To Investigate the Physiological Role of Arcuate Nucleus Cocaine- and Amphetamine- Regulated Transcript in Energy Homeostasis.

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Department of Investigative Medicine
Faculty of Medicine
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For my mum and dad,
brothers and sisters
Abstract

Cocaine- and amphetamine- regulated transcript (CART) was originally identified as a mRNA transcript upregulated in rats in response to administration of cocaine and amphetamine. CART is widely expressed in the central nervous system (CNS) with high levels of expression in hypothalamic nuclei such as the arcuate nucleus (ARC).

CART was initially thought to act as an anorectic peptide since it is coexpressed with the anorectic neuropeptide pro-opiomelanocortin (POMC) in the ARC. In addition, intracerebroventricular (ICV) administration of CART (55-102) peptide inhibits feeding and administration of anti-CART antibody results in stimulation of feeding. However, subsequent studies have suggested CART may also act as an orexigen since injection of CART (55-102) specifically into the ARC and ventromedial nucleus (VMN) of the hypothalamus results in a significant increase in food intake. These data suggest CART acts through both anorectic and orexigenic circuits. Given the importance of the hypothalamus in the regulation of energy homeostasis, and the role of the ARC in integrating peripheral signals, it is essential to elucidate the role of ARC derived CART. In order to elucidate CART’s true physiological role in the ARC I used a combination of genetic approaches. I generated a recombinant Adeno-associated virus (rAAV) expressing CART antisense (CART-AS) and a transgenic mouse model which utilises the POMC promoter to drive expression of CART-AS.

In the transgenic CART-AS model mice exhibited a significantly higher body weight relative to control animals, no significant difference in food intake was observed. In addition, mice expressing the CART-AS transgene demonstrated a reduction in uncoupling protein-1 (UCP-1) mRNA expression in brown adipose tissue (BAT) which is suggestive of decreased thermogenesis. This may explain the observed increase in body weight in the transgenic mice. Bilateral intra-ARC injections of rAAV-CART-AS resulted in a significant increase in cumulative food intake and body weight gain compared to control animals. There was no significant difference in activity or metabolism levels.

The data presented in my thesis provides an important contribution to understanding the role of CART within the ARC. The results from my genetic studies appear to suggest that ARC derived CART has an anorectic role.
Declaration of Contributors

The majority of the research and work described in this thesis was performed by the author. All collaboration and assistance is described below:

Chapter 3:
The pronuclear injection of transgene constructs was carried out by the MRC Transgenic Core Facility, Hammersmith Hospital.
The radioimmunoassays in this chapter were carried out under the supervision of Professor Mohammad Ghatei and Dr Michael Patterson (Department of Investigative Medicine).

Chapter 4:
Competent bacteria and recombinant AAV were prepared by Dr. J. Gardiner (Department of Investigative Medicine).
Studies involving the CLAMS were done with assistance from Dr. N. Semjonous and Dr J. Cooke (Department of Investigative Medicine).
Injection of recombinant AAV into the arcuate nucleus was carried out in collaboration with Dr J. Cooke (Department of Investigative Medicine).
The radioimmunoassays in this chapter were carried out under the supervision of Professor Mohammad Ghatei and Dr Michael Patterson (Department of Investigative Medicine).

All in house radioimmunoassays were established and maintained by Professor M. Ghatei (Department of Investigative Medicine).
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Finally, I would like to thank my family who have been an unending source of support and encouragement.
### Abbreviations

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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>5-HT</td>
<td>5-hydroxytryptamine</td>
</tr>
<tr>
<td>2-AG</td>
<td>2-Arachidonyl glycerol</td>
</tr>
<tr>
<td>2-DG</td>
<td>2-Deoxy-D-Glucose</td>
</tr>
<tr>
<td>AAV</td>
<td>Adeno-Associated Virus</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA Carboxylase</td>
</tr>
<tr>
<td>aCSF</td>
<td>artificial Cerebrospinal Fluid</td>
</tr>
<tr>
<td>ACTH</td>
<td>Adrenocorticotropic Hormone</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenovirus</td>
</tr>
<tr>
<td>AEA</td>
<td>Anandamide</td>
</tr>
<tr>
<td>Arg</td>
<td>Arginine</td>
</tr>
<tr>
<td>AgRP</td>
<td>Agouti-related Protein</td>
</tr>
<tr>
<td>AMP</td>
<td>Adenosine Mono-Phosphate</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AP</td>
<td>Area Postrema</td>
</tr>
<tr>
<td>ARC</td>
<td>Arcuate Nucleus</td>
</tr>
<tr>
<td>AS</td>
<td>Antisense</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine Triphosphate</td>
</tr>
<tr>
<td>BAC</td>
<td>Bacterial Artificial Chromosome</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown Adipose Tissue</td>
</tr>
<tr>
<td>BGH</td>
<td>Bovine Growth Hormone</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>bp</td>
<td>base pairs</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>CAC</td>
<td>CART-Antisense-CART</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic Adenosine Monophosphate</td>
</tr>
<tr>
<td>CART</td>
<td>Cocaine- and Amphetamine-Regulated Transcript</td>
</tr>
<tr>
<td>CCK</td>
<td>Cholecystokinin</td>
</tr>
<tr>
<td>cDNA</td>
<td>copy DNA</td>
</tr>
<tr>
<td>CLAMS</td>
<td>Comprehensive Laboratory Animals Monitoring System</td>
</tr>
<tr>
<td>CMV</td>
<td>Cytomegalovirus</td>
</tr>
<tr>
<td>CNS</td>
<td>Central Nervous System</td>
</tr>
</tbody>
</table>
CRH  Corticotropin Releasing Hormone
CRE  cAMP Response Element
CSF  Cerebrospinal Fluid
Cys  Cysteine
DA  Dopamine
DMEM  Dubecco's Modified Eagle Medium
DMN  Dorsomedial Nucleus
DMV  Dorsal Motor Nucleus of the Vagus
DNA  Deoxyribonucleic Acid
dNTP  Deoxynucleotide Triphosphate
dsRNA  double stranded RNA
DTT  Dithiothreitol
DVC  Dorsal Vagal Complex
EDTA  Ethylenediaminetetraacetic acid
eGFP  Enhanced Green Fluorescent Protein
ELISA  Enzyme-Linked ImmunoSorbent Assay
ES cell  Embryonic stem cell
FCS  Fetal Calf Serum
FMM  Formamide
GABA  γ-Aminobutyric Acid
GDW  Glass Distilled Water
GEE  Generalised Estimation Equation
GFP  Green Fluorescent Protein
GHRH  Growth Hormone Releasing Hormone
GHS-R  Growth Hormone Secretagogue Receptor
GIT  Gastrointestinal Tract
GLP-1  Glucagon-Like Peptide-1
GLUT  Glucose Transporter
Gly  Glycine
GnRH  Gonadotropin-releasing Hormone
GTE  Glucose Tris EDTA
GTT  Glucose Tolerance Test
HIV  Human Immunodeficiency Virus
HPT  Hypothalamo-Pituitary Thyroid
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
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<td>ICV</td>
<td>Intracerebroventricular</td>
</tr>
<tr>
<td>IE gene</td>
<td>Immediate early gene</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>i.p.</td>
<td>intra-peritoneal</td>
</tr>
<tr>
<td>IPSC</td>
<td>Inhibitory Post Synaptic Current</td>
</tr>
<tr>
<td>IRS</td>
<td>Insulin Receptor Substrate</td>
</tr>
<tr>
<td>ITR</td>
<td>Inverted Terminal Repeat</td>
</tr>
<tr>
<td>ITT</td>
<td>Insulin Tolerance Test</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus Kinase</td>
</tr>
<tr>
<td>K-ATP</td>
<td>Potassium-ATP</td>
</tr>
<tr>
<td>LB</td>
<td>Lysogeny Broth</td>
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<td>Lateral Hypothalamus</td>
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<tr>
<td>Lys</td>
<td>Lysine</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen Activated Protein Kinase</td>
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<tr>
<td>MCH</td>
<td>Melanin- Concentrating Hormone</td>
</tr>
<tr>
<td>MTII</td>
<td>Melanotan II</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-Morpholino)propanesulphonic Acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>MSH</td>
<td>Melanocyte Stimulating Hormone</td>
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<tr>
<td>NAc</td>
<td>Nucleus Accumbens</td>
</tr>
<tr>
<td>NE</td>
<td>Norepinephrine</td>
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<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
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<tr>
<td>NSE</td>
<td>Neurone Specific Enolase</td>
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<tr>
<td>NTS</td>
<td>Nucleus of the Solitary Tract</td>
</tr>
<tr>
<td>OC</td>
<td>Optic Chiasm</td>
</tr>
<tr>
<td>OCT</td>
<td>Optimum Cutting Temperature</td>
</tr>
<tr>
<td>OD</td>
<td>Optical Density</td>
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<tr>
<td>pA</td>
<td>Polyadenylation signal</td>
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<tr>
<td>PAC</td>
<td>P1- Derived Artificial Chromosome</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PC</td>
<td>Prohormone Convertase</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
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<tr>
<td>PDX-1</td>
<td>Pancreatic Duodenal Homeobox 1</td>
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<tr>
<td>PEI</td>
<td>Polyethylenimine</td>
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<tr>
<td>Acronym</td>
<td>Full Form</td>
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<td>---------</td>
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<tr>
<td>PeV</td>
<td>Periventricular Nucleus</td>
</tr>
<tr>
<td>PFA</td>
<td>Perifornical Area</td>
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<tr>
<td>PFC</td>
<td>Prefrontal cortex</td>
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<td>Phe</td>
<td>Phenylalanine</td>
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<td>PI3K</td>
<td>Phosphatidylinositol-3-Kinase</td>
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<td>POMC</td>
<td>Proopiomelanocortin</td>
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<td>PP</td>
<td>Pancreatic Polypeptide</td>
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<td>PVN</td>
<td>Paraventricular Nucleus</td>
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<tr>
<td>PYY</td>
<td>Peptide Tyrosine Tyrosoine</td>
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<tr>
<td>rAAV</td>
<td>Recombinant AAV</td>
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<tr>
<td>RER</td>
<td>Respiratory Exchange Ratio</td>
</tr>
<tr>
<td>RIA</td>
<td>Radioimmunoassay</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic Acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA interference</td>
</tr>
<tr>
<td>RT</td>
<td>Reverse Transcriptase</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error of the Mean</td>
</tr>
<tr>
<td>SN</td>
<td>Substantia Nigra</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interfering RNA</td>
</tr>
<tr>
<td>SOB</td>
<td>Super Optimal Broth</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal Transducers and Activators of Transcription</td>
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<tr>
<td>SSPE</td>
<td>Saline Sodium Phosphate EDTA</td>
</tr>
<tr>
<td>TAE</td>
<td>Tris-Acetate EDTA</td>
</tr>
<tr>
<td>TE</td>
<td>Tris EDTA</td>
</tr>
<tr>
<td>Tfam</td>
<td>Mitochondrial Transcription Factor A</td>
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<tr>
<td>TRH</td>
<td>Thyrotropin Releasing Hormone</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer RNA</td>
</tr>
<tr>
<td>TSH</td>
<td>Thyroid Stimulating Hormone</td>
</tr>
<tr>
<td>Tyr</td>
<td>Tyrosine</td>
</tr>
<tr>
<td>UCP-1</td>
<td>Uncoupling Protein-1</td>
</tr>
<tr>
<td>VMN</td>
<td>Ventromedial Nucleus</td>
</tr>
<tr>
<td>VTA</td>
<td>Ventral Tegmental Area</td>
</tr>
<tr>
<td>WAT</td>
<td>White Adipose Tissue</td>
</tr>
<tr>
<td>WT</td>
<td>Wildtype</td>
</tr>
<tr>
<td>WPRE</td>
<td>Woodchuck hepatitis virus Post-Transcriptional Regulatory Element</td>
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YAC  Yeast Artificial Chromosome
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Chapter 1

General Introduction
1. General Introduction

1.1 Obesity

Obesity is a major public health issue and it is predicted that by 2010 40% of children in Europe will be obese. This increase in obesity is a result of people adopting a more sedentary lifestyle, accompanied by an increase in availability and affordability of highly calorific foods. Obesity has significant effects on morbidity and mortality due to comorbid diseases such as type II diabetes, coronary heart disease and cancer. In turn this will lead to greater strains on health care. The National Audit Office which scrutinises public spending estimates the cost of obesity to the NHS is £50 million with further indirect costs of between £1.7 and £1.9 billion, accounting for around 4% of total NHS spending (Bourn, 2001). There is an urgent need to understand how appetite is regulated in order to identify new targets for the development of effective anti-obesity drugs. Energy balance is a highly regulated process with the hypothalamus and brainstem thought to play a central role in this regulation by integrating short term satiety signals and long term signals of energy store.

1.2 The Hypothalamus (Figure 1.1)

The hypothalamus is one of the main sites, in conjunction with the brainstem and ventral tegmental area (VTA), which regulate food intake and energy balance. The hypothalamus is situated at the base of the brain lying immediately above the pituitary, either side of the third ventricle and it is composed of a number of discrete nuclei (Figure 1.2) which have been implicated in the regulation of energy homeostasis such as; the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial nucleus (VMN), dorsomedial nucleus (DMN), lateral hypothalamus (LH) and perifornical area (PFA). The hypothalamus can be divided into three distinct regions; the lateral, medial and periventricular zones. The hypothesis that the hypothalamus is involved in the regulation of appetite originally came from clinical observations that patients with pituitary tumours, and injuries to the base of the brain, developed obesity. These observations led to experimental lesioning studies of specific hypothalamic nuclei which resulted in changes in feeding. Lesioning of the LH resulted in hypophagia and weight loss whereas lesioning of the ventromedial hypothalamus resulted in hyperphagia and weight gain in rats (Hetherington and Ranson, 1942; Nand and Brobeck, 1951).
Figure 1.1 The hypothalamus. Diagram of the hypothalamus. The blue shaded area is the anterior region of the hypothalamus, the red area is the tuberal region and the green area is the lateral posterior region of the hypothalamus. Hypothalamic nuclei shown are paraventricular nucleus, the lateral and medial preoptic nuclei, anterior nucleus, suprachiasmatic nucleus, supraoptic nucleus, arcuate nucleus, ventromedial nucleus, mammillary body, posterior area and dorsomedial nucleus. Other areas outside the hypothalamus that are present are fornix, thalamus, tuber cinereum, anterior and posterior pituitary, infundibular stalk and optic chiasm. Adapted from (Holmes et al., 2003)
Figure 1.2 Organisation of the hypothalamus. Schematic representation of the organisation of the zones and regions of the hypothalamus (a- area, n-nucleus). Adapted from (Simerly and Paxinos, 1998).
These initial findings laid the foundation of the dual centre hypothesis which stated that feeding was controlled by a satiety centre, the ventromedial hypothalamic nucleus, and a feeding centre, the lateral hypothalamus. However, this is now accepted to be an oversimplification as other hypothalamic and non-hypothalamic areas have been implicated in appetite regulation.

1.2.1 The Arcuate Nucleus
The arcuate nucleus is located at the base of the hypothalamus, close to the median eminence, and is ideally positioned to integrate peripheral signals due to the incomplete blood brain barrier. The ARC has a high density of orexigenic neurones that coexpresses neuropeptide Y (NPY) and agouti related peptide (AgRP). Immunohistochemistry has demonstrated approximately 95 percent of AgRP neurones coexpress NPY in the ARC (Broberger et al., 1998). A second dense neuronal population coexpresses the anorectic pro-opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) neuropeptides. Double label in situ hybridisation studies showed that over 90% of ARC POMC neurones contain CART mRNA (Elias et al., 1998). Both of these major neurone populations have been heavily implicated in the control of food intake. Food restriction results in an increase in NPY and AgRP mRNA expression and a decrease in the expression of POMC and CART mRNA in the rodent ARC (Mizuno et al., 1999; Swart et al., 2002). The ARC is also a site of expression of other neurotransmitters and neuropeptides such as galanin (Skofitsch and Jacobowitz, 1985) and γ-amniobutyric acid (GABA) (Cowley et al., 2001; Hentges et al., 2004) and glutamate (Boulland et al., 2004).

Neuronal projections from the ARC to various hypothalamic and non-hypothalamic brain areas involved in the control of food intake is consistent with the ARC being well positioned to integrate signals of energy status. For example, POMC and AgRP neuronal fibres originating in the ARC project to the VMN, DMN, PVN and LH (Elmquist et al., 1998; Elmquist et al., 1999). Beyond the hypothalamus, ARC neurones have been identified projecting to the brainstem, VTA and nucleus accumbens (NAc). These non-hypothalamic brain regions have also been linked with a role in appetite (Haskell-Luevano et al., 1999; Jacobowitz and O'Donohue, 1978; Joseph and Michael, 1988). The ARC also possesses glucose responsive neurones (Muroya et al., 2005; Parton et al., 2007; Wang et al., 2004) and receptors for the circulating hormones leptin and insulin (Hakansson et al., 1996; Obici et al.,...
suggesting that the ARC can sense and transduce signals from peripheral hormonal and nutrient factors.

1.2.2 The Paraventricular Nucleus
The paraventricular nucleus sits adjacent to the top of the third ventricle and receives neuronal input from brain areas which include the ARC, LH and amygdala. The neurones in the PVN can be divided into two components; the magnocellular component and the parvocellular component. The magnocellular component contains oxytocin and arginine vasopressin expressing neurones which project to the neural lobe of the posterior pituitary. The parvocellular component contains a range of neuropeptides including corticotropin releasing hormone (CRH), which causes anorexia, and thyrotropin releasing hormone (TRH) which reduces food intake in addition to controlling the release of adrenocorticotropic hormone (ACTH) and thyroid stimulating hormone (TSH) from the pituitary (Vijayan and McCann, 1977; Vogel et al., 1979). The presence of neuropeptides in the PVN which reduce food intake is indicative of the PVN being involved in the regulation of energy homeostasis. Evidence for the importance of the PVN in appetite control comes from PVN lesioning studies which resulted in the development of hyperphagia and obesity whereas stimulation of the PVN led to a decrease in feeding in rats (Hetherington and Ranson, 1942). Later research involved the administration of feeding related peptides into the PVN which complemented the initial findings from lesioning studies. Intra-nuclear injection into the PVN of the orexigenic peptide NPY (Stanley et al., 1985) or ghrelin (Wren et al., 2001) resulted in increased food intake and the injection of the anorectic cholecystokinin (CCK) resulted in a decrease in food intake (Blevins et al., 2000) suggesting the PVN can mediate both orexigenic and anorexigenic signals. These studies highlight a physiological role for the PVN in food intake.

1.2.3 The Ventromedial Nucleus
The ventromedial nucleus lies above the ARC and can be divided into two separate regions, the dorsomedial and ventrolateral areas. Laser scanning photostimulation of mouse brain slices show that the VMN sends excitatory innervations to POMC neurones of the ARC (Sternson et al., 2005). The VMN also receives innervations from the brainstem and medial zone of the hypothalamus. Afferent and efferent connections of the VMN with other brain
regions associated with appetite control suggest that the VMN plays a part in this regulation. Evidence for the physiological involvement of the VMN in appetite regulation was initially suggested in VMN lesioning studies which resulted in hyperphagia and obesity in rats relative to sham treated animals (Hetherington and Ranson, 1942). Leptin and insulin levels were also raised in the VMN lesioned rats (Suga et al., 1999). In addition, injection of NPY peptide into the VMN results in a robust increase in feeding, and fasting has been associated with increased NPY peptide concentration in the rat VMN (Bouali et al., 1995). Evidence detailing the VMN as a site of action of NPY, coupled with results from lesioning studies suggests a role for the VMN in the control of appetite.

1.2.4 The Lateral Hypothalamus

The lateral hypothalamus is positioned laterally to the VMH and has also been implicated in the control of feeding, a role that was initially suggested by the ablation of the LH which resulted in decreased food intake and weight loss in rats (Milam et al., 1980). The neuropeptides melanin concentrating hormone (MCH) and orexins A and B, which have been shown to increase feeding, have been identified in the LH. MCH and the orexins are expressed in separate but spatially overlapping neuronal populations in the perifornical region of the LH and zona incerta. Fasted rats show an increase in hypothalamic MCH mRNA expression and injection of MCH into the lateral ventricle of rats leads to a stimulation of food intake (Qu et al., 1996). Genetic models of MCH provide evidence of a physiological role for the MCH peptide in energy homeostasis. Mice with targeted deletion of MCH are lean (Shimada et al., 1998) and MCH overexpressing mice are hyperphagic, obese and insulin resistant (Ludwig et al., 2001) which is consistent with MCH being an orexigenic peptide. The orexin peptides have also been linked with an orexigenic role since rats fasted for 48 hours show an increase in prepro-orexin mRNA and intracerebroventricular (ICV) administration of orexin-A and -B increase food intake (Sakurai et al., 1998; Yamanaka et al., 2000). However, the orexin peptides are thought to have a range of functions within the central nervous system (CNS) due to the LH orexin neurones projecting widely within the brain. For example, orexin neurones from the LH project to the VTA (Zheng et al., 2007), which is suggestive of communication between the hypothalamus and reward pathway via the VTA. In addition, it is thought that the orexin peptides mediate the sleep-wake cycle and locomotor activity since ICV administration of orexin-A in rats is accompanied by an increase in arousal during the normal rodent sleep period and increased activity levels such as
grooming (Hagan, 1999). The orexin mediated increase in arousal may contribute to the increase in food intake associated with the orexin peptides since increased periods of wakefulness allow more opportunities for rodent feeding. Electrophysiology studies suggest that the orexin peptides mediate their effects on arousal through the locus coeruleus (LC), a modulator of attentional state and stress, since application of orexin to brain slices of the LC results in increased firing of the noradrenergic neurones from this nucleus (Hagan, 1999). The presence of the orexigenic MCH and orexin peptides in the LH strongly links this nucleus with a role in regulating food intake.

1.2.5 Brainstem
The brainstem plays an important role in the regulation of energy homeostasis. The brainstem senses and integrates signals from circulating peripheral factors released from the gastrointestinal tract and is considered to be a primary site of regulation of gastrointestinal function (Jobst et al., 2004). The brainstem nuclei that have been implicated in the regulation of energy homeostasis are the nucleus of the solitary tract (NTS), dorsal motor nucleus of the vagus (DMV) and the area postrema (AP). These are collectively known as the dorsal vagal complex (DVC).

The AP, like the ARC, possesses an incomplete blood brain barrier and is thus able to respond to circulating peripheral orexigenic and anorectic signals. Injection of the peripheral hormone ghrelin into the brainstem results in hyperphagia, implying the brainstem can respond to peripheral hormones (Faulconbridge et al., 2003). The NTS is thought to integrate viscerosensory information originating from the gastrointestinal tract via vagal nerve inputs which then project to other hypothalamic nuclei such as the PVN, ARC and LH (Jobst et al., 2004). POMC neurones have been shown to project from the brainstem to the hypothalamus, although these POMC neurones do not coexpress CART (Ellacott et al., 2006) and NPY neurones have been shown to project from the NTS to the PVN. Collectively this suggests that the melanocortins and NPY play a role in mediating stimuli from the gastrointestinal tract via the brainstem. The role of the brainstem seems to be one of integrating gastrointestinal signals for meal initiation and termination whereas the role of the hypothalamus is one of regulating long term energy balance.
1.2.6 Ventral Tegmental Area
The ventral tegmental area lies medial to the substantia nigra (SN), between the caudal hypothalamus and brainstem. The VTA is one of the main sites from which dopamine (DA) neurones project to other brain regions including the NAc, prefrontal cortex (PFC) and LH (Margolis et al., 2006). The dopaminergic neuronal projections from the VTA to the NAc and subsequently to the ventral pallidum form the mesolimbic dopamine pathway, which is implicated in mediating the effects of reward and addiction. In addition to dopaminergic neurones, GABAergic and glutamatergic neurones have also been identified in the VTA. Electrophysiological studies confirmed the presence of GABA and glutamate innervation from the VTA to the NAc and PFC (Carr and Sesack, 2000; Chuhma et al., 2004).

Various substances associated with reward and addiction such as cocaine, amphetamine and opioids, have been shown to mediate their rewarding effects through the VTA since intravenous administration of these compounds results in increased dopamine release in the rat VTA. In addition, self administration of cocaine by rats is reduced upon pretreatment with dopamine receptor antagonists administered into the NAc and VTA (Caine and Koob, 1994; Wise, 2004). Peripheral administration of cocaine and amphetamine is associated with a decrease in feeding whereas opioid administration is linked with an increase in feeding (Balopole, 1979; Hsieh et al., 2005; Glass et al., 1999; Yun et al., 2004). It is hypothesised that the VTA may mediate these effects on appetite since injection of ghrelin into this nucleus increases food consumption confirming the ability of the VTA to mediate increases in food intake (Naleid et al., 2005). These data together suggest the VTA mediates the hedonistic component of food intake.

1.3 Neuropeptide Signalling
The ARC is one of the main sites at which peripheral signals of long term energy store, such as leptin and insulin, and short term signals of satiety are integrated within the CNS. These peripheral signals act at specific receptors in the ARC to transduce a neuronal response to other brain areas. The main neuropeptides involved in this signal transduction include the melanocortins, NPY and CART.
1.3.1 The Melanocortin System

The melanocortin system consists of agouti related peptide, the endogenous melanocortin antagonist and several neuropeptides derived from posttranslational processing of pro-opiomelanocortin; these include α, β, and γ- melanocortin stimulating hormone (MSH), β endorphin and adrenocorticotropin hormone. POMC is expressed in the ARC and immunoreactive fibres from POMC expressing neurones project to other hypothalamic nuclei involved in appetite regulation including the PVN, DMN and LH (Elias et al., 1998; Elmquist et al., 1998; Elmquist et al., 1999). In addition, POMC is also present in the pituitary and NTS. In the periphery POMC has been detected by in situ hybridisation in the testes, ovary, placenta and liver (Bicknell, 2008; Gizang-Ginsberg and Wolgemuth, 1985; Chen et al., 1986).

The melanocortin system has five G-protein coupled melanocortin receptors (MC1R-MC5R). These receptors act through adenylyl cyclase to generate the secondary messenger cyclic adenosine monophosphate (cAMP). The MC1R was the first receptor to be cloned and is expressed exclusively in melanocytes where it has a role in determining coat colour in mice. The MC2R is specific for ACTH and is expressed in the adrenal cortex and adipocytes. The MC3R and MC4R are both implicated in energy homeostasis (Chen et al., 2000; Marsh et al., 1999) with MC3R limited to the hypothalamus and brainstem whereas MC4R is more widely distributed throughout the CNS (Mountjoy et al., 1994; Huszar et al., 1997). Mice which lack functional MC3R show an increase in fat mass and a decrease in lean mass without significant changes in food intake or body weight (Chen et al., 2000). In contrast, mice with targeted deletion of MC4R develop maturity onset obesity associated with hyperphagia, hyperinsulinemia and hyperglycemia (Huszar et al., 1997).

Antagonism at the melanocortin receptors was identified from studies using the agouti mouse (A<sup>y</sup>/a). The agouti gene was cloned in 1992 and encodes a 131 amino acid peptide (Bultman et al., 1992). The expression of agouti is normally restricted to the hair follicle where it is a paracrine factor secreted during the hair growth cycle. Agouti acts as an antagonist at the MC1R causing a switch in synthesis from eumelanin to phaeomelanin. In the A<sup>y</sup>/a mouse a chromosomal rearrangement occurs that results in the promoter and the first non coding exon of the closely linked raly gene being spliced to the exon of the agouti gene. As a result, expression of the agouti gene is under the control of the raly promoter and is consequently ectopically expressed. The ectopic expression of the agouti gene produces a severe phenotype.
with the A/y mouse having a yellow coat colour and hyperphagia leading to severe obesity. Hyperinsulinemia and hyperglycemia are also characteristics of the agouti mouse (Bultman et al., 1992). The agouti obese phenotype is thought to be a result of antagonism of the hypothalamic MC4R (Fan et al., 1997).

An agouti like peptide was identified from studies involving the screening of cDNA sequences with homology to agouti (Shutter, 1997). The AgRP gene was isolated in 1997 based on its 40 amino acid cysteine rich carboxyl terminal homology to agouti (Ollmann et al., 1997) and is expressed at high levels in the ARC and adrenal gland and at lower concentration in the testis, lung and kidney. The hyperphagic effect of AgRP was demonstrated by ICV administration in rodents (Rossi et al., 1998; Small et al., 2001) and receptor binding studies demonstrate that AgRP is a competitive antagonist of the MC4R (Fan et al., 1997). The current data supports the hypothesis that α-MSH and AgRP are involved in the control of food intake and that the relationship between the two peptides is an antagonistic one mediated by the melanocortin receptor.

The physiological importance of the melanocortin system in the regulation of appetite has been further emphasised by transgenic studies. Overexpression of POMC in mice increases α-MSH production leading to a reduction in food intake and reduced adiposity. This is in contrast to mice with a targeted deletion of the POMC gene which are hyperphagic and have reduced energy expenditure leading to obesity (Yaswen et al., 1999). Transgenic mice which overexpress human AgRP exhibit an obese phenotype, identical to the agouti mouse, but with normal pigmentation (Ollmann et al., 1997). This confirms the importance of AgRP and α-MSH in the physiological regulation of appetite. In contrast, mice with a targeted deletion of the AgRP gene exhibit normal body composition and food intake. Furthermore, the transgenic mice also display similar levels of suppression of food intake as wildtype mice upon leptin administration (Qian et al., 2002). The lack of a phenotype in mice with a targeted deletion of the AgRP gene may be attributed to developmental compensation. Following from this, genetic studies have been conducted which have studied the effect of temporal ablation of AgRP neurones. One study deleted the mitochondrial transcription factor A (Tfam) in AgRP neurones with the result being progressive post natal cell death (Xu, 2005). Another study attempted to temporally control the ablation of the AgRP neurones using diphtheria toxin (Gropp, 2005). Both studies demonstrated that the loss of AgRP produced a robust phenotype of hypophagia and leanness. However, many neurones express
more than one neuropeptide, so ablation of the AgRP neurone may also ablate other neurotransmitters and neuropeptides that are colocalised with AgRP, consequently whether the resulting phenotype is due to AgRP is hard to state. Data from transgenic studies, coupled with the pharmacological data are indicative of the melanocortin system being important in energy homeostasis.

The relevance of the melanocortin system in humans is highlighted in genetic studies which examine the phenotype resulting from mutations within this system. Congenital POMC deficiency in humans presents with several clinical features. There is failure of adrenal steroidogenesis which is due to the loss of action of ACTH at the MC2R. In addition subjects have pale skin and red hair due to the loss of α-MSH action at the MC1R and obesity due to the loss of its stimulation of MC3R and MC4R. Additionally, these subjects suffer from mild central hypothyroidism (Krude et al., 2003). Conversely, mutations in the human AgRP gene have been associated with increased susceptibility to anorexia nervosa (Vink et al., 2001). Interestingly, human studies have also implicated β-MSH as a potential regulator of food intake, rodents lack the necessary cleavage site to generate β-MSH hence research has mainly focused on the function of α-MSH. A heterozygous mutation disrupting a conserved pair of basic amino acids at the prohormone processing site Arg236Gly between β-MSH and β-endorphin results in the fusion protein of β-MSH/β-endorphin which binds to the MC4R, but has a reduced ability to activate the receptor. This mutation was found in 0.9% of individuals with early onset obesity compared with 0.2% of normal weight individuals (Biebermann et al., 2006). Another heterozygous mutation in the POMC gene affecting the processing of β-MSH has been identified. The Tyr221Cys mutation results in decreased binding of the peptide to the MC4R and occurs in 0.9% of obese individuals relative to 0.08% of normal weight individuals (Lee et al., 2006). The human data together with that from rodent models provides strong evidence of the melanocortin system in the regulation of appetite.

1.3.2 Neuropeptide Y
Neuropeptide Y was first isolated and sequenced from porcine brain and is distributed in the central and peripheral nervous system (Tatemoto et al., 1982). NPY is a 36 amino acid peptide synthesised from a 98 amino acid propeptide, proNPY. The propeptide consists of a 29 amino acid signal sequence, a Gly-Lys-Arg processing site and a 30 amino acid carboxyl terminal flanking peptide. NPY is highly conserved and is identical between rat and human.
The site of expression and projection of NPY neurones is suggestive of a role in energy homeostasis since arcuate NPY neurones innervate other hypothalamic nuclei involved in energy homeostasis including the PVN, DMN and LH (Jobst et al., 2004). Research indicates an orexigenic role for NPY as ARC NPY mRNA expression was upregulated, and NPY peptide release from the PVN increased, in response to food deprivation (Kalra et al., 1991). NPY peptide concentration normalises on re-feeding following a fast (Swart et al., 2002). An orexigenic role for NPY was further confirmed by chronic ICV administration of NPY which resulted in hyperphagia in rats (Zarjevski et al., 1993).

Within the ARC fluorescent double immunolabelling studies demonstrate colocalisation of GABA with NPY in approximately 33% of NPY neurones (Horvath et al., 1997). The NPY/GABA interneurones were found to exert an inhibitory tone on the POMC neurone population. This was demonstrated by Cowley et al where electrophysiology was used to determine the effect of leptin on POMC neurones. Leptin was found to depolarise 72 out of 77 POMC neurones and this was shown to be, in part, mediated by NPY/GABA neurones. GABA mediated inhibitory post synaptic currents (IPSC) were observed in POMC cells and leptin was found to reduce the frequency of IPSC in GABAergic neurones by 25% (Cowley et al., 2001). The evidence of GABAergic projections from orexigenic neurones that can reduce anorectic POMC/CART neuronal firing is consistent with the NPY being an orexigenic agent.

Physiological evidence of a role for NPY in feeding comes from NPY content and release studies. NPY peptide content and release is significantly increased during the dark phase, the period of nocturnal feeding in rodents. ICV administration of NPY antibodies during the dark phase blocks this nocturnal feeding, emphasising the physiological orexigenic role of NPY. Further physiological evidence of NPY’s involvement in the regulation of appetite is presented in genetic studies. Administration of recombinant adeno-associated virus (rAAV) expressing NPY antisense mRNA into the rat ARC resulted in a decrease in food intake and reduced body weight gain (Gardiner et al., 2005). Attempts to establish a transgenic line with targeted deletion of NPY have resulted in mice with no obvious phenotype, although this is generally attributed to developmental compensation (Erickson et al., 1996). Experiments designed to ablate NPY/AgRP neurones postnatally have confirmed this neuronal population as being important in energy homeostasis since ablation of NPY/AgRP neurones by
administration of diphtheria toxin in adult rats resulted in rapid starvation (Luquet et al., 2005).

NPY acts via a family of G protein coupled receptors known as Y1R-Y5R. Mice with targeted deletion of these receptors have been useful as a means to investigate the role of the Y receptor subtype. The Y1 and Y5 receptors are thought to be the main mediators of the orexigenic effects of NPY. Daily food intake and NPY stimulated feeding are only slightly decreased in Y1R deficient mice (Pedrazzini et al., 1998) and the central administration of Y1R selective antagonist inhibits the feeding effects of exogenous NPY (O'Shea et al., 1997). The Y5 receptor has also been implicated in the control of food intake since the mice with a targeted deletion of the Y5R exhibit a blunted response to ICV administration of NPY and develop mild obesity with age (Marsh et al., 1998). Furthermore, the Y5R antagonist, Velneperit, has been developed and is currently in phase III clinical trials. Collectively, these data implicate a role for NPY in the control of feeding.

1.4 Cocaine- and Amphetamine -Regulated Transcript

Cocaine- and amphetamine -regulated transcript was originally identified as an mRNA transcript upregulated in the rat striatum in response to intraperitoneal (i.p) administration of cocaine or amphetamine (Douglass et al., 1995).

1.4.1 Cocaine- and Amphetamine -Regulated Transcript Gene

The CART gene is composed of three exons and two introns. The CART peptide is highly conserved across species with approximately 80% nucleotide homology between rat and human CART cDNA and between mouse and rat homology is 98% (Douglass and Daoud, 1996). The conserved nature of the CART sequence suggests that it plays an important role in the different species. The carboxyl terminus of the CART peptide is the most conserved region and contains six cysteine residues which form three disulphide bonds. The formation of disulphide bridges maintains the tertiary structure of the protein and are essential to CART bioactivity (Thim et al., 1998).
1.4.2 Cocaine- and Amphetamine-Regulated Transcript Peptide

In the rat and mouse CART DNA sequence two different polyadenylation sites have been identified which consequently leads to the transcription of two pre-mRNA transcripts, one of 700bp and the other of 900bp in length. Due to alternate splicing of a 39bp sequence from the coding region of exon two the translation of the CART mRNA results in the two pre-propeptides (pre-proCART) which contains a 27 amino acid leader sequence. The leader sequence is removed by posttranslational cleavage which results in two proCART peptides of 102 (long form) and 89 (short form) amino acids. The ProCART peptides are processed further by posttranslational cleavage resulting in different sized CART fragments. The sites at which the proCART peptide is cleaved are thought to be at positions where pairs of basic amino acids occur (Figure 1.3). Two bioactive forms of CART have been identified, CART (55-102) and CART (62-102) (Thim et al., 1999) which are highly expressed in the ARC (Kristensen, 1998). The nomenclature of CART (55-102) and CART (62-102) is based on the amino acid residues number from the long form of CART peptide.

CART (55-102) has been isolated from the ovine hypothalamus and is generated from cleavage at the pair of basic amino acid residues Lys53-Arg54 (Spiess et al., 1981). It is this fragment of CART that is commonly used in rodent studies. The prohormone convertase (PC) enzymes PC2 and PC1/3, which are expressed at high levels in the ARC, have been implicated in mediating the cleavage of proCART into its bioactive fragments (Schafer et al., 1993). The role of prohormone convertase enzymes in the processing of CART was confirmed by cell line transfection studies. The CART fragments that resulted from the processing of proCART by the two PC enzymes in the cell transfection study were identified by high performance liquid chromatography (HPLC) (Dey et al., 2003). PC2 was shown to be more efficient at generating CART (55-102) and the sole generator of CART (62-102). PC1/3 was shown to be responsible for the generation of additional intermediate CART fragments (Dey et al., 2003). The resulting fragments from the processing of proCART that have been shown to be biologically active in rats are CART (55-102) and CART (62-102).
Figure 1.3 Schematic representation of the primary translation product of CART. Fragments are labelled according to nomenclature based on both the shorter 89 amino acid splice variant and the longer 102 amino acid variant. Adapted from (Thim et al., 1999)

1.4.3 Cocaine- and Amphetamine -Regulated Transcript Localisation

CART is expressed in several hypothalamic nuclei. *In situ* hybridisation and immunohistochemical staining has shown that CART is abundant in the periventricular nucleus (PeV), PVN, the supraoptic nucleus (SON), ARC, the zona incerta and the LH (Vrang et al., 1999).

CART peptide was detected in the ARC by immunohistochemistry. Double label *in situ* hybridisation shows that over 90% of CART neurones also express POMC mRNA in the rat ARC (Elias et al., 1998). There was no evidence of CART colocalising with NPY (Vrang et al., 1999). In the human infundibular nucleus CART immunoreactivity is not found in the POMC neurones, but is found in approximately one third of NPY/AgRP containing neurones (Menyhert et al., 2007).

CART neurones in the PVN, DMN and the posterior PeV coexpress TRH (Elias et al., 2001) but CART containing neurones were not found to colocalise with CRH within the PVN. This suggests that CART may play a role in the regulation of thyroid hormone release through the pituitary (Kristensen, 1998).

The largest group of CART neurones within the hypothalamus are found in the zona incerta and LH where CART has been shown to colocalise extensively with MCH, but not with the
orexin peptides. In the zona incerta MCH and CART colocalise in almost 100% of neurones (Elias et al., 2001). In the medial LH a similar degree of colocalisation is present, however, in the lateral and ventral areas of the LH approximately 70% of MCH neurones coexpress CART (Elias et al., 2001).

Studies investigating the sites of CART expression and identifying neuropeptides that are colocalised in CART neurones implicates CART as having a wide ranging role in the control of energy homeostasis. It may appear contradictory for CART to colocalise with orexigenic peptides such as MCH and anorectic peptide such as TRH and POMC. However, this may suggest that CART acts in competing orexigenic and anorectic circuits within the hypothalamus.

1.4.4 Physiological Role of Cocaine- and Amphetamine -Regulated Transcript

1.4.4.1 Cocaine- and Amphetamine -Regulated Transcript and the Reward System
CART has been implicated in mediating the rewarding effects of psychostimulants. The mechanism of reward and addiction is thought to be mediated through the mesolimbic dopamine pathway (see section 1.2.6). CART immunoreactivity has been identified in this pathway, specifically in the VTA and NAc. Furthermore, CART mRNA expression in the NAc has been shown to be increased in response to i.p administration of cocaine in adult male rats (Hunter et al., 2005). This suggests CART plays a role in mediating reward and addiction through the VTA and NAc. In addition, VTA immunostaining studies demonstrate CART containing neurones synapse with DA and GABAergic neurones (Ivechia-Adams et al., 2002). The physical interaction between the CART and DA neurones suggests that CART may modulate firing of DA neurones within the reward pathway. The current data suggests a role for CART in the reward pathway, specifically in the VTA and NAc.

1.4.4.2 Cocaine- and Amphetamine -Regulated Transcript and the Stress Response
Stress causes an increase in firing of the CRH neurones in the PVN. CRH neurones stimulates the release of ACTH from the pituitary which subsequently acts on the adrenal gland to stimulate the release of glucocorticoids which are associated with an increase in feeding and appetite, possibly through interference of leptin action at the hypothalamus.
(Solano and Jacobson, 1999). In addition, glucocorticoids have also been linked with an increase in salience for pleasurable activities such as increased sucrose consumption in rats (Dallman, 2003; Laugero et al., 2002). CART is thought to have a role in mediating the stress response since ICV administration of CART peptide results in an increase in plasma concentration of ACTH and corticosterone (Vrang, 2000). Furthermore, adrenalectomy in rats, which removes endogenous glucocorticoids, results in a decrease in CART mRNA expression in the PVN and ARC which is partially reversed by glucocorticoid replacement (Balkan et al., 2001). Collectively the data suggests a feedback loop between CART and corticosteroideal secretion since CART has been shown to increase plasma glucocorticoid concentrations and the loss of glucocorticoids results in a reduction in CART mRNA expression.

1.4.5 Peripheral Cocaine- and Amphetamine -Regulated Transcript
Stanley et al found circulating CART levels follow a diurnal pattern in rats. Radioimmunoassay (RIA) was used to measure plasma CART (55-102) -like immunoreactivity, CART peptide concentration was found to be higher in the dark phase, which is the phase most associated with feeding in rodents (Stanley et al., 2004). This suggests that increased plasma CART may promote feeding in the dark phase. However, peripheral CART administration has been shown to have no effect on feeding indicating that circulating CART is not involved in the regulation of food intake (Jensen et al., 1999).

1.4.6 Central Cocaine- and Amphetamine -Regulated Transcript and Food Intake
Hypothalamic CART is thought to be an anorectic agent. ICV administration of CART (55-102) peptide inhibits feeding in satiated and fasted rats. In addition, the orexigenic effect associated with ICV administration of NPY is inhibited by CART in a dose dependent manner, which is consistent with CART acting as an anorectic peptide (Kristensen, 1998). Physiological data further supports an anorectic role for endogenous CART peptide since ICV administration of anti-CART antibody, which blocks endogenous CART, resulted in stimulation of overnight feeding (Lambert, 1998; Kristensen, 1998). The evidence from ICV studies and CART antibody studies is further affirmed by injection of rAAV expressing CART cDNA into the third ventricle of rats. Expression of the CART transgene was demonstrated in cells of the third ventricle and median eminence. Throughout the seven
month study rAAV-CART injected rats displayed decreased food intake and body weight gain (Qing and Chen, 2007).

Aja et al also showed that ICV administration of CART was accompanied by a reduction in food intake and c-fos expression, a marker of neuronal activation, in areas of the brain associated with energy homeostasis (Aja et al., 2001). Collectively the data is indicative of CART having an inhibitory physiological effect on food intake. However, Aja et al reported that administration of CART into the third ventricle was also associated with movement tremors as a result of CART peptide diffusing to the fourth ventricle, an area that is linked with motor control, suggesting the effect of food intake may be secondary to an effect on motor control. However, when CART was prevented from diffusing to the fourth ventricle by means of blocking the aqueduct with the use of a plug, a reduction in food intake was still observed without the movement tremors (Aja et al., 2001). This suggests that movement tremors cannot fully explain the reduction in food intake following third ventricle administration of CART peptide.

While ICV injection of CART (55-102) has been consistently shown to reduce food intake, there is conflicting data regarding the effect of CART (55-102) following injection into specific hypothalamic nuclei. Studies suggest that CART has an orexigenic role in certain nuclei. Injection of 0.04nmol CART (55-102) into the rat ARC and VMN produced a delayed but significant increase in feeding between 1 and 4 hours post injection. Significant increases in feeding were seen after injection of CART at a higher dose (0.2nmol) into the ARC, VMN, PVN, LH, DMN and SON. In contrast, injection of CART peptide into the 3rd ventricle inhibited food intake (Abbott et al., 2001; Kong et al., 2003). Injection of CART into specific hypothalamic nuclei was not associated with any movement tremors (Abbott et al., 2001). Furthermore, CART stimulated the release of NPY and inhibited α-MSH release from hypothalamic explants (Stanley et al., 2001) which is consistent with CART acting as an orexigen. The data on the role of CART is controversial since CART has been linked with both anorectic and orexigenic roles in the regulation of energy homeostasis. Further investigation is needed to elucidate the physiological role of CART within the hypothalamus and its constituent nuclei.
1.4.7 Energy Expenditure

In addition to its role in food intake, CART has also been implicated in the control of energy expenditure. Triple-labelling fluorescent *in situ* hybridisation and immunofluorescence studies demonstrated that CART containing neurones which coexpress α-MSH synapse with TRH neurones, suggesting CART plays a role in the regulation of TRH release (Fekete et al., 2000). TRH is linked with a role in energy expenditure through mediating release of TSH from the pituitary which in turn stimulates the release of thyroxine (T4) and tri-iodothyronine (T3) from the thyroid gland (Mittler et al., 1969). In agreement with this anatomical data administration of CART peptide has been demonstrated to result in an increase in TRH release from hypothalamic explants (Rondeel et al., 1992). A role for CART in energy expenditure was further confirmed in studies by Kong et al which investigated the effect of chronic overexpression of CART cDNA in the arcuate nucleus of rats by gene delivery (Kong et al., 2003). Twenty five days post injection of CART cDNA expressing plasmid into the ARC, brown adipose tissue (BAT) uncoupling protein (UCP-1) mRNA was 80% higher in treated rats relative to the control animals (Kong et al., 2003). Chronic administration of CART peptide into the ARC has been demonstrated to cause hyperphagia and to increase thermogenesis. Hyperphagia is suggestive of CART acting as an orexigen whereas the increase thermogenesis is consistent with CART acting as an anorectic peptide, hence CART seems to be acting as both an anorectic and an orexigenic peptide. However, this may be explained by the observation by Kong et al that rats exposed to a temperature of 4°C over 20 days demonstrated a 124% increase in ARC CART mRNA (Kong et al., 2003). Therefore CART mediated hyperphagia and increased thermogenesis suggests that CART is involved in cold adaptation.

1.4.8 Transgenic Models

Transgenic models have been used in an attempt to investigate the physiological role of CART in energy homeostasis. Mice with targeted deletion of the CART gene were generated by Asnicar et al and exhibited no body weight difference relative to wildtype mice when fed a standard chow diet. However, these mice did display an increase in body weight and food intake relative to wildtype mice when maintained on a high fat diet which suggests that CART may protect against diet induced obesity (Asnicar et al., 2001).
A second strain of mice with a targeted deletion of the CART gene was generated by Wierup et al and they exhibited an increase in body weight relative to control animals with transgenic mice being 14% heavier than wildtype mice at 40 weeks of age when fed standard chow (Wierup et al., 2005). Insulin secretion was also measured in these mice since CART mRNA expression has been identified by immunocytochemistry and in situ hybridisation in the pancreatic nerve fibres in the normal mouse pancreas (Wierup et al., 2004). Mice with a targeted deletion of CART displayed impaired insulin secretion and it was hypothesised that this was a result of β cell dysfunction. This was confirmed by immunocytochemistry which showed mice with a targeted deletion of CART displayed age dependent reduction in immunoreactivity of pancreatic duodenal homeobox 1 (PDX-1) transcription factor, which is important in β cell development and insulin gene expression, and glucose transporter 2 (GLUT2), which is a major determinant of cellular glucose uptake. The absence of GLUT2 and PDX-1 is associated with defective insulin secretion and glucose tolerance (Wierup et al., 2005; Wierup, 2006). The impairment in insulin secretion was observed before an increase in body weight presented suggesting CART directly affects islets cells.

The data from mice with targeted deletion of CART suggests an anorectic physiological role for CART. However, CART peptide is widely expressed in the brain and the periphery and the phenotype of mice with body wide deletion of CART is an amalgamation of the effect of the loss of CART peptide throughout the organism. Therefore it is difficult to conclude a precise role for CART in a specific brain region.

### 1.4.9 Differential Regulation of Cocaine- and Amphetamine -Regulated Transcript

Studies have suggested that CART acts both as an anorectic and an orexigenic agent in the control of energy homeostasis. CART mRNA expression is reduced in rats fasted for 48 hours implicating it with an anorectic role, although, intra-ARC injection of CART peptide causes an increase in feeding which is suggestive of CART acting as an orexigenic factor. To reconcile the data which demonstrates the two competing functions of CART it has been hypothesised that CART is differentially regulated within the hypothalamus. Abbot et al has suggested that CART may be involved in two appetite circuits, one orexigenic and one anorectic (Abbott et al., 2001). Interestingly, POMC neurones in the ARC have been shown to mainly coexpress glutamate but a proportion of POMC neurones coexpress the GABA
neurotransmitter, suggesting that POMC neurones within the ARC may have stimulatory and inhibitory functions (Hentges et al., 2004).

1.4.10 Cocaine- and Amphetamine -Regulated Transcript in Humans
The need to understand the role of CART is underlined by human genetic studies. In humans, a heterozygous CART missense mutation has been identified which cosegregates with obesity through three generations of an Italian family. The missense mutation results in an amino acid substitution of leucine to phenylalanine at codon 34 (Leu34Phe) within the amino terminal CART region. Obese individuals with this mutation have low resting metabolic rates. The leucine residue at codon 34 precedes two lysine residues which may be a site for proteolysis by the PC enzymes. The Leu34Phe mutation may alter the susceptibility of CART to proteolysis and effect energy expenditure (del Giudice et al., 2001). The mutation may reduce the ability of the prohormone convertase enzymes to catalyse proteolysis at this site, alternatively, the mutation may alter the structure of the proCART peptide making it more susceptible to proteolysis. Depending on the effect of the mutation of posttranslational processing the concentration of CART peptide fragments will be either increased or decreased. The exact effect of the Leu34Phe mutation on CART peptide processing is unclear as CART peptide concentration was not measured in the affected individuals (del Giudice et al., 2001). A second study by Yamada et al studied polymorphisms in the CART gene to investigate whether there was a link to obesity. The 5’ flanking region of the CART gene was screened for associations between polymorphisms and obesity. The 5’ flanking region of the CART gene was sequenced. Six polymorphic sites were identified A-->G at -156, T-->C at -390, T-->G at -484, G-->T at -915, G-->C at -929 and C-->T at -962. The nucleotide substitution at -156 was significantly associated with greater body mass index (BMI) and the allele frequency of the -156 variant was significantly higher in obese subjects. The polymorphism at -156 site may be linked with a genetic predisposition to obesity (Yamada et al., 2002).

1.5 Neurotransmitters
Several chemical neurotransmitters have been implicated in the control of food intake. In some circumstances the neurotransmitters have been demonstrated to colocalise with the
neuropeptides described previously which may allow multiple responses through the same neuronal population.

1.5.1 Glutamate and \(\gamma\)-aminobutyric acid

L-Glutamate is the main excitatory and \(\gamma\)-aminobutyric acid is the main inhibitory neurotransmitter in the CNS and both have been implicated in the control of food intake. Injection of glutamate into the LH results in feeding in satiated rats and this feeding is inhibited by the administration of glutamate receptor antagonists (Stanley et al., 1993; Stanley et al., 1996). GABA is also involved in the control of feeding. GABA expressed in ARC NPY neurones exerts an inhibitory tone on ARC POMC neurones (Cowley et al., 2001). Furthermore, ICV administration of GABA agonists cause an increase in feeding in rodents which is reversed by ICV administration of GABA receptor antagonists (Stratford and Kelley, 1997; Ebenezer, 1990). Collectively the evidence suggests that both GABA and glutamate are involved in appetite regulation and for this reason a GABA receptor antagonist named Zonisamide has been developed by Pharma as an anti-obesity drug and is currently in phase III clinical trials.

1.5.2 Monoamine Neurotransmitters

The main monoamine neurotransmitters that have been linked with a role in potentiating food intake are dopamine, norepinephrine (NE) and 5-Hydroxytryptamine (5-HT). Dopamine is thought to increase food intake since administration of dopamine receptor antagonists results in a reduction in feeding (Clifton et al., 1991). DA plays a prominent role in mediating an increase in food intake through the mesolimbic pathway since restoration of DA to the NAc in dopamine deficient mice restores feeding (Szczypka et al., 2001). 5-HT has been implicated as having a suppressive effect on food intake since injection of exogenous 5-HT into the PVN, VMH and DMN leads to a reduction in food intake (Leibowitz and Alexander, 1998). As a result 5-HT has been targeted as a treatment for obesity. Sibutramine is a serotonin and norepinephrine reuptake inhibitor which reduces food intake by inducing the sense of satiety and preventing the decline in metabolic rate that is associated with a low calorie diet by increasing the concentration of 5-HT in the synapse. Sibutramine has been shown to cause dose dependent weight loss in humans in addition to improved lipid and glucose profiles (Blackburn and Smith, 1997; Kim et al., 2003) although the drug has now
been withdrawn for use in the UK by the Medicines and Healthcare Products Regulatory Agency after the drug was linked with an increase in heart attacks and strokes. NE has been shown to both stimulate and inhibit food intake. There are 5 types of adrenergic receptor, $\alpha_1$, $\alpha_2$, $\beta_1$, $\beta_2$ and $\beta_3$. Activation of hypothalamic $\alpha_1$, $\beta_2$ and $\beta_3$ adrenergic receptors results in a decrease in food intake, whereas stimulation of $\alpha_2$ leads to an increase in food intake (Goldman et al., 1985). In fact, Eli Lilly have developed a $\beta_3$ receptor agonist, LY362884, which is currently in phase III clinical trials. Evidence suggests that the monoamine neurotransmitters can mediate effects on food intake through actions at the hypothalamus and the reward pathway. Interestingly, a drug named tesofensine, which acts by blocking the reuptake of the DA, 5-HT and NE is in phase III clinical trials as an anti-obesity therapy.

### 1.5.3 Endocannabinoids and Opioids

Endocannabinoids and opioids have both been shown to effect food intake. Intra-nuclear administration of the endocannabinoids anandamide (AEA) and 2-arachidonyl glycerol (2-AG), and the opioids $\beta$-endorphin and dynorphin, promote feeding in rodents confirming a stimulatory effect on food intake by the opioids and endocannabinoids (Kirkham et al., 2002; Hao et al., 2000; Baile et al., 1986; Glass et al., 1999). In addition, ICV administration of $\beta$-endorphin in rats has been shown to reduce sympathetic nerve activity to intrascapular BAT in a dose dependent fashion (Egawa et al., 1993). Collectively the evidence suggests that the endocannabinoids and opioids have a stimulatory effect on food intake. The ability of the cannabinoid receptor 1 (CB1) to mediate the feeding effect of endocannabinoids has made it an attractive target for anti-obesity therapy. Rimonobant is a drug that acts as a CB1 receptor antagonist which prevents endogenous endocannabinoid action at the CB1 receptor with the result being a reduction in food intake. However, the approval for rimonobant was withdrawn early in 2009 by the European Medicine Agency’s due to evidence linking an increase in the prevalence of psychiatric conditions in patients taking the drug (Christensen et al., 2007).

### 1.6 Peripheral Signals of Adiposity (Figure 1.4)

The brain integrates a number of peripheral signals as part of its role in regulating energy homeostasis. These peripheral hormones include signals of long term energy store, such as leptin and insulin, and short term signals of satiety from the gastrointestinal tract.
1.6.1 Leptin

Leptin is synthesised in the white adipocytes and released into the circulation at a level proportional to body fat and as such is an indicator of peripheral energy stores (Considine et al., 1996). Chronic administration of leptin results in a decrease in food intake, fat mass, and body weight (Halaas et al., 1995). Leptin is a product of the *ob* gene, but prior to the discovery of the *ob* gene, the existence of leptin was first suggested by parabiosis studies in obese, hyperphagic *ob/ob* mice (Coleman, 1973). *Ob/ob* mice carry a mutation in the *ob* gene resulting in a truncated inactive version of leptin which results in an obese phenotype (Zhang et al., 1994). Further to this, Animal models with mutations in the leptin receptor (Ob-R), namely *db/db* mouse and the *fa/fa* rat, showed compromised leptin signalling, diabetes and obesity, which is consistent with the phenotype observed in the *ob/ob* mice which are deficient in the leptin protein (Chua, Jr. et al., 1996; Lee et al., 1996). The leptin receptor is widely expressed in the hypothalamus including the ARC, PVN and VMN (Mercer et al., 1996). Within the ARC the Ob-R is expressed on both NPY/AgRP and POMC/CART neurones (Hakansson et al., 1996) and c-fos activation has been demonstrated in these neurones following intraperitoneal and intravenous administration of leptin which suggests that leptin acts on both populations of neurones (Elmquist et al., 1997). Leptin action on ARC neuropeptides was confirmed in subsequent studies. Electrophysiology demonstrated that action potential frequency in the NPY/AgRP neurones decreased upon administration of leptin, confirming leptin inhibition of neuronal firing (Takahashi and Cone, 2005). Additionally, in the *ob/ob* mouse CART mRNA expression was found to be significantly decreased in the ARC and to a lesser extent in the DMN and LH, suggesting that leptin activates POMC/CART neurones (Kristensen, 1998). This was confirmed in studies which showed that chronic peripheral leptin administration over 10 days increased CART mRNA expression in the ARC and LH of *ob/ob* mice, a reduction in food intake was also observed in the leptin treated group (Kristensen, 1998). Many of the CART expressing neurones in the ARC project to the thoracic spinal cord and Elias et al demonstrated leptin activation of these neurones which provides a possible mechanism by which leptin stimulates the sympathetic nervous system (Elias et al., 1998). Collectively, the evidence detailing the action of leptin on hypothalamic neuropeptides confirms a functional role for leptin at the ARC. The importance of leptin in humans has been highlighted by a few cases of congenital leptin deficiency with the result being hyperphagia and obesity that can be corrected by treatment with recombinant leptin (Farooqi et al., 1999). However, in the majority of obese patients there is a high
circulating level of leptin suggesting the development of leptin resistance (Heymsfield et al., 1999).

1.6.2 Insulin

Insulin is produced in the Islets of Langerhans within the pancreas and was the first peptide to be identified as an adiposity signal (Porte, Jr. and Woods, 1981) since levels circulate relative to body fat. Insulin exerts its effects centrally in a similar way to leptin. Chronic ICV infusion of insulin into streptozotocin induced diabetic rats over six days, at levels that did not affect plasma glucose levels, decreased adiposity and body weight, which is suggestive of insulin acting as an anorectic peptide in the CNS (Sipols et al., 1995). The insulin receptor is widely expressed throughout the brain with particularly high levels of expression in the ARC NPY and POMC neurones (Baskin et al., 1987) suggesting that insulin acts centrally by modulating ARC neuropeptides. ICV administration of insulin has been found to reduce food intake and was also associated with the activation of the POMC/CART neurones and the inhibition of NPY neuronal firing within the ARC (Sipols et al., 1995). It is thought that the main action of insulin on the POMC/CART neurones is through the melanocortin system since ICV administration of insulin induces an increase in POMC mRNA expression but not in CART mRNA (Fekete, 2006). However, in states of fasting which are characterised by reduced leptin and insulin concentrations, rodents demonstrate a decrease in CART mRNA expression. Furthermore, chronic ICV administration of CART is accompanied by a decrease in plasma insulin concentration (Rohner-Jeanrenaud, 2002) which is suggestive of CART exerting an inhibitory effect on plasma insulin release. The physiological importance of central insulin signalling in energy homeostasis is highlighted by mice with brain specific deletion of the insulin receptor in which the mice develop an obese phenotype (Bruning et al., 2000). A second study using antisense oligonucleotides specific for the insulin receptor confirmed these observations. A reduction in insulin receptor expression resulted in an obese phenotype which is consistent with results from mice with targeted deletion of the insulin receptor. In addition, there was an increase in expression of NPY and AgRP and adiposity (Obici et al., 2002). The current evidence confirms a role for insulin acting on the CNS as a signal of long term energy store.
Figure 1.4  Integration of peripheral signals by the arcuate nucleus. Diagram showing two populations of arcuate nucleus neurones, AGRP/NPY and POMC/CART neurones. The AGRP/NPY neurones increase food intake and decrease energy expenditure. The POMC/CART neurones inhibit feeding and increase energy expenditure. These neurones are regulated by circulating peripheral factors. Leptin and insulin circulate at levels proportional to body fat and inhibit AGRP/NPY neurones and activate POMC/CART neurones. Ghrelin is released from the stomach and activates AGRP/NPY neurones leading to an increase in food intake. Ghrs, Growth hormone secretagogue receptor; Y1r/Y2r, neuropeptide Y receptor; Lepr, leptin receptor; Mc3r/Mc4r, melanocortin 3/4 receptor;
1.6.3 Integration of Insulin and Leptin Signalling

Leptin and insulin have been shown to mediate similar actions within the CNS, for example both insulin and leptin have been shown to reduce food intake when administered ICV (Fekete, 2006). Therefore, it has been hypothesised that due to leptin and insulin mediating similar actions they may act through a common signalling pathway. Activation of the insulin receptor results in its autophosphorylation. The phosphorylated sites on the insulin receptor act as a binding domain for the insulin receptor subunit (IRS) protein. Once the IRS protein is bound to the intracellular domain of the insulin receptor it is also phosphorylated. Following the activation of the IRS proteins downstream targets are activated, one of which is phosphatidylinositol-3-kinase (PI3K). Conversely, leptin acts through the Janus Kinase (JAK) signal transducers and activator of transcription (STAT) signalling pathway, specifically the JAK2 and STAT3 proteins. STAT proteins act as transcription factors and are thought to mediate the decrease in POMC and CART gene expression that accompany leptin administration (Banks et al., 2000; Vaisse et al., 1996). However, studies using hypothalamic explants demonstrate that leptin causes hyperpolarisation of hypothalamic neurones through activation of potassium ion channels in the neuronal membrane. The effect on ion permeability cannot be accounted for by STAT3 induced alterations in gene expression due to the short time frame between leptin administration and activation of ion channels, this suggests that leptin may act through additional signalling pathways to that of the JAK-STAT pathway (Spanswick et al., 1997). Confirmation came from studies involving a targeted substitution of specific tyrosine amino acids with the amino acid serine on the leptin receptor. The amino acid Tyr 1138 is essential for the recruitment of the STAT 3 protein; hence, by introducing a mutation at this site the STAT3 leptin signalling pathway is blocked. If the JAK-STAT pathway was the only pathway through which leptin signalled then mice with a mutation at Tyr1138 should display a similar phenotype to the \textit{db/db} mouse which has a targeted deletion of the leptin receptor. Instead mice with a mutation at Tyr1138 show normal NPY mRNA expression levels and improved glucose tolerance. In addition, other leptin receptor mediated signalling pathways such as the mitogen activated protein kinase (MAPK) signal cascade were still active in mice with a mutation at the Tyr1138 position, suggesting that leptin can signal through an alternate pathway independent of the STAT protein (Bates, 2003; Banks et al., 2000). The leptin receptor is also thought to activate the IRS-PI3K component of the insulin signalling pathway via JAK mediated phosphorylation which is independent of the STAT3 protein since immunoprecipitation has demonstrated that the JAK2 protein can phosphorylate and activate IRS and PI3K proteins (Carvalheira et al., 2005;
Kellerer et al., 1997; Bjorbaek et al., 1997). This was confirmed using rat hypothalamic slices in which leptin and insulin were shown to reduce the firing on the same potassium-ATP (K-ATP) channels in the ARC (Spanswick et al., 2000). The ability of either leptin or insulin to reduce the firing of neurones from hypothalamic slices was blocked by the administration of the PI3K inhibitor wortmannin (Spanswick et al., 1997; Spanswick et al., 2000). Studies on hypothalamic explants confirm that the action of both leptin and insulin to reduce neuronal firing is through PI3K since PI3K appears to be necessary for regulating the permeability of K-ATP ion channels in the neuronal membrane. Failure to properly activate PI3K may form the basis of leptin resistance.

Evidence of crossover in the signalling pathway of leptin and insulin allows for integration of signals of long term energy status and may also serve as a control point for the anorectic effects of these peptides.

1.6.4 Gastrointestinal Hormones

The orexigenic peptide ghrelin, and short term signals of satiety originating from the gastrointestinal tract (GIT) signal through the hypothalamus and brainstem.

Ghrelin is a 28 amino acid acylated peptide which is released from the oxyntic cells of the stomach and increases food intake through binding to the growth hormone secretagogue receptor (GHSR) (Kojima et al., 1999) which is expressed in the hypothalamus and brainstem (Guan et al., 1997; Date et al., 2002). ICV and peripheral administration of ghrelin results in increased food intake and body weight in rodents, confirming an orexigenic role for ghrelin (Tschop et al., 2000). It is thought that NPY mediates the observed increase in food intake associated with ghrelin since 90% of activated ARC neurones resulting from peripheral administration of ghrelin were found to express NPY (Wang et al., 2002). The orexigenic effect of ghrelin may be mediated by the vagal nerve projection to the brainstem (Jobst et al., 2004). The GHSR has been identified on the vagus nerve and peripheral administration of ghrelin increases neuronal activation in the NTS and AP. In addition, vagotomised rats do not respond to peripheral administration of ghrelin which highlights the importance of the vagus nerve in mediating the effect of peripheral ghrelin (Date et al., 2002). Physiological evidence of ghrelin’s orexigenic role is provided by transgenic models. Transgenic overexpression of
ghrelin leads to the development of hyperphagia and reduced glucose and leptin sensitivity which is consistent with ghrelin being an orexigenic peptide (Bewick et al., 2009).

Peptide tyrosine tyrosine (PYY) is a 36 amino acid peptide (PYY1-36) which is cleaved by the enzyme dipeptidyl peptidase IV into its more active form PYY3-36. PYY is released from the enteroendocrine L cells lining the distal gastrointestinal tract which is released post prandially. The largest increases in PYY are observed following ingestion of high fat meals (Adrian et al., 1985) which is consistent with PYY being an anorectic peptide. Peripheral administration of PYY3-36 in rodents, and intravenous administration of PYY3-36 in humans result in decreased food intake and body weight (Batterham et al., 2002; Batterham et al., 2003; Degen et al., 2005). The food reducing effect of PYY is thought to be mediated through the Y2R which is an auto-inhibitory receptor expressed on the ARC NPY neurones. This hypothesis is supported by a lack of response to PYY3-36 administration in the Y2R knockout mouse (Batterham et al., 2002). Collectively the evidence demonstrates that PYY plays a critical role in meal termination by acting through the Y2R at the ARC to decrease food intake.

Pancreatic polypeptide (PP) is a 36 amino acid peptide which is synthesised in the PP cell of the pancreas and released postprandially. In the gut cholecystokinin (CCK) is primarily produced in the I cells and, like PP, is also released post prandially. Peripheral administration of PP and CCK have both been shown to decrease food intake in rodents implicating both peptides with an anorexigenic function (Asakawa et al., 2003; Gallmann et al., 2006). The actions of both PP and CCK are thought to be mediated through actions at the brainstem by inhibiting gastric emptying which is consistent with the brainstem being a primary site for integration of gastrointestinal signals (Schmidt et al., 2005; Wu et al., 2002). CCK receptors have been identified in the vagal afferents, a main site of projection to the gastrointestinal tract (Moran et al., 1986; Moran and Kinzig, 2004) and PP binds with high affinity to the Y4 receptor which is found in the brainstem (Whitcomb et al., 1990). In further support of integration of PP signal at the brainstem, vagotomised rats result in elimination of meal related secretion of PP (Rogers et al., 1996).

Glucagon like peptide 1 (GLP-1) is released from the L cell of the gastrointestinal tract and exists in two bioactive forms, GLP-1 (7-37) and GLP-1 (7-36). GLP-1 acts as an incretin, stimulating insulin release in a glucose dependent manner, in addition to inhibiting the
secretion of glucagon (MacDonald et al., 2002; Willms et al., 1996)}. Intravenous infusion of
GLP-1 to normal and obese subjects reduced food intake in a dose dependent fashion
(Verdich et al., 2001) and ICV administration in rodents of the potent GLP-1 antagonist
exendin 9-39 increased food intake (Turton et al., 1996). Collectively suggesting a
physiological role for GLP-1 in energy homeostasis.

The data collectively suggests that peptides from the gastrointestinal tract can relay
information on the current status of fuel availability and these signals are integrated at the
ARC and brainstem.

1.7 Summary
As has been discussed the ARC is a major site within the hypothalamus for integrating
peripheral signals of energy stores and satiety. The transduction of peripheral signals into
neuronal responses is mediated through the orexigenic neuropeptides NPY and AgRP as well
as the anorectic peptide POMC. Additionally, evidence suggests that CART plays an
important role in appetite regulation. However, the exact physiological role of CART is
difficult to define since in different experimental paradigms CART has been shown to
increase and decrease food intake. Given that antibody blockade of CART peptide results in
an increase in food intake, and the importance of mutations in the CART gene in human
familial obesity, it is important to understand the true physiological role of CART. In order to
elucidate the physiological role of CART more complex genetic paradigms need to be used to
pick apart the role of CART in particular hypothalamic nuclei.

1.8 Aim of Thesis
I aim to investigate the role of ARC CART by characterising the phenotype that results from
a physiological reduction in CART peptide concentration specifically in this nucleus. To
achieve a reduction in CART peptide concentration I will use CART antisense expression
which will be targeted to the ARC by rAAV and BAC transgenesis.
Chapter 2

General Materials and Methods
2 Materials and Methods

2.1 Materials
Unless otherwise stated all chemicals were purchased from VWR, Poole, Dorset, UK and all enzymes from New England Biolabs, Hitchin, Herts, UK.

2.2 Methods

2.2.1 Total RNA extraction – Tri-reagent method

Materials
Tri-Reagent (Helena Biosciences, Tyne and Wear, UK)
1-Bromo-Chloro-Propane (Sigma-Aldrich, Poole, Dorset, UK)
Isopropanol
Absolute Ethanol
Sterile distilled water (GDW)

Method
Total RNA was extracted using Tri-Reagent according to the manufacturer’s protocol. Tissue (up to 100mg) was homogenised in 1ml Tri-Reagent, transferred to sterile 1.5ml eppendorf tubes and incubated for five minutes at room temperature. When extracting RNA from cells, cell media was removed and 5ml tri reagent added per 75cm$^3$ flask (Nunc, Thermo Fisher Scientific, Denmark). The flask was shaken gently to detach the cells from the flask wall. Cells were then removed and divided into 1.5ml eppendorfs and incubated for five minutes at room temperature.

One hundred microlitres of bromo-chloro-propane was added per millilitre of tri reagent and mixed vigorously. The mixture was incubated at room temperature for ten minutes, then centrifuged at 12000g for fifteen minutes at 4°C (centrifuge 5417 C/R, Eppendorf, Hamburg, Germany). The upper aqueous phase was transferred to a clean eppendorf tube, precipitated with 0.5ml isopropanol for ten minutes at room temperature then centrifuged at 12000g for ten minutes at 4°C (centrifuge 5417 C/R, Eppendorf, Hamburg, Germany). The supernatant was removed and the pellet washed with 75% (v/v) ethanol in GDW, air dried and
resuspended in 400µl of GDW. RNA concentration was determined by spectrophotometry. The RNA was diluted 1:100 in GDW and 1ml placed into a quartz cuvette (VWR). The absorbance was read at 260 and 280nm, and the concentration calculated using the following formula:
concentration (µg/ml) = (A260 x dilution factor) x 40.

2.2.2 RNA Denaturing Gel

Materials
Agarose, type II-A (Sigma-Aldrich, Poole, Dorset, UK)
40% (v/v) Formaldehyde (VWR)
20x MOPS pH 7.0 (appendix I)
   0.4M 3-(N-Morpholin)propanesulphonic acid (MOPS)
   0.1M sodium acetate
   0.02M EDTA
   0.04% (v/v) formaldehyde
10mg/ml Ethidium bromide (VWR)
DENAT (appendix I)
   70% (v/v) formamide (appendix I)
   3.5% (v/v) formaldehyde
   1.5x MOPS
Gel loading buffer (appendix I)
   25% (v/v) glycerol
   0.1% (w/v) orange G
   25mM EDTA
100x TE pH 7.5 (appendix I)
   1M Tris Base
   0.1M EDTA
2M Sodium acetate pH 5.2 (appendix I)
Sterile distilled water (GDW)
Method
A 1% (w/v) agarose denaturing gel was prepared in 1x MOPS buffer with 7.5% (v/v) formaldehyde. Following quantification by spectrophotometry, the RNA samples were ethanol precipitated with 0.1 volumes sodium acetate pH 5.2 and 2.5 volumes of ice cold absolute ethanol. The samples were precipitated at -20°C for a minimum of one hour then centrifuged at 12000g for ten minutes at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany). The supernatant was removed and the pellet air dried under vacuum for five minutes. RNA pellets were resuspended in the appropriate volume of GDW to a final concentration of 5mg/ml. One micro litre of this solution was added to 12µl of DENAT. The samples were denatured at 65°C for five minutes and 3µl gel loading buffer added. The samples were loaded onto the gel and run in 1x MOPS buffer with 7.5% (v/v) formaldehyde at 150V for approximately forty minutes until the samples had ran 10cm. The gel was then stained in 1x TE with 0.01% (v/v) ethidium bromide on a shaking platform for thirty minutes, then de-stained overnight in 1x TE to allow visualisation of the 18S and 28S ribosomal bands under UV light. The presence of these bands indicated that the RNA had not degraded.

2.2.3 Northern Blot Hybridisation
Northern blotting is a technique to identify and quantity RNA in tissues. The method is adapted from Southern Blotting, a technique developed in the 1970s to quantify DNA (Southern, 1975). A denaturing formaldehyde gel is used to size fractionate RNA fragments. The RNA is then transferred to a nylon membrane by capillary action and the membrane baked to increase the membrane to RNA binding strength

2.2.3.1 Electrophoresis and Immobilisation of Nucleic Acids (Lehrach et al., 1977)

Materials
20x SSC (appendix I)

3M sodium chloride (appendix I)
0.3M tri-sodium citrate pH 7.0

Hybond-N (Amersham Biosciences)
Method
A denaturing formaldehyde gel was prepared and 50µg of total RNA was denatured and loaded onto the gel as described in section 2.2.2. Following electrophoresis, the RNA was transferred from the gel to Hybond-N membrane by capillary action overnight. The gel was inverted and placed into a stack constructed as illustrated in Figure 2.1. After transfer, the filter was baked at 80°C for 1-2 hours to covalently link the RNA to the filter.

![Diagram of Northern blotting](image)

**Figure 2.1 Northern blotting.** Apparatus used for the transfer of RNA by capillary action from a gel to Hybond-N membrane prior to northern blot analysis.

### 2.2.3.2 Hybridisation of $^{32}P$ Labelled Probe to the Filter
Probes for northern blot analysis were prepared from pBluescript containing the appropriate insert, prepared as described in section 2.2.7. To prepare fragments for labelling, pBluescript was cut with BamHI and EcoRI and the fragment excised from the gel and electroleuted (section 2.2.7.1 and 2.2.7.2). Fragments were labelled using random primer labelling. Pre-hybridisation is carried out before the membrane is bound to radiolabelled probe; this blocks...
irreversible non-specific binding of the probe to the membrane. Following hybridisation, a number of increasingly stringent washes are performed to remove non-specifically bound probe.

### 2.2.3.3 Random Primer Labelling of DNA Fragments (Feinberg and Vogelstein, 1983)

**Materials**

DNA probe fragment

5x ABC Buffer: (appendix I)
- 12.5mM magnesium chloride
- 125mM Tris-HCl, pH 8.0
- 23µM 2-mercaptoethanol
- 50µM dATP (Pharmacia)
- 50µM dGTP (Pharmacia)
- 50µM dTTP (Pharmacia)
- 1M N-2-hydroxyethylpiperazine-N’-2-ethanesulphonic acid (HEPES), pH 6.6 (Sigma-Aldrich, Poole, Dorset, UK)
- 34µg/ml random deoxynucleotide hexamers (Amersham Bioscience)
- 10mg/ml BSA, fraction V (Sigma-Aldrich, Poole, Dorset, UK)
- [α-³²P]-dCTP: 10Ci/ml, 3000Ci/mmol (Amersham Bioscience)

DNA polymerase I, Klenow fragment 9U/µl (Amersham Bioscience)

Sephadex G50 (appendix I)

1x TE (appendix I)

**Method**

Twenty nanograms of DNA in a total volume of 15µl of GDW was boiled for five minutes. After boiling, the solution was made up to a total volume of 25µl containing 1XABC buffer, 2mg/ml BSA, 10µCi dCTP and 1U Klenow. The reaction was incubated at 37°C for at least one hour. Incorporation of radiolabelled nucleotide into the DNA probe was measured using a mini Sephadex G50 column. The column was prepared by plugging a glass Pasteur pipette with glass wool, and adding Sephadex G50. The labelling reaction volume was made up to 200µl with 1x TE and loaded onto the column. The column was then eluted with 1x TE and
200µl fractions collected. The fractions were counted and the percentage incorporation calculated. The two fractions with the greatest radiolabel incorporation were pooled.

2.2.3.4 Hybridisation of Northern Filters

Materials

32P labelled probe (section 2.2.3.2)

Hybridisation buffer (appendix I)

- 0.25M sodium phosphate buffer
- 5% (w/v) SDS
- 0.5% (w/v) dried milk
- 2.5mM EDTA
- 25µM ATA (appendix I)

Amasino wash buffer (appendix I)

- 0.25M sodium phosphate pH 7.2
- 1mM EDTA
- 2% (w/v) SDS

Universal wash buffer (appendix I)

- 0.2x SSPE
- 0.2% (w/v) SDS

Method

The baked filter was placed in a heat sealed polythene bag and pre-hybridised in 20ml hybridisation buffer at 60°C for a minimum of two hours. Half the labelled probe (section 2.2.3.2) was boiled for 5 minutes and added to 20ml fresh hybridisation buffer. The pre-hybridisation solution was removed, the hybridisation mix added and the filter incubated overnight at 60°C. Non-specifically bound probe was removed from the filter by washing in decreasing concentrations of sodium chloride. Three twenty minute washes at 60°C were carried out in Amasino wash buffer followed by a further three twenty minute washes at 60°C in Universal wash buffer. The filter was then sealed in a clean plastic bag and exposed to a storage phosphor screen (Molecular Dynamics, Amersham, UK). Radiolabelled RNA was visualised using a Storm imaging system and ImageQuant software (Molecular Dynamics, Amersham, UK).
2.2.3.5 Filter Stripping
Following hybridisation with a radiolabelled probe, northern filters can be stripped of radioactivity and re-used with minimal loss of RNA. Most northern filters can be stripped and re-probed five or six times depending on the abundance of the transcript of interest.

Materials
100 x TE (appendix I)
20% (w/v) SDS (appendix I)

Method
The filter was stripped by incubation in 200 ml of 1xTE/0.5%SDS for 30 minutes in a water bath at 80°C. The filter was then exposed overnight to a phosphoimager plate to ensure that all radioactive probe had been removed.

2.2.3.6 Oligo dT Labelling
A radiolabelled oligo dT probe was prepared which hybridises to the polyA tail of mRNA. This allows mRNA to be quantified.

Materials
20ng oligo dT template
20U/µl Terminal deoxytransferase (Promega)
5x reaction buffer (as supplied)
GDW
\([\alpha^{32}P]dATP\) 10Ci.ml, 3000Ci/mmol (GE healthcare)
Sephadex G50 (appendix I)
1x TE
Hybridisation buffer (appendix I)
20x SSPE (appendix I)
20% (w/v) SDS (appendix I)
Milk power
Nonidet
Method
Twenty nanograms of oligo dT template were added to 1x reaction buffer with 20U of terminal transferase in a final volume of 20µl with GDW. The reaction was incubated at 37°C for one hour. A mini sephadex G50 column was used to measure incorporation of the labelled nucleotide, as described in section 2.2.3.3.

The baked filter was prehybridised in hybridisation buffer (5xSSPE, 0.2% milk powder, 0.2% nonidet) for at least 2 hours at room temperature on a shaking platform. Hybridisation solution was prepared by adding one tenth of the labelled probe to 10ml hybridisation buffer. Hybridisation buffer was removed and replaced with hybridisation solution containing labelled probe. This was incubated overnight at room temperature. A series of washes were carried out to remove non-specifically bound probe. Filter was washed twice in 5x SSPE with 0.2% SDS for 5 minutes, then twice in 2x SSPE with 0.2% SDS for 30 minutes at room temperature. Filter was then exposed to a phosphorimager screen and bands were quantified using ImageQuant software (Molecular Dynamics, Amersham, UK).

2.2.4 Reverse Transcription

Materials
5mg/ml RNA
10mM dNTPs (Amersham Biosciences) (appendix I)
5x Reverse transcriptase buffer (Promega)
   250mM Tris-HCl, pH 8.3
   2560mM KCl
   50mM Magnesium Chloride
   2.5mM Spermidine
   50mM Dithiothreitol (DTT)
Avian myoblastoma virus reverse transcriptase (RT) 10U/µl (Promega)
200ng/µl Oligo dT (12-18) (Amersham Biosciences)

Method
The reaction was set up in a final volume of 20µl containing the following: 1mg/ml RNA, 1x RT buffer, 1mM dNTPs and 10mg/ml oligo dT. The solution was heated to 65°C for five
minutes and allowed to cool to room temperature for thirty minutes. Ten units of RT were added and the reaction incubated at 42°C for one hour. The reaction was then used in a polymerase chain reaction (PCR).

2.2.5 Polymerase Chain Reaction

Materials
10x Taq Buffer (Sigma-Aldrich, Poole, Dorset, UK)
   - 500mM Potassium Chloride
   - 100mM Tris-HCl, pH 9.0
   - 15mM Magnesium chloride
   - 1% (v/v) Triton x100
20µM oligonucleotide primers (Oswel DNA services)
Taq DNA polymerase (Sigma-Aldrich, Poole, Dorset, UK)

Method
cDNA fragments were amplified by PCR using primers corresponding to nucleotides specific to the target of interest. Ten microlitres of the reverse transcription reaction was added to a tube containing 1x Taq buffer, 0.2mM dNTPs, and 200nM oligonucleotide primers. The reaction was heated to 95°C for five minutes after which time 5U of Taq DNA polymerase were added. The reaction was cycled thirty times through the following temperatures: 95°C for forty seconds, 55°C for forty seconds, 72 °C for one minute. After completion of the reaction, 10µl of the PCR product was analysed by gel electrophoresis on a 1% (w/v) TAE/agarose gel.

2.2.6 Agarose Gel Electrophoresis

Materials
Agarose, type II-A medium (Sigma-Aldrich, Poole, Dorset, UK)
50x TAE (appendix I)
   - 2M Tris-Acetate pH 8.5
   - 0.05M EDTA pH 8.0
10mg/ml Ethidium bromide  
DNA marker (BRL 1Kb ladder, Invitrogen Life Technologies)  
Gel loading buffer (appendix I)  

Method  
A 1% (w/v) agarose gel was prepared by dissolving the agarose in 1x TAE using a microwave oven. The gel was cooled to 45°C and ethidium bromide added to a final concentration of 0.5µg/ml. Once set, the gel was placed into an electrophoresis tank containing 0.5x TAE with 0.5µg/ml ethidium bromide. Three microlitres of loading buffer were added to 10µl of PCR product, 1µl of DNA marker was added to 9µl GDW and treated in the same way. Samples were loaded onto the gel and electrophoresed at 10V/cm. The DNA was visualised by illumination with UV light (300nm).

2.2.7 Cloning of DNA (Sambrook, 2000)  
Among the most important advances in the field of molecular biology was the ability to cut DNA at specific sites and to ligate the cut ends. DNA is cut using restriction endonucleases, isolated from bacteria, which recognise specific target sites within double stranded DNA and cleave it. Equally important is the ability to ligate these fragments. This relies on the use of a virus-derived enzyme that catalyse the formation of phosphodiester bonds between a free 5’ phosphate and a free 3’ hydroxy group.

2.2.7.1 Restriction Endonuclease Digestion of DNA  

Materials  
Restriction endonuclease: various enzymes were used and will be covered in the relevant sections. All restriction enzymes were purchased from New England Biosciences with the exception of restriction enzyme Bsp119I which was purchased from Fermentas.

10x restriction buffer (as supplied)  
Phenol /chloroform/Iso-amyl alcohol (IAA) (appendix I)  
2M sodium acetate, pH 5.2 (appendix I)  
Absolute Ethanol
4U/μl Shrimp alkaline phosphatase (SAP) (Amersham Biosciences)

Method
DNA was dissolved in GDW and restriction buffer added to give a final concentration of 1x. Restriction endonuclease was added to five times excess; the volume of enzyme added was kept below 10% of the final volume. The reaction was incubated for at least one hour at 37°C. If the digested DNA was a plasmid, 8U of SAP were added which removes 5’ phosphates and prevents self-ligation of the plasmid. The reaction was extracted with an equal volume of phenol/chloroform/IAA and the phases separated by centrifugation for three minutes at 12,300g at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany). The DNA was precipitated by addition of 0.1 volumes of sodium acetate pH5.2, and 2.5 volumes of cold absolute ethanol and incubated at -20°C for at least one hour. DNA was recovered by centrifugation for seven minutes at 12,300g at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany).

2.2.7.2 Electroelution of DNA fragments
After restriction digestion, purification of the DNA fragment of interest from other contaminating fragments is often advantageous. This is particularly true for plasmids, since restriction digests are not 100% efficient and a small amount of closed circular DNA can produce a high background during transformations. The DNA was therefore size fractionated by electrophoresis on an agarose gel and the DNA of interest electroeluted.

Materials
50x TAE (appendix I)
Dialysis tubing (appendix I)
Gel loading buffer (appendix I)
DNA marker

Method
A 1% (w/v) agarose gel was prepared as described in 2.2.6. The DNA was dissolved in GDW and 0.25 volumes of loading buffer added. 1μl of DNA marker was added to 9μl of GDW and treated in the same way. The samples were loaded onto the gel and electrophoresed at 10 V/cm. The DNA was visualised by illumination with UV light
The band of interest was excised from the gel and placed into a piece of dialysis tubing sealed at one end with a clip. Four hundred microlitres of 0.5x TAE was added to the gel slice, air was excluded from the tubing and the end sealed with a clip. The DNA was eluted from the gel slice by electrophoresis at 20V/cm for 20 minutes. The TAE was removed from the bag, phenol/chloroform extracted and the DNA recovered by ethanol precipitation (section 2.2.7.1). The DNA was dissolved in GDW and quantified, either by spectrophotometry or using an agarose gel. To quantify DNA spectrophotometrically it was diluted 1:100 in GDW and 1ml placed into a quartz cuvette (VWR). The absorbance was read at 260 and 280nm (UV-160 spectrophotometer, Shimadzu, Kyoto, Japan). The reading at 280nm gives an indication of the purity of the DNA sample as phenol absorbs more strongly at 280nm than DNA. The concentration of DNA was calculated using the following formula:

$$\text{concentration (μg/ml)} = (A_{260} \times \text{dilution factor}) \times 50.$$  

For small quantities of DNA, using an agarose gel is more convenient, although not as accurate. This involves electrophoresing an aliquot of the DNA alongside standards of known concentration and estimating the concentration of the sample from its relative luminescence under UV illumination (300nm).

### 2.2.7.3 Ligation of DNA fragments

**Materials**

- T4 DNA ligase (6 units/µl)
- 10x ligase buffer:
  - 500mM Tris-HCl
  - 100mM magnesium chloride
  - 100mM dithiothreitol (DTT)
  - 10mM ATP
  - 250µg/ml acetylated bovine serum albumin (acetylated BSA)
Method
DNA ligation was used to insert the DNA fragments of interest (inserts) into plasmids. All the fragments ligated had overhanging ends, which increases the efficiency of ligation as the ends are held juxtaposed by hydrogen bonding between the complementary bases. The method was identical for all combinations of plasmids and inserts. Twenty nanograms of plasmid were dissolved in GDW and a fourfold molar excess of the insert added. Reaction buffer was added to a final concentration of 1x and 1µl of T4 DNA ligase added to give a final volume of 10 µl. The reaction was incubated at 16 ºC overnight. The ligation reaction was then used to transform bacteria.

2.2.8 Production of Competent Bacteria (Hanahan, 1983)
Gram negative bacteria such as E. coli have a cell wall that prevents entry of exogenous DNA. To allow entry of plasmids into the bacteria, they have to be rendered competent to take up DNA. There are several ways of achieving this, including electroporation and treatment with cations. The method of choice for Xl1-Blue cells is treatment with cations, which is reliable and relatively easy. The method of choice for JC8111 bacteria is electroporation.

2.2.8.1 Production of Competent Bacteria using Cations

Materials
XL1-Blue cells (Stratagene Ltd, La Jolla, CA, U.S.A.)
LB (appendix I)
10mg/ml Tetracycline (Sigma-Aldrich, Poole, Dorset, UK) in absolute ethanol (appendix I)
TFB I: (appendix I)
  30mM potassium acetate
  100mM rubidium chloride
  10mM calcium chloride (appendix I)
  50 mM manganese chloride
  15% (v/v) glycerol
TFB II: (appendix I)
  10mM MOPS (Sigma-Aldrich, Poole, Dorset, UK)
75mM calcium chloride (appendix I)
10mM rubidium chloride
15% (v/v) glycerol

Method
One hundred millilitres of LB supplemented with 5µg/ml of tetracycline (LB<sub>tet</sub>) was inoculated with a colony of XL1-Blue and incubated overnight, with vigorous shaking, at 37°C. One millilitre of this overnight culture was inoculated into 100ml of fresh LB<sub>tet</sub> (prewarmed to 37°C) and incubated at 37°C, with vigorous shaking, until the bacteria were in log phase growth (OD<sub>580</sub> = 0.4-0.5). The bacteria were recovered by centrifugation at 800g for fifteen minutes at 4°C (HB-4 rotor in RC-5B super speed centrifuge, Du Pont). The bacteria were resuspended in 40ml of ice cold TFB I and incubated on ice for ten minutes. The bacteria were recovered as above and resuspended in 4ml of TFB II, then incubated on ice for a further fifteen minutes. They were then aliquoted into 50µl volumes and stored at -70°C.

2.2.8.2 Production of Competent Bacteria using Electroporation

Materials
*E.coli* JC8111 (LGC Standards, Middlesex, UK) c
LB (appendix I)
H<sub>2</sub>O (ice cold)
10% (v/v) glycerol in GDW (ice cold)
SOC medium (appendix I)

Method.
Five millilitres of LB was inoculated with a single colony of the JC8111 strain of *E.coli* and grown overnight at 37°C with moderate shaking. From this overnight culture, 2.5ml was used to inoculate 500ml of fresh LB (prewarmed to 37°C) and incubated at 37°C with vigorous shaking until the bacteria were in the log phase of growth (OD<sub>600</sub> = 0.5-0.6). The cells were then chilled in an ice water bath for fifteen minutes before being centrifuged at 2000g for twenty minutes at 4°C (HB-4 rotor in RC-5B super speed centrifuge, Du Pont). The supernatant was removed and the pellet resuspended in 5ml ice-cold water. Following
resuspension of the cells a further 5ml of ice-cold water was added and mixed well before a further centrifugation cycle at 2000g for twenty minutes at 4\(^\circ\)C (HB-4 rotor in RC-5B super speed centrifuge, Du Pont). This step was repeated once more and the resulting cell pellet resuspended in 40mls of ice-cold 10% (v/v) glycerol and mixed well. The cells were then centrifuged at 2000g for 10 minutes at 4\(^\circ\)C (HB-4 rotor in RC-5B super speed centrifuge, Du Pont). The volume of the resulting pellet was estimated and an equal volume of ice-cold 10% (v/v) glycerol was added and the pellet resuspended. The cells were then aliquoted into 50µl volumes and stored at -80\(^\circ\)C until ready for use in transformation by electroporation (see section 4.4.2).

### 2.2.8.3 Transformation of Competent XL1-Blue Bacteria

Although treating bacteria with cations renders them competent to take up DNA, to obtain efficient transformation requires several additional steps. The initial step, incubation on ice, is believed to allow attachment of the plasmid to the bacterial cell membrane. The heat shock step is believed to allow efficient transport of the plasmid into the bacteria. The incubation following the heat shock allows expression of the resistance genes before exposure to the antibiotic.

**Materials**
- LB (appendix I)
- LB(antibiotic) plates (section 2.2.9)
- Plasmid DNA
- Competent bacteria (section 2.2.8.1)

**Method**

An aliquot of frozen competent bacteria was thawed on ice, 10ng of plasmid (5µl ligation reaction) added and the mixture incubated on ice for twenty minutes. The reaction was then incubated at 42\(^\circ\)C for forty seconds followed by incubation on ice for two minutes. Two hundred microlitres of LB were added and the reaction incubated at 37\(^\circ\)C for thirty minutes. Simultaneously, agar plates supplemented with the appropriate antibiotic were dried at 37\(^\circ\)C for 10 minutes. The volume of transformed bacteria added to the plate varied depending on the source of the DNA (up to 200µl per 90mm Petri dish). The bacteria were spread on the surface of the agar, the plate inverted and incubated at 37\(^\circ\)C overnight.
2.2.9 Production of Agar Plates

After transformation of bacteria with plasmid DNA, the bacterial culture was grown on an agar plate containing an antibiotic. The antibiotic allows selection of bacteria which carry the plasmid of interest.

Materials
LB (appendix I)
Bacto-Agar (Difco Laboratories, MI, USA)
Ampicillin 100mg/ml in GDW (appendix I)
Chloramphenicol 25mg/ml in ethanol (appendix I)
Spectinomycin 50mg/ml in GDW (appendix I)
Petri dishes 90mm (Plastiques Gosslin, Hazebrouck, France)

Method
7.5g of Bacto-Agar was added to 500ml of LB and autoclaved at 120°C for twenty minutes to melt and sterilise the agar. The agar was then cooled to below 50°C before addition of 250µl of the required antibiotic. The agar was then poured into Petri dishes to just cover the bottom surface. The agar was then flame before the lids were placed on the Petri dishes. Once the agar had set the plates were wrapped in parafilm and stored upside down at 4°C.

2.2.10 Small Scale Preparation of Plasmids (Sambrook, 2000)

Initially, plasmids were normally isolated on a small scale culture to allow several clones to be analysed simultaneously. To isolate plasmids from bacteria it is necessary to disrupt the cell wall to release the plasmid, and at the same time to remove contaminating proteins, genomic DNA and RNA. The method used involved disruption of the cell wall by treatment with alkali and SDS followed by precipitation of bacterial debris with sodium acetate. This step also removes most of the genomic DNA, since in bacteria this is anchored to the cell wall. The RNA is removed at a later stage by treatment with RNase A.

Materials
LB (appendix I)
0.2 M sodium hydroxide /1% (w/v) SDS (appendix I)
2M sodium acetate, pH 5.2 (appendix I)
Method
Two millilitres of LB\textsubscript{(antibiotic)} were inoculated with a single bacterial colony and incubated overnight at 37\textdegree C with vigorous shaking. Five hundred microlitres of this culture was added to an equal volume of 80\% (w/v) glycerol and stored at -70\textdegree C. The rest of the culture was centrifuged for three minutes at 12,300g at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany) to pellet the bacteria. The supernatant was discarded and the pellet resuspended in 100\mu l of GTE. Two hundred microlitres of alkaline SDS was added and the sample incubated on ice for five minutes, followed by the addition of 150\mu l of 5M potassium acetate and a further five minute incubation on ice. The precipitated bacterial debris was removed by centrifugation for five minutes at 12,300g at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany). 350\mu l of supernatant was then transferred to a clean tube. An equal volume of phenol/chloroform/IAA was added and the sample mixed. The phases were separated by centrifugation for five minutes at 12,300g at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany) and the supernatant transferred to a new clean tube. The DNA was precipitated by the addition of 0.6 volumes of propan-2-ol and incubation at room temperature for ten minutes. The DNA was recovered by centrifugation for seven minutes at 12,300g at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany) and then dissolved in 100\mu l of GDW. 0.2 M sodium acetate and 250\mu l of absolute ethanol (-20\textdegree C) were added and the solution incubated at -20\textdegree C for at least one hour. After precipitation the DNA was recovered by centrifugation at 12,300g at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany) and then dissolved in 10-20\mu l GDW.
2.2.11 Large Scale Plasmid Purification

Materials
LB (appendix I)
50,000 U/mg Lysozyme (Sigma-Aldrich, Poole, Dorset, UK)
GTE (appendix I)
10mg/ml DNase free RNAse A in GDW (Sigma-Aldrich, Poole, Dorset, UK)
0.2 M sodium hydroxide /1% (w/v) SDS (appendix I)
2M sodium acetate, pH 5.2 (appendix I)
5M potassium acetate, (appendix I)
Phenol/chloroform/IAA (appendix I)

Method
Following small scale preparation of the plasmid the size of the insert was confirmed by restriction digest (section 2.2.7.1) and agarose gel electrophoresis (section 2.2.6). A large scale preparation of the plasmid was carried out prior to purification using a caesium chloride gradient. A small quantity of bacteria containing the plasmid with the correct size insert was inoculated into 500ml LB_{(ampicillin)} and incubated at 37°C overnight with vigorous shaking. The bacteria were recovered by centrifugation for eight minutes at 3000g (4000rpm in HS-4 rotor in RC-5B super speed centrifuge, Du Pont) at 4°C. The pellet was resuspended in 25ml GTE supplemented with 2mg/ml lysozyme and incubated at room temperature for five minutes. Fifty millilitres of 0.2 M sodium hydroxide /1% (w/v) SDS solution was added, the sample mixed and incubated on ice for five minutes. Then, 38ml of 5M potassium acetate was added, the sample mixed and incubated on ice for a further ten minutes. The bacterial debris was removed by centrifugation for 15 minutes at 9000g (7000rpm in HS-4 rotor in RC-5B super speed centrifuge, Du Pont) at 4°C. The supernatant was transferred to a clean tube and 0.6 volumes propan-2-ol added. The sample was incubated on ice for 15 minutes, and the DNA recovered by centrifugation for 15 minutes at 9000g at 4°C (7000rpm in HS-4 rotor in RC-5B super speed centrifuge, Du Pont). The pellet was dissolved in 10ml 1x TE. RNAse A was added at a concentration of 0.1mg/ml and the reaction incubated at 37°C for 30 minutes. The reaction was extracted with an equal volume of phenol/chloroform and the phases separated by centrifugation for 20 minutes at 10000g at 4°C. The DNA was recovered by addition of 0.1 volumes 2M sodium acetate pH 5.2, one volume propan-2-ol, and incubation at -20°C for at least one hour.
2.2.11.1 Caesium Chloride Gradient Purification

Large scale plasmid purification was carried out using a caesium chloride gradient (Sambrook, 2000). This method depends on the decrease in density of nucleic acids when they bind ethidium bromide. Because ethidium bromide binds by intercalation into the DNA, it causes unwinding of the helix. In closed circular DNA the increased super coiling of the plasmid limits binding of ethidium bromide, and the plasmid has a higher buoyant density than linear (genomic) or nicked plasmids. This difference allows separation of the plasmid on a caesium chloride density gradient.

Materials
TES (appendix I)
Caesium chloride
10mg/ml ethidium bromide
Propan-2-ol, caesium chloride saturated (appendix I)
1x TE (appendix I)

Method
The DNA obtained from section 2.2.11 was recovered by centrifugation for 20 minutes at 24,000g (12,000 rpm in a HB4 rotor of a RC5B super speed centrifuge, Du Pont) at 4°C, and then dissolved in 8.25ml of TES containing 8.4 grams of caesium chloride. 150µl of ethidium bromide was added and the solution mixed. The sample was loaded into a polyalumina tube (Ultracrimp, Du Pont), overlaid with paraffin oil, the tube sealed and centrifuged for 16 hours at 20°C and 216,518g (60,000rpm in a T-8100 rotor in a Sorvall SE100 centrifuge). After centrifugation, the DNA bands were visualised by UV illumination and the band containing the closed circular DNA removed using an 18-gauge needle and a 2ml syringe. The ethidium bromide was removed from the plasmid by repeated extraction with an equal volume of caesium chloride saturated propan-2-ol, until both phases were colourless. The DNA was precipitated by addition of two volumes of GDW and six volumes of room temperature absolute ethanol. The DNA was recovered by centrifugation for 15 minutes at room temperature and 24,000g (12,000 rpm in a HB4 rotor of a RC5B super speed centrifuge, Du Pont), the supernatant removed and the pellet dissolved in 0.4 ml GDW. The DNA was ethanol precipitated, recovered by centrifugation for 10 minutes at 12,300g (12,000 rpm in a HB4 rotor of a RC5B super speed centrifuge, Du Pont), dissolved in 1ml GDW and quantified by spectrophotometry (see section 2.2.7.2)
2.2.12 Radioimmunoassay

Radioimmunoassay (RIA) was used to measure the amount of CART (55-102) and α-MSH immunoreactivity. The technique relies upon competition between a radiolabelled antigen and an unlabelled antigen for antibody binding sites. A fixed amount of antibody is added to samples along with a fixed amount of $^{125}$I labelled antigen. The ratio of bound to unbound radiolabelled antigen gives a measure of how much unlabelled antigen is present in the sample. The unbound antigens are separated from the antigen-antibody complex and the amount of label in both is measured using a gamma counter. Separation of bound and unbound antigen can be carried out using the charcoal adsorption method. The unbound antigen is small enough to become trapped in the charcoal whereas the antibody-bound antigen is larger and remains in solution. Separation of bound and unbound antigen can also be achieved using a secondary antibody. A standard curve is constructed using known amounts of unlabelled peptide.

2.2.12.1 Assay Protocol

$^{125}$Iodine labelled peptides were prepared by Professor M. Ghatel and purified by high pressure liquid chromatography (HPLC). The details of the antisera used for each assay are described below. Assays were performed in a total volume of 350µl in 0.06M phosphate buffer (0.05M Na$_2$HPO$_4$.2H$_2$O, 0.006M KH$_2$PO$_4$, 0.01M disodium-EDTA.2H$_2$O, 0.008M NaN$_3$), at pH 7.4 with 0.3% BSA, unless otherwise stated.

All samples were assayed in duplicate. To prevent inter assay variation all test samples for a particular peptide were assayed within the same radioimmunoassay. The general structure of the RIA is outlined in Table 2.1.

Non specific binding tubes enable the assessment of label integrity. The half and two times tubes indicate whether greater sensitivity could be achieved by using half or twice the volume of label. Zero tubes are placed at regular intervals throughout the assay and enable assessment of assay drift. Standard curves were prepared using synthetic peptide in assay buffer, and added in duplicate at concentrations of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100 fmol/tube. A second standard curve towards the end of the assay included 20µl of 0.5M acetic acid per standard. The peptide concentration of tissue extract samples was determined from this standard curve. Tissue extracts were assayed neat, diluted 1 in 10, and 1 in 100, at a
volume of 20µl. Radiolabel was diluted to 180-200 counts/ 10 seconds/ 50µl in assay buffer. The assays were incubated for 72 hours at 4°C. Free and bound peptides were separated using charcoal absorption. Immediately prior to centrifugation, 500µl of 0.01% Triton-X-100 solution was added to each tube, and the samples centrifuged at 1500g, 4°C, for 20 minutes. For charcoal absorption, the free fraction was separated using 4 mg of charcoal per tube suspended in 250µl of 0.06M phosphate buffer with gelatine, added immediately prior to centrifugation. The samples were then centrifuged at 1500g, 4°C, for 20 minutes (RHK4B rotor, Jouan KR-4, DJB labcare, UK). Bound and free label were separated and both the pellet and supernatant counted for 180 seconds using a β counter (model NE1600, Thermo Electron Corporation). Peptide concentrations in the test samples were calculated using a non-linear plot (RIA Software, NE Technology) with reference to the standard.

<table>
<thead>
<tr>
<th>Tube</th>
<th>Buffer (µl)</th>
<th>Ab (µl)</th>
<th>Label (µl)</th>
<th>Standard/sample (µl)</th>
<th>Acetic acid (0.5%) (µl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NSB</td>
<td>300</td>
<td>-</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>½ x label</td>
<td>275</td>
<td>50</td>
<td>25</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2 x label</td>
<td>200</td>
<td>50</td>
<td>100</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Zero</td>
<td>250</td>
<td>50</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Standard 1-15µl</td>
<td>250/230</td>
<td>50</td>
<td>50</td>
<td>1-15</td>
<td>0/20</td>
</tr>
<tr>
<td>Standard 20µl</td>
<td>230/210</td>
<td>50</td>
<td>50</td>
<td>20</td>
<td>0/20</td>
</tr>
<tr>
<td>Standard 30µl</td>
<td>220/200</td>
<td>50</td>
<td>50</td>
<td>30</td>
<td>0/20</td>
</tr>
<tr>
<td>Standard 50µl</td>
<td>200/180</td>
<td>50</td>
<td>50</td>
<td>50</td>
<td>0/20</td>
</tr>
<tr>
<td>Standard 100µl</td>
<td>150/130</td>
<td>50</td>
<td>50</td>
<td>100</td>
<td>0/20</td>
</tr>
<tr>
<td>Zero</td>
<td>250</td>
<td>50</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tissue extracts</td>
<td>230</td>
<td>50</td>
<td>50</td>
<td>20</td>
<td>-</td>
</tr>
<tr>
<td>Excess Antibody</td>
<td>-</td>
<td>300</td>
<td>50</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 2.1 General Structure of a Radioimmunoassay. A Table outlining the general structure of a RIA. NSB, non-specific binding tube; Zero, tube which lacks any sample; The values highlighted in red detail the volume of buffer adjusted for acetic acid.
CART antiserum showed 100% cross reactivity with rat and mouse CART (55-102) and 20% cross reactivity with CART (62-102) and less than 0.1% cross reactivity with CART 91-39). The sensitivity of the CART assay was 1.25 fmol/ml. The intra- and inter assay variation was 8 and 9% respectively. The α-MSH antiserum showed 100% specific for α-MSH. The antibody was less than 0.0018% cross reactive with ACTH, γ-MSH, β-MSH and β-lipoprotein. The sensitivity of the assay was 1 fmol/ml. The intra- and inter assay variation was 7 and 8% respectively.

2.2.13 Confirmation of Gene Transfer by In situ Hybridisation

In situ hybridisation is a technique which allows detection of mRNA within a tissue whilst maintaining structural integrity. A complimentary probe is hybridised to sectioned tissue mounted on poly-lysine coated slides. Probes can be radiolabelled or labelled with compounds such as digoxygenin (DIG). In situ hybridisation was carried out using a $^{35}$S labelled riboprobe. The primer sets used to generate specific riboprobes are detailed in the relevant sections. All riboprobes were prepared in pBluescript plasmid.

2.2.13.1 Production of Radiolabelled RNA Probe for In situ Hybridisation

Materials

- 200µg/µl Template plasmid linearised by digestion with BamHI for antisense or EcoRI for sense
- 100mM dithiothreitol (DTT) (Promega, UK)
- 30U/µl RNase inhibitor
- 10x nucleotide mix: 10mM of each ATP, UTP, GTP (GE Healthcare)
- 20U/µl T7 RNA polymerase (Promega, UK)
- 20U/µl T3 RNA polymerase (Promega, UK)
- 5x RNA polymerase buffer (Promega, UK)
- 7.5U/l DNase I (Amersham Biosciences)
- 10x DNase buffer
  - 800mM tris/HCl, pH7.4
  - 120mM MgCl$_2$
  - 40mM CsCl$_2$
- CTPαS$[^{35}$S] 46TBq/mmol,0.46GBq/ml (Perkin Elmer, Massachusetts, USA)
5M Ammonium acetate (NH₄Ac)
Absolute Ethanol
GDW

Methods
Radiolabelled template probe was produced using an *in vitro* transcription reaction. Two hundred nanograms of template was added to a reaction containing 10mM DTT, 1x nucleotide mix, 1x polymerase buffer, 30U RNase inhibitor and 0.69MBq in a total volume of 18µl. Finally, 20U RNA polymerase was added (T3 to the anti-sense reaction and T7 to the sense reaction) and this was incubated at 37°C for 2 hours. After incubation, 1µl DNase buffer and 7.5U DNaseI were added and the reaction incubated for a further 15 minutes at 37°C. The reaction was precipitated by adding 12µl ammonium acetate and 80µl ice cold ethanol. After one hour at -20°C the reaction was centrifuged at 8000g for seven minutes at room temperature (12084 rotor, Sigma 1-14 microfuge, Sigma, UK), the supernatant removed and the pellet resuspended in 200µl GDW. The activity of 1µl radiolabelled probe was measured on a microbeta counter (Perkin Elmer).

### 2.2.13.2 *In situ* hybridisation

**Materials**
Optimum Cutting Temperature (OCT) embedding compound (Bright Instruments, Huntingdon, UK)
Poly-lysine coated slides (VWR)
0.8% (v/v) Formaldehyde (VWR) solution in 0.01M PBS
0.01M PBS (appendix I)
100% acetic anhydride (VWR)
0.1M triethanolamine pH8.0 (TEA) (appendix I)
20x Saline sodium citrate (SSC) (appendix I)
3M NaCl
70% (v/v) Ethanol in GDW
Absolute Ethanol
Chloroform
Hybridisation buffer (appendix I)
50% (v/v) formaldehyde
60mM NaCl
80mM Tris/HCl pH7.5
4mM EDTA
100mM DTT (Promega, UK)
0.1mg/ml yeast tRNA (Sigma-Aldrich, Poole, Dorset, UK)
2% (w/v) dextran sulphate (Sigma-Aldrich, Poole, Dorset, UK)
1x RNase buffer
10mM Tris pH8.0
1mM EDTA
0.5M NaCl
RNaseA (appendix I)

Methods

Animals were killed by CO₂ asphyxiation. Brains were removed immediately, mounted onto cork discs with Optimum Cutting Temperature embedding compound (Bright Instruments, 148 Huntingdon, Cambridgeshire, U.K.) and rapidly frozen in isopentane chilled to -80°C. The brains were stored at -80°C until being sectioned. Thirteen micrometer sections were cut using a cryostat at -25°C (Bright Instrument Company, Huntingdon, Cambridgeshire, U.K.). The sections were mounted onto poly-L-lysine coated slides and stored at -80°C until hybridisation. In situ hybridisation was performed on every third slide. Sections were fixed in 0.8% (v/v) formaldehyde solution in 0.01M PBS on ice for 20 minutes. Slides were washed twice in 0.01M PBS for 5 minutes. The sections were acetylated in 0.25% (v/v) acetic anhydride in 0.1M TEA for 10 minutes, rinsed briefly in 0.01M PBS and dehydrated by immersion for three minutes in 70% (v/v) ethanol in GDW. Finally, slides were delipidated in chloroform for 5 minutes and allowed to air dry for several hours. Hybridisation buffer was supplemented with 1x10⁶MBq ³⁵S labelled probe and 70µl added to each slide. A coverslip was placed over each slide and slides were hybridised overnight at 60°C in a humid environment. The following day, the slides were washed in a series of washes with decreasing salt content to remove non-specifically bound probe. Slides were washed in 4x SSC and gently agitated to remove cover slips, slides were then washed four times in 4x SSC for 5 minutes. Slides were RNase treated by incubation in 1x RNase buffer containing 100µg/ml RNaseA for 30 minutes at 37°C. Slides were washed twice in 10mM DTT/2x SSC for 5 minutes. Slides were then washed for 10 minutes in 10mM DTT/1x SSC, then 10mM
DTT/0.5x SSC. Finally, slides were washed for 30 minutes in 10mM DTT/0.1M SSC at 60°C, rinsed in 10mM DTT/0.1x SSC and dehydrated in 70% ethanol then 100% ethanol for 3 minutes. Slides were exposed to Bio-Max film (Kodak, Hemel Hempstead, Herts, UK). After 4 days film was developed and transgene expression determined by observation of hybridisation within specific regions.
Chapter 3

Modification of a POMC Containing Bacterial Artificial Chromosome to Drive CART Antisense Expression Specifically in POMC Expressing Neurones

in vivo
3. Modification of POMC Containing Bacterial Artificial Chromosome to drive Cocaine- and Amphetamine -Regulated Transcript Antisense Expression Specifically in POMC Expressing Neurones in vivo

3.1 Introduction
Analysis of the complete human genome suggests the presence of approximately 20,000 genes. The functions of only several hundred of these genes are actually known. Sequence homologies and extrapolation from invertebrate studies can suggest the functions of unknown genes but their in vivo functions are difficult to predict.

Prior to the development of genomic technologies the analysis of mammalian gene function was limited to the rare spontaneous genetic mutations that produced inactivation or modification of a gene leading to identifiable phenotypes. Transgenic technologies and reverse genetics have developed as invaluable tools for investigating gene function in vivo. The mouse is an ideal organism for use in transgenic studies of complex genetic traits. A number of factors have contributed to the popularity of the mouse; there is extensive homology between the human and mouse genome and the relative short life span of the mouse, coupled with the availability of mouse embryonic stem (ES) cells and the ease of controlled breeding make the mouse an attractive model organism.

Genomic technologies can generally be placed in two categories; those which alter the endogenous gene by homologous recombination in ES cells (targeted transgenesis), and those which insert exogenous genes into the genome (additive transgenesis). Gene targeting in ES cells has allowed either the inactivation of genes in vivo to produce ‘knockouts’ or the insertion of functional mutations to produce ‘knock-ins’. Insertion of exogenous genes into the genome is often termed transgenesis and has allowed the insertion of extra copies of a gene to produce animal models which overexpress a particular protein. Advances in transgenic technologies allow genetic manipulations to study gene function which are otherwise impossible in other organisms. Null mutations, as well as subtle missense or gain of function mutations, can be introduced into any gene in the mouse germ line using homologous recombination based gene targeting technology. The use of Cre-Lox technology also makes it possible to conditionally activate or inactivate gene expression in vivo (Sauer, 1998). This can be achieved both spatially in a subset of cells and temporally with the use of
promoters known to switch on at a specific point or with the use of drugs to activate gene expression such as the case of the tet-on and tet-off system (St-Onge et al., 1996).

3.1.1 The Transgene

The generation of transgenic animals is an established technique with the first step being the selection of the transgene. The transgene consists of a promoter and a specific cDNA transcript, often the cDNA encodes all or part of a particular protein of interest, but it can also be a genomic fragment which contains all or part of a gene, or an antisense fragment (Shuto et al., 2002). The cDNA is sub-cloned downstream of a suitable promoter which will determine the level, specificity and temporal pattern of expression. Promoters such as cytomegalovirus (CMV) promoter can be used for ubiquitous expression whereas other promoters like neurone specific enolase (NSE) are tissue specific. When a transgene expression pattern is required to mirror that of the endogenous gene, the genes own promoter can be used.

The transgene is introduced into fertilised mouse eggs by pronuclear injection (Figure 3.1). Once inside the pronucleus, the transgene either integrates into the genome in a random fashion or is degraded by exonucleases. Transgenes typically integrate into the genome as tandem repeats (head to tail concatamers) at a single locus. The exact mechanism of integration remains unknown but is thought to coincide with the occurrence of double strand breaks within the nuclear DNA during the injection procedure. The ova is then transplanted into a pseudo-pregnant foster mother and a normal pregnancy follows (Ittner and Gotz, 2007). The transgene replicates with the endogenous chromosome becoming part of the forming embryo. Embryos which contain integrated transgenes usually acquire several copies of the transgene. Integration of the transgene usually occurs early in development, at the single cell stage, so most or all of the cells in the adult mouse contain the transgene. If integration occurs after a few rounds of cell division, the transgene will only be present in a subset of cells thus creating a mosaic transgenic adult mouse. If the transgene is present in cells comprising the germ line, it will be transmitted to subsequent generations of mice. Mice which successfully transmit the transgene are known as founder mice (Ittner and Gotz, 2007).
Figure 3.1 Generation of a transgenic mouse colony. (A) Typical cDNA transgene sub-cloned into a plasmid containing a promoter which will drive its expression in vivo. (B), Schematic diagram of the steps required to create a transgenic mouse colony. The transgene is introduced into the fertilised mouse egg by pronuclear injection where it integrates into the mouse genome in a random fashion. The ova is then transplanted into a foster mother and normal pregnancy follows. Transgenic mice are mated with wildtype mice to generate separate transgenic lines.
3.1.2 Transgene Copy Number and Position Effects

Whilst the design of the transgene can be optimised there are other factors which can affect expression that cannot be controlled for in the design of the transgene construct. One such factor is the number of copies of the transgene that integrate into the host genome.

Each founder mouse derived from one pronuclear injection is unique. One key difference between founder mice is the number of copies of the transgene that integrate into the genome which can range from 1 to greater than 50. The expression of the transgene is normally correlated to the number of copies integrated, although not necessarily in a linear relationship. The copy number, while being uncontrolled, can be useful in terms of gene dosage studies (Koike et al., 1994).

Another important factor affecting transgene expression is known as the ‘chromosome position effect’. Genes can span several hundred kilobases (kb) of DNA although only a small part of this actually codes for the protein. Even genes that have small coding regions can have important regulatory sequences located a great distance from the coding region, the tyrosinase gene being one example. In the mouse tyrosinase gene a melanoma cell specific enhancer was identified approximately 12kb upstream of the tyrosinase gene by DNaseI hypersensitive site mapping (Ruth Ganss et al., 2007). In addition, spatiotemporal expression of human agouti related peptide (AgRP) has been found to be part regulated by the intron sequence of the neighbouring ATPase gene (ATP6V0D1) (Ilnytska et al., 2009).

Because the transgene integrates randomly into the mouse genome following pronuclear injection, the genomic sequences flanking the site of integration can have a marked effect upon transgene expression; enhancing, reducing or abolishing it completely. For example integration of the transgene near the break point between heterochromatin and euchromatin could result in position effect variegation, the extent of variegation would depend on the distance of the gene from the breakpoint (Weiler and Wakimoto, 1995). Transgene integration can also disrupt endogenous promoter elements and protein coding regions resulting in spurious phenotypes (Cases et al., 1995).

A number of strategies have been developed to overcome these position effects and increase the probability of optimal transgene expression following random integration. Genes are organised on chromosomes as contiguous but independent units called expression domains.
Expression domains include regulatory elements which are necessary to control gene expression both spatially and temporally. The inclusion of all regulatory elements relevant for a given expression domain would ensure optimal transgene expression. The manipulation and analysis of large genes has been limited by the insert capacity of standard cloning vectors. Vectors such as Yeast Artificial Chromosomes (YAC), Bacterial Artificial Chromosomes (BAC) and P1 Artificial Chromosomes (PAC) however are able to incorporate large genomic segments of DNA, and would therefore be expected to contain whole expression domains. Other strategies that have been developed to optimise transgene expression after random integration into the genome include the incorporation of intron sequences in the transgene construct to improve transcription efficiency (Choi et al., 1991; Brinster et al., 1988). The inclusion of a polyadenylation (pA) signal in the modification cassette also increases the efficiency of expression of the transgene (Azzoni et al., 2007). The polyadenylation signal was identified as a 3’ flanking sequence downstream of the AAUAAA poly A consensus sequence (Ribeiro et al., 2004). The most commonly used polyadenylation signal is from the bovine growth hormone (BGH) gene which has been shown to be as effective as the SV40 polyadenylation signal (Azzoni et al., 2007). The inclusion of specific sequences such as locus control regions and insulators have also been shown to improve transcription (Lisowski and Sadelain, 2007).

### 3.1.3 Yeast, P1 and Bacterial Artificial Chromosomes

YACs possess the largest insert cloning capacity being able to maintain genomic fragments of up to 1Mb (Burke et al., 1987). Due to their large size and linear nature they are difficult to handle in the laboratory using conventional techniques. However the main problem with using YACs is that they suffer from insert instability and rearrangements leading to the possibility of contamination of the transgene with endogenous yeast chromosome, a phenomenon which complicates purification.

PACs do not suffer from insert instability and are easy to handle in the laboratory setting. PACs are capable of maintaining inserts between 70-100kb (Ioannou et al., 1994). BACs can accommodate larger inserts in the region of 300kb (Shizuya et al., 1992). Being circular in nature they are more resistant to mechanical shearing and can be isolated using conventional plasmid purification protocols. Due to the large size of BACs they should contain all the necessary upstream and downstream regulatory elements to drive accurate *in vivo* expression.
if the gene of interest is positioned near the centre of the genomic insert. This should reduce
the likelihood of transgene expression being affected by position effects. To date BACs and
PACs have been used to create genomic libraries of many species.

3.1.4 Bacterial Artificial Chromosome Modification

Methods for modifying BACs rely on three basic features. The first step is the restoration of
competence for homologous recombination to the BAC host strain which is achieved by the
reintroduction of the *E. coli* recombination genes. This is necessary since homologous
recombination is required for the targeted modification of the selected BAC by the transgene
containing modification cassette. BACs are commonly maintained in DH10B *E. coli* strains
that have a targeted deletion of the recombination gene *RecA*, to reduce the chance of
sequence rearrangements in the BAC as a result of recombination. The next stage is the
introduction of a shuttle vector or linear DNA fragment that carries the desired reporter or
modification cassette flanked by sequences homologous to the genomic DNA carried in the
BAC (Burke et al., 1987). The flanking sequences are used to target the modification cassette
into a precise site on the genomic DNA insert in the BAC. Recombinants are selected and
screened for precise integration. Thirdly, unwanted sequences such as antibiotic resistance
cassettes used for screening successful recombinants are removed through a second
recombination event, or excised by using an appropriate site specific recombinase such as
Cre. The modification cassette can then be expressed in transgenic animals. Modification
protocols have enabled the insertion of marker genes such as enhanced green fluorescent
protein (eGFP) (Liu et al., 2003), recombination enzymes such as Cre recombinase (DeFalco
et al., 2001) and toxins into the BAC.

3.1.5 RecE and RecT (ET) Cloning (Figure 3.2)

In 1998 Stewart et al showed that PCR-amplified linear double stranded (ds) DNA, flanked
by short sequences of homology >42bp could be efficiently targeted to a plasmid by
electroporating the dsDNA into *recBC sbcA* strains (Zhang et al., 1998). The *sbcA*
mutation activates expression of *recE* and *recT* genes (Hall et al., 1993). The *recE* gene encodes for
exonuclease VIII whilst the *recT* encodes for the recT protein, these proteins promote
homologous pairing and strand exchange in reactions containing linear duplex DNA and
homologous, circular DNA as substrates. These recombination functions enable genomic
DNA to be modified directly with PCR-generated linear dsDNA targeting cassettes, rather than using targeting cassettes that carry long homologies generated by multi-step sub cloning (Zhang et al., 1998). To allow for ET cloning, the pGETrec plasmid was developed which contains the recE and recT and gam genes coordinated under the control of an arabinose inducible promoter (P_{BAD}) promoter (Narayanan et al., 1996). This allows temporal control over the expression of the rec genes, and consequently over homologous recombination, since the P_{BAD} promoter can be induced by addition of arabinose. Activation of the recE, recT and gam genes makes the host cell competent for homologous recombination. The recE and recT genes induce a recombination event between the modification cassette and its target on a BAC. The gam gene encodes the gam protein which inhibits the recBCD enzyme complex. This enzyme functions as a DNA exonuclease by binding to double strand breaks in DNA and degrades both strands simultaneously (Amundsen et al., 1986). Inhibition of recBCD prevents the recBCD dependent destruction of the targeting cassette. Efficiency of the recombination event has been shown to be increased by using longer homology arms. The efficiency of recombination is also dependent on the distance between homology arms on the host genome, optimal distances range between 7bp and 1200bp (Zhang et al., 1998). Successfully modified BACs are selected by the antibiotic resistance gene conferred by the modification cassette. Removal of the antibiotic resistance gene from the modified BAC is desirable since antibiotic genes have been shown to produce phenotypes in transgenic and knockout mice. Removal of the antibiotic resistance gene is achieved by a site specific recombinase such as Cre recombinase (Mejia and Larin, 2000). Cre is a 38 kDa protein product of the cre gene from bacteriophage P1 which is necessary and sufficient to catalyse recombination between two loxP sites in the P1 genome. LoxP is a 34bp site that consists of 13bp inverted repeats flanked by an 8bp non palindromic core which gives directionality. Cre recognises loxP and recombination is catalysed between pairs of loxP sites. Cre has been shown to be functional in yeast and mammals. It is by using site specific Cre recombinase that the antibiotic resistance gene is excised. Cre activity is introduced to the BAC containing cell by introduction of a plasmid that can controllably and transiently express Cre recombinase e.g. by a temperature sensitive promoter. Removal of the antibiotic gene is then screened for by positive and negative selection and confirmed by PCR. The BAC DNA can then be purified for pronuclear injection.
Figure 3.2 ET Cloning. Diagram outlining the steps in ET cloning used to modify BACs. The modification cassette with homology arms is constructed in a typical cloning vector by PCR, restriction digests and ligation. The modification cassette is then isolated from the cloning vector by restriction digest and electroporated into the BAC host cell with the pGETrec plasmid. Incubation of the host cells with arabinose activates recE and recT and gam genes on pGETrec. recE and recT catalyse recombination between homology arms and the BAC. The modified BAC can then be isolated purified and injected into the pronucleus to create a transgenic line.
3.1.6 Antisense RNA

Antisense has enormous potential as a technology in the study of gene function. The general basis of antisense is the inhibition of gene expression through binding of a complementary ribonucleotide transcript to a specific mRNA sequence. Natural antisense was first described in prokaryotes where it was found to down regulate gene expression. Natural antisense has also been described in eukaryotes for example the αI collagen in the chicken chondrocyte (Neckers and Whitesell, 1993).

Antisense technology provides a methodology for reducing gene expression in targeted tissue through the use of specific promoters. One example of this is expression of Glucocorticoid II receptor antisense, under the control of a neurofilament L promoter, which has been shown to reduce expression of the glucocorticoid II receptor by 50-70% in specific brain areas and by 30-50% in the liver (Pepin, 1992). The antisense sequence is an inversion of the sense strand of the target gene. When choosing the antisense sequence the general trend is that the longer the antisense constructs the more effective it is as an inhibitor. The inhibition of β glucuronidase was shown to be more effective when a 1.4kb antisense was used relative to a 350bp antisense transcript. In both cases the MT-1 promoter, which is a weak constitutive promoter, was used to drive the expression of the antisense (Ao et al., 1991). Small antisense transcripts are useful when they are directed against a target which has a number of closely related units. There are a number of promoters that are available to drive antisense expression. The choice of promoter very much depends on the required transgene expression pattern for example the CMV is a ubiquitous promoter, whereas the Cyp1a-1 promoter is induced fourfold by dioxin.

The mechanism by which antisense RNA blocks the expression of the target gene is not entirely clear. It is thought that the antisense binds to the target sense strand and forms an RNA duplex within the cell nucleus. The RNA duplex becomes a target of the enzyme adenosine deaminase. The adenosine deaminase converts adenosine residues in the RNA duplex to inosine residues and causes the RNA duplex molecule to unwind (Kumar and Carmichael, 1997). The inosine residues are recognised by nuclear proteins, for example p54nrb, which bind inosine containing RNA and results in nuclear retention of the RNA transcripts through attachment to the nuclear matrix (Zhang and Carmichael, 2001).
The use of such technology allows for the long term knock down of a gene of interest in order to determine the physiological consequences. Therefore this methodology can be applied to study the physiological role of cocaine- and amphetamine-regulated transcript (CART) within the arcuate nucleus. CART antisense (CART-AS) expression under the control of the pro-opiomelanocortin (POMC) expression domain will result in reduced CART mRNA expression specifically in POMC expressing neurones.

3.1.7 Hypothesis

Hypothesis: A BAC containing the POMC expression domain will drive accurate and specific expression of cocaine- and amphetamine-regulated transcript antisense in POMC neurones.

3.1.8 AIM

1. To identify a BAC likely to contain the POMC expression domain.

2. To modify the BAC using ET cloning so that the POMC expression domain drives the expression of the CART-AS in a transgenic animal.

3. To create transgenic lines by pronuclear injection of the modified POMC BAC and to characterise the resulting transgenic lines.
3.2 Materials and Methods

3.2.1 Bacterial Artificial Chromosome DNA Purification
The large size of BAC DNA makes them more vulnerable to shearing by mechanical forces than small cloning vectors. Extra care and slightly modified techniques are therefore required when isolating BAC DNA from their host *E.coli* cells.

3.2.1.1 Small Scale Purification of Bacterial Artificial Chromosome DNA
BACs were initially isolated by small scale culture to allow several clones to be analysed simultaneously. To isolate plasmids from bacteria it is necessary to disrupt the cell wall to release the plasmid, and at the same time to remove contaminating proteins, genomic DNA and RNA.

Materials
Lysogeny Broth (LB) (appendix I)
25mg/ml Chloramphenicol (Cm) (appendix I)
0.2 M sodium hydroxide / 1% (v/v) SDS solution in GDW (appendix I)
5M potassium acetate, pH 4.6 (appendix I)
Absolute Ethanol
GTE (appendix I):
   25 mM Tris-HCl, pH 8.0
   10 mM EDTA
   50mM glucose

Method
Five millilitres of LB with 12.5µg/ml chloramphenicol were inoculated with a single bacterial colony and incubated overnight at 37°C with vigorous shaking. Five hundred microlitres of this culture were added to an equal volume of 80% (v/v) glycerol and stored at -70°C. The rest of the culture was centrifuged for three minutes at 12300g at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany) to pellet the bacteria. The supernatant was discarded and the pellet resuspended in 100µl of GTE. Two hundred microlitres of 0.2M NaOH/ 1% (v/v) SDS solution were added and the sample incubated on ice for five minutes, followed by the addition of 150µl of 5M potassium acetate and a further five minute
incubation on ice. The precipitated bacterial debris was removed by centrifugation for five minutes at 12300g at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany) and 350µl of supernatant were transferred to a clean tube. An equal volume of phenol/chloroform/IAA was added and the sample mixed. The phases were separated by centrifugation for three minutes at 12300g at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany) and the upper phase transferred to a fresh tube. The DNA was precipitated by the addition of 0.6 volumes of propan-2-ol and incubation at room temperature for ten minutes. The DNA was recovered by centrifugation for seven minutes at 12300g at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany) and dissolved in 100µl of GDW. Ten microlitres of 2M sodium acetate and 250µl of absolute ethanol (-20°C) were added and the solution incubated at -20°C for at least one hour. The DNA was recovered by centrifugation for seven minutes at 12300g at room temperature (F45-12-11 microfuge, Eppendorf, Hamburg, Germany), then washed three times with 500µl of 70% (v/v) ethanol in GDW before air drying and resuspending in 20µl GDW. The DNA can then be used in follow on protocols such as restriction digests (see section 2.2.7.1).

3.2.1.2 Large Scale Purification of Bacterial Artificial Chromosome DNA

Materials
Qiagen Large Construct Kit
Sterile GDW

Method
The manufactures protocol was modified slightly to give optimal results. A small quantity of bacteria containing the plasmid of interest (see section 3.2.1.1) was inoculated into 500 ml of LB(Cm) and incubated at 37°C overnight with vigorous shaking. The bacteria were recovered by centrifugation for fifteen minutes at 6000g (HS-4 rotor in RC-5B super speed centrifuge, Du Pont) at 4°C. The pellet was resuspended in 40ml of ice cold solution P1 before addition of 40ml of solution P2, the sample was mixed by gently inverting six to eight times and incubated for five minutes at room temperature. Forty millilitres of solution P3 was then added. The sample mixed by inversion and the lysate incubated on ice for thirty minutes. The neutralised lysate was then centrifuged at 20,000g for thirty minutes at 4°C (JA-18 rotor, Beckman J2-21 centrifuge). The supernatant containing the BAC DNA was removed and re-
centrifuged at 20,000g for fifteen minutes (JA-18 rotor, Beckman J2-21 centrifuge). The DNA supernatant was filtered through the folded filter paper provided in the kit. The resulting solution was precipitated using 0.6 volumes of room-temperature isopropanol (approx 36mls) and immediately centrifuged at 15,000g (Beckman JA-18 rotor, Beckman J2-21 centrifuge) for thirty minutes at 4°C. The pellet was then washed in 5ml 70% (v/v) ethanol in GDW and centrifuged at 15,000g (Beckman JA-18 rotor, Beckman J2-21 centrifuge). After air drying the pellet for two to three minutes the pellet was re-dissolved in 9.5ml of buffer EX, and 200µl of ATP-dependent exonuclease plus 300µl of ATP solution added. This solution was incubated for one hour at 37°C before addition of 10mls of buffer QS and run through an equilibrated Qiagen-tip 500. The tip was washed with two x 30mls of QC buffer and the DNA eluted with 15mls of buffer QF warmed to 65°C. The eluted DNA was precipitated with 10.5ml room-temperature isopropanol and centrifuged at 15,000g (Beckman JA-18 rotor, Beckman J2-21 centrifuge). The DNA pellet was then washed with 5ml of room-temperature 70% (v/v) ethanol, centrifuged at 15,000g (Beckman JA-18 rotor, Beckman J2-21 centrifuge), and resuspended in 100µl of GDW.

3.2.2 Construction of Cocaine- and Amphetamine -Regulated Transcript Antisense Modification Cassette (Figure 3.4)

When constructing the CART-AS modification cassette for insertion into the POMC BAC by homologous recombination there were several considerations to be taken into account such as the position of the homology arms, ensuring that the antisense fragment is not compromised by endogenous BAC sequence and the inclusion of elements thought to increase transgene transcription, such as intron sequences. Homology arm 1 was designed so that it was homologous to the noncoding sequence at the 5’ end of exon 1 of the POMC gene. This positioning of homology arm 1 ensured that there would be minimal endogenous POMC sequence transcribed with the antisense sequence whilst at the same time not disrupting possible transcription control elements. The presence of additional sequences to that of the CART-AS may affect the specificity with which the antisense will bind to its target sense strand. The distance between the homology arms should be kept to a minimum in order to reduce possible disruption to the endogenous BAC sequence, for this reason homology arm 2 was designed so it was homologous to the end of POMC exon 2 and a small section of the non coding sequence following exon 2. The design of homology arm 2 also maintained the intron sequence between exon 2 and 3 of the POMC gene (Figure 3.3). It has been
demonstrated that the presence of intronic sequences can increase expression of the transgene (Brinster et al., 1988; Choi et al., 1991). The cloning of a polyadenylation signal 3’ of the CART-AS sequence enhances transcription and nuclease protection of the transcript (Azzoni et al., 2007). Additionally, the positioning of the polyadenylation signal adjacent to the CART-AS directs termination of transcription to this point and ensures that the remaining POMC sequence is not present in the antisense transcript. The result being that the specificity of the antisense is not compromised by additional endogenous POMC sequence. A floxed antibiotic gene must be included to screen for successful recombinants and then allow for its subsequent removal.
Figure 3.3 Strategy used to modify the POMC BAC. Homologous recombination between the modification cassette and the POMC BAC was catalysed by the pGETrec plasmid so that the POMC expression domain could drive the expression of the CART-AS. The POMC BAC regulatory elements and exon 1-3 (E1-E3) are shown. Homology arm 1 from the modification cassette undergoes recombination with exon 1 in the POMC domain. Homology arm 2 of the modification cassette undergoes recombination with exon 2 in the POMC domain.
A BAC containing the POMC gene near the centre of its insert was chosen for modification with CART-AS (RP24-034C3 BAC). A flow diagram for the construction of the modification cassette is shown in Figure 3.5. The homology arms were generated by PCR (see section 2.2.5) using the POMC BAC as the template. Homology arm 1 (414bp) was amplified using primers corresponding to nucleotides 89353-89372 (5’primer) and 89749-89771 (3’primer) in the RP24-034C3 BAC (http://www.ensembl.org/index.html). Homology arm 2 (282bp) was amplified using primers that correspond to nucleotides 93333-93352 (5’ primer) and 93067-93089 (3’primer) in the RP24-034C3 BAC (http://www.ensembl.org/index.html). The CART-AS fragment was amplified by RT-PCR firstly a reverse transcriptase reaction (see section 2.2.4) was carried out using RNA extracted from mouse hypothalamus, followed by a PCR reaction (see section 2.2.5) from the resulting RT mixture using primers corresponding to nucleotides 32-62 (5’Primer) and 421-441 (3’primer) in the mouse CART mRNA sequence (accession number NM_013732). The PCR reaction yielded products of 369bp and 408bp. The two PCR products are due to alternate splicing in exon 2 on the CART gene. The primer set amplifies this region so both transcripts were produced. The BGH polyadenylation signal (230bp) was isolated from the pTRCGW plasmid (kind gift from Dr J. Gardiner) (Figure 4.1) using the restriction enzymes SacI and NotI (see section 2.2.7.1.). The products from the restriction digest were run on a 1% agarose gel (see section 2.2.6) and the 230bp BGH polyadenylation signal was excised from the gel and purified by electroelution (see section 2.2.7.2.).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Oligonucleotide sequence</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arm1</td>
<td>5’ AGC TGG TAG CCA TTG GGG AAA TCT GCG ACA</td>
<td>NheI</td>
</tr>
<tr>
<td></td>
<td>3’ AGC TGG ATC CCT GTC GCT CTT CTC TCT TCT</td>
<td>BamHI</td>
</tr>
<tr>
<td>Arm2</td>
<td>5’ AGC TTT CGA AAG TGC TGA GGA GTC CTC ACA</td>
<td>Bsp119I</td>
</tr>
<tr>
<td></td>
<td>3’ AGC TGC GGC CGC AGA GCA ACC TGC TGG TAT GT</td>
<td>NotI</td>
</tr>
<tr>
<td>CART AS</td>
<td>5’ AGC TCG GTC CGC AGA ACC ATG GAG AGC TCC C</td>
<td>SacI</td>
</tr>
<tr>
<td></td>
<td>3’ AGC TGG ATC CCC TTC ACA AGC ACT TCA AGA GG</td>
<td>BamHI</td>
</tr>
</tbody>
</table>

Table 3.1 Primers for PCR amplification of homology arm 1 and homology arm 2 using the BAC containing POMC as a template. Primers are shown for CART-AS using mouse hypothalamus RNA as template. The restriction sites are shown in bold with the corresponding restriction enzyme.
The PCR product for homology arm 1 and the cloning vector pFastBAC Dual (Figure 3.6) were cut with NheI and BamHI restriction enzymes (see section 2.2.7.1). The products from the restriction digest were run on a 1% agarose gel (see section 2.2.6.) and the linearised plasmid and homology arm were excised from the gel and purified by electroelution (see section 2.2.7.2). Homology arm 1 was sub-cloned by ligation (see section 2.2.7.3) into the linearised pFastBAC Dual cloning vector to form pFASTBAC/ARM1. The pFASTBAC/ARM1 plasmid and the amplified CART-AS were then cut in a restriction digest with BamHI and SacI, run on a 1% agarose gel and electroeluted. The 369bp CART-AS cut PCR product was sub-cloned into the pFASTBAC/ARM1 plasmid to produce pFASTBAC/ARM1/CARTAS. The pFASTBAC/ARM1/CARTAS vector was then cut with SacI and NotI restriction enzymes. The linearised plasmid was then electroeluted and the BGH polyadenylation signal, which was isolated from the pTRCGW plasmid, was sub-cloned into pFASTBAC/ARM1/CARTAS to form pFASTBAC/ARM1/CARTAS/pA.

Homology arm 2 and pFASTBAC/ARM1/-CARTAS/pA were then cut by the restriction enzymes NotI and Bsp119I. After the linearised pFASTBAC/ARM1/-CARTAS/pA and homology arm 2 were electroeluted, homology arm 2 was sub-cloned into the plasmid to form pFASTBAC/ARM1/CARTAS/pA/ARM2. A 2.15kb NotI fragment containing a floxed spectinomycin resistance gene was isolated from plasmid pDLOX5 (a kind gift from Dr C Huxley) (Figure 3.6). The spectinomycin gene and pFASTBAC/ARM1/CARTAS/pA/ARM2 were cut with NotI. The spectinomycin gene was then sub-cloned between homology arm 2 and polyadenylation signal to form pFASTBAC/ARM1/CARTAS/pA/Spec/ARM2. The final plasmid was purified by large scale purification (see section 3.2.1.2) and the integrity of the modified cassette checked by DNA sequencing (Advanced Biotechnology Centre, Imperial College, Faculty of Medicine, Charing Cross Campus, London).
Figure 3.4 Schematic of the CART-AS modification cassette. The homology arms were amplified by PCR using the POMC BAC as a template. The antisense (CART-AS) gene was amplified from mouse hypothalamus RNA. The bovine growth hormone polyadenylation signal (pA) was cut from the pTRCGW plasmid (kind gift from Dr J. Gardiner). The spectinomycin gene was cut from the pDLOX5 plasmid.
Homology arm 1 was subcloned into the NheI and BamHI restriction sites in the cloning vector pFASTBac Dual. This was then followed by the CART-AS gene into the BamHI and SacI sites on the same vector. The pA signal was cloned into the same vector at the SacI and NotI sites. Homology arm 2 was then subcloned into the NotI and Bsp119I sites. Finally the spectinomycin gene was isolated in a NotI fragment from pDLOX5 and subcloned into the NotI site of the vector containing the homology arms, antisense gene and pA signal.
Figure 3.6 pFASTBAC Dual. A map of the vector pFASTBAC Dual and its multiple cloning sites. Pcmv, cytomegalovirus promoter; BGH pA, bovine growth hormone polyadenylation signal; f1 ori, origin of replication for F1 filamentous phage; SV40 ori, origin of replication for SV40; Sv40 pA, SV40 polyadenylation signal; pUC ori, pUC origin of replication; HSV tk pA, herpes simplex virus thymidine kinase polyadenylation signal; Tn7L, left element of transposon 7; Tn7R right element of transposon 7; Pp10, P10 promoter, Pph, polyhedrin promoter.
Figure 3.7 pDLOX5 Plasmid. Map of plasmid pDLOX5 showing Spectinomycin resistance cassette (Sp), restriction sites, T7 T3 transcription promoters, chloramphenicol resistance gene (Cm) and LoxP sites.
3.2.3 Modification of the POMC Bacterial Artificial Chromosome by ET Cloning

Materials

Agarose (Sigma-Aldrich)

LB (appendix I)

Ampicillin 100mg/ml in GDW (Sigma) (appendix I)

Chloramphenicol 25mg/ml in ethanol (Sigma) (appendix I)

Spectinomycin 50mg/ml in GDW (Sigma) (appendix I)

10% (v/v) Glycerol in GDW (appendix I)

10% (w/v) L-arabinose (Sigma) in GDW (appendix I)

10X Taq buffer (Sigma)

20µM oligonucleotide primers (Oswel DNA service, University of Southampton, Southampton, U.K.)

Taq DNA polymerase (Sigma)

0.1cm electrode gap cuvette (BTX, San Diego, CA)

Methods

A strategy for modifying the POMC BAC is shown in Figure 3.8. The CART-AS modification cassette was isolated from pFASTBacDual/ARM1/CARTAS/-pA/SPEC/HOM2 by digesting the plasmid with NheI and Bsp119I restriction enzymes (see section 2.2.7.1). The DNA was excised and run on a 1% agarose gel (see section 2.2.6) and the modification cassette was electroeluted (see section 2.2.7.2).

Initially the pGETrec plasmid (Figure 3.9) was electroporated into DH10B E.coli containing the POMC BAC. To prepare electrocompetent cells 1ml of culture grown overnight in LB with 12.5µg/ml chloramphenicol (Cm) was seeded into fresh LB with Cm at a ratio of 1:100 and grown for four to five hours at 37°C with shaking. When the optical density (OD) of the cells reached 0.5-0.6, measured at 580nm using a spectrophotometer, the cells were centrifuged at 800g for ten minutes at 4°C (HB-4 rotor in RC-5B super speed centrifuge, Du Pont). The cell pellet was washed twice by resuspending in ice cold 10% (v/v) glycerol and centrifuging at 4,000g for 10 minutes at 4°C (HB-4 rotor in RC-5B super speed centrifuge, Du Pont) and then repeating. After the final wash, the pellet was resuspended in 100µl of ice cold 10% (v/v) glycerol. 40µl of the cell suspension and 10ng of pGETrec was used per electroporation. Electroporation conditions were: 1.8kV, 186Ω, 25F in 0.1cm electrode gap.
cuvette using an Electro Cell Manipulator 600 (BTX). Following electroporation the cells were immediately removed into 960µl of prewarmed (37°C) LB and incubated for two hours at 37°C with shaking before being plated on LB + Chloramphenicol 12.5µg/ml + Ampicillin 100µg/ml plates (see section 2.2.9) which selects for *E.coli* cells which contain the POMC BAC and pGETrec plasmid.

The isolated and eluted CART-AS modification cassette was then electroporated into the DH10B *E.coli* strain containing the POMC BAC and pGETrec plasmid. Electrocompetent cells were prepared as described above except that after seeding the overnight culture in LB+Cm+Amp and culturing for three hours, 10% (w/v) L-arabinose was added to the cells to a final concentration of 0.2% (v/v) to induce the arabinose inducible P$_{BAD}$ promoter on pGETrec plasmid. Induction of the P$_{BAD}$ promoter on pGETrec drives production of recE and recT which catalyse homologous recombination between the modification cassette and the POMC BAC. Cells were cultured for 40 minutes in the presence of L-arabinose, then centrifuged at 4,000g for ten minutes at 4°C (HB-4 rotor in RC-5B super speed centrifuge, Du Pont) and washed twice in ice cold 10% (v/v) glycerol. 40µl of cell suspension and 400ng of the isolated CART-AS modification cassette was used for each electroporation as described above. Electroporated cells were incubated in prewarmed (37°C) LB for three hours at 37°C with shaking then plated onto LB agar plates with chloramphenicol and spectinomycin 50µg/ml.

Three colonies were screened for successful recombination by PCR (see section 2.2.5) after small scale POMC BAC DNA isolation. Three PCR primer sets were used to check correct integration of the CART-AS modification cassette; the 5’ end was checked using a forward primer outside the region of homology arm 1 and the forward primer for CART-AS. Screening for correct 3’ integration was confirmed using forward primer for homology arm 2 and the reverse primer outside the region of homology arm 2. Integration of the complete construct was checked using the forward primer outside the region of homology arm 1 which corresponds to nucleotides 89171-89191 of the RP24-034C3 BAC and the reverse primer outside the region of homology arm 2 which corresponds to nucleotides 93411-93431 of the RP24-034C3 BAC (Table 3.2). PCR was carried out in 1x taq buffer, 0.2mM dNTPs, 200nM oligonucleotides and with 5U of taq per reaction. The PCR reaction was heated to 95°C for fifteen minutes before being cycled thirty times through the following temperatures: 95°C for forty seconds, 55°C for forty seconds, 72°C for two minutes.
To remove the spectinomycin gene a Cre-reaction was performed using plasmid pJM2545 (a kind gift from Dr. C. Huxley) in the DH10B *E.coli* strain containing the CART-AS modified BAC. Electrocompetent cells were prepared from cells with the modified BAC as above, and 20ng of plasmid pJM2545 electroporated into the cells. After electroporation cells were incubated in LB + 0.5mM IPTG to induce Cre expression. Following a 60 minute incubation at 37°C shaking, the cells were plated onto LB + 12.5µg/ml chloramphenicol and incubated for 75 minutes at 30°C and then finally overnight at 42°C. The colonies were cultured into 96 well plates containing LB agar + chloramphenicol or spectinomycin. Clones which exhibited chloramphenicol resistance and sensitivity to spectinomycin were checked by PCR after small scale BAC DNA isolation (see section 3.2.1.1). The same primer sets were used to check correct integration of the modification cassette and to show successful removal of the spectinomycin resistance gene. Successful integration was followed by large scale BAC DNA isolation (see section 3.2.1.2)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
</tr>
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<tbody>
<tr>
<td>OUTARM1</td>
<td>TAG AGT TTG GGC ACA GAA GG</td>
</tr>
<tr>
<td>OUTARM2</td>
<td>GTT GAT TTG GGA GCC AAA GG</td>
</tr>
</tbody>
</table>

**Table 3.2** Primers used to check correct integration of the CART-AS modification cassette
Figure 3.8 Strategy used to modify the POMC BAC. Homologous recombination between the modification cassette and the POMC BAC was catalysed by the pGETrec plasmid so that the POMC expression domain could drive the expression of the CART-AS. The POMC BAC regulatory elements and exon 1-3 (E1-E3) are shown. Homology arm 1 from the modification cassette undergoes recombination with exon 1 in the POMC domain. Homology arm 2 of the modification cassette undergoes recombination with exon 2 in the POMC domain.
Figure 3.9 Map of pGETrec plasmid. The recE, recT and gam genes are under the control of the PBAD promoter. PBAD, PBAD promoter; ampR, ampicillin resistance gene, Pc prom, PC promoter; P3 prom, P3 promoter; M13 ori, origin of replication for M13; pBR322, origin of replication for pBR322; araC, arabinose promoter.
3.2.4 Generation of Transgenic Mice and Genotyping PCR

Materials
Tail digestion buffer:
- 6.7mM Tris-HCL
- 1.66mM (NH₄)₂SO₄
- 0.67mM MgCl₂
- 0.5% (v/v) Triton X-100
- 1% (v/v) β-mercaptoethanol (added just before use)
- 20mg/ml Proteinase K in GDW (appendix I)
- 10X Taq buffer (sigma)
- 20µM oligonucleotide primers (Oswel DNA service, University of Southampton, Southampton, U.K.)
- Genomic Red Taq DNA polymerase (Sigma)

Method
DNA of the modified POMC BAC was isolated using a Qiagen large construct kit and microinjected at low concentration (0.5-1 ng/µl) into pronuclei of fertile mouse (c57BL/6J x CBA/Ca) oocytes. Oocytes were cultured from and then implanted into foster females using standard techniques. All DNA injections and mouse manipulations were carried out by the MRC Transgenic unit at Hammersmith Hospital, London, UK. Offspring resulting from these injections were weaned and genotyped by PCR (see section 3.3) at three weeks of age. DNA was isolated from a 1mm tail biopsy by digestion in 100µl of tail digestion buffer. The reaction was incubated at 95°C for 10 minutes then 1mg/ml proteinase K was added and the reaction incubated for 2 hours at 55°C. Proteinase K was inactivated with a final ten minutes at 95°C. Digested tails were centrifuged in a microfuge to settle the debris and 1µl of supernatant added to a 25µl PCR reaction. Primers designed to amplify a cDNA fragment from the vector portion of the POMC BAC, pBeloBAC11, were used (Table 3.3). PCR was carried out in 1x Red Taq buffer, 0.2mM dNTPs, 200nM oligonucleotides with 3U of Genomic Red Taq per reaction, the PCR reaction was heated to 95°C for fifteen minutes before being cycled thirty times through the following temperatures: 95°C for thirty seconds, 55°C for thirty seconds, 72°C for thirty seconds. 20µl of PCR reactions were analysed on a 1% agarose gel (see section 2.2.6).
Table 3.3 Primer set used for genotyping PCR to identify transgenic offspring.

<table>
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<td></td>
<td>3’ GTGCCTGGAGAAATGCTCTT</td>
</tr>
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</table>

3.2.5 Animals
As a result of pronuclear injection several founder mice were identified by genotyping PCR which contained the transgene (CART-AS 3A, CART-AS 4A, CART-AS 5A, CART-AS 6A, CART-AS 13A and CART-AS 18A). These were maintained as separate transgenic lines. Mice were maintained in cages under controlled temperature (21-23°C) and light (eleven hours light/thirteen hours dark) with ad libitum access to food (RM3 diet, SDS UK Ltd.) and water. Animal procedures were approved under the British Home Office Animals Scientific Procedures Act 1986 (Project licence numbers 70/6270 and 70/6402). From the identified founder mice, line CART-AS 13A was selected for further characterisation. Although line CART-AS 5A was the only other line to produce transgenic offspring they failed to exhibit a body weight or food intake phenotype (data not shown).

3.2.6 Food Intake and Body Weight
Food intake and body weight were measured two times per week at 0900h (Adam Equipment, Milton Keynes, UK).

3.2.7 Measurement of Uncoupling Protein-1 mRNA Expression in Brown Adipose Tissue
One hundred milligrams of brown adipose tissue (BAT) was homogenised per 1ml tri reagent and total RNA extracted as described in section 2.2.1. RNA viability was checked using RNA denaturing gel (section 2.2.2). BAT uncoupling protein (UCP-1) mRNA expression was determined by northern blot analysis (section 2.2.3) of 30µg of BAT RNA. A $^{32}$P labelled UCP-1 DNA probe was generated using a primer set specific for the mouse UCP-1 gene and these are detailed in Table 3.4 (accession number BC012701).
### Table 3.4 Primer sequence for UCP-1 DNA probe. The highlighted sequence details the restriction site associated with the particular primer. UCP1 5’ primer corresponds to nucleotides 368-396 and UCP1 3’ primer corresponds to nucleotides 661-687 of the mouse UCP1 gene (accession number BC012701).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCP1 5’</td>
<td>AGCTGGATCCAAGGCCAGGCTCCAGTACT</td>
<td>BamHI</td>
</tr>
<tr>
<td>UCP1 3’</td>
<td>AGCTGAATTCCGTTTGATCCCATGCAGATG</td>
<td>EcoRI</td>
</tr>
</tbody>
</table>

### 3.2.8 Measurement of Cocaine- and Amphetamine-Regulated Transcript peptide from punch biopsy

Mice were killed via CO₂ inhalation, and death confirmed by dislocation of the cervical vertebrae. Following confirmation of death animals were decapitated and brains removed. Brains were then placed on filter paper with the ventral surface facing upwards so that the hypothalamus was visible, and sections lateral to the hypothalamus on either side were cut away using a razorblade. The remaining block of brain was then frozen using isopentane cooled on dry ice. Frozen brains were mounted upon a freezing sled microtome (Shandon Southern Products Ltd, Runcorn, UK) so that a sagittal section of brain was facing upwards. Sections were then removed from the brain until a section approximately 0.675mm from the midline was exposed (as identified using a mouse brain atlas (Figure 3.10)). The brain was then removed from the sled and flipped over so that the opposite sagittal section was exposed and the process repeated. The remaining section of brain was then mounted onto a slide. Slides containing brain sections were kept on dry ice to re-freeze sections and nuclei were identified using the Allen mouse brain atlas. Paraventricular nucleus (PVN) biopsies were removed using a 0.5mm diameter neuropunch (Harvard apparatus, Edenbridge, UK), arcuate nucleus (ARC)/ventromedial nucleus (VMN) biopsies were removed using a 1.0mm diameter neuropunch (Harvard).

Peptide extraction was carried out immediately on all punch biopsies using acetic acid extraction. Localisation and tissue content of punch biopsies are summarised in Figure 3.10.
3.2.9 Acetic Acid Extraction of Peptide

Materials:
0.5M Acetic Acid
Water bath (100°C)

Method
0.5M acetic acid was warmed in a boiling water bath. 500µl of warmed acid was added to each eppendorf containing biopsy tissue which was subsequently placed in the boiling water bath for 20 minutes, ensuring that holes are pierced in the top of the eppendorf to release any pressure. After incubation in the water bath the sample is stored at -20°C until required. CART Activity was then assayed for using radioimmunoassay (RIA) (see section 2.2.12)
Figure 3.10 Location of punch biopsies in the mouse brain.

Mouse brains were placed on a sledge microtome in the sagittal orientation, sections were removed from the brain until a section located approximately 0.68mm from the midline was exposed (as identified using the Allan mouse brain atlas 2008). This process was then repeated on the opposite side of the brain. Punch biopsies were then collected from the remaining slice of brain and homogenised for use in the CART assay. Punch biopsies were collected from:

A) VMH punch biopsy, containing VMN and ARC areas. Diameter 1mm.

B) PVN punch biopsy, containing PVN only. Diameter 0.5mm.

3.3 Results

3.3.1 Identification of POMC Containing Bacterial Artificial Chromosome

Using the UCSC genome browser (http://genome.ucsc.edu) a BAC clone was identified which contained the POMC gene at the centre of its insert. The insert was therefore likely to contain all the necessary regulatory sequences required to replicate POMC like expression \textit{in vivo} (Figure 3.11).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure311.png}
\caption{\textit{In Silico} identification of POMC containing BAC. Screen shot from the UCSC genome browser highlighting the BAC RP24-340C3 which was selected for modification. The position of the POMC gene within the BAC is shown in the box. The RP24-340C3 BAC was selected because of the central position of the POMC gene thus maximising the possibility that all of the necessary regulatory elements are present.}
\end{figure}
3.3.2 Production of the Cocaine- and Amphetamine -Regulated Transcript Antisense Modification Cassette

The components of the modification cassette were generated separately before being cloned into the pFASTBAC Dual vector. Homology arm 1 (~414bp) and homology arm 2 (~282bp) were successfully generated by PCR using primer sets Arm1 and Arm2 (see Table 3.1) and the POMC BAC as a template. The primer set for homology arm 1 contained NheI and BamHI restriction sites. The primer set for homology arm 2 contained NotI and Bsp119I restriction sites. The inclusion of these restriction sites in the primer sequence allowed for cloning into the pFASTBAC Dual vector. Mouse hypothalami total RNA was used as the template for production of the CART-AS insert. An RT reaction was first performed followed by a PCR reaction using the CART-AS primer set (see Table 3.1). The CART-AS primer set included the sequence for the BamHI and SacI restriction enzyme cut sites to facilitate cloning. The resulting products from the PCR reactions can be seen in Figure 3.12. The BGH polyadenylation signal was isolated from the pTRCGW plasmid (Figure 4.1) (a kind gift from Dr J. Gardiner) using the SacI and NotI restriction enzymes. A 2.15kb fragment containing the spectinomycin gene was digested from the pDLOX plasmid (a kind gift from Dr C Huxley) using NotI restriction enzymes.

The components of the modification cassette were sub-cloned into the pFASTBAC Dual vector. To confirm successful incorporation of the inserts into the completed modification cassette restriction digest analysis was performed. The correct integration of homology arm 1 was demonstrated using restriction enzymes Nhe1 and BamHI (Figure 3.13). The resulting digest yielded a product of 414bp indicating successful integration of homology arm 1. There were additional products resulting from the restriction digest; digestion of the spectinomycin gene, due to a BamHI restriction site, yielded products of either 680bp or 2630bp which subsequently yielded a plasmid backbone of 7589bp or 5639bp respectively. The different product sizes resulting from the restriction digest are due to the presence of a BamHI restriction site within the spectinomycin gene and the orientation in which the spectinomycin gene was cloned into the modification cassette. Correct integration of the CART-AS insert was confirmed by the presence of a 369bp product resulting from a restriction digest using the enzymes BamHI and SacI (Figure 3.13). The resulting restriction digests yielded additional products of 300bp or 2261bp due to the presence of the BamHI restriction site within the spectinomycin gene.
This consequently means that the plasmid backbone was either 8003bp or 6053bp. Restriction digest using SacI and NotI showing correct integration of the BGH polyadenylation signal yielded a product of 230bp (Figure 3.14). In addition, a 2.15kb product corresponding to the spectinomycin gene resulted from NotI restriction digest. This was due to NotI restriction sites flanking the spectinomycin gene. A product of 6303bp corresponding to the plasmid backbone was also present. Confirmation of integration of homology arm 2 was confirmed by restriction digest using NotI and Bsp119I (Figure 3.14). The resulting restriction digest yielded a product of 282bp which corresponded to homology arm 2. In addition, a 2.15kb product which corresponded to the spectinomycin gene and a 6355bp product which corresponded to the plasmid backbone were also produced. The results from the restriction digest show that 3 colonies (colonies A, B and E) had correct insert incorporation. Colony B was selected and sequenced confirming that no mutations were present.

3.3.3 Recombination between the Cocaine- and Amphetamine-Regulated Transcript Antisense Modification Cassette and the POMC Bacterial Artificial Chromosome

The CART-AS modification cassette was introduced into the POMC BAC by homologous recombination. Three clones were found to have resistance to chloramphenicol and
spectinomycin. To check for correct integration of the modification cassette a PCR strategy was employed which used three primer sets (1-3) (see Table 3.1 and Table 3.2); primer set (1) POMC out1/POMC out2, (2) POMC out 1/ CART AS F (3) POMC out 2/ Arm 2 F. Two of the clones exhibited the correct 5’ and 3’ prime integration of homology arms 1 and 2 which was confirmed by PCR using primer set (1) POMC out1/POMC out2 where a PCR product of approximately 3.69kb indicated correct integration of the homology arms and the presence of the spectinomycin resistance gene in the modified BAC. The same primer set using the wildtype unmodified BAC as a template gave a PCR product of approximately 4.26kb equal to both homology arms and the POMC gene. The difference in size of products resulting from the PCR reaction for the modified and unmodified BAC is due to the modification cassette being 561bp shorter than the endogenous POMC sequence it replaced via homologous recombination. Primer set (2) POMC out 1/ CART AS F yielded a product of ~957bp in the modified BAC, no product was produced for the wildtype BAC and (3) POMC out 2/ Arm 2 F yielded a product of ~362bp for the modified and unmodified BAC (Figure 3.15).

A successfully modified clone, clone 3, was chosen for the removal of the spectinomycin gene. Following exposure to Cre recombinase 96 clones were picked for positive and negative selection. One was found to be sensitive to spectinomycin and resistant to chloramphenicol. The clone was analysed for the removal of the spectinomycin gene by PCR using primer set 1-3. The PCR product from primer set 1 was approximately 1549bp, compared to a clone containing the spectinomycin gene which would be 3.69kb in length. Using primer sets 2 and 3 produced PCR products of 957bp and 362bp respectively indicating that the spectinomycin gene (~2.15kb) had been successfully removed from the clone and the construct remained intact with no rearrangements occurring (Figure 3.16). This clone was isolated, grown on a large scale and purified for pronuclear injection. Before pronuclear injection the large scale purified BAC was examined for rearrangements by PCR using the strategy employed above (Figure3.17).
Figure 3.13 Restriction digest from completed construct from small scale preparations. (A) The expected results from restriction digest for homology arm 1 (shown in green) and CART-AS (shown in black) are shown. Due to the presence of a BamHI restriction site in the spectinomycin gene alternate products can arise when performing restriction digests which depend on the orientation that the spectinomycin gene was subcloned into the modification cassette, both variations are shown. Correct integration of homology arm 1 yields a product of 414bp. In addition to this a product of either 680bp or 2630bp is produced which is due to a BamHI restriction site in the spectinomycin gene. This in turn resulted in a product of either 7589bp or 5639bp which corresponds to the plasmid backbone. Correct integration of CART-AS yields products of 369bp. In addition to this products of either 300bp or 2261bp are produced due to a BamHI restriction site in the spectinomycin gene. A product of 8003bp or 6053bp is produced which corresponds to the plasmid backbone. (B) Isolated mini prep DNA from six colonies was checked for the correct integration of each component of the modification cassette by restriction digest. Colonies A, B and E showed correct integration. Colony B was selected for sequencing prior to BAC modification. L = 1kb ladder; A-F- mini prep A-F.
Figure 3.14 Restriction digest of completed construct from small scale preparations. (A) The expected results from restriction digest for polyadenylation signal and spectinomycin (shown in yellow) and homology arm 2 and spectinomycin (shown in pink) are shown. Correct integration of the pA tail will yield a product of 230bp. In addition to this a product of 2.15kb spectinomycin is produced as a result of using the NotI enzyme in the digest and a product of 6303bp corresponding to the plasmid backbone. Confirmation of the homology arm 2 by restriction digest using NotI and Bsp119I will produce digest products of 282bp for arm 2 additionally products of 2.15kb corresponding to the spectinomycin gene and 6355bp for the backbone. (B) Isolated mini prep DNA from six colonies was checked for the correct integration of each component of the modification cassette by restriction digest. Colonies A, B and E showed correct integration. Colony B was selected for sequencing prior to BAC modification. L = 1kb ladder; A-F- mini prep A-F.
Figure 3.15 Analysis of 5’ and 3’ integration in the modified POMC BAC. Primer set 1-3 were used to analyse integration in the modified BAC. The expected PCR products are shown for a successfully modified BAC (shown in red) and wildtype BAC (shown in orange) (A). Primer set 1 yields a product of 3.69Kb in the modified BAC and 4.26kb in the unmodified BAC. This is due to the modification cassette being 561bp shorter than the sequence it replaces via homologous recombination. Primer set 2 yields a product of 957bp in the modified BAC. Primer set 3 yields a product of 362bp in the modified and unmodified BAC. Three clones were found to be resistant to chloramphenicol and spectinomycin and were analysed using primer sets 1-3 (B). Clone 1 showed correct 3’ integration but lacked correct 5’ integration. Clone 2 and 3 showed correct 5’ and 3’ integration. Clone 3 was selected to remove spectinomycin. L= 1kb ladder; 1 = PCR product from primer set 1; 2 = PCR products from primer set 2; 3 = PCR products from primer set 3; N.C= negative water control; WT=wild type POMC BAC; C1-3= clone 1-3.
Figure 3.16 Removal of spectinomycin from Clone 3. Primer set 1-3 was used to confirm the removal of the spectinomycin gene. The expected PCR products are shown for a modified BAC with spectinomycin gene successfully removed (shown in red) and a wildtype BAC (shown in orange) (A). Primer set 1 yields a PCR product of 1549bp in the modified clone and 4260bp for the wildtype POMC BAC. The change in size from 3699bp to 1549bp is due to the removal of 2.15kb spectinomycin gene. Primer set 2 yields a PCR product of 957bp for the modified clone. No product is present for the unmodified POMC BAC as the primer cannot bind to the CART-AS sequence. Primer set 3 PCR product is 362bp for both the wildtype and the modified BAC indicating no rearrangements had occurred. The removal of spectinomycin gene from clone three was confirmed by PCR using primer sets 1-3 (B). The clone was isolated, grown on a large scale and purified for pronuclear injection.

L= 1kb ladder; 1 = PCR product from primer set 1; 2 = PCR products from primer set 2; 3 = PCR products from primer set 3; N.C= negative water control; WT=wild type POMC BAC; C3= clone 3.
Figure 3.17 Checking correct arrangement in CART-AS modification cassette prior to pronuclear injection. Primer set 1-3 was used to confirm the removal of the spectinomycin gene from clone 3 prior to pronuclear injection. The expected PCR products are shown for a modified BAC with spectinomycin gene successfully removed (shown in red) and a wildtype BAC (shown in orange) (A). Primer set 1 yields a PCR product of 1549bp in the modified clone and 4260bp for the wildtype POMC BAC. The change in size from 3699bp to 1549bp is due to the removal of 2.15kb spectinomycin gene. Primer set 2 yields a PCR product of 957bp for the modified clone. No product is present for the unmodified POMC BAC as the primer cannot bind to the CART-AS sequence. Primer set 3 PCR product is 362bp for both the wildtype and the modified BAC indicating no rearrangements had occurred. The removal of spectinomycin gene from clone three was confirmed by PCR using primer sets 1-3 (B). The clone was isolated, grown on a large scale and purified for pronuclear injection. L= 1kb ladder; 1-3 = PCR product from primer set 1-3; N.C= negative water control; WT=wild type POMC BAC; C3= clone 3.
3.3.4 Creation of Cocaine- and Amphetamine -Regulated Transcript Antisense Transgenic Mice

The modified CART-AS containing BAC was microinjected into the pronuclei of c57BL/6JxCBA/Ca oocytes by the MRC core transgenic facility at a concentration of 1ng/µl. The injected ova were then implanted into foster females using standard techniques. Tail biopsies were obtained from offspring of the foster mother at 3 weeks of age and their genotype analysed by PCR (see section 3.2.4). Founder mice were identified by a PCR product of 600bp using the BeloBAC primers (Table 3.3). No PCR product will be produced from the reaction using the BeloBAC primers if the mice are wildtype. Four male (CART-AS 3A, CART-AS 4A, CART-AS 5A, CART-AS 6A) and two female transgenic founder mice (CART-AS 13A, CART-AS 18A) were produced as a result of pronuclear injection from a total of 20 mice (Figure 3.18). This represents a transgene integration rate of approximately 30%. The founders were mated with wildtype c57BL/6J mice and each line maintained separately. Subsequent offspring from CART-AS 3A line produced 1 transgenic mouse from a total litter of 4 mice but failed to produce any subsequent litters containing transgenic offspring which suggests the transgene did not incorporate into the germline. CART-AS 5A line produced 19 transgenic mice from a total of 39 mice over five litters. CART-AS 13A produced 44 transgenic mice from a total of 90 mice over fourteen litters. Lines CART-AS 18A, CART-AS 4A and CART-AS 6A did not produce any transgenic offspring. The CART-AS 13A line was used in subsequent characterisation studies. The remaining CART-AS lines were terminated.
Figure 3.18 Identification of transgenic mice by PCR. Tail biopsies obtained at weaning were subjected to a genotyping PCR protocol. 6 transgenic mice, 3-6 and 13 and 18, were identified by a PCR product of 600bp using the BeloBAC primer set. Non transgenic mice produced no PCR product. L= ladder, N.C=water control, WT=positive control, 1-20= mouse tail genotyping DNA (1-12 male and 13-20 female).
3.3.5 Effect on Food Intake and Body Weight
Food intake and body weight were measured from weaning to 32 weeks of age in transgenic mice expressing CART-AS (n= 4) and compared with wildtype litter mates (n= 3). At the start of the study the average body weight of wildtype mice in this study was fractionally higher than the average body weight of the male transgenic mice being 21.64 ± 0.35g and 21.06 ± 0.3g respectively, although this was not significant (P = 0.25, unpaired two tail Students t-test). From week six onwards the transgenic mice exhibited a higher body weight than control animals. Male CART-AS mice were found to have a significantly increased body weight and body weight gain over the 27 week period when analysed using the generalised estimation equation (GEE) (P = <0.001). At the conclusion of the study CART-AS expressing mice exhibited a mean body weight that was 15% higher than that of the control group (Figure 3.19). However, there was no significant difference in cumulative food intake during the study (Figure 3.20).
Figure 3.19 Effect on (A) body weight and (B) body weight gain in mice expressing CART-AS. Body weight of male mice expressing CART-AS (n=4) (shown in blue) and male wildtype (WT) litter mates (n=3) (shown in red) were measured over a 27 week period after weaning. Values shown as mean ± SEM ***P<0.001 as analysed by GEE and compared to control animals over whole time period analysed.
Figure 3.20 Effect on cumulative food intake of CART-AS expression in male mice relative to wildtype litter mate controls. Food intake was measured in CART-AS mice (n=4) (shown in blue) and wildtype (WT) mice (n=3) (shown in red) up to 32 weeks of age. There was no significant difference between the transgenic mice and wildtype mice over the whole time period when analysed using the GEE.
3.3.6 Measurement of Cocaine- and Amphetamine -Regulated Transcript Peptide from Punch Biopsies

CART peptide concentration was measured in ARC/VMN and PVN tissue biopsies by RIA from CART-AS (n= 10) and wildtype mice (n= 13) at eight weeks of age. No significant difference in CART peptide concentration in the ARC/VMN tissue biopsies (P = 0.22 using an unpaired Students t-test) were identified with mean peptide concentration measuring 890.3 ± 67.5 fmol/punch biopsy from transgenic mice and 995.6 ± 104.4 fmol/punch biopsy from the wildtype mice (Figure 3.21). There was a trend towards decreased CART peptide expression in tissue from the PVN in the CART-AS mice when compared with wildtype litter mates (P = 0.07 using an unpaired Students t-test) with average CART peptide concentration being 106.1 ± 21.3 fmol/punch and 158 ± 24.7 fmol/punch respectively which represents a reduction in CART peptide concentration in the order of 30% in the transgenic mice, however this did not reach significance (Figure 3.21).
Figure 3.21 CART peptide concentrations in (A) ARC/VMN punch biopsy and (B) PVN punch biopsy. Concentration of CART peptide was measured by RIA from punch biopsies taken from the ARC/VMN and the PVN taken from CART-AS expressing male mice (n=10) (shown in blue) and wildtype (WT) litter mates (n=13) (shown in red) at 8 weeks of age. Values shown as mean ± SEM.
3.3.7 Measurement of Uncoupling Protein-1 mRNA Expression in Brown Adipose Tissue

UCP-1 mRNA expression was measured in BAT from wildtype (n=2) male mice and male mice expressing CART-AS (n=4) at 32 weeks of age. Expression of CART-AS in the ARC appears to have resulted in a significant decrease in UCP-1 mRNA expression in BAT relative to control animals (P = 0.016) as measured by an unpaired Students t-test (Figure 3.22.).

Figure 3.22 Effect of ARC CART-AS on UCP-1 mRNA expression in mice. BAT UCP-1 mRNA expression was measured in male mice expressing CART-AS (n=4, shown in blue) and from their wildtype (WT) litter mates (n=2, shown in red) at 32 weeks of age. There was a significant reduction in UCP-1 mRNA expression as measured by unpaired Students t-test. Values shown as mean ± SEM *P<0.05.
3.4 Discussion

3.4.1 Choosing an Artificial Chromosome
The RP24_340C3 BAC was chosen for modification with the CART-AS construct as it contains the POMC gene at the centre of its insert to maximise the chance of all regulatory elements that are necessary for accurate POMC like expression being present. Barsh et al recently used a POMC BAC clone to drive expression of green fluorescent protein (GFP) specifically in POMC neurones. The BAC used by Barsh et al contained approximately 60kb of 5’ flanking sequence and 30kb of 3’ flanking sequence (Xu et al., 2005) which suggests all the necessary regulatory elements to recapitulate POMC like expression in vivo are contained within this fragment. Since the RP24_340C3 BAC contained approximately 84kb of 5’ flanking sequence and approximately 70kb of 3’ flanking sequence it can be concluded that it too contains all the POMC regulatory elements needed to drive accurate POMC like expression in vivo.

3.4.2 Antisense
I chose to use antisense technology to reduce CART peptide concentration in the ARC as it is a technique which has been successfully used previously in the central nervous system (CNS). In addition, antisense offers a high level of control over the expression of endogenous genes relative to other techniques such as RNA interference (RNAi). The use of RNAi to study gene function has many advantages since gene silencing is directed using a 21-23 nucleotide fragment that is homologous to the target gene. This allows for almost any gene to be targeted. In addition, the generation of the desired nucleotide fragment is less time consuming than other transgenic methods such as BAC transgenesis. However, the main disadvantage with using RNAi is that it requires the ubiquitous RNA polymerase 3 promoter which restricts the level of tissue specificity that can be obtained using this method. Furthermore, the use of RNAi has been associated with stimulation of the immune response in cells via the interferon pathway which could potentially complicate the interpretation of any resulting phenotype as the activation of cytokine pathways have been demonstrated to alter food intake (Buchanan, 2007). Cre/Lox technology is another method that can be used to target the knockdown of a specific gene, it can also be used to target a specific tissue, but there is greater complexity involved in such undertaking, for example two transgenic mouse lines need to be maintained and interbred. One transgenic line must be generated that
contains the target gene flanked by loxP sequences; this is achieved by modifying ES cells which is technically difficult and expensive due to the requirement of growth factors such as fibroblast growth factor to maintain the cells in pluripotency (Johnson et al., 2008). A second line must contain Cre recombinase under the control of a tissue specific promoter; this line is generated by pronuclear injection. Tissue specific deletion of the gene of interest only occurs when these lines are crossed. Cre can be expressed in most cell types making it a broadly applicable technique. In addition, if cre/lox is used to produce a mouse with the targeted deletion of a gene then the gene function will be lost in 70-80% of cells whereas antisense will reduce gene expression by a maximum of 50% in all cells. Therefore overall there is probably a greater reduction in gene function using cre/lox compared with antisense. However, this method is both time consuming and expensive due to the generation and maintenance of several transgenic lines.

3.4.3 Designing the Modification Cassette

The CART-AS modification cassette was designed to incorporate features that have been shown to be advantageous for homologous recombination efficiency. Homology regions of 42bp have been shown to be effective for recombination, however homology arms of a larger size have been shown to improve recombination efficiency (Zhang et al., 1998). The homology arms used in the modification of the POMC gene were designed to be 300-400bp since this length of homology arm has been successfully used in previous BAC transgenesis studies conducted within the Department of Investigative Medicine (Bewick, 2005). An additional consideration when designing the modification cassette is the distance between the two homology arms, this distance should be kept to a minimum to avoid the potential loss of endogenous regulatory sequences which may result in inaccurate POMC like transgene expression. Importantly, a distance of less than 7bp separating the homology arms has been shown to reduce recombination efficiency. The distance between the two homology arms in the POMC gene was 3.3kb.

The POMC gene is composed of three exons and the homology arms were designed so that homology arm 1 was homologous to the untranslated sequence at the 5’ end of exon one and homology arm 2 was designed so that it was homologous to the 3’ end of exon two. This will result in a recombination event that removes the POMC sequence between the homology arms and inserts the modification cassette. As a result of insertion of the modification cassette
at this site the remaining POMC sequence was maintained, this included the intron sequence following exon two and also exon three. The inclusion of the intron sequence is important as this has been shown to improve transcription efficiency, although the mechanism by which this is achieved is unclear (Brinster et al., 1988; Choi et al., 1991).

The BGH polyadenylation signal was cloned downstream of the CART-AS fragment using SacI and NotI restriction enzymes. The inclusion of a polyadenylation signal has been shown to increase transcription efficiency and ensures that mature transcription will terminate after the antisense sequence (Zhang et al., 1998; Ribeiro et al., 2004). Termination of transcription after the antisense fragment prevents the endogenous POMC sequence being included in the CART-AS mRNA transcript. The presence of additional sequences other than that required for antisense action may have the result of reducing the binding efficiency of the antisense to its target sense strand.

### 3.4.4 Production of Transgenic Mice

The number of transgenic mice arising from each set of pronuclear injection can vary significantly as the insertion of the transgene is a random integration event. The DNA was purified and diluted to a concentration of 1ng/µl in order to reduce toxicity and it is also the optimum concentration for transgene integration.

Typically between 5-30 % of the founder (F0) generation arising from pronuclear injection are transgenic, although it is not unexpected to observe integration rates outside of this range. Pronuclear injection of the CART-AS construct resulted in six out of twenty mice possessing the transgene; CART-AS 3A, CART-AS 4A, CART-AS 5A, CART-AS 6A, CART-AS 13A, CART-AS 18A, representing an integration rate of 30%. These founder mice were then mated with wildtype c57BL/6J mice to initiate separate transgenic colonies. The F1 generation resulting from mating of the founder mice resulted in offspring in the following ratios; Line CART-AS 13A produced male and female transgenic offspring with an overall transgene integration ratio of 48% calculated over 14 litters. This consisted of 41% transgenic male mice and 30% transgenic female mice. The proportion of offspring possessing the transgene would be expected to fall in the range dictated by Mendelian inheritance which is 25%. However, rates much higher than this were observed in the CART-AS 13A line which may be a result of the transgene conferring a selective advantage over wildtype litter mates,
alternatively the modified BAC may have incorporated at more than one site in the genome of the founder mouse. Importantly, the numbers of litters used to calculate transgene transmission are low and if the same calculation was performed including many more generations the rate of transgene transmission would be expected to be closer to that dictated by Mendelian inheritance.

The founder line CART-AS 5A produced transgenic offspring but these did not exhibit any significant phenotype differences relative to their wildtype litter mates which may be caused by position effects leading to the silencing of transgene expression, although any position effect should be reduced by using BAC transgenesis. Alternatively the lack of an observable phenotype may be a result of a low copy number of the BAC integrating into the genome of the founder mouse, as a consequence the level of CART-AS expression may not have been high enough to produce a phenotype. The founder line CART-AS 3A produced transgenic offspring in the ratio of 25% in the first litter, but failed to produce subsequent transgene containing offspring, this is most likely to be the result of mosaic transgene integration where the transgene integrates late in embryonic development and consequently is only present in a subset of cells. Lines CART-AS 18A, CART-AS 6A and CART-AS 4A did not produce transgene containing litters suggesting the transgene failed to integrate into the germline. This is not uncommon and is a result of transgene integration into the host genome after several cycles of cell division meaning the transgene is expressed in a subset of cells of the founder mouse, but not in the germline of the founder mouse so is not transmitted to subsequent generations.

3.4.5 Characterisation of Mice Expressing Arcuate Nucleus Cocaine- and Amphetamine-Regulated Transcript Antisense

Mice expressing ARC CART-AS from line CART-AS 13A were studied for possible differences in phenotype relative to wildtype litter mates.

3.4.5.1 Tissue Characterisation

CART peptide was measured in tissue biopsies of specific hypothalamic nuclei namely the ARC/VMN and the PVN. The male mouse brains were removed as described in section 3.2.8 at 8 weeks of age and the concentration of CART peptide measured by RIA. There was no
significant difference in the concentration of CART peptide in ARC/VMN tissue biopsies between the transgenic and wildtype mice. However, there was a trend for CART peptide concentration to be lower in the transgenic mice relative to control mice in biopsies from the PVN, but did not reach significance (P = 0.07 as measured by unpaired Students t-test).

A potential explanation for the reduction in CART peptide concentration in the PVN, but not in the ARC tissue biopsies, may be due to the spatial difference in the site of synthesis and storage of CART neuropeptide within the POMC neurone since limited CART expression has been identified in the VMN. The cell body of the POMC neurones is found in the ARC and is the site at which the CART peptide is synthesised. However, the CART peptide is stored at the synaptic button of the POMC neurones. The synaptic button of POMC neurones are found at sites within the CNS that POMC neurones terminate in, this includes the PVN, lateral hypothalamus (LH) and dorsal medial nucleus (DMN) (Elmquist et al., 1998; Elmquist et al., 1999). Therefore, in mice expressing the CART-AS transgene it is not surprising to observe a larger reduction in CART peptide concentration at the site of the synaptic button of POMC neurones where the CART peptide is most abundant and this may explain why a greater reduction in CART peptide concentration was observed in tissue biopsies from the PVN compared with the ARC/VMN. Therefore, it would be interesting to investigate the concentration of CART peptide in tissue biopsies from the LH and DMN to establish if CART peptide concentration is reduced at these sites.

3.4.5.2 Food Intake and Body Weight

Food intake and body weight were measured in male mice over a 27 week period following weaning. The results show a significant increase in the body weight of male mice expressing CART-AS. However, the increase in body weight was not due to an increase in food consumption as there were no differences in cumulative food intake between control and transgenic animals.

Previous studies have suggested that CART acts as an orexigenic agent since study paradigms which chronically increase CART peptide in the ARC result in increased food intake and body weight (Abbott et al., 2001; Kong et al., 2003). The studies conducted by Kong et al and Abbot et al investigated the effect of an increase in ARC CART peptide concentration using intra-nuclear injection and polyethylenimine (PEI) gene delivery of a
CART expressing plasmid. Both of these techniques cause a local increase in CART peptide and consequently will only affect neurones within the ARC. As a result of direct injection of CART peptide into the ARC non-CART producing neurones which express the CART receptor will be activated. In addition, CART neurones may potentially be affected through activation of auto-receptors expressed on the CART expressing neurones. The use of gene delivery within the CNS has been found to mainly affect inter-neurones within the nuclei targeted (private communication Dr J. Gardiner). This approach will be likely to increase CART release specifically in the targeted nucleus. This is in contrast to the transgenic approach I have used. A reduction in CART expression in POMC neurones will affect CART release from all POMC projections which includes the ARC but also projections to other hypothalamic nuclei. For example, I found a reduction in CART peptide concentration in the PVN (see section 3.3.5). The different paradigms used between my studies and those by Kong et al and Abbot et al may account for the difference in phenotype reported.

Conversely, transgenic studies have been reported which support my findings of CART having an anorectic role in energy homeostasis. Two models of mice with a targeted deletion of the CART gene have been generated. In the model generated by Wierup et al mice deficient in CART peptide exhibited increased body weight with no significant difference in food intake (Wierup et al., 2005). The phenotype of mice generated by Wierup at al which had a targeted deletion of CART the gene is in agreement with the phenotype demonstrated by mice expressing the CART-AS transgene. A second model was generated by Asnicar et al in which the CART deficient mice exhibited increased body weight and food intake when maintained on a high fat diet (Asnicar et al., 2001). My data suggests a decrease in CART peptide in POMC neurones does not alter food intake in transgenic mice maintained on a diet of regular chow. However, a difference in food intake may have presented if the mice were fed a high fat diet. A potential explanation for the different food intake between the Asnicar et al model and my CART-AS model could be due to the extent to which CART peptide concentration was reduced. The phenotype of CART-AS transgenic mice is attributed solely to the reduction of CART peptide concentration in POMC neurones. The phenotype of the CART knockout mouse is a result of loss of CART gene function throughout the whole organism which may affect other sites within the CNS to cause an increase in food intake.
3.4.5.3 Energy Expenditure

BAT was dissected and UCP-1 mRNA measured by northern blot analysis in male mice at 32 weeks of age. UCP-1 is a surrogate marker for thermogenesis (Margareto et al., 2001). UCP-1 is found in the inner mitochondrial membrane where it allows electrons to flow back across the mitochondrial membrane, bypassing ATP synthase, resulting in the dissipation of the energy stored in the electrochemical gradient as heat (Nicholls and Locke, 1984). Northern blot analysis demonstrated a significant decrease in UCP-1 mRNA expression in transgenic male mice. Therefore the resulting reduction in thermogenesis may account for the observed increase in body weight in CART-AS expressing mice given that they did not exhibit an increase in food intake. The data presented in this chapter suggests that the reduction in UCP-1 mRNA is a result of reduced CART peptide expression in POMC neurones which project to the PVN. This hypothesis is supported by previous studies which have demonstrated that CART administration into the PVN leads to an increase in UCP-1 mRNA through stimulation of sympathetic outflow (Wang et al., 2000).

A potential mechanism by which the CART peptide in POMC neurones may affect energy expenditure could be through modulation of thyrotropin releasing hormone (TRH) release. The PVN is the site of TRH expressing neurones and the main role for TRH is to stimulate the release of thyroid stimulating hormone (TSH) from the anterior pituitary gland. TSH stimulates the synthesis and release of thyroxine (T4) and tri-iodothyronine (T3) from the thyroid gland (Mittler et al). Thyroid function has been linked with energy expenditure since decreased thyroid function in humans is associated with decreased metabolic rate and increased obesity. It would be interesting to investigate whether a reduction in CART peptide in POMC neurones at the PVN is involved in modulating TRH release and subsequently UCP-1 mRNA expression and thyroid function. Current evidence suggests a role for CART in the regulation of TRH. Triple labelling fluorescent in situ hybridisation and immunofluorescence studies demonstrate that CART neurones which also stain positive for POMC, are in contact with TRH neurones in the PVN, suggesting a physical link between CART and TRH neurones (Fekete et al., 2000). Pharmacological evidence suggests a link between CART and TRH release since ICV administration of CART suppresses a fasting induced decrease in TRH in the PVN which suggests that CART acts to stimulate the release of TRH (Fekete et al., 2000). Furthermore, the administration of CART peptide to hypothalamic explants has been shown to increase TRH release, confirming a functional role for CART in the control of TRH release (Fekete et al., 2000). It would be interesting to
investigate proTRH mRNA expression, TSH release and the concentration of thyroid hormone in the CART-AS transgenic mice to establish the potential for CART to regulate energy expenditure through modulation of TRH release and UCP-1 mRNA expression. In addition, it would also be important to study energy expenditure via indirect calorimetry to investigate whether a reduction in activity levels in mice expressing the CART-AS transgene may account for the observed increase in body weight.

In summary, male mice expressing CART-AS specifically in POMC neurones exhibited increased body weight relative to control animals without a significant difference in food intake. A reduction in ARC CART peptide was not demonstrated, but a trend towards reduced CART peptide in the PVN was observed and may account for the reduction in energy expenditure and the resulting increase in weight gain detected in the transgenic mice. These data suggest CART peptide in POMC neurones may be a more important regulator of energy expenditure than food intake, at least in male mice.
Chapter 4

Manipulation of Arcuate Nucleus CART Using rAAV Expressing CART Antisense

4.1 Introduction
Studies in the previous chapter demonstrated how gene expression can be altered in mice using bacterial artificial chromosome (BAC) transgenesis. The application of BAC transgenesis allowed CART-AS expression to be targeted to pro-opiomelanocortin (POMC) expressing cells, resulting in a reduction in cocaine- and amphetamine -regulated transcript (CART) peptide concentration. However, within the arcuate nucleus (ARC) CART is not solely coexpressed in POMC neurones, CART is also colocalised with dynorphin (Elias et al., 2001). The use of recombinant adeno-associated virus (rAAV) addresses some of the questions that are unanswered by BAC transgenesis. rAAV expressing CART antisense (CART-AS) will reduce CART peptide expression in all CART expressing neurones rather than exclusively in POMC cells. The main advantage of using rAAV is the temporal control of transgene expression since it can be administered at different stages in the animal life cycle. This avoids the potential for developmental compensation which can mask the true role of a gene and is a potential confounding factor for germline modification of the genome. This chapter demonstrates how rAAV expressing CART-AS can be used to reduce endogenous CART peptide concentration in the rat ARC.

4.2 Gene Transfer and Expression Methods
The transfer of foreign DNA into cells can be achieved by employing the use of a viral vector. The choice of vector to use will depend on such factors as the target tissue and the length of time transgene expression is required.

4.2.1 Viral Gene Delivery Vectors
Viral gene delivery vectors have been the method of choice for many in vivo gene transfer studies as there are established delivery methods and they can produce long term transgene expression.
4.2.1.1 Herpes Simplex Virus
Herpes simplex virus (HSV) was the first virus to be used in gene transfer studies. HSV has a double stranded (ds) DNA genome which is 150kb long. The virus is rendered replication defective by mutation of immediate early (IE) genes which are required for viral packaging and replication. The main disadvantages of using HSV in gene transfer are the difficulties in maintaining long term gene expression due to cellular toxicity of the virus and the low efficiency of transfection relative to other viral vectors (Neve and Geller, 1995). Furthermore a major factor for consideration is the containment level required for use in the laboratory since it is pathogenic.

4.2.1.2 Lentivirus
Lentivirus belongs to the retrovirus family with the first lentiviral gene transfer vectors being derived from human immunodeficiency virus (HIV) (Miller, 1993). Lentiviruses possess the gag, pol and env genes that are common to all retroviruses which code for structural proteins, viral enzymes such as reverse transcriptase and envelope glycoproteins which are flanked by long terminal repeat elements (LTR) (Miller, 1993). For use as a gene transfer agent the lentivirus is rendered replication defective by the deletion of the gag, pol and env genes, generating a cloning site of approximately 8kb. Once the lentivirus has entered the cell the virus faithfully integrates the sequence flanked by the LTR elements into the host genome, this is true in both dividing and non dividing cells (Naldini et al., 1996). Integration of the transgene into the host cell genome allows for long term gene expression, making it an attractive vector for use in gene transfer studies. The main disadvantage associated with using lentivirus in gene transfer is one of safety. When manufacturing a replication deficient lentiviral vector there is the potential for recombination between the replication defective plasmid and the packaging vector resulting in a replication active virus. As a result, use of lentivirus in gene transfer requires a higher laboratory containment level than that needed for other viral vectors. The ability of a laboratory to meet the necessary containment level is not always practical or desirable, especially as alternative viral vectors are available.

4.2.1.3 Adenovirus
Adenovirus (Ad) consists of a linear dsDNA genome of approximately 36kb encased in an icosahedral capsid. The Ad genome contains four early genes (E1-E4) that are expressed after
infection which are involved in regulating viral and cellular gene expression, viral replication and inhibition of cellular apoptosis. For use in gene transfer Ad is rendered replication defective by deletion of the E1 and E3 genes which provides a cloning site of approximately 8kb. The E1 region contains genes that encode proteins necessary for the expression of genes E2-E4. Replication defective virions are produced in cell culture using a cell line that provides the necessary E1 peptides in *trans* such as the human embryonic kidney (HEK) 293 cell line (Graham et al., 1977). Ad are useful in gene delivery studies as they can infect dividing as well as non dividing cells (Wilson, 1996), however, there are disadvantages associated with using the Ad viral vector. Ad does not integrate into the host genome and provokes an immune response due to Ad viral genes also being transcribed by the host cell. This restricts the potential for long term transgene expression *in vivo* from a period of weeks to months (Ehrengruber, 2001; Slack and Miller, 1996; Yang et al., 1996). Ad has also been shown to be toxic at high titres and infects glial cells preferentially to neuronal cells which limit its use in neuronal studies in the central nervous system (CNS). Modified Ad with deletions in the E2-E4 genes have been manufactured in an attempt to reduce the immune effect of Ad (Gao et al., 1996).

### 4.2.1.4 Adeno-associated Virus

Adeno-associated virus (AAV) is a member of the dependovirus family. A helper virus, commonly adenovirus or herpes virus is required to produce lytic infection by AAV. Over one hundred AAV serotypes have been identified with AAV serotype 2 (AAV-2) being the most studied. AAV-2 has a 4.7 kb single stranded linear DNA genome. It has been shown to preferentially infect neuronal cells rather than glial cells making it an attractive vector for use in neuronal gene transfer studies in the CNS (Bartlett et al., 1998). The AAV genome consists of two open reading frames (ORF) which are flanked by 145bp inverted terminal repeats (ITR). The left ORF contains the *rep* (replication) gene which produces four proteins Rep78, 68, 52 and 40. These genes are required for DNA replication and regulate gene expression in the presence and absence of a helper virus (Pereira et al., 1997). The rep proteins are also thought to direct integration of viral DNA at chromosome 19 on the human genome. This is thought to be possible due to a short sequence on human chromosome 19 being homologous to the rep protein binding sequence on the ITR. The right ORF contains the *cap* (capsid) gene which produces viral capsid proteins VP1, VP2 and VP3.
The viral particle enters the cell via binding to a receptor, in the case of AAV-2 this has been identified as the heparin sulphate proteoglycan receptor (Summerford & Samulski, 1998). Once the virus has entered the cell it translocates to the nucleus, uncoats and releases its viral DNA. Upon infection of the host cell by AAV the virus can integrate into the genome in a latent state or a productive state. For AAV to enter the productive life cycle a helper virus is required. If the helper virus is not present a latent infection occurs (Laughlin et al., 1986).

4.2.1.4.1 Recombinant Adeno-associated Virus

rAAV is adapted from AAV. To generate a virus suitable for use in gene transfer the majority of the endogenous viral genome is removed including the rep and cap genes, rendering the virus replication defective and creating a cloning site of approximately 4.5kb. The ITRs remain in place as they are needed for packaging and integration of the transgene into the host genome. Additional elements are also required to produce efficient transgene expression. These elements include a strong promoter to drive transgene expression, one example is the constitutively active cytomegalovirus (CMV) promoter. The woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) and bovine growth hormone (BGH) polyadenylation sequence are also required to drive transgene expression as they have been shown to enhance transgene expression in vivo (Azzoni et al., 2007; Ribeiro et al., 2004; Xu et al., 2003). These elements are flanked by the ITRs. Vectors are available which contain the ITRs and the additional elements needed for efficient transgene expression. One such plasmid that has been widely used within the Department of Investigative Medicine is the pTRCGW plasmid (Figure 4.1). The first stage of rAAV preparation is to modify the pTRCGW plasmid by subcloning the transgene of interest into the vector, downstream of the CMV promoter. This is achieved using standard molecular biology techniques; PCR, restriction digests, ligation and transformation. The modified vector is then transfected into HEK 293 cells. A helper plasmid is also transfected. The helper plasmid encodes the adenoviral helper genes, E2A and E4, in addition to the genes necessary for viral amplification and packaging; the rep and cap genes. The lack of sequence homology between the plasmid containing the modification cassette and the helper plasmid means that homologous recombination between the two plasmids is not possible. Following viral replication and packaging the HEK cells are lysed and the recombinant virus is isolated and purified.
4.2.1.4.2 Using Recombinant AAV to Study the Physiological Role of Cocaine- and Amphetamine -Regulated Transcript Peptide in the Arcuate Nucleus

AAV has many advantages over other viruses for gene transfer. It is the safest viral vector in terms of pathogenicity in humans thus requiring a lower level of containment for use in the laboratory. In addition rAAV does not stimulate an immune response when administered in vivo (Ehrengruber, 2001). Further to this different serotypes of AAV have been shown to have specificity for certain tissues. The rAAV serotype 2 has been shown to preferentially infect neuronal cells making it ideal for use in neuronal studies in the brain (Kaplitt, 1994; Peel, 2000). In addition, the use of rAAV is associated with high levels of stable gene expression. Injection of β-galactosidase expressing rAAV into muscle resulted in β-galactosidase positive-staining muscle fibres for up to 2 years (Fisher, 1997). The potential for maintenance of long term transgene expression, the lack of immune response associated with administration of rAAV and the availability of serotypes which preferentially infect neuronal cells make it the vector of choice for studying the physiological effect of CART-AS expression in the ARC.

4.2.1.4.3 Advantages and Disadvantages of using Recombinant AAV and Bacterial Artificial Chromosome Transgenesis

BAC transgenesis utilises whole expression domains to drive transgene expression. In the previous chapter a POMC BAC was modified to drive expression of CART-AS which allows for transgene expression to be specifically targeted to cells which express POMC, for example the POMC/CART neurones in the ARC. BAC transgenesis can allow for gene dosage studies to be conducted in order to investigate the effect of different levels of transgene expression. This is possible because the number of modified BACs which integrate into the germline may vary between each founder mouse (see section 3.1.2). However, there are disadvantages to using BAC transgenesis. Germline manipulation has the potential for developmental compensation which can result in a lack of phenotype. In addition, it is a time consuming and expensive process to generate the modified BAC and maintain several lines of transgenic mice.

There are several advantages to using rAAV to study gene function. rAAV allows for temporal control of transgene expression since it can be administered at various stages in the life cycle of the animal. This reduces the chance of developmental compensation. Although if
rAAV is administered early in the life cycle a degree of developmental compensation can occur due to the plastic nature of synaptic connections (Bouret et al., 2004). rAAV is normally delivered directly to the target site, especially in studies involving the CNS, which offers a degree of spatial control of transgene expression. There are disadvantages to using rAAV. Administration of rAAV deep within the hypothalamus requires the use of an injector which may damage areas that it passes through. In addition, there is an inherent degree of variability associated with administration of rAAV in terms of the exact site of injection in each animal and the number and type of neurones that are infected by the virus.

In summary, BAC transgenesis is useful for targeting transgene expression to a specific neuronal population throughout development, for example the effect of CART-AS in the POMC neurone. Gene dosage studies can also be used to establish the effect of differing levels of transgene expression. The use of rAAV allows for the temporal control of transgene expression at a specific target site, such as the ARC. Therefore to establish the physiological role for CART both approaches can be used as they address different questions which when analysed together can offer a physiological overview of the role of the CART peptide in the ARC.

4.3 AIMS:

1) To test the ability of a CART-AS plasmid to reduce CART peptide levels in a CART producing cell line in vitro.

2) To use the subsequent plasmid to produce rAAV expressing CART antisense RNA.

3) To use rAAV-CART-AS to investigate the physiological effect of chronic down regulation of CART signalling in the ARC on energy homeostasis.
4.4 Materials and Methods

4.4.1 Modification of the pTRCGW Plasmid

The pTRCGW plasmid was selected for modification with the appropriate transgene as it contains the CMV promoter, WPRE and the ITRs necessary to produce efficient transgene expression by rAAV in vivo. The first stage in modification of the pTRCGW plasmid (a gift from Professor J. Verhaagen, The Netherlands Brain Institute, Amsterdam) was to generate CART-AS cDNA. Using rat total hypothalami RNA CART-AS cDNA fragments were amplified by RT-PCR (see section 2.2.4) using the CART primer set detailed in Table 4.1. The primer set was designed to amplify full length CART. Due to alternate splicing of the CART mRNA transcript the PCR will produce two fragments of 721bp and 760bp (accession number NM_0171101).

<table>
<thead>
<tr>
<th>Primer Name</th>
<th>Primer Sequence</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>5’CARTAS</td>
<td>AGC-TTG-TAC-AAG-CGA-GGA-AGT-CCA-GCA-CCA</td>
<td>BsrGI</td>
</tr>
<tr>
<td>3’CARTAS</td>
<td>AGC-TGG-ATC-CCG-GGT-TAT-GAT-GTC-ATC-TGC</td>
<td>BamHI</td>
</tr>
</tbody>
</table>

Table 4.1. Primers for PCR amplification of CART-AS for use in rAAV. The highlighted sequence details the restriction site associated with the particular primer. The 5’ primer corresponds to nucleotides 1-20, and the 3’ primer corresponds to nucleotides 741-760 in the rat CART prepropeptide mRNA (accession number NM_0171101).

The 721bp PCR product was subcloned (see section 2.2.7) into the pTRCGW plasmid (Figure 4.1). The first step in subcloning was to cut the CART-AS PCR product and the pTRCGW plasmid using the restriction enzymes BamHI and BsrGI. The use of the restriction enzymes BamHI and BsrGI linearises the pTRCGW plasmid and removes the green fluorescent protein (GFP) gene. The products from the restriction digest were run on a 1% (w/v) agarose gel (see section 2.2.6). The linearised plasmid and the cut CART-AS cDNA fragment were excised from the gel and electroeluted (see section 2.2.7.2). The cDNA fragment was subcloned (see section 2.2.7) into the linearised plasmid by ligation reaction which results in the modified pTRCCART-AS plasmid. The ligated plasmid was then transformed into competent bacteria (see section 2.2.8.1).
Figure 4.1 pTRCGW Plasmid. Map showing the plasmid pTRCGW. ITR, inverted terminal repeats. CMVprom, cytomegalovirus promoter. Gfp, Green fluorescent protein gene. WPRE, woodchuck hepatitis virus posttranscriptional regulatory element. polyA, polyadenylation sequence. AmpR, ampicillin resistance gene.

4.4.2 Transformation of Competent Bacteria

Methods for transformation of bacterial cells include incubation in divalent cations (see section 2.2.8.1) and electroporation. Electroporation involves exposing cells to an electric field, thus increasing the permeability of the cells. Pores are momentarily formed in the cell membrane which allows entry of DNA into the cell. JC8111 bacteria are used in the preparation of pTRCCART-AS plasmid since they lack recombination genes which means the ITRs are not removed by the bacteria. Since JC8111 cells are resistant to transformation by divalent cations, electroporation is used.
Materials
Competent JC8111 bacteria (see section 2.2.8.2)
5mg/ml Glycogen (Ambion, Warrington, UK)
SOB (appendix I)
Products from ligation (section 4.4.1)
LB agar plate with ampicillin (see section 2.2.9)
0.1cm electrode gap cuvette (BTX, San Diego, CA)

Methods
Ligated DNA from section 4.4.1 was precipitated at -20°C for one hour with 2.5 volumes absolute ethanol, 0.1 volumes 2M sodium acetate (NaAc) pH 5.2 (see section 2.2.2) and 1µl glycogen. An aliquot of frozen competent JC8111 bacteria (see section 2.2.8.2) was thawed on ice and then transferred into a 0.1cm electrode gap cuvette. Five microlitres of the resuspended ligation mix was added to the cuvette and this was electroporated. Electroporation conditions were: 1.8kV, 186Ω, 25F in 0.1cm electrode gap cuvette using an Electro Cell Manipulator 600 (BTX, CA). Eight hundred microlitres of warmed SOB was added and the entire volume incubated at 37°C for an hour with shaking. An agar plate containing ampicillin was warmed and 200µl SOB containing the bacteria was then spread evenly across the surface and incubated overnight at 37°C.

Twelve clones were selected and initially grown using small scale culture (see section 2.2.10) which allowed for the analysis of several clones simultaneously. The clones were subjected to restriction digest analysis using BamHI and BsrGI restriction enzymes (see section 2.2.7.1). One clone was selected which had been successfully transformed with the modified plasmid (pTRCCART-AS). The selected clone was grown using large scale amplification (see section 2.2.11).

4.4.3 Testing of Cocaine- and Amphetamine -Regulated Transcript Antisense Plasmid in vitro
The pTRCCART-AS plasmid was initially tested in vitro to ensure that the CART-AS mRNA reduced CART peptide release. RIN1056a CART cells were used in cell culture
experiments. RIN1056a CART cells are RIN1056a cells that have been transfected with the pcDNA3-CART plasmid and express full length CART (Kong et al., 2003).

4.4.4 Tissue Culture

Materials
Dulbecco’s Modified Medium (DMEM) (Gibco, Paisley, UK)
2.5% (w/v) Trypsin (Invitrogen)
Fetal Calf Serum (FCS) (Invitrogen)
Versene (appendix I)

Methods
Cells were cultured in 75cm$^3$ flasks (Nunc, Thermo Fisher Scientific, Denmark) with 10ml DMEM (Gibco, Paisley, UK) supplemented with 10% (v/v) FCS and 5% (v/v) penicillin and streptomycin. The cells were incubated at 37°C in a 5% CO$_2$ atmosphere. Medium was changed every 2 to 3 days and the cells sub-cultured when 70-80% confluent using 0.25% (v/v) trypsin in versene. Briefly, the medium was aspirated from the flask and the cells washed with versene containing 0.25% (v/v) trypsin. This was aspirated and the cells incubated at room temperature with fresh versene/trypsin until they detached from the flask. The trypsin was inactivated by the addition of 10ml fresh medium and the cells recovered by centrifugation for 5 minutes at 100g at room temperature (Mistral 1000, MSE, UK). The cells were resuspended in fresh medium and transferred to new flasks at a dilution of 1:2.

4.4.5 Polyethylenimine Transfection
Polyethylenimine (PEI) has been used widely in vitro (Lungwitz et al., 2005; Neu, 2005) and the low cost associated with this method makes it an attractive method for gene transfer. However, the high charge density associated with high molecular weight PEI complexes results in toxicity to the cell, a major limitation for use in vivo (Godbey, 1999).
4.4.5.1 Polyethylenimine Preparation

Materials
Water free 25kDa PEI (Sigma-Aldrich)
GDW
1M HCl

Methods
To prepare 0.1M PEI, 450mg of high molecular weight, water free PEI was dissolved in 80ml GDW. The solution was adjusted to pH7 with 1M HCl and the volume made up to 100ml using GDW. The PEI solution was then sterilised by passage through a 0.2µm filter. Prepared PEI was stored at room temperature for a maximum of 2 weeks.

4.4.5.2 Optimisation of Transient Transfection using Polyethylenimine and β-galactosidase

To optimise the transfection procedure cells were transfected with the pCMVβ plasmid (appendix 3). Successfully transfected cells appear blue when incubated in 5-bromo-4-chloro-3-indolyl-galactopyranoside (X-gal) and can be visualised under a light microscope. The main factor which affects the efficiency of transfection using PEI is the ratio between the amount of DNA and PEI nitrogen equivalents.

Materials
1mg/ml pCMVβ
0.1M PEI (prepared as described in section 4.4.5.1)
10% (w/v) glucose solution

Methods
Twenty four hours prior to transfection, RIN1056a CART cells were plated at a density of 1x10⁴ cells/cm onto poly-L-lysine coated six well plates (Nunc, Thermo Fisher Scientific, Denmark). Transfections were carried out when the cells were 60% confluent. To optimise the transfection process cells were transfected using 2µg of pCMVβ plasmid DNA with 0, 3, 6, 9, 12 or 15 nitrogen equivalents. This was then repeated using 4µg of pCMVβ plasmid DNA. Plasmid DNA was prepared in 5% (w/v) glucose solution. Three wells were
transfected for each treatment. The 0.1M PEI solution at the appropriate ratio equivalents was slowly added to the DNA solution, vortexed for 30 seconds and allowed to stand at room temperature for 10 minutes before use. The amount of PEI required was calculated according to the ratio of PEI amine nitrogen equivalents to DNA phosphate where 1µl of 0.1M PEI = 100nmol amine nitrogen and 10µg of DNA = 30nmol DNA phosphate. Cells were incubated at 37°C in a humid atmosphere containing 5% CO₂. After three hours the medium containing the transfection mixture was removed and replaced with fresh medium. Forty eight hours post transfection the medium was removed and β-galactosidase gene expression analysed.

4.4.5.3 β-Galactosidase Staining
This method was used to visualise the cells transfected with pCMVB using PEI.

Materials
50 mg/ml X-gal solution (appendix I)
0.1% (v/v) glutaraldehyde in 0.01M PBS (Sigma-Aldrich)
0.01M Phosphate Buffered Saline (PBS) (Sigma-Aldrich) (appendix I)

Methods
Cells were washed twice with 0.01M PBS and then fixed in approximately 2ml 0.1% (v/v) glutaraldehyde for 10 minutes at room temperature. Glutaraldehyde was removed and cells were washed twice in 0.01M PBS and incubated in the dark at room temperature in approximately 1ml of 0.4mg/ml X-gal in 0.01M PBS solution for 24 hours. Expression of β-galactosidase was indicated by the presence of an indigo blue colour on examination by light microscopy.

4.4.6 Transient Transfection with pTRCCART-AS using Polyethylenimine
Following optimisation of transient transfection using PEI, RIN1056a CART cells were transfected with 4µg pTRCCART-AS or pTRCGW (control cells) at 12 PEI nitrogen equivalents as described in section 4.4.5.2 (n= 6 wells). Two days following transfection the media was removed and fresh media added, the fresh media was collected and frozen at -20°C until radioimmunoassay (RIA) (see section 2.2.12) for CART (55-102)-like immunoreactivity at 1, 4 and 6 hours was performed.
4.4.7 Stable Transfection with pTRCCART-AS using Polyethylenimine

Stable transfection allows for selection of successfully transfected cells by cotransfecting the plasmid of interest with a plasmid which confers antibiotic resistance. Therefore, cells which express the transgene will survive in antibiotic containing medium. RIN1056a CART cells were transfected with pTRCCART-AS in combination with pcDNA3 (appendix 3) a plasmid containing a neomycin (Geneticin) resistance gene. Cells were cotransfected with two plasmids as integration usually occurs in tandem. To ensure the CART-AS plasmid was incorporated into selected cells pTRCCART-AS was added in excess to the neomycin resistance plasmid.

Materials
RIN1056a CART cells
1mg/ml pTRCCART-AS
1.3 mg/ml pcDNA3
DMEM (Gibco, Paisley, UK)
50mg/ml Geneticin (Sigma-Aldrich)

Methods
RIN1056a CART cells were plated at a density of 1x10^4 cells/cm onto poly-L-lysine coated six well plates (Nunc, Thermo Fisher Scientific, Denmark). Transfections were carried out when the cells were 60% confluent. Cells were transfected with 10µg pTRCCART-AS and 25ng pcDNA3 using PEI at 12 PEI nitrogen equivalents, as described in section 4.4.6. After 2 days the medium was replaced with DMEM with the addition of 10ml geneticin per 500ml DMEM. Cells were maintained in this medium. RIN1056a CART cells that were stably transfected with the CART antisense plasmid are referred to as CART antisense CART (CAC) cells.

4.4.7.1. Confirmation of pTRCCART-AS Plasmid in RIN1056a CART Cells following a Stable Transfection by Northern Blot Analysis

To confirm the presence of pTRCCART-AS in the transfected cells northern blot analysis was performed. Two clones were selected (CACA and CACB) and cells from each clone were grown to 90% confluence and RNA extracted using tri reagent (see section 2.2.1) and the viability of the RNA was confirmed using a 1% denaturing formaldehyde gel (see section
2.2.2). A northern blot was then carried out to identify and quantify the RNA (see section 2.2.3) using a WPRE DNA probe (see Table 4.2). A 310bp WPRE DNA probe (accession number J04514) was prepared using the primer set detailed in Table 4.2 (see section 2.2.3).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Restriction Site</th>
</tr>
</thead>
<tbody>
<tr>
<td>WPRE 5’</td>
<td>AGC-TGG-ATC-CGC-TAT-GTG-GAT-ACG-CTG-CTT</td>
<td>BamHI</td>
</tr>
<tr>
<td>WPRE 3’</td>
<td>AGC-TGA-ATT-CGG-AAT-TGT-CAG-TGC-CCA-ACA</td>
<td>EcoRI</td>
</tr>
</tbody>
</table>

**Table 4.2** Table detailing the primer sequence for the WPRE DNA probe. The highlighted sequence details the restriction site for the particular primer. The WPRE 5’ primer corresponds to nucleotides 1161-1180, and the WPRE 3’ primer corresponds to nucleotides 1450-1471 in the woodchuck hepatitis B virus genome (accession number J04514).

### 4.4.7.2 Measuring Cocaine- and Amphetamine -Regulated Transcript Expression in RIN1056a CART cells that have been transfected with pTRCCART-AS

CART (55-102) -like immunoreactivity was measured in each clone. The clones CACA and CACB were plated onto 24 well plates (Nunc, Thermo Fischer Scientific, Denmark) and grown overnight to around 90% confluence. The media was removed and fresh media was added. The media was then removed after 1, 2, 4, 6 and 8 hours and stored at -20°C until radioimmunoassay for CART (55-102) -like immunoreactivity (see section 2.2.12) (n=6 well per time point were analysed).

### 4.4.8 Production of Recombinant AAV

AAV was produced by Dr. J. Gardiner (Department of Investigative Medicine) using the two plasmid system. Briefly, host cells (HEK293) are co-transfected with the rAAV plasmid (Figure 4.1) and a helper plasmid pDG (appendix 3) using calcium phosphate. There is no homology between the two plasmids so they cannot recombine and form a wild-type virus. This results in the production of rAAV particles. To release the particles cells are lysed by freeze thawing and the rAAV produced is purified.
4.4.8.1 Cell maintenance
HEK 293 cells were maintained as described in section 4.4.4.

4.4.8.2 Calcium Phosphate Transfection

Materials
HEK 293 cells
1mg/ml pTRCCART-AS
1mg/ml pDG (appendix 3)
10x HEBS buffer:
1.36M NaCl
0.05M KCl
0.007M Na$_2$HPO$_4$.2H$_2$O
0.2M HEPES (appendix I)
0.125g glucose
2M CaCl$_2$ (appendix I)

Methods
HEK 293 cells were sub-cultured into a ten chamber cell factory (L x W x H (mm): 335 x 205 x 190, culture area: 6867cm$^2$) (Nunc, Thermo Fischer Scientific, Denmark) at 2.5x10$^6$ cells per chamber and incubated in 2L of DMEM with 10% (v/v) FCS at 37°C in an atmosphere of 5% CO$_2$. Twenty four hours later, when the cells were 50% confluent, the culture medium was replaced and the transfection was performed after a two hour incubation period at 37°C in an atmosphere of 5% CO$_2$. The transfection mixture was prepared for the cell factory, it consisted of 560µg pTRCCART-AS and 1.68mg pDG, in 12 ml of 1xHEBS buffer in 52ml GDW. Immediately prior to transfection, 7.2ml 2M CaCl$_2$ was added and mixed gently. The transfection mixture was allowed to stand for four minutes at room temperature and added drop wise to the cell medium while gently swirling the cell factory. Cells were incubated for six hours at 37°C in 5% CO$_2$ atmosphere.
4.4.8.3 Recovery and Purification of Recombinant AAV

Materials
0.01M PBS (appendix I)
Sepharose Column (GE Healthcare, UK)
1.32g/ml Iodixanol (Life Sciences Technology, Eggenstein, Germany)
0.3x PBS/0.1M NaCl
0.3x PBS/2mM EDTA
2M MgCl$_2$
250U/µl Benzonase (Novagen, Nottingham, UK)
Lysis buffer (appendix I)

Methods
After 48 hours incubation, following transfection, cell medium was removed and cells were washed using 500ml PBS and then harvested using 500ml PBS/2mM EDTA, the remaining cells were removed using a further 500ml PBS/2mM EDTA. Cells were then centrifuged at 5000g for 10 minutes at 4°C (HB-4 rotor in RC-5B super speed centrifuge, Du Pont) washed in a further 150ml PBS and then re-centrifuged at 5000g for 10 minutes at 4°C (HB-4 rotor in RC-5B super speed centrifuge, Du Pont). Cells were then resuspended in a total of 60ml lysis buffer in 50ml falcon tubes (15mls in each falcon tube). Cells were exposed to 3 freeze thaw cycles which consisted of ten minutes in dry ice/ethanol bath followed by incubation in a 37°C waterbath for 10 minutes. 7.3µl 2M MgCl$_2$ and 1.2µl Benzonase were then added to each falcon tube and incubated at 37°C for 30 minutes. The cells were then centrifuged at 2000g for 10 minutes at 4°C (Mistral 1000 rotor, MSE UK) to pellet cell debris. The supernatant was removed and loaded into four 50ml polyallomer tube (Beckmann, CA) and under layered with iodixanol gradient (15%, 25%, 40% and 60% iodixanol). The tubes were then topped up with lysis buffer, heat sealed and centrifuged at 69,000g for 1 hour at 18°C (Type 70Ti rotor in a Sorvall SE100 centrifuge). To remove the rAAV particles the top of each tube was punctured with an 18G needle and rAAV collected by puncturing the tube, and collecting a 5ml fraction from the 60/40% interface of the iodixanol gradient. The fractions from the 4 tubes were pooled, added to 20ml PBS and loaded onto a Sepharose column (GE Healthcare, UK) (pre-washed with 25ml 0.1M NaCl/0.3x PBS and 50ml 0.3x PBS). rAAV was then washed with 50ml 0.3x PBS and eluted with 15ml 350mM NaCl/0.3xPBS. The elutant was transferred to an Apollo Concentrator (Orbital Biosciences, MA, USA)
containing 10ml PBS and centrifuged at 2000g for 5 minutes at room temperature (Mistral 1000 rotor, MSE UK). The Apollo concentrator is an ultrafiltration device used for the concentration or purification of proteins. The flow through was discarded and a further 5ml PBS added below and 19.5ml PBS above the filter. The tube was then recentrifuged at 2000g for 5 minutes at room temperature (Mistral 1000 rotor, MSE UK) and the 0.5ml rAAV remaining in the filter was aliquoted into siliconized eppendorf tubes (Sigma-Aldrich). The viral titre was quantified by dot blot analysis (section 4.4.8.4.)

4.4.8.4 Determination of Total Viral Particle Titre by Dot Blot Analysis
The level of transgene expression is affected by the rAAV titre so total viral particle number was determined by dot blot analysis. Dot blot analysis is based in similar principles to Southern blots; a specific probe can be used to quantify a specific DNA or RNA species in a sample. Rather than being separated by size and transferred to a membrane, the sample being analysed is placed directly onto a membrane. The DNA or RNA species present in the sample can be detected and quantified using radiolabelled probes.

Materials
rAAV viral preparation (section 4.4.8.3)
50ng/µl pTRCGW (for use as a standard)
Solution A (appendix I)
  10mM Tris/HCl pH8.0
  10mM MgCl2
  10µg/ml DNaseI
Solution B (appendix I)
  10mM Tris/HCl pH8.0 (appendix I)
  100mM NaCl
  10mM EDTA (appendix I)
  0.5% (w/v) SDS
  1mg/ml proteinase K
Phenol/chloroform(appendix I)
3M sodium acetate (appendix I)
Absolute Ethanol
5mg/ml Glycogen (Ambion, Warrington, UK)
Denaturing solution (appendix I)
Neutralising solution (appendix I)
Hybridisation buffer (appendix I)
Amasino wash buffer (appendix I)
Universal wash buffer (appendix I)

Methods

Forty five microlitres of solution A was added to 5µl rAAV virus stock and incubated for 30 minutes at 37°C. Two hundred microlitres of solution B was added and incubated at 55°C for 30 minutes before the DNA was extracted by adding an equal volume of phenol/chloroform (see section 2.2.7.1). Ethanol precipitation of the DNA was performed by adding 1/10 volume NaAc, 40µg glycogen and 2.5 volumes ice cold ethanol. After 20 minutes at -70°C the solution was centrifuged at 8000g at 4°C (centrifuge 5417 C/R, Eppendorf, Hamburg, Germany), the supernatant removed, the pellet washed in 75% cold ethanol and air-dried. The pellet was resuspended in 10µl GDW and 1µl applied to a nylon membrane (Hybond-N). A series of dilutions of pTRCGW (50ng, 25ng, 10ng, 5ng, 2.5ng, 1ng, 0.5ng, 0.25ng and 0.1ng) were also applied to the membrane to act as a standard curve. The membrane was washed for 5 minutes in denaturing solution, then washed twice for 5 minutes in neutralising solution. The membrane was then baked at 80°C for 2 hours. The baked membrane was placed in a heat sealed polythene bag with 20ml of pre-hybridisation solution and incubated at 60°C for a minimum of two hours (see section 2.2.3.2). The WPRE probe (Table 4.2) was then prepared as described in section 2.2.3.3, boiled for five minutes and then added to the 20ml pre-hybridisation buffer. Hybridisation was carried out overnight at 60°C. The following day the membrane was washed as described in section 2.2.3. The membrane was then placed on a phosphorimager screen overnight. Radiolabelled areas were visualised and quantified by image densitometry using ImageQuant software (Molecular Dynamics). Quantification was performed by comparing viral DNA to known amounts of pTRCGW in the standard curve.
4.4.9 Recombinant AAV Mediated Transfer of Cocaine- and Amphetamine-Regulated Transcript Antisense in the Arcuate Nucleus

4.4.9.1 Animals

Adult male Wistar rats (Charles River, Bicester, UK) were maintained in individual cages under controlled temperature (21-23°C) and light (12 hours light, 12 hours dark) with ad libitum access to food and water. All animal procedures were approved under the Animal Scientific Procedures Act 1986. For all studies rats were randomised into groups of equal mean body-weight (within 2g). Body-weight and food intake were measured at least three times per week. Food was weighed on balances accurate to 0.01g (Adam Equipment, Milton Keynes, UK). Animals which were considered unwell were excluded from studies.

4.4.9.2 Intra-nuclear Injection of Recombinant AAV

Animals were anaesthetised by intraperitoneal (i.p.) injection of xylazine (Rompun 12mg/kg, Bayer UK Ltd, Bury St Edmonds, UK) and ketamine (Ketalar 60mg/kg, Parke-Davis, Pontypool, UK) in a 2:5 ratio. Prophylactic antibiotics were administered to prevent post-operative infection, an intraperitoneal injection of amoxicillin sodium (Amoxil, 37.5mg/kg) and flucloxacillin (37.5mg/kg) was given prior to surgery. Animals were then placed in a Kopf stereotaxic frame (David Kopf instruments, supplied by Clark Electromedical Instruments, Kent, UK) with the incisor bar set at 3mm below the interaural line. The head was cleaned with 10% (w/v) povidone/iodine solution (Betadine, Seton Scholl Healthcare, UK). A 1.5cm incision was made in the scalp and the skull surface exposed by removal of the periosteum. A hole was drilled using a stereotactic electric drill (David Kopf Instruments). In order for rAAV to be injected bilaterally, two holes were drilled, one each side of the bregma according to the co-ordinates of Paxinos and Watson (Paxinos and Watson, 1998) as shown in Table 4.3.

<table>
<thead>
<tr>
<th></th>
<th>Posterior/Anterior from bregma</th>
<th>Lateral from bregma</th>
<th>Cannula length</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arc</td>
<td>-3.8mm</td>
<td>± 0.5mm</td>
<td>9.5mm</td>
</tr>
</tbody>
</table>

Table 4.3 Table showing the co-ordinates for intra-nuclear injections into the rat arcuate nucleus.
A 26-gauge stainless steel cannula (Plastic One inc.) was lowered into the hypothalamus. The 33-gauge stainless steel injector (Plastic One inc.) was connected by polythene tubing (inner diameter 0.5mm, outer diameter 1mm) to a Hamilton syringe (Fisher Scientific, Leicestershire, UK) in a Harvard infusion pump (Harvard Apparatus, Massachusetts, USA). The tubing was filled with water and an air bubble drawn up to separate the experimental compound from the water. Injection of rAAV was carried out while the animals remained anaesthetised, at a rate of 12µl per hour, until 1µl rAAV-CART-AS (11.8ng) (n=23) or rAAV-eGFP (n=18) had been injected, via a stainless steel injector projecting 1mm beyond the tip of the cannula. Following injection of the appropriate volume the injector was left in place for 5 minutes before removal. The cannula was removed and used to guide the second injection for bilateral injection. The rats were rehydrated by an i.p. injection of 0.9% sodium chloride (5ml/rat). Animals received a single subcutaneous injection of buprenorphine (45mg/kg, Schering-Plough Corp, Welwyn Garden City, UK) for analgesia post-operatively. The incision was closed with Ethilon 5.0 sutures (Johnson and Johnson, Livingston, UK) which were removed after 5 to 7 days. Following injection, animals were returned to their home cages with a pre-weighed amount of chow.

4.4.9.3 Confirmation of Transgene Expression by In situ Hybridisation

Transgene expression was investigated in male Wistar rats injected bilaterally into the ARC with rAAV-CART-AS (n=3) or rAAV-eGFP (n=2).Brains were removed and in situ hybridisation (see section 2.2.13.2) performed using a $^{35}$S labelled 248bp CART riboprobe which was generated using the primer set detailed in Table 4.4 (accession number NM_017110). Transgene expression was investigated in animals 26 days post-injection. In the remaining animals, food intake and body weight were measured with transgene expression being investigated at the end of the study, 130 days post-injection.
Table 4.4 Table detailing the primer sequence used to generate the CART riboprobe. The highlighted sequence details the restriction site for the particular primer. The CART 5’ primer corresponds to nucleotides 300-318, and the CART 3’ primer corresponds to nucleotides 529-548 in the CART mRNA sequence (accession number NM_017110).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>CART5'</td>
<td>AGC-TGA-ATT-CCA-TGT-GTG-ACG-CTG-GAG-AGC</td>
<td>EcoRI</td>
</tr>
<tr>
<td>CART3'</td>
<td>AGC-TGG-ATC-CTG-AAG-CAG-CAG-GGA-AAG-AGC</td>
<td>BamHI</td>
</tr>
</tbody>
</table>

4.4.10 Characterisation of Phenotype Resulting from Intra-arcuate Injection of Recombinant AAV- Cocaine- and Amphetamine -Regulated Transcript Antisense

4.4.10.1 Effect on Food Intake and Body Weight
Male Wistar rats were maintained as described in section 4.4.9.1. Food intake and body-weight were measured three times per week at 0900h, rAAV-CART-AS (n=10) and rAAV-eGFP (n=8).

4.4.10.2 Effect on Neuropeptide Release from Hypothalamic Slices
Male Wistar rats that were injected bilaterally into the Arc with rAAV-CART AS (n=10) or rAAV-eGFP (n=8) (see section 4.4.9.2) were killed 26 days post injection and the release of hypothalamic neuropeptides measured using the static explant system. Male Wistar rats were killed and their brains immediately removed. Each brain was mounted with the ventral surface uppermost on a precooled block which was then placed in a vibrating microtome (Microfield Scientific Ltd. Dartmouth, Devon, U.K.) and placed in artificial cerebrospinal fluid (aCSF) (appendix I) which was equilibrated with 95% O₂ and 5% CO₂. A 1.9mm slice was taken from the basal hypothalamus to include the MPOA, SON, PVN, DMN, VMN, LH and ARC. The hypothalamic blocks were transferred to Sarstedt tubes (Sarstedt Ltd., Leicester, UK) each containing 1ml aCSF. The tubes were placed on a platform in a water bath maintained at 37°C for two hours. After a 2 hour equilibration period, hypothalami were incubated for 45 minutes in 600µl aCSF (basal period), followed by exposure to 56mM KCl, for 45 minutes, where aCSF was prepared with K⁺ substituted for Na⁺. After each period,
aCSF solution was removed and immediately stored at -20°C for analysis. Samples were analysed by RIA (see section 2.2.12) for CART (55-102) and α-MSH –like immunoreactivity. Mean neuropeptide release was compared between animals injected with rAAV-CART-AS and rAAV-eGFP.

4.4.10.3 Metabolic Characterisation using the Comprehensive Laboratory Animal Monitoring System

The Comprehensive Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, Ohio, USA) is an automated metabolic cage system that allows simultaneous and continuous measurement of food intake, locomotor activity and metabolic parameters such as CO₂ production and O₂ consumption. Animals were individually housed in plexiglass cages (Columbus instruments) with water and food available ad libitum. Side-feeders containing powdered RM1 food (SDS Ltd, Witham, UK) rested on balances directly linked to a computer measuring food intake every 30 minutes. Cumulative food intake was automatically calculated. The locomotor activity of each individually housed animal was measured by the number of optical photo-beams (Opto M3, Columbus Instruments) broken in a given sampling period. Cumulative activity counts along the x and z axes were recorded simultaneously every 30 minutes and were used to determine horizontal X ambulatory (XAMB) and Z Total (ZTOT) activity scores. XAMB measures the number of consecutive beams broken from horizontal movement of the animal, whereas ZTOT detects rearing movements. Sensors measuring O₂ and CO₂ levels (Columbus Instruments) were calibrated to a gas standard (CO₂: 0.5%, O₂: 20.5%, N₂: 79%, BOC Gases, Guildford, Surrey, UK). Room concentration of O₂ and CO₂ were calculated from air passed through a reference cage. The range of detection of O₂ is 18.9-21.2% and CO₂ is 0-0.8% with a variation less than 1% for both gases. These background gas levels were used as a reference for sample air sequentially obtained from all animal cages, at a flow rate of 0.6L/min. Each cage was sampled for 1 minute at 30 minute intervals. Background gas levels were re-recorded every 8 cages in order to maintain an accurate control for all the indirect calorimetry readings. The O₂ consumption and CO₂ production were corrected for body-weight in each animal. Respiratory exchange ratio (RER) was calculated as the change in CO₂ production (VCO₂) divided by change in oxygen consumption (VO₂). RER is used as an indicator of fat oxidisation, reduced RER is associated with increased fat oxidation and increased RER is associated with increased carbohydrate utilisation.
Male Wistar rats injected with rAAV-CART-AS (n=10) or rAAV-eGFP (n=8) (described in section 4.4.9.2) were analysed in metabolic cages for 3 days. This was carried out 125 days post rAAV injection. Animals were allowed ad libitum access to food for the first two study days since fasting induces an increase in voluntary locomotor activity (Nishimura et al., 1996). On the third study day fasting blocks were placed in the feeders to prevent access to food. This was because food intake is associated with postprandial thermogenesis (Ricquier, 2006) which is a process whereby energy is expended digesting and processing food that has been consumed. Therefore to avoid the confounding effects of food intake on VCO$_2$ and VO$_2$ measurements animals were fasted during these measurements. Body-weight was measured each morning between 0900 and 1000h, and this was used to normalise O$_2$ consumption and CO$_2$ production data. All parameters were measured every 30 minutes.

4.4.10.4 Tissue Analysis
Male Wistar rats injected with rAAV-CART-AS (n=10) or rAAV-eGFP (n=8) were killed by CO$_2$ asphyxiation 130 days post-injection. Blood was removed by cardiac puncture into lithium heparin tubes containing 0.07µg aprotinin (10,000 KIU/ml, Nordic Pharma, Reading, UK), these were centrifuged at 12000g for 15 minutes at 4°C (centrifuge 5417 C/R, Eppendorf, Hamburg, Germany), the plasma was removed and stored at -70°C. Epididymal white adipose tissue (WAT) and brown adipose tissue (BAT) from the intra-scapular region was dissected out and weighed. BAT was snap frozen in liquid nitrogen and stored at -70°C for further analysis.

4.4.10.5 Measurement of Uncoupling Protein-1 mRNA Levels in Brown Adipose Tissue
BAT was homogenised in 1ml Tri-reagent and total RNA was extracted (see section 2.2.1). BAT uncoupling protein-1 (UCP-1) mRNA levels were determined by northern blot analysis (see section 2.2.3) using 50µg of total BAT RNA and a $^{32}$P labelled 307bp UCP-1 DNA probe which was produced using the UCP-1 primer set in Table 4.5 (accession number M11814).
Table 4.5 Table detailing the primer sequence for the UCP-1 DNA probe. The highlighted sequence details the restriction site of the particular primer. The primer UCP-1 5’ corresponds to the nucleotides 350-369 and the primer UCP-1 3’ corresponds to nucleotides 638-657 of the rat mitochondrial BAT mRNA sequence (accession number M11814).

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer Sequence</th>
<th>Restriction site</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCP-1 5’</td>
<td>ACG-TGG-ATC-CTG-TCT-TAG-GGA-CCA-TCA-CCA</td>
<td>BamHI</td>
</tr>
<tr>
<td>UCP-1 3’</td>
<td>ACG-TGA-ATT-CGC-ATT-GTA-GGT-CCC-AGT-GTA</td>
<td>EcoRI</td>
</tr>
</tbody>
</table>

4.4.10.6 Measurement of Plasma Leptin Levels
Plasma leptin levels were measured in animals injected with rAAV-CART-AS (n=10) and rAAV-eGFP (n=8) 130 days post-injection. Leptin levels were measured by enzyme linked immunosorbent assay (ELISA) (CrystalChem, Illinois, USA) according to the manufacturer’s instructions. Briefly, the antibody coated microplate was washed twice with 300ml wash buffer. Forty five microlitres of sample diluent was added to each well along with 50µl guinea pig anti-leptin serum. Samples (5µl) and standards (0, 0.2, 0.4, 0.8, 1.6, 3.2, 6.4 and 12.8ng/ml) were added and the microplate covered and incubated overnight at 4ºC. The contents of each well were aspirated and wells washed five times with 300µl wash buffer. One hundred microlitres of anti-guinea pig immunoglobulin G (IgG) enzyme conjugate was added to each well and the microplate covered and incubated for 3 hours at 4ºC. Contents were removed and each well was washed seven times in 300µl wash buffer. One hundred microlitres of enzyme substrate solution was added to each well and allowed to react for 30 minutes at room temperature in the dark. Finally, 100µl enzyme reaction stop solution was added to each well and the absorbance was measured using a plate reader. Absorbance was measured at 450 and 360nm, and the final value calculated by subtracting the A_{360} value from the A_{450} value. Concentrations of standards were plotted on a semi-log scale and the sample concentrations interpolated from this graph.
4.5 Results.

4.5.1 Production of the pTRCCART-AS Plasmid
The CART-AS insert was generated separately before being subcloned into the pTRCGW plasmid to produce the modified pTRCCART-AS plasmid vector. Rat hypothalami total RNA was used as a template for the production of the CART-AS insert. A RT reaction was first performed (see section 2.2.4) followed by a PCR reaction (see section 2.2.5) using the CART primer set detailed in Table 4.1. The resulting PCR produced products of 760bp and 721bp. The 721bp CART PCR product was used in the production of rAAV-CART-AS. The CART primer set included the sequence for the BsrgI and BamHI restriction enzyme cut sites to facilitate cloning. The resulting product from the PCR reaction is shown in Figure 4.2.

![Figure 4.2 PCR production of CART-AS. Results from the PCR reaction to generate CART-AS using PCR primer set detailed in Table 4.1. Lane 1-1kb ladder; Lane 2- CART-AS DNA (721bp and 760bp).](image)

4.5.2 Optimisation of Transient Transfection using Polyethylenimine
RIN1056a CART cells were transfected with the pCMVβ plasmid. Different PEI nitrogen equivalents in combination with different DNA amounts were used. Low transfection efficiency (<5%) was observed following transfection with 3, 6 and 15 PEI equivalents in
combination with 2µg pCMVβ (data not shown). Transfection with 9 or 12 PEI equivalents was repeated in combination with 2 and 4µg pCMVβ. Results for this are shown in Table 4.6.

<table>
<thead>
<tr>
<th>PEI Equivalents</th>
<th>µg pCMVβ</th>
<th>Mean % cells transfected (± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>0 2 4</td>
<td>0 8 ± 0.16 30±1.35 0 7.5±0.34 35±0.88</td>
</tr>
<tr>
<td>12</td>
<td>0 2 4</td>
<td></td>
</tr>
</tbody>
</table>

**Table 4.6** Effect of PEI:DNA ratio and DNA concentration on transfection efficiency in RIN1056a CART cells. Values are given as mean percentage of cells transfected ± SEM. **P<0.01, ***P<0.001 compared with control cells when analysed using a one way-ANOVA followed by a post-hoc Turkeys multiple comparison test.

### 4.5.3 Northern Blot Analysis of RIN1056a CART cells following Stable Transfection with the pTRCCART-AS Plasmid

Northern blot analysis using a WPRE DNA probe (Table 4.2) detected WPRE mRNA expression in both CAC cells lines. No WPRE mRNA expression was detected in RIN1056a cells. (Figure 4.5).

![Northern blot analysis of RNA from RIN1056a CART cells following stable transfection with pTRCCART-AS.](image)

**Figure 4.3** Northern blot analysis of RNA from RIN1056a CART cells following stable transfection with pTRCCART-AS. RIN1056a (lane 1), CACA (lane 2), CACB (lane 3), RIN1056a CART (lane 4) cells probed with a $^{32}$P- dCTP WPRE DNA probe.
4.5.4 Transient Transfection with pTRCCART-AS using Polyethylenimine

Transient transfection of RIN1056a CART cells with 4µg pTRCCART-AS, using PEI at 12 nitrogen equivalents, did not have a significant effect on CART (55-102)-like immunoreactivity when measured by RIA (Figure 4.3) and analysed using an unpaired Students t-test (at 1 hour P = 0.86, at 4 hour P = 0.90 and at 6 hour P = 0.93).

4.5.5 Stable Transfection with pTRCCART-AS using Polyethylenimine

Stable transfection of RIN1056a CART cells with pTRCCART-AS and 0.25ng pcDNA3 significantly reduced production of CART (55-102)-like immunoreactivity for up to 8 hours (n=6 wells per time point). Release of CART (55-102)-like immunoreactivity was significantly reduced at 1, 2, 4, 6 and 8 hours compared to release from RIN1056a CART cells. CART release between 0-8h was 22.21 ± 1.43 fmol/µl [RINA1056a CART], 17.02 ± 1.00 fmol/µl [CACA], P< 0.05, 16.38 ± 0.10 fmol/µl [CACB], P< 0.05)) (Figure 4.4).
Figure 4.4 Release of CART (55-102)-like immunoreactivity from cells transiently transfected with pTRCCART-AS. Release of CART (55-102)-like immunoreactivity at 1, 4 and 6 hours from RIN1056a CART cells and RIN1056a CART cells transfected with 4µg pTRCCART-AS using 12 PEI nitrogen equivalents Values represent mean ± SEM (n=6 wells).
Figure 4.5 CART release from RIN1056a CART cells and CAC cells. Release of CART (55-102)-like immunoreactivity at 1, 2, 4, 6 and 8 hours from RIN1056a CART cells and CART antisense CART (CAC) cells were studied. The clones CACA and CACB were stably transfected with 10µg pTRCCART-AS and 0.25ng pcDNA3 plasmid, using 12 PEI nitrogen equivalents. Values represent mean ± SEM, *P<0.05, **P<0.01 (n=6 wells per time point) when compared with RIN1056a CART cells using one way ANOVA followed by a post-hoc Turkeys multiple comparison test.
4.5.6 Determination of Total Viral Particle Titre by Dot Blots Analysis
Dot blot analysis using linear regression of standard curve values revealed 1µl of rAAV-CART-AS fraction contained 11.8ng/µl viral DNA (Figure 4.6). This is $2.91 \times 10^{13}$ virions per ml (appendix 4).

![Figure 4.6 Dot blot analysis of rAAV-CART-AS.](image)

**Figure 4.6 Dot blot analysis of rAAV-CART-AS.** Total viral particle titre was detected using a $^{32}$P labelled WPRE DNA probe. A standard curve of 50ng, 25ng, 10ng, 5ng, 2.5ng, 1ng, 0.5ng, 0.25ng, 0.1ng pTRCGW were analysed. Duplicate samples, neat, 1:10 and 1:50 dilutions are also shown.

4.5.7 Recombinant AAV Mediated Transfer of Cocaine- and Amphetamine-Regulated Transcript Antisense in the Arcuate Nucleus

4.5.7.1 Confirmation of Gene Transfer by In situ Hybridisation
*In situ* hybridisation was carried out on brain slices from rats that had been injected with rAAV-CART-AS using a $^{35}$S labelled CART sense probe (Table 4.4). The CART sense probe is required to detect any CART antisense that is being expressed as a result of injection of rAAV-CART-AS into the ARC. The CART sense probe will bind in a complementary fashion to CART-AS mRNA, but will not bind to endogenous CART mRNA. To confirm the specificity of detection of CART-AS expression by the CART sense probe an *in situ* hybridisation was performed on rat brain slices that were not injected with rAAV-CART-AS (Figure 4.7).
Figure 4.7  *In situ* hybridisation demonstrating rAAV-CART-AS expression. *In situ* hybridisation using $^{35}$S labelled CART riboprobe showing expression of CART-AS in 13µm coronal sections of a male Wistar rat brain approximately -3.8mm from bregma. Brains were removed 125 days after bilateral intra-Arc injection. Binding of CART sense riboprobe, which is used to detect CART-AS expression, in a rat injected with rAAV-CART-AS is shown in (A) and (C), CART sense riboprobe used to detect CART-AS expression is shown in an untreated rat (B) highlighting the specificity of the CART sense probe. Binding of the CART sense probe is indicated by a black arrow. The binding area of the CART-AS probe shown in (A) is enlarged in (C). The scale bar  is 2mm.
4.5.7.2 Effect of Cocaine- and Amphetamine -Regulated Transcript Antisense on Food Intake

Bilateral intra-ARC injection of rAAV-CART-AS significantly increased cumulative food intake over the 124 days measured, compared to animals injected with rAAV-eGFP (P<0.05 when analysed by the generalised estimation equation (GEE) (Figure 4.8).

**Figure 4.8 Cumulative food intake.** Effect of bilateral intra-ARC injection of rAAV-CART-AS on cumulative food intake in male Wistar rats. Animals were injected with rAAV-CART-AS (n=10) (shown in blue) or rAAV-eGFP (n=8) (shown in green) and cumulative food intake measured for 124 days post-injection. Values shown as mean ± SEM. *P<0.05 compared to animals injected with rAAV-eGFP and analysed using the GEE over whole time period.
4.5.7.3 Effect of Cocaine- and Amphetamine -Regulated Transcript Antisense on Body Weight

Rats injected bilaterally with rAAV-CART-AS exhibited a significant increase in body weight and body weight gain relative to the control group over the 124 days measured when analysed using the GEE (P<0.05) (Figure 4.9).
Figure 4.9 Body weight and body weight gain. Effect of bilateral intra-ARC injection of rAAV-CART-AS on body-weight (A) and body-weight gain (B) in male Wistar rats. Animals were injected with rAAV- CART-AS (n=10) (shown in blue) or rAAV-eGFP (n=8) (shown in green) and body-weight and body-weight gain recorded for 124 days post-injection. Values shown as mean ± SEM. *P<0.05 compared to animals injected with rAAV-eGFP and analysed using the GEE over the whole time period.
4.5.7.4 Effect on Neuropeptide Release from Hypothalamic Slices

There was no significant difference in the basal release of CART (P = 0.65 using unpaired Students t-test) or α-MSH (P = 0.81 using unpaired Students t-test) -like immunoreactivity in rAAV-CART-AS injected rats when compared with animals injected with rAAV-eGFP (Figure 4.10).

However, injection of rAAV-CART-AS into the rat ARC resulted in a significant reduction in potassium stimulated release of CART (55-102)-like immunoreactivity and a trend towards reduced potassium stimulated release of α-MSH-like immunoreactivity when compared to control animals (CART, 1.34 ± 0.20fmol/µl [eGFP], 0.95 ± 0.10fmol/µl [CART-AS], P=0.03, α-MSH, 0.015 ± 0.001fmol/µl [eGFP], 0.011 ± 0.002fmol/µl [CART-AS], P=0.1, using an unpaired t-test) (Figure 4.10).

4.5.7.5 Metabolic Characterisation

Bilateral intra-ARC injection of rAAV-CART-AS had no significant effect on VO$_2$ (P = 0.51 fed state, P = 0.84 fasted state when analysed using the GEE), VCO$_2$ (P = 0.51 fed state, P = 0.72 fasted state when analysed using the GEE) or RER (P = 0.72 fed state, P = 0.92 fasted state when analysed using the GEE) in fasted or ad libitum fed animals over the 24 hour period recorded by the CLAMS (Figure 4.11).

No significant differences in XAMB movement were detected in fasted or ad libitum fed animals (P = 0.69 fed state, P = 0.51 fasted state when analysed using the GEE) (Figure 4.12). No significant differences in food intake were recorded during the ad libitum fed day, although a trend towards increased food intake was observed in rAAV-CART-AS animals (P = 0.076 when analysed using the GEE) (Figure 4.12).
Figure 4.10 Effect of rAAV-CART-AS on potassium stimulated release of CART and αMSH. Effect of bilateral intra-ARC injection of rAAV-CART-AS on basal release of CART (55-102) (A) and α-MSH (C) –like immunoreactivity. Potassium stimulated release of CART (55-102) (B) and α-MSH (D) –like immunoreactivity was also measured. Animals were injected with rAAV-CART-AS or rAAV-eGFP and potassium stimulated release of CART (55-102)-like immunoreactivity and α-MSH like immunoreactivity measured from ex-vivo hypothalamic explants 26 days post-injection. Neuropeptide release was measured by RIA and shown as mean ± SEM (n=8-10 per group) *P<0.05 by unpaired Students t-test.
Figure 4.11 Metabolic characterisation. Effect of bilateral intra-ARC injection of rAAV-CART-AS on metabolic parameters in fasted and fed male Wistar rats. Animals were injected with rAAV-CART-AS (shown in blue) or rAAV-eGFP (shown in green) and placed in CLAMS cages 125 days post-injection. Oxygen consumption (A and B), carbon dioxide production (C and D) and RER (E and F) were measured from 9am for 24 hours, results shown as ad libitum fed period on the left and a fasted period on the right. The grey area indicates the dark phase. Values shown as mean ± SEM (n = 8-10 per group)
Figure 4.12 Effect of rAAV-CART-AS on activity and food intake. Effect of bilateral intra-ARC injection of rAAV-CART-AS on activity and food intake. Animals were injected with rAAV-CART-AS (shown in blue) or rAAV-eGFP (shown in green) and placed in CLAMS cages 125 days post-injection. XAMB activity was measured in ad libitum fed (A) and fasted (B) animals from 9am for 24 hours. Cumulative food intake (C) was also measured in ad libitum fed animals. The grey area indicates the dark phase. Values shown as mean ± SEM (n = 8-10 per group).
4.5.7.6 Tissue Analysis
Bilateral intra-ARC injection of rAAV-CART-AS had no significant effect on intrascapular BAT weight at 130 days post injection when compared to animals injected with rAAV-eGFP (n=8-10). Rats injected with rAAV-CART-AS had a mean BAT weight of 0.78 ± 0.04g and control animals exhibited a mean BAT weight of 0.70 ± 0.39g (P = 0.96 using an unpaired Students t-test). In addition, there was no significant difference when BAT tissue weight was corrected for body weight (P = 0.75 using unpaired Students t-test).

Bilateral intra-ARC injection of rAAV-CART-AS had no significant effect on WAT weight at 130 days post injection when compared to animals injected with rAAV-eGFP (n=7-9). Mean epididymal WAT weight in rats injected with rAAV-CART-AS was 12.23 ± 1.13g and rats injected with rAAV-eGFP exhibited a mean WAT weight of 9.79 ± 0.5g (P = 0.11 using an unpaired Students t-test). Furthermore, there was no significant difference when WAT weight was corrected for body weight (P = 0.14 using unpaired Students t-test) (Figure 4.13).

4.5.7.7 Measurement of Uncoupling Protein-1 mRNA levels in Brown Adipose Tissue
Northern blot was used to measure UCP-1 mRNA expression in rats injected with either rAAV-CART-AS or rAAV-eGFP 130 days post injection. There was no significant difference in BAT UCP-1 mRNA expression between animals injected with rAAV-CART-AS, which exhibited a mean expression level of 0.65 ± 0.047 AU, and the rAAV-eGFP control group, which displayed mean UCP-1 mRNA expression levels of 0.5 ± 0.079 AU (P = 0.22 unpaired Students t-test) (n=8-10). UCP-1 expression was normalised using oligodT and calculated using imageQuant software (Figure 4.14).

4.5.7.8 Plasma Leptin Levels
Bilateral intra-ARC injection of rAAV-CART-AS had no significant effect on plasma leptin levels (P = 0.64 using unpaired Students t-test) as measured by ELISA at 130 days post injection compared to animals injected with rAAV-eGFP (n=8-10). Rats injected with rAAV-CART-AS displayed a mean plasma leptin concentration of 7.21 ± 0.71ng/ml and rats in the control group had a mean plasma leptin concentration of 6.30 ± 0.66ng/ml (Figure 4.15).
Figure 4.13 Tissue weight analysis. Effect of bilateral intra-ARC injection of rAAV-CART-AS on WAT (A) and BAT (B) weight. WAT (C) and BAT (D) weight corrected for body-weight are also shown. Animals were injected with rAAV-CART-AS or rAAV-eGFP and epididymal WAT pads and intrascapular BAT removed and weighed 130 days post-injection. Values shown as mean ± SEM (n = 8-10 per group).
Figure 4.14 UCP-1 mRNA expression. Effect of bilateral intra-ARC injection of rAAV-CART-AS on UCP-1 mRNA levels in BAT (n = 8-10 per group). UCP-1 mRNA levels were measured by northern blot and quantified using ImageQuant software. Values shown as mean ± SEM.
Figure 4.15 Plasma leptin. Effect of bilateral intra-ARC injection of rAAV-CART-AS on plasma leptin levels. Animals were injected with rAAV-CART-AS or rAAV-eGFP and plasma leptin measured 130 days post-injection by ELISA. Values shown as mean ± SEM (n = 8-10 per group).
4.6 Discussion

4.6.1 Productions of Recombinant AAV Construct

The pTRCGW plasmid was chosen for modification with CART-AS because it has been successfully used within the Department of Investigative Medicine in previous neuronal gene transfer studies (Gardiner et al., 2005) and contains the necessary sequence elements required for \textit{in vivo} rAAV transgene expression, including the ITRs which are required for transgene integration into the host genome. The pTRCGW vector contains the CMV promoter which is used to drive expression of the CART-AS transgene. The CMV promoter is a strong constitutively active promoter and has been shown to drive transgene expression for up to a year following rAAV gene delivery (Guy et al., 1999). Additional elements in the pTRCGW plasmid are the WPRE which has been shown to enhance \textit{in vivo} transcription of mRNA (Xu et al., 2003) and the BGH polyadenylation sequence which stabilises the mRNA transcript, protecting it from degradation (Ribeiro et al., 2004). An antibiotic resistance gene is also present in the pTRCGW plasmid to allow for selection of successfully modified clones, but is positioned outside the region flanked by the ITR elements to ensure that it is not transferred into the host genome with the transgene. The first stage in preparation of the rAAV plasmid was to generate the CART-AS transgene by RT-PCR. Due to alternate splicing in the CART transcript two CART fragments (760bp and 721bp) were produced in the resulting PCR. The 721bp CART fragment produced by a PCR reaction was subcloned downstream of the CMV promoter to produce the modified pTRCCART-AS plasmid. This fragment was chosen because this is the more abundant form of CART.

4.6.2 \textit{In vitro} Testing pTRCCART-AS in RIN1056a CART cells

RIN1056a CART cells are rat pancreatic insulinoma cells that have been transfected with a CART transgene and consequently express CART peptide. Successful transfection of pTRCCART-AS plasmid was confirmed by northern blot analysis. RNA was analysed from RIN1056a, RIN1056a CART and CAC cells. A northern blot was carried out using a WPRE probe since WPRE is a component of the pTRCCART-AS plasmid, but is not endogenous to RIN1056a CART cells. This confirmed the presence of the pTRCCART-AS plasmid in the transfected cell lines.
Initially a transient transfection was performed so the cells would express the transgene for a limited time. The transient transfection was optimised using β-galactosidase expressing plasmid pCMVβ (appendix 3). This allowed for successfully transfected cells to be visualised as they stain blue when treated with X-gal as a result of the β-galactosidase enzyme cleaving the galactoside from the indole ring of X-gal. The highest transfection efficiency was obtained using 4µg pCMVβ with 12 PEI equivalents which resulted in approximately 35% of cells being successfully transfected.

When the transient transfection was carried out using pTRCCART-AS plasmid no difference in the release of CART (55-102) -like immunoreactivity compared with control cells was observed. The lack of a reduction in CART (55-102) -like immunoreactivity may be a consequence of the nature of transient transfection. As can be seen from the optimisation studies involving β-galactosidase only a proportion of cells are successfully transfected and there is no method of selection for these cells. As a result, any subtle knock down in CART peptide caused by cells expressing the CART-AS transgene may be masked by the high portion of cells which have not been transfected and consequently exhibit normal CART peptide expression.

To overcome these difficulties a stable transfection procedure was used to ensure the majority of cells contained the CART-AS expressing plasmid. Cotransfection was carried out using the pTRCCART-AS plasmid and a plasmid expressing the neomycin resistance gene to allow for selection of successfully transfected cells. Due to the use of cotransfection there is a possibility some of the cells which were transfected with the neomycin resistance plasmid were not successfully transfected with the pTRCCART-AS plasmid. These cells would remain when exposed to geneticin but would not express the CART-AS transgene. However, as pTRCCART-AS was transfected in 200 fold excess of the neomycin resistance plasmid this is unlikely to occur frequently. The amount of CART (55-102) -like immunoreactivity released from cells following a stable transfection with the pTRCCART-AS plasmid, of which there were two clones, CACA and CACB, was significantly lower than release from the RIN1056a CART cells. In the first 2 hours, CAC cells produced around 50% less CART (55-102)-like immunoreactivity than RIN1056a CART cells. The difference in CART (55-102) release between the cell lines did diminish over time, with a 30% reduction in CART (55-102) -like immunoreactivity seen in CAC cells at 8 hours. This was potentially due to a
reduction in the rate of CART (55-102) release from all the cell lines, which may reflect a negative feedback response to the high levels of CART (55-102) in the bathing media.

4.6.3 Recombinant AAV Injection in the Arcuate Nucleus

Following on from confirmation of CART-AS action in vitro, the pTRCCART-AS plasmid was used to produce rAAV particles for use in vivo. Dot blot analysis was used to quantify the total viral particle for in vivo injection. Animals were injected with rAAV-CART-AS or rAAV-eGFP into the arcuate nucleus to elucidate the physiological effect of a reduction in ARC CART peptide concentration. Recombinant AAV serotype 2 was used in this study since it preferentially infects neuronal cells rather than glial cells (Bartlett et al., 1998). In addition rAAV has been widely used in gene transfer studies within the brain with no reports of toxicity and a low immune reaction (Tenenbaum et al., 2004).

The coordinates chosen for rAAV injection into the ARC were based upon those used by Paxinos and Watson and were confirmed by pilot studies which involved injecting ink into the hypothalamus to confirm the accuracy of the co-ordinates that would be used (see appendix 2).

4.6.4 Recombinant AAV Injection and Detection in vivo

In order to confirm expression of the transgene in the ARC in situ hybridisation was carried out to detect transgene derived mRNA species using a radiolabelled CART probe. Expression of CART-AS mRNA was demonstrated 125 days post injection in the ARC confirming expression of the CART-AS transgene. Expression of rAAV transgenes have been shown to increase gradually following injection and reach a detectable peak approximately 2 weeks post injection (Flannery et al., 1997). The slow increase in transgene expression is thought to be due to inefficient second strand synthesis by rAAV since in the absence of the helper virus rAAV relies on the host DNA repair mechanism to generate the second strand for integration (Ferrari et al., 1996).
4.6.5 Characterisation of Animals Injected with Recombinant AAV- Cocaine- and Amphetamine -Regulated Transcript Antisense in the Arcuate Nucleus

4.6.5.1 Food Intake and Body weight

Food intake and body weight of rats injected with rAAV-CART-AS and rAAV-eGFP were measured over a 4 month period. The results show that there was a significant increase in cumulative food intake, body weight and body weight gain in the rAAV-CART-AS treated group relative to the rAAV-eGFP group. This suggests heavier animals had increased food intake compared to those with a lower body weight which is consistent with studies by Chen et al which demonstrated that intracerebroventricular (ICV) injection of rAAV expressing CART resulted in a decrease in food intake and body weight in rats (Qing and Chen, 2007).

Previous studies have demonstrated arcuate nucleus CART to have an orexigenic role. Kong et al and Abbot et al showed that intra-ARC injections of CART peptide resulted in increased food intake (Kong et al., 2003; Abbott et al., 2001). In addition, Kong et al demonstrated that PEI transfer of a CART expressing plasmid, pcDNA3-CART, into the ARC chronically overexpressed CART peptide and resulted in an increase in food intake and body weight. I used rAAV expressing CART-AS to down regulate physiological CART peptide expression and the results suggest that CART has an anorectic role. The different results obtained between the studies may support the hypothesis of CART existing in two circuits within the ARC, one of which is orexigenic in nature and a second ARC circuit which causes anorectic actions. Alternatively, results reported by Kong et al and Abbot et al following administration of CART peptide to the ARC may be the result of activation of pre-synaptic auto-receptors expressed on CART containing neurones. The activation of the auto-receptors may follow as a result of the ARC CART peptide concentration being raised beyond physiological concentrations. This may cause a decrease in release of either CART or α-MSH from synaptic terminals of POMC neurones. This may explain why an increase food intake was observed in both studies conducted by Kong et al and Abbot et al and in my study. Whilst a receptor for CART has not yet been identified there is precedent for hypothalamic neuropeptides self-regulating their release through actions on presynaptic auto-receptors. The Y2 receptor has been shown to act as an auto-inhibitory receptor for neuropeptide Y (NPY) release. Localisation studies have identified Y2 receptors in NPY neurones in the ARC (Broberger et al., 1997) and administration of the Y2 receptor antagonist BIIE0246 to rat hypothalamic slices shows results in a reduction in the release of NPY (King et al., 2000).
Additional factors need to be considered when comparing the results from my studies using rAAV-CART-AS and those reported by Kong et al which used PEI to transfect neurones with a CART expressing plasmid. The use of PEI will lead to the infection of neuronal cells as well as glial cells, which have a role in synapse development and plasticity (Pfrieger, 2002). Therefore the observed increase in cumulative food intake and body weight gain reported by Kong et al may be in part due to CART expression in glial cells. Although glial cells are unlikely to release the CART neuropeptide, the expression of CART may disrupt the normal function of the glial cells. Furthermore, utilising a vector to overexpress the CART peptide will result in CART peptide expression and release from the synaptic terminals of all transfected neurones even if they do not normally express endogenous CART. Given that the interactions between CART and specific receptors are yet to be identified there is the potential that CART peptide release from normally non CART producing neurones will act on receptor which CART would not normally interact with, for example CART may act as an antagonist at the postsynaptic neurone. In my studies I have used rAAV serotype 2 which results in the preferential infection of neuronal cells. In addition, whilst rAAV expressing CART-AS will not discriminate in the type of neurone it infects, the antisense will only have an effect on neuropeptide release in CART producing neurones.

4.6.5.2 Potassium Stimulated Release
Exposure of neurones to high concentrations of potassium ions induces depolarisation of neuronal membranes and the release of intracellular contents. Hypothalamic explants from rats injected with rAAV-CART-AS and rAAV-eGFP were exposed to high levels of potassium ions and the resulting peptides that were released were measured by RIA (see section 2.2.12). Potassium stimulated release of CART (55-102)-like immunoreactivity from hypothalamic explants was significantly reduced in animals injected with rAAV-CART-AS in the ARC compared to rAAV-eGFP controls, which is consistent with CART-AS expression in the ARC. There was a trend towards reduced α-MSH although this was not significant. There was no significant difference in the basal release of α-MSH or CART (55-102) –like immunoreactivity suggesting that results from potassium stimulated release are not due to a decrease basal leakage of the neuropeptides in rats injected with rAAV-CART-AS. Recent electrophysiological evidence suggests that CART may in fact stimulate the release of α-MSH (private communication with Dr K Murphy). Therefore a reduction in potassium stimulated release of CART (55-102) –like immunoreactivity may account for a non
significant reduction in α-MSH release. Alternatively, CART may exert a direct effect on food intake.

4.6.5.3 Energy Expenditure

Analysis of rAAV-CART-AS and rAAV-eGFP injected animals when in the CLAMS showed no difference in activity levels, RER, oxygen consumption or carbon dioxide production. This suggests that reduced activity or energy expenditure did not contribute to the increase in body weight that was observed. Indirect calorimetry measurements in mice with targeted deletion of the CART gene (Asnicar et al., 2001) and in diet induced obese rats injected with rAAV overexpressing CART (Qing and Chen, 2007), support the observations that neither an increase in or decrease in CART peptide alter activity levels.

WAT from the epididymal fat pad and BAT from the intrascapular region was removed from the animals that were injected with rAAV-CART-AS and rAAV-eGFP to act as markers of whole body adiposity and energy expenditure. There was no significant difference in WAT weight from the rAAV-CART-AS treated group which was consistent with there being no significant difference in plasma leptin levels between the rAAV-CART-AS group and the control animals. This is in keeping with previous studies by Chen et al using rAAV to overexpress CART since there was no significant difference in fat mass between treated and control animals, although a decrease in lean mass was reported (Qing and Chen, 2007).

There was no significant difference in UCP-1 mRNA expression or BAT weight in the rAAV-CART-AS treated rats relative to the levels observed in the rAAV-eGFP control animals. However, in contrast to my observations, Kong et al did report that chronic upregulation of CART peptide expression resulted in an increase in BAT UCP-1 mRNA expression. As has been discussed, rAAV-CART-AS will affect neuropeptide release only in those neurones that express CART mRNA. In contrast using a vector to overexpress CART may result in the release of CART peptide from the synaptic terminals of non CART expressing neurones. Since CART and non CART producing neurones are affected this may account for why Kong et al observed an increase in UCP-1 mRNA expression, which is consistent with anorectic factor, and an increase in food intake which is consistent with CART being an orexigen, although Kong et al attributes the effect to a role for CART in cold adaptation (Kong et al., 2003).
As has been discussed there is a hypothesis that CART may be differentially regulated within the hypothalamus (Abbott et al., 2001) and for this reason it is important to study the effect of CART in specific nuclei to fully dissect the role of CART. By using rAAV-CART-AS it is possible to observe the effect of chronic down regulation of ARC CART peptide expression which has several advantages over other approaches. Kong et al utilised PEI to transfect cell in the ARC with a vector to overexpress CART (Kong et al., 2003). However, PEI infects neurones and glial cells. In studies conducted as part of this these neurones have been specifically targeted using rAAV serotype 2 which negates the possible involvement of glial cells in any phenotype analysis. In my thesis I have chosen to study the effect of a reduction in CART peptide expression in the ARC which is more physiologically relevant since this does not lead to supra-physiological concentrations of CART peptide in the ARC that may activate CART auto-receptors involved in mediating negative feedback. In addition, rAAV-CART-AS, due to the use of the constitutive CMV promoter, will result in the expression of CART-AS in all infected neurones. However, CART-AS expression will only affect neuropeptide release from neurones which express CART. This is not necessarily the case when using vectors to overexpress CART peptide since the CART peptide will be expressed and released from all neurones that are transfected with the vector whether they normally express endogenous CART or not. Due to there being no identified CART receptor it is not possible to conclude whether CART release from neurones which do not normally express CART will have an effect on phenotype.

In summary, rAAV expressing CART-AS was injected into the rat arcuate nucleus to establish a physiological role for ARC CART in energy homeostasis. Expression of CART-AS resulted in a reduction in CART peptide levels in the ARC which resulted in increased cumulative food intake, body weight and body weight gain over a four month period. Potassium stimulation of hypothalamic explants demonstrated a significant reduction in release of CART (55-102) –like immunoreactivity which may account for a non significant reduction in α-MSH in rats injected with rAAV-CART-AS. It appears that the effect of CART-AS on body weight is through increased food intake since there was no significant difference in energy expenditure between the rAAV-CART-AS and rAAV-eGFP injected animals.
Chapter 5

General Discussion and Future Work
5. General Discussion and Future Work

5.1 Discussion
The hypothalamus is an important area of the central nervous system (CNS) involved in the control of energy homeostasis. The arcuate nucleus (ARC) is one of several nuclei that constitute the hypothalamus, and it has been shown to be essential in the integration of peripheral signals of energy store status (Hakansson et al., 1996; Obici et al., 2002; Parton et al., 2007). It is by further understanding the role of hypothalamic neuropeptides that a better understanding of the physiological processes that underlie obesity can be attained and this may offer the potential for the development of anti-obesity therapies. The aim of this thesis was to elucidate the physiological role of arcuate nucleus derived cocaine- and amphetamine–regulated transcript (CART) in energy homeostasis.

CART was originally identified in the rat striatum as a mRNA transcript that was upregulated in response to peripheral administration of cocaine and amphetamine (Douglass et al., 1995) which is also associated with a decrease in food intake (Balopole, 1979; Hsieh et al., 2005). CART is widely expressed within the CNS (Hunter et al., 2005; Vrang et al., 1999) including the hypothalamic paraventricular nucleus (PVN), ARC and lateral hypothalamic nucleus (LH) (Vrang et al., 1999). Early studies linked CART with a role in regulating food intake since CART mRNA expression was shown to be upregulated by leptin (Kristensen, 1998) and intracerebroventricular (ICV) administration of recombinant CART peptide resulted in an inhibition of feeding in rats (Kristensen, 1998). Since these early studies, attempts have been made to elucidate the true physiological role of CART in energy homeostasis. However, attempts to define CART as an anorectic or an orexigen have proved inconclusive. Administration of anti-CART antibody, which blocks endogenous CART, resulted in stimulation of overnight feeding (Lambert, 1998). In addition, CART peptide has been shown to reduce feeding upon ICV administration in satiated and fasted rats (Kristensen, 1998). These data are consistent with CART acting as an anorectic peptide. However, ICV administration of CART peptide was associated with movement tremors which were a result of diffusion of CART peptide to the fourth ventricle (Aja et al., 2002) which suggested that the observed reduction in food intake was secondary to the effects of CART on motor behaviour. The effect of CART on motor behaviour is unlikely to fully account for the reduction in feeding since a reduction in food intake was still observed when diffusion of CART peptide to the fourth ventricle was blocked (Aja et al., 2002). Interestingly, CART has
also been implicated in an orexigenic role since intra-nuclear administration of CART peptide in rats resulted in increased feeding (Abbott et al., 2001). Administration of 0.2nmol of CART peptide into the rat ARC, PVN, LH and dorsal medial nucleus (DMN) resulted in an increase in feeding (Abbott et al., 2001). Due to the conflicting data which has implicated CART as both an anorectic and orexigenic factor the true role of CART remains unclear. Instead the data gathered so far may serve to highlight the complexity in the physiological function of CART. This has given rise to the hypothesis that CART may be differentially regulated within the hypothalamus with CART being expressed in two circuits, one orexigenic and one anorectic (Abbott et al., 2001).

The aim of my thesis was to investigate the physiological role of ARC CART peptide in energy homeostasis. In order to achieve this I sought to study the phenotype resulting from a reduction in CART peptide concentration in the rodent arcuate nucleus. I chose to target the CART peptide since no receptor for CART has been identified.

To date, studies investigating the role of CART have mainly focussed on studying the phenotype that results from an increase in CART peptide which may have the result in producing a non physiological phenotype (Kong et al., 2003; Kristensen, 1998). Although there have been studies which have investigated the effect of loss of CART gene function these studies utilised transgenic methods to achieve a whole body deletion of the CART gene (Asnicar et al., 2001; Wierup et al., 2005). Therefore given that CART is expressed in the periphery and CNS and may potentially be differentially regulated within the brain, the loss of CART peptide throughout the organism does not provide evidence for a conclusive role for hypothalamic CART. For these reasons I have chosen to downregulate endogenous CART peptide expression specifically in the ARC. To produce a reduction in ARC CART peptide concentration I utilised two methods of genetic manipulation to target CART-AS expression. Initially I generated a CART-AS expressing transgenic mouse line by bacterial artificial chromosome (BAC) transgenesis to reduce CART peptide concentrations specifically in pro-opiomelanocortin (POMC) expressing cells. In the second part of my thesis I reduced ARC CART peptide concentration using recombinant adeno-associated virus (rAAV) expressing CART-AS.

In the first part of my thesis I set out to selectively express the CART-AS transgene in POMC neurones. In order to achieve this, a BAC was identified which contained the POMC gene at
the centre of its insert as this increases the likelihood that all the necessary regulatory elements required for driving accurate POMC like expression are present. Barsh et al demonstrated accurate POMC like expression of eGFP using a BAC containing 60kb 5’ flanking sequence and 30kb of 3’ flanking sequence (Xu et al., 2005). I chose to use the RP24_340C3 BAC and since the amount of flanking sequence present in the RP24_340C3 BAC (84kb 5’ flanking sequence and 70kb 3’ flanking sequence) was greater than that used by Barsh et al it is likely that it will drive accurate POMC like expression pattern. Following the selection of the BAC, homologous recombination, driven by the pGETrec plasmid, was used to modify the POMC BAC so that it contained the CART-AS transgene. The successfully modified BAC was then injected into the pronuclei of fertilised mouse eggs from which founder mice were identified and used to set up separate transgenic lines. Expression of the CART-AS transgene in POMC neurones appeared to reduce CART peptide release at the synaptic terminals of POMC neurones which includes projections within the ARC and to other areas within the hypothalamus (Elmquist et al., 1998; Elmquist et al., 1999). For example I found a reduction in CART peptide concentration in PVN tissue biopsies.

Expression of the CART-AS transgene in POMC neurones significantly increased body weight in male mice maintained on regular chow when compared with wildtype litter mates. The increased body weight appeared to be due to decreased thermogenesis since brown adipose tissue (BAT) uncoupling protein-1 (UCP-1) mRNA expression was reduced with no significant difference in food intake. A reduction in CART peptide release at the PVN, and UCP-1 mRNA expression, is consistent with previous studies which have demonstrated that injection of CART peptide into the PVN results in increased UCP-1 mRNA expression through stimulation of sympathetic outflow (Wang et al., 2000). These data suggest that CART peptide in POMC neurones has a catabolic role in energy homeostasis, and that this role may be more important in the control of thermogenesis than food intake.

In the second part of my thesis I set out to reduce CART peptide expression in the ARC using rAAV (serotype 2) expressing CART-AS. The use of rAAV allows temporal control of transgene expression as the viral vector can be administered at any stage in the life cycle of the rodent. This allows delivery of the transgene to the adult circumventing the problem of developmental compensation which can occur in BAC transgenesis and is a confounding factor in determining any potential phenotype. In addition, rAAV serotype 2 preferentially infects neuronal cells (Bartlett et al., 1998) and this coupled with the use of the constitutive
cytomegalovirus (CMV) promoter to drive CART-AS expression caused the transgene to be expressed in all neuronal cells that the virus infected. Therefore the reduction in CART peptide release is not confined to POMC expressing neurones as is the case in the CART-AS transgenic mice. It is important to note that although rAAV does not discriminate in the neurones it infects, CART-AS expression will only affect neurones which express the CART peptide.

Injection of rAAV-CART-AS into the rat ARC resulted in increased body weight in rats which is consistent with the findings from the transgenic CART-AS mouse model. However, the increase in body weight resulting from rAAV-CART-AS injection appeared to be due to increased food intake. Analysis of parameters governing energy expenditure did not reveal any significant difference in activity levels, oxygen consumption or BAT UCP-1 mRNA expression which is consistent with the findings reported by Chen et al following ICV injection of rAAV expressing CART (Qing and Chen, 2007). Chen et al also reported a decrease in food intake and body weight (Qing and Chen, 2007). These data support the findings from the CART-AS expressing mice which suggest that CART acts as an anorectic factor. Furthermore, results from hypothalamic explants raise the possibility that CART may influence release and/or storage of α-melanocortin stimulating hormones (MSH). Previous studies using hypothalamic explants have demonstrated interaction between ARC neuropeptides since administration of 1, 10 and 100nmol neuropeptide Y (NPY) to the explant system resulted in an increase in release of agouti related peptide (AgRP) (Dhillon et al., 2002). It would therefore be interesting to confirm whether interaction between CART and POMC was of physiological significance.

The experiments performed as part of my thesis provide additional information regarding the role of the CART peptide within the ARC. Collectively, these data suggests that a reduction in CART peptide, specifically in POMC neurones and in the ARC, supports the hypothesis of CART having a catabolic role in energy homeostasis. However, results from CART-AS expressing mice suggests that the role of CART in the POMC neurone is one that is important in the regulation of energy expenditure whereas the effect of rAAV-CART-AS expression on body weight appears to be due to increased food intake.
The different causes behind the observed increase in body weight in the two rodent models used in this thesis may be explained by the different sites at which CART peptide expression was reduced. CART-AS expressing mice displayed a reduction in CART peptide release from POMC neurone projections in the ARC and other hypothalamic nuclei. Therefore the data suggests that CART peptide in POMC neurones is involved in regulating energy expenditure. In contrast, given that 90% of CART neurones in the ARC express α-MSH, there are 10% of CART neurones where peptide release is not reduced in the transgenic mice, but are reduced in the rAAV-CART-AS model (Elias et al., 1998). It is possible that POMC derived CART neurones are important in energy expenditure, and additional ARC CART neurones are important in the control of food intake. An alternative explanation is that rAAV is more effective at reducing CART peptide concentration in the ARC and therefore in rAAV-CART-AS injected animals CART peptide is reduced below a threshold which is not achieved using BAC transgenesis and results in the increase in food intake.

The studies conducted as part of my thesis implicate CART with a catabolic role, and several studies have been conducted which support these findings, such as the study by Chen et al in which rAAV expressing CART was injected ICV in rats and was shown to reduce food intake and body weight with no effect on activity levels (Qing and Chen, 2007). In addition, Wierup et al developed a line of transgenic mice with a targeted deletion of the CART gene which exhibited an increase in body weight but with no significant difference in food intake. The authors suggest that this may be due to a decrease in energy expenditure (Wierup et al., 2005) and is consistent with my findings from the CART-AS expressing mice described in this thesis.

A second model generated by Asnicar et al showed that mice with a targeted deletion of the CART gene resulted in an increase in food intake and body weight when fed a high fat diet (Asnicar et al., 2001). The reported increase in food intake in mice deficient in the CART peptide is in keeping with results from studies I have conducted involving injection of rAAV-CART-AS. However, mice expressing the CART-AS transgene did not exhibit an increase in food intake. This may be explained by CART-AS mice exhibiting reduced CART peptide expression in the 90% of CART neurones that are colocalised with α-MSH (Elias et al., 1998). In contrast rAAV-CART-AS injected animals, like the mice with targeted deletion of CART, have reduced CART peptide concentration in all CART expressing neurones within the ARC. The reduction in CART peptide in the 10% of ARC CART neurones which are not
colocalised with α-MSH may account for why the increase in food intake was not observed in mice expressing the CART-AS transgene. An alternative explanation may be that rAAV-CART-AS, and mice with a targeted deletion of the CART gene, reduce CART peptide concentration below the threshold needed to affect food intake whereas CART-AS expressing mice generated by BAC transgenesis are not as efficient at reducing CART peptide concentration. Although Asnicar et al did not find any difference in energy expenditure when compared with wildtype mice, humans which have a mutation at leu34phe in the CART gene display a reduced metabolic rate which suggests a role for CART in energy expenditure (del Giudice et al., 2001).

However, studies have also implicated CART as an orexigenic agent since experimental paradigms which chronically increase CART peptide in the ARC result in increased food intake and body weight (Abbott et al., 2001; Kong et al., 2003). This is in contrast to the results I have obtained which suggest that a reduction in CART peptide in the ARC results in an increase in body weight, and in the case of rAAV-CART-AS also an increase in food intake. These conflicting data may be explained by the way in which CART is targeted to the ARC. Administration of CART peptide will result in activation of non CART producing neurones which express the CART receptor. In addition, an increase in CART peptide may activate pre-synaptic auto-receptors expressed on CART neurones which would cause a reduction in CART peptide release from the synaptic terminal as a result of negative feedback. The same is true when using a vector which chronically overexpresses CART peptide. Kong et al utilised polyethylenimine (PEI) to transfect cells in the ARC with a plasmid to cause an increase in CART peptide expression. The consequence of using peptide overexpression to investigate peptide function is that non CART expressing neurones will synthesise and release the CART peptide. This may result in the activation of neurones which would not normally be activated by CART. Hence the increase in food intake and body weight reported in studies investigating an increase ARC CART peptide concentration may be, in part, a product of reduced CART either through activation of auto-receptors, or through activation of neurones which do not express endogenous CART. In choosing to use the POMC expression domain to drive CART-AS expression in mice and rAAV serotype 2 to ensure neurones are preferentially infected, a greater specificity is achieved compared with other studies discussed here. In addition, the methods that I have employed ensure that neuropeptide release will only be affected in cells which express CART since the antisense
mRNA will only bind to sense CART mRNA therefore neuropeptide release will not be affected in cells which do not express CART.

CART is still the least understood neuropeptide in the ARC and further studies need to be conducted once a CART receptor has been identified. Studies involving the CART receptor may provide a further insight into the theory that suggests CART acts both as an orexigenic and an anorectic factor since different CART receptor subtypes may exist to mediate the two CART appetite circuits.

5.2 Future Work

5.2.1 Bacterial Artificial Chromosome transgenesis Cocaine- and Amphetamine - Regulated Transcript Antisense Model

Further work is needed to fully characterise the CART-AS mouse model. Initially it will be important to confirm the site of CART-AS transgene expression by *in situ* hybridisation. CART has been shown to colocalise with POMC in the ARC, retrochiasmatic nucleus and pituitary and *in situ* hybridisation will highlight any expression in these areas which will need to be taken into account when analysing the phenotype (Elias et al., 1998; Stanley et al., 2004). Following this, the level of transgene expression can be determined using quantitative *in situ* hybridisation.

Given that analysis of tissue biopsies from the PVN demonstrated a reduction in CART peptide it would be interesting to investigate whether CART peptide concentration was reduced in other hypothalamic nuclei to which POMC/CART neurones project, such as the LH and DMN. In addition it would also be interesting to investigate whether CART-AS expression altered the release of other neuropeptides in these nuclei. The content and release of the neuropeptides CART, POMC, AgRP and NPY from whole hypothalami and tissue biopsies from specific hypothalamic nuclei will be investigated by a static hypothalamic explant incubation system. The explants will be incubated in artificial cerebrospinal fluid (aCSF) to determine basal release prior to stimulation with potassium. Upon stimulation CART, α-MSH, POMC and NPY immunoreactivity will be measured by radioimmunoassay (RIA).
Previous studies have demonstrated that CART neurones that coexpress POMC synapse with thyrotropin releasing hormone (TRH) neurones in the PVN (Fekete et al., 2000). In addition, injection of CART peptide into the PVN has been shown to result in an increase in BAT UCP-1 mRNA expression (Wang et al., 2000). In light of this evidence, coupled with my findings that CART-AS expressing mice displayed reduced CART peptide concentration in the PVN tissue biopsies and reduced BAT UCP-1 mRNA expression, the effect of reduced CART peptide concentration in POMC neurones on energy expenditure needs to be investigated further. It will be interesting to study proTRH mRNA expression by quantitative PCR to establish if reduced TRH expression may account for decreased BAT UCP-1 mRNA expression. Further to this, TRH release stimulates the release of thyroid stimulating hormone (TSH) from the pituitary which causes an increase in circulating thyroid hormones. Therefore, TSH release and the plasma concentration of T3 and T4 thyroid hormone from CART-AS expressing mice should be measured by RIA. It would also be important to study energy expenditure via indirect calorimetry to establish any differences in activity levels or oxygen consumption between transgenic and wildtype mice. This can be studied using the CLAMS which are detailed in section 4.4.

Due to the ARC being a site at which peripheral hormones can signal on the status of energy stores circulating plasma leptin should be measured using a standard leptin enzyme linked immunosorbent assay (ELSIA) to investigate the possibility of a feedback mechanism between CART and leptin. Nutrients have also been shown to act at the hypothalamus as peripheral signals of immediate fuel availability, for example, glucose sensing neurones have been identified in the ARC (Parton et al., 2007). Glucose sensing is thought to occur through the kir6.2 protein channel subunit and the kir6.2 protein subunit has been identified on the POMC neurone (Parton et al., 2007). Therefore it would be interesting to investigate whether a reduction in CART peptide in the POMC neurones affected the ability of mice expressing the CART-AS transgene to sense and respond to glucose. This could be investigated using standard glucose tolerance tests (GTT) and insulin tolerance tests (ITT).
5.2.2 Recombinant AAV- Cocaine- and Amphetamine -Regulated Transcript Antisense Model

There is scope for further investigation of the effect of intra-ARC injection of rAAV-CART-AS. Considering the result from the hypothalamic explant study which suggests a potential for CART to affect α-MSH release it would be interesting to investigate whether this was physiological and whether CART also affects NPY or AgRP release. This could be addressed by studying the effect of CART-AS expression on the release of α-MSH, NPY and AgRP from ARC tissue biopsies using a static explant system. The tissue will be incubated in aCSF to determine basal release before measuring potassium stimulated release of neuropeptides. In addition, it would be useful to carry out electrophysiological studies on ARC neurones to establish if animals expressing CART-AS exhibit increased action potential frequency on the NPY/AgRP neurones relative to control animals which could potentially explain the increase in food intake in rAAV-CART-AS injected animals. To put this into context, NPY/GABA neurones within the ARC have been shown to inhibit the POMC/CART neurone population (Cowley et al., 2001) so it is possible that CART interneurones may exert inhibitory tones on the orexigenic neurone population, either directly or through stimulation of α-MSH.

5.3 Conclusion

Collectively the data presented in this thesis supports the hypothesis of ARC CART having a catabolic role in energy homeostasis. Although further work is needed in the characterisation of the CART-AS mouse model the data gathered regarding the effect of CART on thermogenesis is consistent with previous studies involving CART administration into the PVN. This suggests that the CART-AS expressing mouse is a promising model to study the role of CART peptide. However, given the wide distribution of CART within the brain, the fact that CART has been implicated in mediating stress and the effect of psychostimulants, coupled with the conflicting data showing CART to act as an anorectic and orexigenic peptide, it raises questions about the suitability of targeting CART as a possible anti-obesity therapy. Drugs which affect peptides that are involved in multiple systems can have adverse side effects, one example is rimonobant. The drug rimonobant was found to reduce food intake but approval was withdrawn following reports of a link between psychiatric disorders. However, it may be more practical to target CART as an anti-obesity therapy once a CART receptor and its subtypes have been identified since it may then be possible to target an antagonist to a specific receptor subtype that mediates the orexigenic effect of CART peptide.
Although even this may be problematic, CART has been shown to cause movement disorders upon diffusion to the fourth ventricle following ICV administration of CART in rodents.


Appendices
**Appendix 1 - Solutions**

**ABC buffer**
Combine solution A, B and C in the following proportions - 100:250:150, and store at -20°C.

**Solution A**
Add 1ml of 125mM MgCl2 and 1.25M Tris (pH8.0) to 18µl Mercaptoethanol, 5µl 100mM dATP, 5µl 100mM dTTP and 5µl dGTP.

**Solution B**
2M HEPES pH6.6 (titrated with 4M NaOH)

**Solution C**
Random oligonucleotides at 112µg/ml

**Artificial CSF (aCSF) stock solutions**
- NaHCO3 (0.1M) 4.62g dissolved in 500ml GDW
- NaCl (0.63M) 18.43g dissolved in 550ml GDW
- Na2HPO4.2H2O (0.09M) 1.56g dissolved in 100ml GDW
- MgSO4.7H2O (0.09M) 2.2g dissolved in 100ml GDW
- CaCl2 (0.07M) 0.792g dissolved in 100ml GDW
All stock solutions were stored at 4°C

**aCSF**
20ml NaHCO3 (0.1M), 20ml NaCl (0.63M), 1ml Na2HPO4.2H2O (0.09M), 10ml KCl (0.56M), 1ml MgSO4.7H2O (0.09M), 90mg glucose, 1ml aprotinin, 17.6mg ascorbic acid were made up to 100ml with GDW. The solution was gassed with O2 and kept on ice.

**Alkaline SDS (0.2M NaOH/1%SDS):**
Mix 2ml 10M sodium hydroxide, 5ml 20% SDS and 93ml autoclaved distilled water.
Amasino wash buffer

Mix 250ml 1M Na phosphate buffer pH 7.2, 2ml 500mM EDTA and 100ml 20% SDS with 650ml of distilled water.

5M ammonium acetate:
Dissolve 385g CH$_3$.COONH$_4$ in 600ml autoclaved distilled water, then make up to 1l.

100mg/ml ampicillin:
Dissolve 1g in 10ml autoclaved distilled water, sterilise by passage through a 0.2µm filter and distribute into 1ml aliquots.

10% l-arabinose:
Dissolve 1g of L-arabinose in 10ml autoclaved distilled water. Sterilise by passage through a 0.2µm filter.

2.5mM ATA:
Dissolve 0.1183g ATA in 100ml autoclaved distilled water.

Caesium chloride-saturated isopropanol:
Vigorously mix 100g CsCl$_2$, 100ml autoclaved distilled water, and 100ml propan-2-ol and leave to settle.

2M calcium chloride:
Dissolve 5.88g of CaCl$_2$.2H$_2$O in 20ml distilled water and sterilise by passage through a 0.2µm filter. Store in 1ml aliquots at -20°C.

25mg/ml chloramphenicol
Dissolve 250mg of chloramphenicol in 10ml of molecular biology grade ethanol.

Denaturing solution (DNA):
Dissolve 43.5g of NaCl in GDW, add 25ml of 10MNaOH and make up to 500ml with GDW.
DENAT (RNA denaturing solution):
Mix 1ml of formamide, 300µl formaldehyde and 100µl 20x MOPS.

Dextran coated charcoal:
Add 2.4g of charcoal and 0.24g of dextran to 100ml of phosphate buffer with gelatine, and mix for twenty minutes at 20°C.

Dialysis tubing:
Dialysis tubing is cut into ~10cm lengths, immersed in 2% (w/v) NaHCO₃/1mM EDTA and boiled for ten minutes. Tubing is then rinsed in distilled water then boiled for five minutes in 1mM C₁₀H₁₄O₈Na₂.2H₂O. After cooling, tubing is stored in solution at 4°C.

Gel loading buffer:
Mix 3.125ml 80% glycerol, 50µl 0.5M EDTA, and 10mg orange G in 6.075ml of autoclaved distilled water.

dNTP mix for PCR and reverse transcription:
Mix 10µl each of the 100mM dGTP, dCTP, dATP, and dTTP in 60µl of autoclaved distilled water and aliquot into 20µl batches.

0.5M ethylenediaminetetra-acetic acid (EDTA):
Dissolve 186.1g C₁₀H₁₄O₈Na₂.2H₂O in 800ml autoclaved distilled water and adjust to pH 8.0 with 1M NaOH. Make up to 1l with water.

Formamide (de-ionised):
Add 2.7g Duolite MB 6113 (Merck) to 100ml formamide and mix until duolite turns brown (~1 hour). Remove Duolite by passage through Whatman GF/C filters, aliquot and store at -20°C.

10% glycerol in water:
Add 10ml glycerol to 90ml of distilled water.
80% glycerol in water:

Add 80ml glycerol to 20ml of distilled water.

GTE:

Mix 2.5ml 1M Tris-HCl pH 8.0, 2ml 0.5M EDTA, and 5ml 18% glucose and make up to 100ml. Sterilise by passing through a 0.2µm filter.

2x HEPES-buffered saline (HBS):

dissolve 1.6g NaCl, 75mg KCl, 21mg, Na₂HPO₄, 0.22g dextrose and 1g HEPES in a total volume of 90 mls of water. Adjust to pH 7.05 with 0.5M sodium hydroxide and volume to 100 mls with water. Sterilise by passing through a 0.22 micron filter and store in 5 ml aliquots at -20ºC.

Hybridisation buffer for Northern and dot blot

Dissolve 0.5g dried milk powder and 0.5ml EDTA (500mM stock) in 48ml GDW, leave at 37ºC to dissolve. Allow to cool then add 25ml of Na Phosphate buffer (1M stock), 25ml SDS (20% stock), and 1ml ATA (2.5mM stock)

25mg/ml IPTG:

Dissolve 100mg of IPTG in 4ml autoclaved distilled water. Sterilise by passage through a 0.2µm filter.

LB culture media:

Dissolve 5g sodium chloride, 10g tryptone and 5g yeast extract to 1l of distilled water. Adjust to pH 7.5 with 10M sodium hydroxide and sterilise by autoclaving.

Lysis buffer

Add 8.765g NaCl, 6.055g Tris, make up to 1l with GDW and adjust to pH8.5
20x MOPS:
Dissolve 83.6g 3-(N-Morpholino)propanesulphonic acid (MOPS), 8.1g sodium acetate, 7.4g C_{10}H_{14}O_{8}Na_{2}.2H_{2}O and 2.5ml formaldehyde in 800ml water. Adjust to pH 7.0 with 10M sodium hydroxide and make up to 1l.

Neutralising solution
Dissolve 43.5g sodium chloride and 60.2g Trizma base in 430ml GDW. Adjust pH to 7.5 by addition of 70ml hydrochloric acid.

0.01M phosphate buffered saline (PBS):
Dissolve 8.7g NaCl, 0.272g KH_{2}PO_{4}, and 1.41g Na_{2}HPO_{4}.2H_{2}O in 800ml of distilled water. Adjust to pH 7.5 and make up to 1l.

0.01M PBS/0.1% Na Azide/ 0.1% BSA:
Dissolve 1g Na azide and 1g of BSA in 100ml of 0.01M PBS, then make up to 1l.

PBS/Tween:
Add 0.25ml Tween 20 to 500ml 0.01M PBS.

Phenol/chloroform/IAA:
Mix equilibrated phenol, chloroform and iso-amyl alcohol in the ratio 25:24:1 and store under 0.1M Tris-HCl, pH 8.0.

Phosphate buffer (RIA buffer):
48g of Na_{2}HPO_{4}.2H_{2}O, 4.13g KH_{2}PO_{4}, 18.61g C_{10}H_{14}O_{8}Na_{2}.2H_{2}O, 2.5g NaN_{3} were dissolved in 5l of distilled water that had been boiled and allowed to cool, the pH was measured to confirm it was 7.6 ± 0.1 and the buffer was stored at 4°C.
Phosphate buffer with gelatin:

The buffer was produced as above with 12.5g of gelatin dissolved in the boiling water then cooled before the addition of the other reagents

3M potassium acetate:

Dissolve 294.4g CH₃COOK in 500ml distilled water, add 115ml glacial acetic acid and make up to 1l with water.

20mg/ml proteinase K:

Dissolve 20mg for every 1ml of autoclaved distilled water and store at -20°C.

10mg/ml RNase A:

Dissolve 100mg RNase A in 10ml 10mM Tris-HCl, pH 7.5/15mM NaCl and boil for fifteen minutes. Allow to cool and distribute in to 1ml aliquots.

Sephadex G₅₀:

Add 8g fine grade Sephadex G-50 beads (diameter: 20-80µm) (Sigma), 2ml 100x TE and 0.1ml 20% SDS to 200ml distilled water and autoclave to expand the beads.

SOB

Dissolve 20g bactotryptone, 5g yeast extract, 0.5g NaCl in 1l GDW, pH to 7.4 and autoclave. Before use add 20ml 1M MgSO₄.

2M sodium acetate, pH 5.2:

Dissolve 164.1g CH₃COONa in 800ml autoclaved distilled water, adjust to pH 5.2 with glacial acetic acid and make up to 1l with water.

5M sodium chloride:

Dissolve 292.2g NaCl in 1l of distilled water.

0.75M sodium citrate:

Dissolve 220.5g Na₃C₆H₅O₇·2H₂O in 1l autoclaved distilled water and adjust to pH 7.0.
20% sodium dodecyl sulphate (SDS):
Add 200g SDS to 800ml autoclaved distilled water, heat to 60°C while stirring. Allow to cool and make up to 1l with water.

10M sodium hydroxide:
Dissolve 400g of NaOH in 500ml autoclaved distilled water. Once dissolved make up to 1l with water.

0.2% sodium hydroxide 1% SDS
Add 3ml 10M NaOH to 140ml GDW, mix and add 7.5ml 20% SDS.

0.5M sodium phosphate, pH 6.8:
Dissolve 71g Na$_2$HPO$_4$.2H$_2$O in 1l distilled water and 41.4g NaH$_2$PO$_4$.H$_2$O in 600ml distilled water. Then mix.

50mg/ml spectinomycin:
Dissolve 0.5g of spectinomycin in 10ml of autoclaved distilled water. Sterilise by passage through a 0.2µm filter and distribute into 1ml aliquots.

20x SSC:
Dissolve 1,753g NaCl and 882g Na$_3$C$_6$H$_5$O$_7$.2H$_2$O in 8l distilled water, adjust to pH 7.0 and make up to 10l.

20x SSPE:
Dissolve 210.4g NaCl, 14.2g Na$_2$HPO$_4$ and 7.4g C$_{10}$H$_{14}$O$_8$Na$_2$.2H$_2$O in 700ml of distilled water, adjust to pH 7.7 with 10M NaOH and make up to 1l with water.
50x TAE:
Dissolve 242g Trizma base in 843ml distilled water. Add 57ml glacial acetic acid and 100ml 0.5M C_{10}H_{14}O_{8}Na_{2}2H_{2}O and mix.

100x TE:
Dissolve 121.1g Trizma base and 3.7g C_{10}H_{14}O_{8}Na_{2}2H_{2}O in 800ml autoclaved distilled water. Adjust to pH 7.5 with hydrochloric acid and make up to 1l.

0.1M TEA Buffer:
Dissolve 18.6g C_{6}H_{15}NO_{3}.HCl (triethanolamine-hydrochloride) in 800ml of distilled water, pH adjusted to 8.0 with hydrochloric acid and the volume adjusted to 1l with water.

TES Buffer:
Mix 25ml 1M Tris-HCl pH 8.0, 5ml 5M NaCl, and 5ml C_{10}H_{14}O_{8}Na_{2}2H_{2}O. Make up to a final volume of 500ml with autoclaved distilled water.

100mg/ml tetracyclin:
Dissolve 50mg in 10ml ethanol and distribute in 1ml aliquots.

Transformation buffer II (TFBII):
Dissolve 418mg MOPS, 3.28g CaCl_{2}.2H_{2}O and 242mg RbCl in autoclaved distilled water. Add 37.5ml 80% glycerol and adjust the volume to 200ml with water. The solution is sterilised by passing through a 0.2µm filter.

Transformation buffer I (TFBI):
Dissolve 589mg CH_{3}.COOK, 2.418g RbCl, 1.979g CaCl_{2}.2H_{2}O and 438mg MnCl_{2}.4H_{2}O in autoclaved distilled water. Add 37.5 ml 80% glycerol and adjust the volume to 200ml with water. Sterilise by passing through a 0.2µm filter.
0.1MTris/HCl pH 8.2:
   Dissolve 12.1g of Trizma base in 1l of distilled water, adjust pH to 8.2 with HCl.

1M Tris-HCl pH 7.5:
   Dissolve 121.1g Trizma base in 800ml of distilled water. Adjust to pH 7.5 with HCl and make up to 1l.

2M Tris-HCl, pH 8.0:
   Dissolve 121.1g Trizma base in 450ml of distilled water. Adjust to pH 8.0 with HCl and make up to 500ml.

Universal wash buffer:
   Mix 10ml 20% SDS and 10ml 20x SSPE with 980ml of distilled water.

Versene:
   Dissolve 16g NaCl, 0.4g KCl, 2.88g Na₂HPO₄.2H₂O, 1.2g C₁₀H₂₄O₈Na₂.2H₂O and 0.4g KH₂PO₄ to 1L water, add 3 mls phenol red (Sigma) and make up to 2L with water. Sterilise by autoclaving.

X-Gal solution
   Add 1.5ml 0.1M K₃Fe(CN)₆, 1.5ml 0.1M K₄Fe(CN)₆.3H₂O, 60µl 1M MgCl₂ and 240µl X-Gal 50mg/ml (Promega) and make up to 30ml with 0.01M PBS (Sigma-Aldrich)
Appendix 2 – Ink Injections in the Rat Arcuate Nucleus

Ink injection in the rat arcuate nucleus using coordinates of Paxinos and Watson (Posterior/anterior from bregma -3.8mm, lateral from bregma ± 0.5mm). Magnification 4X using Nikon eclipse 50i. The scale bar represents 100µm.
Appendix 3 - Plasmids

pcDNA3 (neomycin resistance plasmid)

http://www.pkclab.org/PKC/vector/pcDNA3.gif

pDG

(Grimm, 2003)
pCMVβ

http://www.clontech.com/images/products/1184_fig1.gif
Appendix 4 – Calculation of Virion Concentration

Calculation of virion concentration for rAAV-CART-AS:

Dot blot analysis revealed 1µl rAAV-CART-AS fraction contained 11.8ng/µl viral DNA.

The molecular weight of rAAV is 975000.
Therefore the number of moles of rAAV in 11.8ng of viral DNA is 1.21x10^{-14}

Avagadro’s number is 6.022x10^{23}
Therefore the number of rAAV single stranded DNA particles is 7288164103
rAAV is double stranded, so this number is multiplied by 2, hence the number of DNA particles per 11.8ng of rAAV-CART-AS is 1.457x10^{10}

Taking into account of dilution factors the number of rAAV particles per µl is 2.9x10^{10}
Therefore the number of rAAV particles in 11.8ng is 2.91x10^{13} particles/ml