represents $p<0.001$, t-test. (N=6).

5.3.2 The role of PKC

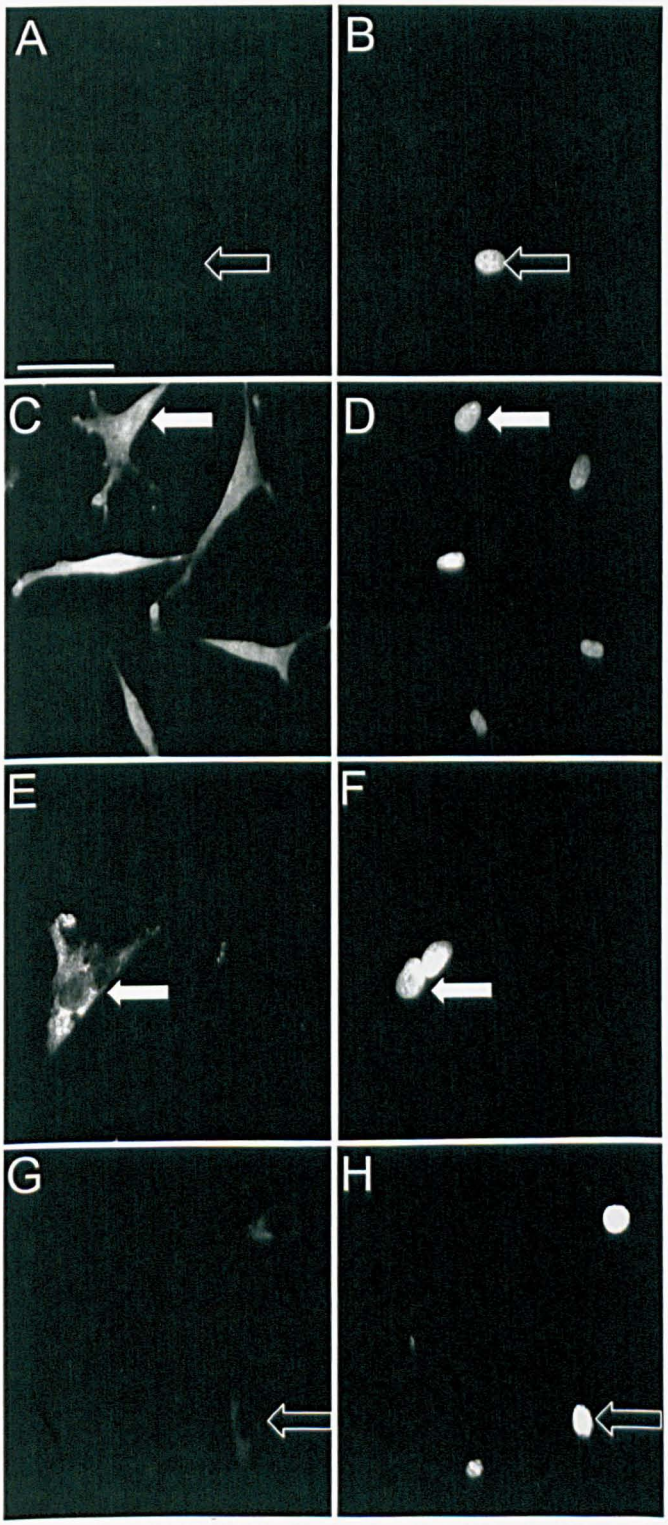
5.3.2.1 CCK activates PKC in VAN

The hypothesis that CCK might regulate CART expression via PKC was explained initially by showing stimulation with a phorbol ester, PMA, which stimulates PKC activity, was able to increase CART abundance in cultured VAN (figure 5.6.C). Further evidence that PKC plays a role in regulating CART downstream of CCK was that the PKC inhibitor, Ro-32-0432, blocked CCK induced CART synthesis (figure 5.6.D). PMA was then shown to phosphorylate CREB after 30 minutes (figure 5.7.C). CCK increased the abundance of phosphoCREB in the nuclei of cultured neurons and this was also inhibited by Ro-32-0432 (figure 5.7.D). Furthermore PMA increased luciferase activity to a similar level as seen with CCK (results not shown), while Ro-3204-32 significantly inhibited luciferase activity ($p<0.01$, t-test; figure 5.8).

5.3.2.2 CCK regulates PKC-alpha

In order to demonstrate that stimulation with CCK was capable of causing PKC translocation from the cytoplasm to the plasma membrane, thereby indicating PKC activation, a PKC-alpha tagged GFP construct was transfected into cultured VAN. Images using time-lapse microscopy were taken pre- and post- CCK stimulation. In unstimulated cells PKC-alpha was visible in the cytoplasm of the

Figure 5.6. CCK induces CART expression through activation of PKC. Immunocytochemistry of cultured VAN after treatment with A) and B) SF medium, C) and D) SF medium with 10nM CCK, E) and F) SF medium 100nM PMA stimulation, G) and H) SF medium with 1μM Ro-32-0432 and 10nM CCK. Images A, C, E and G were observed with CART antibody. Images B, D, F, and H are stained with DAPI. Full arrows indicate positive CART-immunoreactivity. Empty arrows indicate negative CART neurons. Scale bar= 50μm. Representative images from 5 independent experiments.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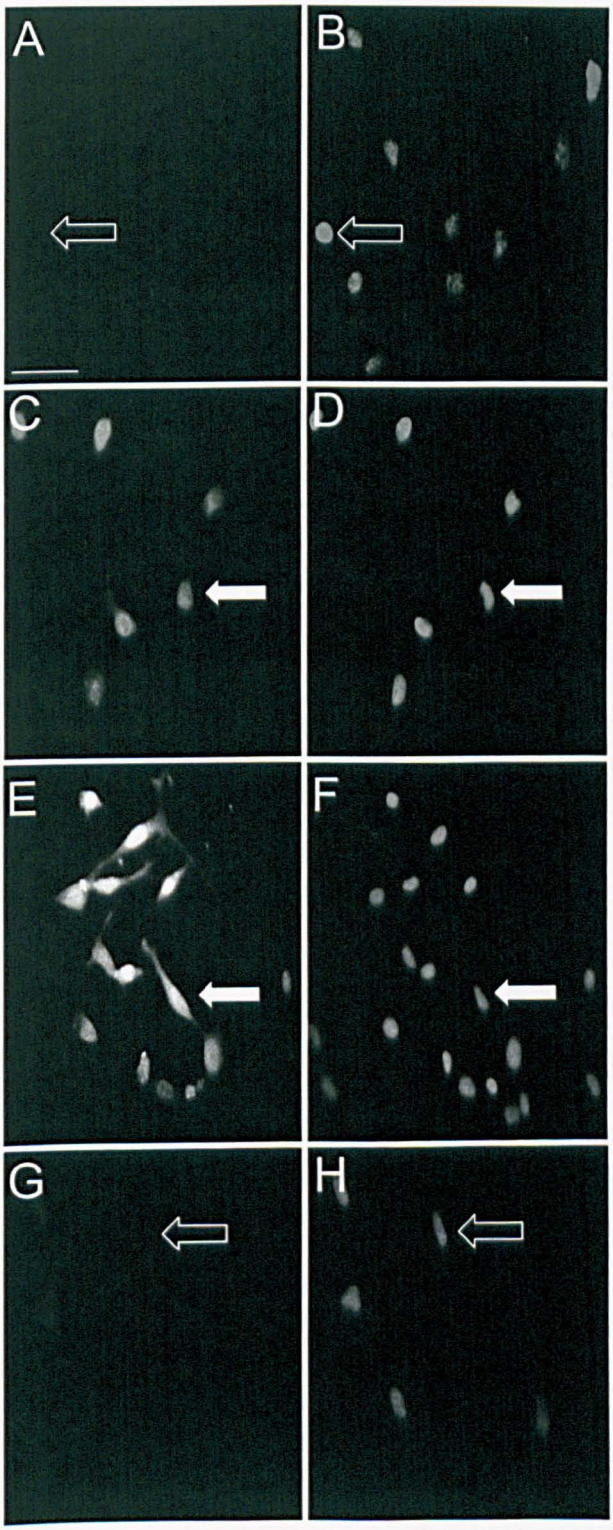
B
C
D
E
F
G
H

Figure 5.7. CCK induces CREB phosphorylation through activation of PKC. Immunocytochemistry of cultured VAN after A) and B) SF media, C) and D) SF media with 10nM CCK, E) and F) SF media with 100nM PMA stimulation. G) and H) SF medium with 1μM Ro-32-0432 with 10nM CCK. Images A, C, E and G show staining with phosphoCREB antibody. Images B, D, F, and H show staining with DAPI. Full arrows represent nuclear phosphoCREB-immunoreactivity. Empty arrows represent empty phosphoCREB neurons. Scale bar= 50μm. Representative images from 5 independent experiments.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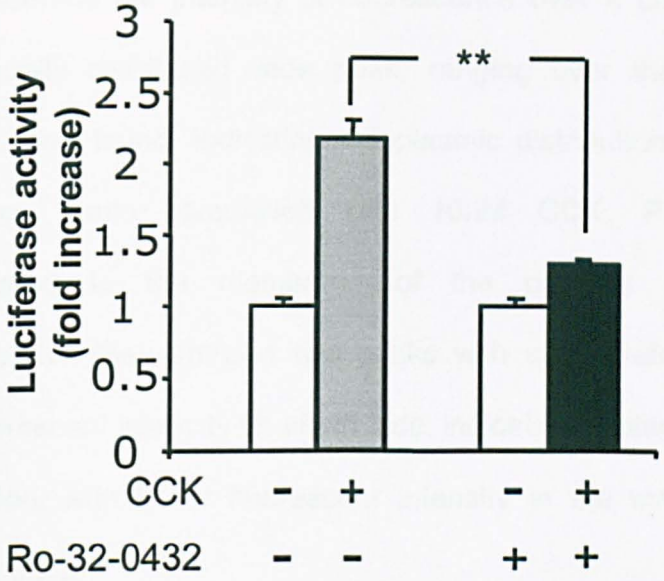


Figure 5.8. CCK acts via PKC to regulate CART promoter activity. Luciferase assay of VAN transfected with 3451CART LUC promoter luciferase reporter after SF medium (1), SF medium with 10nM CCK (2.1), 1μM Ro-32-0432 (1), and SF medium 1μM Ro-32-0432 and 10nM CCK (1.2). ** represents $p<0.01$, t-test. (n=6)

(figure 5.9.A). This was quantified using the computer program Image J to determine the intensity of fluorescence over a chosen distance. One equally distributed wide peak, ranging over the width of the process, was found, indicating cytoplasmic distribution (figure 5.9.B). Following 10min stimulation with 10nM CCK, PKC-alpha had translocated to the membrane of the process (figure 5.9.C). Quantification then showed two peaks with an elevated centre, with high fluorescent intensity on either side, indicative of plasma membrane distribution, with lower fluorescent intensity in the cytoplasmic area (figure 5.9.D).

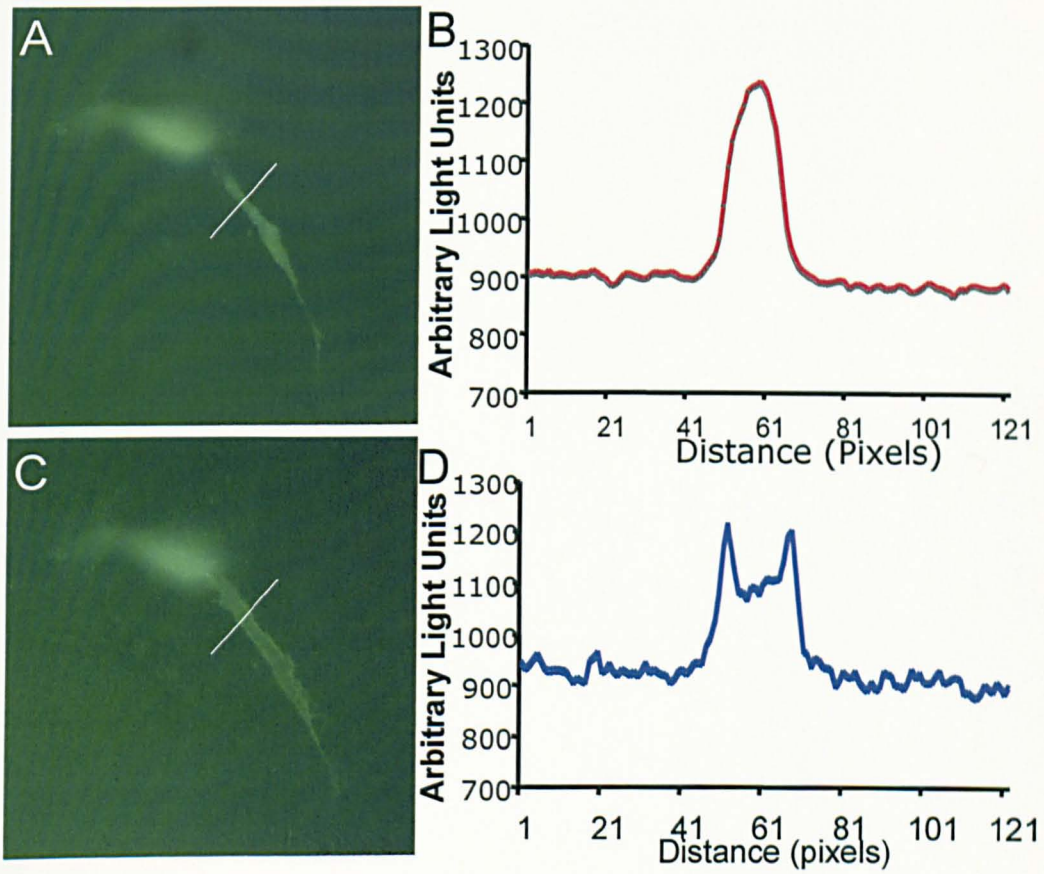


Figure 5.9. CCK causes PKC-alpha translocation to the plasma membrane. Time-lapse image of cultured VAN transfected with PKC-alpha. Immunocytochemistry A) before addition of CCK C) after 10 minutes 10nM CCK . Quantification of fluorescence over 121 pixels B) before administration of CCK D) after 10 minutes of 10nM CCK. Representative samples from 3 independent experiments.

5.4 DISCUSSION

The main conclusions of the work in this chapter are that CCK regulates CART via activation of CREB. There is evidence that PKC is involved in this pathway, possibly in phosphorylating CREB. Other studies, aimed at identifying which transcription factors may be involved in the up-regulation of CART mRNA, identified CREB as a putative mediator of the transcriptional activation (Lakatos *et al.*, 2002).

In vivo cocaine and amphetamine have been found to activate the cyclic AMP/PKA/CREB pathway in brain regions, such as the nucleus accumbens, and this led to increased levels of phosphoCREB (Walters *et al.*, 2003). In a recent publication, Jones and Kuhar demonstrated that CART expression is regulated via the cyclic AMP/PKA/CREB pathway *in vivo*. Furthermore, when administered into the nucleus accumbens, forskolin increased CART mRNA levels and this effect was attenuated by both H89 and Rp-cyclic AMPS, both inhibitors of PKA (Walters *et al.*, 2003).

Using a dominant negative mutant of CREB, A-CREB, it was demonstrated in this chapter that CCK acts via CREB to induce CART expression in VAN. A-CREB contains the CREB leucine zipper dimerisation domain, but lacks the basic domain required for DNA binding, which is replaced by an acidic extension. Therefore, A-CREB heterodimerises with CREB family members and prevents their binding to DNA (Ahn *et al.*, 1998). A-CREB has been successfully used before

to inhibit CREB DNA binding activity in other cell systems (Chepurny *et al.*, 2002; Dworet and Meinkoth, 2006).

Approximately 8% of neurons were transfected with the A-CREB vector, and in these neurons the action of CCK on CART expression was inhibited, while untransfected cells in the same cultures exhibited elevated CART expression in response to CCK. In control experiments using a GFP-encoding vector, transfected cells exhibited an increase in CART abundance in response to CCK similar to controls. Further evidence for the role of CREB in regulating CART transcription was provided using CART-promoter luciferase-reporter constructs. Transfection of A-CREB along with 3451CART LUC completely inhibited CCK-induced CART promoter activity. E1A, when transfected with 3451CART LUC also inhibited CCK-induced CART promoter activity. E1A is an adenovirus oncoprotein which has been found to inhibit phosphoCREB activity by binding to CBP, specifically blocking recruitment of RNA Pol II to the promoter.

The proximal promoter region of the CART gene contains transcription factor binding sites which are conserved between rats, mice, and humans (Barrett 2001). Analysis of the mouse CART gene 5' upstream sequence by Dominguez *et al.* identified a region containing a cluster of transcription factor binding sites, including a CRE site, two AP1, two SP1 sites, and several AP2 sites, along with a TATA-like sequence and an E-box. A STAT- response element was

found present in an overlapping STAT/CRE/AP2 site. Further upstream a putative binding site for the pituitary specific transcription factor Pit-1 was described (Dominguez *et al.*, 2002).

The regulatory element for cyclic AMP-mediated transcription is localised to reside between 102 and 641 bases upstream of the putative transcriptional start site. The predictive sequence analysis reveals a potential CRE binding site between these two locations of the mouse CART promoter, which resides at the same position relative to the initiation codon found in the rat promoter. Support for our finding that CREB regulates CART promoter activity comes from two groups. Barrett et al have demonstrated that the enhanced expression of CART due to elevated levels of cyclic AMP is mediated through the CRE binding site, because deletion of mutation of this site abolished responsiveness to forskolin stimulation (Barrett *et al.*, 2001). Furthermore, Dominguez *et al.* used supershift assays with antibodies raised against phosphorylated CREB in GH3 cells (Dominguez *et al.*, 2002). Although, they express CART mRNA, GH3 cells are a rat pituitary cell line and have very little to do with events involved in appetite regulation. Nevertheless, this provides support for the hypothesis that CCK regulates CART synthesis via CREB phosphorylation in the nodose ganglion. The present findings now provide a direct link between a specific and relatively well understood

regulator of satiety, CCK, and control of expression in a relatively well defined target of CCK, namely VAN.

Sequence analysis of a 3.4-kb mouse genomic DNA identified a 320bp region, located immediately upstream from the transcriptional start site that is highly conserved between the mouse and human CART gene. This region contains several transcription factor binding sites and thus suggests that the mouse and human CART gene could be transcriptionally regulated by the same transcription factors. For example, the overlapping STAT/CRE/AP1 site, located 148 nucleotides upstream from the predicted site of transcription initiation, is completely conserved between mouse and human (Barrett *et al.*, 2001). Additionally, the conservation of these binding sites suggests that these transcription factors play an important role in the regulation of the CART gene. Theoretically, the STAT/CRE/AP1 element could allow complex transcriptional regulation via CREB and cJun proteins as well as by STAT proteins (Shaywitz and Greenberg, 1999). Composite CRE/AP1 sites have been found in a number of genes involved in neurotransmitter synthesis, including dopamine b-hydroxylase (Shaskus *et al.*, 1992), prodynorphin (Messersmith *et al.*, 1996), proenkephalin (Comb *et al.*, 1986), and CCK (Hansen *et al.*, 1999). This has not been studied yet; however it could be of particular interest as this could be a mechanism by which leptin stimulates CART mRNA transcription, since the leptin receptor signals through the Janus

kinases/STAT pathway. The leptin receptor is found to co-localise with CCK1R in VAN in 50% of neurons, and its expression is not dependent on the animals feeding state (Burdyga et al, 2002). The presence of the SP1 adjacent to the STAT-response element in the CART gene promoter sequence adds additional support for the possible role of leptin in regulation of CART, since SP1 has been shown to play a role in mediating the STAT response (Look *et al.*, 1995). Leptin is another important regulator of CART mRNA; in fact when leptin signaling is altered it affects CART mRNA expression. Mice lacking the *leptin* gene (*ob/ob* mice) were shown to have a reduction in CART mRNA expression in the arcuate nucleus of the hypothalamus (Kristensen *et al.*, 1998). Expression of CART mRNA was restored in the *ob/ob* mice with daily injections of recombinant leptin (Kristensen et al., 1998). It would be interesting to see what role leptin plays in regulating CART expression in VAN.

There was little difference between the luciferase activity of the 3451CART LUC and the 3100CART LUC constructs. However, there was a significant decrease in luciferase activity between the 3100CART LUC and the 620CART LUC. Although there is nearly 1500bp separating the two constructs, the only consensus sequence identified was a POU homeodomain. This homeobox is at position 818 in the mouse CART promoter. The POU domain was first identified in 1988. It was originally defined on the basis of a common DNA binding domain

in the mammalian factors Pit-1, Oct-1, and Oct-2 as well as the nematode protein Unc-86. Pit-1 is widely described as a pituitary specific transcription factor (Lin *et al.*, 1994; Rhodes *et al.*, 1996; Andersen *et al.*, 1997) and so may not play much of a role in the regulation of CART expression in VAN. Nevertheless, Pit-1 involvement is supported by experiments where a DNA/protein complex was formed between GH3 nuclear extracts and an oligonucleotide containing the CART Pit-1 sequence; this complex was super-shifted by a Pit-1 antibody (Dominguez, 2006). Alternatively, oct-1 is a transcription factor expressed in all tissue types and could therefore play a role in regulating the POU homeobox. Other transcription factors of the POU family involved in sensory neurons, such as Brn-3, could also play a role in regulating CART expression.

The transduction pathways are commonly comprised of cascades of intracellular signaling protein kinases. These kinases play an important role in the subsequent activation/phosphorylation of the transcription factor, CREB. PhosphoCREB-induced gene transcription is widely reported in neuronal processing of various extracellular signals. Additionally, it has been reported that the co-coordinated phosphorylation of CREB is initiated by the rapid phosphorylation of MAP kinase, as well as other activated protein kinases, such as calcium/calmodulin protein-dependent kinase II, PKA, and PKC (Kawasaki *et al.*, 2004; Miletic *et al.*, 2004; Fang *et al.*, 2005). A

number of groups have found that exogenous CCK produced an increase in the phosphorylation of the transcription factor CREB at Ser133, with the dose of 2 μg/kg CCK producing the maximal effect. (Hansen *et al.*, 2004; Sutton *et al.*, 2004; Sutton *et al.*, 2005)

Interestingly, I found that CCK was able to activate PKC-alpha. In pancreatic acinar cells, 100 pM CCK stimulated the translocation of PKC-α, -δ, and -ε but not -ζ to a membrane fraction. (Dominguez and Kuhar, 2004). In VAN, I demonstrated that a transfected GFP-tagged PKC-alpha construct translocated to the plasma membrane within 5min of CCK administration. This led to the hypothesis that CCK could activate the classical PKC to phosphorylate CREB. This hypothesis is supported by the fact that PKC has been reported in VAN by a number of other groups (Ikeda *et al.*, 2005; Matsumoto *et al.*, 2005), and that PKC has been found to be involved in CREB phosphorylation in a number of other systems (Wu *et al.*, 2005; Aggarwal *et al.*, 2006).

In order to demonstrate a role for PKC in regulating CART, I used the phorbol ester, PMA, which has been widely used as an activator of PKC (Sansbury *et al.*, 1997). In this chapter, it is demonstrated that PMA increases both CART abundance and CREB phosphorylation in cultured VAN. In addition the PKC inhibitor, Ro-32-0432, prevents both CCK-induced CART expression and CCK-induced CREB phosphorylation.

Therefore, this chapter provides a number of different lines of evidence suggesting that in VAN, CCK leads to the phosphorylation of CREB via PKC, and that these events regulate the activity of the CART promoter.

CHAPTER SIX

Mechanisms of CART down-regulation

6.1 INTRODUCTION

The primary aim of this chapter is to determine the role of orexigenic hormones in CART expression. It is well established that the orexigenic hormone, ghrelin is secreted in the absence of food and stimulates appetite. The same is true of orexin-A. It has been shown that VAN constitutively express orexin (OxR1) and ghrelin (GHS1R) receptors (Burdyga *et al.*, 2003; Burdyga *et al.*, 2006). Given the actions of these orexigenic peptides and the localisation of their receptors on VAN, these hormones could have the potential to down-regulate CART expression. They could achieve this by inhibiting CREB or by activating an inhibitory transcription factor. If they were to regulate CART expression, then by what intracellular signalling mechanism do they elucidate their effects.

6.2 METHODS

Nodose ganglia were dissected as described in chapter 2.2.1.1, and cells were dissociated as described in chapter 2.2.5.

Immunohistochemistry was performed as described in chapter 2.2.5.1, using the following primary antibodies: rabbit anti-CART, rabbit anti-phosphoCREB, rabbit anti-CREB; and the following secondary antibodies: Donkey anti-Rabbit conjugated to TxR or FITC, as appropriate. Cells were cultured in FM for 3-4 day, and then transferred to SF medium with or without CCK, orexin-A (OxA), ghrelin (Gh) alone or in combination for 30-120min.

Luciferase assays were performed as described in chapter 2.2.5.4. VAN were cultured in FM for 3-4 days, and transfected for 24hr as described in chapter 2.2.5.2 with the following constructs: ACREB, 3451CART-LUC, 3100CART-LUC, 620CART-LUC, 120CART-LUC, and PKC-epsilon GFP. Cells were cultured in FM for 3-4 day, and then transferred to SF medium with or without CCK for 120min, with OxA alone for 120min, or with OxA for 30min followed by CCK for 120min.

6.3 RESULTS

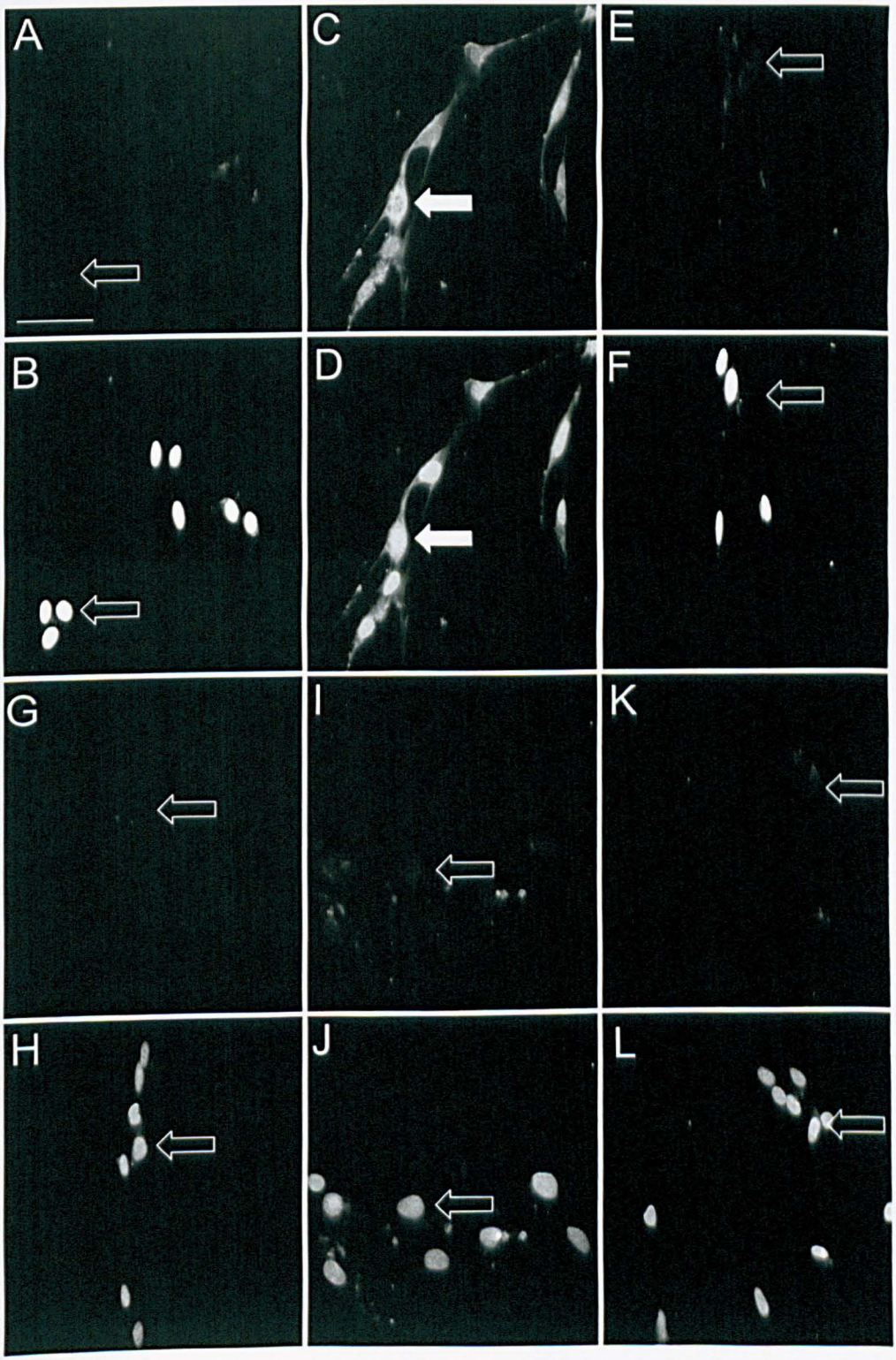
6.3.1 Effects of orexin-A and ghrelin on CART expression

Initially the effect of 10nM orexin-A or 10nM ghrelin on CART abundance in cultured VAN was examined. Orexin-A and ghrelin each inhibited CCK-induced expression of CART in neurons cultured in SF medium as detected by immunocytochemistry (figure 6.1). As previously described, VAN cultured in SF medium (figure 6.1.A) have low CART abundance, which is increased upon the addition of 10nM CCK for 2hr (figure 6.1.B). Treatment with either orexin-A or ghrelin alone had little to no effect on the abundance CART (figure 6.1.E and 6.1.G respectively). However pre-incubation with either 10nM ghrelin or 10nM orexin-A for 30min before the addition of 10nM CCK inhibited CCK-induced CART expression in VAN (figure 6.1.I and 6.1.K).

The promoter activity of CART was also inhibited by orexin-A using a luciferase promoter-reporter assay (figure 6.2). In the presence of 10nM CCK, 3451CART LUC promoter activity was significantly ($p<0.001$, t-test) increased by 2.5-fold compared to unstimulated transfected cells. However when the transfected cells were pre-incubated with orexin-A for 30min prior to the addition of CCK, the promoter activity was significantly ($p<0.01$, t-test) decreased to 1.7-fold compared to cells that were stimulated with CCK in the absence of orexin-A. Pre-incubation with orexin-A corresponds to a 47% reduction

in the activity of the CART promoter compared to cells receiving CCK alone.

Figure 6.1. Orexin-A and ghrelin inhibit CCK induced CART abundance. Immunocytochemistry of cultured VAN after treatment with A) and B) SF medium, C) and D) SF medium with 10nM CCK, E) and F) SF medium with 10nM OxA, G) and H) SF medium with 10nM Gh, I) and J) SF medium with 10nM OxA and 10nM CCK, and K) and L) SF medium with 10nM Gh and 10nM CCK. Images A), C), E), G), I), and K) stained with CART antibody. Images B), D), F), H), J) and L) show overlap of both CART and DAPI staining. Full arrows indicate positive CART-immunoreactivity. Empty arrows indicate negative CART neurons. Scale bar=50μm. Representative images from 6 independent experiments.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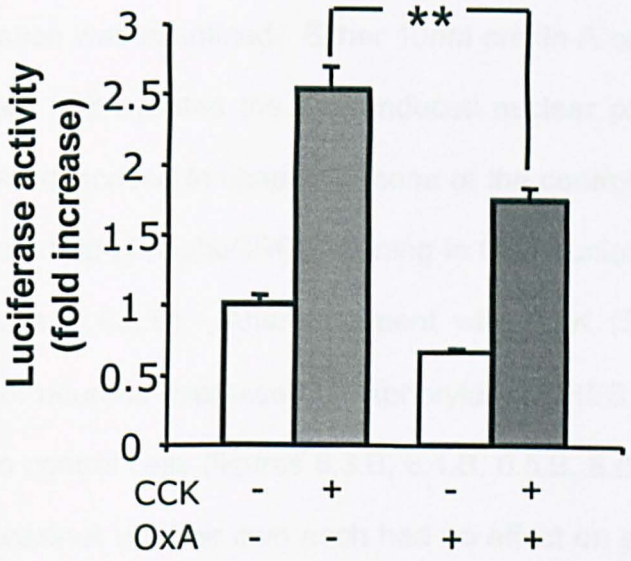

Figure 6.2. Orexin-A inhibits CCK induced CART promoter activity. Cultured VAN transfected with 3451CART LUC, cultured 2hr in SF medium, with the addition of 10nM CCK, 10nM OxA, or preincubated with 10nM OxA and 10nM CCK. All values normalised to 1. ** represents $p<0.01$, t-test. (N=6).

6.3.2 Effects of orexin-A and ghrelin on CREB

In view of the evidence that CCK regulates CART expression via phosphoCREB, the action of orexigenic hormones on CREB phosphorylation was examined. Either 10nM orexin-A or 10nM ghrelin was sufficient to prevented the CCK-induced nuclear phosphorylation of CREB. As described in chapter 4, none of the control cells cultured in SF medium had phosphoCREB staining in their nuclei (6.3.A, 6.4.A, 6.5.A, 6.6A, and 6.8.B). After treatment with CCK (30min, 10nM), 53.8±0.5% of neurons expressed phosphorylated CREB in their nuclei compared to control cells (figures 6.3.B, 6.4.B, 6.5.B, 6.6B, and 6.8.B). Ghrelin or orexin-A on their own each had no effect on phosphoCREB compared to controls (figures 6.3.E, 6.4.E, 6.5E, 6.6.E, and 6.8.B), but unexpectedly, 56±1.0% of cells still expressed phosphoCREB when pre-treated with 10nM orexin-A or ghrelin for 30 minutes before administration of CCK (30min, 10nM). Interestingly only 8.2±0.8% of these cells retained phosphoCREB within the nucleus, whereas the rest of the 47.5±2.3% of the neurons expressed phosphoCREB in the cytoplasm ($p<0.001$, t-test; figures 6.3.G and I, 6.4.G and I, 6.5.G and I, 6.6.G and I and 6.8.B).

Total CREB expression was found in the nucleus of 95±0.1% of all cells. Under SF condition 9.8±2.1% of these neurons expressed total CREB outside the nucleus (figure 6.7.A and 6.8.A). The same was seen in cells treated with CCK (10nM, 2hr; 10.0±0.7%) compared

to untreated cells (figure 6.7B and 6.8.A). Orexin-A or ghrelin alone slightly increased the number of cells containing this pool of total CREB (16.0+0.6%; figure 6.6) (figure 6.7.C and G and 6.8.A.). Interestingly, pre-treatment with either ghrelin or orexin-A prior to CCK administration, increased the number of cells expressing total CREB outside the nucleus to 48.1±1.3% ($p<0.001$, t-test), which may explain the phosphoCREB in the cytoplasm seen in the previous experiment (figure 6.7.H and I and 6.8.A).

Figure 6.3. Orexin-A inhibits CCK induced nuclear CREB phosphorylation after 30min. Immunocytochemistry of cultured VAN after 30min treatment with A) and B) SF medium, C) and D) SF medium with 10nM CCK, E) and F) SF medium with 10nM OxA, G) and H) SF medium with OxA and 10nM CCK, and I) magnified image of G). Images A), B), C), and D) are stained against phosphoCREB antibody. Images E), F), G) and H) are stained with DAPI. Full arrows indicate positive phosphoCREB-immunoreactivity. Empty arrows indicate negative phosphoCREB neurons. Scale bar=50μm. Representative images from 6 independent experiments.

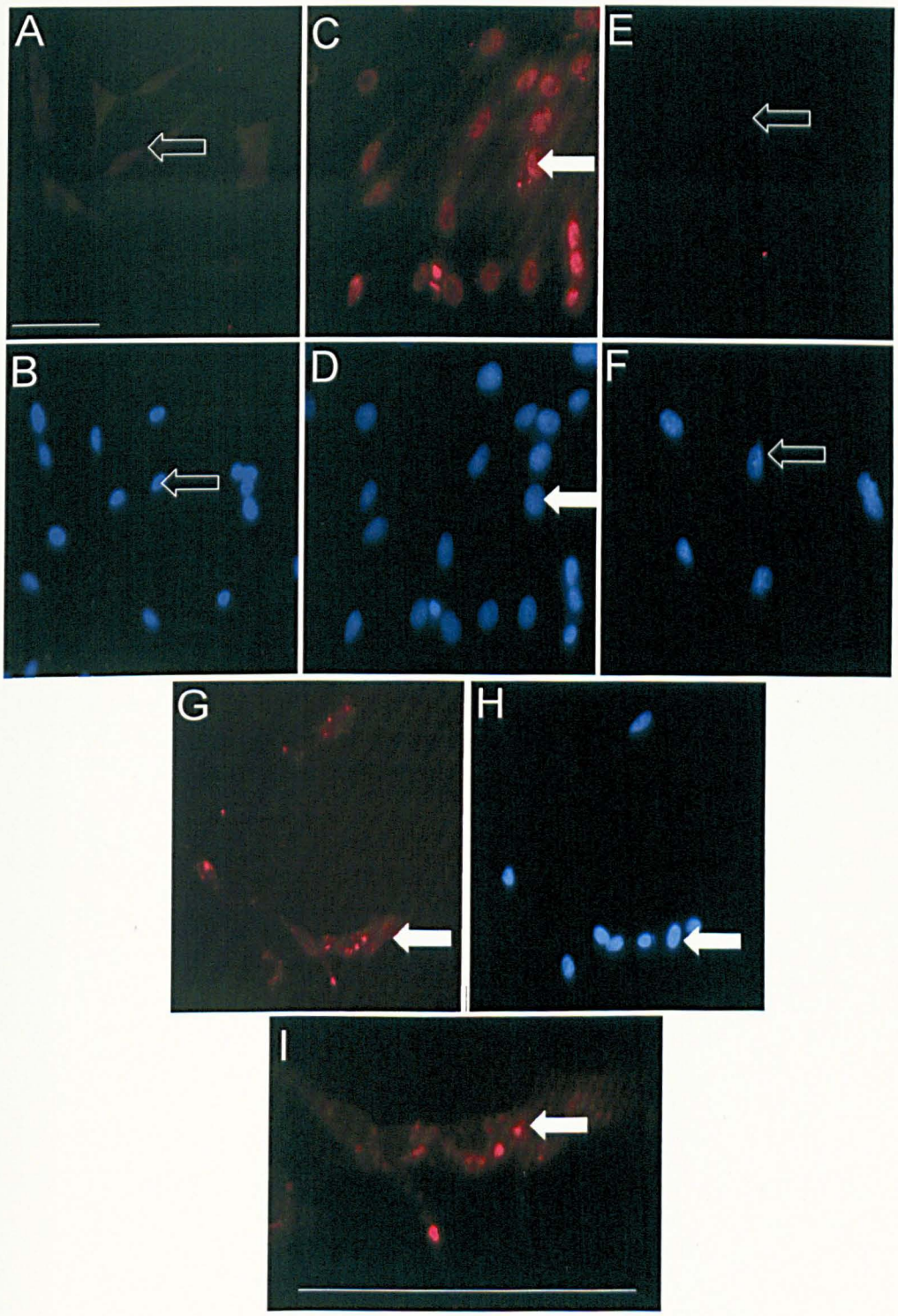
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Figure 6.4. Orexin-A inhibits CCK induced nuclear CREB phosphorylation after 2hr. Immunocytochemistry of cultured VAN after 2hr treatment with A) and B) SF medium, C) and D) SF medium with 10nM CCK, E) and F) SF medium with 10nM OxA, G) and H) SF medium with OxA and 10nM CCK, and I) magnified image of G). Images A), B), C), and D) are stained against phosphoCREB antibody. Images E), F), G) and H) are stained with DAPI. Full arrows indicate positive phosphoCREB-immunoreactivity. Empty arrows indicate negative phosphoCREB neurons. Scale bar=50μm. Representative images from 6 independent experiments.

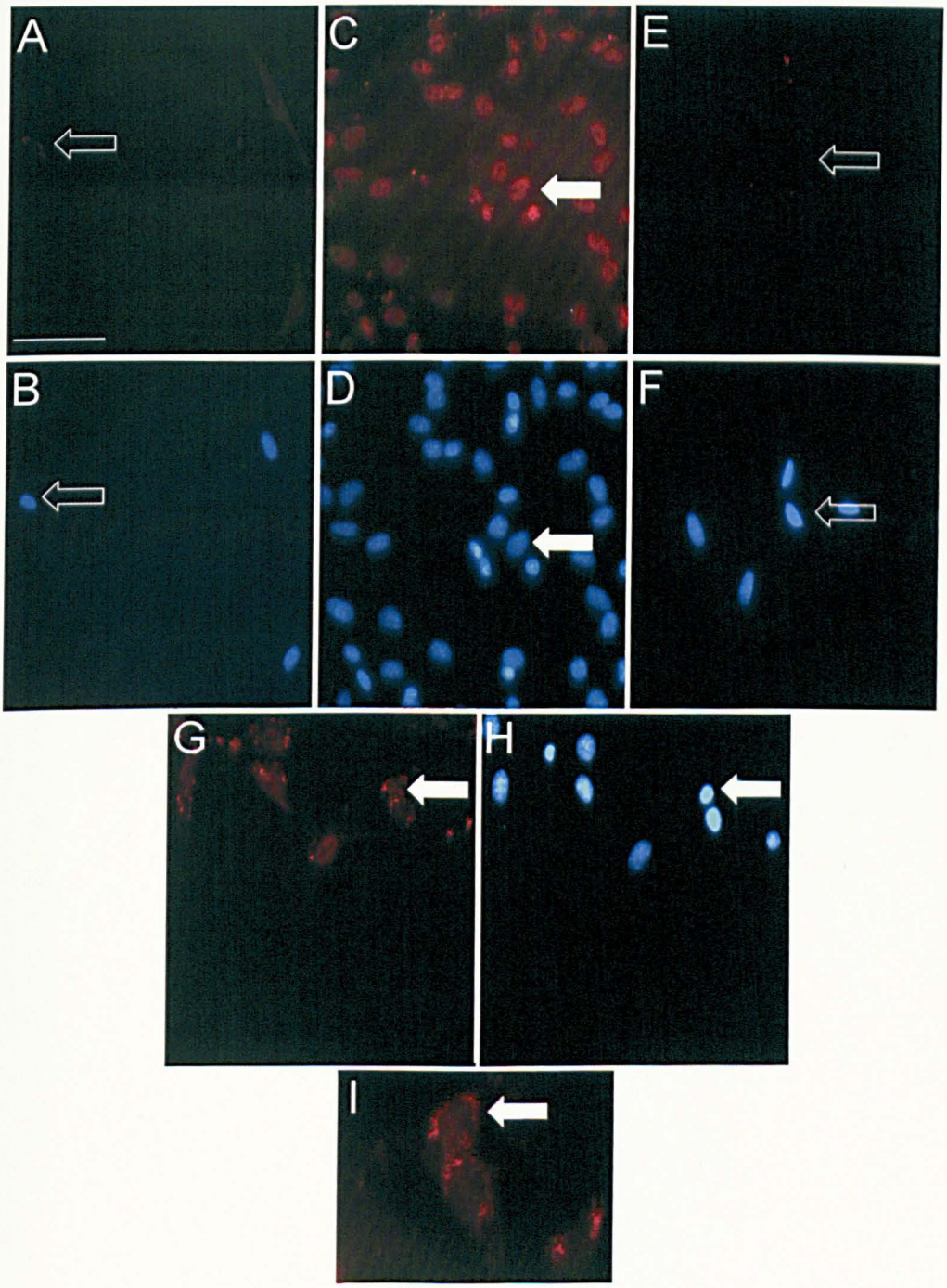
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Figure 6.5. Ghrelin inhibits CCK induced nuclear CREB phosphorylation after 30min. Immunocytochemistry of cultured VAN after 30min treatment with A) and B) SF medium, C) and D) SF medium with 10nM CCK, E) and F) SF medium with 10nM Gh, G) and H) SF medium with Gh and 10nM CCK, and I) magnified image of G). Images A), B), C), and D) are stained with phosphoCREB antibody. Images E), F), G) and H) are stained with DAPI. Full arrows indicate positive phosphoCREB-immunoreactivity. Empty arrows indicate negative phosphoCREB neurons. Scale bar=50μm. Representative images from 6 independent experiments.

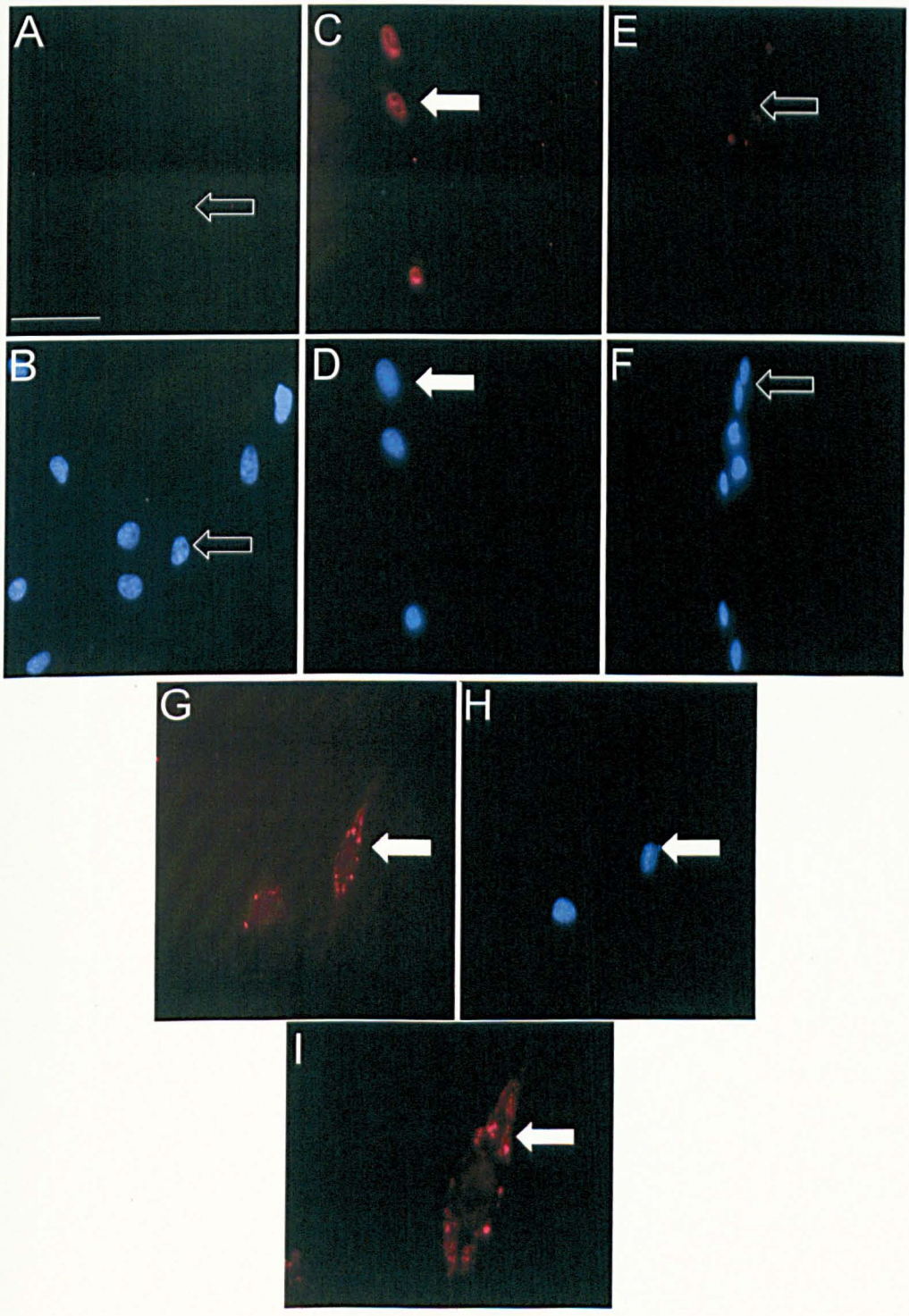
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Figure 6.6. Ghrelin inhibits CCK induced nuclear CREB phosphorylation after 2hr. Immunocytochemistry of cultured VAN after 2hr treatment with A) and B) SF medium, C) and D) SF medium with 10nM CCK, E) and F) SF medium with 10nM Gh, G) and H) SF medium with Gh and 10nM CCK, and I) magnified image of G). Images A), B), C), and D) are stained with phosphoCREB antibody. Images E), F), G) and H) are stained with DAPI. Full arrows indicate positive phosphoCREB-immunoreactivity. Empty arrows indicate negative phosphoCREB neurons. Scale bar=50μm. Representative images from 6 independent experiments.

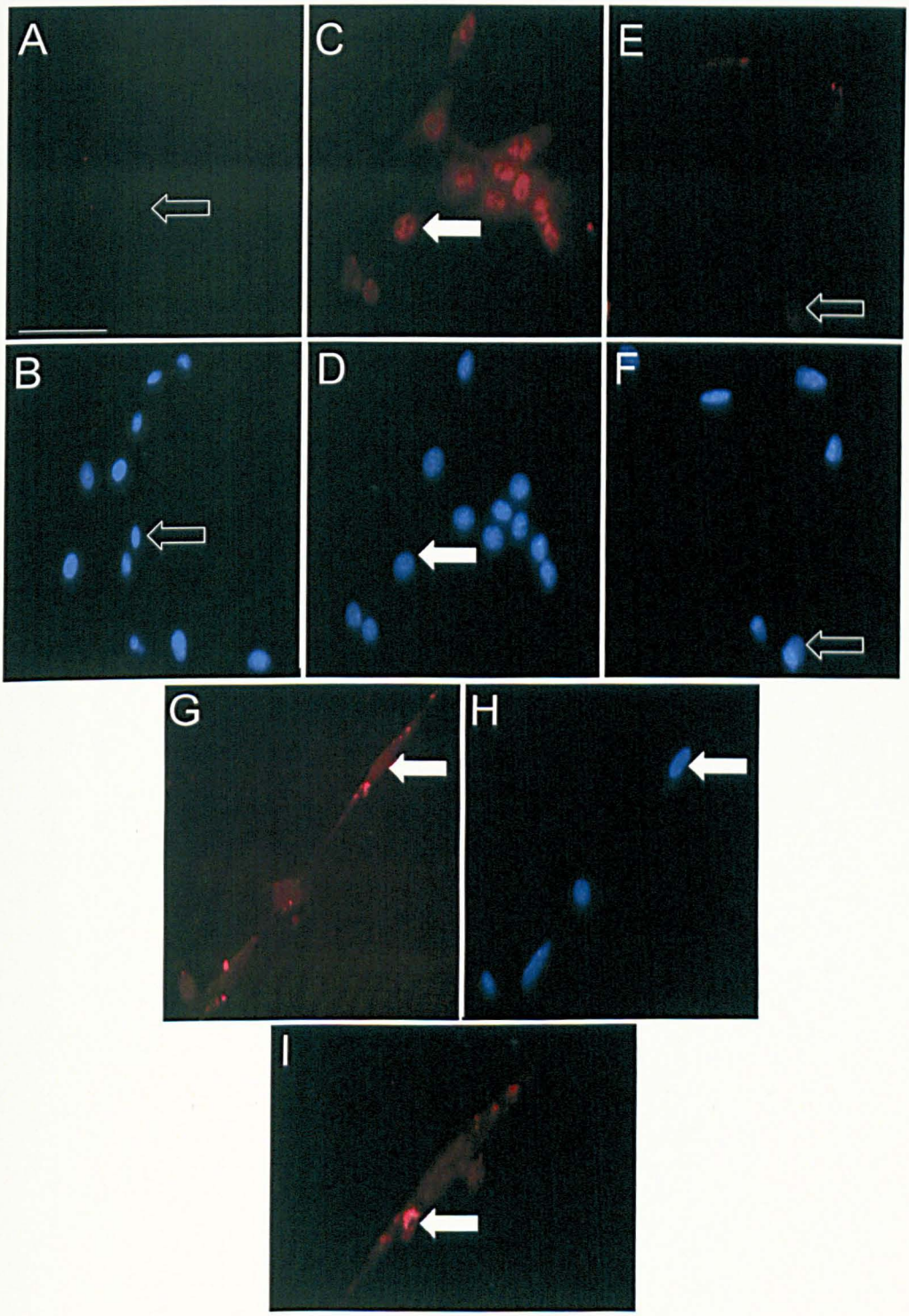
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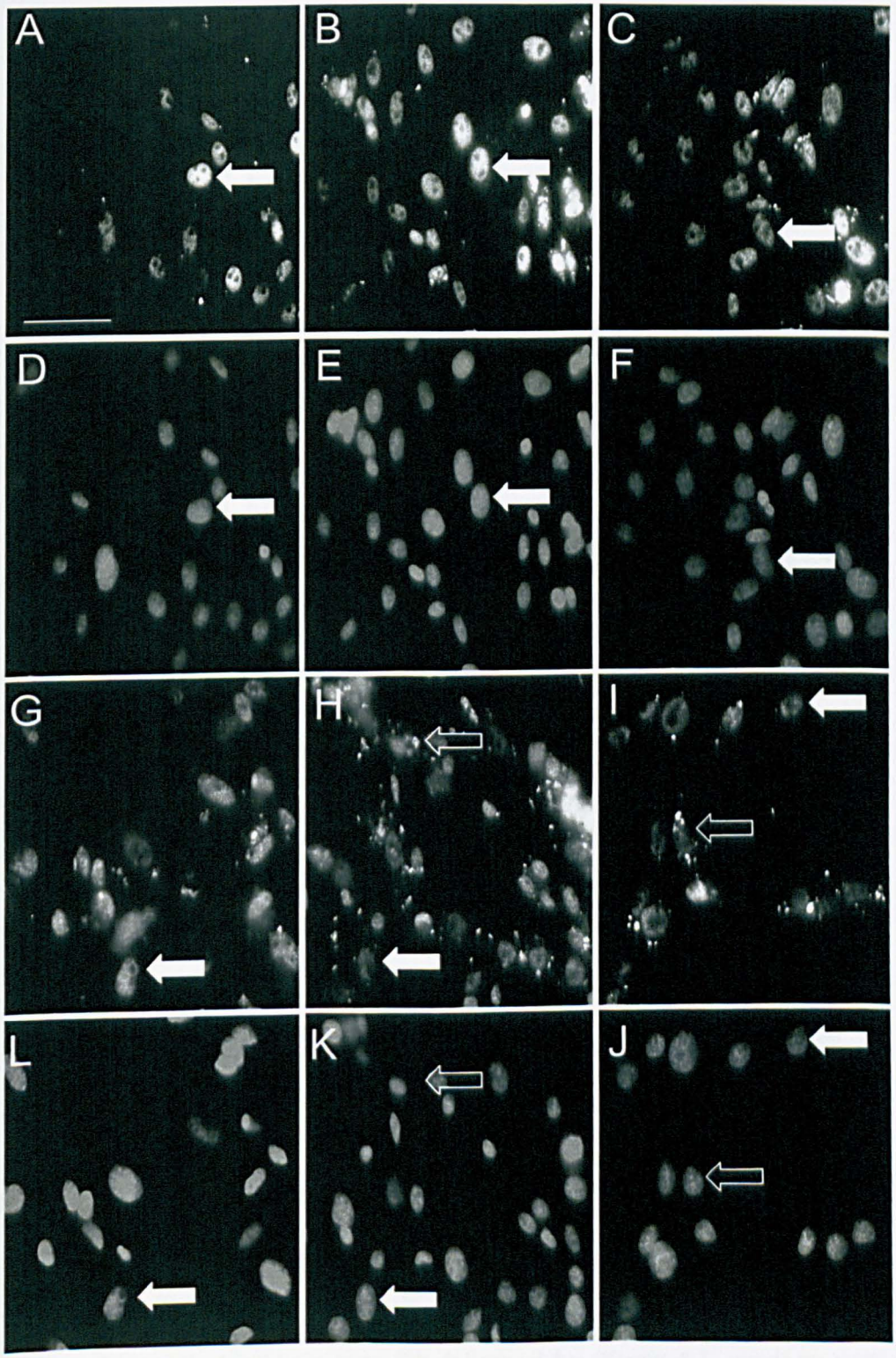
Figure 6.7. Orexin-A and ghrelin create a pool of total CREB outside the nucleus. Immunocytochemistry of cultured VAN after treatment with A) and D) SF medium, B) and E) SF medium with 10nM CCK, C) and F) SF medium with 10nM OxA, G) and L) SF medium with10nM Gh, H) and K) SF medium with OxA and 10nM CCK, and I) and J) SF medium with 10nM Gh and 10nM CCK. Images A), B), C), G), H, and I) are stained with total CREB antibody. Images D), E, F), L), K, and J) show DAPI staining. Full arrows indicate pool of total CREB inside nucleus. Empty arrows indicate pool of total CREB outside nucleus. Scale bar=50μm. Representative images from 6 independent experiments.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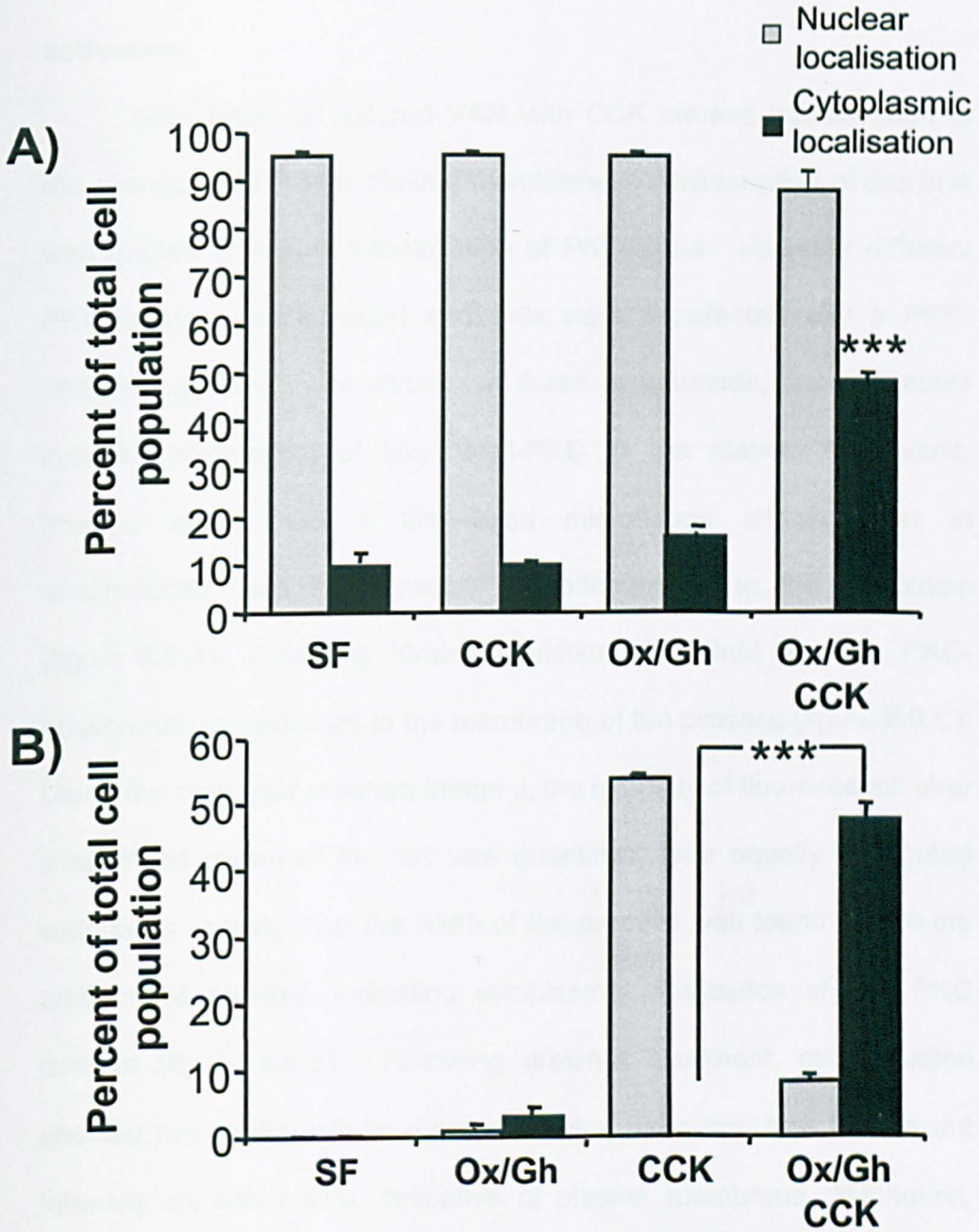


Figure 6.8. Orexin-A and ghrelin increase the cytoplasmic localisation of both phosphoCREB and total CREB. Quantification of nuclear and cytoplasmic expression of A) total CREB B) phosphoCREB in cultured VAN treated with 60min SF medium, 30min SF medium followed by 30min SF medium with 10nM CCK, 30min SF medium followed by 30min SF medium with 10nM OxA or Gh, 30min SF medium with 10nM OxA or Gh followed by 30min SF medium with 10nM CCK. *** represents $p<0.001$, t-test. (N=6).

6.3.3 Effects of orexin-A and ghrelin on PKC translocation and activation

Stimulation of cultured VAN with CCK caused translocation of the classical PKC to the plasma membrane. Administration of orexin-A was unable to induce translocation of PKC-alpha. However different PKC isoforms were tested and cells were transfected with a PKC-epsilon tagged GFP construct. In these experiments, orexin-A could induce translocation of this novel-PKC to the plasma membrane. Images taken from a time-lapse microscope showed that in unstimulated cells PKC-epsilon is predominantly in the cytoplasm (figure 6.9.A). Following 10min stimulation with 10nM orexin-A, PKC-epsilon had translocated to the membrane of the process (figure 6.9.C). Using the computer program Image J, the intensity of fluorescence over a specified region of the cell was quantified. One equally distributed wide peak ranging over the width of the process was found before the addition of orexin-A indicating cytoplasmic distribution of this PKC isoform (figure 6.9.B). Following orexin-A treatment, quantification showed two peaks with an elevated centre, indicating high fluorescent intensity on either side, indicative of plasma membrane distribution, with lower fluorescent intensity in the cytoplasm (figure 6.9.D).

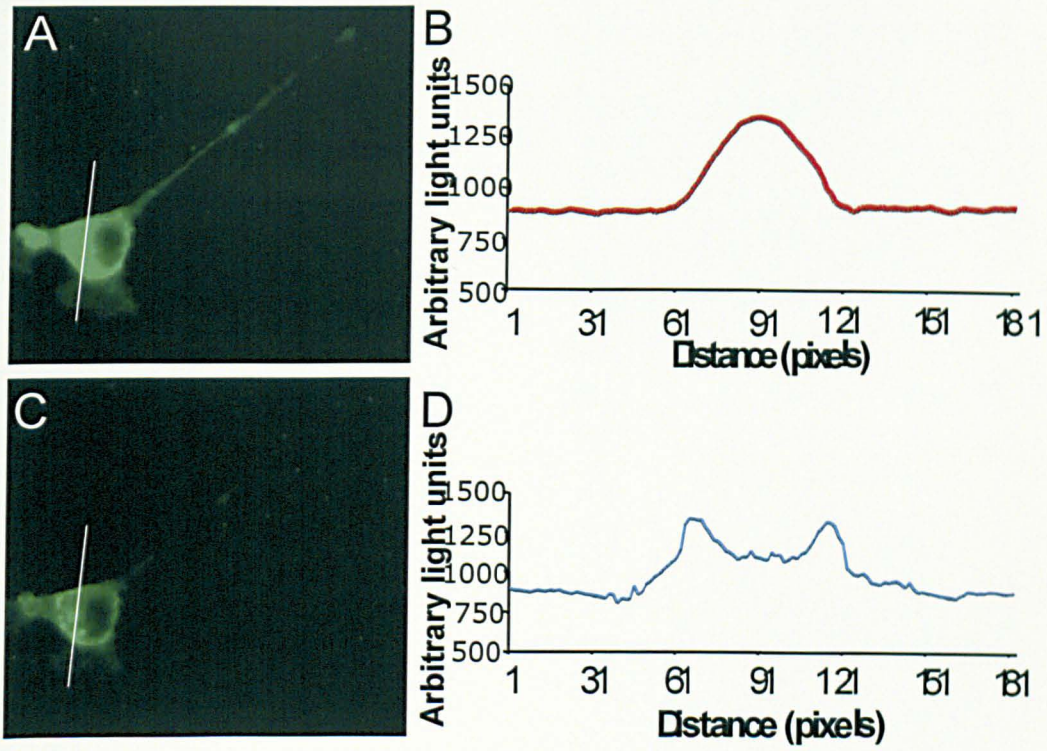


Figure 6.9. Orexin-A causes PKC-epsilon translocation to the plasma membrane. Time-lapse image of cultured VAN transfected with PKC-epsilon. Immunocytochemistry (A) before addition of OxA (C) after 10min OxA. Quantification of fluorescence over 181 pixels (B) before administration of OxA (D) after 10min of OxA. Representative sample from 3 independent experiments.

6.4 DISCUSSION

There is evidence that ghrelin requires an intact vagus nerve to stimulate food intake (Asakawa *et al.*, 2001), indicating that one site of action of ghrelin is the vagus nerve. In addition, GHS1R as well as OxR1 have been shown to localise on nodose ganglia and the expression of these receptors is not dependent on the feeding state of the animal (Burdyga *et al.*, 2002; Burdyga *et al.*, 2003; Burdyga *et al.*, 2006). One of the main findings of this chapter is that orexigenic hormones, such as ghrelin and orexin-A, can inhibit CCK-induced CART expression in VAN.

In the immediate postprandial period, there are declining plasma concentrations of ghrelin and rising concentrations of CCK (McLaughlin *et al.*, 1999; Cummings *et al.*, 2001). Orexigenic hormones have been shown to inhibit the effects of CCK (Date *et al.*, 2005). Orexin-A has been found to counteract CCK-induced reduction in feeding when injected i.c.v. into brain (Asakawa *et al.*, 2002). Previous studies have also shown that ghrelin and orexin-A have acute effects in inhibiting vagal afferent nerve discharge (Date *et al.*, 2002; Burdyga *et al.*, 2003). However, since CCK has been demonstrated to stimulate vagal afferent discharge (Raybould and Lloyd, 1994), these data are compatible with the idea that vagally-mediated orexigenic effects of peripheral ghrelin and orexin-A are involved in suppression of the effects of CCK (Burdyga *et al.*, 2003).

In this chapter it has been demonstrated that orexin-A and ghrelin inhibit CCK-induced CART abundance. Orexigenic hormones might therefore act at least in part to induce appetite by inhibiting CART synthesis. Luciferase assay studies showed that CCK stimulated CART promoter activity and that this activation was inhibited by orexin-A. This suggests that ghrelin and orexin-A exert their effects, at least partly, by inhibiting CART synthesis at a transcriptional level.

I have found that in the presence of CCK phosphoCREB is predominantly absent from the cytoplasm of VAN. Other groups have also reported that immunostained cells and immunoblotting of subcellular fractions show that CREB is almost exclusively nuclear in both unstimulated and stimulated cells (Waeber and Habener, 1991; Ginty *et al.*, 1993; Hagiwara *et al.*, 1993). Having demonstrated that the orexigenic hormones regulated CART transcriptionally it was hypothesised that these hormones were preventing CREB phosphorylation. Surprisingly, CREB phosphorylation was still increased in response to CCK even in the presence of ghrelin and orexin-A. However, these hormones prevented nuclear localisation phosphoCREB in the presence of CCK. Interestingly, there was also a pool of total CREB protein found outside the nucleus in the presence of either ghrelin or orexin-A, which probably relates to the phosphorylated pool of CREB. The accumulation of phosphoCREB in the cytoplasm suggested that these orexigenic hormones could be interfering with

nuclear transport of phosphorylated CREB. Hence, evidence is provided in this chapter to suggest that the mechanism of action though which orexigenic hormones could be down-regulating CART synthesis, is by inhibiting nuclear localisation of phosphoCREB. Other groups have also identified CREB localisation in the cytoplasm. Chalovich *et al.* found phosphoCREB was increased in the cytoplasm and decreased in the nucleus of 6-OHDA-treated neuronal cells (Chalovich *et al.*, 2006).

Regulation of nuclear transport is tightly regulated. The transport of proteins and RNAs into and out of the nucleus occurs through the nuclear pore complex, a large macromolecular structure embedded in the double membrane of the nuclear envelope (Fried and Kutay, 2003; Weis, 2003; Mosammaparast and Pemberton, 2004). A large, evolutionarily conserved family of transport factors, the karyopherin-β family, mediates the import and export of most proteins, ribosomal subunits and some RNAs. Most karyopherins mediate either nuclear import or nuclear export, and all karyopherins interact directly with their cargoes, although some also use adapter proteins. The best-characterised adapter protein is the evolutionarily conserved protein, karyopherin-α (Goldfarb *et al.*, 2004). Karyopherins that mediate import bind to their cargoes in the cytoplasm via recognition of the nuclear localization signal (NLS). The karyopherin : cargo complex translocates through the nuclear pore complex via interactions with

nuclear pore complex proteins. Once in the nucleus, the karyopherin encounters RanGTP, and the ensuing karyopherin : RanGTP complex leads to dissociation of the cargo from the karyopherin, whereby the karyopherin is recycled back to the cytoplasm. Conversely, karyopherins that mediate export bind cargo in the nucleus via recognition of a nuclear export signal. Karyopherin binding to export cargo occurs co-operatively with RanGTP, resulting in a karyopherin : cargo : RanGTP ternary complex. Hence, Ran regulates both the assembly and the disassembly of karyopherin : cargo complexes. Ran-mediated nuclear import is inhibited by introducing a dominant negative mutant of Ran or by wheat germ agglutinin, which binds carbohydrate groups on nuclear pore proteins (Carey *et al.*, 1992; Moore and Blobel, 1992). CREB contains a nuclear localisation signal within its DNA recognition sequence between amino acids 286 and 295 (Waeber and Habener, 1991). However, a nuclear export signal has not currently been identified within the CREB sequence; although, two leucine-rich domains near the C-terminus have the potential to function as a nuclear localisation signal.

In the presence of the orexigenic hormones after the addition of CCK, phosphoCREB was localised to the cytoplasm. The mechanism by which the orexigenic hormones induce this effect is currently unknown, but could involve the regulation of nuclear transport of CREB. The orexigenic hormones could therefore either prevent phosphoCREB

from being imported into the nucleus or induce export of phosphoCREB from the nucleus. The caveat of the idea that phosphoCREB is exported from the nucleus, is that it would need to be replaced in the nucleus by newly synthesized CREB as quickly as the phosphoCREB was removed. This would imply that a significant decrease in nuclear total CREB protein would be found in VAN. Evidence from figures 6.8 and 6.9, suggests that the total CREB population is found in the nucleus of the vast majority of the cells even in the presence of the orexigenic hormones.

Stevenson *et al.* demonstrate that inhibiting Ran-mediated nuclear import of CREB led to the appearance of phosphoCREB in the cytoplasm after depolarization in vascular smooth muscle cells (Stevenson *et al.*, 2001). This report raises the possibility that CREB is inhibited from entering the nucleus in the presence of ghrelin or orexin-A. It has been demonstrated by Stevenson *et al.*, that inhibitors of nuclear import elicit increased cytoplasmic phosphoCREB and decreased CREB-mediated transcriptional responses in smooth muscle cells. Inhibitors of nuclear import have no effect on the sub-cellular distribution of total CREB or CRE-mediated transcription when muscle cells are stimulated by cyclic AMP. In contrast, CRE transcriptional response to platelet-derived growth factor receptor or membrane depolarisation was inhibited when nuclear import was blocked, contributing to cytoplasmic accumulation of phosphoCREB (Stevenson

et al., 2001). This is something that could also be performed in the VAN cultures.

In chapter 5, it was demonstrated that CCK-induced CART expression was modulated by PKC-alpha. Hence, PKC was found to play an important role in phosphorylating CREB. Therefore I wanted to test the effects that the orexigenic hormones would exhibit on PKC activation. Orexin-A did not activate the classical PKC, PKC-alpha. I did not test the effects of orexin-A on CCK induced PKC-alpha translocation, but I did observe that orexin-A could induce translocation of the novel PKC, PKC-epsilon, which was not activated by CCK. The presence of endogenous PKC-epsilon has previously been reported in VAN. Interestingly it has been found to be activated by ghrelin (Mousseaux *et al.*, 2006). Mousseaux *et al.* provided several lines of evidence indicating that ERK1/2 activation was related to PLC and novel PKC-epsilon. Using an inhibitor, U-73122, they demonstrated that the PKC pathway was necessary for ghrelin mediated ERK1/2 activation in CHO cells (Mousseaux *et al.*, 2006). It is possible that orexin-A activation of PKC-epsilon plays a role in the regulation of CREB translocation in and out of the nucleus, although the mechanisms are currently unclear.

In conclusion I have found that orexigenic hormones inhibit CCK-induced CART synthesis. The mechanism by which this occurs seems to involve depletion of phosphoCREB in the nucleus.

CHAPTER SEVEN

Orexigenic hormones and receptors

7.1 INTRODUCTION

Under fasting conditions the CB1R, and MCH1 receptor, as well as MCH itself are each up-regulated *in vivo* in nodose ganglion neurons (Burdyga *et al.*, 2004; Burdyga *et al.*, 2006). Moreover each of these genes are expressed in neurons that also express CCK1R. CCK down-regulated expression of each of these proteins after fasting for more that 18hr (Burdyga *et al.*, 2006). It is unclear, however, whether these genes are also differently regulated in VAN cultures.

Activation of MCH and endocannabinoid pathways in the hypothalamus are associated with stimulation of food intake. It seems possible therefore that under some circumstances VAN might switch their phenotype from predominantly activating satiety mechanisms to appetite stimulating mechanisms. The work in this chapter was directed at determining whether a switch in phenotype could be triggered in cultured neurons, thereby opening the way to studies of cellular mechanisms.

7.2 METHODS

Nodose ganglia were dissected as described in chapter 2.2.1.1, and cells were dissociated as described in chapter 2.2.5.

Immunohistochemistry was performed as described in chapter 2.2.5.1, using the following primary antibodies: rabbit anti-CART, rabbit anti-CB1R, goat anti-MCH, and mouse anti-Flag; and the following secondary antibodies: donkey anti-rabbit conjugated to TxR, donkey anti-mouse conjugated to FITC, or donkey anti-goat conjugated to FITC or TxR, as appropriate. VAN were cultured in FM for 3-4 days, and then transferred to SF medium with or without CCK, OxA, and Gh alone or in combination for 85min-10hr.

Cells were also transfected 24hr as described in chapter 2.2.5.2 with the ACREB construct and transferred to SF medium for 8hr followed by incubation with CCK for 2hr.

7.3. RESULTS

7.3.1 VAN express CB1R

Immunohistochemical analysis showed VAN cultured for 3hr in SF medium expressed CB1R in a perinuclear localisation (figure 7.1.C). After 6hr in SF medium this expression was still in the perinuclear space (figure 7.1.E). However after 10hr of SF culture CB1R was localised to punctuate structures in the cytoplasm indicating vesicles (figure 7.1.G, and magnified 7.1.I). Neurons cultured for 10hr in SF medium in the presence of CCK did not exhibit detectable CB1R (figure 7.1.A).

7.3.2 VAN express both MCH and CART

Dual staining using antibodies to CART and MCH, revealed that after 8hr incubation in SF medium CART expression was undetectable whereas MCH immunoreactivity was found in 53.0±0.9% in a perinuclear compartment with some in large vesicles (figure 7.2.K). This may correspond to the Golgi apparatus. Addition of CCK (10nM, 2hr) inhibited MCH expression while elevating CART expression to 60.0±0.8% of VAN localised in punctate structures (figure 7.2.J). Interestingly when neurons were incubated for 6.5hr in SF medium followed by 85min in SF medium with CCK, both MCH and CART were found to co-localise with 71.2±4.3% of CART positive neurons. There was also a small population of CART only cells (10.0±1.0%),

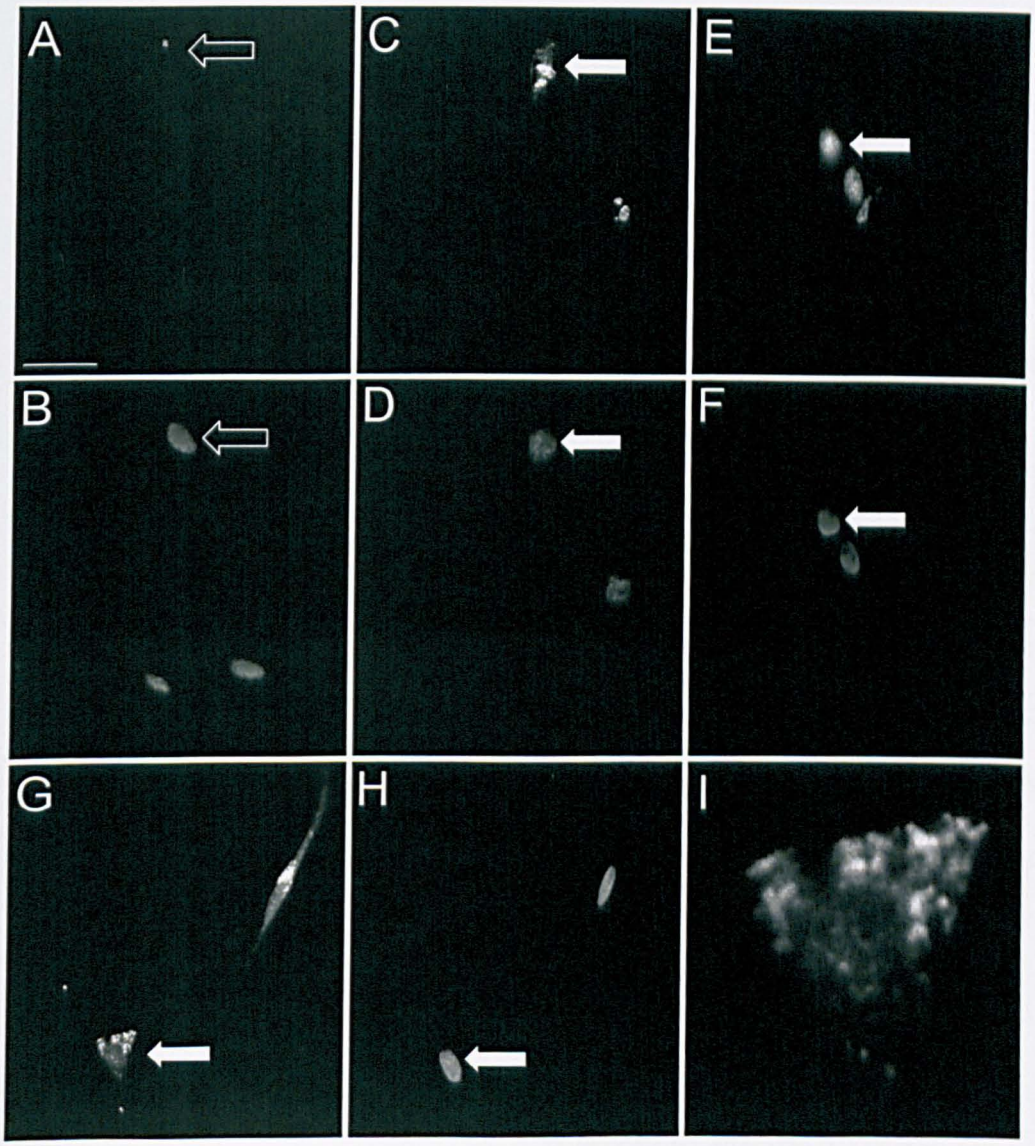
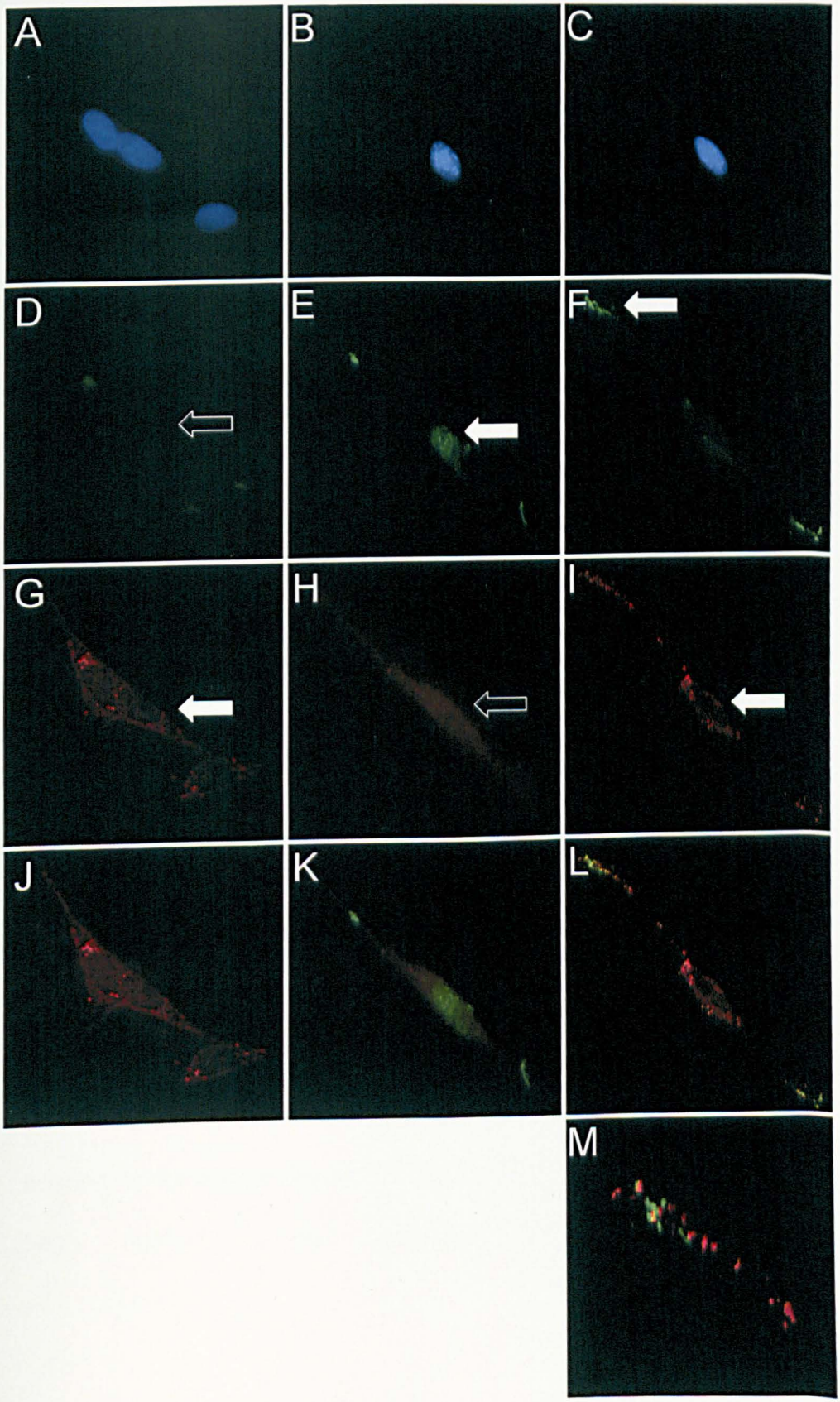


Figure 7.1. CCK down-regulates CB1R expression. Immunocytochemistry of cultured VAN after treatment with A) and B) 10hr SF medium with 10nM CCK, C) and D) 7hr SF medium with 10nM CCK, E and F) 4hr SF medium with 10nM CCK, G), H) and I) 10hr SF medium. Images A), C) , E), F) and I) stained with CB1R antibodies. Images B), D), F) and H) stained with DAPI. Scale bar=50μm. Representative images from 6 independent experiments.

Figure 7.2. CCK switches between CART and MCH in VAN. Immunocytochemistry of cultured VAN after treatment with A), D), G) and J) 6hr SF medium followed by 120min SF medium with 10nM CCK, B), E), H) and K) 8hr SF medium, C), F), I), L) and M) 6hr 25min SF medium followed by 85min SF medium with 10nM CCK. Images A), B) and C) stained with DAPI. Images D), E) and F) stained with MCH antibody. Images G), H), and I) stained with CART antibody. Images J), K), L), and M) show overlap of both CART- and MCH-immunoreactivity. Full arrows indicate positive immunoreactivity. Empty arrows indicate negative neurons. Scale bar=50μm. Representative images from 6 independent experiments.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and a smaller population of MCH only cells (3.2±0.7%). However, while MCH expression was predominantly found at terminal processes, CART expression was more widely distributed (figure 7.2.L). Higher magnification of one of the processes illustrates that there is very little co-localisation within the vesicles (figure 7.2.M).

7.3.3 Effects of orexin-A and ghrelin on MCH in VAN

Culturing VAN for 6hr in SF medium followed by 30min pre-incubation with either orexin-A or ghrelin before administration of 2hr CCK inhibits CART expression (7.3.C and G) whereas under the same conditions, MCH expression remains elevated (7.3.B and F). It would appear therefore that in addition to inhibiting CCK-induced CART expression, orexin-A and ghrelin are both able to inhibit CCK inhibition of MCH expression.

7.3.4 Effects of CREB on MCH in VAN

Neurons transfected with the dominant negative mutant of CREB, ACREB, maintain their MCH expression when cultured for 6hr in SF medium and incubated 2hr with 10nM CCK. As seen in figure 7.4.A neurons express ACREB, as indicated by staining with the flag epitope on the vector (figure 7.4.C). Both of the cells staining with the flag antibody also express MCH (figure 7.4.B).

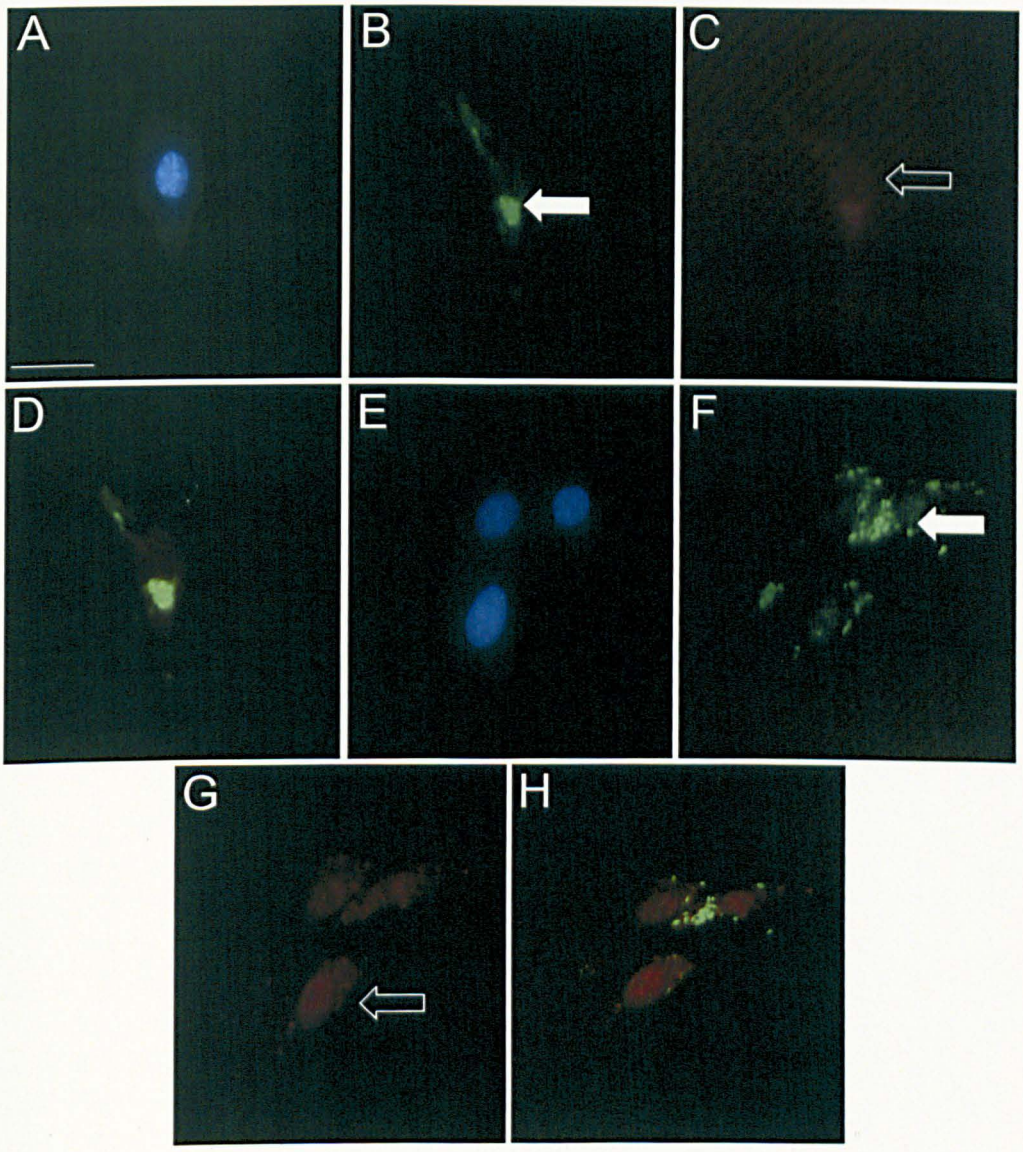

Figure 7.3. Orexin-A and ghrelin inhibit CCK inhibition of MCH. Immunocytochemistry of cultured VAN after treatment with A), B), C) and D) 6hr SF medium followed by 2hr 10nM Ox-A and 10nM CCK, E), F), G) and H) 6hr SF medium followed by 2hr 10nM ghrelin and 10nM CCK. Images A) and E) are stained with DAPI. Images B) and F) are stained with MCH antibodies. Images C) and G) are stained with CART antibodies. Images D) and H) show overlap of both CART and MCH antibodies. Scale bar=50µm. Full arrows indicate positive MCH immunoreactivity. Empty arrows indicate empty CART neuron. Representative images from 6 independent experiments.

The transfection efficiency was 8.2±0.5%, and of these cells the vast majority (96.6±1.3%) co-expressed ACREB and MCH. The inhibition of CREB can therefore prevent the CCK induced inhibition of MCH expression, indicating a possible role for CREB in the pathway.

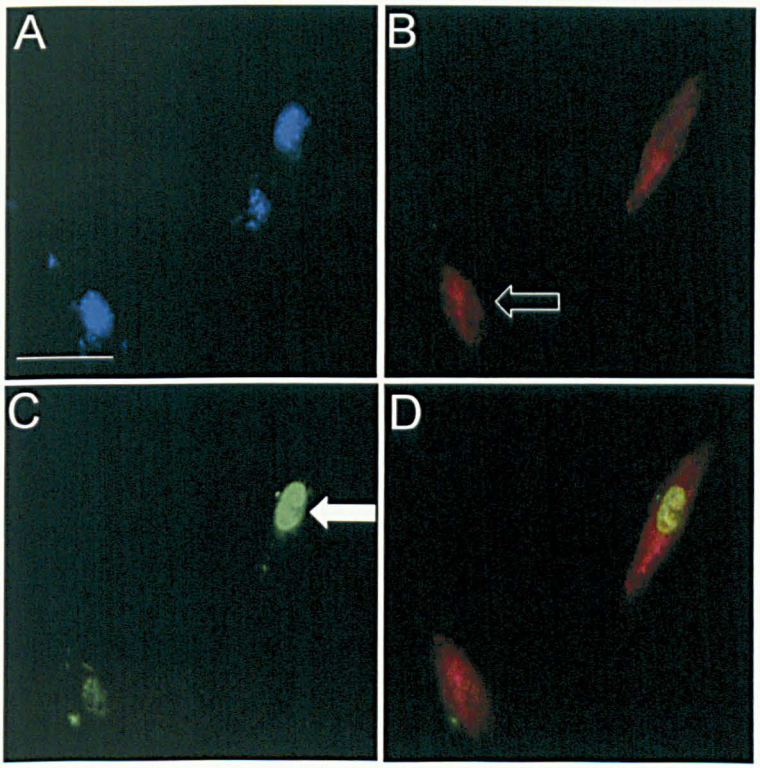

Figure 7.4. CREB down-regulates MCH. Immunocytochemistry of cultured VAN after 24hr transfection with ACREB. VAN were then cultured in SF medium for 8hr followed by 2hr CCK and stained with A) DAPI B) MCH antibodies C) Flag antibodies (indicating presence of ACREB plasmid) D) Overlay of Flag and MCH overlap. Full arrow indicates ACREB positive cell. Empty arrow indicates positive MCH-immunoreactivity. Scale bar=50μm. Representative images from 6 independent experiments.

7.4 DISCUSSION

It has been known for many years that cannabinoids are associated with stimulation of appetite (Mechoulam and Hanu, 2001) with both central and peripheral sites of action. Recent work indicates that the effect of peripherally administered endocannabinoids on appetite is mediated by VAN, suggesting a role in modulating gut–brain signalling (Gomez *et al.*, 2002). Two types of cannabinoid receptor have been characterised, CB1R and CB2R. The latter appears to be expressed predominantly by immune cells, whereas CB1R is expressed by many central and peripheral neurons, including VAN (Pertwee, 1997; Freund *et al.*, 2003; Partosoedarso *et al.*, 2003). Cota *et al.* have shown that CART colocalises with CB1R in the PVN, LH and DMN (Cota *et al.*, 2003). VAN express the CB1R, and importantly expression is increased by fasting and down-regulated by CCK (Burdyga *et al.*, 2004). In cultured VAN, CB1R was expressed within 3hr in a perinuclear compartment. Similarly to *in vivo* results, in which CB1R was found up-regulated within 12hr (Burdyga *et al.*, 2004), CB1R was found in vesicles after 8-10hr in cultured VAN.

These receptors expressed by VAN, which are implicated in control of food intake, are also expressed by hypothalamic neurons. In addition, there are a variety of other peptide transmitter systems found in hypothalamic neurons that so far have not been implicated in the peripheral signalling pathways that modulate food intake (Woods *et al.*,

1998; Schwartz *et al.*, 2000). One example is MCH, which is associated with stimulation of food intake (Collins and Kym, 2003; Forray, 2003). The majority of work on MCH has focused on the idea that it acts at the level of the hypothalamus to regulate food intake. However, it has been reported that MCH occurs in the intestine, raising the possibility of a peripheral component to the biology of MCH control of appetite (Hervieu *et al.*, 1996a; Hervieu *et al.*, 1996b). The present findings show that MCH and CB1R are expressed in VAN, suggesting an additional site of action for this peptide in controlling food intake.

In hypothalamus, CART and MCH are expressed in different hypothalamic neurons: the CART-expressing neurons are thought to be involved in inhibition of food intake, while MCH-expressing neurons stimulate food intake. It is interesting, therefore, that in nodose ganglia the same neurons can express both peptides. Importantly, however, while CCK stimulates CART expression it inhibits MCH expression; the two peptides are not found in the same vesicles, and it is only for a very brief period following CCK stimulation that the separate vesicle populations containing CART and MCH are identifiable within the same neurons. The present data suggest an additional mechanism by which ghrelin inhibiting the vagal actions of CCK could further reduce the satiety response to CCK after limited energy availability. Ghrelin prevented CCK inhibition of MCH after 6hr in SF medium. Since the GHS1R has high constitutive activity (Dass *et al.*, 2003), it suggests

that it maintains a tonic restraint on CCK1R activation. As described in chapter 6, vagally-mediated orexigenic effects of peripheral ghrelin and orexin-A involve suppression of the effects of CCK. The present data extend the findings to include stabilisation of the expression of MCH into the postprandial period. They indicate potentially longer-lasting effects on vagal afferent nerve function than are suggested by the acute responses shown by electrophysiological studies.

To date there have not been any mechanistic studies on how MCH expression is regulated in VAN, although other groups have found that MCH and phosphoCREB colocalise in 40% of hypothalamic neurons in fasted rats (Mogi *et al.*, 2005). After 24hr of food deprivation, CRE-mediated transcription was robustly up-regulated in MCH neurons but not in the adjacent orexin-A neurons (Georgescu *et al.*, 2005). For this reason the results demonstrating that CREB plays a role in regulating MCH expression in VAN is particularly interesting. VAN transfected with ACREB prevented CCK inhibition of MCH expression. This indicates that CCK inhibits MCH by phosphorylating CREB. I found however, that blocking CREB alone was not sufficient to induce MCH expression. So the mechanism by which MCH is up-regulated is still largely unknown.

Since CCK abolishes MCH expression within 2hr, it is unlikely that CCK only inhibits MCH transcription, as MCH would still be seen traveling through the secretory pathway. It is possible therefore that

CCK targets MCH for degradation, possibly by activating proteases. However, this hypothesis has not been studied, and further work is required to determine the mechanism by which CCK and CREB inhibit MCH.

CHAPTER EIGHT

Discussion

8.1 SUMMARY

The main findings described in this thesis are that the action of CCK in regulating CART in VAN is mediated by activation of PKC that in turn leads to phosphorylation of CREB and this appears to be a key regulatory of CART expression (see figure 8.1). Specifically,

(A) Food withdrawal decreased CART expression *in vivo*.

(B) In cultured neurons serum starvation reduced CART.

(C) In both cases, the loss of CART immunoreactivity was reversed by CCK. The loss of CART was not due to a generalised failure of protein synthesis since at least two proteins associated with orexigenic functions, the regulatory peptide MCH and the CB1R, exhibited increased expression.

(D) Immunocytochemical experiments demonstrated that phorbol ester phosphorylated CREB and induced CART synthesis, and that this was reversed by Ro-32-0432. In the presence of CCK, transfected PKC-alpha translocated to the plasma membrane.

(E) In luciferase studies CART promoter activity in response to CCK was inhibited by a dominant negative mutant CREB, ACREB, as well as a CBP inhibitor, E1A.

(F) VAN transfected with ACREB lacked CART immunoreactivity in the presence of CCK. Moreover, mutating the CRE site of the CART promoter inhibited its promoter activity.

(G) Up-regulating of CART by CCK was found to be inhibited by orexigenic hormones by a mechanism that appears to involve nuclear export of phosphoCREB (see figure 8.1).

(H) Immunocytochemistry demonstrated that MCH and the CB1R are up-regulated in VAN in the absence of CCK (see figure 8.2). MCH was found to co-localise with CART in VAN and again CREB was identified as a mediator.

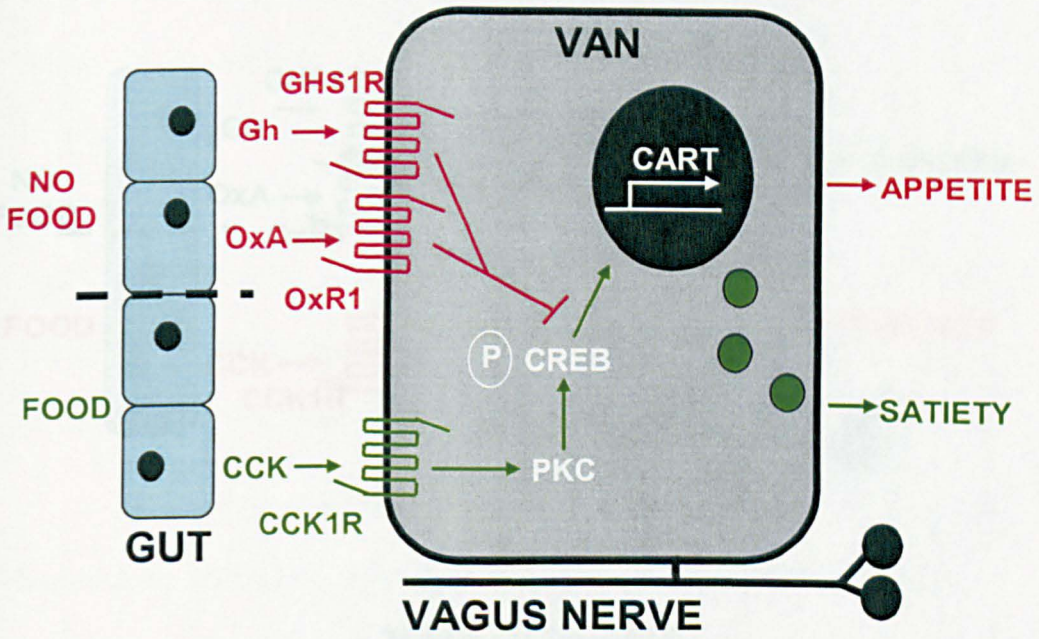

Figure 8.1. Model of CART regulation in VAN. Green pathway indicates up-regulation of CART expression in the presence of food. Red pathway indicates down-regulation of CART expression in the absence of food.

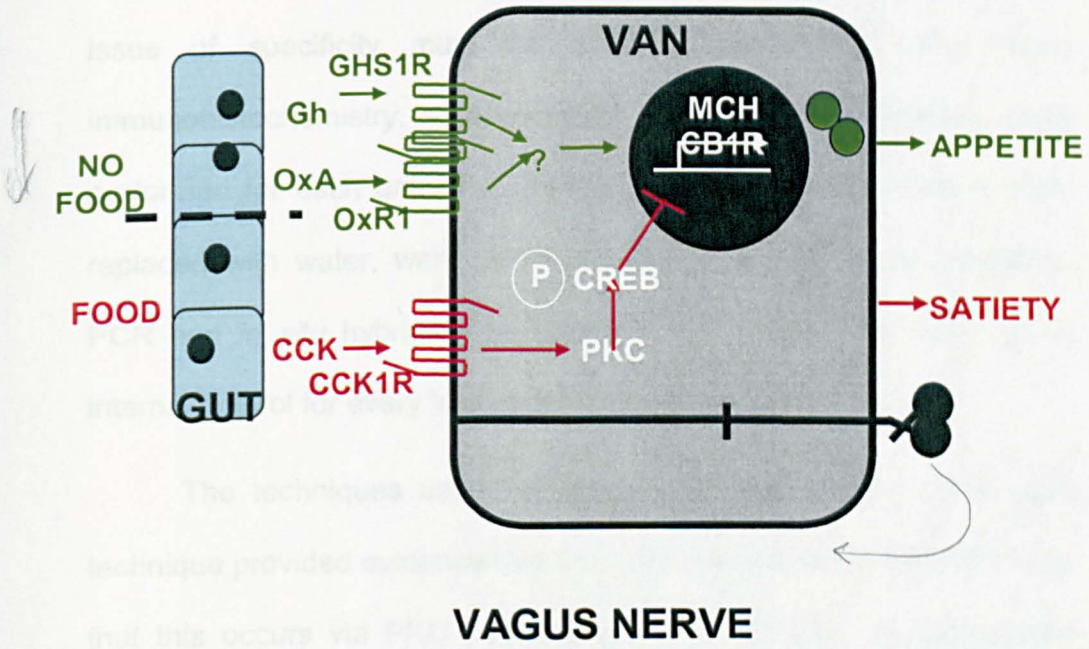

Figure 8.2. Model of MCH/CB1R regulation in VAN. Green pathway indicates up-regulation of MCH/CB1R expression in the absence of food. Red pathway indicates down-regulation of MCH/CB1R expression in the presence of food.

8.2 TECHNIQUES

The present work was based on both *in vivo* and *in vitro* studies of VAN. Immunohistochemical experiments were used extensively together with luciferase assays, PCR and *in situ* hybridisation. The issue of specificity must be carefully scrutinised when using immunohistochemistry. Absorption controls of antibodies were performed for each antibody. Water controls, in which primers were replaced with water, were performed in every reverse transcriptase-PCR and *in situ* hybridisation experiment. Renilla was used as an internal control for every luciferase antibody experiment.

The techniques used complemented each other in that each technique provided evidence that CCK regulates CART expression and that this occurs via PKC phosphorylation of CREB. In subsequent studies, Western blot and Radioimmunoassay would be useful to use, to quantify changes in CART protein expression. Moreover, electrophoretic mobility shift assay should now be used to demonstrate transcription factors activity on the CART promoter. It is however, important to note the low protein levels in nodose ganglia, and that these techniques do not lend themselves to study of this ganglion.

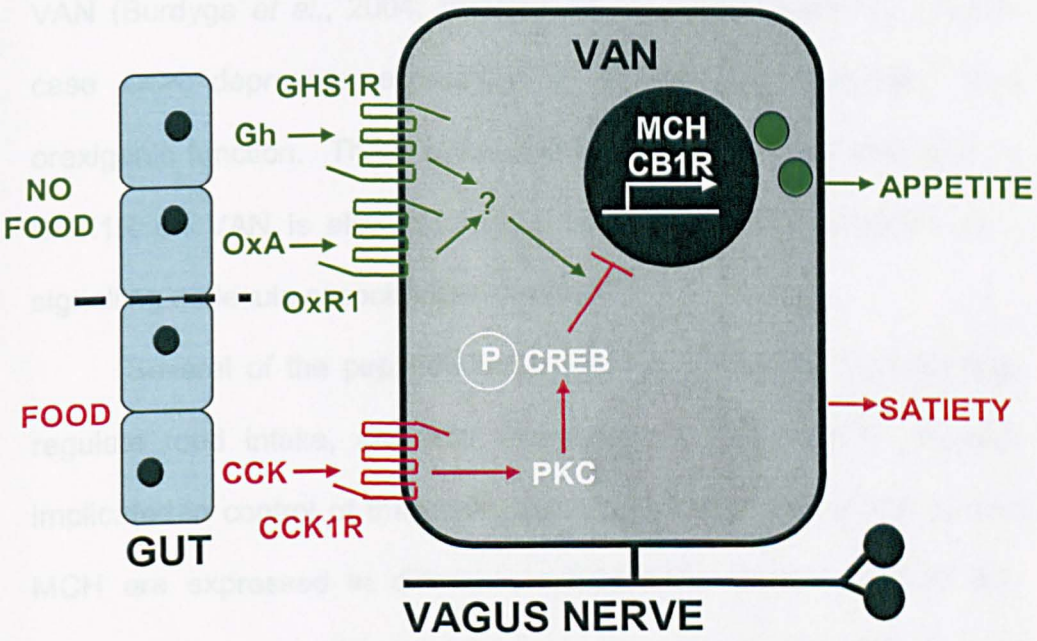

Figure 8.2. Model of MCH/CB1R regulation in VAN. Green pathway indicates up-regulation of MCH/CB1R expression in the absence of food. Red pathway indicates down-regulation of MCH/CB1R expression in the presence of food.

The finding that CCK regulates CART expression adds a new dimension to proposed hypothesis that CART could be a peptide mediator of the satiety effects of CCK in VAN (Broberger *et al.*, 1999; Hokfelt *et al.*, 2001). CCK has previously been found to regulate the expression of signalling molecules associated with feeding behaviour in VAN (Burdyga *et al.*, 2004; Burdyga *et al.*, 2006); however, in each case CCK depresses expression of a molecule associated with orexigenic function. These present findings suggest that activation of CCK1R on VAN is also associated with increased expression of a signalling molecule associated with satiety.

Several of the peptides and receptors expressed by VAN that regulate food intake, are also expressed in hypothalamic neurons implicated in control of the same behaviour. Interestingly, CART and MCH are expressed in different hypothalamic neurons: the CART-expressing neurons are thought to be involved in inhibition of food intake while MCH-expressing neurons stimulate food intake. It is interesting, therefore, that in nodose ganglia the same neurons can express both peptides. Importantly, however, while CCK stimulates CART expression it also inhibits MCH expression; both CART and MCH are not found in the same vesicles and it is only for a very brief period following CCK-stimulation when the separate vesicle populations containing CART and MCH are identifiable within the same neurons. The CART/MCH switch within these neurons is presumably part of a

mechanism that allows control of meal size to be influenced by prior energy intake so that after energy restriction there is a transient and compensatory increase in food intake and a corresponding acceleration of gastric emptying to ensure rapid delivery of food to the intestine.

8.4 FUTURE WORK

The site of CCK action in regulating CART abundance remains to be determined. CCK could act at CCK1R located on VAN terminals, or at receptors located near the cell bodies in the nodose ganglia via the circulation. Further experimental work is required to answer this question. Similarly, the targets of CART once released from VAN are currently unclear. Appleyard et al demonstrated that i.p. administration of CCK induced c-fos gene expression in NTS POMC neurons suggesting that they are activated by afferent neurons stimulated by the satiety hormone (Appleyard *et al.*, 2005). There is also evidence that CCK activation of POMC neurons could regulate the activation of the neuronal melanocortin-4 receptor (Fan *et al.*, 2004). Work could be done to test whether CART has an effect on this pathway.

Another interesting result that needs further investigation is the observation that the orexigenic hormones prevent nuclear phosphoCREB. Again, the mechanism by which this occurs is unclear. As described in the chapter 6 discussion, it is possible that orexin-A and ghrelin inhibit nuclear import. In order to test this hypothesis we could inhibit members of the nuclear import machinery in the presence of CCK to see if the same pattern of phosphoCREB localisation is observed. Once we have identified the key players that regulate import of CREB to the nucleus, we could determine whether the orexigenic hormones inhibit their activity.

I have demonstrated that in cultured VAN, MCH, as well as the CB1R is expressed in VAN in the absence of CCK. Having demonstrated this it will now be possible to study the mechanism of expression. This may involve producing a MCH-promoter luciferase-reporter construct to identify the transcription factors involved in the regulation of MCH. In addition, since the orexigenic hormones ghrelin and orexin-A have successfully been demonstrated to up-regulate MCH, the mechanism by which they cause up-regulation of MCH could be studied. Electrophysiology could be used to determine whether the changes in CB1R and CCK1R effect the neurons firing or produce some other change resulting in either synaptic plasticity or terminal changes.

The observation that MCH is down-regulated within 120min indicates that CCK does not inhibit MCH via a transcriptional route, as it would take longer for MCH to travel through the secretory pathway. The hypothesis that proteases are involved needs to be investigated. It will be interesting to see if protease inhibitors could prevent CCK inhibition of MCH. If proteases were found to play a role in inhibiting MCH, it would be interesting to determine if CREB activates these proteases in VAN.

As discussed earlier it will be hugely important to discover the CART receptor. Much work has already been put into the discovery of this receptor with little avail. Once the receptor has been found it will

be important to clone it and sequence it. Localising the receptor will be an important step, as it will enable us to determine the role of CART more efficiently.

CHAPTER NINE

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