

THE MECHANISM OF ENERGY EXPENDITURE OF OXYNTOMODULIN

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1.1 ABSTRACT

The gut hormone oxyntomodulin is an attractive potential treatment for obesity as it reduces food intake and increases energy expenditure in rats and in man. However, the mechanisms by which OXM increases energy expenditure are not known.

Oxyntomodulin has a short half-life *in vivo*, so long-acting analogues are being developed for both obesity pharmacotherapy and to help us understand the physiology of OXM. This thesis uses the novel oxyntomodulin analogue, OX2, to investigate the energy expenditure effects of oxyntomodulin.

The first part of this thesis shows that OX2 increases energy expenditure acutely, as directly measured in CLAMS metabolic cages. Metabolic cage and pair-feeding studies show this increase in energy expenditure is sustained with prolonged administration of the oxyntomodulin analogue. The increase in energy expenditure occurs via activation of the glucagon receptor, as shown in studies using the GLP-1 antagonist Ex9-39, and comparing OX2 to the GLP-1 specific analogue OX2-Glu3.

Metabolic cage data shows that oxyntomodulin increases energy expenditure via altering metabolic processes rather than physical activity. The second part of this thesis investigates which pathways are affected by OXM. Using 6-hydroxydopamine to cause a chemical sympathectomy, this thesis demonstrates that the sympathetic nervous system is important in controlling the energy expenditure effects of OXM. Chronic administration of OX2 suppressed many metabolic pathways, including brown adipose tissue thermogenesis and organ growth. However, protein metabolisms and lipolysis were both increased, leading to a reduction in both body fat and body protein. The final part of this thesis then shows OX2 has acute, as well as chronic, effects on protein metabolism.

The findings in this thesis provide a basis for further investigations into the metabolic effects of oxyntomodulin. The thesis also underlines the importance of understanding these processes before oxyntomodulin analogues can be used in the treatment of obesity.

1.2 ACKNOWLEDGEMENTS

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My greatest thanks is owed to my husband Pete. Your support makes everything I do possible. I'm just glad you now know that rats can't vomit.

This thesis is dedicated to Moo and Pip. You are the bookends to my PhD, the reason I get out of bed in the morning, the best things that have happened to me.

1.3 DECLARATON OF ORIGINALITY

All work in this thesis was conceived and designed by the author, Rebecca Scott. The work of all other groups has been referenced throughout this thesis. A complete list of all references is found at the end of this thesis.

1.4 COPYRIGHT DECLARATION

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1.5 DECLARATION OF CONTRIBUTORS

The work in this thesis was conceived and designed by the author. The author undertook all the work with collaborations as detailed below:

Chapters 1 & 2

The cAMP accumulation bioactivity assays were undertaken with the assistance of LJ Ball and V Pathak.

Chapter 5

The propranolol studies were undertaken with the assistance of Samantha Price.

Dissection and Surgery

Dissection of animals in chapters 4, 5, 6 and 7, and insertion of Alzet pumps in chapters 3 and 4 were carried out with the kind assistance of many members of the Department for Investigative Medicine.

1.6 ABBREVIATIONS

6-OHDA	6-hydroxydopamine
ANOVA	Analysis of variance
AP	Area postrema
ARC	Arcuate nucleus
ASS	Argininosuccinate synthetase
ATGL	Adipose triglyceride lipase
ATP	Adenosine triphosphate
BAT	Brown adipose tissue
BMI	Body mass index
BMR	Basal metabolic rate
cAMP	Cyclic AMP
CCK	Cholecystokinin
CLAMS	Comprehensive lab animal monitoring system
CPS	Carbamoyl phosphate synthetase
CTX	C-terminal telopeptide
Dio2	Deiodinase 2
DIT	Diet-induced thermogenesis
DMH	Dorsomedial hypothalamus
DPPIV	Dipeptidyl peptidase IV
EDTA	Ethylenediaminetetraacetic acid
EE	Energy expenditure
Ex4	Exendin 4
Ex9-39	Exendin 9-39
FGF21	Fibroblast growth factor 21
FI	Food intake
fT3	Free triiodothyronine
fT4	Free thyroxine
G6Pase	Glucose-6-phosphatase
GCG	Glucagon

GIP	Glucose-dependnet insulinotropic polypeptide
GLP-1	Glucagon-like peptide 1
GLP-2	Glucagon-like peptide 2
GRPP	Glicentin-related pancreatic peptide
HSL	Hormone-sensitive lipase
ICV	Intracerebroventricular
IGF-1	Insulin-like growth factor 1
IP	Intraperitoneal
IP1/2	Intervening peptide 1/2
KO	Knockout
mRNA	Messenger ribonucleic acid
NE	Norepinephrine
NICE	National Institute for Health and Care Excellence
NST	Non-shivering thermogenesis
NTS	Nuclues of the tractus solitarius
OXM	Oxyntomodulin
P1NP	N-terminal propeptide of type 1 procollagen
PC1/2/3	Proconvertrase 1/2/3
PCR	Polymerase chain reaction
PEPCK	Phosphoenolpyruvate carboxykinase
PF	Pair-fed
PP	Pancreatic polypeptide
PPARGC1a	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha
PPGDs	Pre-proglucagon Derivatives
PS	Signalling peptide
PVN	Paraventricular nucleus
PYY	Peptide tyrosine tyrosine
RER	Respiratory exchange ratio
RIA	Radioimmunoassay
RNA	Ribonucleic acid
SC	Subcutaneous

SEM	Standard error of the mean
SERCA	Sarcoendoplasmic reticulum calcium ATPase
SLN	Sarcolipin
SNS	Sympathetic nervous system
UCP-1	Uncoupling protein 1
VO ₂	Oxygen consumption
WAT	White adipose tissue

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Chapter 1

General Introduction

1.1 INTRODUCTION

The world is facing an obesity epidemic. According to Public Health England, more than a quarter of adults in the UK were classified as obese in 2014 (https://www.noo.org.uk/NOO_about_obesity/adult_obesity/UK_prevalence_and_trends). This affects the health, wealth and social cohesion of society. Obesity is associated with multiple co-morbidities, including diabetes, heart disease and cancer. It's an expensive problem too: the direct cost to the NHS was estimated in the Foresite Report to be £4.2 billion in 2007 (https://www.noo.org.uk/NOO_about_obesity/economics) and this is likely to have risen since. When the associated factors of increased social care, reduced attendance at work and loss of productivity are taken in to account, the indirect cost of obesity to the economy has been estimated at £15.8 billion (*ibid*).

For individuals, body weight is determined by the balance between energy consumed and energy expended. The traditional concept was that, for a given individual, homeostatic mechanisms within the body enable maintenance of a constant body weight despite changes in energy supply in the environment. However, this concept has developed to incorporate the knowledge that all animals, when given access to excess, energy-dense food, will consume more than they need in order to meet their energy demands; the excessively consumed energy is stored within adipose depots, and expansion of these depots causes an increase in body weight (Rothwell and Stock, 1982, Ravussin et al., 1994). This has the evolutionary advantage of maintaining energy supplies over the longer term, in times of famine.

However, in humans, the effects of this otherwise useful physiological drive are compounded by recent environmental changes which mean that food is more palatable, more readily available, in bigger portions, as well as a physical environment which requires less activity in day-to-day living (Christakis and Fowler, 2013, Obesity, 2016, Report). These environmental changes are coupled with a culture that, in the developed world at least, has a higher tolerance of obese body shapes than previously. These factors gradually shift the energy balance equation and lead to chronic obesity in epidemic proportions.

Tackling obesity is therefore a major priority for many governments. In 2014, the National Institute for Health and Care Excellence (NICE) published guidance for the treatment of obesity in the UK. It recommends initial treatment with diet and lifestyle modification, followed by pharmacological intervention, and finally, bariatric surgery (NICE, 2014). These three aspects of obesity management will be further discussed below.

1.2 MANAGEMENT OF OBESITY

1.2.1 DIET BEHAVIOUR AND LIFESTYLE MODIFICATION

Caloric restriction is the primary way in which most people attempt to lose weight. A variety of structured programmes have been designed to assist in this, and typically they do cause significant weight loss in the short term (Hemmingsson et al., 2012, Jebb et al., 2011, Rock et al., 2007). However, in the long-term, most people regain their weight (Franz et al., 2007, MacLean et al., 2011); this is because the body seems to defend the previously gained higher weight through increased appetite, changes in peripheral signals of energy status and reduced energy expenditure (MacLean et al., 2011).

Increased physical activity is recommended by several public health institutions, including NHS Livewell and the American College of Sports Medicine. However, the weight loss attainable by physical activity, unless at very high intensity, is minimal – usually only about 2kg for an adult (Donnelly et al., 2009, Swift et al., 2014, Franz et al., 2007). Of course, there are other benefits to maintaining an active lifestyle, such as improved glucose control, lipid profile and quality of life, and it can help to maintain weight loss (Swift et al., 2014). But though individuals may lose significant amounts of weight in response to enhanced physical activity, there is little evidence that enhanced physical activity across the population will solve the obesity epidemic.

1.2.2 PHARMACOLOGICAL INTERVENTION

Many drugs have been developed over the years for treatment of obesity. However, most have been abandoned due to lack of significant efficacy, or more frequently due to side-effects (Rodgers et al., 2012) (Table 1-1).

Currently only two pharmacological treatments are licensed for treatment of obesity in the UK: orlistat and liraglutide.

Orlistat is a pancreatic lipase inhibitor that reduces fat absorption from the gut. Weight loss is modest – approximately 1kg per month for the first 4 months, typically followed by an increase in body weight back to baseline over 3 years while continuing treatment (Douglas et al., 2015). There are also significant side effects: more than 80% of patients suffer some gastrointestinal disturbance (including diarrhoea and oily faeces) which prevents many people continuing with treatment (Finer et al., 2000).

The long-acting GLP-1 analogue liraglutide, marketed as Saxenda (Novo Nordisk), has recently been licensed in the UK as another anti-obesity pharmacotherapy. The pharmacology of GLP-1 shall be

discussed further below. Clinical trials lasting up to one year show subjects given Saxenda lose significant amounts of weight, with more than 60% of subjects losing more than 5% of their body weight and more than 30% losing 10% of initial body weight (Astrup et al., 2009, Pi-Sunyer et al., 2015). However, over 70% of subjects experience side effects, mostly nausea and vomiting, though these are often transient (Astrup et al., 2009, Pi-Sunyer et al., 2015). As Saxenda has only recently been licensed in the UK, the efficacy in routine clinical practice is still to be determined, but early signs suggest it may offer a moderately effective alternative in the treatment of obesity.

DRUG	MECHANISM OF ACTION	REASON NOT IN USE
Amphetamine/Phentermine	Central acting sympathomimetics	Cardiovascular risk Abuse potential
Fenfluramine/Dexfenfluramine Combined Phentermine/Fenfluramine	Serotonin-releasing agents	Cardiac valvulopathy
Sibutramine	Noradrenaline and serotonin uptake inhibitor	Cardiovascular risk
Rimonabant	Cannabinoid CB1 receptor antagonist	Psychiatric problems
Lorcaserin	Serotonin 2C receptor agonist	Never licensed in the UK due to concerns of tumour growth, psychiatric disorders and valvulopathy
Phentermine/Topiramate (Qsymia)	Combined sympathomimetic and anti-epileptic	Cardiovascular safety Psychiatric effects Teratogenic concerns

Table 1-1 Selected drugs which have been developed for the treatment of obesity which are no longer in clinical use in the UK.

1.2.3 BARIATRIC SURGERY

There are several types of bariatric surgery: gastric banding involves reversible placement of an inflatable silastic band around the stomach, limiting the amount of food that can be eaten; sleeve gastrectomy involves surgical excision of most of the stomach, leaving a pouch approximately 150ml in volume, which also acts by reducing capacity for food; the most common, and most efficacious surgery, however, is the Roux-en-Y gastric bypass. In this procedure, most of the stomach is excised, and a small gastric pouch is produced. This is joined to part of the distal small intestine to allow continuity of digestion. The stomach remnant and the bypassed intestine are also joined via an

anastomosis to the distal small intestine, allowing all the stomach, biliary and pancreatic juices to come in to contact with the intestine, but later in the digestive process.

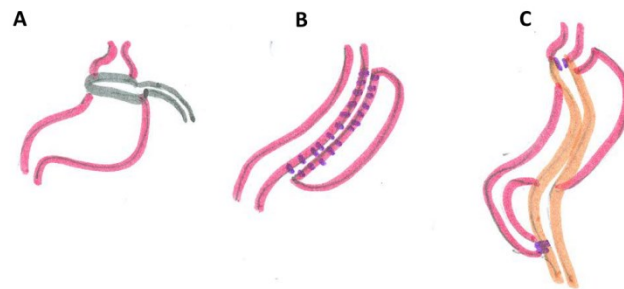


Figure 1.1 Types of Bariatric Surgery

This diagram shows the 3 most common forms of bariatric surgery: gastric banding (A), sleeve gastrectomy (B) and Roux-en-Y gastric bypass (C).

Roux-en-Y gastric bypass, which causes a combination of restriction of food intake, and alteration in digestive pattern, is the most successful long-term treatment available for obesity. In follow up studies with patients up to twenty years after undergoing the procedure, there is an 18% loss of body weight from baseline, with added health benefits of reduced incidence of diabetes, myocardial infarction and stroke (Sjostrom, 2013). However, gastric bypass is not without risk; at 30 days, there is still a 0.15-0.6% chance of mortality and approximately 5% chance of serious morbidity, despite improvements over recent years (Young et al., 2015, Marsk et al., 2009). Moreover, bariatric surgery is expensive, with gastric bypass costing approximately £10,000-15,000 per person. There is also limited availability due to lack of specially-trained staff and appropriate surgical facilities; therefore, bariatric surgery is unlikely to be a realistic treatment for everyone who suffers from obesity.

The efficacy of Roux-en-Y gastric bypass, when compared to the purely restrictive forms of bariatric surgery, is thought to be due in part to the changes in gut hormones after the bypass. It is well-recognised that the gut is an endocrine organ, producing multiple hormonal signals which inform the brain of the energy status of the body and affect ongoing food consumption. The most common of these hormones are detailed below (Table 1-2). As can be seen, apart from the hormone ghrelin, all these hormones are satiety signals, and physiologically act to reduce food intake when sufficient nutrients have been consumed. However, in obesity, the levels of the satiety signals are suppressed, thus promoting further food consumption in the obese individual. After gastric bypass, in contrast, many of the levels of these hormones are increased, so that the individual feels more sated more easily, leading to long-term weight loss.

HORMONE	PRODUCED BY	FUNCTION	CHANGES IN OBESITY	CHANGES AFTER BARIATRIC SURGERY
Cholecystokinin (CCK)	I Cells Duodenum	Suppresses food intake	Initially increased, reduced in morbid obesity	Increased post-prandial response
Ghrelin	X/A Cells Stomach	Increases food intake	Lower basal level but levels remain higher after eating	Reduced
Glucagon-like peptide 1 (GLP-1)	L Cells Ileum	Suppresses food intake	Lower fasting levels and blunted post-prandial response	Increased post-prandial response
Glucose-dependent insulinotropic polypeptide (GIP)	K Cells Jejunum	Suppresses food intake	Inconsistent observations	Reduced
Oxyntomodulin	L cells Ileum	Suppresses food intake		Increased post-prandial response
Pancreatic polypeptide (PP)	PP cells pancreas	Suppresses food intake	Suppressed post-prandial response	Reduced
Peptide tyrosine tyrosine (PYY)	L Cells Ileum	Suppresses food intake	Reduced fasting levels and suppressed post-prandial response	Increased post-prandial response

Table 1-2 Gut hormones and changes after bariatric surgery

1.3 THE PREPROGLUCAGON DERIVATIVES:

As seen, many different gut hormones are affected by bariatric surgery. One family of these hormones is the pre-proglucagon derivatives (PPGDs), which include GLP-1, glucagon and oxyntomodulin. GLP-1 is elevated after bariatric surgery; it is also the foundation of the pharmacological therapy Saxenda. As GLP-1 has proven itself as an obesity pharmacotherapy, the other PPGDs are of great interest in the development of further pharmacological treatments for obesity. This family of hormones shall be detailed further below.

The preproglucagon gene is found on chromosome 2. The translated 180 amino-acid polypeptide is expressed in the alpha-cells of the pancreas, L-cells of the intestine, and within the brain. Within the pancreas, the enzyme prohormone convertase 2 converts this polypeptide predominantly into glucagon, with residual glicentin-related pancreatic peptide and major proglucagon fragment. In the intestine and brain, prohormone convertase 1/3 cleaves the prohormone into GLP-1 and oxyntomodulin, and the residual related peptides of glicentin and GLP-2.

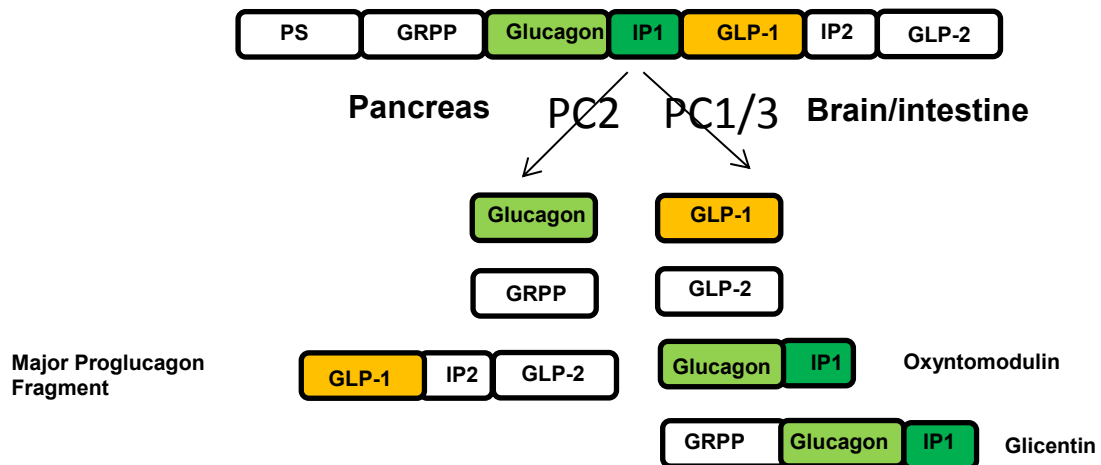


Figure 1.2 Processing of the pre-proglucagon gene.

In the pancreas, prohormone convertase 2 (PC2) cleaves the peptide to produce glucagon, with glicentin-related pancreatic polypeptide (GRPP) and the major proglucagon fragment as remnants. In the brain and intestine, prohormone convertase 1/3 acts to produce GLP-1 and oxyntomodulin, with small amounts of GLP-2 and glicentin. PS: signaling peptide; GRPP: glicentin-related pancreatic peptide; IP1: intervening peptide 1; IP2: intervening peptide 2; PC2: prohormone convertase 2; PC1/3: prohormone convertase 1/3.

1.3.1 GLUCAGON-LIKE PEPTIDE 1 (GLP-1)

As described, GLP-1 is produced by proconvertase 1/3 in the L-cells of the ileum, and within multiple areas of the brain, particularly the hypothalamus and the brainstem (Larsen et al., 1997, Eissele et al., 1992). GLP-1 is found in two main forms in the plasma: GLP-1(1-37) and GLP-1(7-37). GLP-1(7-36) is only found at low levels in the plasma (0.6-1.0pmol/l)(Dai et al., 2008); however, only this form has biological activity (Mojsov et al., 1987, Matsuyama et al., 1988).

The primary physiological function of GLP-1 is thought to be as an incretin. It enhances insulin release in response to an oral glucose load above the release of insulin seen with the same load given IV (Perley and Kipnis, 1967, Wang et al., 1995), and when given exogenously, also increases insulin release (Perley and Kipnis, 1967, Mojsov et al., 1987, Kreyman et al., 1987). Other effects of GLP-1 in the pancreas include increasing beta cell mass (Lamont et al., 2012, Farilla et al., 2002), reducing beta cell apoptosis (Li et al., 2005, Li et al., 2003) and increasing insulin mRNA expression

in beta cells (Fehmann and Habener, 1992, Drucker et al., 1987). The incretin effect of GLP-1 underlies the proliferation and success of GLP-1 analogues as a treatment for diabetes. At least 5 have been licensed for use, including Exenatide (Byetta/Bydureon, AstraZeneca); Liraglutide (Victoza, NovoNordisk); Lixisenatide (Lyxumia, Sanofi); Albiglutide (Tanzeum, GlaxoSmithKline) and Dulaglutide (Trulicity, Eli Lilly).

In addition to the incretin effects, GLP-1 can reduce food intake. The anorectic effect of GLP-1 was first established in 1996: Turton *et al.* showed that ICV administration of GLP-1 inhibits food intake in rats (Turton et al., 1996). Peripheral infusions of GLP-1 also reduce food intake in man (Flint et al., 1998, Flint et al., 2001). Moreover, GLP-1 has been established as an endogenous satiety signal at physiological levels: levels rise after eating (Elliott et al., 1993), while central blockade of GLP-1 receptors increases food intake (Turton et al., 1996). This anorectic effect is seen clinically: in the first clinical trial of liraglutide as a treatment for diabetes, patients also significantly reduced their body weight (VilSBoll et al., 2007), and a large-scale audit of GLP-1 analogue use in patients in everyday clinical practice showed a real-world reduction in body weight in those on either Exenatide or Liraglutide (Ryder et al., 2011). This has led, as mentioned above, to the licensing of high-dose Liraglutide (Saxenda, NovoNordisk) as a treatment specifically for obesity; in one trial over 20 weeks, it caused, on average, 9kg weight loss; more recently, a study over 56 weeks caused an average loss of 8kg (Astrup et al., 2009, Pi-Sunyer et al., 2015). Unfortunately, the anorectic effect of GLP-1 is complicated by associated gastrointestinal side effects, namely diarrhoea and nausea, and these are the predominant reasons for trial withdrawal and treatment cessation (VilSBoll et al., 2007, Astrup et al., 2009, Ryder et al., 2011). This limits the success and efficacy of GLP-1 analogues as obesity therapies.

In contrast to the clear effect of GLP-1 on food intake, the physiological and pharmacological role of GLP-1 in energy expenditure (EE) remains undetermined. Studies using the long-acting, naturally-occurring, GLP-1 analogue Exendin 4 show a reduction in energy expenditure in mice, as measured by indirect calorimetry (Baggio et al., 2004). In contrast, the GLP-1 analogue Liraglutide increases energy expenditure in mice, as demonstrated when administered at a low dose in a pair-feeding experiment (Heppner et al., 2015, Beiroa et al., 2014). At higher doses, however, the anorectic effect of liraglutide eliminated any change in EE (Heppner et al., 2015). In man, there is the same ambiguity. Several human studies show no change in energy expenditure after infusion of GLP-1 (Tan et al., 2013, Bagger et al., 2015, Flint et al., 2001). In contrast, one study comparing treatment with Liraglutide plus Metformin with Metformin alone showed treatment with Liraglutide plus Metformin for one year caused a significant increase in fat-free mass adjusted resting metabolic

rate in man (Beiroa et al., 2014); furthermore 4 weeks treatment with Liraglutide alone caused a tendency to increase energy expenditure in type 2 diabetics (Horowitz et al., 2012). There are also conflicting data as to the effect of GLP-1 on brown adipose tissue (BAT) induced thermogenesis, a surrogate marker of energy expenditure. ICV of GLP-1 administration has been shown to increase BAT temperature and molecular markers of BAT activation in mice (Lockie et al., 2012, Beiroa et al., 2014), though this has not been replicated with peripherally administered Liraglutide (Heppner et al., 2015). Those studies which claim GLP-1 elevates energy expenditure consistently use a GLP-1 analogue (Liraglutide), and often require this to be administered centrally to have an effect. Therefore, it is unclear that there is any physiological effect of native GLP-1 in energy expenditure.

The GLP-1 receptor is a member of the family B class of G-protein coupled receptors. Activation predominantly causes downstream signalling through production of cAMP (Thorens, 1992, Dillon et al., 1993), though also through calcium signalling and some PKA-independent actions (Flamez et al., 1999, Drucker et al., 1987, Bode et al., 1999, Buteau et al., 1999). The GLP-1 receptor is widely distributed in the gut, brain, pancreas, kidney, heart and lung (Wei and Mojsov, 1995, Yamato et al., 1997, Dunphy et al., 1998, Farr et al., 2016). The incretin effect predominantly occurs through stimulatory actions on the β cells in the pancreas (Mojsov et al., 1987, Drucker et al., 1987). However, GLP-1 receptors are also expressed at a low-level in α cells of the islets, where GLP-1 suppresses glucagon release, which may also contribute to the incretin effect (De Marinis et al., 2010).

The anorectic effects of GLP-1 appear to rely on a complex circuit involving multiple brain structures. The role of central GLP-1 receptors was first suggested by Turton *et al.* where central but not peripheral administration of GLP-1 reduced food intake in rats (Turton et al., 1996). In the same study, administration of GLP-1 caused an increase in *c-Fos* expression (a marker of neuronal activation) in the paraventricular nucleus of the hypothalamus (PVN), an important structure in reducing food intake (Sims and Lorden, 1986), as well as in the amygdala. These results have been replicated, with other studies showing additional activation in the dorsomedial hypothalamus (DMH), arcuate nucleus (ARC), area postrema (AP), nucleus of the tractus solitarius (NTS) and parabrachial nucleus (vanDijk et al., 1996, Abbott et al., 2005). Functional imaging in rodents has shown GLP-1 alters activity in similar areas, including the PVN, VMH and AP (Parkinson et al., 2009, Chaudhri et al., 2006). The vagus nerve also contributes to the anorectic circuit: transection of the brainstem or vagotomy reduces the anorectic effect of GLP-1 (Ruttimann et al., 2009, Abbott et al., 2005); however, this pathway is not essential for the anorectic effect of GLP-1 as other studies fail to show a reduction in the effect of GLP-1 after capsaicin-induced vagotomy (Zhang and Ritter, 2012). It

is becoming increasingly clear that GLP-1 also activates areas of the brain involved in the motivational salience and rewarding properties of food (van Bloemendaal et al., 2014, De Silva et al., 2011, Farr et al., 2016, Akesson et al., 2007), and administration of GLP-1 reduces consumption specifically of palatable food in rats as well as food-motivated behaviour and food-reward behaviour (Richard et al., 2015). It may therefore be that altering the rewarding aspects of food contributes to the anorectic effects of GLP-1.

1.3.2 GLUCAGON

Glucagon is another derivative of the preproglucagon gene. This 29 amino-acid polypeptide is produced through proconvertase 2 in the alpha cells of the islets of Langerhans in the pancreas.

The primary functions of glucagon are to raise glucose levels and counteract the actions of insulin. This occurs predominantly via activation of glycogenolysis and gluconeogenesis (Levine, 1965, Curnow et al., 1975). The main stimulus for glucagon secretion from the pancreas is hypoglycaemia. This is detected by hypothalamic nuclei, particularly the VMH (Chan et al., 2011, Zhu et al., 2010), and relayed to the pancreas by the parasympathetic nervous system (Seoane-Collazo et al., 2015, Taborsky and Munding, 2012, Jansen et al., 1997). Systemic sympathoadrenal activation also stimulates glucagon secretion during hypoglycaemia (Taborsky and Munding, 2012, Banarier et al., 2002), as do changes in intra-islet glucose levels (Banarier et al., 2002).

However, there are situations where glucagon levels rise despite normal plasma glucose. These include stress-induced hyperglycaemia, which is commonly seen in Intensive Care Unit patients (Harp et al., 2016), burns (Nygren et al., 1995) and sepsis (Ishida and Hinshaw, 1986). These are all highly catabolic situations. One function therefore of glucagon may be to maintain fuel supplies to tissues during these stressful situations. This is supported by glucagon's effects on protein metabolism. It stimulates protein metabolism, increasing ureagenesis and reducing plasma amino acids by stimulating uptake into hepatocytes (Fitzpatrick et al., 1977, Boden et al., 1996, Fehlmann et al., 1979). The resultant carbon skeletons can enter the gluconeogenic pathway and maintain glucose supply to a variety of organs including the brain (Fitzpatrick et al., 1977, Noda et al., 1988, Boden et al., 1996).

There has been some suggestion that glucagon enhances lipid breakdown, which could also be used to fuel tissue metabolism and gluconeogenesis in catabolic situations, though this has not been consistently demonstrated. For example, Arafat *et al.* showed an increase in non-esterified fatty acids (a surrogate marker of lipolysis) after infusion of glucagon in both lean healthy and type 1 diabetic participants (Arafat et al., 2013), as well as enhanced glycerol release (another surrogate of

lipolysis) in isolated adipocytes (Arafat et al., 2013). In contrast, Xiao *et al.* found glucagon infusions did not affect triglyceride or fatty acid levels, though the VLD1 ApoB100 fraction production and catabolism rate was affected (Xiao et al., 2011). Glucagon is responsible for β -oxidation and ketogenesis through activation of the carnitine acetyltransferase system (Foster and McGarry, 1982, Longuet et al., 2008).

In addition to its effects on metabolism, the cardiac effects of glucagon support its role in stress/fight or flight response. It has positive chronotropic and inotropic effects, which are independent of the sympathetic nervous system, but which facilitate oxygen and nutrient delivery around the body to support metabolic processes (Regan et al., 1964, Stuesse et al., 1982).

Acutely, glucagon reduces food intake in both man and rodents (Geary et al., 1993, Geary and Smith, 1982, Schulman et al., 1957, Quinones et al., 2015, Parker et al., 2013). However, these studies use non-physiological levels of glucagon, and the role of glucagon in the physiological control of appetite is not clear. When lower dose glucagon is given, no effect on food intake is observed (Cegla et al., 2014). Glucagon receptor antagonists do not affect food intake in rodents, again suggesting that there may not be a physiological role for glucagon in food intake (O'Harte et al., 2014, McShane et al., 2014). However, glucagon does affect energy expenditure. Salter showed that glucagon reduces body weight gain in rats to a greater degree than pair-fed controls, suggestive of increased energy expenditure (Salter, 1960); Dicker *et al.* demonstrated a direct increase in energy expenditure as measured by elevated oxygen consumption in rats (Dicker et al., 1998). This has been recapitulated in man, with infusions of glucagon increasing energy expenditure as measured by indirect calorimetry (Salem et al., 2016, Tan et al., 2013).

In contrast to the statement above about the effect of glucagon on food intake, one study showed that low dose glucagon increased food intake (Hell and Timo-laria, 1985) – however this may reflect the importance of the increased energy expenditure effect, leading to an initial compensatory hyperphagia, with the anorectic effect only developing at higher doses; a similar phenomenon is seen in thyrotoxic patients.

The glucagon receptor is another member of the family B class of GPCRs. It is a $G_{\alpha s}$ G-protein subunit coupled receptor, and activation leads to an increase in intracellular levels of cyclic AMP (cAMP) and calcium (Jelinek et al., 1993). The effects of glucagon may be mediated by FGF21, in that glucagon causes release of FGF21 from hepatocytes, and many of the metabolic effects of glucagon are lost in FGF21 knock-out mice (Cyphert et al., 2014, Habegger et al., 2013). Glucagon receptor expression is widespread in the body; the highest concentration is in the liver and kidney, but

receptors have also been found in the gut, adipose tissue, the spleen, testes, adrenal glands and thymus (Dunphy et al., 1998, Hansen et al., 1995).

The hepatic glucagon receptor is fundamental for the effects of glucose on tolerance (Sloop et al., 2004). The anorectic effect is mediated predominantly through the brain stem, as anorectic doses increase *c-Fos* in the NTS, AP and amygdala (Parker et al., 2013). Energy expenditure is a multifactorial process, though how glucagon enhances energy expenditure is not fully understood. The hyperglycaemia associated with glucagon prevents its pharmacological use in the treatment of obesity.

1.3.3 OXYNTOMODULIN

Oxyntomodulin (OXM) is a further derivative of the pre-proglucagon gene, produced by prohormone convertase 1. It is a 37-amino acid peptide, which consists of the same sequence as glucagon plus an octapeptide tail. The predominant source of oxyntomodulin is the L-cells of the ileum, where it is co-secreted with GLP-1. Endogenous oxyntomodulin probably acts as a satiety signal: its levels rise post-prandially, with secretion in proportion to calorie intake (Ghatei et al., 1983, Lequellerc et al., 1992). Small amounts of oxyntomodulin are also found in nerves of the nucleus of the tractus solitarius (NTS), so it may act as a neurotransmitter (Larsen et al., 1997). Furthermore, oxyntomodulin stimulates insulin release from the pancreas. Its main role is probably in acting as an incretin, and fine-tuning blood glucose levels; it dose-dependently reducing blood glucose following a glucose tolerance test (Du et al., 2012).

Pharmacological studies show oxyntomodulin is a potential treatment for obesity. In Wynne *et al.*, overweight and obese subjects self-administered oxyntomodulin subcutaneously three times per day for 4 weeks. This caused a 2.4% reduction in body weight, which was significantly greater than the 0.5% weight loss seen in the placebo group (Wynne et al., 2005).

Oxyntomodulin's potential role as an obesity treatment is of particular research interest as it reduces weight though both a reduction in food intake and an increase in energy expenditure. Dakin *et al.* showed intracerebroventricular injections of oxyntomodulin daily for 7 days in rats caused a significant reduction in food intake. The injections also caused a significant loss of body weight, and more weight loss than in a pair-fed group, whose food intake was restricted to that of the OXM group but only had saline injections. The difference between these two groups represents an elevation in energy expenditure (Dakin et al., 2002). In man, three-times daily subcutaneous injections of oxyntomodulin in a 'free living' environment increased activity-related energy expenditure as measured by an Actiheart monitor (calculated from a combination of heart rate and accelerometry), as well as a reduction in food intake (Wynne et al., 2006). Therefore oxyntomodulin

is a very attractive potential obesity treatment, as unlike other anti-obesity drugs, it tackles both sides of the energy equation, reducing food intake and increasing energy expenditure. At present, approximately 11 anti-obesity drugs based on oxyntomodulin are in development (Tschop et al., 2016).

Native oxyntomodulin has a very short half-life in the circulation, being degraded by DPPIV and neprilysin (Zhu et al., 2003, Druce et al., 2009, Baldissera et al., 1988). Many groups have therefore developed long-acting oxyntomodulin analogues in order to investigate both the physiological properties of OXM and to demonstrate the potential of OXM as an obesity therapy (Druce et al., 2009, Liu et al., 2010, Lynch et al., 2014, Kerr et al., 2010, Bianchi et al., 2013, Day et al., 2009, Henderson et al., 2016). This thesis will similarly use an oxyntomodulin analogue to investigate the physiological properties of OXM.

To date, no specific oxyntomodulin receptor has been discovered. Rather, OXM acts via both the GLP-1 and glucagon receptors. Affinity of OXM for both the GLP-1 and GCG receptors is lower than either of the native ligand (between 30-50x lower) (Druce et al., 2009, Baldissera et al., 1988, Dakin et al., 2001); and the potency at both receptors is 10-100x lower, as measured by ability to stimulate cAMP accumulation (Baggio et al., 2004, Baldissera et al., 1988, Dakin et al., 2001, Price et al., 2015a, Henderson et al., 2016). The different metabolic effects of OXM are thought to be mediated through activation of both the glucagon and GLP-1 receptors.

1.3.3.1 OXYNTOMODULIN AT THE GLP-1 RECEPTOR

Many rodent studies have suggested the anorectic effect of OXM is via activation of the GLP-1 receptor. Co-administration of the GLP-1 antagonist Ex9-39 blocks the anorectic effect of OXM (Dakin et al., 2001, Dakin et al., 2004, Baggio et al., 2004). The anorectic effect of OXM is attenuated in GLP-1 receptor knock-out mice (Baggio et al., 2004). Peripherally administered OXM induces *c-Fos* activation in the same brain areas (PVN, NST and AP) as peripherally administered Ex4 (Baggio et al., 2004). However, functional imaging in mice suggests that OXM and GLP-1 act via distinct pathways, with OXM changing activity in the ARC, PVN and supraoptic nucleus, compared to just the PVN and VMH following GLP-1 administration (Chaudhri et al., 2006).

The GLP-1 receptor also mediates the incretin effect of OXM. In wild-type mice, OXM dose-dependently reduces glucose excursion after a glucose load; however, this glucose-lowering ability is completely lost in GLP-1r knock-out mice (Maida et al., 2008). Oxyntomodulin can be modified to act only at the GLP-1 receptor using a single amino acid switch (glutamine-3 to glutamate-3, a.k.a. OXM-Glu3 (Pocai et al., 2009), rather than having activity at both the GLP-1 and glucagon receptors.

Following this modification, the glucose lowering effects are actually enhanced (Du et al., 2012). The GLP-1 receptor also helps OXM prevent β cell apoptosis (Maida et al., 2008).

1.3.3.2 OXYNTOMODULIN AT THE GLUCAGON RECEPTOR

As described above, while activation of the GLP-1 receptor mediates the incretin effect of OXM, activation of the glucagon receptor tempers the glucose lowering effect of OXM. A higher dose of OXM than OXM-Glu3 is required to cause reduce the glucose excursion in an intraperitoneal glucose tolerance test (Du et al., 2012). In a hyperglycaemic clamp, the glucose infusion rate was higher in animals given OXM-Glu3 than those given OXM, implying OXM-Glu3 increases glucose disposal compared to OXM (Du et al., 2012). This is possibly due to glucagon-receptor activation causing glycogenolysis, which prompts glucose release (Kosinski et al., 2012). Glucagon receptor activation also enables the ketogenic effect of OXM (Du et al., 2012).

Though the GLP-1 receptor mediates much of the anorectic effect of OXM, activation of the glucagon receptor may also have a role. Pocai *et al.* developed a long-acting oxyntomodulin analogue, DualAG, and a corresponding analogue GLPAG with the single amino acid switch of Gln to Glu at the third residue, eliminating its efficacy at the glucagon receptor (Pocai et al., 2009). Though the GLPAG caused a reduction in food intake, this reduction was even greater in the DualAG group, suggesting activation of the glucagon receptor did reduce food intake.

The glucagon receptor also mediates some of the extra metabolic effects of oxyntomodulin, notably on heart rate. Oxyntomodulin has similar chronotropic effects to glucagon which occur through direct action on the myocardium and are independent of the autonomic nervous system (Sowden et al., 2007, Mukharji et al., 2013a).

1.3.3.3 OXYNTOMODULIN AND ENERGY EXPENDITURE

Though many of the effects of oxyntomodulin are clearly mediated through either the glucagon or GLP-1 receptors, the source of the energy expenditure effect is not obvious. Kosinski *et al.* showed that in DIO mice, OXM and OXM-Glu3 reduced food intake to a similar degree, but OXM caused greater weight loss – which would suggest an energy expenditure effect due to the additional glucagon activity in the OXM group (Kosinski et al., 2012). In contrast, Lockie *et al.* showed that the increase in BAT thermogenesis caused by central administration of OXM was lost in GLP-1 receptor knock-out mice, suggesting the GLP-1 receptor was important for thermogenesis (Lockie et al., 2012). It should be noted that neither of these studies used direct measures of energy expenditure to determine the role of the glucagon and GLP-1 receptors in the energy expenditure effect of oxyntomodulin. Further studies are clearly therefore required to investigate the receptors which mediate the energy expenditure effects of OXM: this thesis will include such studies.

1.4 COMPONENTS OF ENERGY EXPENDITURE

This thesis also aims to investigate the metabolic processes by which oxyntomodulin enhances energy expenditure. Investigating the underlying mechanisms of energy expenditure is complex, since many different processes are involved in animals. An appreciation of these is essential if energy expenditure is to be understood. These processes are detailed below:

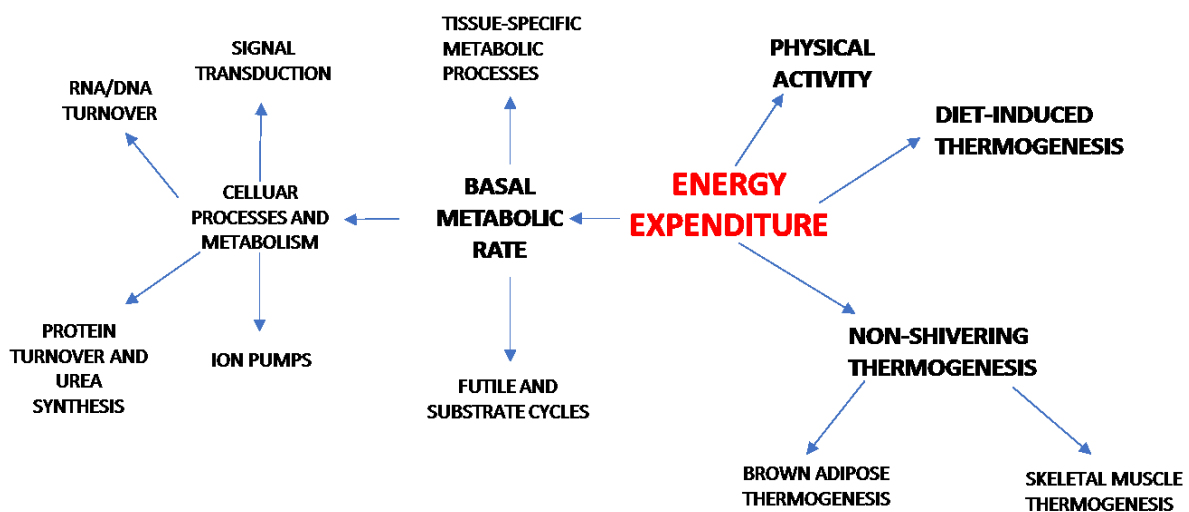


Figure 1.3 Diagram of the components of energy expenditure in animals.

1.4.1 PHYSICAL ACTIVITY

The most obvious means of energy expenditure is overt physical activity. Multiple studies have suggested that part of the reason for the obesity epidemic is our increasingly sedentary lifestyle (Hill and Peters, 1998, Stubbs and Lee, 2004). The NHAPS study from 2004 showed that in the USA, less than 1% of daily energy expenditure was due to 'exercise', while 'driving the car' accounted for more than 10% of the energy expended (Dong et al., 2004). There is capacity for physical activity to be increased to help elevate energy expenditure and treat obesity; however, as many studies have shown, and as described above, very few people manage to maintain increased levels of exercise that cause a significant loss of weight (Franz et al., 2007).

Beyond conscious physical activity, skeletal muscle increases energy expenditure in cold environments by causing shivering; indeed as rats are exposed to progressively colder temperatures, oxygen consumption and shivering increase in parallel (Gautier, 2000). Mammals have a much higher skeletal muscle mass compared to similar sized reptiles, and it is probably the ability of this muscle to produce heat that enabled endotherms to develop (Rowland et al., 2015).

1.4.2 NON-SHIVERING THERMOGENESIS

Shivering creates heat through involuntary muscle contraction. However, the body can produce heat without muscle contraction, in a series of processes known as non-shivering thermogenesis (NST).

One key effector of NST is brown adipose tissue (BAT). BAT is so called because it is brown in colour due to a high density of mitochondria, and a high level of vascularity. The key marker of BAT is that its mitochondria possess the protein Uncoupling Protein 1 (UCP-1). Normally, mitochondria produce a proton gradient across the inner mitochondrial membrane, by the movement of electrons along the electron transport chain. The protons then flow back across the membrane via ATP-synthase, which harnesses the released energy as ATP. UCP-1, on the other hand, is essentially a channel which allows the protons to flow back across the mitochondrial membrane without going via ATP-synthase; the energy is therefore released as heat.

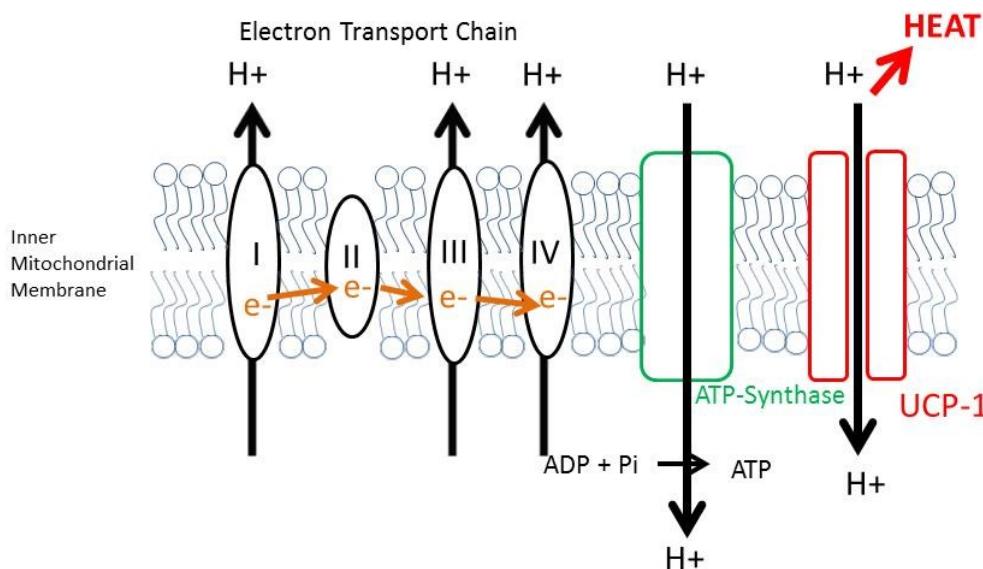


Figure 1.4 Diagram of the ATP and heat production in the inner mitochondrial membrane.

The electron transport chain causes movement of protons across the membrane. These then flow back down the electron gradient. Typically, the protons move via ATP-synthase, and the energy of the protons is captured in high-energy bonds in ATP. However, in the mitochondria of brown adipose tissue, UCP-1 allows protons to move across the membrane without passing through ATP-synthase; the resultant energy as they move down the electron gradient is then lost as heat.

BAT is sympathetically innervated, controlled by specific areas in the hypothalamus and brainstem, including the nucleus tractus solitarius, paraventricular nucleus and lateral hypothalamus (Song et al., 2005, Cano et al., 2003). BAT expresses β_3 adrenoreceptors, and it is through these receptors that it is activated by the sympathetic nervous system (SNS) (Xiao et al., 2015, Mirbolooki et al.,

2014, Vaughan and Bartness, 2012). The main stimulus for BAT activation is cold (Smith and Roberts, 1964, Cypess et al., 2009) but it can also be directly activated by noradrenaline, β_3 agonists, and other sympathomimetics (Mirbolooki et al., 2014, Mirbolooki et al., 2013, Krief et al., 1993, Ootsuka et al., 2011).

BAT is found in large quantities in small rodents (Page and Babineau, 1950), and in human infants (Dawkins and Scopes, 1965), where the large surface area-to-volume ratio means thermogenesis is key to allow survival. However, 3 seminal studies in 2009 showed that there are significant amounts of BAT in adult humans (Lichtenbelt et al., 2009, Virtanen et al., 2009, Cypess et al., 2009), and that it can be activated. This has led to interest in the possible role of BAT as a treatment for obesity. It has been predicted that full activation of 50g of BAT could equate to 20% of resting energy expenditure, which would imply a loss of 4.1kg fat per year (Virtanen et al., 2009, Cypess et al., 2009, Yoneshiro et al., 2011).

Recently, attention has focused on BAT as a source of NST; but other tissues can also cause NST. One such tissue is skeletal muscle. The predominant enzyme in skeletal muscle is myosin ATPase; this allows hydrolysis of ATP to produce muscle contraction; the speed of contraction is related to ATPase activity (Barany, 1984). However, skeletal muscle fibres also contain sarcoendoplasmic reticulum calcium ATPase (SERCA) and sarcolipin (SLN). SERCA is a pump which uses ATP to move calcium ions from the cytosol to the sarcoplasmic reticulum during muscle relaxation. SLN uncouples the hydrolysis of ATP from the movement of calcium: ATP is broken down without calcium accumulation (Smith et al., 2002), leading to the energy from ATP being dissipated as heat. This uncoupling is an important source of NST, with SLN knockout mice unable to increase their energy expenditure during cold exposure, leading to rapid death from hypothermia (Bal et al., 2012). This shows the importance of NST as a source of energy expenditure for physiological stability, as well as the importance of muscle in the basal metabolic rate (Zurlo et al., 1990).

1.4.3 DIET INDUCED THERMOGENESIS

Diet induced thermogenesis (DIT) is defined as the increase in energy expenditure after feeding above the basal fasting metabolic rate, divided by the energy content of the food consumed. It comprises the energy-consuming processes that occur after ingestion, such as digestion and absorption of nutrients, initial metabolism, and storage of the metabolites until they are further processed. This can equate to anything from 8-27% of man's daily energy expenditure, and possibly even more in rodents, though it varies significantly between individuals (Jequier and Schutz, 1983, Rothwell and Stock, 1982, Levine, 2002). Moreover, it changes with the macronutrient content of the food, being greater for protein, then carbohydrate, then fat (Westerterp, 2004).

1.4.4 BASAL METABOLIC RATE

Basal metabolic rate (BMR) is the minimum energy requirement of an organism at rest to maintain baseline cellular integrity and processes. This is the biggest contributor to energy expenditure, accounting for about 60% of an individual's daily expenditure (Levine, 2002). Age, sex, thyroid status and lean body mass all affect BMR (Cunningham, 1980, Johnstone et al., 2005).

A variety of processes contribute to BMR. There are processes that are common to most cells, such as Na⁺/K⁺ATPase to maintain ion gradients across the cell membrane, and cytosolic protein synthesis and RNA/DNA synthesis, which require ATP; there are also processes that are tissue-specific, such as hepatic gluconeogenesis or cardiac cell excitation (Buttgereit and Brand, 1995). The contribution of different tissues to basal metabolic rate depend on both their specific metabolic requirements and their size: both brain and skeletal muscle have overall the same energy expenditure because despite its small mass, the brain is very metabolically active (Rolfe and Brown, 1997).

The metabolic processes detailed above require ATP, and oxygen is contemporaneously consumed to replenish the ATP. There are also some cellular processes that use oxygen without consuming ATP, such as oxidation within peroxisomes, and mitochondrial proton leak, which also contribute to BMR (Rolfe and Brown, 1997).

1.4.5 FUTILE SUBSTRATE CYCLES

Within basal metabolic processes, there is a subset of processes known as futile substrate cycles. These involve two or more reactions which work in a cyclical fashion; in the interconversion between compounds, there is the net hydrolysis of ATP but no overall change in metabolites because it is a cycle. Examples include the conversion of glucose to glucose-6-phosphate and back, and phosphofructokinase-1/fructose 1,6-bisphosphatase and the reverse (Phillips et al., 1995, Berg et al., 2002). Modelling has suggested there are more than 15,000 substrate cycles (Gebauer et al., 2012). Energy expenditure through these cycles can vary dramatically. The role of these futile cycles is not fully understood, but hypotheses include improving metabolic regulation and thermogenic regulation (Qian and Beard, 2006, Miyoshi et al., 1988). Indeed it has been proposed that a futile cycle between fructose 6-phosphate and fructose diphosphate using phosphofructokinase and fructose diphosphatase is essential in maintaining thorax temperature in bumblebees during the short periods of time that they rest to feed, to allow them to continue to fly (Newsholme et al., 1972).

1.4.6 MANIPULATION OF ENERGY EXPENDITURE BY HORMONES

The energy expenditure processes described above can be affected by endogenous and exogenous hormones. Physical activity may be manipulated by thyroid status: in a study of euthyroid obese and overweight postmenopausal women, thyroid-stimulating hormone levels were inversely associated with physical-activity induced energy expenditure (Rondeau et al., 2010). Patients with subclinical hypothyroidism demonstrated impaired response to exercise (Caraccio et al., 2005). Some researchers also suggest thyroid hormone levels will influence spontaneous physical activity level, with increased small movements/fidgeting in hyperthyroidism, and the converse in hypothyroidism; these small movements are not picked up on measures of traditional physical activity and are therefore difficult to assess directly (Danforth and Burger, 1984).

Basal metabolic rate is also readily affected by hormone levels. Thyroid status accounts for some of the variance in basal metabolic rate in man (Johnstone et al., 2005), and thyroid hormones affect many of the processes that contribute to BMR. Tri-iodothyronine increases levels of the Na⁺/K⁺ATPase (Azuma et al., 1993, Danforth and Burger, 1984), while thyroid hormone also alters skeletal muscle fibre type and levels of the calcium pump SERCA, affecting muscular contractility and efficiency; for example, hypothyroid rats have a reduction in fast-twitch myosin (Soukup and Diallo, 2015, Sayen et al., 1992). There is reduced and increased protein turnover in hypo- and hyperthyroidism respectively, with reciprocal changes in energy requirements (Danforth and Burger, 1984, Muller and Seitz, 1984, Carter et al., 1981). Thyroid hormones also affect futile cycles: tracer studies in rats showed that the futile cycling between glucose and glucose 6-phosphate is increased in hyperthyroid and reduced in hypothyroid states (Okajima and Ui, 1979).

Catecholamines also modulate many of the cellular processes which contribute to basal metabolic rate. Adrenaline infusions increase nitrogen excretion in man, suggesting an effect on protein turnover (Ratheiser et al., 1998) as well as having an acute effect on Na⁺/K⁺ATPase activity (Ewart and Klip, 1995). In contrast, repeated administration of glucocorticoids down-regulates many cellular systems such as immune processes and cellular proliferation (Barnes, 2006, Coutinho and Chapman, 2011, Rogatsky et al., 1997, Gronemeyer, 1992), which may underlie the reduction in energy expenditure and increased obesity seen with chronic glucocorticoid exposure.

Non-shivering thermogenesis is susceptible to endocrine manipulation: exogenous catecholamines can enhance BAT thermogenesis in rodents (Mirbolooki et al., 2014, Mory et al., 1984), while local thyroid metabolism through expression of the deiodinase type-2 gene is essential for BAT-thermogenesis (de Jesus et al., 2001, Hall et al., 2010). In man, adrenergic and thyroid stimulation of NST via skeletal muscle is probably more important than BAT activation.

1.5 THESIS SUMMARY AND AIMS

This introduction shows that oxyntomodulin and its analogues are attractive as potential anti-obesity therapies as they can reduce food intake and increase energy expenditure at the same time. However, the way oxyntomodulin increases energy expenditure is not understood. As multiple processes are involved in energy expenditure, it is important to have a systematic approach to the investigation of energy expenditure by any drug. This thesis will endeavour to take such an approach to the mechanisms underlying the energy expenditure effects of oxyntomodulin.

This thesis will therefore:

- Confirm that a novel oxyntomodulin-like dual-analogue at the GLP-1 and glucagon receptor, OX2, does indeed increase energy expenditure.
- Determine which receptor mediates this energy expenditure.
- Understand which metabolic processes are affected by OXM to cause this elevation in energy expenditure.

Chapter 2

Energy Expenditure Effects Of Oxyntomodulin Analogues

2.1 INTRODUCTION

As shown, obesity is a major international health concern. Several pharmaceutical companies are developing analogues of oxyntomodulin (i.e. dual agonists at the glucagon and GLP-1 receptor) as a potential treatment for obesity (Tschop et al., 2016). Oxyntomodulin is an attractive target because it produces a negative energy balance by both reducing food intake and increasing energy expenditure.

Dakin *et al.* used a pair-feeding paradigm to show that rats given oxyntomodulin ICV twice daily lost more weight than animals whose food intake was restricted to match that of the oxyntomodulin group but which only received saline injections. This extra weight loss was attributed to an increase in energy expenditure (Dakin et al., 2002). Subsequently Wynne *et al.* showed that thrice daily subcutaneous injections of oxyntomodulin increased activity-related energy expenditure in man (Wynne et al., 2006).

The measurement of energy expenditure *in vivo* is complex. The 'gold standard' is to measure an individual's oxygen consumption, as through aerobic respiration, oxygen consumption is directly related to energy expenditure. In rodents, metabolic cages are used to assess oxygen consumption. These are sealed cages, with constant input and removal of oxygen and carbon dioxide. The concentrations of both gases are monitored at the inlet and outlet ports, enabling a measure of oxygen consumption and carbon dioxide production.

Metabolic cages also allow measurement of other metabolic parameters: food intake, locomotor activity, and the respiratory exchange ratio (RER) (a measure of the fuel that is being oxidised at a given time). These features allow an examination of the underlying mechanism for the energy expenditure. For example, if oxygen consumption increases in parallel with an increase in physical activity, then it would suggest that locomotion is the source of the increased energy expenditure.

There are limitations to the system (Speakman, 2013). Within CLAMS metabolic cages, 16 rat metabolic cages are connected within one system. This limits the ability to power studies. There can be no additional environmental enrichment in the cages, which makes them more stressful than their home cages; this may affect metabolism. And while the cages can measure physical activity, most movement by rodents is to search novel environments and seek food; however, the cages are small and food easily accessible, so will change normal behaviour in rodents.

An alternative approach to looking at chronic energy expenditure is through pair-feeding. Pair-feeding studies involve a control group, a peptide group, and a pair-fed group. Body weight and food

intake are measured daily. The peptide group is injected daily with peptide while the control and pair-fed groups are injected daily with vehicle. The peptide and control groups have unlimited access to standard laboratory chow throughout the experiment. The control and peptide groups receive their first injection, and 24 hours later, the pair-fed group is injected; however, they are only given access to the median amount of food consumed by the peptide group in the preceding 24 hours. As the peptide and pair-fed groups consume the same amount of food each day, any additional weight loss in the peptide group represents the energy expenditure effect of the peptide.

This approach has several advantages when compared to metabolic cage studies. More animals can be included, enabling more groups to be studied. Animals remain in their home cages, so the stressful effects of metabolic cages are avoided. Comparison of the peptide and pair-fed groups allows examination of both the anorectic effects of drugs and their energy expenditure effects. However, there are limitations to the pair-feeding paradigm. Changes in body weight and food intake are not direct measurements of energy expenditure but surrogate markers. Body weight can be affected by other processes e.g. hydration or gastrointestinal emptying, which are not measured in the pair-feeding set up. Because food is only presented once per day, and typically during the light phase, the behaviour of the pair-fed animals changes, such that they often eat all their food at once soon after food is presented, in contrast to the continuous consumption of food during the dark phase in control animals. And as pair-feeding involves comparison of food intake and body weight over a 24-hour period, acute changes in energy expenditure, over minutes and hours, may be missed.

No study has yet directly measured increased energy expenditure after administration of native OXM. Studies in metabolic cages by Baggio *et al.* failed to show an increase in oxygen consumption in mice given ICV injections of oxyntomodulin (Baggio *et al.*, 2004). In humans, indirect hood calorimetry (which, like metabolic cages, directly measures oxygen input and carbon dioxide output), failed to show an increase in energy expenditure after injections of OXM (Wynne *et al.*, 2006).

One of the difficulties of studying the effects of oxyntomodulin is that it has a very short half-life, lasting approximately 6 -12 minutes in the circulation (Schjoldager *et al.*, 1988, Kervran *et al.*, 1990, Baldissera *et al.*, 1988). Any physiological response to exogenous oxyntomodulin will therefore be very brief and difficult to capture. To overcome the difficulties associated with the short half-life of oxyntomodulin, several groups have developed oxyntomodulin analogues. By making specific amino-acid changes, the peptide can be made resistant to degradation *in vivo*. Amino acid alterations also affect the potency of oxyntomodulin, improving efficacy and potency at both the GLP-1 and glucagon receptors. Increased energy expenditure has been shown directly in studies using these

analogues. Liu *et al.* showed a direct increase in energy expenditure, as measured by increased oxygen consumption for 6 hours, in rats continually infused with the oxyntomodulin analogue OXM6421 over an 8-day period (Liu *et al.*, 2010). Similarly elevated energy expenditure has been shown in metabolic cages in mice given 3 different oxyntomodulin analogues (Lynch *et al.*, 2014, Henderson *et al.*, 2016, Day *et al.*, 2009); Henderson *et al.* confirm this elevation in energy expenditure with a pair-feeding study in DIO mice.

The underlying mechanisms by which oxyntomodulin increases energy expenditure are not established at present. Liu *et al.* and Day *et al.* showed no change in physical activity in rats given an oxyntomodulin analogue; they suggested the enhanced energy expenditure was due to changes in underlying basal metabolic processes, but gave no further suggestions as to which processes were affected (Liu *et al.*, 2010, Day *et al.*, 2009). In contrast, Lynch *et al.* found their OXM analogue increased physical activity in mice during the dark phase, and suggested this accounted for the increased energy expenditure (Lynch *et al.*, 2014). However, the increase in oxygen consumption in this study occurred over both the light and dark phases, i.e. not obviously correlated to the increased locomotion. This suggests other processes may be also increase energy expenditure.

2.1.1 OXYNTOMODULIN ANALOGUES OX2 AND OX2-GLU3

This chapter will first introduce a novel dual GLP1 and glucagon receptor ligand, OX2, while chapter 3 also uses the analogue OX2-Glu3. Both analogues have been designed by Professor S Bloom (Imperial College, London) as part of a drug-discovery programme. Both are based on the sequence of native oxyntomodulin. For full sequences, please see Section 9.1, Appendix A: Peptide Sequences.

OX2 is 35 amino-acids in length. Compared to native oxyntomodulin, there are 5 amino-acid substitutions between residues 16-27. Some of these substitutions are derived from the sequence for either GLP-1 or exendin-4, and therefore increase activity of OX2 at the GLP-1 receptor. The other changes improve potency at the glucagon receptor. There are also changes in the octapeptide tail of oxyntomodulin which enable the peptide to form a complex with zinc chloride. This is used as a diluent so that a subcutaneous depot is formed, which prolongs the action of OX2 *in vivo*.

The N-terminal of oxyntomodulin is essential for its efficacy at the glucagon receptor. OX2-Glu3 is identical to OX2, but the third residue is changed from glutamine to glutamate, a change which lowers affinity of the peptide for the glucagon receptor while retaining activity at the GLP-1 receptor (Kosinski *et al.*, 2012, Pocai *et al.*, 2009, Price *et al.*, 2015b, Price *et al.*, 2015a).

Within this chapter, initial studies will compare OX2 to native OXM *in vitro* and *in vivo* to demonstrate OX2 is an appropriate analogue to use to investigate the physiological properties of

OXM. I shall then examine if OX2 increases energy expenditure, both directly by examining oxygen consumption in metabolic cages, and indirectly in a pair-feeding study. The metabolic cage studies will allow initial investigations into the roles of locomotor activity and basal metabolic processes in the energy expenditure effects of OX2. These investigations will be further developed in the subsequent chapters of this thesis, with the aim to elucidate the mechanisms underlying the energy expenditure caused by dual GLP-1 and glucagon receptor agonists.

2.2 CHAPTER SUMMARY AND AIMS

This chapter aims to

- Confirm the *in vitro* action of OX2 as an oxyntomodulin analogue by demonstrating its functional activity at both the GLP-1 and glucagon receptors, and comparing its potency to native oxyntomodulin.
- Compare *in vivo* the pharmacokinetic properties of OX2 and OXM, and their acute effects on food intake and body weight in rats.
- Demonstrate that OX2 increases energy expenditure with direct measurement of oxygen consumption.
- Characterise the metabolic changes caused by OX2, as described in CLAMS metabolic cages.
- Demonstrate the energy expenditure effect of OX2 by pair-feeding.

2.3 METHODS

2.3.1 PEPTIDES

The oxyntomodulin analogue OX2 was designed by Professor S Bloom (Imperial College, London). Based on the sequence of native oxyntomodulin, the peptide was altered to have a longer half-life and greater stability. The sequences of all peptides used in this thesis are found in Appendix A (see 9.1, page 214). OX2 was custom synthesised by Insight Biotechnology Limited (Wembley, UK), using an automated fluorenylmethyloxycarbonyl (Fmoc) solid phase peptide synthesis method with each amino acid sequentially added from the C to the N terminus.

Peptides were cleaved from the resin and then purified using reverse phase preparative high performance liquid chromatography (HPLC) followed by lyophilisation. Peptide purity was determined by reverse-phase HPLC and by matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS). All peptides supplied had a purity of >95%.

OX2 was designed to have a prolonged period of action when dissolved in a zinc-chloride containing vehicle. Unless otherwise stated, throughout this thesis OX2 is administered in this vehicle which prolongs its action.

2.3.1.1 *IN VITRO* STUDIES

2.3.1.2 CELL MAINTENANCE

The cells used in this thesis are CHO cells, stably over-expressing the human GCG (hGCG), human GLP-1 (hGLP1) and rat GCG (rGCG) receptors, and HEK cells, stably over-expressing the rat GLP-1 (rGLP-1) receptor. All cells are maintained at 37°C with 5% CO₂. All HEK cells were maintained in media containing Dulbecco's Modified Media (DMEM) (Gibco® by Life Technologies), supplemented with 100U/ml penicillin and 100µg/ml streptomycin and 10% fetal bovine serum (Gibco® by Life Technologies); the CHO cell media was additionally supplemented with 0.1mM non-essential amino acids and 25mM HEPES (pH 7.3, both Sigma-Aldrich). Cells were passaged every 2-3 days when at 80% confluence.

2.3.1.3 cAMP ACCUMULATION BIOACTIVITY ASSAY

cAMP accumulation was measured using a cAMP dynamic 2 assay (Cisbio Assays, Codolet, France). This is based on Homogenous Time-Resolved Fluorescence (HTRF® technology). CHO cells were detached from their flasks using 0.05% trypsin-EDTA (Gibco® by Life Technologies), while HEK cells were detached using 0.02% EDTA (Sigma Aldrich). Cells were added to a 96-well plate at a density of 10000 cells/well (8×10^5 cells/ml) in serum-free media. The peptides were reconstituted in serum-

free media containing IBMX (3-isobutyl-1-methylxanthine, Sigma-Aldrich), and added at different concentrations to the cells. Following the manufacturer's protocol, the cells were then incubated at room temperature for exactly 30 minutes. 12.5µl of the cAMP labelled dye d2 was then added, followed immediately by a monoclonal anti-cAMP antibody labelled with Eu3+-Cryptat. The assay was further incubated at room temperature for one hour, before the fluorescence ratio (665nm/620nm) was determined using a Spectra Max® i3x plate reader (Molecular Devices, Sunnyvale, CA). The concentration of cAMP produced was then compared to a cAMP standard curve performed at the same time.

2.3.2 IN VIVO STUDIES

All animal procedures undertaken were approved by the British Home Office Animal (Scientific Procedures) Act 1986 (Project Licences 70/7236 and 70/7596).

2.3.2.1 ANIMAL HUSBANDRY

Adult male Wistar rats (Charles River, Margate, UK) were single-housed in individual cages under controlled temperature environment (21-23°C) with a 12:12 hour light-dark cycle (lights on at 0700). All animals had *ad libitum* access to water. In the metabolic cages, animals had unlimited access to powdered RM1 diet, while in their home cages, they had access to pelleted RM1 diet (Special Diet Services, Witham, UK). Prior to all experiments, animals were extensively handled and acclimatised to subcutaneous (SC) injections to limit the non-specific stress effects of handling during any study.

2.3.2.2 PHARMACOKINETICS OF OX2

Four male rats were injected with a single SC injection of 6mg of OX2 in 30µl of zinc chloride diluent at a 1:1 molar ratio concentration of peptide to zinc. Injections were performed in fed rats at the start of the light phase. Blood samples were collected from the rats via tail venesection into 1.5ml microtubes that had been flushed with heparin and contained 4µl of 25x protease inhibitor (Sigma-Aldrich). Samples were taken before the SC injection, at time 0, and then at 30 minutes, 3, 24, 48, 72 and 168 hours. Samples were then centrifuged at 4°C for 8 minutes at 10,000g; the plasma was removed and stored at -20°C.

OX2 levels in the plasma were measured using a sensitive in-house radioimmunoassay. The assay was performed in 700µl of 0.06M phosphate EDTA buffer containing 0.3% bovine serum albumin at pH7.4. The antibody (R6241) had been raised in rabbits against a glucagon (1-6) fragment, coupled to glutaraldehyde and used at a final dilution of 1:1000. This antibody cross-reacted specifically with the N-terminal glucagon sequence common in oxyntomodulin analogues including OX2. ¹²⁵

glucagon was produced by direct iodination and purified by reverse phase high-pressure liquid chromatography. The assay was incubated for 3 days at 4°C and separated into free and antibody bound label using charcoal separation. Both pellet and supernatant were counted using a γ -counter (model NE1600, NE Technology Ltd, Reading UK) for 180 seconds, and the ratio of free to bound label calculated. All samples were assayed in duplicates, and the concentration of OX2 compared to a standard curve prepared from purified OX2.

2.3.3 THE ACUTE EFFECT OF OXM AND OX2 ON FOOD INTAKE AND BODY WEIGHT IN FASTED RATS

Rats were randomised into 3 groups of equal average body weight (average body weight 537g, range 481-587g) (n=8-9). All animals were fasted overnight from 1600. At 0800, animals were weighed and received a single SC injection of either vehicle, OXM (40nmol/kg) diluted with zinc chloride in a 1:1 molar ratio, or OX2 (40nmol/kg) diluted with zinc chloride in a 1:1 molar ratio. At the same time, pre-weighed standard pelleted laboratory chow was returned to the cages. Food weight was re-measured at 30 minutes and 2, 8 and 24 hours after injection; body weight was reassessed at 24 hours post-injection.

2.3.4 METABOLIC CAGES

The metabolic cages used in this work are the Comprehensive Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments, Columbus, Ohio). CLAMS cages are enclosed metabolic monitoring cages. The cages have a food hopper that rests on a highly-sensitive balance, allowing precise monitoring of food consumption by animals. There are infrared beams that transect all of the chambers' axes; movement is assessed by counting the number of 'beam breaks' that occur within an interval. Conventionally, the number of breaks of the beam in the X-axis is used to record physical activity. The cages also include an open circuit calorimeter, which measures oxygen consumption (VO_2) and carbon dioxide production (VCO_2). Measurements of all parameters are taken for 1 minute every 20 minutes. From measurement of VO_2 and VCO_2 , the respiratory exchange ratio (RER) and 'heat', a measure of energy expenditure, can be calculated. RER is the ratio of the rate of carbon dioxide production compared to the rate of oxygen consumption. RER reflects the fuel source the animal is using; metabolizing pure carbohydrates gives an RER of 1.0, metabolizing pure fat gives an RER of 0.7, and protein causes an RER of 0.82 (Lusk, 1924, Frayn, 1983). Heat, given in kilocalories (kcal/hr), is calculated by multiplying the VO_2 by a calorific value derived from the RER; consequently, heat is directly proportional to VO_2 . As VO_2 is directly related to aerobic metabolism, this value is typically used as an expression of energy expenditure, rather than the derived 'heat' value.

The animals remain in the metabolic cages throughout the study, apart from when they are briefly removed for measurement of body weight, and injection of any experimental substances.

2.3.4.1 THE EFFECT OF OX2 ON RAT METABOLISM AS MEASURED BY METABOLIC CAGES

16 rats were randomised to two groups of equal mean body weight (average body weight 266g, range 246-319g). On day 0, all animals were placed in the CLAMS cages, to become acclimatised to the new cage. All animals had free access to food throughout the experiment, and were not fasted. On days 1, 2 and 3 they received subcutaneous injections at 0830 of vehicle or OX2 (40nmol/kg). At the same time body weight was measured. On day 4, all animals were returned to their home cages.

In view of the natural diurnal changes in energy expenditure, physical activity and RER that occur in rats, I repeated the above protocol, but with injections of OX2 or vehicle at 1630.

2.3.5 3-DAY PAIR-FEEDING STUDY WITH OX2

24 rats were randomised to three groups (n=8) by body weight (mean body weight 414g, range 387-470). The control and peptide group received daily SC injections of vehicle and OX2 (40nmol/kg) for 3 days at 0830 starting on day 0. The pair-fed group received daily injections of saline from day 1, but food intake was restricted to the mean intake of the OX2 group over the previous 24 hours. Therefore the vehicle and OX2 groups were freely fed throughout, while the pair-fed group had restricted access to food through the study. Food intake and body weight were measured daily at 0830.

2.3.6 STATISTICAL ANALYSIS

All data throughout this thesis are expressed as mean $\pm$ SEM.

All statistical analysis in this thesis was conducted using Prism V7 (GraphPad Software Inc). The specific statistical test used, plus any post-hoc analysis, is described in each experiment.

2.4 RESULTS

2.4.1 KEY RESULTS FOR CHAPTER 2

- OX2 is an appropriate analogue to study the pharmacology of OXM as:
 - OX2 is more potent at both the GLP-1 and glucagon receptors than OXM, but has a similar preference for the glucagon receptor over the GLP-1 receptor.
 - OX2 has prolonged activity *in vivo* when compared to OXM.
 - OX2 caused an increase in energy expenditure from 2-12 hours after a single SC dose, as measured by oxygen consumption in CLAMS cages; the increase in energy expenditure was confirmed in the pair-feeding study.
 - OX2 had no effect on physical activity despite increasing energy expenditure.
 - OX2 caused a rapid reduction in body weight in rats, but had inconsistent effects on food intake.
 - OX2 caused an acute reduction in respiratory exchange ratio after a single SC dose.
-

2.4.2 COMPARING OX2 WITH NATIVE OXM

2.4.2.1 OXM AND OX2 ON cAMP PRODUCTION AT THE GLUCAGON AND GLP-1 RECEPTORS.

This study examined the potency of OXM, OX2, glucagon and GLP-1 by looking at the ability of these peptides to stimulate cAMP production in cells over-expressing glucagon or GLP-1 receptors (rat and human). At the rat glucagon receptor, OXM was approximately 37 times less potent than glucagon; OX2 was also less potent, but only 4 times so. At the rat GLP-1 receptor, OXM was 89 times less potent than GLP-1, while OX2 was 18 times less potent. Both OXM and OX2 were more active at the glucagon receptor than the GLP-1 receptor.

	RAT GCGr		RAT GLP-1r	
	cAMP EC ₅₀ (nM) ± SEM	Ratio to native peptide (arbitrary units)	cAMP EC ₅₀ (nM) ± SEM	Ratio to native peptide (arbitrary units)
GLP-1	ND	ND	1.1 ± 0.3	1
GLUCAGON	4.2 ± 3.2	1	ND	ND
OXM	157.0 ± 70.0	37	93.0 ± 30.0	89
OX2	17.5 ± 3.2	4.2	18.2 ± 4.3	17

Table 2-1 Summary of the mean EC₅₀ values for the natural ligands and the analogue OX2 at the rat glucagon receptor and GLP-1 receptor.

CHO cells over-expressing the rat GLP-1 receptor, and HEK cells over-expressing the rat GCG receptor were incubated with peptides over a range of concentrations and then cAMP levels quantified by HTRF® technology. EC₅₀ values shown are the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the peptide EC₅₀/native peptide EC₅₀. ND – not detectable.

A similar picture was seen at the human GCG and GLP-1 receptors. OXM was 6 times less potent than native glucagon at the human glucagon receptor; OX2 was 4 times less potent. At the GLP-1 receptor, OXM was 16 times less potent than GLP-1, while OX2 was 9 times less potent. OXM and OX2 again appeared to have slightly greater potency at the glucagon receptor than the GLP-1 receptor.

	HUMAN GCGr		HUMAN GLP-1r	
	cAMP EC ₅₀ (nM) ± SEM	Ratio to native peptide (arbitrary units)	cAMP EC ₅₀ (nM) ± SEM	Ratio to native peptide (arbitrary units)
GLP-1	>1000	ND	23.3 ± 1.1	1.0
GLUCAGON	0.3 ± 0.2	1.0	ND	ND
OXM	2.0 ± 0.2	6.0	376 ± 147.7	16
OX2	1.3 ± 0.9	4.4	208.0 ± 106	9.0

Table 2-2 Summary of the mean EC₅₀ values for the natural ligands and the analogue OX2 at the human glucagon receptor and GLP-1 receptor.

CHO cells over-expressing either the human GLP-1 or glucagon receptor were incubated with a peptides over a range of concentrations and then cAMP levels quantified by HTRF® technology. EC₅₀ values shown were the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the peptide EC₅₀/native peptide EC₅₀. ND – not detectable.

2.4.2.2 PHARMACOKINETICS OF OXM

OX2 was detected throughout the study, with a peak value at 48 hours after injection. Plasma values persisted at approximately half this value to 7 days post-injection (Figure 2.1).

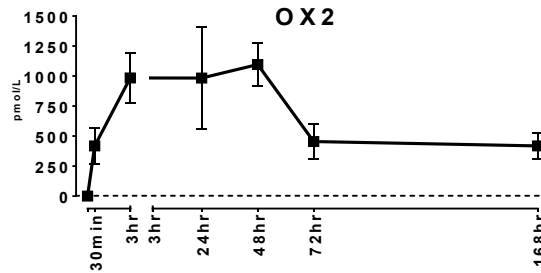


Figure 2.1 Pharmacokinetic profile of a single SC injection of OX2.

OX2 was administered at 6mg per rat into male Wistar rats, in a diluent containing a 1:1 molar ratio of zinc chloride. Plasma levels were taken from tail venesection at the time-points stated, and assessed by in-house radioimmunoassay. Data presented as mean $\pm$ SEM (pmol/L).

In contrast, oxyntomodulin administered in a similar study (courtesy of J. Minnion, unpublished data), produces a very high peak at 30 minutes, but is undetectable by 24 hours.

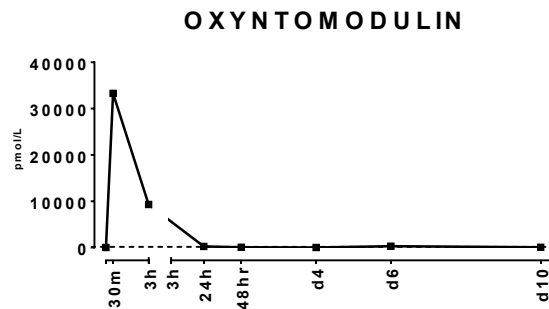


Figure 2.2 Pharmacokinetic profile of a single SC injection of OXM

OX2 was administered at 2mg per rat into male Wistar rats, in a diluent containing a 1:1 molar ratio of zinc chloride. Plasma levels were taken from tail venesection at the time-points stated, and assessed by in-house radioimmunoassay. Data presented as mean $\pm$ SEM (pmol/L).

2.4.2.3 THE ACUTE EFFECT OF OXM AND OX2 ON FOOD INTAKE AND BODY WEIGHT IN FASTED RATS

In rats who had been fasted overnight and subsequently administered a single dose of vehicle, OXM or OX2, over 24 hours, there was no significant difference in food intake in any of the groups. However, there was a tendency for both the OXM and OX2 groups to eat less than the vehicle group (average food intake $\pm$ SEM (g), vehicle: 42.7 ± 1.5 g; OXM: 40.8 ± 1.3 g; OX2: 39.2 ± 1.4 g) (Figure 2.3A). There was a significant effect of time period, and a significant interaction of time-period and injection, over the 0-30 minute, 30 minute-2hr, 2-8hr and 8-24hr periods (ordinary two-way ANOVA, effect of treatment, $F(2,91)=1.7$, $p=0.2$; effect of time period, $F(3,91)=556.7$, $p<0.0001$; interaction, $F(6,91)=3.3$, $p<0.01$). There was no significant difference in food intake between groups in the 0-30 minute, 30 minute-2 hour or 2-8 hour intervals (0-30 minutes, average food intake $\pm$ SEM (g), vehicle: 4.8 ± 1.2 g; OXM: 4.2 ± 0.4 g; OX2: 5.7 ± 0.3 g; 30 minutes-2 hours, average food intake $\pm$

SEM (g), vehicle: 6.5 ± 1.0 g; OXM: 5.2 ± 0.5 g; OX2: 4.8 ± 0.6 g; 2-8 hours average food intake $\pm$ SEM (g), vehicle: 1.8 ± 0.7 g; OXM: 3.5 ± 1.0 g; OX2: 3.2 ± 0.6 g). However, in the 8-24 hour time interval, the OX2 group ate significantly less than the vehicle group (average food intake $\pm$ SEM (g), vehicle: 29.7 ± 1.5 g; OXM: 27.3 ± 1.1 g; OX2: 24.6 ± 1.2 g; vehicle vs. OX2, $p < 0.05$) (Figure 2.3C). Body weight gain over 24 hours was significantly less in the OX2 group only compared to the vehicle group (average body weight change $\pm$ SEM (g): vehicle: 19 ± 1.4 g; OXM: 16 ± 1.6 g; OX2: 3 ± 3.6 g; vehicle vs. OX2, $p < 0.001$) (Figure 2.3B).

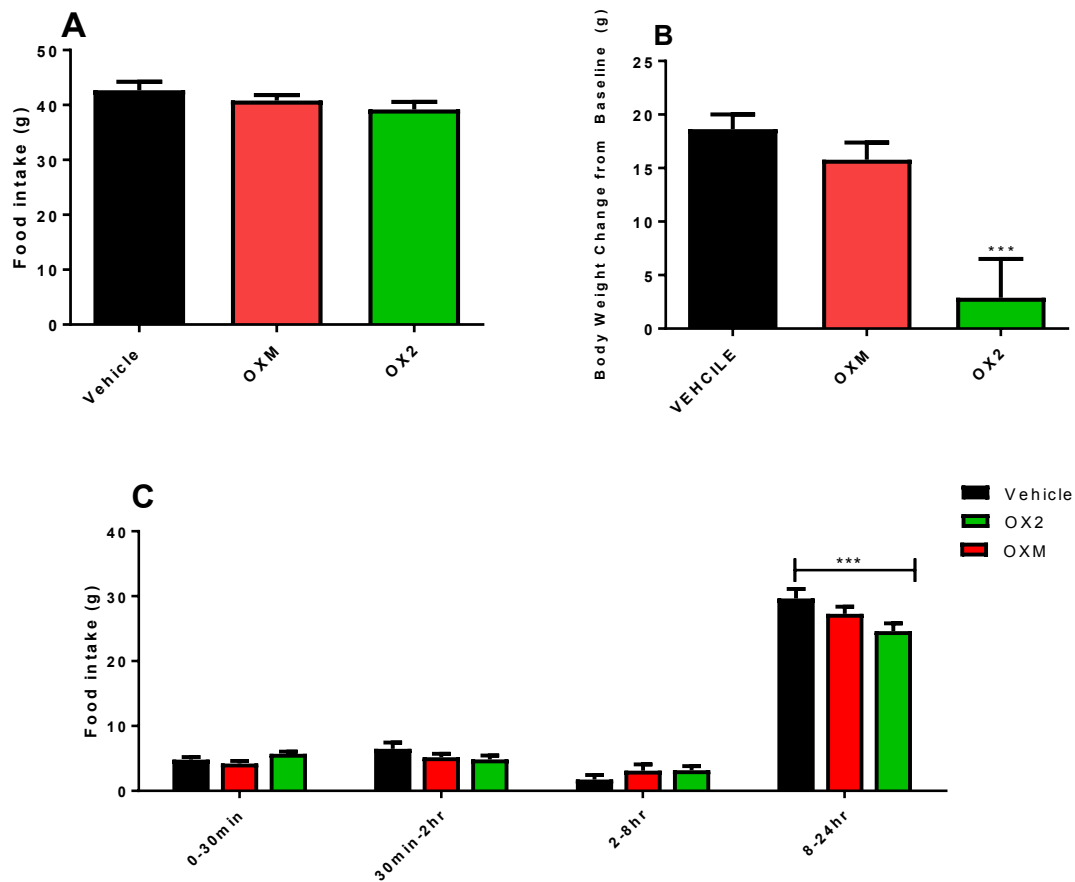


Figure 2.3 Comparison of OXM and OX2 on food intake and body weight in over-night fasted rats.

Cumulative food intake over 24 hours (A), cumulative body weight change from baseline over 24 hours (B) and cumulative food intake over specific time periods (C) in male Wistar rats fasted overnight from 1600, and then given a single SC injection of vehicle, OXM (40nmol/kg in zinc chloride in a 1:1 molar ratio) or OX2 (40nmol/kg in zinc chloride in a 1:1 molar ratio). Groups have the same average baseline body weight (537g). Data presented as mean $\pm$ SEM (g). Statistical analysis of total food intake undertaken using an unpaired t-test, and analysis of cumulative body weight change undertaken using a Kruskal-Wallis test. Analysis of food intake over each separate time period undertaken using an ordinary 2-way ANOVA with Tukey's multiple comparison test. *** $p < 0.001$.

2.4.3 EFFECTS OF OX2 AS MEASURED BY CLAMS METABOLIC CAGES

2.4.3.1 EFFECTS OF OX2 ON FOOD INTAKE AND BODY WEIGHT IN RATS

Following 3 injections of OX2 or vehicle at 0830 in CLAMS cages, the OX2 group consumed significantly less food than the vehicle group when given free access to food (average food intake $\pm$ SEM (g): vehicle: 97.9 ± 3.5 g; OX2: 74.3 ± 5.7 g; $p < 0.01$) (Figure 2.4 A). This equated to a reduction in food intake of 24% in the OX2 group compared to the vehicle group over the course of the experiment. The anorectic effect was most pronounced straight after each injection, significantly suppressing food intake in each light phase (average food intake in the light phase of day 1 after the 1st injection, mean $\pm$ SEM(g), vehicle: 0.4 ± 0.1 g; OX2: 0.1 ± 0.1 g; $p < 0.01$; average food intake in the light phase of day 2 after the 2nd injection, mean $\pm$ SEM (g), vehicle: 0.4 ± 0.1 g; OX2: 0.2 ± 0.1 g, $p < 0.05$; average food intake in the light phase in day 3 after the 3rd injection, mean $\pm$ SEM(g), vehicle: 0.3 ± 0.1 g; OX2: 0.1 ± 0.1 g; $p < 0.01$) (Table 2-3 and Figure 2.4B). There was also a significant reduction in food intake in the OX2 group in the dark phase after the first injection, but no significant difference in the dark phases after the 2nd or 3rd injections (average food intake in the dark phase of day 1 after the 1st injection, mean $\pm$ SEM(g), vehicle: 1.6 ± 0.1 g; OX2: 0.9 ± 0.3 g; $p < 0.05$; average food intake in the dark phase of day 2 after the 2nd injection, mean $\pm$ SEM(g), vehicle: 1.7 ± 0.1 g; OX2: 1.4 ± 0.1 g; average food intake in the dark phase of day 3 after the 3rd injection, mean $\pm$ SEM(g), vehicle: 1.6 ± 0.1 g; OX2: 1.2 ± 0.2 g)(Table 2-3 and Figure 2.4B).

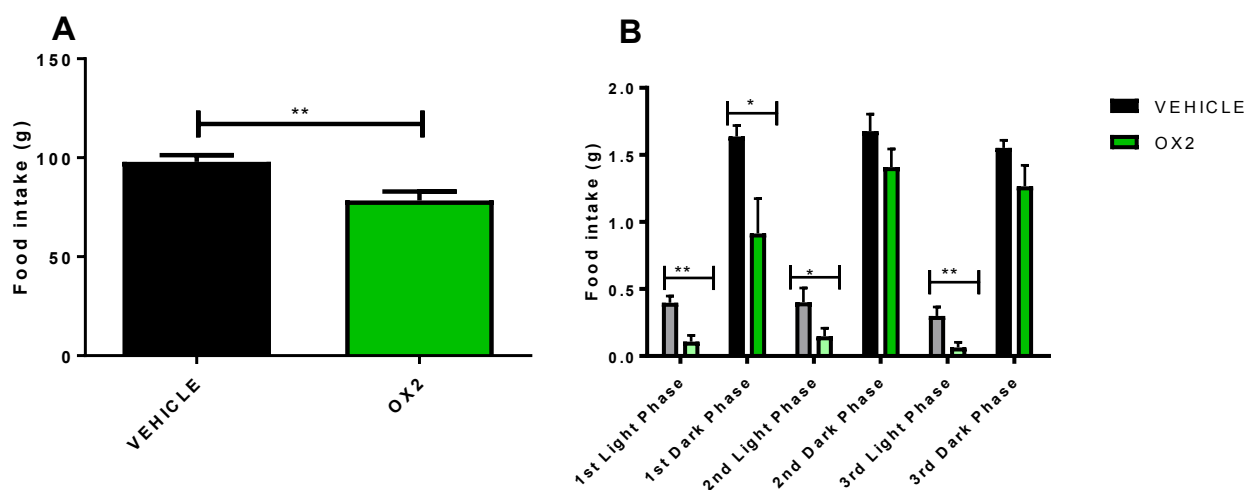


Figure 2.4 Effect of 3 daily injections of OX2 on food intake in rats as measured by CLAMS metabolic cages

Mean total food intake over the course of the experiment (A) and mean food intake over each light and dark phase (B) in rats given injections of vehicle or OX2 (40nmol/kg) at 0830 daily for 3 days in CLAMS metabolic cages, with unrestricted access to food. Data expressed as mean $\pm$ SEM (g). $n = 7-8$ /group. Statistical analysis undertaken using unpaired t-tests for each time-period. * $p < 0.05$, ** $p < 0.01$ vs vehicle.

TIME PHASE	Food intake, mean $\pm$ SEM (g)		STATISTICAL ANALYSIS (unpaired t-test for each time phase)
	Vehicle	OX2	
Light phase after 1 st injection	0.4 $\pm$ 0.1	0.1 $\pm$ 0.1	$p < 0.01$
Dark phase after 1 st injection	1.6 $\pm$ 0.1	0.9 $\pm$ 0.3	$p < 0.05$
Light phase after 2 nd injection	0.4 $\pm$ 0.1	0.2 $\pm$ 0.1	$p < 0.05$
Dark phase after 2 nd injection	1.7 $\pm$ 0.1	1.4 $\pm$ 0.1	ns
Light phase after 3 rd injection	0.3 $\pm$ 0.1	0.1 $\pm$ 0.1	$p < 0.01$
Dark phase after 3 rd injection	1.6 $\pm$ 0.1	1.2 $\pm$ 0.2	ns

Table 2-3 Average food intake in each light and dark phase in male Wistar rats given an injection of either vehicle or OX2 (40nmol/kg) at 0830 each day in CLAMS metabolic cages.

Both groups started at the same baseline body weight. At the end, of the experiment, the OX2 group weighed significantly less than the vehicle group (mean final body weight $\pm$ SEM (g), vehicle: 282 $\pm$ 5.2g; OX2, 252 $\pm$ 10.3g; $p < 0.05$). OX2 caused a reduction in absolute body weight with a loss of 7.7% from baseline, while vehicle caused an increase of 4.7% body weight over the three days; this led to a significant difference in change in body weight from baseline between the two groups (average change in body weight from baseline $\pm$ SEM (g), vehicle: 13 $\pm$ 1.2g; OX2: -21 $\pm$ 5.2g) (Figure 2.5).

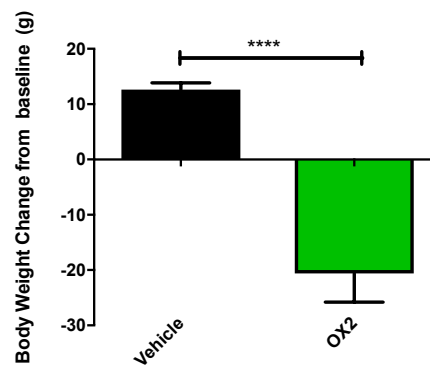


Figure 2.5 Effect of 3 daily injections of OX2 on body weight change from baseline in rats in as measured by CLAMS metabolic cages.

Vehicle or OX2 (40nmol/kg) were administered at 0830 daily for three days to rats single housed in CLAMS metabolic cages. Animals in both groups started with the same average baseline body weight (266g) and had unrestricted access to food. Data presented as mean $\pm$ SEM. n = 8/group. Statistical analysis performed using an unpaired t-test. **** $p < 0.0001$ vs vehicle.

2.4.3.2 EFFECT OF OX2 ON OXYGEN CONSUMPTION IN RATS

In fed rats in CLAMS metabolic cages, both groups showed a spike in oxygen consumption directly after each injection of vehicle or OX2 (Figure 2.6A). However, the rise in oxygen consumption was sustained in the OX2 group. The increase in oxygen consumption persisted for about 12 hours after each injection; therefore the total oxygen consumption increased in the OX2 group in each light period when compared to the vehicle group (light phase after 1st injection, average total oxygen consumption $\pm$ SEM (ml/kg), vehicle: 23879 ± 929 ml/kg; OX2: $26669 \pm 974 \pm 75.0$ ml/kg; $p=0.06$; light phase after 2nd injection, average total oxygen consumption $\pm$ SEM (ml/kg), vehicle: 23105 ± 656 ml/kg; OX2: 26452 ± 1187 ml/kg; $p<0.05$; light phase after 3rd injection, average total oxygen consumption $\pm$ SEM (ml/kg), vehicle: 23600 ± 753 ml/kg; OX2: 27224 ± 1088 ml/kg; $p<0.05$) (Figure 2.6B and Table 2-4).

In the dark phase after each injection, oxygen consumption was not significantly different between the groups (average oxygen consumption in the dark phase after the 1st injection, mean $\pm$ SEM (ml/kg), vehicle: 26232 ± 833 ml/kg; OX2: 27890 ± 1066 ml/kg; average oxygen consumption in the dark phase after the 2nd injection, mean $\pm$ SEM (ml/kg), vehicle: 24412 ± 839 ml/kg; OX2: 24254 ± 1292 ml/kg; average oxygen consumption in the dark phase after the 3rd injection, mean $\pm$ SEM (ml/kg), vehicle: 24792 ± 934 ml/kg; OX2: 23583 ± 1575 ml/kg) (Table 2-4).

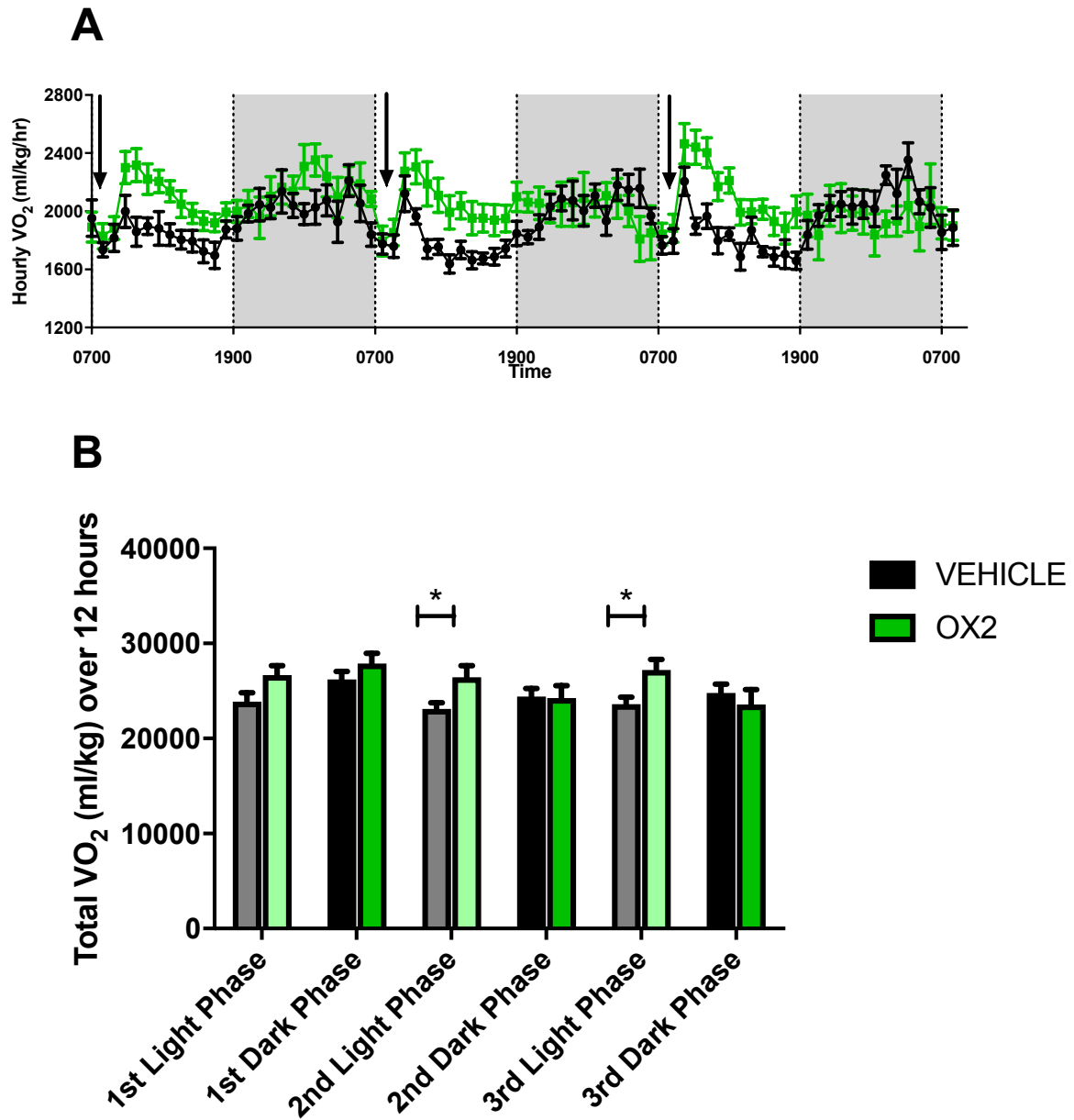


Figure 2.6 The effect of 3 daily injections of OX2 on oxygen consumption in rats as measured in CLAMS metabolic cages. Hourly oxygen consumption (A) and total oxygen consumption over the 12 hours of the light or dark phases (B) in male Wistar rats single housed in CLAMS metabolic cage, injected with vehicle or OX2 (40nmol/kg) at 0830 daily for 3 consecutive days and had unrestricted access to food. Data expressed as mean $\pm$ SEM. $n=7-8$. Arrows show when injections occurred. Shaded area represents the dark phase. Statistical analysis performed using unpaired t-tests at each time period. * $p<0.05$ vs vehicle.

TIME PHASE	Oxygen consumption (ml/kg $\pm$ SEM)		STATISTICAL ANALYSIS (unpaired t-test for each time phase)
	Vehicle	OX2 (40nmol/kg)	
Light phase after 1 st injection	23879 $\pm$ 929	26669 $\pm$ 974	$p=0.06$
Dark phase after 1 st injection	26232 $\pm$ 833	27890 $\pm$ 1066	ns
Light phase after 2 nd injection	23105 $\pm$ 656	26452 $\pm$ 1187	$p<0.05$
Dark phase after 2 nd injection	24412 $\pm$ 839	24254 $\pm$ 1292	ns
Light phase after 3 rd injection	23600 $\pm$ 753	27224 $\pm$ 1088	$p<0.05$
Dark phase after 3 rd injection	24792 $\pm$ 934	23583 $\pm$ 1575	ns

Table 2-4 Average oxygen consumption in each light and dark phase in male Wistar rats given an injection of either vehicle or OX2 (40nmol/kg) at 0830 each day.

2.4.3.3 EFFECT OF OX2 ON PHYSICAL ACTIVITY IN RATS

No significant difference or pattern of modified behaviour for physical activity was observed between the groups over the whole experiment, nor during any light or dark phase (Figure 2.7B and Table 2-5).

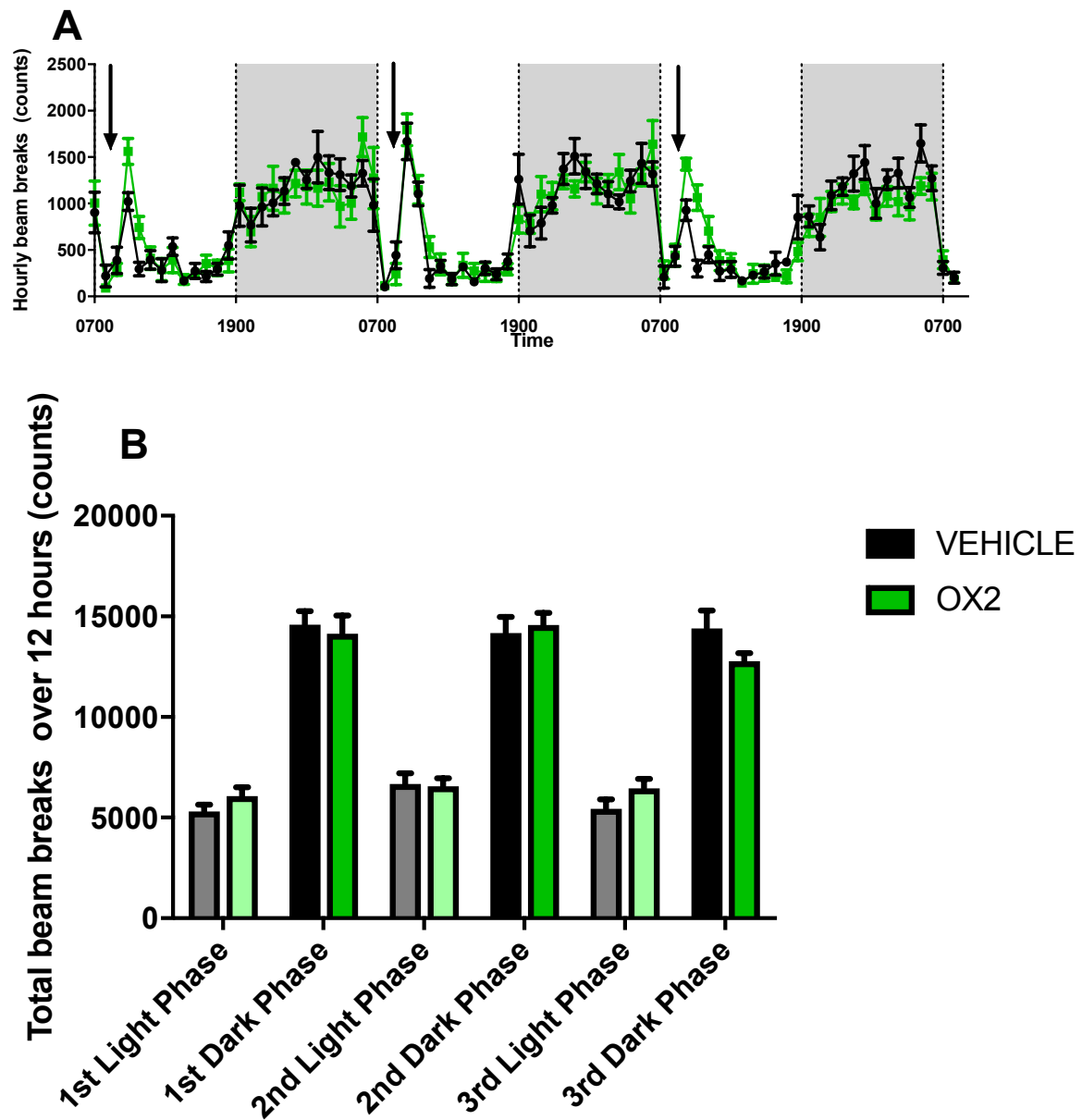


Figure 2.7 The effect of 3 daily injections of OX2 on physical activity in rats as measured in CLAMS metabolic cages. Hourly beam breaks (A) and total beam breaks over the 12 hours of the light or dark phases (B) in male Wistar rats single-housed in CLAMS metabolic cage, injected with vehicle or OX2 (40nmol/kg) at 0830 daily for 3 consecutive days, and had unrestricted access to food. Data expressed as mean $\pm$ SEM. $n=7-8$. Arrows show when injections occurred. Shaded area represents the dark phase. Statistical analysis performed using Mann-Whitney U tests at each time-period.

TIME PHASE	Beam breaks, mean counts $\pm$ SEM		STATISTICAL ANALYSIS (Mann-Whitney U test for each time period)
	Vehicle	OX2 (40nmol/kg)	
Light phase after 1 st injection	5309 $\pm$ 332.7	6075 $\pm$ 439.9	ns
Dark phase after 1 st injection	14598 $\pm$ 657.1	14135 $\pm$ 905.8	ns
Light phase after 2 nd injection	6681 $\pm$ 523.2	6562 $\pm$ 401.3	ns
Dark phase after 2 nd injection	14182 $\pm$ 791.2	14567 $\pm$ 609	ns
Light phase after 3 rd injection	5438 $\pm$ 478.5	6460 $\pm$ 461.4	ns
Dark phase after 3 rd injection	14411 $\pm$ 882.4	12770 $\pm$ 399.5	ns

Table 2-5 Average physical activity in each light and dark phase in male Wistar rats given an injection of either vehicle or OX2 (40nmol/kg) at 0830 each day

2.4.3.4 EFFECT OF OX2 ON RESPIRATORY EXCHANGE RATIO IN RATS

Administration of OX2 (40nmol/kg) caused an overall reduction in the average RER compared to vehicle in all light and dark phases (average RER in the light phase after the 1st injection, mean $\pm$ SEM, vehicle: 0.93 $\pm$ 0.004; OX2: 0.90 $\pm$ 0.011, $p < 0.01$; average RER in the light phase after the 2nd injection, mean $\pm$ SEM, vehicle: 0.91 $\pm$ 0.012; OX2: 0.85 $\pm$ 0.015, $p < 0.01$; average RER in the light phase after the 3rd injection, mean $\pm$ SEM, vehicle: 0.93 $\pm$ 0.007; OX2: 0.90 $\pm$ 0.012, $p < 0.05$; average RER in the dark phase after the 1st injection, average RER $\pm$ SEM, vehicle: 1.01 $\pm$ 0.012; OX2: 0.93 $\pm$ 0.03, $p < 0.05$; average RER in the dark phase after the 2nd injection, mean $\pm$ SEM, vehicle: 1.01 $\pm$ 0.007; OX2: 0.95 $\pm$ 0.02, $p < 0.05$; average RER in the dark phase after the 3rd injection, mean $\pm$ SEM, vehicle: 1.01 $\pm$ 0.011; OX2: 0.95 $\pm$ 0.02, $p < 0.05$)(Figure 2.8 B, and Table 2-6).

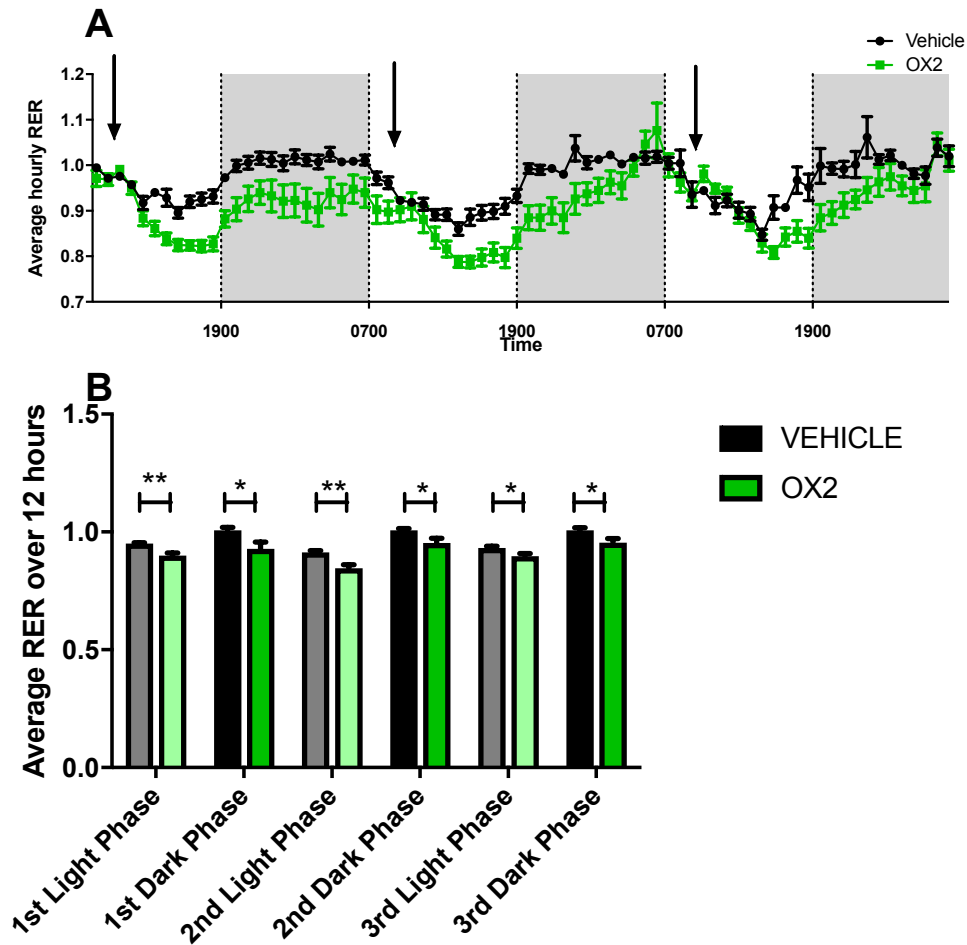


Figure 2.8 The effect of 3 daily injections of OX2 on respiratory exchange ratio in rats as measured in CLAMS metabolic cages.

Hourly RER (A) and average RER over the 12 hours of each of the light and dark phase (B) in male Wistar rats single housed in CLAMS metabolic cage, injected with vehicle or OX2 (40nmol/kg) at 0830 daily for 3 consecutive days. Data expressed as mean $\pm$ SEM. $n=7-8$. Arrows show when injections occurred. Shaded area represents the dark phase. Statistical analysis performed using unpaired t-tests at each time-period. * $p < 0.05$, ** $p < 0.01$ vs vehicle.

TIME PHASE	Respiratory Exchange Ratio mean $\pm$ SEM		STATISTICAL ANALYSIS (unpaired t-test for each time phase)
	Vehicle	OX2(40nmol/kg)	
Light phase after 1 st injection	0.95 $\pm$ 0.004	0.90 $\pm$ 0.011	$p < 0.01$
Dark phase after 1 st injection	1.01 $\pm$ 0.012	0.93 $\pm$ 0.030	$p < 0.05$
Light phase after 2 nd injection	0.91 $\pm$ 0.008	0.85 $\pm$ 0.015	$p < 0.01$
Dark phase after 2 nd injection	1.01 $\pm$ 0.007	0.95 $\pm$ 0.020	$p < 0.05$
Light phase after 3 rd injection	0.93 $\pm$ 0.007	0.90 $\pm$ 0.012	$p < 0.05$
Dark phase after 3 rd injection	1.01 $\pm$ 0.011	0.95 $\pm$ 0.018	$p < 0.05$

Table 2-6 Average RER in each light and dark phase in male Wistar rats given an injection of either vehicle or OX2 (40nmol/kg) at 0830 each day

2.4.3.5 EFFECT OF OX2 ON RATS WHEN INJECTED BEFORE THE START OF THE DARK PERIOD AS MEASURED IN METABOLIC CAGES

Over the course of 3 injections, OX2 caused a significant reduction in food intake compared to vehicle (average food intake $\pm$ SEM (g), vehicle: 75.9 ± 1.8 g; OX2: 41.0 ± 6.1 g; $p < 0.001$) (Figure 2.9A). From the same baseline body weight in each group (298g), OX2 caused a loss of body weight, while vehicle did not (average body weight change from baseline, mean $\pm$ SEM(g), vehicle: 2 ± 1.3 g; OX2: -48 ± 5.8 g; $p < 0.0001$); this lead to a significant difference in change in body weight from baseline between groups (Figure 2.9B).

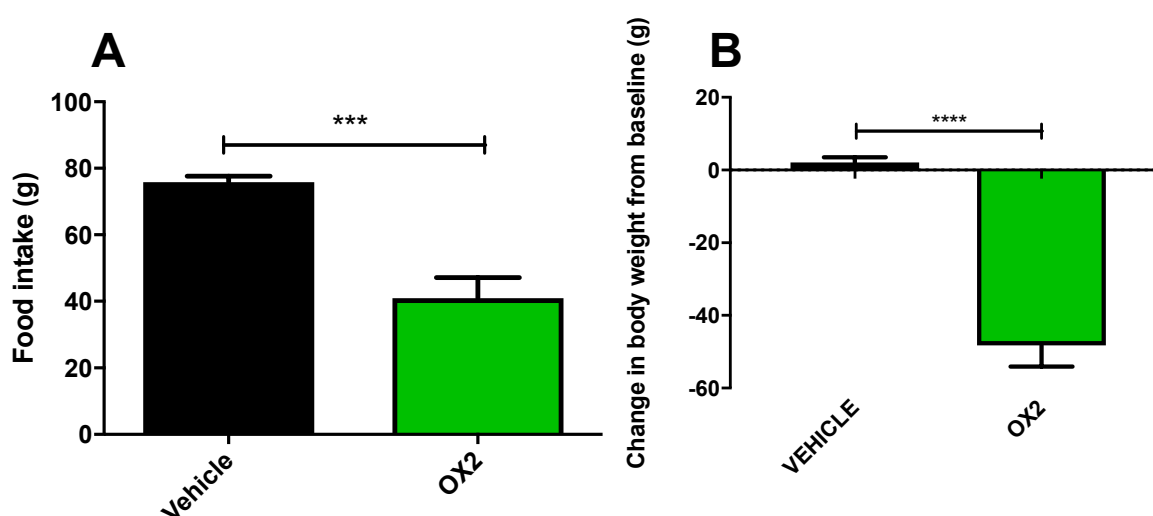


Figure 2.9 Effect of OX2 on food intake and body weight in rats when injected before the onset of the dark phase. Vehicle or OX2 (40nmol/kg) were administered at 1630 daily for 3 days to 16 male Wistar rats in CLAMS metabolic cages. Baseline body weight 298g. Mean total food intake (A) and mean body weight change from baseline (B) during the experiment. Data expressed as mean $\pm$ SEM (g). $n = 8$ /group. Statistical analysis undertaken using unpaired t-test. *** $p < 0.001$, **** $p < 0.0001$.

Though not as pronounced as with injections at 0800, OX2 increased oxygen consumption in the 12 hours after each injection at 1630. This increase was non-significant after the 1st and 3rd injections (average oxygen consumption in the 12 hours after the 1st injection, mean $\pm$ SEM (ml/kg), ml/kg, vehicle: 22236 ± 1162 ml/kg; OX2: 24691 ± 1640 ml/kg, $p = 0.2$; average oxygen consumption in the 12 hours after the 3rd injection, mean $\pm$ SEM (ml/kg), vehicle: 12753 ± 875 ml/kg; OX2: 10773 ± 610 ml/kg, $p = 0.08$), but was significant after the 2nd injection (average oxygen consumption in the 12 hours after the 2nd injection, mean $\pm$ SEM (ml/kg), vehicle: 24382 ± 1125 ml/kg; OX2: 29399 ± 1656 ml/kg $p < 0.05$)(Figure 2.10 and Table 2-7).

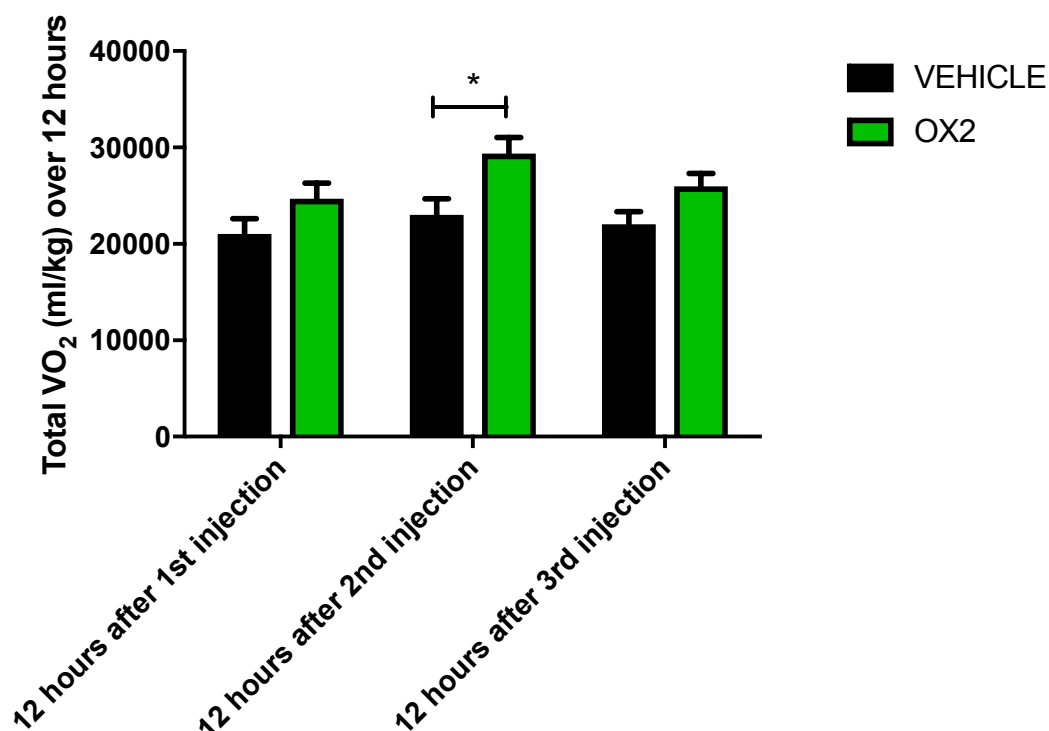


Figure 2.10 Effect of OX2 on oxygen consumption in rats when injected before the dark phase.

Oxygen consumption for 12 hours after injection of vehicle or OX2 (40nmol/kg) at 1630 daily for 3 days to 16 male Wistar rats in CLAMS metabolic cages, with free access to food. Data expressed as mean $\pm$ SEM. Statistical analysis undertaken by unpaired t-test after each injection. * $p < 0.05$ vs vehicle.

TIME PHASE	Oxygen Consumption (ml/kg $\pm$ SEM)		STATISTICAL ANALYSIS (unpaired t-test at each time period)
	Vehicle	OX2 (40nmol/kg)	
12 hours after 1 st injection	22236 $\pm$ 1162	24691 $\pm$ 1640	$p=0.2$
12 hours after 2 nd injection	24382 $\pm$ 1125	29399 $\pm$ 1656	$p<0.05$
12 hours after 3 rd injection	12753 $\pm$ 875	10773 $\pm$ 610	$P=0.08$

Table 2-7 Average oxygen consumption in rats for 12 hours after an injection of either vehicle or OX2 (40nmol/kg) at 1630 each day.

OX2 had a no significant effect on physical activity in the 12 hours after each injection, though there was a tendency for reduced movement in the OX2 group compared to the vehicle group (average physical activity for the 12 hours after the 1st injection, mean beam breaks $\pm$ SEM (counts), vehicle: 11550 $\pm$ 830 counts; OX2: 9377 $\pm$ 900 counts; average physical activity for the 12 hours after the 2nd injection, mean beam breaks $\pm$ SEM (counts), vehicle: 13158 $\pm$ 1027 counts; OX2: 11540 $\pm$ 811 counts; average physical activity for the 12 hours after the 2rd injection, mean beam breaks $\pm$ SEM(counts), vehicle: 12753 $\pm$ 875 counts; OX2: 10773 $\pm$ 610 counts) (Figure 2.11 and Table 2-8).

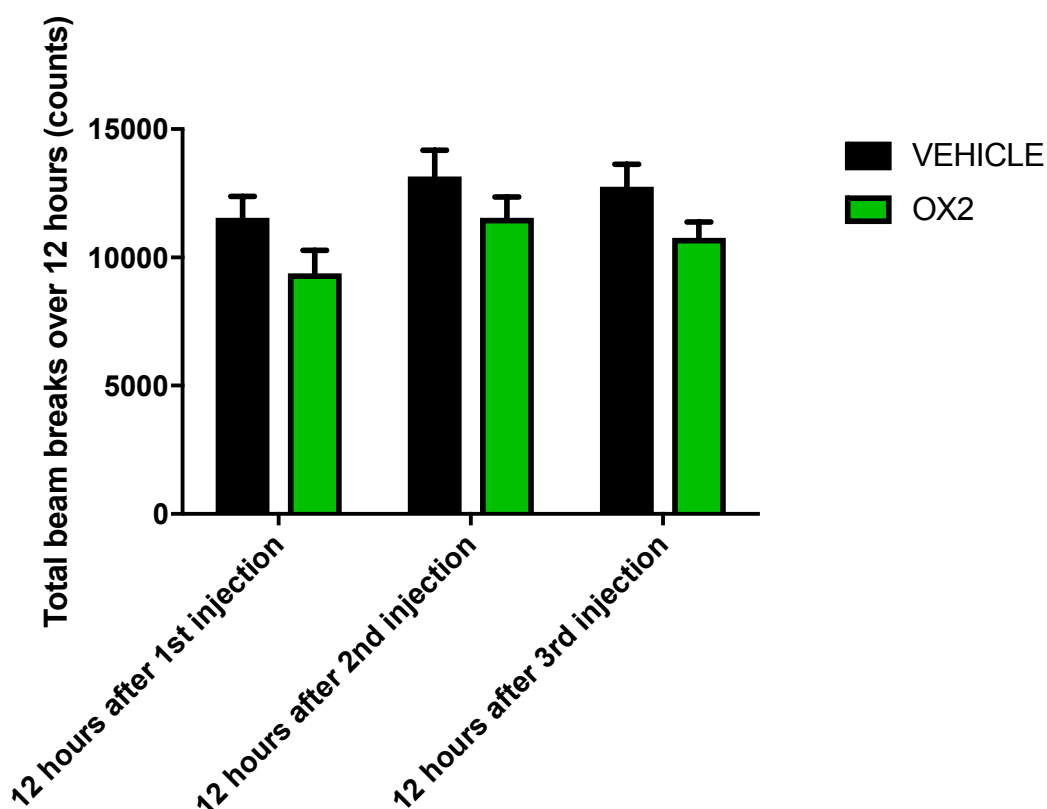


Figure 2.11 Effect of OX2 on physical activity in rats when injected before the dark phase.

Beam breaks for 12 hours after injection of vehicle or OX2 (40nmol/kg) at 1630 daily for 3 days to 16 male Wistar rats in CLAMS metabolic cages with free access to food. Data expressed as mean $\pm$ SEM. Statistical analysis undertaken by unpaired t-tests after each injection.

TIME PHASE	Physical Activity (beam breaks $\pm$ SEM)		STATISTICAL ANALYSIS (unpaired t-test at each time period)
	Vehicle	OX2 (40nmol/kg)	
12 hours after 1 st injection	11550 $\pm$ 830	9377 $\pm$ 900	p=0.09
12 hours after 2 nd injection	13158 $\pm$ 1027	11540 $\pm$ 811	p=0.2
12 hours after 3 rd injection	12753 $\pm$ 875	10773 $\pm$ 610	p=0.08

Table 2-8 Average physical activity in rats for 12 hours after an injection of either vehicle or OX2 (40nmol/kg) at 1630 each day.

The respiratory exchange ratio was suppressed by OX2 for the 12 hours after each injection (average RER for the 12 hours after the 1st injection, mean $\pm$ SEM, vehicle: 1.00 $\pm$ 0.002; OX2: 0.91 $\pm$ 0.012, p <0.0001; average RER for the 12 hours after the 2nd injection, mean $\pm$ SEM, vehicle: 1.00 $\pm$ 0.003; OX2: 0.86 $\pm$ 0.020, p <0.0001; average RER for the 12 hours after the 3rd injection, mean $\pm$ SEM, vehicle: 1.00 $\pm$ 0.005; OX2: 0.87 $\pm$ 0.019, p <0.0001) (Figure 2.12 and Table 2.9).

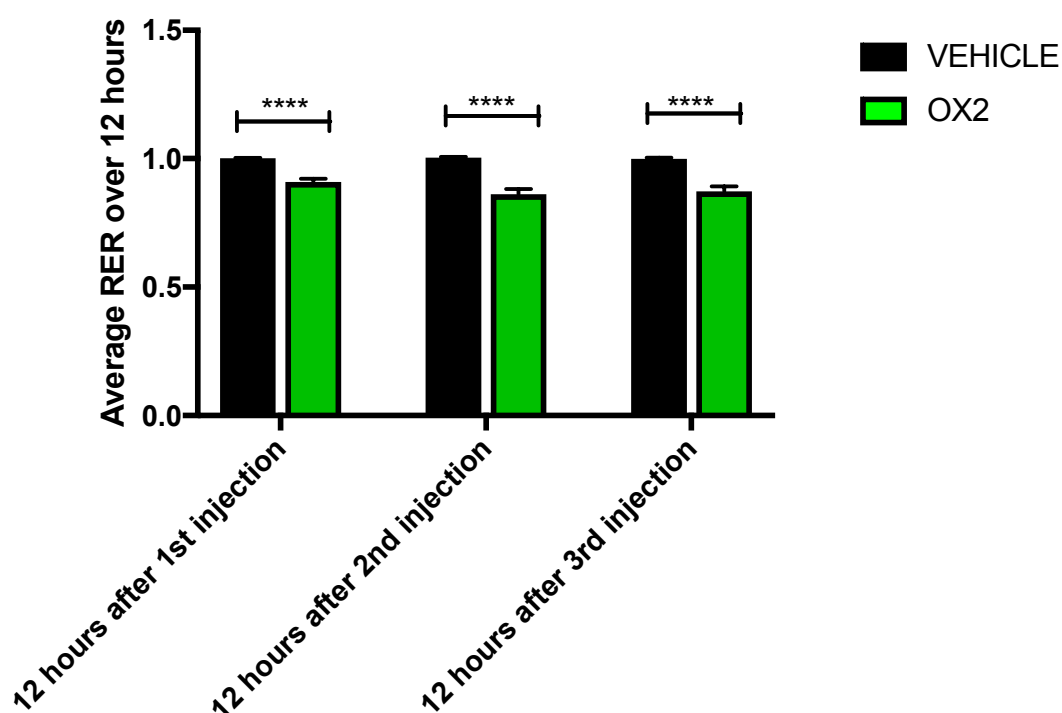


Figure 2.12 Effect of OX2 on RER in rats when injected before the dark phase.

Average RER for the 12 hours after each injection in 16 male Wistar rats receiving daily injections of vehicle or OX2 (40nmol/kg) for 3 days in CLAMS metabolic cages at 1630. Animals had free access to food throughout. n=7-8. Data expressed as mean ± SEM. Statistical analysis undertaken by unpaired t-test. **** $p < 0.0001$ vs vehicle.

Time Phase	RER (mean ± SEM)		Statistical analysis (unpaired t-test at each time period)
	Vehicle	OX2 (40nmol/kg)	
12 hours after 1 st injection	1.00 ± 0.002	0.91 ± 0.012	$p < 0.0001$
12 hours after 2 nd injection	1.00 ± 0.003	0.86 ± 0.020	$p < 0.0001$
12 hours after 3 rd injection	1.00 ± 0.005	0.87 ± 0.019	$p < 0.0001$

Table 2-9 Average RER in rats for 12 hours after an injection of either vehicle or OX2 (40nmol/kg) at 1630 each day.

2.4.4 PAIR-FEEDING STUDY WITH OX2 IN RATS

Daily injections of OX2 (40nmol/kg) significantly increased food intake compared with the control group; over the experiment, the OX2 group ate 9% more food than the vehicle group, though both groups had free access to food. The pair-fed group was unable to match this increased food intake, and overall food intake did not differ significantly from either the vehicle or OX2 groups (average food intake ± SEM (g), vehicle: 85.5 ± 1.8g; OX2: 92.8 ± 2.6g; pair-fed: 88.9 ± 0.99g; vehicle vs. OX2, $p < 0.05$) (Figure 2.13 A).

Both the vehicle and pair-fed groups gained weight during the experiment, while the OX2 group lost weight. The change in weight from baseline in the OX2 group was significantly different from both

the vehicle and pair-fed groups (average body change from baseline $\pm$ SEM(g), vehicle: 8 ± 1.6 g; OX2: -8 ± 2.2 g; pair-fed: 7 ± 1.8 g; vehicle vs. OX2 $p < 0.0001$, pair-fed vs. OX2 $p < 0.0001$, Figure 2.13B). After 3 days, the OX2 group weighed 4% less than the vehicle group, and 3% less than the pair-fed group.

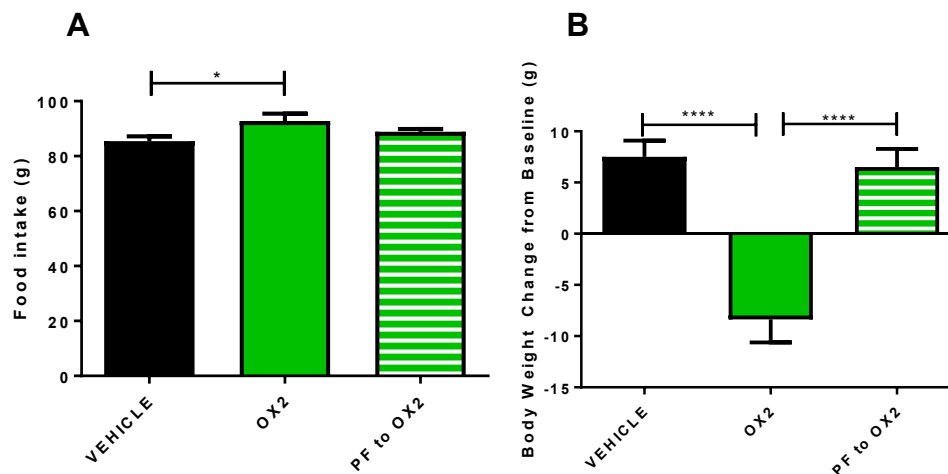


Figure 2.13 3-day pair-feeding study in rats with OX2.

Cumulative food intake (A) and bodyweight change from baseline (B) in male Wistar rats after 3 days of injections of vehicle, OX2 (40nmol/kg) or pair-feeding to OX2 group. Food and body weight were measured daily at 0830. N=8. Mean initial body weight in each group was 414g. Data shown as the mean $\pm$ SEM. Statistical analysis carried out using one-way ANOVA with post hoc tests Tukey's multiple comparison test, * $p < 0.05$, **** $p < 0.0001$.

2.4.5 SUMMARY TABLE OF KEY EFFECTS OF OX2

FOOD INTAKE IN THE FASTED RAT	REDUCED OVER 8-24 HRS COMPARED TO VEHICLE BUT NO OVERALL CHANGE COMPARED TO VEHICLE OR OXM FROM 0-24 HOURS	Figure 2.3
FOOD INTAKE IN THE FED RAT	REDUCED IN CLAMS COMPARED TO VEHICLE INCREASED OVER 3 DAYS IN PAIR-FEEDING COMPARED TO VEHICLE	Figure 2.4 & Figure 2.9 Figure 2.13
BODY WEIGHT IN THE FED RAT	REDUCED IN CLAMS COMPARED TO VEHICLE REDUCED IN PAIR-FEEDING COMPARED TO BOTH VEHICLE AND PAIR-FED ANIMALS	Figure 2.5 & Figure 2.9 Figure 2.13
OXYGEN CONSUMPTION	INCREASED IN CLAMS COMPARED TO VEHICLE	Figure 2.6 & Figure 2.10
PHYSICAL ACTIVITY	NO EFFECT IN CLAMS COMPARED TO VEHICLE	Figure 2.7 & Figure 2.11
RESPIRATORY EXCHANGE RATIO	REDUCED IN CLAMS COMPARED TO VEHICLE	Figure 2.8 & Figure 2.12

Table 2-10 Summary Table of Key Effects of OX2.

2.5 DISCUSSION

The studies in this chapter showed that the novel oxyntomodulin analogue OX2 reduced food intake and increased energy expenditure, and that this occurs via changes in basal metabolic processes.

Oxyntomodulin is a dual agonist at the GLP-1 and glucagon receptors. However, it is less efficacious at both the GLP-1 and GCG receptors than either native hormone (Table 2-1 and Table 2-2); (Druce et al., 2009). It has a very short half-life in circulation, in the order of minutes (Schjoldager et al., 1988), which limits the ability to study OXM physiology.

To overcome this limitation, analogues of OXM have been developed by several groups. OX2 is one such analogue. OX2 stimulates cAMP production at both the glucagon and GLP-1 receptors, confirming that it is a dual-agonist at the GLP-1 and GCG receptors (Table 2-1 and Table 2-2). OXM activates the GCG receptor with slightly greater efficacy than the GLP-1 receptor, as shown by a lower EC₅₀ ratio with the native hormone at the GCG receptor compared to the GLP-1 receptor; OX2 appears to also favour the GCG receptor to a similar degree. However, though OX2 is less potent than either GLP-1 or GCG at the respective receptors, it is more potent at both receptors than native OXM, as seen by lower EC₅₀ values at all receptors

One modification from OXM to OX2 is the addition of amino acids at the C-terminal that enable the peptide to complex with zinc in a subcutaneous depot. This significantly extends its half-life. As seen in the subcutaneous pharmacokinetic study, OX2 is still detectable one week after a single injection (Figure 2.1). Previous work from our laboratory (Figure 2.2), and predictions made given the known half-life of OXM, show no peptide would be present after approximately 3 hours (Schjoldager et al., 1988, Wynne et al., 2006).

The doses of OX2 required to be detectable by RIA (6mg per rat) are substantially higher than the doses required for a measurable *in vivo* effect. The prolonged half-life was also demonstrated *in vivo* in the acute feeding study. Though there was no significant difference in food intake between groups in the 0-30 minute time period, the OXM group did eat somewhat less than the vehicle and OX2 groups; in contrast, the anorectic effect of the OX2 group was only significantly demonstrable in the 8-24 hour interval (Figure 2.3A).

Work in this chapter confirms the findings of previous groups that oxyntomodulin analogues increase energy expenditure (Lynch et al., 2014, Liu et al., 2010). This increase in energy expenditure was directly measured in the CLAMS metabolic cages, where injections of OX2 caused, on average, a 14% increase in oxygen consumption when compared to vehicle (Figure 2.6). The pair-feeding study offered indirect evidence of energy expenditure, as the OX2 group lost significantly more body

weight than the pair-fed group (Figure 2.13). The acute feeding study also indirectly suggested an increase in energy expenditure in the OX2 group, as there was no significant difference in food intake overall between the vehicle, OXM and OX2 groups, but a significant reduction in body weight in the OX2 group (Figure 2.3B).

The elevation in oxygen consumption started immediately after injection of OX2, peaking at approximately 4 hours after injection and persisting for 12 hours (Figure 2.6). This profile remained regardless of the time of injection. This was a different time course from the anorectic effect of OX2, where food intake suppression was not seen until about 8 hours after the injection (Figure 2.3). This reinforces the idea that the energy expenditure and anorectic effects of OXM are mediated through different pathways, and potentially through different receptors.

The metabolic cage studies also help determine the aetiology of the energy expenditure. There is no increase in physical activity associated with the elevation in oxygen consumption with OX2 when injections are administered at 0830 (Figure 2.7 and Table 2-5). This supports the findings of Liu *et al.* that oxyntomodulin analogues increase energy expenditure through processes other than physical activity (Liu *et al.*, 2010). However, physical activity shows a marked diurnal variation in rats, with elevated movement in the dark phase associated with food seeking behaviour (Figure 2.7A). As the energy expenditure effects of OX2 appear to only last for 12 hours after injection, any effect on physical activity may be missed with injections at the start of the light phase. This may explain why twice daily dosing of an OXM-type analogue increased physical activity in the study by Lynch *et al.* (Lynch *et al.*, 2014), as the analogue was still present in the circulation during the dark phase. When the injections of OX2 were delivered at 1630, in order that peptide would still be present in the dark phase, the increase in oxygen consumption persisted again for 12 hours after injection (Figure 2.10 and Table 2-7). However locomotor activity was suppressed over the same period (Figure 2.11 and Table 2-8). This adds to the suggestion that oxyntomodulin increases energy expenditure through basal metabolic rate or non-shivering thermogenesis not physical activity. It also highlights other potential pharmacological effects of oxyntomodulin. The reduction in movement may be a consequence of the anorectic effect of OX2 suppressing the food-seeking behaviour in rats. Alternatively, it may reflect a sensation of nausea, causing altered behaviour in the rat; nausea and vomiting are common effects of initiation of GLP-1 agonists in humans. As rats cannot vomit, the sensation of nausea may manifest itself in reduced movement. Finally, the reduction in movement may be compensatory behaviour in response to the elevation in energy expenditure that the OX2 causes; the rats may move less to conserve energy by other means. These different possibilities cannot be determined from this study.

OX2 caused a notable change in respiratory exchange ratio, with a suppression in RER throughout the day and night, regardless of the time of injection (Figure 2.8, Table 2-6, Table 2-9 and Figure 2.12). Other OXM analogues cause a similar reduction in RER in mice (Lynch et al., 2014, Henderson et al., 2016). RER reflects which fuel is being oxidised by the body, and therefore being utilised for any metabolic processes. It is calculated as ratio of carbon dioxide production to oxygen consumption, and pure carbohydrate metabolism results in an RER of 1.00, whereas fat metabolism results in an RER of 0.70. Most studies only refer to carbohydrate and fat metabolism, based on tables established by Lusk in 1924 (Lusk, 1924). However, it is important to note that protein metabolism also affects RER, with pure protein metabolism giving a ratio of 0.82. Determining the relative contribution of protein metabolism requires a measure of urinary nitrogen, not available in CLAMS metabolic cages, and is therefore rarely described in *in vivo* studies (Frayn, 1983).

The reduction in RER may reflect the anorectic effect of OX2 and a need to use other fuel sources for metabolic processes (this accounts for the natural diurnal variation in RER, where a switch from carbohydrate to fat and protein metabolism occurs during the day when animals are resting). Alternatively, the reduction in RER may occur if OX2 directly upregulates metabolic pathways that affect fat and protein metabolism, switching to fat and protein oxidation despite sufficient carbohydrate availability. This is suggested by the very rapid change in RER following the first injection of OX2 (Figure 2.8 and Figure 2.12) – at this point animals are still well fed so a reliance on fat and protein metabolism would not be expected. The effect of OX2 on protein and fat metabolic pathways will be further analysed in chapters 6 and 7 of this thesis.

Comparison of the results of the CLAMS metabolic cage and pair-feeding studies in this chapter highlight some of the difficulties in measuring energy expenditure, as well as the importance of using multiple approaches to study energy expenditure effects of compounds. There was a notable difference in the effect of OX2 on food intake in the pair-feeding and CLAMS studies. OX2 in metabolic cages significantly suppressed food intake after the first injection, and this anorectic effect persisted for the three injections (Figure 2.4). In contrast, in the pair-fed study there was a slight increase in food intake in the OX2 group when compared to the vehicle group, despite receiving the same number of OX2 injections at the same dose (Figure 2.13). This was associated with only a 4% reduction in body weight in the OX2 group in the pair-feeding study, compared to an 11% reduction in body weight in the OX2 group in the metabolic cages (Figure 2.5 and Figure 2.13). Though CLAMS metabolic cages allow a direct measure of energy expenditure, they are very stressful environments, which lack the comfort and enrichment of normal rat cages. One study suggests acclimatisation to metabolic cages is not seen even after animals have remained in the cages for 3 weeks (Kalliokoski et

al., 2013). Stress has a notable effect on food intake, energy expenditure, and the efficacy of endogenous gut hormones (Marti et al., 1994, Abbott et al., 2006, Kalliokoski et al., 2013). Given the anorectic effect of OX2 seen in both the CLAMS studies, and in the acute feeding study where rats were moved and handled regularly (Figure 2.3), but not in the pair-feeding study, it appears that stress augments the anorectic effects of OX2. Though not possible to determine from the studies in this chapter, stress may also affect the energy expenditure effects of OXM. Further studies could look at the effect of OX2 administration on the hypothalamic-pituitary-adrenal stress axis, and if this affects weight loss and energy expenditure.

2.6 CONCLUSIONS

This chapter confirms that OX2 is a dual agonist at the glucagon and GLP-1 receptors, though with a greater potency than native oxyntomodulin, and a prolonged half-life when given as a subcutaneous depot. The studies in this chapter confirm that OX2 increases energy expenditure, and that this is not via an increase in physical activity. The studies also suggest that OX2 causes a change in fuel oxidation. Which metabolic processes are affected by OX2, and which receptor mediates these effects, will be investigated in the remaining chapters of this thesis.

Chapter 3

Receptors Responsible For The Increase Energy Expenditure Effect of Oxyntomodulin

3.1 INTRODUCTION

To date, neither rodent nor human studies have found an oxyntomodulin-specific receptor. Rather, oxyntomodulin is an agonist at both the glucagon and GLP-1 receptors, though is less potent than either cognate ligand.

As covered in the General Introduction, different actions of oxyntomodulin are ascribed to its different receptor activity. Activation of pancreatic GLP-1 receptors mediates the incretin effect of oxyntomodulin (Maida et al., 2008, Du et al., 2012), while activation of the glucagon receptor by oxyntomodulin tempers its glucose-lowering effects, probably through glycogenolysis (Du et al., 2012, Kosinski et al., 2012). Glucagon receptor activation is also responsible for the chronotropic effects of OXM (Sowden et al., 2007, Mukharji et al., 2013a). Activity at both the GLP-1 and glucagon receptors contribute to OXM's anorectic effects, though the GLP-1 receptor is the main mediator (Dakin et al., 2001, Dakin et al., 2004, Baggio et al., 2004, Pocai et al., 2009).

The receptor or receptors responsible for the energy expenditure effect of oxyntomodulin has not been determined. If OXM is compared to OXM-Glu3, a peptide with reduced activity at the glucagon receptor, the weight loss effect is reduced without any effect on food intake, suggesting the glucagon activity mediates an energy expenditure effect (Kosinski et al., 2012). Conversely, the thermogenic effect of centrally administered OXM is lost in GLP-1 knockout mice, suggesting the GLP-1 receptor mediates this effect (Lockie et al., 2012).

There are two experimental approaches that have been used to investigate receptor activity of oxyntomodulin *in vivo*. The first is to use mice with germ-line mutations which prevent expression of specific receptors. Several studies have used GLP-1 or glucagon knock-out mice to investigate oxyntomodulin physiology (Lockie et al., 2012, Baggio et al., 2004, Du et al., 2012, Sowden et al., 2007, Maida et al., 2008, Mukharji et al., 2013b). However, mice with germ-line alterations in the glucagon or GLP-1 receptors have compensatory changes in other components of the enteroendocrine axis. For example, GCG receptor knockout mice have raised GLP-1 levels compared to wild-type mice (Ali et al., 2011, Conarello et al., 2007). Therefore, these may not be reliable models for investigating either the physiology of oxyntomodulin or pharmacological intervention on this axis.

The second approach is to pharmacologically block the receptor. Exendin 9-39 (Ex9-39) is a robust and reliable antagonist at the GLP-1 receptor (Thorens et al., 1993). However, due to the need for very high doses of Ex9-39 to block the GLP-1 receptor, studies investigating the role of Ex9-39 on the effect of oxyntomodulin *in vivo* have all used centrally administered Ex9-39 (Dakin et al., 2001,

Baggio et al., 2004, Dakin et al., 2004). These studies therefore demonstrate the effect only of blocking central GLP-1 receptors, and not peripheral GLP-1 receptors. However, understanding the effects of activation of peripheral GLP-1 receptors by OXM is essential, both as OXM is produced in the periphery, and because any pharmacological treatment based on OXM will be administered in the periphery.

Pharmacological blockade of the glucagon receptors is also difficult. The commercially available antagonist des-His1-[Glu9]-Glucagon (1-29) amide does block the rat glucagon receptor, but has a short half-life and poor potency, making it difficult to use for *in vivo* experiments. Many long-acting glucagon antagonists are being developed for the treatment of diabetes. However, after liaison with Merck, Sharp & Dohme Corp. (Kenilworth, USA) who kindly made available glucagon receptor antagonists which are not commercially available, I determined that these antagonists are specific to the human glucagon receptor, and therefore inappropriate for the *in vivo* experiments used to investigate the energy expenditure effects of OXM.

Many groups use an alternative pharmacological approach to investigate the role of the GLP-1 and GCG receptors in the action of OXM. They compare the native hormone (or its derived analogues) with the identical peptide, but with a single amino-acid switch which eliminates its activity at the glucagon receptor. Switching glutamine at residue 3 (Gln3) to glutamate (Glu3) lowers affinity of the peptide for the glucagon receptor by several orders of magnitude, while retaining activity at the GLP-1 receptor (Kosinski et al., 2012, Pocai et al., 2009, Price et al., 2015a). The advantage of this approach is that both peptides can be administered at the same concentration and in the same way, enabling an easy comparison of the effects of OXM or its analogues with or without glucagon activity. However, Price *et al.* have recently shown that *in vitro*, some Glu-3 peptides can act as competitive antagonists at the glucagon receptor in rats, thereby blocking the effect of endogenous glucagon (Price et al., 2015b). *In vivo*, these specific analogues caused an increase in body weight with no concurrent increase in food intake. There was no direct measure of energy expenditure in this study, but the authors hypothesize that the increase in body weight may be due to suppression of energy expenditure normally caused by endogenous glucagon. This antagonist property may therefore exaggerate any differences in energy expenditure seen in groups given an OXM analogue and its Glu-3 pair.

Having noted these limitations, in this chapter I will endeavour to determine which receptor is responsible for the energy expenditure effect of OXM. Peripherally administered Ex9-39 will be used to blockade the GLP-1 receptor, while comparison of OX2 with OX2-Glu3 will determine the effect of the glucagon receptor.

Previous studies have only used indirect measures of energy expenditure to look at which receptor is responsible for the energy expenditure effect of OXM. Kosinski *et al.* assessed energy expenditure by comparing relative food intake and body weight change in wild-type and knock-out mice given OXM and OXM-Glu3 (Kosinski *et al.*, 2012), while Lockie *et al.* measured brown adipose tissue temperature in wild-type and GLP-1 knockout mice given OXM (Lockie *et al.*, 2012). This chapter will use direct measures of energy expenditure, by measuring oxygen consumption in metabolic cages. Therefore, these studies should allow for a reliable assessment of which receptor is responsible for the energy expenditure effect of OX2.

3.2 CHAPTER SUMMARY AND AIMS

This chapter will:

- Investigate the role of both the glucagon and GLP-1 receptors in the energy expenditure caused by OX2, as measured by indirect calorimetry, by
 - Blocking activity at the glucagon receptor by using an OX2/OX2-Glu3 switch.
 - Blocking activity at the GLP-1 receptor by using the GLP-1 antagonist Ex9-39.
- Investigate the role of both the glucagon and GLP-1 receptors in other metabolic processes affected by OX2, as measured in metabolic cages.

3.3 METHODS

3.3.1 PEPTIDES

As described previously, all oxyntomodulin analogues were designed by Professor S Bloom (Imperial College, London) and synthesized by Insight Biotechnology Limited (Wembley, UK). The peptides utilized in this study were OX2, and OX2-Glu3, which had a single amino acid change from glutamine to glutamate at position 3. The sequence of both peptides is seen in Appendix A (see 9.1, page 214). *In vivo*, both OX2 and OX2-Glu3 were administered in a diluent containing zinc chloride. The activity of OX2-Glu3 *in vitro* at both the glucagon and GLP-1 receptors was determined using a cAMP accumulation bioactivity assay as previously described (2.3.1.3). Exendin (9-39) was purchased from Bachem (Bubendorf, Switzerland).

3.3.2 IN VIVO STUDIES

All animal procedures undertaken were approved by the British Home Office Animal (Scientific Procedures) Act 1986 (Project Licences 70/7236 and 70/7596).

3.3.2.1 ANIMAL HUSBANDRY

Adult male Wistar rats (Charles River, Margate, UK) were single-housed in individual cages under controlled temperature environment (21-23°C) with a 12:12 hour light-dark cycle (lights on at 0700) conditions. Unless otherwise stated, all animals had *ad libitum* access to water and RM1 diet (Special Diet Services, Witham, UK). Prior to all experiments, animals were extensively handled and acclimatised to SC injections to limit the non-specific stress effects of handling during any study.

3.3.3 THE ROLE OF THE GCG RECEPTOR IN THE ENERGY EXPENDITURE EFFECT OF OX2

32 male Wistar rats were randomized to 3 groups of equal mean body weight (average baseline bodyweight 318g, range 276-356g) (n=10-11). On day 0, all animals were placed in CLAMS metabolic cages. On days 1, 2 and 3, all animals received subcutaneous injections of either vehicle (saline), OX2 (40nmol/kg) or OX2-Glu3 (40nmol/kg) at 0800. This dose was chosen as the same OX2 dose as used in the metabolic cage studies in chapter 2. Animals had free access to food throughout the study. Body weight was measured each day at the same time as the injections were administered. Oxygen consumption, carbon dioxide production, food intake and movement were measured by the CLAMS cages. On day 4, all animals were returned to their home cage.

3.3.4 THE ROLE OF THE GLP-1 RECEPTOR IN THE ENERGY EXPENDITURE EFFECT OF OX2

32 male Wistar rats were randomised to 4 groups of equal mean body weight (average baseline body weight 222g, range 201-245g) (n=8). On day 0, all animals entered CLAMS metabolic cages. Between 1000 and 1200 on day 1, all rats had mini-osmotic pumps (Alzet Osmotic Pumps, Model 1003D, Durect Co., Cupertino, USA) inserted under isofluorane anaesthesia. The pumps delivered either saline or Ex9-39 at 100nmol/kg/hr. This dose was chosen following preliminary studies as to its efficacy at blocking the GLP-1 receptor (See Section 9.2 Appendix B). Immediately after recovery from anaesthesia, the animals were returned to the CLAMS metabolic cages. At 0800 on day 2 and day 3, half of the animals receiving saline, and half of those receiving Ex9-39, were given a SC injection of vehicle, while the remaining animals received a SC injection of OX2 (40nmol/kg). Animals had free access to food throughout the period in CLAMS. This was the same dose as delivered in the metabolic cages studies in Chapter 2. At 0900 on day 4 animals were returned to their home cages. Body weight was measured daily.

3.4 RESULTS

3.4.1 KEY RESULTS FOR CHAPTER 3

- Oxyntomodulin increases energy expenditure via the glucagon receptor demonstrated by:
 - No increase in energy expenditure when the analogue OX2-Glu3 is administered, which lacks activity at the glucagon receptor.
 - Persistent increase in oxygen consumption when OX2 is co-administered with the GLP-1 antagonist Ex9-39.

3.4.2 *IN VITRO* ACTIVITY OF OX2 AND OX2-GLU3 AT THE GLUCAGON AND GLP-1 RECEPTORS

OX2-Glu3 caused no detectable accumulation of cAMP at the rat GCG receptor, confirming that the Gln-Glu switch removes activity at the glucagon receptor. OX2-Glu3 was 12 times less potent than GLP-1 at the rat GLP-1 receptor; this is slightly more potent than OX2 (Table 3-1).

	RAT GCGr		RAT GLP-1r	
	cAMP EC ₅₀ (nM) ± SEM	Ratio to native peptide (arbitrary units)	cAMP EC ₅₀ (nM) ± SEM	Ratio to native peptide (arbitrary units)
GLP-1	ND	ND	1.1 ± 0.3	1
GLUCAGON	4.2 ± 3.2	1	ND	ND
OXM	157.0 ± 70.0	37	93.0 ± 30.0	89
OX2	17.5 ± 3.2	4.2	18.2 ± 4.3	17
OX2-GLU3	ND	ND	12.1 ± 1.6	12

Table 3-1 Summary of the mean EC₅₀ values for the natural ligands and the analogues OX2 and OX2-Glu3 at the rat glucagon receptor and GLP-1 receptor.

CHO cells over-expressing the rat GLP-1 receptor, and HEK cells over-expressing the rat GCG receptor were incubated with a range of peptide concentrations and then cAMP levels quantified by HTRF® technology. EC₅₀ values are shown as the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the peptide EC₅₀/native peptide EC₅₀. ND – not detectable.

At the human GCGr receptor, OX2-Glu3 did cause cAMP accumulation, but it was 54x weaker than native glucagon, and 13.5x weaker than OX2. At the human GLP-1 receptor, OX2-Glu3 was 5 times weaker than native GLP-1, but again, was slightly more potent than OX2 at this receptor (Table 3-2).

	HUMAN GCGr		HUMAN GLP-1r	
	cAMP EC ₅₀ (nM) ± SEM	Ratio to native peptide (arbitrary units)	cAMP EC ₅₀ (nM) ± SEM	Ratio to native peptide (arbitrary units)
GLP-1	>1000	ND	23.3 ± 1.1	1.0
GLUCAGON	0.3 ± 0.2	1.0	ND	ND
OXM	2.0 ± 0.2	6.0	376 ± 147.7	16
OX2	1.3 ± 0.9	4.4	208.0 ± 106	9.0
OX2-GLU3	16.3 ± 0.4	54	112.1 ± 67	5.0

Table 3-2 Summary of the mean EC₅₀ values for the natural ligands and the analogues OX2 and OX2-Glu3 at the human glucagon receptor and GLP-1 receptor.

CHO cells over-expressing either the human GLP-1 or glucagon receptor were incubated with a range of peptide concentrations and then cAMP levels quantified by HTRF® technology. EC₅₀ values are shown as the mean of at least 3 separate experiments ± SEM. The ratio was calculated by dividing the peptide EC₅₀/native peptide EC₅₀. ND – not detectable.

3.4.3 ROLE OF THE GLUCAGON RECEPTOR IN THE ENERGY EXPENDITURE EFFECT OF OX2

3.4.3.1 COMPARISON OF OX2 and OX2-GLU3 ON FOOD INTAKE AND BODY WEIGHT IN RATS

OX2-Glu3 caused a significant reduction in food intake compared to both vehicle and OX2 groups, though all animals had free access to standard laboratory chow throughout the experiment (average food intake, mean ± SEM (g), vehicle: 96.0 ± 3.6g; OX2: 93.5 ± 2.3g; OX2-Glu3: 85.3 ± 2.4g; vehicle vs OX2-GLU3, $p < 0.05$; OX2 vs OX2-Glu3, $p < 0.05$). This was an 11% reduction in the OX2-Glu3 group compared to vehicle, and a 9% reduction compared to OX2. There was no significant difference in food intake between the OX2 and vehicle groups (Figure 3.1A).

From an equal average baseline body weight (318g), both the vehicle and OX2-Glu3 groups gained weight (4% and 2% weight gain respectively); the OX2 group remained the same average weight as baseline (final body weight, mean ± SEM (g), vehicle: 332 ± 5.5g; OX2: 318 ± 6.8g; OX2-Glu3: 323 ± 4.0g). This change in body weight from baseline was significantly different in the OX2 group compared to the vehicle group (average change in body weight from baseline, vehicle: 13 ± 1.8g; OX2: 1 ± 3.3g; OX2-Glu3: 6 ± 2.5g; vehicle vs OX2, $p < 0.01$) (Figure 3.1B).

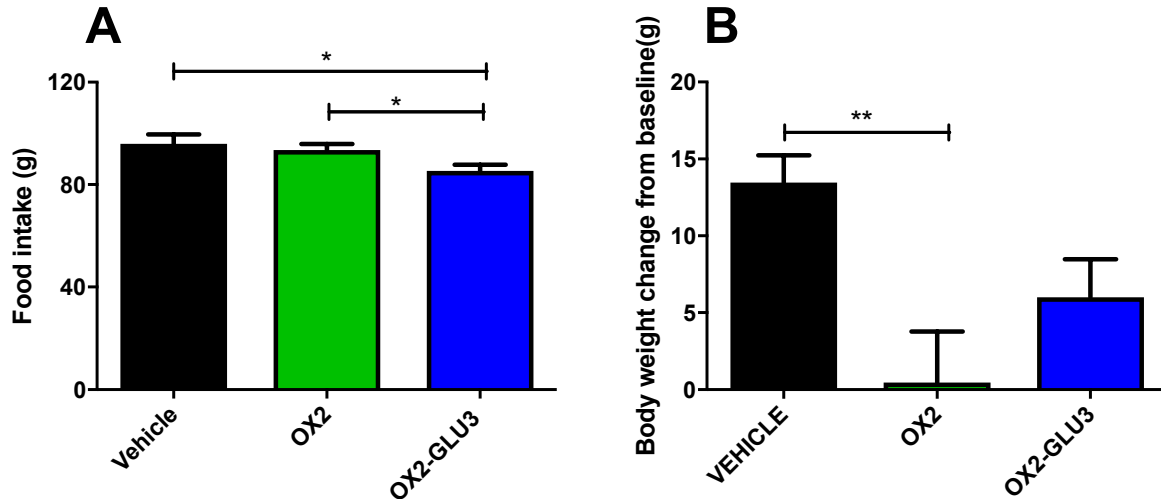


Figure 3.1 Effect of OX2 and OX2-Glu3 on food intake and body weight in rats.

Cumulative food intake (A) and cumulative change in body weight from baseline (B) in male Wistar rats single-housed in CLAMS metabolic cages, receiving a SC injection of vehicle, OX2 or OX2-Glu3 (both at 40nmol/kg) daily for 3 days. Animals had free access to laboratory chow throughout. Data expressed as mean $\pm$ SEM. $n=10-11$. Statistical analysis of food intake used Kruskal-Wallis Test, with *post-hoc* Dunn's multiple comparison test; analysis of body weight change used one-way ANOVA with *post-hoc* Tukey's multiple comparison test. * $p<0.05$, ** $p<0.01$.

3.4.3.2 EFFECT OF OX2 AND OX2-GLU3 ON OXYGEN CONSUMPTION IN RATS

OX2 significantly increased oxygen consumption compared to vehicle in the 12 hours of the light phase after the 1st and 2nd injections, and non-significantly after the 3rd injection. OX2-Glu3 had no effect on oxygen consumption after any injection (average oxygen consumption in the 12 hours after the 1st injection, mean $\pm$ SEM (ml/kg), vehicle: 19896 ± 395 ml/kg; OX2: 21501 ± 403 ml/kg; OX2-Glu3: 20388 ± 536 ml/kg; vehicle vs OX2, $p<0.05$; average oxygen consumption in the 12 hours after the 2nd injection, mean $\pm$ SEM (ml/kg), vehicle: 16583 ± 303 ml/kg; OX2: 19056 ± 314 ml/kg; OX2-Glu3: 17012 ± 468 ml/kg; vehicle vs OX2, $p<0.001$; OX2-Glu3 vs OX2, $p<0.01$; average oxygen consumption in the 12 hours after the 3rd injection, mean $\pm$ SEM (ml/kg), vehicle: 13542 ± 778 ml/kg; OX2: 14728 ± 719 ml/kg; OX2-Glu3: 13791 ± 893 ml/kg) (Figure 3.2 and Table 3-3). This equates to an average increase in oxygen concentration of 11% by OX2 compared to vehicle injections, but only a 2% increase in the OX2-Glu3 group. Oxygen consumption was measured for the 12 hours after injection because it was over this course that OX2 increased oxygen consumption in Chapter 2 (Figure 2.6).

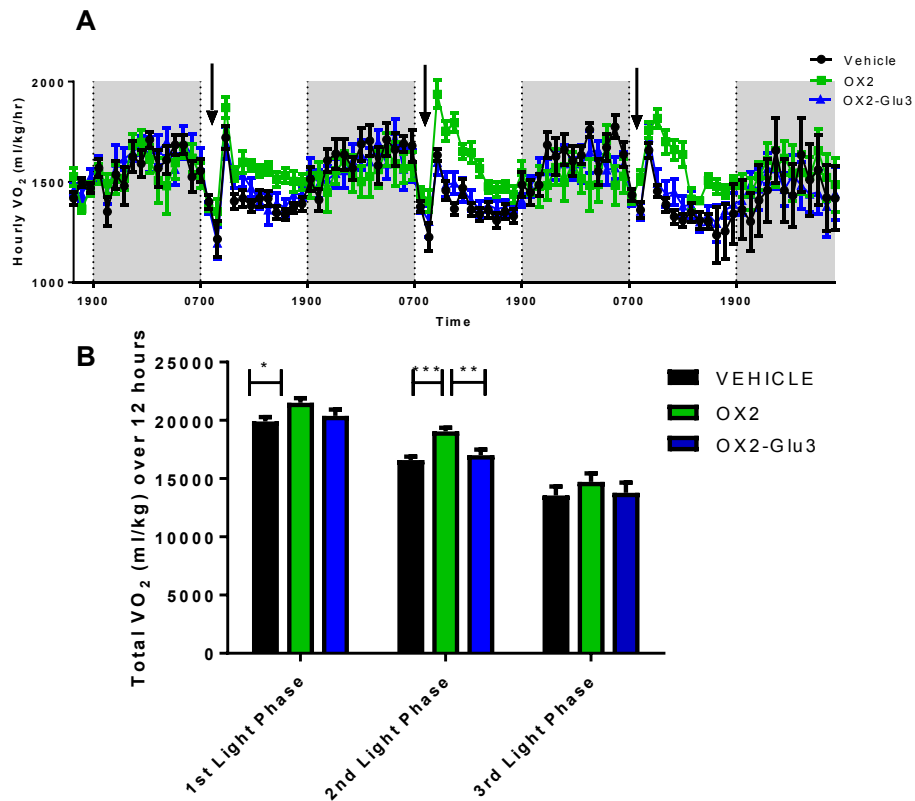


Figure 3.2 Effect of OX2 and OX2-Glu3 on oxygen consumption.

Hourly oxygen consumption (A) and total oxygen consumption in the 12 hours after an injection (B) in male Wistar rats injected with vehicle, OX2 (40nmol/kg) or OX2-Glu3 (40nmol/kg) at 0800 daily for 3 days in CLAMS metabolic cages. Animals had free access to standard laboratory chow throughout. Data expressed as mean $\pm$ SEM. $n=9-11$. Shaded area represents the dark phase. Statistical analysis after each injection was performed using one-way ANOVA with post-hoc Tukey's multiple comparison test. * $p<0.05$ ** $p<0.01$, *** $p<0.001$.

TIME PHASE	OXYGEN CONSUMPTION (ml/kg $\pm$ SEM)			STATISTICAL ANALYSIS (one-way ANOVA with post-hoc Tukey's multiple comparison test)
	Vehicle	OX2 (40nmol/kg)	OX2-Glu3 (40nmol/kg)	
Light phase after 1 st injection	19896 $\pm$ 395	21501 $\pm$ 403	20388 $\pm$ 536;	Vehicle vs OX2, $p<0.05$
Light phase after 2 nd injection	16583 $\pm$ 303	19056 $\pm$ 314	17012 $\pm$ 468	Vehicle vs OX2, $p<0.001$; OX2 vs OX2-Glu3, $p<0.01$
Light phase after 3 rd injection	13542 $\pm$ 778	14728 $\pm$ 719	13791 $\pm$ 863	NS

Table 3-3 Average oxygen consumption in rats for 12 hours after an injection of either vehicle, OX2 (40nmol/kg) or OX2-Glu3 (40nmol/kg).

3.4.3.3 EFFECT OF OX2 AND OX2-GLU3 ON PHYSICAL ACTIVITY IN RATS

There was no significant difference in physical activity over the 12 hours after injections between any of the groups (average physical activity in the 12 hours after the 1st injection, mean $\pm$ SEM (counts), vehicle: 4446 ± 224 counts, OX2: 5070 ± 359 counts; OX2-Glu3: 4781 ± 459 counts; average physical activity in the 12 hours after the 2nd injection, mean $\pm$ SEM (counts), vehicle: 4285 ± 298 counts, OX2: 4953 ± 303 counts; OX2-Glu3: 4474 ± 339 counts; average physical activity in the 12 hours after the 3rd injection, mean $\pm$ SEM (counts), vehicle: 3515 ± 261 counts, OX2: 4380 ± 249 counts; OX2-Glu3: 3739 ± 294 counts) (Figure 3.3 and Table 3-4).

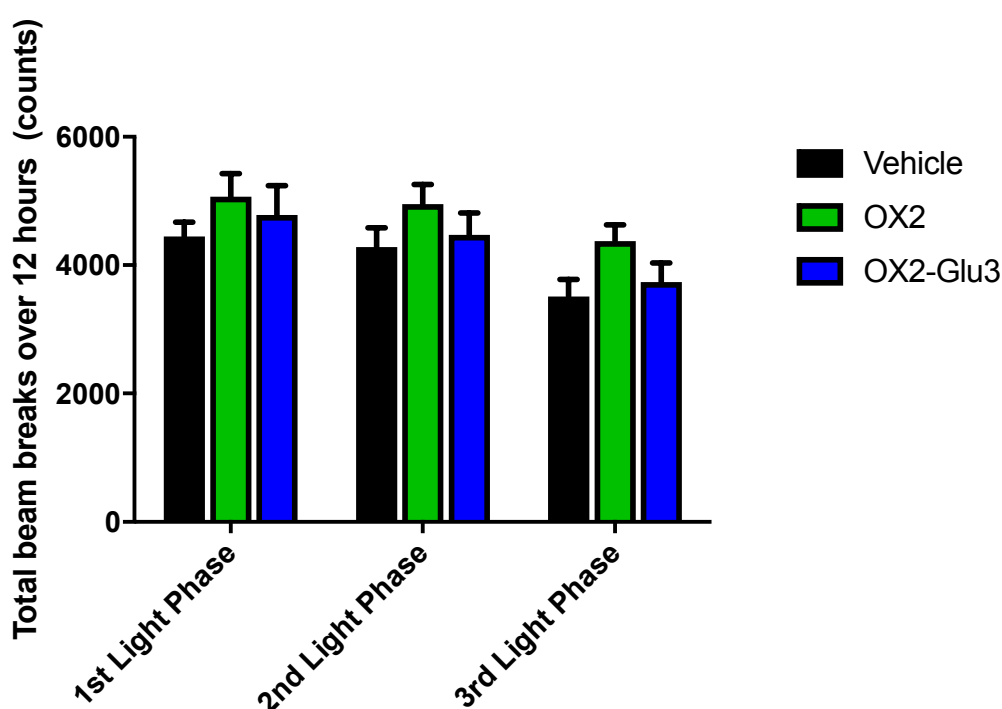


Figure 3.3 Effect of OX2 and OX2-Glu3 on physical activity

Total physical activity in the 12 hours after an injection of vehicle, OX2 (40nmol/kg) or OX2-Glu3 (40nmol/kg) in male Wistar rats single housed in CLAMS metabolic cages and injected at 0800 daily for 3 days. Animals had free access to laboratory chow throughout. Data expressed as mean $\pm$ SEM. n=9-11. Statistical analysis after each injection was performed using one-way ANOVA with post-hoc Tukey's multiple comparison test.

TIME PHASE	BEAM BREAKS, mean $\pm$ SEM (counts)			STATISTICAL ANALYSIS (one-way ANOVA with post-hoc Tukey's multiple comparison test)
	VEHICLE	OX2 (40nmol/kg)	OX2-Glu3 (40nmol/kg)	
Light phase after 1 st injection	4446 $\pm$ 224	5070 $\pm$ 359	4781 $\pm$ 459	NS
Light phase after 2 nd injection	4285 $\pm$ 298	4953 $\pm$ 303	4474 $\pm$ 339	NS
Light phase after 3 rd injection	3515 $\pm$ 261	4380 $\pm$ 249	3739 $\pm$ 294	NS

Table 3-4 Average physical activity in rats for 12 hours after an injection of either vehicle, OX2 (40nmol/kg) or OX2-Glu3 (40nmol/kg).

3.4.3.4 EFFECT OF OX2 AND OX2-GLU3 ON RESPIRATORY EXCHANGE RATIO IN RATS

Overall, despite free access to food throughout, both OX2 and OX2-Glu3 suppressed RER for the 12 hours after injection; OX2-Glu3 to a greater extent than OX2 (average RER over the 12 hours after the 1st injection, mean $\pm$ SEM, vehicle: 1.02 $\pm$ 0.004; OX2: 0.99 $\pm$ 0.009; OX2-Glu3: 0.96 $\pm$ 0.011; vehicle vs. OX2, p <0.05; vehicle vs OX2-Glu3, p <0.001; average RER over the 12 hours after the 2nd injection, mean $\pm$ SEM, vehicle: 1.00 $\pm$ 0.005; OX2: 0.98 $\pm$ 0.008; OX2-Glu3: 0.94 $\pm$ 0.010; vehicle vs OX2-Glu3, p <0.001; average RER over the 12 hours after the 3rd injection, mean $\pm$ SEM, vehicle: 1.01 $\pm$ 0.008; OX2: 1.04 $\pm$ 0.010; OX2-Glu3: 0.98 $\pm$ 0.013).

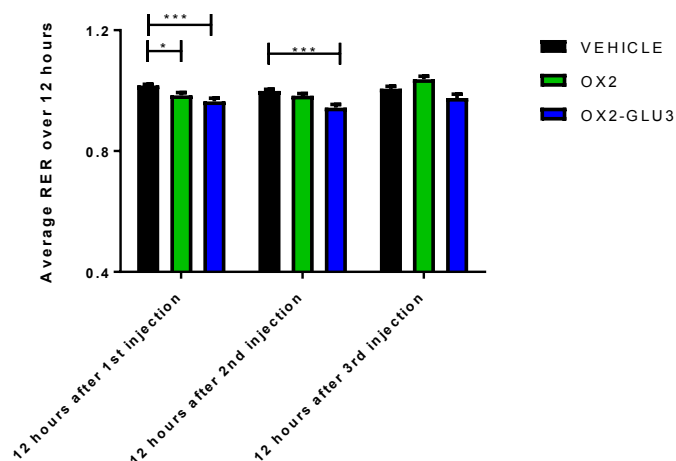


Figure 3.4 Effect of OX2 and OX2-Glu3 on RER

Average RER in the 12 hours after an injection of vehicle, OX2 (40nmol/kg) or OX2-Glu3 (40nmol/kg) in male Wistar rats single housed in CLAMS metabolic cages and injected at 0800 daily for 3 days. Animals had free access to laboratory chow throughout. Data expressed as mean $\pm$ SEM. n =9-11. Statistical analysis after each injection was performed using one-way ANOVA with post-hoc Tukey's multiple comparison test. * p <0.05, *** p <0.001.

TIME PHASE	RER mean $\pm$ SEM			STATISTICAL ANALYSIS (one-way ANOVA with post-hoc Tukey's multiple comparison test)
	VEHICLE	OX2 (40nmol/kg)	OX2-Glu3 (40nmol/kg)	
Light phase after 1 st injection	1.02 $\pm$ 0.004	0.99 $\pm$ 0.009	0.96 $\pm$ 0.011	Vehicle vs OX2, $p < 0.05$ Vehicle vs OX2-Glu3, $p < 0.001$
Light phase after 2 nd injection	1.00 $\pm$ 0.005	0.98 $\pm$ 0.008	0.94 $\pm$ 0.010	Vehicle vs OX2-Glu3, $p < 0.001$
Light phase after 3 rd injection	1.01 $\pm$ 0.008	1.04 $\pm$ 0.010	0.98 $\pm$ 0.013	NS

Table 3-5 Average RER in male Wistar rats singly housed in CLAMS metabolic cages over the 12 hours following a daily SC injection of vehicle, OX2(40nmol/kg) or OX2-Glu3 (40nmol/kg).

	OX2	OX2-GLU3	
FOOD INTAKE	NO EFFECT COMPARED TO VEHICLE	REDUCE FOOD INTAKE COMPARED TO VEHICLE AND OX2 GROUPS	Figure 3.1
BODY WEIGHT	SIGNIFICANT REDUCTION COMPARED TO VEHICLE; NON-SIGNIFICANT REDUCTION COMPARED TO PAIR-FED	NON-SIGNIFICANT REDUCTION COMPARED TO VEHICLE	Figure 3.1
OXYGEN CONSUMPTION	SIGNIFICANT INCREASE COMPARED TO VEHICLE	NO EFFECT COMPARED TO VEHICLE	Figure 3.2
PHYSICAL ACTIVITY	NO EFFECT COMPARED TO VEHICLE	NO EFFECT COMPARED TO VEHICLE	Figure 3.3
RESPIRATORY EXCHANGE RATIO	REDUCED COMPARED TO VEHICLE	REDUCED COMPARED TO VEHICLE	Figure 3.4

Table 3-6 Summary of comparison of effects of OX2 and OX2-Glu3

3.4.4 ROLE OF THE GLP-1 RECEPTOR IN THE ENERGY EXPENDITURE EFFECT OF OX2

3.4.4.1 EFFECT OF EX9-39 AND OX2 ON FOOD INTAKE AND BODY WEIGHT IN RATS

All groups were the same average body weight before surgery (222g), and average body weight between groups was not significantly different in the morning before the first injection (average body weight, mean $\pm$ SEM(g) vehicle: 235 $\pm$ 3.5g; EX9-39: 239 $\pm$ 2.3g).

Following the injections of vehicle or OX2, Ex9-39 had no significant effect on food intake, while the effect of OX2 approached significance; there was no interaction between Ex9-39 and OX2 (ordinary 2-way ANOVA, effect of Ex9-39, $F(1,27) = 1.7$, $p = 0.2$; effect of OX2, $F(1,27) = 3.8$, $p = 0.06$; interaction, $F(1,27) = 0.4$, $p = 0.5$). Although all animals had free access to standard laboratory chow throughout

there was a trend for OX2 to reduce food intake; however, this was mainly in the saline/OX2 group, where food intake was reduced by 11% compared to the saline/vehicle group, compared to only a 3% suppression in the Ex9-39/OX2 group (average food intake, mean $\pm$ SEM (g), saline/vehicle 47.3 ± 2.1 g; Ex9-39/vehicle 48.7 ± 2.6 g; Saline/OX2 group 41.9 ± 2.0 g; Ex9-39/OX2 45.9 ± 1.6 g) (Figure 3.5).

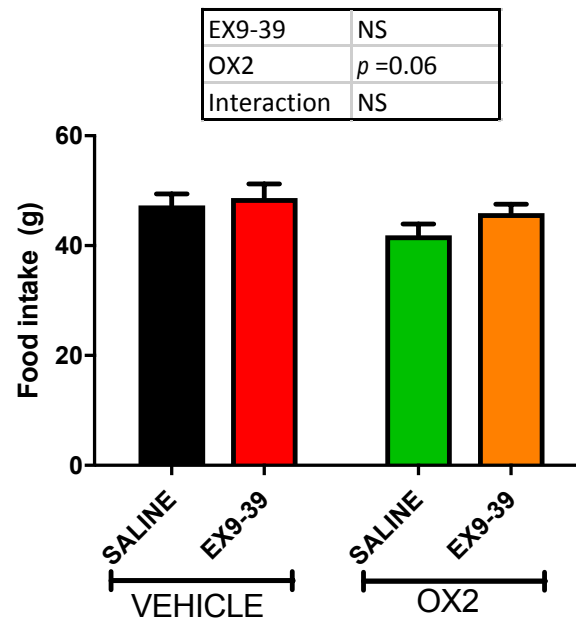


Figure 3.5 Effect of OX2 and Ex9-39 on food intake in rats.

Total food intake after 2 daily injections of vehicle or OX2 (40nmol/kg) in rats with previously inserted subcutaneous mini-osmotic pumps delivering either saline or Ex9-39 (100nmol/kg/hr). Animals had free access to laboratory chow throughout. N=8. Graphs show mean $\pm$ SEM. Statistical analysis undertaken using ordinary 2-way ANOVA.

Following the start of the daily injections, there was no effect of Ex9-39 on body weight change, but OX2 did significantly affect body weight change; there was no interaction between Ex9-39 and OX2 (ordinary 2way ANOVA, effect of Ex9-39, $F(1,28)=1.4$, $p=0.3$; effect of OX2, $F(1,28)=6.3$, $p=0.02$; interaction, $F(1,28)=0.6$, $p=0.4$). OX2 caused significantly less weight gain than vehicle injections; during the 2 days of injections, there was a 4% increase in body weight for the saline/vehicle group, a 3% increase in the Ex9-39/vehicle group, a 2% increase in the saline/OX2 group and a 0% increase in the Ex9-39/OX2 group (average body weight change following injections, mean $\pm$ SEM (g), saline/vehicle: 9 ± 1.3 g; Ex9-39/vehicle: 8 ± 3.0 g; saline/OX2: 5 ± 2.5 g; Ex9-39/OX2: 0 ± 2.2) (Figure 3.6).

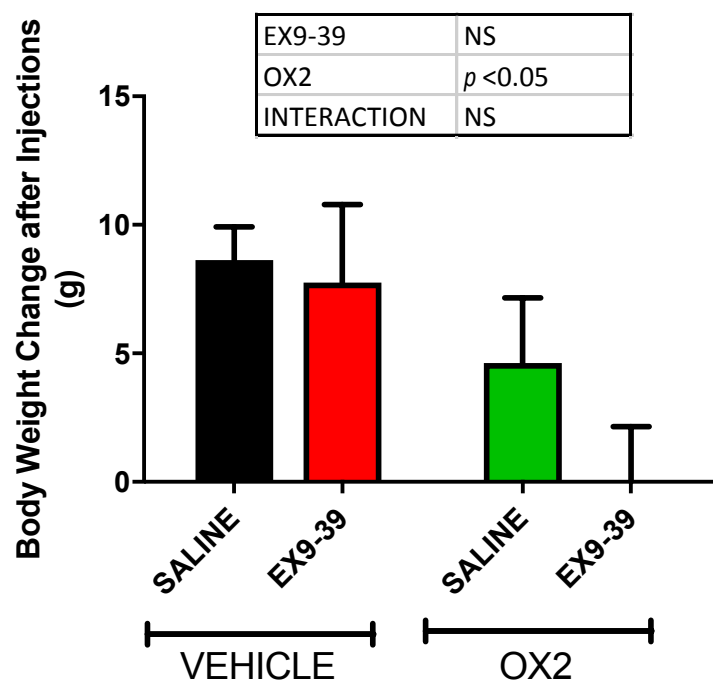


Figure 3.6 Effect of OX2 and Ex9-39 on body weight in rats.

Change in body weight after injections of vehicle or OX2 (40nmol/kg) in rats with previously inserted subcutaneous mini-osmotic pumps delivering either saline or EX9-39 (100nmol/kg/hr). Average body weight before the injections was 235g in the animals with pumps containing vehicle, and 239g in those containing Ex9-39. Animals had free access to laboratory chow throughout. N=8. Graphs shows mean $\pm$ SEM. Statistical analysis undertaken using an ordinary 2-Way ANOVA.

3.4.4.2 EFFECT OF EX9-39 AND OX2 ON OXYGEN CONSUMPTION IN RATS

The effect of both Ex-39 and OX2 on oxygen consumption for the 12 hours after an injection of vehicle or OX2 was examined. OX2 had an effect on oxygen consumption, but there was no effect of EX9-39, and no interaction between Ex9-39 and OX2 (ordinary two way ANOVA of oxygen consumption during the 12 hours after 1st injection, effect of Ex9-39, $F(1,24)=0.03$, $p=0.8$; effect of OX2, $F(1,24)=4.9$, $p=0.04$; interaction, $F(1,24)=0.3$, $p=0.6$; ordinary 2 way ANOVA of oxygen consumption during the 12 hours after the 2nd injection, effect of EX9-39, $F(1,24)=0.01$, $p=0.9$; effect of OX2, $F(1,24)=15.3$, $p=0.0007$; interaction, $F(1,24)=0.2$, $p=0.7$). OX2 caused an increase in oxygen consumption (average total oxygen consumption for the 12 hours after the first injection, $VO_2 \pm SEM$ (ml/kg), saline/vehicle: 22501 ± 544 ml/kg; Ex9-39/vehicle: 22415 ± 860 ml/kg; saline/OX2: 24333 ± 600 ml/kg ml/kg; Ex9-39/OX2: 23818 ± 759 ml/kg; average total oxygen consumption for the 12 hours after the 2nd injection, $VO_2 \pm SEM$ (ml/kg), saline/vehicle: 24499 ± 745 ml/kg; Ex9-39/vehicle: 23638 ± 1036 ml/kg; saline/OX2: 27397 ± 700 ml/kg ml/kg; Ex9-39/OX2: 27840 ± 837 ml/kg) (Figure 3.7 and Table 3-7). This equated to approximately a 10% increase in oxygen consumption in both the saline/OX2 group and Ex9-39/OX2 groups compared to the saline/vehicle group, whereas the EX9-

39/vehicle group showed, on average, a 1% reduction in oxygen consumption compared to the saline/vehicle group.

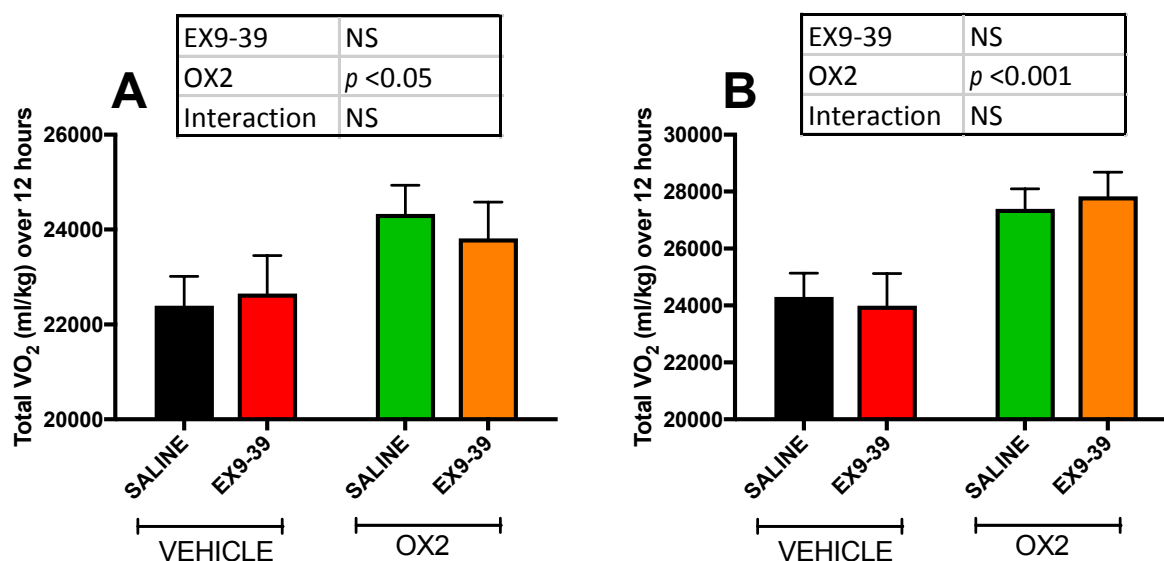


Figure 3.7 Effect of OX2 and EX9-39 on oxygen consumption in rats

Oxygen consumption during the 12 hours of the light phase after the 1st injection (A) and 2nd injection (B) of vehicle or OX2, in rats single-housed in CLAMS metabolic cages. Rats received a daily SC injection of vehicle or OX2 (40nmol/kg) at 0800 for 2 days. Rats previously had subcutaneous mini-osmotic pumps implanted containing saline or EX9-39. Animals had free access to laboratory chow throughout. N=8. Data expressed as mean $\pm$ SEM. Statistical analysis undertaken using ordinary 2-way ANOVA.

	OXYGEN CONSUMPTION (ml/kg $\pm$ SEM)				
TIME PHASE	Injection: vehicle		Injection: OX2		STATISTICAL ANALYSIS (2- way ANOVA)
	SALINE PUMP	EX 9-39 PUMP	SALINE PUMP	EX 9-39 PUMP	
Light phase after 1 st injection	22501 $\pm$ 544	22145 $\pm$ 860	24333 $\pm$ 600	23818 $\pm$ 759	Effect of OX2 $p < 0.05$ Effect of pump = NS Interaction = NS
Light phase after 2 nd injection	24499 $\pm$ 745	23638 $\pm$ 1036	27397 $\pm$ 700	27840 $\pm$ 837	Effect of OX2 $p < 0.001$ Effect of pump = NS Interaction = NS

Table 3-7 Average oxygen consumption in Wistar rats administered OX2 and Ex9-39.

3.4.4.3 EFFECT OF EX9-39 AND OX2 ON PHYSICAL ACTIVITY IN RATS

The effect of EX9-39 and OX2 on physical activity in rats in the 12 hours after the 1st and 2nd injections of either vehicle or OX2 was examined. There was no effect of EX9-39 or OX2 on physical

activity, nor was there an interaction between pump content and injection (2-way ANOVA of 12 hours after 1st injection, effect of pump content, $F(1,28) = 0.1$, $p=0.7$; effect of OX2, $F(1, 28)= 0.1$, $p=0.8$; interaction, $F(1, 28) = 0.3$, $p=0.6$; 2-way ANOVA of 12 hours after 2nd injection, effect of pump content, $F(1,27) = 0.03$, $p=0.9$; effect of OX2, $F(1, 27) = 2.1$, $p=0.2$; interaction, $F(1,27)=3.8$, $p=0.06$) (average total number of beam breaks in the 12 hours after the first injection, mean $\pm$ SEM (counts), saline/vehicle: 5731 ± 375 counts; Ex9-39/vehicle: 5638 ± 476 counts; saline/OX2: 5598 ± 551 counts; Ex9-39/OX2: 6026 ± 365 counts; average total number of beam breaks in the 12 hours after the 2nd injection, mean $\pm$ SEM (counts), saline/vehicle: 4994 ± 365 counts; Ex9-39/vehicle: 4423 ± 289 counts; saline/OX2: 4840 ± 325 counts; Ex9-39/OX2: 5521 ± 296 counts) (Figure 3.8 and Table 3-8).

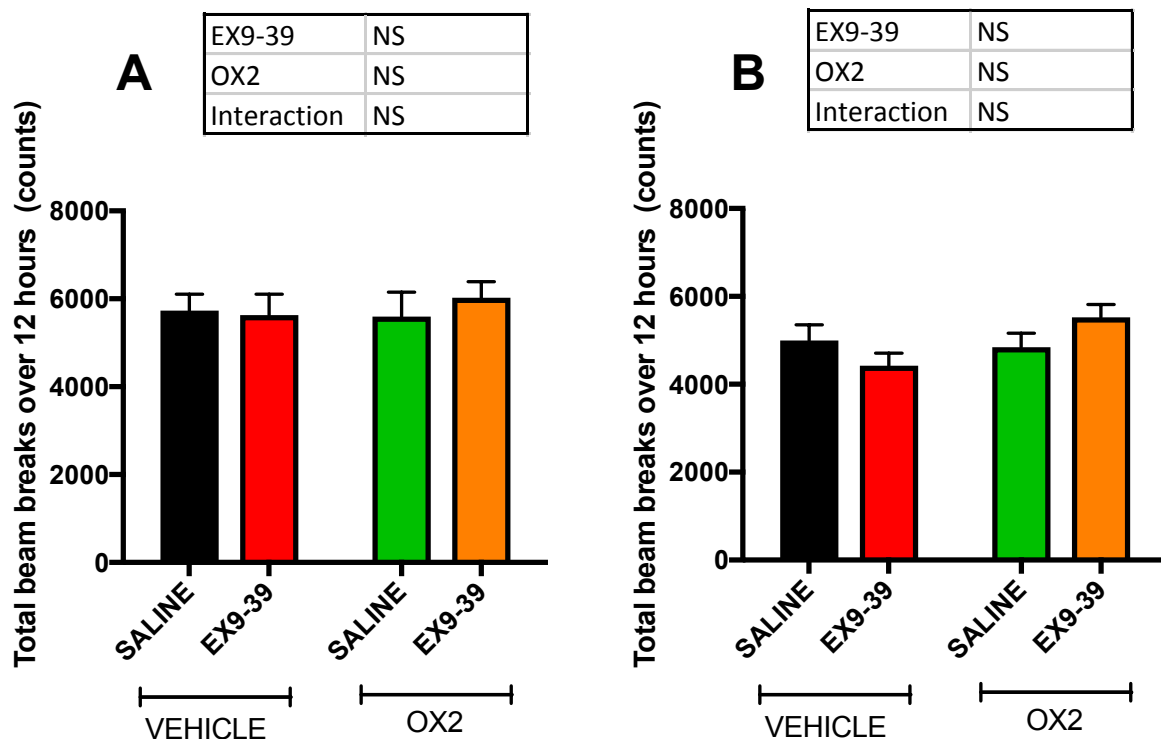


Figure 3.8 Effect of OX2 and Ex9-39 on physical activity in rats.

Physical activity (beam breaks) during the 12 hours of the light phase after the 1st injection (A) and 2nd injection (B) of vehicle or OX2, in rats single-housed in CLAMS metabolic cages. Rats received a daily SC injection of vehicle or OX2 (40nmol/kg) at 0800 for 2 days. Rats previously had subcutaneous mini-osmotic pumps implanted containing saline or EX9-39. Animals had free access to standard laboratory chow throughout. N=8. Data expressed as mean $\pm$ SEM. Statistical analysis undertaken using ordinary 2-way ANOVA.

	BEAM BREAKS mean $\pm$ SEM (counts)				
TIME PHASE	Injection: vehicle		Injection: OX2		STATISTICAL ANALYSIS (2-way ANOVA)
	SALINE PUMP	EX 9-39 PUMP	SALINE PUMP	EX9-39 PUMP	
Light phase after 1 st injection	5731 $\pm$ 375	5628 $\pm$ 476	5598 $\pm$ 551	6026 $\pm$ 365	NS
Light phase after 2 nd injection	4994 $\pm$ 365	4423 $\pm$ 289	4840 $\pm$ 325	5521 $\pm$ 296	NS

Table 3-8 Average physical activity rats administered EX9-39 and OX2.

3.4.4.4 EFFECT OF EX9-39 AND OX2 ON RESPIRATORY EXCHANGE RATIO IN RATS

The effect of both EX9-39 and OX2 on respiratory exchange ratio over the 12 hours after both the 1st and 2nd injections of either vehicle or OX2 was examined. There was no effect of EX9-39 or OX2 injection on RER after either injection, nor was there any interaction between pump content and injection (ordinary 2-way ANOVA of RER during 12 hours after 1st injection, effect of EX9-39 content, $F(1,28) = 0.1$, $p=0.7$; effect of OX2, $F(1, 28)= 0.1$, $p=0.8$; interaction, $F(1, 28) = 0.1$, $p=1.0$; ordinary 2-way ANOVA of RER during 12 hours after 2nd injection, effect of EX9-39 content, $F(1,24) = 0.1$, $p=0.4$; effect of OX2, $F(1, 24) = 0.5$, $p=0.5$; interaction, $F(1,24)=0.1$, $p=0.7$) (average RER in the 12 hours after the first injection, mean $\pm$ SEM, saline/vehicle: 0.84 ± 0.015 ; Ex9-39/vehicle: 0.83 ± 0.011 ; saline/OX2: 0.84 ± 0.012 ; Ex9-39/OX2: 0.83 ± 0.008 ; average RER in the 12 hours after the 2nd injection, mean $\pm$ SEM, saline/vehicle: 0.89 ± 0.016 ; Ex9-39/vehicle: 0.89 ± 0.017 ; saline/OX2: 0.88 ± 0.011 ; Ex9-39/OX2: 0.88 ± 0.015) (Figure 3.9 and Table 3-9).

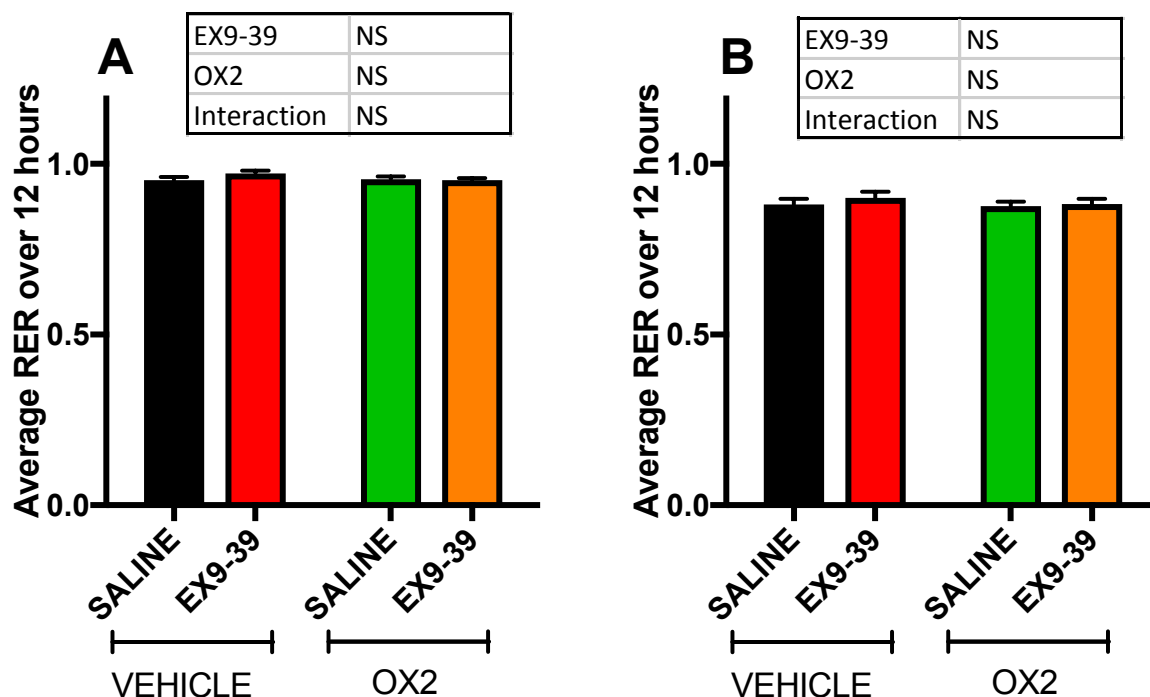


Figure 3.9 Effect of OX2 and EX9-39 on RER in rats.

Average RER during the 12 hours of the light phase after the (A) 1st injection and (B) 2nd injection of vehicle or OX2, in rats single-housed in CLAMS metabolic cages. Rats received a daily SC injection of vehicle or OX2 (40nmol/kg) at 0800 for 2 days. Rats previously had subcutaneous mini-osmotic pumps implanted containing saline or EX9-39. Animals had free access to standard laboratory chow throughout. N=8. Data expressed as mean $\pm$ SEM. Statistical analysis undertaken using ordinary 2-way ANOVA.

	RER, mean ± SEM				
TIME PHASE	Injection: vehicle		Injection: OX2		STATISTICAL ANALYSIS (2 way ANOVA)
	SALINE PUMP	EX9-39 PUMP	SALINE PUMP	EX9-39 PUMP	
Light phase after 1 st injection	0.84 ± 0.015	0.83 ± 0.011	0.84 ± 0.012	0.83 ± 0.008	NS
Light phase after 2 nd injection	0.89 ± 0.016	0.89 ± 0.017	0.88 ± 0.011	0.88 ± 0.015	NS

Table 3-9 Average RER in Wistar rats administered Ex9-39 and OX2.

	SALINE/VEHICLE	EX9-39/VEHICLE	SALINE/OX2	EX9-39/OX2	
FOOD INTAKE	REFERENCE	NO EFFECT	NON-SIGNIFICANT REDUCTION	NO EFFECT	Figure 3.5
BODY WEIGHT	REFERENCE	NO EFFECT	REDUCED	REDUCED	Figure 3.6
OXYGEN CONSUMPTION	REFERENCE	NO EFFECT	INCREASED	INCREASED	Figure 3.7
PHYSICAL ACTIVITY	REFERENCE	NO EFFECT	NO EFFECT	NO EFFECT	Figure 3.8
RESPIRATORY EXCHANGE RATIO	REFERENCE	NO EFFECT	NO EFFECT	NO EFFECT	Figure 3.9

Table 3-10 Summary of comparison of effects of OX2 or vehicle administered in rats receiving infusions of saline or Ex9-3.

3.5 DISCUSSION

This chapter aimed to investigate whether glucagon or GLP-1 receptors, or both, were responsible for the increased energy expenditure caused by OX2, an analogue of oxyntomodulin with an extended duration of biological activity. The studies in this chapter were the first to assess which receptor is responsible by measuring energy expenditure directly in CLAMS metabolic cages. They were also the first to use continuously-administered subcutaneous Ex9-39 to block the GLP-1 receptor, and to compare this with the effects of reducing activity at the glucagon receptor by using an oxyntomodulin analogue and its Glu-3 pair. Together, the studies in this chapter suggest oxyntomodulin increases energy expenditure through activation of the glucagon receptor.

In the OX2-Glu3 study, OX2 increased oxygen consumption in the light phase by an average of 11% when compared to vehicle injections; in the OX2-Glu3 group, the increase in oxygen consumption was only 2%, suggesting that reduction in activity at the glucagon receptor eliminated the energy expenditure increase (Figure 3.2). In the Ex9-39 study, injections of OX2 also increased energy expenditure by 10%; however this was not reduced by GLP-1 receptor blockade (Figure 3.7). The Ex9-39 study also failed to show any role for the GLP-1 receptor in endogenous energy expenditure, as there was no difference in the oxygen consumption following vehicle injections in those with a pump releasing saline or one delivering Ex9-39 (Figure 3.7).

Both studies support the findings in the literature that the GLP-1 receptor mediates the anorectic effect of OXM (Dakin et al., 2001, Dakin et al., 2002, Dakin et al., 2004, Baggio et al., 2004, Maida et al., 2008). In the Ex9-39 study, there was a non-significant reduction in food intake in animals given OX2; however, Ex9-39 limited the anorectic effect, as there is only a 3% reduction in food intake in the Ex9-39/OX2 group (when compared to saline/vehicle group) and an 11% reduction in food intake in the saline/OX2 group (Figure 3.5). Therefore blocking the GLP-1 receptor reduced the anorectic effect of OX2. The OX2-Glu3 study also supported the role of the GLP-1 receptor in reducing food intake as there was a significant reduction in food intake in the OX2-Glu3 group, where the peptide given only had activity at the GLP-1 receptor (Figure 3.1).

This study also supports the findings of Price *et al.* that activity at the glucagon receptor may actually limit the anorectic effects of OXM analogues in rats, by causing a compensatory hyperphagia in response to the increased energy expenditure (Price et al., 2015a). OX2 reduced food intake by only 2% when compared to vehicle injections; OX2-Glu3 reduced food intake by 11% (Figure 3.1); therefore, activity at the glucagon receptor increases food intake. Such a compensatory hyperphagia is seen clinically in patients with thyrotoxicosis, and is the explanation for increased food intake in one study of animals given low-dose glucagon (Hell and Timo-laria, 1985). However, this hyperphagic

effect is only found at low doses of OXM analogues by Price *et al.* At higher doses, a robust anorectic effect is seen; the hyperphagic effect of OXM analogues only occurs over a narrow dose range. Why the anorectic effect increases, whether it is due to increased activity at the GLP-1 or glucagon receptors, has not been determined. The investigation by Price *et al.*, however, does show that the dose of oxyntomodulin analogue is important for its anorectic effect. Future studies are required to see if there is a similar dose-response effect on the energy expenditure effect of OXM analogues. Reducing food intake typically suppresses energy expenditure, so it may be that at higher doses of OXM analogue, as the anorectic effect increases, energy expenditure effects may reduce. This may have implications for the clinical use of oxyntomodulin analogues as anti-obesity therapies. GLP-1 receptor agonists only cause medically significant weight loss (more than 10% of body weight) in 33% of subjects (Ryder *et al.*, 2011, Pi-Sunyer *et al.*, 2015). To achieve significant weight loss in a greater proportion of patients, it may be beneficial to add in glucagon receptor agonism to increase energy expenditure. However, my data, along with published findings such as Price *et al.* suggest these two mechanisms are not independent, and therefore activity at the glucagon and GLP-1 receptors must be carefully balanced to achieve maximal weight loss.

The OX2-GLU3 study suggests that both GLP-1 and glucagon receptors affect metabolism and fuel oxidation. OX2-Glu3 suppresses RER (Figure 3.4). A reduction in RER is also seen with administration of the GLP-1 agonist Ex-4, and therefore it is likely that GLP-1 receptor activation causes a reduction in food intake which necessitates increased fat metabolism (Baggio *et al.*, 2004). OX2 also reduces RER, though to a lesser extent than OX2-Glu3; however, this was seen in the absence of any significant reduction in food intake. This suggests that glucagon receptor activation affects fuel oxidation, increasing either fat or protein metabolism, independently of a reduction in food intake. I investigate this further in Chapter 7 of this thesis.

Interestingly, OX2 does not affect RER in the Ex9-39 study, despite suppressing RER in the OX2-Glu3 study, and in the studies in Chapter 2 (Figure 2.8 and Figure 2.12). However, in the Ex9-39 study, RER is notably lower in the control saline/vehicle animals than in control animals in the other metabolic cage studies (Figure 3.9 and Table 3-9); average RER in the control animals is 0.85 rather than 1.0 in the above OX2-Glu3 study (Figure 3.4) or 0.95 as seen in Chapter 2. Surgery is a physiologically stressful event, and is known to affect metabolism, with an increase in fat and protein metabolism (Burton *et al.*, 2004, Kinney *et al.*, 1970). Even though insertion of a subcutaneous osmotic mini-pump is a relatively minor procedure, this may have been sufficient to alter fuel metabolism and RER in all animals, overwhelming the effects of OX2. This emphasises that there are multiple influences on metabolic processes which must be considered in any physiological study.

Activating the glucagon receptor has been known to increase energy expenditure since the 1950s. Elegant studies in the laboratory of Banting and Best showed that glucagon increases energy expenditure as measured both indirectly in rats, through a pair feeding study, and directly by measuring oxygen consumption (Davidson et al., 1957, Salter et al., 1957). The energy expenditure effect of glucagon has latterly been confirmed in man using indirect calorimetry (Tan et al., 2013, Salem et al., 2016). In contrast, indirect calorimetry has failed to show an increase in energy expenditure after GLP-1 administration in either man or rodents (Flint et al., 2001, Tan et al., 2013, Baggio et al., 2004). Therefore, it is unsurprising that the energy expenditure caused by oxyntomodulin happens via the glucagon receptor. However, despite the wealth of literature on the increase in energy expenditure caused by glucagon, the underlying cellular and molecular mechanisms by which glucagon receptor activation leads to increased energy expenditure have not been determined.

The potential molecular and cellular mechanisms by which oxyntomodulin increases energy expenditure will be investigated in future chapters of this thesis. As the energy expenditure effects of OX2 appear to be mediated via the glucagon receptor, it may also be appropriate to apply these findings to the metabolic effects of glucagon.

3.6 CONCLUSIONS

The studies in this chapter confirm that the increase in energy expenditure caused by oxyntomodulin, as exemplified by the analogue OX2, occurs via the glucagon receptor. They also demonstrate the complex interaction of the glucagon and GLP-1 receptors on food intake in rats, and suggest the two receptors also interact to determine fuel metabolism.

Chapter 4

Oxyntomodulin And UCP-1

4.1 INTRODUCTION

Brown adipose tissue (BAT) is so named because its high density of mitochondria, and relatively prolific vasculature, make it dark in appearance. The primary function of BAT is thermogenesis. This function depends on high levels of expression of the protein uncoupling protein-1 (UCP-1) in the mitochondria of BAT. This contrasts with white adipose tissue (WAT), which is pale in appearance and is primarily a fuel store, with lipids stored as triacylglycerol, and an endocrine organ producing adipokines such as leptin.

UCP-1 is the characteristic molecular signal of brown adipose tissue. Normally, protons pass into the mitochondrial matrix down the electron transport chain, through ATP synthase, and the energy released is stored in high-energy bonds in ATP. UCP-1 uncouples the passage of protons from the synthesis of ATP; instead, the energy is released as heat (see Figure 4.1). Therefore, activation of BAT, leading to enhanced UCP-1 levels and function, causes thermogenesis and increased energy expenditure (Yoneshiro et al., 2011). Prior to the discovery of UCP-1, indirect measures of BAT thermogenesis were used such as BAT weight, mitochondrial weight and DNA content, or interscapular temperature (Dicker et al., 1998, Billington et al., 1987). Now, levels of UCP-1 mRNA are considered the 'gold standard' measurement of BAT activity in rodent models.

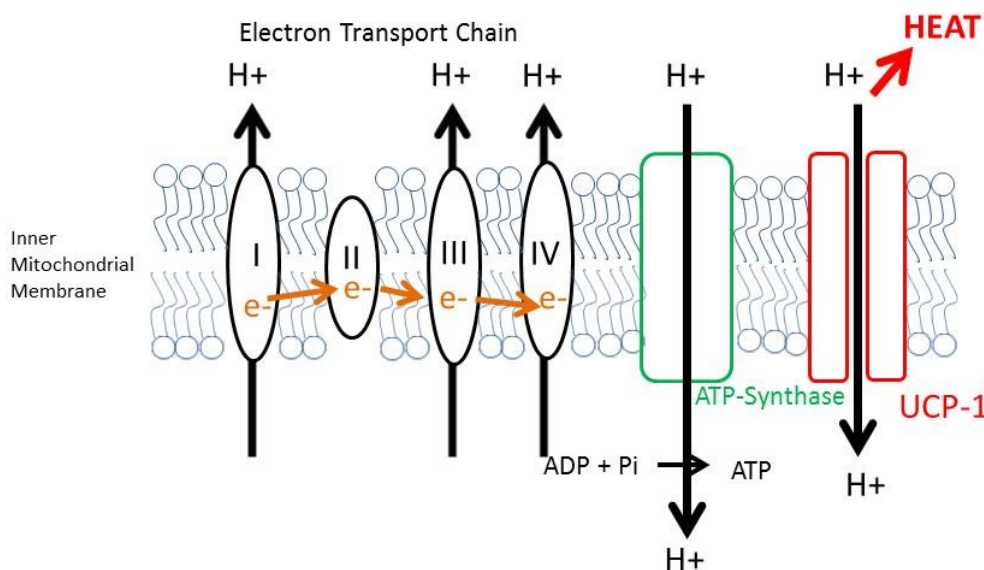


Figure 4.1 Proton transport in the inner mitochondrial membrane.

Movement of electrons along the electron transport chain causes protons to move across the inner mitochondrial membrane. They then flow back across the membrane down the proton gradient. The energy released as they move down the gradient can be captured as the high-energy bonds in ATP via ATP-synthase. If the protons move instead via UCP-1, the energy is not coupled to ATP synthesis and is lost as heat.

Although UCP-1 is considered essential for BAT thermogenesis, many different molecules contribute to the pathways which lead to BAT activation. These include adipose triglyceride lipase (ATGL),

deiodinase 2 (Dio2) and peroxisome proliferator-activated receptor- γ coactivator 1- α (PPARGC1a). ATGL causes the hydrolysis of triacylglycerol in adipose to form diacylglycerol, the initial step in the formation of fatty acids that are used for activation of UCP-1; ablation of ATGL within BAT prevents thermogenesis and converts BAT to a WAT-like tissue (Ahmadian et al., 2011); cold exposure also enhances ATGL levels in the BAT of obese rats (though not in lean rats) (Caimari et al., 2012). Dio2 causes local production of tri-iodothyronine, and is essential for upregulation of UCP-1 levels in BAT (de Jesus et al., 2001). PPARGC1a is a key regulator of thermogenesis by affecting not only UCP-1 levels but other transcription factors in the thermogenic pathway (Sharma et al., 2014, Bargut et al., 2016, Uldry et al., 2006).

For many years, there was little interest in brown adipose, as humans were not thought to possess significant levels of BAT once they reached adulthood. However, 3 seminal papers produced in 2009 used PET scanning to show that adult humans have significant quantities of BAT in the neck area; histological analysis confirmed the presence of UCP-1 in this tissue (Lichtenbelt et al., 2009, Virtanen et al., 2009, Cypess et al., 2009). These studies also showed that BAT in adult man can be activated by exposure to cold. It has been predicted that persistent activation of BAT in adult humans could lead to an energy expenditure equivalent of 79kcal/day/15ml of BAT, or loss of 4.1kg fat per year (Virtanen et al., 2009, Cypess et al., 2009, Yoneshiro et al., 2011, Cypess et al., 2012); therefore, any means of pharmacologically increasing BAT activity could be useful as part of a treatment for obesity.

The main stimulus for BAT activation, in both rodents and man, is exposure to cold (Smith and Roberts, 1964, Cypess et al., 2012). BAT expresses β 3 adrenoreceptors; cold activates the sympathetic nervous system (SNS), which in turn activates BAT via release of norepinephrine. However, in practice, cold exposure is not particularly useful to treat obesity: chronic cold exposure may have no effect on body weight (Lee et al., 2014, Peterson et al., 2016). Many studies have therefore looked at alternative means of activating BAT. Exogenous administration of β 3 agonists has been shown to enhance BAT activation in rodents and humans (Yoshitomi et al., 1998, Xiao et al., 2015, Cypess et al., 2015, Himmshagen et al., 1994); but to date no β 3 agonists have been successful in clinical trials to treat obesity. Historical studies have shown that glucagon has a stimulatory effect on brown adipose tissue. *In vitro* glucagon increases brown adipose tissue oxygen consumption (Joel, 1966, Kuroshima and Yahata, 1979) as well as lipolysis (Joel, 1966). *In vivo*, glucagon acutely enhances brown adipose tissue temperature (Heim and Hull, 1966, Cockburn et al., 1968), while chronic glucagon exposure increases BAT weight (Billington et al., 1987, Yahata et al., 1981, Kikuchi et al., 1992) as well as mitochondrial mass (Billington et al., 1987). To date, however,

no studies have shown that glucagon increases UCP-1 levels in BAT; nor that increase in BAT activity is responsible for the elevated energy expenditure that is seen with glucagon. Moreover, a recent study in man shows no increase in BAT activity, as measured by both supraclavicular thermography and uptake on (18)F-FDG PET/CT, following an infusion of glucagon, despite an increase in energy expenditure as recorded by indirect calorimetry (Salem et al., 2016).

Few studies have investigated the effect of oxyntomodulin on brown adipose. Dakin *et al.* showed that BAT mass is reduced by ICV OXM treatment compared to vehicle, but did not look at measures of BAT activity (Dakin et al., 2002). Lockie *et al.* also looked at the effect of ICV OXM on BAT activity; they showed an increase in sympathetic nerve discharge to BAT, and an increase in BAT UCP-1 levels following OXM infusion (Lockie et al., 2012). However, Day *et al.* found no increase in BAT UCP-1 mRNA following 1 month of treatment with the oxyntomodulin analogue Aib2 C24 lactam 40K (Day et al., 2009).

Although adipose tissue has classically been divided into white and brown types there has been recent interest in a third type of adipose: 'beige' or 'brite' adipose. Initially thought to be ectopic BAT cells found in WAT, these have recently been found to come from a separate precursor pool, and have a separate genetic profile to BAT (Harms and Seale, 2013, Wu et al., 2012).

	BROWN ADIPOSE TISSUE	WHITE ADIPOSE TISSUE	BRITE ADIPOSE TISSUE
ENRICHED GENETIC MARKERS	Zic 1, Lhx8, Eva1, Pdk4, Epst 1	ASC-1 Tcf21 Hoxc8 LPL Leptin	Cd137 Tbx1 Tmem 26 Shox 2 Cited 1
EMBRYONIC PRECURSORS	Myf5 ⁺ cells	Myf5 ⁻ cells	Myf5 ⁻ cells Pdgfr- α ⁺
LOCATION	Interscapular Cervical, axillary and perirenal area in rodents	Subcutaneous Visceral Inguinal Interscapular Retroperitoneal Perigonadal Mesenteric	Supraclavicular Interspersed within WAT in rodents

Table 4-1 Features of brown, white and brite adipose tissue.

Adapted from (Harms and Seale, 2013, Gesta et al., 2007, Walden et al., 2012, Bargut et al., 2016)

Brite adipose tissue is of interest because, under certain circumstances, UCP-1 expression can be induced within these cells, enhancing its thermogenic ability (Wu et al., 2012, Harms and Seale,

2013). Many physiological stimuli, cytokines, hormones and drugs have been shown to induce UCP-1 expression in brite adipose (Forest et al., 2016, Harms and Seale, 2013). Sufficient brite adipose induction can provide enough UCP-1 to increase thermogenesis, and elevate energy expenditure: mice with increased brite adipose have improved metabolic profiles and reduced body weight (Bostrom et al., 2012, Harms and Seale, 2013). Despite multiple groups that have looked at induction of brite adipose, there are no studies into the ability of either oxyntomodulin, or glucagon, to induce brite adipose.

Given the thermogenic ability of UCP-1, both in BAT and in brite adipose, the experiments in this chapter will investigate if the energy expenditure caused by the oxyntomodulin analogue OX2 can be attributed to BAT activation, or to induction of brite adipose cells.

4.2 AIMS

The studies in this chapter will

- Investigate if OX2 increases UCP-1 levels in BAT acutely.
- Determine if chronic administration of OX2 causes continued energy expenditure in rats.
- Examine whether chronic administration of OX2 increases BAT activity or induces brite adipose.
- Determine if BAT or brite adipose tissue is responsible for the elevation in energy expenditure seen with OX2.

4.3 METHODS

4.3.1 PEPTIDES

As previously described, the oxyntomodulin analogue OX2 was designed by Professor S Bloom (Imperial College, London) and custom-synthesised by Insight Biotechnology Limited (Wembley, UK). The beta-3 receptor agonist, CL316,243 disodium salt, was purchased from Tocris Bioscience (Abingdon, United Kingdom).

4.3.2 *IN VIVO* STUDIES

All animal procedures undertaken were approved by the British Home Office Animal (Scientific Procedures) Act 1986 (Project Licences 70/7236 and 70/7596).

4.3.2.1 ANIMAL HUSBANDRY

Adult male Wistar rats (Charles River, Margate, UK) were single-housed in individual cages under controlled temperature environment (21-23°C) with a 12:12 hour light-dark cycle (lights on at 0700) conditions. Unless otherwise stated, all animals had *ad libitum* access to water and RM1 diet (Special Diet Services, Witham, UK). Prior to all experiments, animals were extensively handled and acclimatised to SC injections to limit the non-specific stress effects of handling during the studies.

4.3.2.2 ACUTE EFFECT OF OX2 ON BROWN ADIPOSE TISSUE ACTIVITY

As the studies in chapter 2 showed that OX2 causes a significant increase in oxygen consumption that peaks by 4 hours after injection, the first study in this chapter aimed to see if UCP-1 activity was induced over the same time-course.

16 rats were randomised to two groups of equal mean body weight (338g, range 315-365g). The groups received an injection of either saline or OX2 (40nmol/kg) (the same injection as used in the CLAMS study in chapter 2) (see 2.3.4.1) at 9am. After 4 hours, all animals were culled by CO₂ asphyxiation and cervical dislocation. All animals had been freely fed overnight, and were given unrestricted access to food throughout the experiment. Food weight was measured at the time of injection, and at the time of culling. After culling, BAT was dissected out from all animals, snap-frozen in liquid nitrogen, and stored at -80°C until further processing.

4.3.2.3 EFFECT OF CONTINUOUS OX2 ON ENERGY EXPENDITURE

Though the work in chapters 2 and 3 has shown that acute doses of OX2 elevate energy expenditure, for oxyntomodulin analogues to be useful clinically, chronic administration will be required. As a prelude to the final study in this chapter, I designed this study to determine if continuous

administration of OX2 for 8 days via a subcutaneous osmotic mini-pump caused a continued elevation in energy expenditure.

16 male Wistar rats were divided into 2 groups (n=6-10) of equal mean body weight (mean body weight 387g, range 340-431g). On day 0 all animals had subcutaneous osmotic mini-pumps (Alzet pump model 2002, Durect Corporation, Cupertino USA) inserted into the interscapular region under isofluorane anaesthetic. The pumps delivered either saline or OX2 (80nmol/kg/24 hours). The animals were then returned to their home cages, and had free access to standard laboratory chow throughout. Baseline body weight and food intake were measured at 1600 on the day of the surgery, and at the same time each subsequent day. At 1600 on day 6, the animals entered CLAMS metabolic cages, and metabolic measurements are recorded until 0800 on day 8.

4.3.3 COMPARISON OF THE EFFECT OF CONTINUOUS INFUSION OF OX2 AND β 3 AGONIST IN RATS

Following the above study, I undertook a study to determine if chronic administration of OX2 effects BAT activity and brite adipose induction.

This study involved a modified pair-feeding paradigm. 39 rats were randomised to 4 groups (n=9-10) by body weight (mean body weight 313g, range 266-355g). On day 0, 3 groups of animals had subcutaneous osmotic mini-pumps (Alzet pump model 2002, Durect Corporation, Cupertino USA) inserted into the interscapular region under isofluorane anaesthetic. The mini-osmotic pumps delivered either saline, the oxyntomodulin analogue OX2 (80nmol/kg over 24 hours) or the highly-specific β 3 agonist CL316,243 (1mg/kg over 24 hours). This group was included as a positive control, since CL316243 is known to both activate brown adipose tissue and induce browning of WAT when administered chronically (Xiao et al., 2015, Yoshitomi et al., 1998). At the same time as the pump was inserted, temperature transponders (IPTT-300, Bio Medic Data Systems, Seaford, USA) were inserted into the subcutaneous pocket with the Alzet pump, such that they would overlie the interscapular BAT pad to measure BAT temperature (n=7-10). After pump insertion, animals were returned to their home cages, and had free access to standard laboratory chow. Body weight and food intake were measured daily from 1600 on the day of pump insertion. From day 1 of the study, the fourth group of animals entered the study. They did not have subcutaneous pumps inserted, but their daily food intake was restricted to the median food intake of the OX2 group over the preceding 24 hours as a pair-fed group.

On day 8 of the study, having been freely fed overnight, all animals with pumps were culled using decapitation. Blood was taken in heparinised microtubes, which were centrifuged at 6,000rpm for

10 minutes; the resultant plasma was stored at -80°C. Brown adipose tissue was dissected from the interscapular area, while white adipose tissue was removed from the epididymal fat pad; samples from both were snap-frozen in liquid nitrogen before long-term storage at -80°C. The pair-fed group underwent similar decapitation and dissection on day 9.

4.3.4 mRNA EXTRACTION

Samples were homogenised in 1ml QIAzol Lysis Reagent (Qiagen, Hilden, Germany) using the TissueLyser II (Qiagen, Hilden, Germany). RNA was then extracted from WAT using the RNeasy® Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) as per manufacturer's instruction, while RNA was extracted from BAT using RNeasy Mini Kit (Qiagen, Hilden, Germany). The RNA samples underwent on-column DNase digestion (Purelink DNase, Life Technologies). cDNA was then formed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, USA), as per the manufacturer's instructions. Finally, quantitative real-time PCR was performed in triplicate using the following TaqMan® Gene expression primer sets: UCP-1 primer RN00562126_m1, Dio2 Rn00581867_m1, ATGL Rn01479969_m1; PPARGC1a Rn00580421_m1; Tmem26 Rn01428021_m1; Tbx1 Rn01405401_m1. All genes were normalized to the housekeeping gene 18s.

4.3.5 BROWN ADIPOSE TISSUE NOREPINEPHRINE QUANTIFICATION

Norepinephrine levels (NE) in BAT were determined using a competitive enzyme immunoassay kit (Noreadrenaline Research ELISA™, BA E-5200, Labor Diagnostika Nord GmbH & Co. KG, Germany), following the manufacturer's instructions. In brief, BAT samples were weighed and then homogenised using TissueLyser II (Qiagen, Hilden, Germany), in the presence of a homogenisation buffer containing 0.01M HCl, 1mM EDTA (Sigma-Aldrich, Germany) and 4mM sodium metabisulfite (Sigma-Aldrich, Germany). 100µl of homogenate was diluted 1:10 in homogenisation buffer, and the sample extracted using a boronate affinity gel. It was then acylated and enzymatically converted to quantitative norepinephrine levels. The optical density of the final solution was measured at 450nm, against a reference of 620nm. The amount of norepinephrine was determined against a standard curve, and corrected for the initial dilution; it was then normalised to the amount of BAT initially homogenised.

4.3.6 PLASMA NOREPINEPHRINE QUANTIFICATION

NE levels in plasma were determined using a competitive enzyme immunoassay kit (Noradrenaline Plasma ELISA, BA E-4200, Labor Diagnostika Nord GmbH & Co. KG, Germany), following the manufacturer's instructions. 300ul of plasma was added to a boronate affinity gel extraction plate; it

was then acylated and enzymatically converted to quantitative norepinephrine levels. The absorbance of the final solution was read at 450nm, and the concentration of norepinephrine determined from a standard curve.

4.4 RESULTS

4.4.1 KEY RESULTS FOR CHAPTER 4

- A single dose of OX2 increases BAT activity as demonstrated by increased UCP-1 mRNA at 4 hours.
 - Administration of the β 3 agonist CL316243 for 8 days causes BAT activation and browning of WAT, with a trend for increased BAT UCP-1 mRNA levels, increased BAT temperature, and elevated BAT norepinephrine levels, as well as increased UCP-1 expression in epididymal WAT. However, there was no reduction in body weight compared to controls.
 - Chronic administration of OX2 causes persistent elevation in energy expenditure, as shown by both increased oxygen consumption in CLAMS metabolic cages and increased weight loss compared to a pair-fed group.
 - However, chronic administration of OX2 causes suppression of BAT activity as shown by suppressed UCP-1 mRNA levels and no increase in BAT temperature or BAT norepinephrine levels; there was also no browning of WAT.
 - OX2 does increase sympathetic nervous system activity, with an elevation in plasma norepinephrine levels after 8 days of treatment.
 - This suggests alternative mechanisms are responsible for the increased energy expenditure caused by OX2.
-

4.4.2 ACUTE EFFECT OF OX2 ON BROWN ADIPOSE TISSUE ACTIVITY

Four hours after administration of OX2 to fed animals, there was a significant elevation in all mRNA levels of markers of BAT activity when compared to control animals (average fold change in UCP-1 mRNA, mean $\pm$ SEM, vehicle: 1.0 ± 0.2 ; OX2: 1.6 ± 0.2 ; $p < 0.05$; average fold change in ATGL mRNA, mean $\pm$ SEM, vehicle: 1.0 ± 0.3 ; OX2: 3.2 ± 0.6 ; $p < 0.05$; average fold change in Dio2 mRNA, mean $\pm$ SEM, vehicle: 1.0 ± 0.2 ; OX2: 2.7 ± 0.5 ; $p < 0.05$; average fold change in PPARGC1a mRNA, mean $\pm$ SEM, vehicle: 1.0 ± 0.3 ; OX2: 4.1 ± 1.1 ; $p < 0.05$) (Figure 4.2). Over the same period time, there was no significant difference in food intake between the groups though they had free access to standard laboratory chow (mean food intake, g $\pm$ SEM, vehicle: $3.3g \pm 0.6$; OX2 3.5 ± 0.4 ; $p = \text{NS}$) (Figure 4.3).

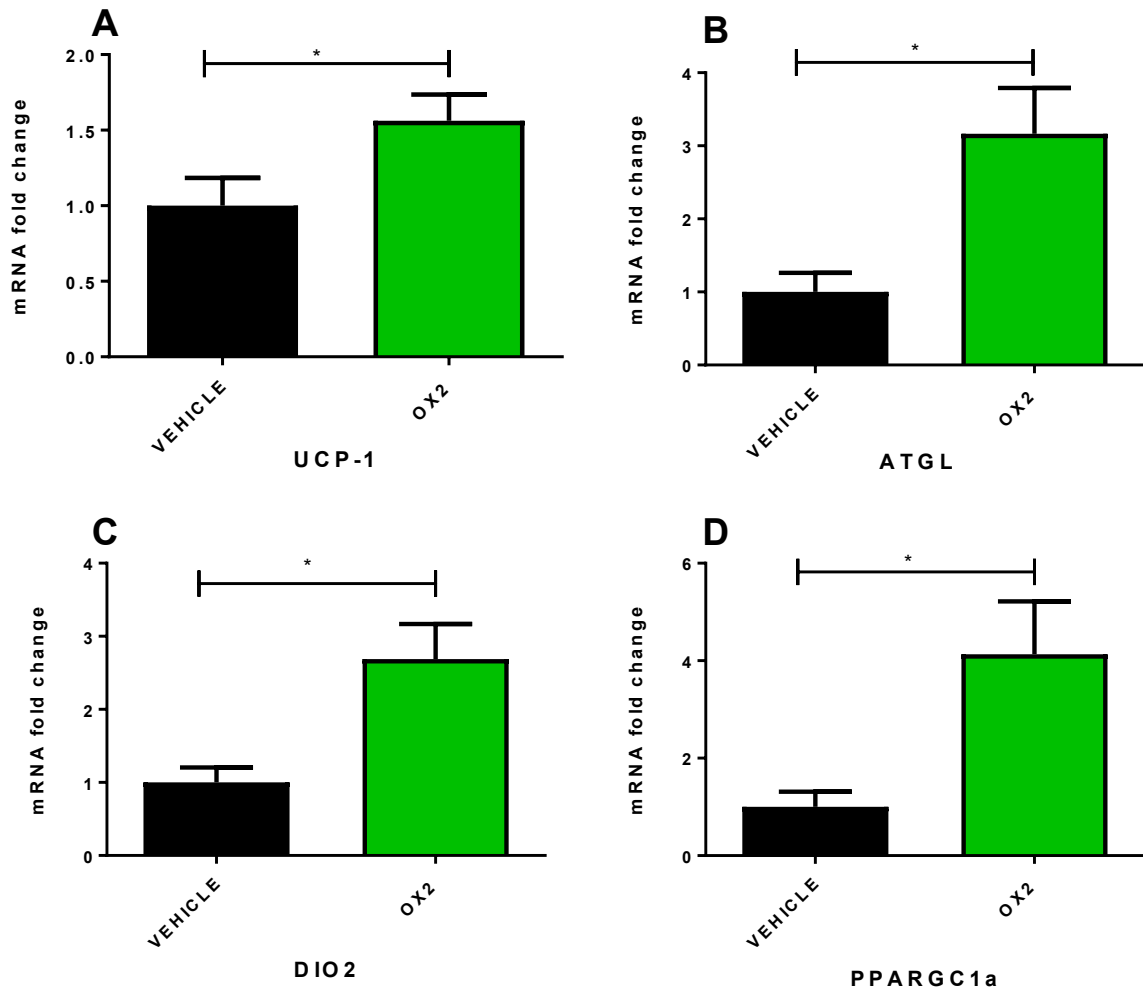


Figure 4.2 Acute effect of OX2 on mRNA levels of markers of BAT activity in rats.

Relative abundance of UCP-1 (A) ATGL (B) Dio2 (C) PGC1a (D) from rats administered SC vehicle or OX2 (40nmol/kg) 4 hours previously. All animals had free access to standard laboratory chow. All mRNA expression normalised to the housekeeping gene 18s. N=6-8. Graph shows mean $\pm$ SEM. Statistical analysis undertaken using unpaired t-tests. * p <0.05.

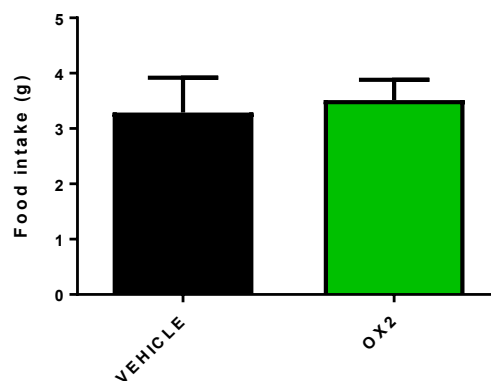


Figure 4.3 Acute effect of OX2 on food intake in rats.

Cumulative food intake in rats administered a single dose of vehicle or OX2 (40nmol/kg) 4 hours previously. N=8. Graph shows mean $\pm$ SEM. Statistical analysis undertaken using unpaired t-test.

4.4.3 EFFECT OF CONTINUOUS OX2 ON ENERGY EXPENDITURE

During the 8 days of continuous OX2 infusion, though all animals had free access to standard laboratory chow, there was a trend to reduced food intake in the OX2 group (average cumulative food intake, mean $\pm$ SEM(g): vehicle, 229.5 $\pm$ 9.1g; OX2, 212.4 $\pm$ 5.2g) (Figure 4.4A). Both groups started the study at the same average baseline body weight of 387g, but over the 8 days of the study, the vehicle group gained, on average, 8% body weight, while the OX2 group lost, on average, 4% of their body weight (average body weight change from baseline, mean $\pm$ SEM(g): vehicle, 34 $\pm$ 4.0g; OX2, -14 $\pm$ 4.3g; $p < 0.0001$). The change from baseline was significantly different between the groups (Figure 4.4B).

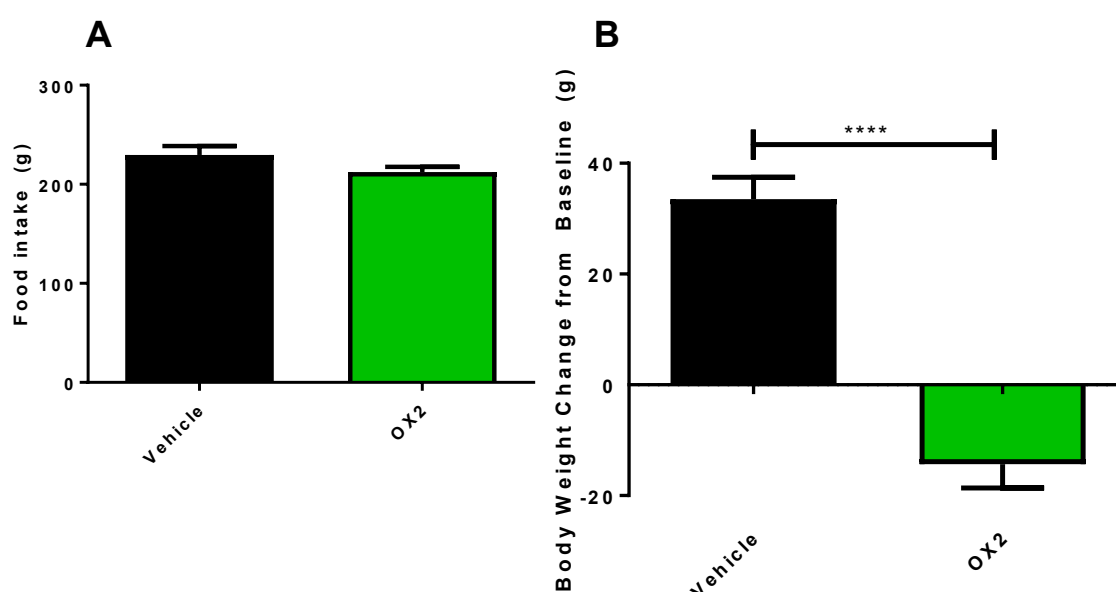


Figure 4.4 Effect of continuous OX2 on food intake and body weight in rats.

The cumulative food intake (A) and body weight change from baseline (B) in rats who have osmotic mini-pumps inserted subcutaneously delivering either saline or OX2 (80nmol/kg/24hrs) for 8 days. Throughout the experiment, animals had free access to standard laboratory chow. N=6-10. Graph shows mean $\pm$ SEM. Statistical analysis undertaken using unpaired t-tests. **** $p < 0.0001$ vs vehicle.

During the period in CLAMS cages, the OX2 group showed elevated energy expenditure, with a significant increase in VO_2 during the 12 hours of the light phase (average oxygen consumption during the 12 hours of the light phase, mean $\pm$ SEM (ml/kg): vehicle, 16957 $\pm$ 421 ml/kg; OX2, 18426 $\pm$ 475 ml/kg, $p < 0.05$) (Figure 4.5). There was no difference in oxygen consumption during the 12 hours of either dark phase the animals spent in CLAMS (average oxygen consumption during the 12 hours of the 1st dark phase in CLAMS, mean $\pm$ SEM (ml/kg): vehicle, 17325 $\pm$ 1191 ml/kg; OX2, 19003 $\pm$ 981 ml/kg, $p = ns$; average oxygen consumption during the 12 hours of the 2nd dark phase in CLAMS, mean $\pm$ SEM (ml/kg): vehicle, 17233 $\pm$ 862 ml/kg; OX2, 17546 $\pm$ 892 ml/kg, $p = ns$).

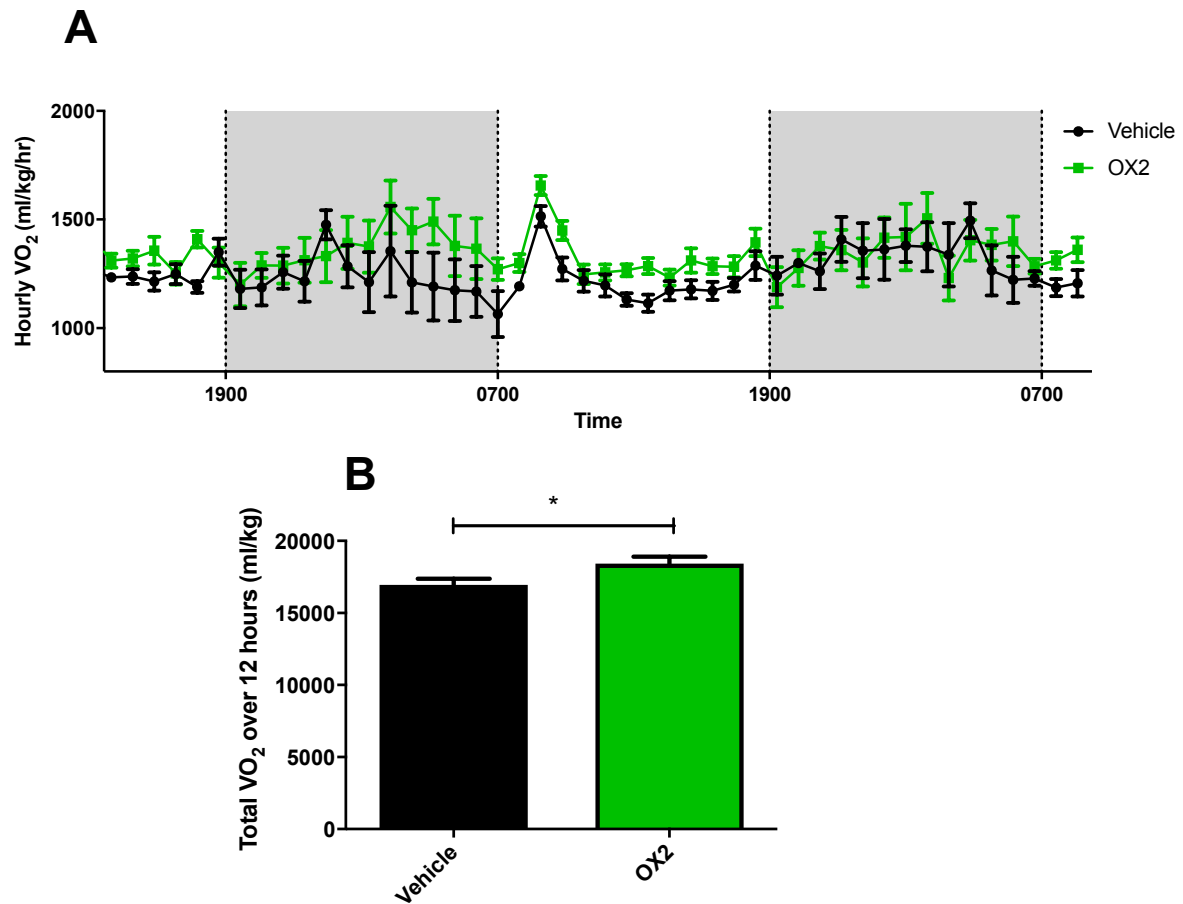


Figure 4.5 Effect of continuous administration of OX2 on oxygen consumption in rats.

Hourly oxygen consumption over days 6-8 (A) and total oxygen consumption during the 12 hours of the light phase of day 7 (B), in rats who had osmotic mini-pumps delivering either saline or OX2 (80nmol/kg/24hrs) inserted subcutaneously on day 0. Animals had free access to standard laboratory chow throughout. N=6-10. Graphs show mean $\pm$ SEM. Statistical analysis undertaken using unpaired t-test. * $p < 0.05$ vs vehicle.

There was no significant difference in physical activity between the two groups at any period (average light phase physical activity, mean beam breaks $\pm$ SEM (counts): vehicle, 4924 ± 517 counts; OX2, 4725 ± 255 counts; $p = \text{ns}$; average physical activity in the 1st dark phase, mean beam breaks $\pm$ SEM (counts): vehicle, 10289 ± 269 counts; OX2, 10425 ± 600 counts; $p = \text{ns}$ average physical activity in the 2nd dark phase, mean beam breaks $\pm$ SEM (counts): vehicle, 10465 ± 520 counts; OX2, 10715 ± 526 counts; $p = \text{ns}$) (Figure 4.6).

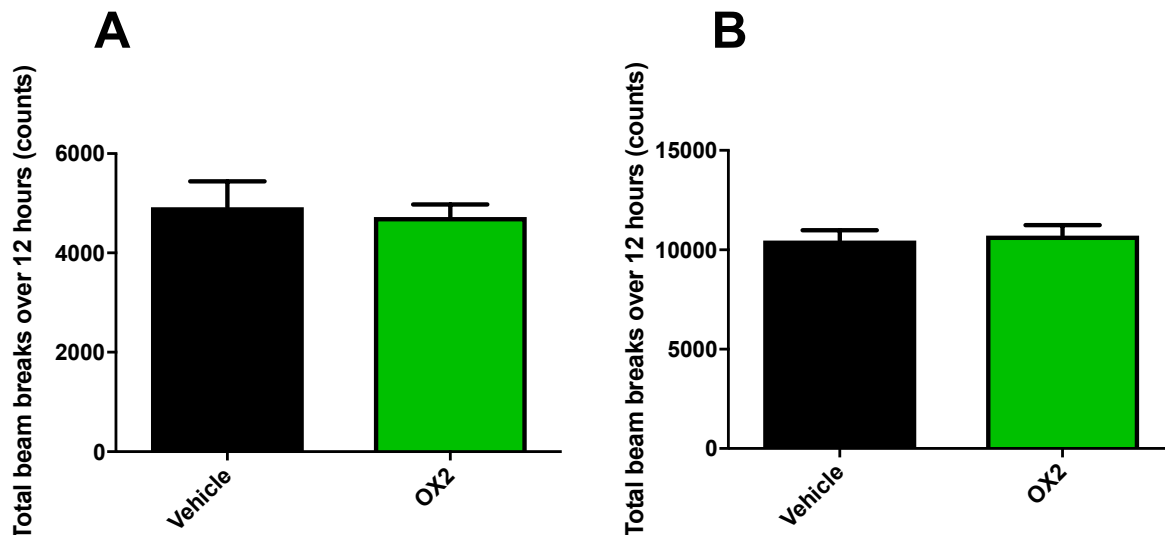


Figure 4.6 Effect of continuous administration of OX2 on physical activity in rats.

Average number of beam breaks during the 12 hours of the light phase (A) and the 12 hours of the 2nd dark phase in CLAMS (B) in rats who had osmotic mini-pumps containing saline or OX2 (80nmol/kg/24hrs) inserted subcutaneously 7 days previously. Animals had free access to standard laboratory chow throughout. N=6-10. Graphs show mean ± SEM. Statistical analysis undertaken using unpaired t-tests.

4.4.4 COMPARISON OF THE EFFECT OF CONTINUOUS INFUSION OF OX2 AND β 3 AGONIST IN RATS

4.4.4.1 EFFECT OF CONTINUOUS INFUSION OF OX2 AND β 3 AGONIST ON FOOD INTAKE AND BODY WEIGHT IN RATS

In animals given free access to standard laboratory chow, an 8-day infusion of OX2 caused a significant reduction in food intake when compared to infusion of vehicle; in contrast there was a tendency for the β 3 group to eat more (average cumulative food intake, mean ± SEM (g), vehicle: 223.1 ± 5.4 g; OX2: 194.9 ± 14.7 g; pair-fed to OX2: 191.9 ± 0.1 g; β 3 agonist: 228.6 ± 5.5 g; vehicle vs OX2, $p < 0.05$; vehicle vs pair-fed to OX2, $p < 0.05$) (Figure 4.7A).

All groups entered the study with the same average baseline body weight (313g). During the 8-days of the study, the vehicle, β 3 and pair-fed groups gained weight; in contrast, the OX2 group lost weight (average body weight change from baseline, mean ± SEM(g), vehicle: 40 ± 3.7 g; OX2: -13 ± 6.2 g; pair-fed: 26 ± 3.3 g; β 3 agonist: 43 ± 1.5 g; vehicle vs OX2, $p < 0.0001$; vehicle vs pair-fed, $p < 0.05$) (Figure 4.7B). This equated to an increase in body weight compared to baseline of 12.8% in the vehicle group, 8.2% in the pair-fed group, and 13.6% in the β 3 agonist group; and a 4.0% decrease in body weight from baseline in the OX2 group.

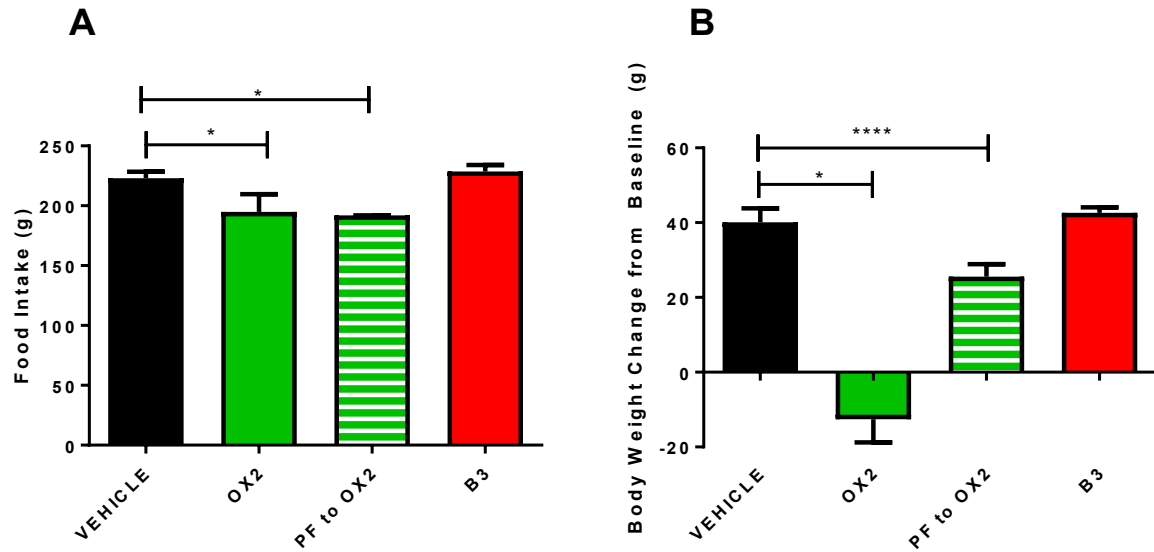


Figure 4.7 Effect of continuous OX2 or β 3 agonist on food intake and bodyweight in rats.

Cumulative food intake (A) and bodyweight change from baseline (B) in male Wistar rats after 8 days of continuous infusion of vehicle, OX2 (80nmol/kg/24 hours) or β 3 agonist (1mg/kg/24 hours) via osmotic mini-pump, or pair-feeding to the OX2 group. Vehicle, OX2 and β 3 groups had unlimited access to standard laboratory chow; the food intake of the pair-fed group was restricted to that of the OX2 group. Food and body weight were measured daily at 1600. N=9-10/group. Mean initial body weight 313g. Data shown as the mean $\pm$ SEM. Statistical analysis carried out using one-way ANOVA and post hoc tests with Dunnett's multiple comparison test comparing to the vehicle control, * p < 0.05, **** p < 0.0001 vs vehicle.

4.4.4.2 EFFECT OF CONTINUOUS INFUSION OF OX2 AND β 3 AGONIST ON BODY TEMPERATURE IN RATS

Over the 8-days of the study, both treatment and time had an effect on BAT temperature, but there was no interaction between time and treatment (2-way ANOVA, effect of treatment, $F(2,85)=4.5$, $p=0.01$; effect of time, $F(3,85)=2.7$, $p=0.05$; interaction, $F(6,85)=1.8$, $p=0.1$). 2 days after pump insertion, OX2 significantly increased BAT temperature; there was also a non-significant increase in BAT temperature in the β 3 group (average BAT temperature, mean $\pm$ SEM ($^{\circ}$ C), vehicle: $36.7 \pm 0.2^{\circ}$ C; OX2: $37.4 \pm 0.2^{\circ}$ C; β 3 agonist: $37.2 \pm 0.2^{\circ}$ C; vehicle vs OX2, $p < 0.05$). At 5 days post-operative, only β 3 significantly increased BAT temperature (average BAT temperature, mean $\pm$ SEM ($^{\circ}$ C), vehicle: $36.6 \pm 0.2^{\circ}$ C; OX2: $36.6 \pm 0.2^{\circ}$ C; β 3 agonist: $37.5 \pm 0.2^{\circ}$ C; vehicle vs β 3, $p < 0.05$) (Figure 4.8 and Table 4-2).

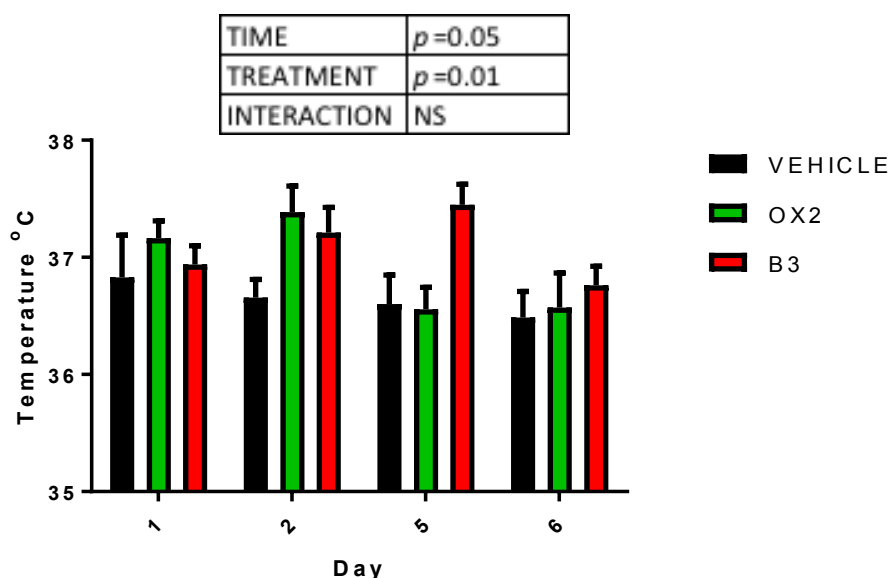


Figure 4.8 Effect of continuous OX2 or B3 agonist on BAT temperature in rats.

Average BAT temperature in male Wistar rats on specific days during 8 days of continuous infusion of vehicle, OX2 (80nmol/kg/24 hours) or $\beta 3$ agonist (1mg/kg/24 hours) via a mini-osmotic pump. Temperature for each rat was a mean of three readings done at 3.30pm using a hand-held reader and an implanted transponder. N=7-10. Data shown as the mean $\pm$ SEM. Statistical analysis carried out using a regular 2-way ANOVA.

POST-OPERATIVE DAY	BAT TEMPERATURE MEAN $\pm$ SEM °C			STATISTICAL ANALYSIS (2-way ANOVA with Dunnett's multiple comparison test)
	VEHICLE	OX2 (80nmol/kg/24hrs)	$\beta 3$ agonist (1mg/kg/24hrs)	
1	36.8 $\pm$ 0.4	37.2 $\pm$ 0.1	36.9 $\pm$ 0.2	NS
2	36.7 $\pm$ 0.2	37.4 $\pm$ 0.2	37.2 $\pm$ 0.2	Vehicle vs OX2, $p<0.05$
5	36.6 $\pm$ 0.2	36.6 $\pm$ 0.2	37.5 $\pm$ 0.2	Vehicle vs $\beta 3$, $p<0.05$
6	36.5 $\pm$ 0.2	36.6 $\pm$ 0.3	36.8 $\pm$ 0.2	NS

Table 4-2 Effect of continuous infusion of vehicle, OX2 or $\beta 3$ agonist on BAT temperature in rats.

4.4.5 EFFECT OF CONTINUOUS INFUSION OF OX2 AND $\beta 3$ AGONIST ON NOREPINEPHRINE LEVELS IN RATS

At the end of 8-days of continuous infusion, OX2 caused a significant elevation in plasma norepinephrine levels, with levels being 1.6 times higher than in the vehicle group; there was no significant difference from vehicle in the pair-fed or $\beta 3$ groups (average plasma norepinephrine levels, mean $\pm$ SEM (ng/ml), vehicle: 6.3 $\pm$ 0.4ng/ml; OX2: 10.0 $\pm$ 1.1ng/ml; pair-fed: 7.2 $\pm$ 0.6ng/ml; $\beta 3$ agonist: 6.8 $\pm$ 0.7ng/ml; vehicle vs OX2, $p<0.01$) (Figure 4.9A).

After 8 days of continuous infusion, there was a tendency for treatment with $\beta 3$ agonist to increase BAT norepinephrine levels; BAT norepinephrine levels were 1.5x higher than in the vehicle group. In contrast, there was no change in BAT norepinephrine levels in either the OX2 or pair-fed groups (average BAT norepinephrine level, mean $\pm$ SEM (ng/g), vehicle: 98 ± 7 ng/g; OX2: 107 ± 14 ng/g; pair-fed: 126 ± 10 ng/g; $\beta 3$ agonist: 143 ± 15) (Figure 4.9B).

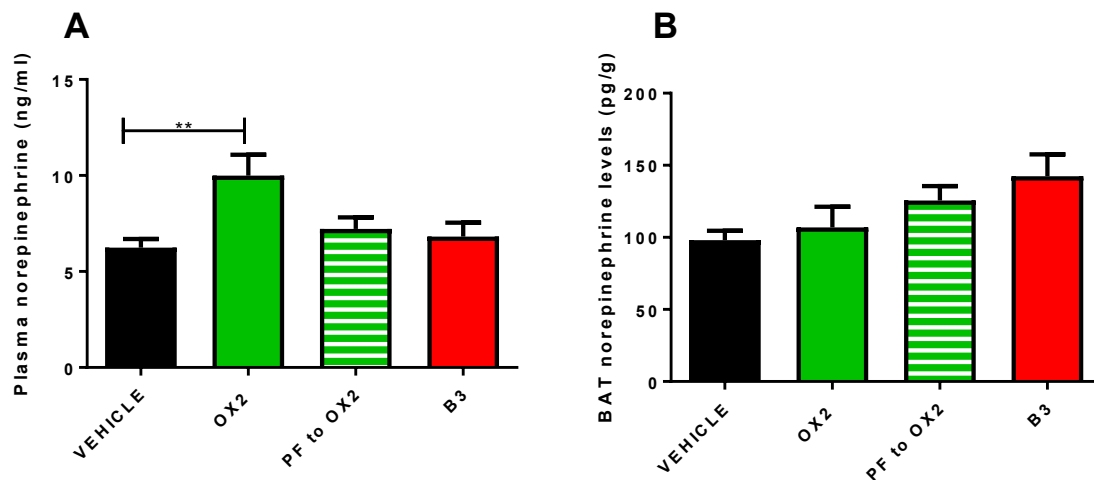


Figure 4.9 Effect of continuous OX2 or $\beta 3$ agonist on norepinephrine levels in plasma and BAT in rats.

Norepinephrine levels in plasma (A) and BAT (B) of male Wistar rats who had received a continuous subcutaneous infusion of vehicle, OX2 (80nmol/kg/24 hours), $\beta 3$ agonist (1mg/kg/24 hours), or who were pair-fed to the OX2 group for 8 days. Vehicle, OX2 and $\beta 3$ groups had unlimited access to standard laboratory chow; the food intake of the pair-fed group was restricted to that of the OX2 group N=9-10. Data shown are mean $\pm$ SEM. Statistical analysis carried out using one-way ANOVA for each time point and post hoc tests with Dunnett's multiple comparison test comparing to vehicle control. ** $p < 0.01$.

4.4.6 EFFECT OF CONTINUOUS INFUSION OF OX2 AND $\beta 3$ AGONIST ON UCP-1 LEVELS IN RATS

After 8 days of continuous administration, $\beta 3$ agonist caused a non-significant increase in BAT UCP-1 mRNA levels by 1.6 fold compared to vehicle, while there was a non-significant suppression of mRNA levels in both the OX2 and pair-fed groups to 0.4x compared to vehicle (average fold change in UCP-1 mRNA, mean $\pm$ SEM, vehicle: 1.0 ± 0.2 ; OX2: 0.4 ± 0.1 ; $\beta 3$: 1.6 ± 0.4 ; pair-fed: 0.4 ± 0.1) (Figure 4.10A). When compared to vehicle, there was no significant difference in levels of either Dio2 or ATGL mRNA levels in any other group (average fold change in ATGL mRNA, mean $\pm$ SEM, vehicle: 1.0 ± 0.3 ; OX2: 1.4 ± 0.5 ; $\beta 3$: 1.4 ± 0.5 ; average fold change in Dio2 mRNA, mean $\pm$ SEM, vehicle: 1.0 ± 0.3 ; OX2: 1.5 ± 0.5 ; $\beta 3$: 1.6 ± 0.5 ; pair-fed: 1.1 ± 0.2) (Figure 4.10B and C). PPARGC1a mRNA levels were significantly elevated by 2.7x in the pair-fed group compared to vehicle, but there was no significant change in the other groups (average fold change in PPARGC1a mRNA, mean $\pm$ SEM,

vehicle: 1.0 ± 0.2 ; OX2: 1.8 ± 0.6 ; $\beta 3$: 1.2 ± 0.4 ; pair-fed: 2.7 ± 0.6 ; vehicle vs pair-fed, $p < 0.05$) (Figure 4.10D).

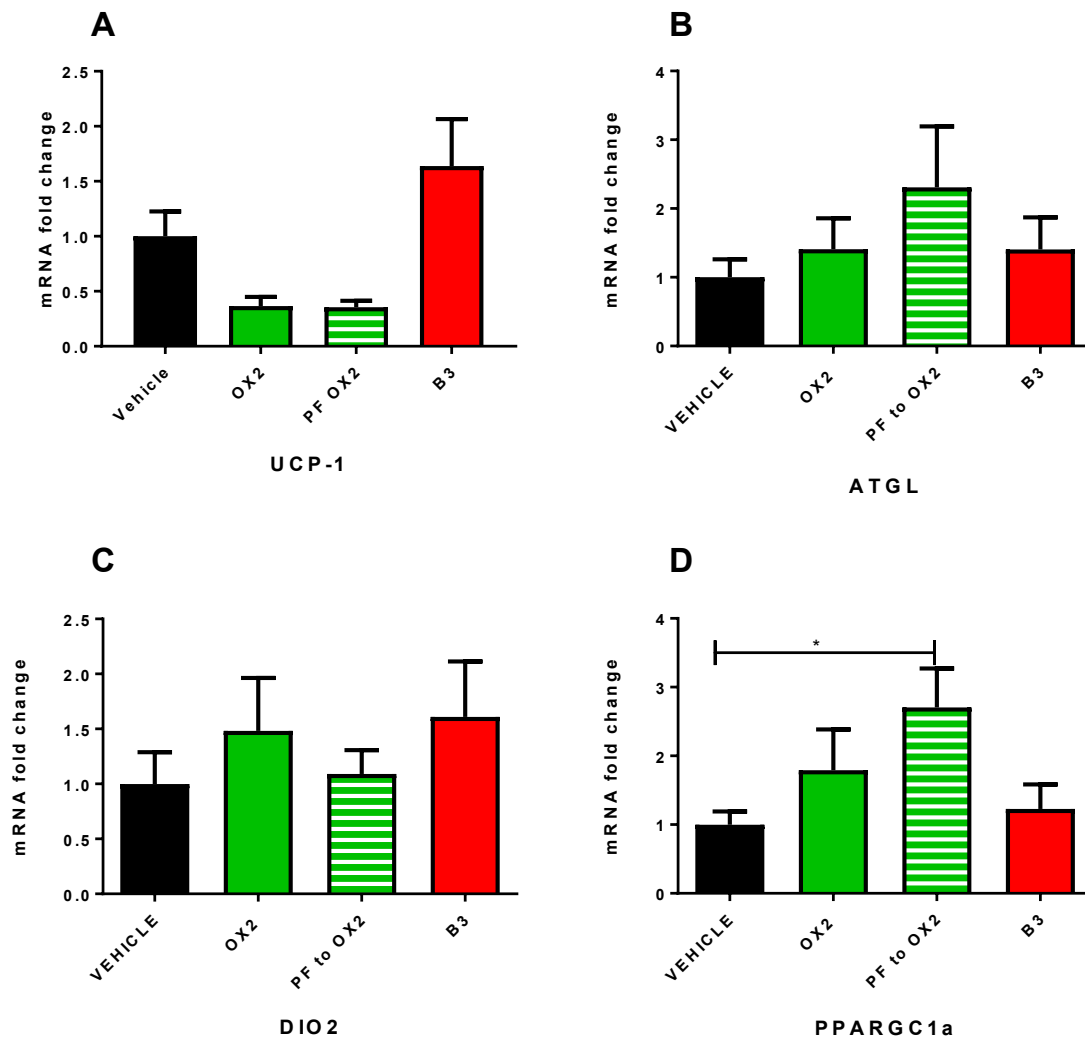


Figure 4.10 Effect of continuous OX2 or $\beta 3$ agonist on mRNA levels of markers of BAT activity.

Relative abundance of UCP-1 (A) ATGL (B) Dio2 (C) PGC1a (D) mRNA in the brown adipose of male Wistar rats who received 8-days continuous subcutaneous infusion of vehicle, OX2 (80nmol/kg/24 hours) or B3 agonist (1mg/kg/24 hours), or who were pair-fed to the OX2 group. The animals were culled after freely eating overnight. All mRNA expression normalised to the housekeeping gene 18s. N=9-10. Data shown are mean fold change mRNA $\pm$ SEM. Statistical analysis carried out using one-way ANOVA and post hoc tests with Dunnett's multiple comparison test. * $p < 0.05$.

$\beta 3$ administration for 8 days caused a significant elevation in WAT UCP-1 mRNA, with an increase in levels more than 190x that seen in vehicle; no other group showed a change in WAT UCP-1 levels (average fold change in UCP-1 mRNA, mean $\pm$ SEM, vehicle: 1.0 ± 0.5 ; OX2: 2.4 ± 1.8 ; $\beta 3$: 193 ± 114 ; pair-fed: 1.1 ± 0.8 ; vehicle vs $\beta 3$, $p < 0.05$) (Figure 4.11A). There were no significant differences in the mRNA levels of the other brite adipose markers, TMEM26 and Tbx1, between any groups (average fold change in TMEM26 mRNA, mean $\pm$ SEM, vehicle: 1.0 ± 0.2 ; OX2: 0.8 ± 0.2 ; $\beta 3$: 1.7 ± 0.3 ; pair-

fed: 1.9 ± 0.4 ; average fold change in Tbx1 mRNA, mean $\pm$ SEM, vehicle: 1.0 ± 0.6 ; OX2: 0.5 ± 0.1 ; $\beta 3$: 2.0 ± 1.5 ; pair-fed: 0.4 ± 0.2) (Figure 4.11B and C).

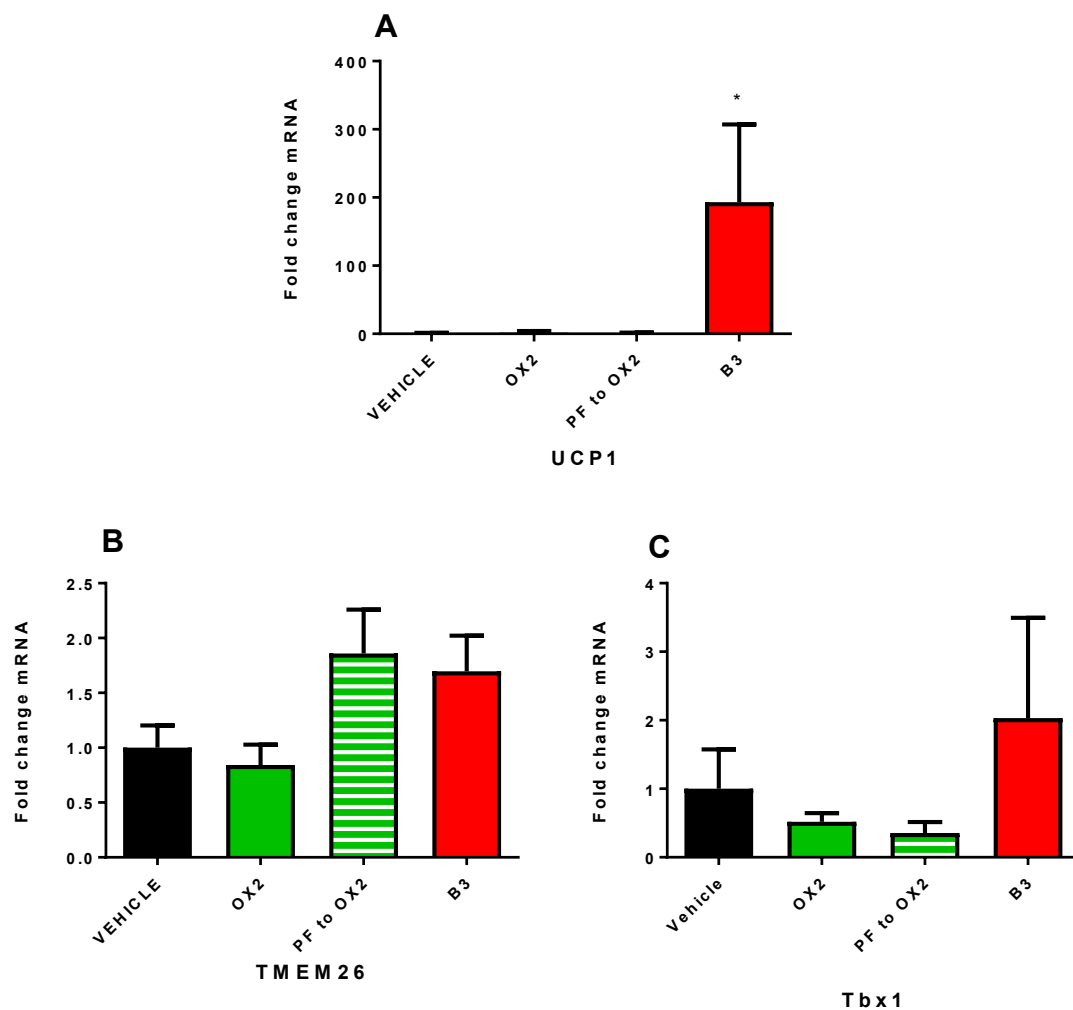


Figure 4.11 Effect of continuous OX2 or $\beta 3$ agonist on mRNA levels of markers of brite adipose.

Relative abundance of UCP-1 (A) TMEM26 (B) and Tbx1 (C) mRNA in the epididymal white adipose of male Wistar rats who received 8-days continuous subcutaneous infusion of vehicle, OX2 (80nmol/kg/24 hours) or B3 agonist (1mg/kg/24 hours), or who were pair-fed to the OX2 group. Animals had free access to standard laboratory chow throughout the experiment. All mRNA expression normalised to the housekeeping gene 18s. N=8-10. Data shown are mean fold change mRNA $\pm$ SEM. Statistical analysis carried out using Kruskal-Wallis and post hoc tests with Dunn multiple comparison test. * $p < 0.05$ vs vehicle.

	SALINE	OX2	Pair-fed to OX2	β 3	
FOOD INTAKE	REFERENCE	REDUCED	REDUCED	NON-SIGNIFICANT INCREASE	Figure 4.7
BODY WEIGHT	REFERENCE	REDUCED REDUCED COMPARED TO PAIR-FED	REDUCED	NO EFFECT	Figure 4.7
INTERSCAPULAR TEMPERATURE	REFERENCE	INCREASED ON DAY 2	NOT MEASURED	INCREASED ON DAY 2 AND 5	Figure 4.8
PLASMA NOREPINEPHRINE	REFERENCE	INCREASED	NO EFFECT	NO EFFECT	Figure 4.9
BAT NOREPINEPHRINE	REFERENCE	NO EFFECT	NO EFFECT	TREND TO INCREASED LEVELS	Figure 4.9
BAT UCP-1 mRNA LEVELS	REFERENCE	NON-SIGNIFICANT SUPPRESSION	NON-SIGNIFICANT SUPPRESSION	NON-SIGNIFICANT ELEVATION	Figure 4.10
BAT ATGL mRNA LEVELS	REFERENCE	NO EFFECT	NO EFFECT	NO EFFECT	Figure 4.10
BAT Dio2 mRNA LEVELS	REFERENCE	NO EFFECT	NO EFFECT	NO EFFECT	Figure 4.10
BAT PPARGC1a mRNA LEVELS	REFERENCE	NO EFFECT	INCREASED	NO EFFECT	Figure 4.10
WAT UCP-1 mRNA LEVELS	REFERENCE	NO EFFECT	NO EFFECT	INCREASED	Figure 4.11
WAT TMEM26 mRNA LEVELS	REFERENCE	NO EFFECT	NO EFFECT	NO EFFECT	Figure 4.11
WAT Tbx1 mRNA LEVELS	REFERENCE	NO EFFECT	NO EFFECT	NO EFFECT	Figure 4.11

Table 4-3 Summary of comparison of effects of 8-days continuous administration of vehicle, OX2, pair-feeding to OX2 and β 3 agonist in rats.

4.5 DISCUSSION

As demonstrated in Chapter 2, a single subcutaneous dose of OX2 in rats caused an increase in oxygen consumption, which reaches a maximum at 4 hours, and persists for 12 hours after the injection (see Figure 4.12).

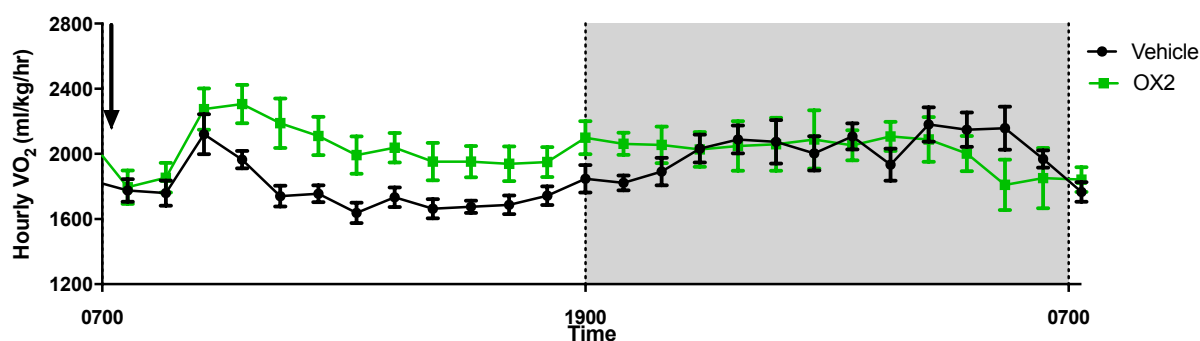


Figure 4.12 Hourly oxygen consumption following as single SC OX2 (40nmol/kg) in rats.

Hourly oxygen consumption by rats in CLAMS metabolic cages after the injection of vehicle or OX2 (40nmol/kg) on day 2. Study described in chapter 2 - see 2.3.4.1.

After a single dose of OX2, UCP-1 mRNA levels were elevated at 4 hours in rat BAT (Figure 4.2A). This suggested the possibility that the acute increase in energy expenditure seen after a single dose of OX2 is due to BAT activation. This was further supported by significant elevation in other markers of BAT activity, namely ATGL, Dio2 and PGC1a (Figure 4.2B, C and D). This could not confirm, however, that oxyntomodulin increases energy expenditure through BAT activation, but prompted the subsequent studies looking at the effect of chronic OX2 on brown adipose activation.

The CLAMS study conducted in this chapter, in which rats had subcutaneous osmotic mini-pumps inserted delivering either saline or OX2, demonstrated that chronic administration of OX2 can cause a sustained increase in energy expenditure. Oxygen consumption remained significantly raised in the OX2 group during the light phase even one week after insertion of the osmotic mini-pump (Figure 4.5). The chronic elevation in energy expenditure by OX2 was supported in the pump pair-feeding study, where animals receiving continuous OX2 via an osmotic mini-pump lost significantly more weight than the equivalent pair-fed group (Figure 4.7). This consistent rise in energy expenditure supports the idea that OXM analogues may be useful as a treatment for obesity.

Though OX2 increased energy expenditure in the light phase, there was no increase in energy expenditure in the dark phase in the OX2 group. This may reflect the difference in behaviour in rats during the light and dark phase. At night, rats are more active (Figure 4.6) and the relative contribution of the basal metabolic processes to energy expenditure is smaller. Therefore, the increase in these processes by OX2 may be masked by the physical-activity related increase in energy

expenditure. There was also a spike in energy expenditure during the morning in both groups that corresponds with the animals being removed from the cages for weighing (Figure 2.6A). This likely reflects a stress-induced increase in energy expenditure (Deboкас and Behren, 1977, McGregor et al., 1994), a feature which must always be considered in studies looking at energy expenditure.

Despite the sustained increase in energy expenditure following OX2 administration, there was no evidence this was due to prolonged activation of BAT, nor through induction of brite adipose tissue.

In the β 3-agonist group (positive-control), there was a trend for increased BAT UCP-1 levels; in contrast, BAT UCP-1 levels were suppressed in the OX2 group (Figure 4.10). There was a rise in BAT norepinephrine levels in the β 3 group, which was to be expected since BAT is sympathetically innervated, so activation should increase BAT norepinephrine. However, there was no increase in the OX2 group (Figure 4.9). Though there was an initial increase in BAT temperature in both OX2 and β 3 groups (seen on day 2 post-operatively), this was only sustained to day 5 post-operatively in the β 3 group (Figure 4.8). Moreover, the β 3 group showed a significant increase in UCP-1 levels in WAT, highly suggestive of induction of brite adipose tissue; no such induction occurred in the OX2 group (Figure 4.11). Given the differences in results between the OX2 and β 3 groups, this would suggest that these two drugs work through different pathways. It suggests that the chronic elevation of energy expenditure by OX2 is not a consequence of a measurable increase in BAT activity or induction of brite adipose tissue.

How then, can this biphasic response of UCP-1 to OX2, with an acute elevation and then chronic suppression, be explained? The acute activation may reflect the role of glucagon as a stress hormone. In Chapter 3, it was shown that OX2 increases energy expenditure via the glucagon receptor. Glucagon is acutely released in response to multiple physiological stresses, including cold (Seitz et al., 1981). This may be an adaptive response to enhance fuel delivery (through glycogenolysis, gluconeogenesis and ketogenesis) to support the response to the stress of cold. This may include upregulating transcription and translation of UCP-1. It can therefore be postulated that acute administration of oxyntomodulin also increases fuel delivery to BAT, and enables UCP-1 upregulation. However, a recent study by Salem *et al.* found no increase in BAT activity during a glucagon infusion in man, as measured by thermal imaging and 18-F FDG PET/CT glucose uptake, despite a detectable increase in energy expenditure as measured by indirect calorimetry (Salem et al., 2016). This suggests there is no acute thermogenic response of BAT to glucagon. Despite UCP-1 mRNA levels being used as the 'gold standard' measure of BAT activation, it must be remembered that changes in UCP-1 mRNA do not actually reflect an increase in heat production. Nedergaard *et al.* summarised BAT adaptation in mice in response to cold, and showed that changes in UCP-1 mRNA

do not mirror whole-body UCP-1 protein levels (Nedergaard and Cannon, 2013). Therefore, the acute increase in UCP-1 mRNA seen in this chapter may have no physiological significance. To further determine if BAT does contribute to the acute energy expenditure effect of OXM, studies should quantify acute changes in UCP-1 protein levels in response to OXM administration, as well as acute changes in BAT temperature.

The suppression of UCP-1 levels following chronic administration of OX2 may be explained as an adaptive response to the increase in other energy-expenditure processes. As chronic OX2 clearly does elevate energy expenditure, but not via BAT, there must be other metabolic pathways which are up-regulated. To divert energy to these pathways, rats may down-regulate their BAT-induced thermogenesis. A similar suppression in UCP-1 levels is seen in mice given the chemical-uncoupler dinitrophenol; this suppression compensates for the increased thermogenesis caused by the drug (Goldgof et al., 2014). The suppression of UCP-1 levels may be compounded by the anorectic effect of chronic OX2. Fasting reduces UCP-1 levels, in what is also likely to be an adaptive response to reduce energy expenditure in an energy-deficient environment (Champigny and Ricquier, 1990). This would also explain why UCP-1 levels were equally suppressed in the OX2 group and the pair-fed group (Figure 4.10).

Though there was no apparent sympathetic activation of BAT in the OX2 group, after 8 days of continuous administration of OX2 there was a significant increase in plasma norepinephrine levels in the OX2 group (Figure 4.9). Levels in the OX2 group were approximately 1.6 times higher than in the vehicle group, suggesting OX2 may cause an increase in general SNS activity. Glucagon also increases sympathetic nervous system activity: peripheral and central administration of glucagon increases activity in sympathetic nerve terminals (Lockie et al., 2012, Krzeski et al., 1989), and the catecholamine-response to an injection of glucagon has been used clinically to detect pheochromocytomas (catecholamine-secreting tumours of the adrenal gland) as it stimulates the sympathetic nervous system (Bisschop et al., 2009, Lawrence, 1967). Moreover, blocking the sympathetic nervous system reduces glucagon-induced oxygen consumption in rats, and limits the hyperglycaemic response to central glucagon (Amir, 1986, Dicker et al., 1998).

Additionally, activation of the sympathetic nervous system increases energy expenditure. Sympathomimetics such as amphetamine, which stimulate the sympathetic nervous system via central mechanisms, elevate energy expenditure (Jones et al., 1992), as does direct activation of adrenoreceptors on peripheral tissues through administration of catecholamines (Atgie et al., 1998, Benthem et al., 1990, Cypess et al., 2012). Given these findings, it may be that OX2 increases activity

in the sympathetic nervous system, and this activation leads to a change in energy expenditure. This hypothesis will be investigated in Chapter 5 of this thesis.

It is interesting to note that, although the $\beta 3$ agonist increased UCP-1 levels in both BAT and WAT, there was no associated weight loss in the $\beta 3$ group. This substantiates previous findings in the literature where, although CL316243 may activate brown adipose tissue, there is overall no change in body weight (Xiao et al., 2015, Yoshitomi et al., 1998). This may be, in part, due to a compensatory increase in food intake; there was a tendency for the $\beta 3$ group to eat more than the vehicle group over the 8 days of infusion (Figure 4.7). Successful obesity treatments may need to combine agents which have an anorectic effect as well as an energy expenditure effect; both features found with OXM. The lack of body weight loss may also reflect the fact that, even when BAT and white adipose are upregulated, the actual energy expenditure caused by UCP-1 mediated thermogenesis may be very small. There was no upregulation in any other markers of BAT activation (ATGL, Dio2 and PPARGC1a) following $\beta 3$ treatment (Figure 4.10); this lack of co-activators may contribute to the paucity of thermogenic effect seen in the $\beta 3$ group. And though there was an increase in UCP-1 levels in the WAT of the $\beta 3$ group, there was no contemporaneous increase in TMEM26 or Tbx1, both markers of white adipose recruitment (Figure 4.11). This suggests that while the small amount of white adipose cells present in the adipose depot may have been activated, there was no increased recruitment of white adipose, so the actual quantity of UCP-1 is likely to be small. The difficulties in obtaining a clinically significant thermogenic effect through BAT activation may explain why, to date, no BAT activator has been used successfully to treat obesity.

Recent emphasis has been placed on the role of BAT in causing increased energy expenditure via non-shivering thermogenesis. However, other tissues (notably skeletal muscle) contribute to NST. This is controlled via the same sympathetic nerve pathways as BAT-mediated NST. Therefore it may be prudent, when looking at BAT-induced thermogenesis, to also look at other mediators of NST. This was recently demonstrated in studies investigating the energy expenditure effects of centrally administered melanocortin agonists. Studies have shown that central administration of the melanocortin agonist MTII causes an increase in energy expenditure, and an increase in BAT activation (Song et al., 2008, Williams et al., 2003). It was therefore hypothesised that the elevation in energy expenditure was caused by BAT activation. However, a recent study by Gavini *et al.* showed that, in addition to BAT activation, central MTII caused an increase in energy loss through skeletal muscle thermogenesis (Gavini et al., 2016). Without specific investigation of skeletal muscle thermogenesis, it would have been possible to assume that the elevation in energy expenditure was solely down to the increase in BAT thermogenesis. Therefore, although there was no evidence that

OXM increases BAT mediated thermogenesis, future studies should look at the effects of OXM on other mediators of non-shivering thermogenesis, such as skeletal muscle.

4.6 CONCLUSIONS

Acute administration of OX2 increases UCP-1 levels in brown adipose tissue. Chronic administration causes a sustained increase in energy expenditure, but is not associated with continued BAT activation. Therefore, other mechanisms by which OXM increases energy expenditure need to be sought.

Chapter 5

The Sympathetic Nervous System

And Energy Expenditure Caused

By Oxyntomodulin

5.1 INTRODUCTION

The sympathetic nervous system (SNS) is a division of the autonomic nervous system. Pre-ganglionic neurons originate from the thoracolumbar region of the spinal cord, and synapse in paravertebral ganglia; long post-ganglionic neurons then diverge across the body to innervate a wide range of tissues. These include brown adipose tissue (as discussed in chapter 4), white adipose tissue, skeletal muscle, the liver, the heart and others. The SNS is responsible for catabolic processes, increasing metabolism in many of these target tissues. This up-regulation in turn allows for rapid response in stressful situations – the typical ‘fight or flight’ response.

Several anti-obesity treatments exploit the increased metabolic rate caused by sympathetic nervous system activation. The sympathomimetics, including phenteramine, diethylpropion and amphetamine, act within the brain to stimulate the sympathetic nervous system, with a subsequent anorectic effect and an increase in metabolic rate (Arch, 1981). Side effects related to activation of the SNS in the periphery (such as tachycardia and xerostomia) partially explain the limited use of sympathomimetics in the treatment of obesity. However, it is their potential as drugs of abuse, and their damaging effects on the cardiovascular system which have prevented their licensing as obesity treatments in the UK. Because of these known issues, it is important to assess whether any new drugs for obesity also activate the SNS.

There are several ways of measuring sympathetic nervous system activity. Recording electrical discharge in specific sympathetic nerve terminals within a single tissue gives direct evidence of sympathetic stimulation of that tissue (Guild et al., 2010). This very precise measurement allows the recording of even transient nerve stimulation. However, it is difficult to extrapolate from a single neuron’s activity to a change in metabolic process or increase in energy expenditure in a whole animal.

This can be navigated by measuring tissue and plasma adrenaline and norepinephrine levels in order to gain a more general perspective on activation of the sympathetic nervous system. Sympathetic neurons release norepinephrine onto their target tissues, and therefore tissue norepinephrine and markers of norepinephrine turnover can be used to reflect relative sympathetic activation within that tissue (Zygmunt and Stanzyk, 2010). The adrenal gland releases adrenaline and norepinephrine into the circulation in response to sympathetic nervous system activation; therefore, plasma levels can reflect systemic activation of the sympathetic nervous system. However, the SNS is exquisitely sensitive to changes in environment; in animals, it is activated in response to stress such as handling or the presence of other animals, which will affect plasma catecholamine levels (Debocas and

Behren, 1977, Niebylski et al., 2012). Levels can also vary dramatically depending on the method by which samples are collected, and this must be considered when results are interpreted (Debocas and Behren, 1977). Finally, though the sympathetic nervous system is activated, as with nerve terminal recordings, this does not necessarily equate to a change in metabolic process or indeed in energy expenditure.

To investigate whether the sympathetic nervous system mediates the effects of a pharmacological intervention, it is possible to block the SNS, and investigate if the effects of a drug persist. Just as there are several different ways to measure activation of the SNS in different ways, so there are different methods to block SNS function (Ravussin and Tataranni, 1996).

The first is surgically disrupting sympathetic neurons. This allows the role of specific sympathetic innervations to be investigated, especially as disruption of the sympathetic nerve on one side of the body allows for an experimental control within the same animal. For example, Morales *et al* denervated one interscapular BAT pad and investigated the subsequent effect of cold exposure of BAT mass, RNA and glucagon receptor expression; they then compared it to the contralateral BAT pad which had intact sympathetic innervation (Morales et al., 2000). This technique enables the assessment of specific sympathetic nerves on metabolic processes within specific tissues.

Alternatively, it is possible to chemically disrupt the whole sympathetic nervous system. Complete, persistent disruption of the sympathetic nervous system (i.e. a chemical sympathectomy) can be performed using toxins such as 6-hydroxydopamine (6-OHDA) or guanethidine. These destroy sympathetic nerve terminals, preventing further sympathetic transmission. These can be administered systemically, and disrupt the global sympathetic nervous system, or can be injected into specific tissues for more directed investigations. Because the nerve terminals are destroyed by the drug, it can take days to weeks for the sympathetic nervous system to recover, and therefore chemical sympathectomy is useful when researchers want to undertake chronic or recurrent investigations of the SNS.

Finally, it is possible to pharmacologically target specific areas of the SNS. Norepinephrine released from sympathetic nerve terminals acts on adrenoreceptors. There are several different types of adrenoreceptor, including α_1 , α_2 , β_1 , β_2 and β_3 receptors. These different receptors have specific tissue distributions and actions. Specific adrenoreceptor blocking agents are available to target these receptors, and this allows for targeted investigations of the sympathetic nervous system. For example, there are cardio-specific β blockers such as atenolol which are used to investigate the heart, whereas non-specific β blockers such as propranolol can be used to investigate more

generalised phenomena such as memory. Most adrenoreceptor blockers act very quickly, but their effects are transient. The speed of onset can be very useful in acute studies, and transience allows animals to recover; however, very high doses are required to produce receptor blockade, and careful dosing strategies are required if prolonged receptor blockade is required.

To date, only one study has investigated the effect of oxyntomodulin on the SNS. Lockie *et al.* monitored activity in sympathetic neurons that innervate BAT, and found acute ICV OXM increased their activity (Lockie et al., 2012). In chapter 4 (Figure 4.9), I demonstrated an increase in plasma norepinephrine levels after administration of OX2, commensurate with an increase in SNS activity. As activation of the sympathetic nervous system is known to elevate energy expenditure (Ravussin and Tataranni, 1996), and because oxyntomodulin and OX2 do increase sympathetic nervous system activity, it is prudent to determine if the sympathetic nervous system mediates the energy expenditure effects of peripherally administered OXM. To do this, studies in this chapter will use two different pharmacological agents to block the SNS, and determine if these reduce the energy expenditure caused by OX2. The two agents used are beta blockade with propranolol, and chemical sympathectomy with 6-OHDA.

Propranolol

Propranolol is a reversible antagonist of the β_1 , β_2 , and to an extent, β_3 receptors. β_1 receptors are found on the heart, white adipose tissue and salivary glands, while β_2 receptors are found on smooth muscle, such as the GI tract, skeletal muscle arteries and bronchial tree, as well as the heart and eye. β_3 receptors are restricted in their distribution, being found on the urinary bladder and brown adipose tissue. The broad distribution of β receptors means propranolol affects many physiological processes, including heart rate, vascular tone, psychological status and bone formation (Bonnet et al., 2008, Granville-Grossman, 1974, Cramb et al., 1984). Clinically, it is used to treat a wide range of conditions, as diverse as arrhythmias, heart failure, tremors, anxiety, migraine and angina.

The non-specific β -blockade afforded by propranolol makes it a commonly used tool to investigate the sympathetic nervous system. However, it has a relatively short half-life of approximately 1 hour when administered by SC injection, necessitating multiple daily injections in some animal studies (Smits and Struykerboudier, 1979).

6-Hydroxydopamine (6-OHDA)

6-OHDA is an analogue of norepinephrine (NE) which undergoes selective uptake into sympathetic nerve termini via dopamine and norepinephrine reuptake transporters. When a critical level is reached within the nerve terminal, it is oxidised to free radicals that then destroy elements of the respiratory transport chain (Sachs and Jonsson, 1975). This prevents the nerve terminals from being able to conduct further action potentials (Kostrzewa and Jacobowitz, 1974). 6-OHDA does not cross the blood-brain barrier, so after intraperitoneal administration, only peripheral sympathetic nerves are affected. It takes several weeks for new sympathetic nerve terminals to form, arising from intact pre-terminal processes; this allows sufficient time to study the effects of sympathectomy on other physiological processes (Kostrzewa and Jacobowitz, 1974). 6-OHDA is administered in a vehicle containing ascorbic acid, so this vehicle is used for control injections.

By using two distinct methods to disrupt the sympathetic nervous system, and by recording both indirect measures of energy expenditure (food intake and body weight), and direct assessment of energy expenditure (through CLAMS metabolic cages), the studies in this chapter aim to determine if the sympathetic nervous system is required for the energy expenditure effects of OX2.

5.2 METHODS

5.2.1 PEPTIDES AND CHEMICALS

As previously described, OX2 was designed by Professor S Bloom (Imperial College, London) and manufactured by Insight Biotechnology Limited (Wembley, UK). Propranolol hydrochloride, 6-hydroxydopamine hydrobromide, EDTA and sodium metabisulphite were purchased from Sigma Aldrich (Dorset, UK).

5.2.2 *IN VIVO* STUDIES

As previously mentioned, all animal procedures undertaken were approved by the British Home Office Animal (Scientific Procedures) Act 1986 (Project Licence 70/7236 and 70/7596). Animal husbandry conditions were as previously described. When in CLAMS metabolic cages, animals had access to powdered RM1 diet, while in their home cages they had access to pelleted RM1 diet (Special Diet Services, Witham, UK).

5.2.2.1 FEEDING STUDY COMBINING PROPRANOLOL AND OX2 IN RATS

40 male Wistar rats were randomised to four groups of equal average body weight (mean body weight 571g, range 482-681g) (n=8-12). For 4 days, two groups received twice daily SC injections of saline at 0800 and 1500; the other two groups received injections of propranolol (26mg/kg) at the same time.

This dose of propranolol was based on other studies using propranolol to block the sympathetic nervous system, as well as preliminary studies undertaken for this thesis suggesting this dose was the maximum tolerated by rats. Soszynski *et al.* found a total of 7.5mg/kg of propranolol reduced the stress-induced elevation in body temperature due to SNS activation for 60 minutes (Soszynski *et al.*, 1996). Dicker *et al.* used 20mg/kg to inhibit the sympathetically-mediated thermogenic response to a stressful injection in rats (Dicker *et al.*, 1998), while Rothwell *et al.* found 20mg/kg reduced basal metabolic rate in fasted rats, and blunted 3-hour re-feeding induced increments in energy expenditure in rats (Rothwell *et al.*, 1982). Therefore, by using 26mg/kg twice daily of propranolol, all these processes of the sympathetic nervous system should be suppressed; moreover, because of the short half-life of propranolol, twice-daily dosing would hopefully cause a degree of beta-blockade throughout the day.

At 1500, one of the saline and one of the propranolol groups received a SC injection of OX2 (40nmol/kg), while the remaining groups received injections of vehicle. The groups are detailed below

GROUP	Beta Blockade (SC, 0800 and 1500)	Peptide (SC, 1500)
1	Saline	Vehicle
2	Saline	OX2 40nmol/kg
3	Propranolol 26mg/kg	Vehicle
4	Propranolol 26mg/kg	OX2 40nmol/kg

Table 5-1). All animals had free access to standard laboratory chow throughout. Body weight and food intake were measured daily at 1500.

GROUP	Beta Blockade (SC, 0800 and 1500)	Peptide (SC, 1500)
1	Saline	Vehicle
2	Saline	OX2 40nmol/kg
3	Propranolol 26mg/kg	Vehicle
4	Propranolol 26mg/kg	OX2 40nmol/kg

Table 5-1 Experimental design in study examining effect of beta-blockade on energy expenditure caused by OX2.

5.2.2.2 FEEDING STUDY COMBINING 6-OHDA INDUCED-SYPATHECTOMY AND OX2 IN RATS

The following study investigated the effect of prior global chemical sympathectomy using 6-hydroxydopamine (6-OHDA) on the food intake and body weight change effects of OX2.

32 rats were randomised to four groups of equal average body weight (mean body weight 306g, range 253-363g) (n=8). Two groups subsequently underwent chemical sympathectomy over 5 days using 6-OHDA, while the others received injections of ascorbic acid (ascorbic acid stabilises 6-OHDA, reducing its rate of decomposition, and is therefore used as both the diluent for 6-OHDA, and for the control injections). On day 7, all groups started a feeding study with OX2. One group with sympathectomy, and one group without, received daily SC injections at 1500 of OX2 (40nmol/kg) for 3 days; the other groups received SC injections of vehicle at the same time. Throughout both the sympathectomy and the feeding study, all animals had free access to standard laboratory chow. Body weight and food intake were measured daily at 1500.

Twenty-four hours after the third injection of OX2 or vehicle, all animals were culled by decapitation, having had free access to food overnight. The spleen was rapidly removed and snap-frozen in liquid nitrogen. It was then stored at -80°C until the norepinephrine levels could be analysed.

The protocol is detailed below:

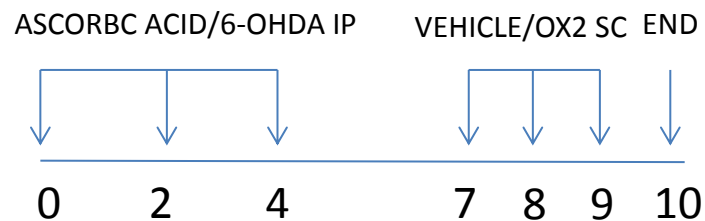


Figure 5.1 Protocol for feeding study combining global sympathectomy with 6-OHDA and OX2.

5.2.2.3 EFFECT OF GLOBAL CHEMICAL SYMPATHECTOMY ON ENERGY EXPENDITURE AND METABOLIC CHANGES CAUSED BY OX2 IN RATS

This study further analysed the effects of global sympathectomy with 6-OHDA on the metabolic effects of OX2 through a study in CLAMS metabolic cages.

Sixteen rats were randomised to two groups of equal average body weight (mean body weight 353g, range 305-399g) (n=8). All animals entered CLAMS metabolic cages at 1500 on day 0 of the experiment. They received daily SC injections of either vehicle or OX2 (40nmol/kg) at 0830 for three days. On day 4, they were returned to their home cages. From day 7-11, all animals underwent chemical sympathectomy using 6-OHDA. On day 14, they again entered CLAMS metabolic cages at 1500, and from day 15, received the same injections of vehicle or OX2 as they had at the start of the experiment, daily for 3 days. On day 18 they were returned to their home cages. Throughout both periods in the CLAMS cages, and during the sympathectomy, all animals had free access to standard laboratory chow.

The protocol is detailed below:

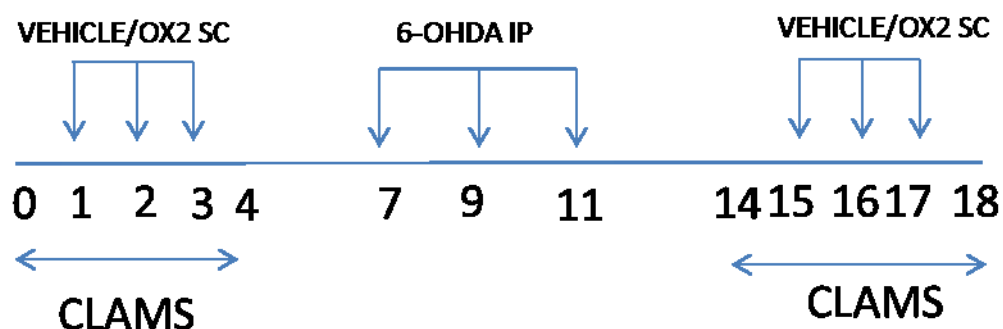


Figure 5.2 Protocol for CLAMS study examining impact of global sympathectomy with 6-OHDA on the metabolic effects of OX2.

5.2.2.3.1 CHEMICAL SYMPATHECTOMY PROTOCOL

Chemical sympathectomy was achieved by administration of 3 doses of 6-hydroxydopamine hydrobromide (6-OHDA) IP at 50mg/kg in 0.1% ascorbic acid on alternate days. During this period all animals had *ad libitum* access to food and water. This protocol was based on doses found in the literature demonstrating significant norepinephrine depletion from a variety of tissues, as well as reducing oxygen consumption in rats (Chaswal et al., 2014, Horvathova et al., 2015, Wang et al., 2013, Finch et al., 1973, Depocas et al., 1984), plus a pilot study showing it to significantly reduce splenic norepinephrine levels. Control animals received 3 x IP injections of 0.1% ascorbic acid over the same period. Ascorbic acid stabilises 6-OHDA, and is therefore used as both a diluent and for control injections.

5.2.2.3.2 CONFIRMATION OF SYMPATHECTOMY

Confirmation of the efficacy of the sympathectomy was undertaken by measuring norepinephrine within the spleen. NE levels were determined using a competitive enzyme immunoassay kit (Norepinephrine Research ELISA BA E-5200, Labor Diagnostika Nord GmbH & Co. KG, Germany), following the manufacturer's instructions. In brief, spleen samples were homogenised using TissueLyser II (Qiagen, Hilden, Germany), in the presence of 1ml homogenisation buffer containing 0.01M HCl, 1mM EDTA and 4mM sodium metabisulfite. The homogenate was diluted 1:100 in homogenisation buffer, and then extracted using a boronate affinity gel. It was then acylated and enzymatically converted to quantitate norepinephrine levels. The optical density of the final solution was measured at 450nm and compared to a norepinephrine standard curve.

Protein levels in each splenic sample were ascertained using the Bicinchoninic Acid Kit (Sigma-Aldrich, Dorset, UK) as per the manufacturer's protocol. Samples of homogenised splenic tissue were diluted 1:75 using homogenisation buffer. 25µl of sample were then added to 200µl of the BCA Working Reagent. Samples were incubated at 37°C for 30 minutes, before being read on a microplate reader at 540nm. Norepinephrine levels within the spleen samples were normalised to the amount of protein in each sample.

5.3 RESULTS

5.3.1 KEY RESULTS FOR CHAPTER 5

- The sympathetic nervous system is essential for the maintenance of basal metabolic rate, as demonstrated by the effects of administering both propranolol and 6-OHDA on food intake and body weight in rats
 - The sympathetic nervous system is involved in the increased energy expenditure caused by OX2, with a lower increase in oxygen consumption after OX2 administration following treatment with 6-OHDA than in control animals.
-

5.3.2 FEEDING STUDY COMBINING PROPRANOLOL AND OX2 IN RATS

When the influence of propranolol and OX2 on food intake was examined in animals with free access to standard laboratory chow, there was a significant effect of propranolol but not OX2 on food intake, and no interaction between OX2 and propranolol (ordinary 2-way ANOVA, effect of OX2 $F(1,36) = 1.4$, $p=0.2$; effect of propranolol, $F(1,36) = 37.9$, $p<0.0001$; interaction, $F(1,36)= 0.3$, $p= 0.6$). Propranolol reduced food intake when compared to saline injections (mean food intake $\pm$ SEM (g), saline/vehicle: 119.4 ± 2.0 g; propranolol/vehicle: 93.7 ± 2.3 g; saline/OX2: 116.5 ± 6.1 g; propranolol/OX2: 85.7 ± 5.7 g) (Figure 5.3A).

All groups started with the same baseline body weight (571g). Over the course of the experiment, OX2 but not propranolol changed body weight, and there was no interaction between OX2 and propranolol (ordinary 2-way ANOVA, effect of OX2, $F(1,36)=38.3$, $p<0.001$; effect of propranolol, $F(1,36) = 0.2$, $p=0.6$; interaction, $F(1,36) = 0.02$, $p=0.9$). Those animals receiving OX2 lost weight, whilst those receiving vehicle injections gained weight from baseline (average body weight change from baseline, mean $\pm$ SEM (g), saline/vehicle: 6 ± 2.7 g; propranolol/vehicle: 3 ± 1.1 g; saline/OX2: -19 ± 4.9 g; propranolol/OX2: -20 ± 4.9 g) (Figure 5.3B). This equates to an increase in body weight of 1% in the saline/vehicle group and 0.5% in the propranolol/vehicle group, and a loss of 3% in the saline/OX2 group and 3.5% in the propranolol/OX2 group.

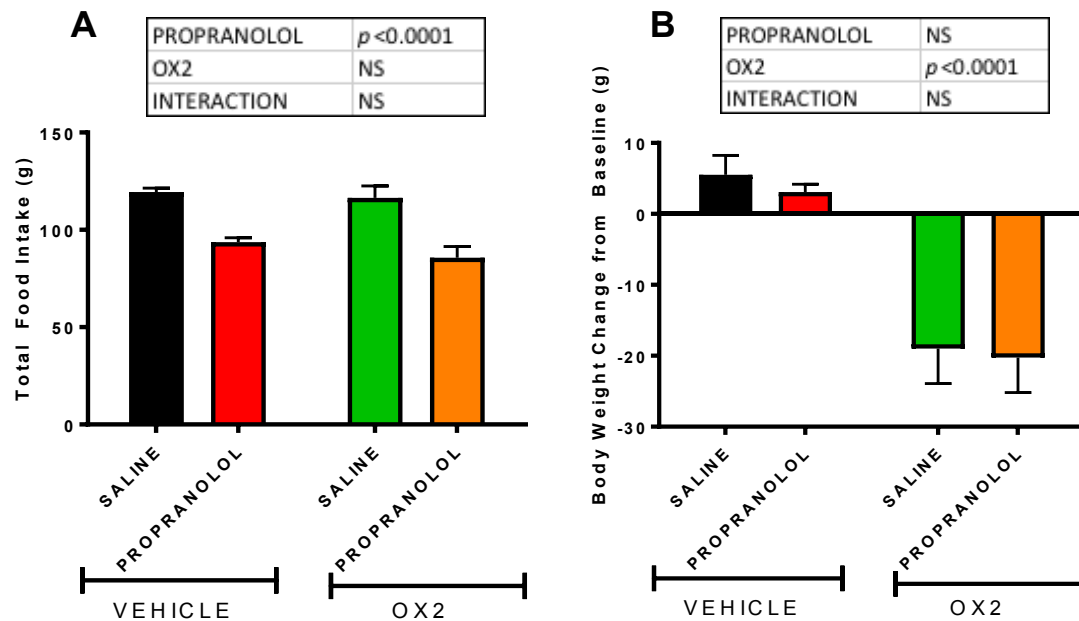


Figure 5.3 Effect of propranolol and OX2 on food intake and body weight change in rats.

Cumulative food intake (A) and body weight change from baseline (B) in male Wistar rats given daily subcutaneous injections of either vehicle or OX2 (40nmol/kg) as well as twice daily subcutaneous injections of saline or propranolol (2.6mg/kg), every day for 4 days. Animals had free access to standard laboratory chow throughout. Average baseline body weight 571g. N= 8-12. Graph shows mean $\pm$ SEM (g). Statistical analysis was undertaken with ordinary two-way ANOVA.

	SALINE/ VEHICLE	PROPRANOLOL/ VEHICLE	SALINE/ OX2	PROPRANOLOL/ OX2	
FOOD INTAKE	REFERENCE	REDUCED	NO EFFECT	REDUCED	Figure 5.3
BODY WEIGHT	REFERENCE	NO EFFECT	REDUCED	REDUCED	Figure 5.3

Table 5-2 Summary of results from feeding study combining propranolol and OX2 in rats.

5.3.3 FEEDING STUDY COMBINING 6-OHDA INDUCED-SYMPATHECTOMY AND OX2 IN RATS

Administration of 6-OHDA significantly reduced food intake by 20% over week 1 of the experiment when compared to ascorbic acid in animals with free access to standard laboratory chow (average food intake over the sympathectomy week, mean $\pm$ SEM (g), ascorbic acid: 203.7 ± 5.0 g; 6-OHDA: 162.0 ± 4.0 g) ($p > 0.0001$). When a two-way ANOVA was undertaken to investigate the effect of the day of the study and 6-OHDA on food intake, there was a significant effect of day of the study, a significant effect of 6-OHDA, and an interaction between day and 6-OHDA (Effect of day, $F(6,210)=10.2$, $p < 0.0001$; effect of 6-OHDA, $F(1, 210) = 101.5$, $p < 0.0001$; interaction, $F(6, 210)= 3.4$, $p = 0.003$). *Post-hoc*, Sidak's multiple comparison tests show that there was a reduction in food intake in the 6-OHDA animals on the days when 6-OHDA was administered (average food intake on

day of first 6-OHDA injection, mean $\pm$ SEM (g), ascorbic acid: 28.5 ± 0.9 g; 6-OHDA: 17.9 ± 2.4 g; $p < 0.0001$; average food intake on the day of the 2nd 6-OHDA injection, mean $\pm$ SEM (g), ascorbic acid: 29.1 ± 0.9 g; 6-OHDA: 19.9 ± 1.6 g; $p < 0.0001$; average food intake on day of the 3rd 6-OHDA injection, mean $\pm$ SEM, (g), ascorbic acid: 27.4 ± 0.9 g; vehicle: 21.9 ± 0.9 g; $p < 0.01$) (Figure 5.4 and Table 5-3).

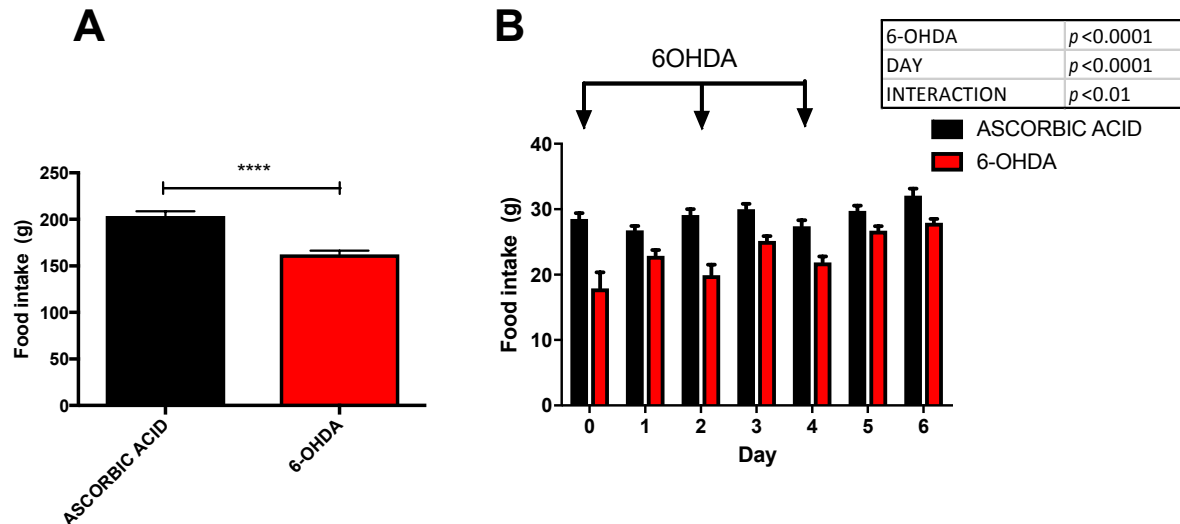


Figure 5.4 Effect of 6-OHDA sympathectomy on food intake in rats.

Cumulative food intake over 7 days (A) and daily food intake (B) in male Wistar rats undergoing chemical sympathectomy with 6-OHDA (50mg/kg IP given on day 0, 2 and 4). Animals had free access to standard laboratory chow throughout. N=16/group. Data shown as the mean $\pm$ SEM. Statistical analysis of cumulative food intake was undertaken with an unpaired t-test, and of daily food intake with an ordinary 2-way ANOVA. **** $p < 0.0001$ vs vehicle.

DAY	Food intake, mean $\pm$ SEM (g)		STATISTICAL ANALYSIS (2-way ANOVA with post-hoc Sidak's multiple comparison test)
	Ascorbic Acid Injections	6-OHDA Injections	
0	28.5 ± 0.9	17.9 ± 2.4	$p < 0.0001$
1	26.8 ± 0.7	22.9 ± 0.9	NS
2	29.1 ± 0.9	19.9 ± 1.6	$p < 0.0001$
3	30.0 ± 0.8	25.2 ± 0.7	$p < 0.05$
4	27.4 ± 0.9	21.9 ± 0.9	$p < 0.01$
5	29.8 ± 0.8	26.7 ± 0.7	NS
6	32.1 ± 1.1	28.0 ± 0.5	NS

Table 5-3 Effect of sympathectomy with 6-OHDA on daily food intake in rats.

All groups started the sympathectomy week with the same average body weight (306g). 6-OHDA administration significantly affected body weight over the sympathectomy week, with those receiving 6-OHDA gaining significantly less weight than those having ascorbic acid injections (average

body weight change from baseline, mean $\pm$ SEM (g) vehicle: 34 ± 2.8 g; 6-OHDA: 9 ± 2.8 g; $p < 0.0001$). At the end of the week, those animals receiving 6-OHDA weighed 9% less than those receiving ascorbic acid (absolute body weight, mean $\pm$ SEM (g), vehicle: 340 ± 6.9 g; 6-OHDA: 311 ± 5.3 g; $p < 0.01$).

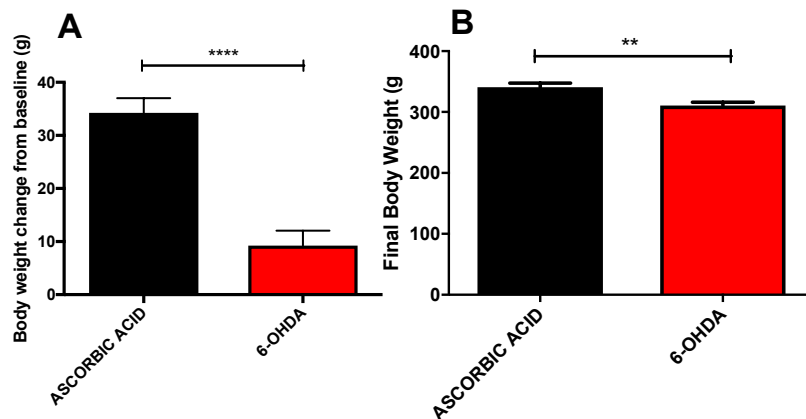


Figure 5.5 Effect of 6-OHDA sympathectomy body weight of rats.

Total body weight change from baseline (A) and final absolute body weight (B) in male Wistar rats 7 days after chemical sympathectomy with 6-OHDA (50mg/kg IP given on day 0, 2 and 4). Animals had free access to standard laboratory chow throughout. N=16/group. Mean initial body weight 306g. Data expressed as mean $\pm$ SEM. Statistical analysis was undertaken using an unpaired t-test. ** $p < 0.01$, **** $p < 0.0001$ vs vehicle.

During the feeding week, all animals had free access to standard laboratory chow. There was an effect of both previous sympathectomy with 6-OHDA and OX2 on food intake, though no interaction of these effects (ordinary 2 way ANOVA, effect of 6-OHDA, $F(1,28) = 7.9$, $p < 0.01$; effect of OX2, $F(1,28)=20.6$, $p < 0.0001$; interaction $F(1,28)=0.01$, $p=0.9$). Both OX2 and previous sympathectomy reduced food intake (average food intake, mean $\pm$ SEM (g), ascorbic acid/vehicle: 112.8 ± 2.4 g; ascorbic acid/OX2: 99.8 ± 4.2 g; 6-OHDA/vehicle: 104.6 ± 1.5 g; 6-OHDA/OX2: 92.2 ± 2.4 g) (Figure 5.6A).

However, only OX2 and not previous 6-OHDA administration had a significant effect on body weight change during the feeding week, and there was no interaction between 6-OHDA and OX2 (ordinary 2-way ANOVA, effect of 6-OHDA, $F(1, 28) = 0.6$, $p=0.4$; effect of OX2, $F(1,28)=82.0$, $p < 0.0001$; interaction, $F(1,28)=0.007$, $p=0.9$). Those animals receiving OX2 lost weight during the feeding week, and those receiving vehicle injections gained weight (average body weight change during feeding study, mean $\pm$ SEM (g), ascorbic acid/vehicle: 19 ± 1.5 g; ascorbic acid/OX2: -15 ± 6.0 g; 6-OHDA/vehicle: 22 ± 1.1 g; 6-OHDA/OX2: -12 ± 3.8 g) (Figure 5.6B). Though sympathectomy did not significantly affect body weight change during the feeding week, there was a tendency for the those

with previous sympathectomy to gain more weight/lose less weight than their ascorbic acid pair: the ascorbic acid/vehicle group gained 5.5% body weight during the feeding week while the 6-OHDA/vehicle group gained 7.0% body weight, and the ascorbic acid/OX2 group lost 4.4% body weight during the feeding week, while the 6-OHDA/OX2 group lost only 3.9% body weight.

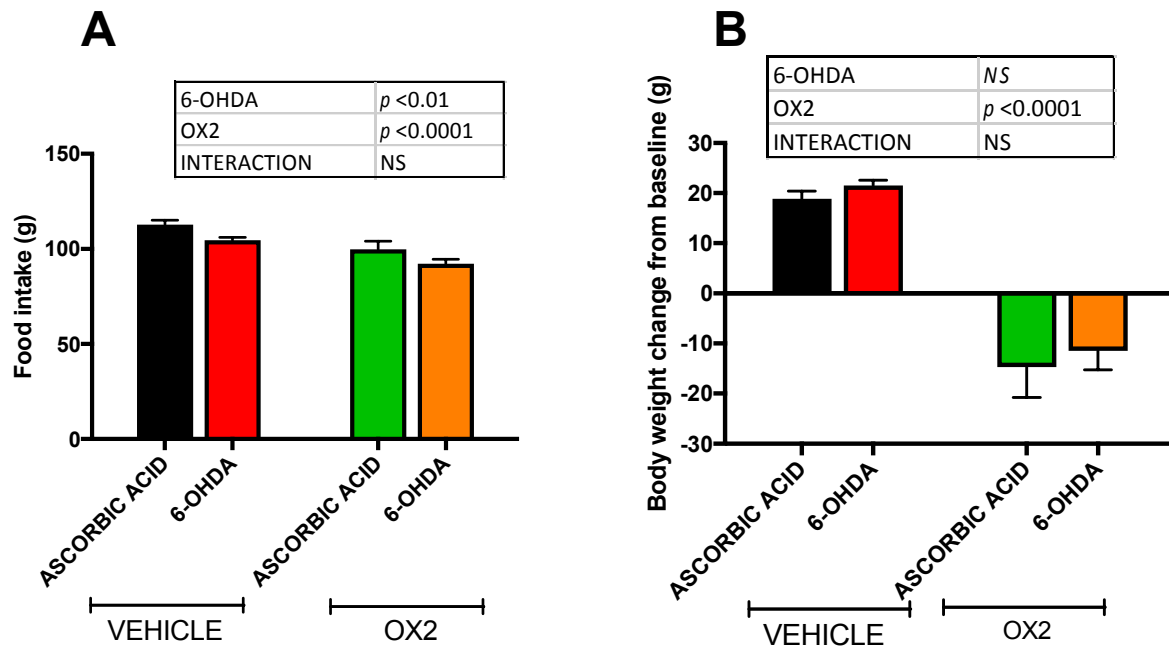


Figure 5.6 Effect of combining 6-OHDA sympathectomy and OX2 on food intake and body weight in rats.

Total food intake during feeding study (A) and bodyweight change from baseline during feeding study (B) in male Wistar rats receiving daily injections of vehicle or OX2 (40nmol/kg) after chemical sympathectomy with 6-OHDA or control injections. All animals had free access to standard laboratory chow throughout. Food and body weight were measured daily at 1500. N=8/group. Data shown as the mean $\pm$ SEM. Statistical analysis was carried out using ordinary two-way ANOVA.

5.3.4 CONFIRMATION OF SYMPATHECTOMY USING 6-OHDA

At the end of this experiment, the splenic norepinephrine levels were measured as a marker of sympathectomy efficacy. Administration of 6-OHDA (50mg/kg IP in 3 doses) had a significant effect on splenic norepinephrine levels, though OX2 did not, nor was there an interaction between 6-OHDA and OX2 (ordinary 2-way ANOVA, effect of 6-OHDA, $F(1,27) = 25.4$, $p < 0.0001$; effect of OX2, $F(1,27) = 2.3$, $p = 0.1$; interaction, $F(1,27) = 0.6$, $p = 0.5$). 6-OHDA significantly reduced levels of norepinephrine in the spleen by approximately 80% (average norepinephrine levels per mg of splenic protein, mean $\pm$ SEM ng/mg: 13 $\pm$ 3.5ng/mg; ascorbic acid/OX2: 19 $\pm$ 3.4g; 6-OHDA/vehicle: 2 $\pm$ 1.1g; 6-OHDA/OX2: 4 $\pm$ 0.8g).

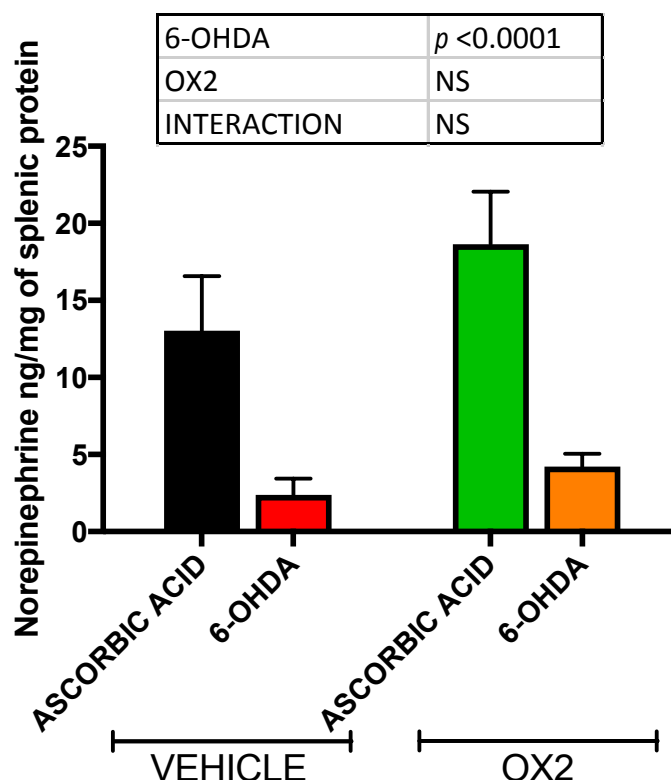


Figure 5.7 Effect of combining 6-OHDA sympathectomy and OX2 on splenic norepinephrine levels.

Splenic norepinephrine levels in male Wistar rats after administration of 6-OHDA and OX2. On day 0, 2 and 4, rats in the 6-OHDA groups received IP injections of 6-OHDA (50mg/kg), or the vehicle ascorbic acid. On days 7-9, they had daily SC injections of either zinc-chloride containing vehicle, or OX2 (40nmol/kg). On day 10, all animals were culled by decapitation and the spleen rapidly removed and frozen prior to norepinephrine analysis. The animals had free access to food throughout, including from final injection until they were culled. N=8. Graph shows mean $\pm$ SEM. Statistical analysis was undertaken using ordinary 2-way ANOVA.

	ASCORBIC ACID/ VEHICLE	6-OHDA/ VEHICLE	ASCORBIC ACID/ OX2	6-OHDA/ OX2	
FOOD INTAKE	REFERENCE	REDUCED	NO EFFECT	REDUCED	Figure 5.6
BODY WEIGHT	REFERENCE	NO EFFECT	REDUCED	REDUCED	Figure 5.6
SPLenic NOREPINEPHRINE	REFERENCE	REDUCED	NO EFFECT	REDUCED	Figure 5.7

Table 5-4 Summary of results from feeding study combining 6-OHDA and OX2 in rats.

5.3.5 EFFECT OF GLOBAL CHEMICAL SYMPATHECTOMY ON ENERGY EXPENDITURE AND METABOLIC CHANGES CAUSED BY OX2 IN RATS

When the effect of OX2 before and after sympathectomy was compared in metabolic cages, OX2 had a significant effect on food intake, but 6-OHDA did not, nor was there any interaction between OX2 and 6-OHDA, in animals given free access to standard laboratory chow (ordinary 2-way ANOVA, effect of 6-OHDA, $F(1,26)=1.8$, $p=0.2$; effect of OX2, $F(1,26) = 34.7$, $p<0.0001$; interaction $F(1,26)=$

0.03, $p=0.9$). OX2 significantly reduced food intake when compared to vehicle injections, and did to a similar effect both before and after 6-OHDA administration (average food intake, mean $\pm$ SEM (g), pre-sympathectomy/vehicle: 101.0 ± 3.1 g; pre-sympathectomy/OX2: 67.1 ± 6.9 g; post-sympathectomy/vehicle: 94.1 ± 1.4 g; post-sympathectomy/OX2: 58.1 ± 8.1 g) (Figure 5.8A). This equated to a 33% reduction in food intake by OX2 compared to vehicle pre-sympathectomy, and a 39% reduction by OX2 compared to vehicle post-sympathectomy.

OX2 but not 6-OHDA administration also had a significant effect on body weight change from the baseline, and again there was no interaction between OX2 and 6-OHDA (ordinary 2-way ANOVA, effect of 6-OHDA, $F(1,28)=1.6$, $p=0.2$; effect of OX2, $F(1,28)=78.7$, $p<0.0001$; interaction, $F(1,28)=0.6$, $p=0.5$). OX2 caused a significant reduction in body weight from the baseline during week 1 and again during week 3 (average body weight change from baseline at the start of the week, mean $\pm$ SEM (g), pre-sympathectomy/vehicle: 9 ± 1.3 g; pre-sympathectomy/OX2: -33 ± 6.4 g; post-sympathectomy/vehicle: 6 ± 1.2 g; post-sympathectomy/OX2: -43 ± 7.8 g) (Figure 5.8B). At the start of the first week, both groups had an average body weight of 353g; after 3 injections, the vehicle group weighed, on average, 363g, while the OX2 group lost weight to an average of 315g; this equated to an increase in body weight of 3% in the vehicle group, and a reduction in body weight of 11% in the OX2 group. After the sympathectomy, the vehicle group weighed, on average, 375g and the OX2 group weighed 367g. After a further 3 injections of vehicle or OX2, the vehicle group weighed, on average, 388g, which was an increase in body weight by a further 3%; in contrast the OX2 group weighed 327g, which was a loss of a further 11% body weight.

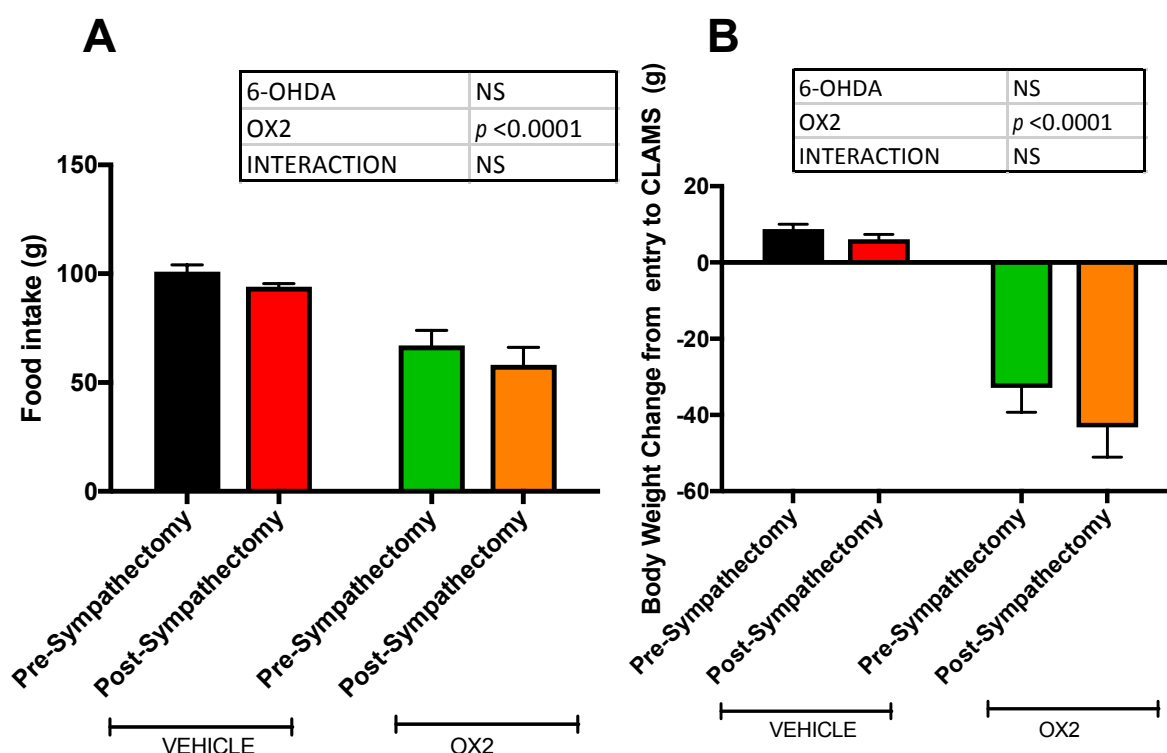


Figure 5.8 Effect of sympathectomy and OX2 on food intake and body weight in rats in CLAMS metabolic cages. Cumulative food intake (A) and body weight change from baseline (B) in rats receiving daily SC injections of vehicle or OX2 (40nmol/kg) for 3 days while in CLAMS metabolic cages, both before and after chemical sympathectomy with 6-OHDA. Animals had free access to standard laboratory chow throughout the study. N=8. Graph shows mean $\pm$ SEM (g). Statistical analysis was undertaken using ordinary 2-way ANOVA.

When total oxygen consumption during each period in the CLAMS cages was compared, there were significant effects of both OX2 and 6-OHDA on oxygen consumption, as well as a significant interaction between OX2 and 6-OHDA (ordinary 2-way ANOVA, effect of 6-OHDA, $F(1,26) = 47.8$, $p < 0.0001$; effect of OX2, $F(1,26) = 54.6$, $p < 0.0001$, interaction, $F(1,26) = 11.6$, $p < 0.01$). Oxygen consumption was reduced in both groups after sympathectomy. In contrast, OX2 enhanced oxygen consumption when compared to vehicle injections; however, OX2 caused a much smaller elevation in oxygen consumption after the sympathectomy than before (average oxygen consumption during the period in CLAMS metabolic cages, mean $\pm$ SEM (ml/kg), pre-sympathectomy/vehicle: 114382 ± 4587 ml/kg; pre-sympathectomy/OX2: 159843 ± 4777 ml/kg; post-sympathectomy/vehicle: 99626 ± 4065 ml/kg; post-sympathectomy/OX2: 116405 ± 3004 ml/kg) (Figure 5.9 and Table 5-5). Pre-sympathectomy, over the total period in CLAMS, the OX2 group consumed 39% extra oxygen compared to the vehicle group; after sympathectomy, the relative increase was only 17% in the OX2 group.

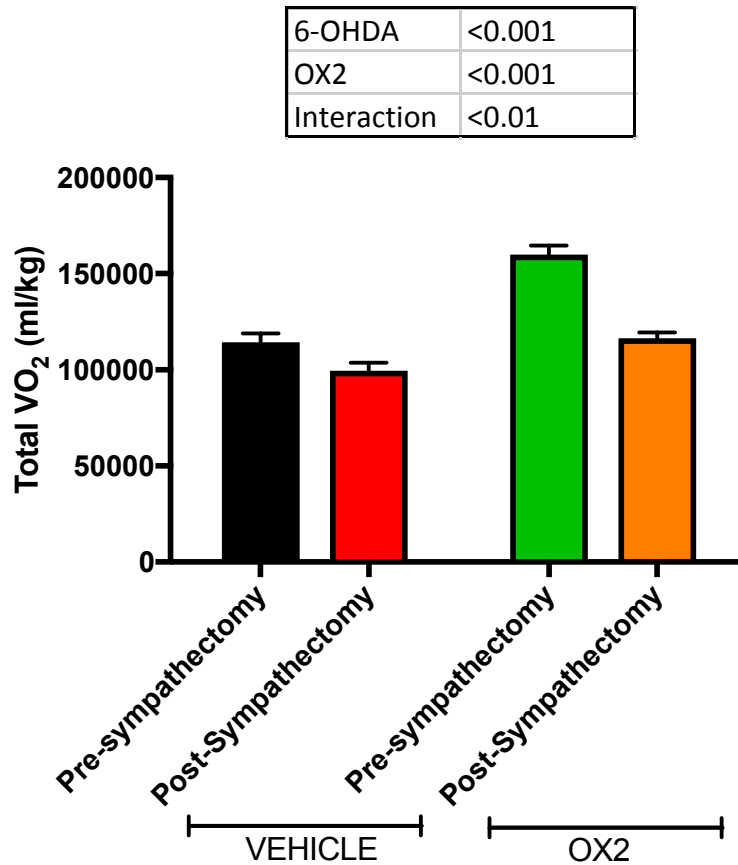


Figure 5.9 Effect of sympathectomy and OX2 on oxygen consumption in rats in CLAMS metabolic cages.

Total oxygen consumption in rats receiving daily SC injections of vehicle or OX2 (40nmol/kg) for 3 days while in CLAMS metabolic cages, both before and after chemical sympathectomy with 6-OHDA. Animals had free access to standard laboratory chow throughout. N=8. Graph shows mean $\pm$ SEM (g). Statistical analysis was undertaken using ordinary 2-way ANOVA.

VO ₂ mean $\pm$ SEM ml/kg			
PRE-SYMPATHECTOMY		POST-SYMPATHECTOMY	
VEHICLE	OX2	VEHICLE	OX2
114382 $\pm$ 4587 ^a	159843 $\pm$ 4777 ^{acd}	99626 $\pm$ 4065 ^{bd}	116405 $\pm$ 300 ^{bc}

Table 5-5 Effect of sympathectomy and OX2 on oxygen consumption in rats in CLAMS metabolic cages.

Statistical analysis was undertaken using ordinary 2-way ANOVA, with post-hoc Sidak's multiple comparison test. Significant differences exist between groups with the same superscript letter.

Neither treatment with 6-OHDA nor OX2 had an effect on physical activity in rats in metabolic cages, nor was there any interaction (ordinary 2-way ANOVA, effect of 6-OHDA, $F(1,28)=1.4$, $p=0.3$; effect of OX2, $F(1,28)=0.2$, $p=0.7$; interaction, $F(1,28)=0.6$, $p=0.6$) (Figure 5.10 and Table 5-6).

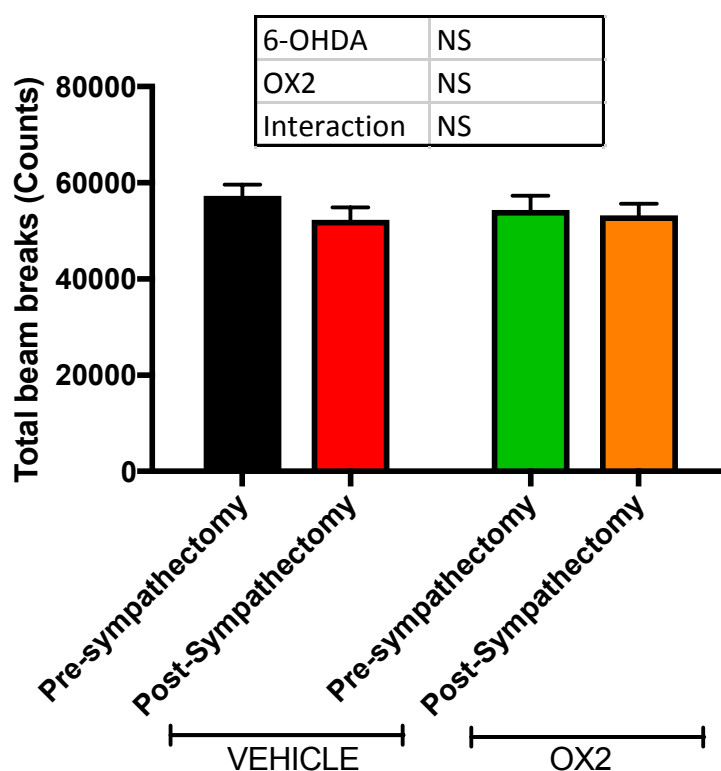


Figure 5.10 Effect of sympathectomy and OX2 on physical activity in rats in CLAMS metabolic cages.

Total physical activity, as measured by beam breaks in the X-axis, in rats receiving daily SC injections of vehicle or OX2 (40nmol/kg) for 3 days while in CLAMS metabolic cages, both before and after chemical sympathectomy with 6-OHDA. Animals had free access to standard laboratory chow throughout. N=8. Graph shows mean $\pm$ SEM (g). Statistical analysis was undertaken using ordinary 2-way ANOVA.

Physical activity, mean $\pm$ SEM (counts)			
PRE-SYMPATHECTOMY		POST-SYMPATHECTOMY	
VEHICLE	OX2	VEHICLE	OX2
57311 $\pm$ 2321	54337 $\pm$ 2994	52328 $\pm$ 2576	53267 $\pm$ 2393

Table 5-6. Effect of sympathectomy and OX2 on physical activity in rats in CLAMS metabolic cages

OX2 had an effect on average RER during each period in CLAMS, whereas treatment with 6-OHDA did not, nor was there an interaction between OX2 and 6-OHDA (ordinary 2-way ANOVA, effect of OX2 $F(1,26)=24.4$, $p<0.0001$; effect of 6-OHDA, $F(1,26)=0.4$, $p=0.5$; interaction, $F(1,26) = 0.04$, $p=0.8$). OX2 reduced RER when compared to vehicle both before and after sympathectomy (average RER, mean $\pm$ SEM, pre-sympathectomy/vehicle: 0.964 ± 0.008 , pre-sympathectomy/OX2: 0.895 ± 0.016 post-sympathectomy/vehicle: 0.957 ± 0.009 ; post-sympathectomy/OX2: 0.883 ± 0.023) (Figure 5.11 and Table 5-7).

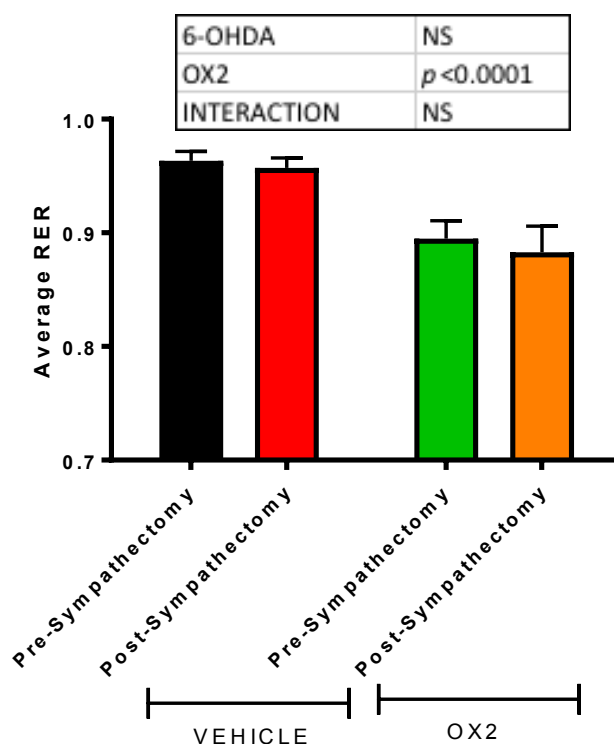


Figure 5.11 Effect of sympathectomy and OX2 on RER in rats in CLAMS metabolic cages.

Average respiratory exchange ratio in rats receiving daily SC injections of vehicle or OX2 (40nmol/kg) for 3 days while in CLAMS metabolic cages, both before and after chemical sympathectomy with 6-OHDA. Animals had free access to standard laboratory chow throughout. N=8. Graph shows mean $\pm$ SEM (g). Statistical analysis was undertaken using ordinary 2-way ANOVA.

RER, mean $\pm$ SEM			
PRE-SYMPATHECTOMY		POST-SYMPATHECTOMY	
VEHICLE	OX2	VEHICLE	OX2
0.964 ± 0.008^{ad}	0.895 ± 0.01^{ac}	0.957 ± 0.009^{bc}	0.883 ± 0.023^{bd}

Table 5-7 Effect of sympathectomy and OX2 on RER in rats in CLAMS metabolic cages.

Statistical analysis undertaken using ordinary 2-way ANOVA, with post-hoc Sidak's multiple comparison test. Significant differences exist between groups with the same superscript letter.

	ASCORBIC ACID/ VEHICLE	6-OHDA/ VEHICLE	ASCORBIC ACID/ OX2	6-OHDA/ OX2	
FOOD INTAKE	REFERENCE	NO EFFECT	REDUCED	REDUCED	Figure 5.8
BODY WEIGHT	REFERENCE	NO EFFECT	REDUCED	REDUCED	Figure 5.8
OXYGEN CONSUMPTION	REFERENCE	NO EFFECT	INCREASED	INCREASED BUT LESS THAN ASCORBIC ACID /OX2	Figure 5.9
PHYSICAL ACTIVITY	REFERENCE	NO EFFECT	NO EFFECT	NO EFFECT	Figure 5.10
RESPIRATORY EXCHANGE RATIO	REFERENCE	NO EFFECT	REDUCED	REDUCED	Figure 5.11

Table 5-8 Summary of results of sympathectomy and OX2 in rats in CLAMS metabolic cages.

5.4 DISCUSSION

The studies in this chapter confirm that the sympathetic nervous system has an important role in determining basal metabolic rate, and that disruption of the SNS reduces baseline energy expenditure. They also provide the first direct evidence that the sympathetic nervous system mediates some of energy expenditure effect of OXM, but that it is not the exclusive mechanism for this process.

Propranolol has been previously shown to reduce metabolic rate and energy expenditure (Belza et al., 2009, Buemann et al., 1992, Christin et al., 1989, Rothwell et al., 1982). This was confirmed in this chapter. Propranolol, at the dose used in this study, caused rats to eat significantly less food than control animals (Figure 5.3). However, those rats given propranolol did not lose significantly more weight than controls. This suggests that there must have been compensation for the reduced food intake, with a contemporaneous reduction in energy expenditure.

Chemical sympathectomy also reduces basal metabolic rate (Villanueva et al., 2002, Lin, 1979, Depocas et al., 1984). In the feeding study investigating the effect of 6-OHDA and OX2, treatment with 6-OHDA significantly reduced food intake, but did not affect body weight change; indeed, there was a trend for the 6-OHDA group to gain weight/lose less weight when compared to the equivalent ascorbic acid pair (Figure 5.6). As with the propranolol study, this would suggest a compensatory reduction in energy expenditure accompanied the anorectic effect. The metabolic cage study confirmed the reduction in metabolic rate by sympathectomy with 6-OHDA as all rats showed a reduction in oxygen consumption after treatment with 6-OHDA (Figure 5.9 and Table 5-5). Moreover, as 6-OHDA had no effect on physical activity, this suggests the reduction in energy expenditure was via changes in basal metabolic rate (Figure 5.10 and Table 5-6).

The metabolic cage study directly confirmed that OX2 increases energy expenditure, as there was a significant increase in oxygen consumption when compared to vehicle (Figure 5.9 and Table 5-5). The other studies offer indirect confirmation that OX2 increases energy expenditure by comparing food intake and body weight change. In the propranolol study, OX2 caused a significant reduction in body weight without affecting food intake when compared to the vehicle group – as in a pair-feeding paradigm, this would suggest an increase in energy expenditure causes the loss of body weight (Figure 5.3). In the feeding study with 6-OHDA and OX2, OX2 did cause a small reduction in food intake compared to the vehicle control, but a much greater reduction in body weight, which is highly suggestive of an increase in energy expenditure (Figure 5.6).

The feeding study combining 6-OHDA and OX2 also supports the findings from Chapter 4 (paragraph 4.4.5) that OX2 stimulates the sympathetic nervous system. 6-OHDA significantly reduced splenic norepinephrine levels in the feeding study, confirming that 3 doses of 6-OHDA significantly reduced SNS activity (Figure 5.7). However, it is notable that there was a trend for increased norepinephrine in the spleen of animals given OX2 when compared to the vehicle pair. Prior to sympathectomy, norepinephrine levels per gram of splenic protein were 1.4x higher after OX2 compared to vehicle, and after sympathectomy, they were 1.8x higher, even though the levels were reduced by more than 80% from pre-sympathectomy (Figure 5.7). This relative increase in norepinephrine levels suggests OX2 does increase splenic SNS activity, even if it is only the residual activity following sympathectomy.

The metabolic cage study's results were also congruent with the idea that the sympathetic nervous system is the mechanism by which, at least in part, OX2 increases energy expenditure. There is an interaction between 6-OHDA and OX2 treatment, with the increase in energy expenditure caused by OX2 significantly blunted by treatment with 6-OHDA (Figure 5.9 and Table 5-5). OX2 causes a 40% increase in oxygen consumption pre-sympathectomy, and only a 17% increase after sympathectomy. This suggests that an intact sympathetic nervous system is required for OX2 to fully increase energy expenditure.

Interestingly, there was no interaction between beta-blockade and OX2 on body weight and food intake in rats in the associated feeding study (Figure 5.3), or between 6-OHDA and OX2 in the feeding study investigating combining global chemical sympathectomy with 6-OHDA (Figure 5.6). An interaction might be expected if blocking the sympathetic nervous system did affect the energy expenditure effect of OX2. For example, if propranolol did reduce the EE effect of OX2, then the amount of body weight loss in the propranolol/OX2 group might be expected to be less than that of the saline/OX2 group despite the same food intake, reflecting the fact that the food consumed was less easily burnt up. There was a suggestion of this in the feeding study investigating the effect of 6-OHDA and OX2: though statistically non-significant, the 6-OHDA/OX2 group ate less than the ascorbic acid/OX2 group but their absolute weight loss was slightly less, suggesting they also had lower energy expenditure (Figure 5.6). The lack of statistical significance here may reflect the fact that food intake and body weight are indirect measures of energy expenditure, and can be affected by multiple processes beyond energy expenditure, making it harder to distinguish the effects that are specifically due to energy expenditure. Including groups pair-fed to the ascorbic acid/OX2 and 6-OHDA/OX2 groups may have shown the changes in energy expenditure. Nevertheless, the effect of

6-OHDA on the energy expenditure caused by OX2 was demonstrated in the CLAMS metabolic cages, demonstrating the value in directly measuring energy expenditure.

Incomplete blockade of the SNS may be an explanation for the lack of interaction between propranolol and OX2 on food intake and body weight change, despite suggestions that propranolol reduces energy expenditure while OX2 increases it (Figure 5.3). A single dose of OX2 increases energy expenditure for 12 hours, whereas propranolol has a half-life of only 1 hour (Smits and Struykerboudier, 1979). Despite twice daily administration of propranolol, the blockade of beta-receptors may not persist for the duration of OX2 activity. So although propranolol may have reduced metabolic rate and limited the effect of OX2 on oxygen consumption, propranolol's action would have been transient and resulted in the lack of statistical interaction between propranolol and OX2. Further studies could use subcutaneous mini-pumps to continuously deliver propranolol to provide constant β -blockade while investigating the role of the SNS on the energy expenditure effects of OXM.

Surprisingly, when measured in metabolic cages, treatment with 6-OHDA did not affect RER, although OX2 did (Figure 5.11 and Table 5-7). White adipose tissue is sympathetically innervated, and activation causes an increase in lipolysis (Villanueva et al., 2002), while SNS blockade can reduce lipolysis and fat oxidation (Buemann et al., 1992). It might therefore be expected that 6-OHDA would cause RER to be elevated as lipolysis is reduced. The failure of 6-OHDA to increase RER in this study may reflect an insufficient disruption of sympathetic innervation of white adipose tissue; alternatively, it may reflect obligate lipid metabolism if all carbohydrate stores are used up, or even a switch to protein metabolism. Future studies could include a measurement of norepinephrine levels or turnover within white adipose to determine the efficacy of WAT sympathectomy, as well as more detailed examination of the type of fuel being metabolised.

Though the studies detailed demonstrated that OX2 does increase sympathetic nervous system activity, and that this does mediate some of the enhanced energy expenditure caused by OX2, they do not determine which downstream metabolic processes are stimulated by the SNS to cause the increase in energy expenditure.

It is known the SNS can increase energy expenditure through activation of brown adipose tissue. In rodents, sympathetic activation of brown adipose tissue may be important in overall energy expenditure; bilateral denervation of BAT in mice reduced energy expenditure and enhanced fat deposition (Dulloo and Miller, 1984). However, the studies in Chapter 4 on the effect of OX2 on brown adipose tissue showed that this is not the route by which OX2 increases energy expenditure.

In man, BAT is less important for SNS-induced energy expenditure: the sympathomimetic ephedrine increases energy expenditure without contemporaneous activation of BAT (Cypess et al., 2012). Skeletal muscle may instead be the mediator of SNS-induced energy expenditure. The sympathetic nervous system innervates skeletal muscle, and activation of the SNS increases energy expenditure via non-shivering thermogenesis in skeletal muscle in rats (Gavini et al., 2016, Gavini et al., 2014). One study in man calculated that non-shivering thermogenesis in skeletal muscle could account for more than 50% of the ephedrine-induced increase in oxygen consumption, whereas BAT activation would only be responsible for 14 % (Astrup et al., 1985). It may therefore be that changes in skeletal muscle metabolism are the route by which OXM-induced SNS activation increases energy expenditure. Future studies could therefore look specifically at changes in sympathetic activity in the skeletal muscle of animals given OXM, and whether there are associated changes in skeletal muscle metabolism that could account for the changes in energy expenditure. Knowledge about how the SNS affects energy expenditure through other processes is still incomplete, but as this knowledge grows, it could be applied to the effects of OXM to determine how this increases energy expenditure.

5.5 CONCLUSIONS

The studies in this chapter show that the sympathetic nervous system controls basal metabolic rate, and that OX2 causes SNS activation, and the energy expenditure effects of OX2 are, in part mediated via SNS activation. OXM is therefore also likely to increase energy expenditure via the SNS. However, the downstream metabolic pathways via which OXM-induced SNS activation then increase energy expenditure are still to be elucidated.

Chapter 6

Oxyntomodulin And Metabolic Processes

6.1 INTRODUCTION

Total energy expenditure is a combination of the metabolic expenditure of all tissues in the body. In rats, the metabolic rate of different tissues varies dramatically, but most notably, white adipose has a much lower metabolic rate than most other tissues (Assaad et al., 2014); this feature is also seen in humans where fat-free mass has a much higher metabolic rate than fat mass (Nelson et al., 1992).

Ideally, a treatment of obesity would only cause the loss of the metabolically inactive white adipose, with preservation of metabolically active lean tissue. Low and very-low calorie diets may fail because they cause a significant loss of fat-free mass as well as fat mass (Chaston et al., 2007), leading to a reduction in basal metabolic rate (Leibel et al., 1995), which makes further weight loss, and weight loss maintenance, much more difficult. Research is increasingly demonstrating the importance of body composition over absolute body weight for risks of multiple metabolic and cardiovascular diseases, and even for severe cognitive impairment (Papachristou et al., 2015). Work in this chapter will therefore first determine the effect of long-term administration of the oxyntomodulin analogue OX2 on rat body composition, in order to determine its utility as a safe weight-loss drug.

Many different metabolic processes and pathways contribute to an individual's basal metabolic rate. These include tissue-specific pathways such as hepatic and renal gluconeogenesis; muscle contraction; and urea synthesis in the liver (Rolfe and Brown, 1997). These can be affected by physiological demand, but also by pharmacological intervention. Therefore, studies in this chapter will also look at tissue-specific metabolic processes, to determine how these are affected by chronic administration of OX2. This may give insight into how OX2 elevates energy expenditure, but also suggest potential side effects that might occur in response to chronic administration of OXM analogues.

6.2 AIMS

1. To determine how the oxyntomodulin analogue OX2 affects body composition in Wistar rats
2. To investigate the effects of the oxyntomodulin analogue OX2 on the metabolic processes involved in growth, glucose metabolism, fat metabolism and protein metabolism.

6.3 METHODS

6.3.1 PEPTIDES

As previously described, the oxyntomodulin analogue OX2 was designed by Professor S Bloom (Imperial College, London), and synthesized by Insight Biotechnology Limited (Wembley, UK). OX2 was delivered in a long-acting formulation in all studies in this chapter.

6.3.2 *IN VIVO* STUDIES

All animal procedures undertaken were approved by the British Home Office Animal (Scientific Procedures) Act 1986 (Project Licences 70/7236 and 70/7596).

6.3.2.1 ANIMAL HUSBANDRY

Adult male Wistar rats (Charles River, Margate, UK) were single-housed in individual cages under controlled temperature environment (21-23°C) with a 12:12 hour light-dark cycle (lights on at 0700) conditions. Unless otherwise stated, all animals had *ad libitum* access to water and RM1 diet (Special Diet Services, Witham, UK). Prior to all experiments, animals were extensively handled and acclimatised to SC injections to limit the non-specific stress effects of handling during any study.

6.3.3 EFFECT OF OX2 ON BODY COMPOSITION IN RATS

27 male Wistar rats were randomised to three groups of equal mean body weight (mean body weight 356g, range 323-407g). Groups 1 and 2 received daily SC injections of vehicle or OX2 (40nmol/kg) at 0900 for 22 days, with *ad libitum* access to standard laboratory chow. The third group had daily injections of vehicle, but were pair-fed to the OX2 group. Food was presented daily at 0900.

After the final injection, the animals were fasted from 1600. The following morning, a blood sample was taken for blood glucose measurement using a Contour Blood Glucose Meter (Bayer, Switzerland). The animals were then culled by CO₂ asphyxiation followed by cervical neck dislocation. Prior to cervical dislocation, body length was measured from the nose to the base of the tail. The right epididymal white adipose tissue depot, right inguinal white adipose tissue depot, interscapular brown adipose tissue, right gastrocnemius muscle and right kidney were dissected and weighed, and the right tibia was dissected and its length measured. All the tissues were then returned to the carcass, which was stored at -20°C until body composition analysis.

6.3.3.1 BODY COMPOSITION ANALYSIS

Body composition was determined through saponification in potassium hydroxide with ethanol. The frozen carcass was weighed and an equal volume of 3M potassium hydroxide and 65% ethanol (v/v)

was added. This was subsequently incubated in a sealed container at 70°C for 5 days. The resultant liquid was strained to remove any solid material, before being made up to 1L with 100% ethanol. Aliquots were then labelled and stored until glycerol and protein concentrations were determined.

Glycerol content was measured using a glycerol assay kit (Randox GY 105). The resultant levels were subsequently corrected to determine the grams of glycerol per carcass. This was then converted to triglyceride content, assuming the molecular weight of a triglyceride molecule is 885g/mol, compared to 92g/mol for glycerol.

Protein concentration was determined using a Modified Lowry Kit (ThermoFischer Scientific 23240). Dilutions were again corrected to give the grams of protein per carcass.

6.3.4 EFFECT OF OX2 ON TISSUE-SPECIFIC METABOLIC PROCESSES

24 male Wistar rats were randomised to three groups of equal mean body weight (mean weight 353g, range 337-384g). Groups 1 and 2 received daily SC injections of vehicle or OX2 (40nmol/kg) at 0900 for 22 days, with *ad libitum* access to standard laboratory chow. The third group had daily injections of vehicle, but were pair-fed to the OX2 group. Food was presented daily at 0900.

After the penultimate injection, the animals were transferred to renal metabolic cages, and their urine and faeces was collected for 16 hours. The animals were subsequently returned to their home cages. After the final injection, the animals were fasted from 1600. They were culled the following morning by CO₂ asphyxiation and cervical dislocation. Blood was collected by immediate cardiac puncture. One sample was placed in a plain eppendorf and clotted for 30 minutes at room temperature, before being centrifuged at 6000rpm for 10 minutes. The resultant serum was frozen immediately in liquid nitrogen. The remaining blood was placed in a vacutainer containing potassium-EDTA and aprotinin. These samples were immediately centrifuged at 6000rpm for 10 minutes, and the resulting plasma frozen in liquid nitrogen. Samples of brown adipose, epididymal and inguinal white adipose, gastrocnemius, and liver were dissected and snap frozen in liquid nitrogen. All tissue, serum and plasma samples were then kept at -80°C until further processing.

6.3.4.1 mRNA EXTRACTION AND QUANTIFICATION

Brown adipose, liver and skeletal muscle samples were all processed using PureLink® RNA mini kit (Ambion, ThermoFischer Scientific), while white adipose was processed using RNease Lipid Tissue Mini Kit (Qiagen) as previously described. All samples underwent on-column DNase (Purelink DNase, Life Technologies). Subsequently cDNA was formed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Waltham, USA).

Quantitative polymerase chain reaction was then undertaken using the following TaqMan gene-expression primer sets: argininosuccinate synthetase Rn00565808_g1; carbamoyl phosphate synthetase Rn00567109_m1; glucose-6-phosphatase Rn00689876_m1; phosphoenolpyruvate carboxykinase Rn01529014_m1; fibroblast growth factor 21 (FGF21) Rn00590706m1; Atrogin 1 Rn00591730m1; MuRF1 Rn00590197_m1; hormone-sensitive lipase Rn00689222_m1. All samples were normalised to the endogenous housekeeping gene 18s.

6.3.4.2 PLASMA ANALYSIS

Plasma analytes were measured using the following assay kits, following the manufacturers' instructions:

- Free thyroxine and free tri-iodothyronine ELISA (CB-E05076r and CB-E05079r, Cusabio, Wuhan, China)
- Glycerol GPO-PAP (GY 105, Randox GY 105)
- β -hydroxybutyrate colorimetric assay (CAY700190, Cayman Chemical, Ann Arbor, USA)
- IGF-1 Quantikine ELISA (MG100, R&D Systems, Abingdon, UK)
- L-amino colorimetric assay (MAK002, Sigma-Aldrich)
- Urea levels were measured on an Abbott Architect autoanalyser.

6.3.4.3 SERUM ANALYSIS

Serum analytes were measured using the following assay kits, following the manufacturer's instructions:

- CTX-1 EIA (AC-06F1, Immunodiagnostic Systems Limited, Boldon, UK)
- P1NP EIA (AC-33F, Immunodiagnostic Systems Limited, Boldon, UK)

6.3.4.4 URINE ANALYSIS

Urine urea levels were measured using the Abbott Architect autoanalyser.

6.3.5 STATISTICAL ANALYSIS

All data is presented as mean $\pm$ SEM. All data analysis was undertaken using Prism 7.0 (Graphpad).

6.4 RESULTS

6.4.1 KEY RESULTS FOR CHAPTER 6

- OX2 administration for 22 days caused a significant change in body composition with
 - Reduced body weight and body length
 - Lower body fat levels
 - A reduction in muscle mass and absolute body protein, though no change in percentage of body weight due to protein
- This was accompanied by increased markers of both protein and fat metabolism, including the urea cycle, markers of proteolysis, hormone sensitive lipase and ketogenesis.
- Other metabolic pathways were down-regulated, including growth hormone axis, bone turnover, the thyroid axis and brown adipose tissue thermogenesis.
- These findings together suggest multiple non-essential metabolic pathways are suppressed, and fuel stores in muscle and fat are used up, to ensure diversion of energy to the metabolic pathways stimulated by OX2.
- Protein metabolism may be one of the metabolic pathways upregulated by OX2 which causes an increase in energy expenditure.

6.4.2 EFFECT OF CHRONIC OX2 ON BODY COMPOSITION IN RATS

6.4.2.1 EFFECT OF CHRONIC OX2 ON FOOD INTAKE AND BODY WEIGHT IN RATS

Repeated injections of OX2 caused a significant reduction in food intake when compared to vehicle (average food intake over the 22 days of the study, mean $\pm$ SEM (g), vehicle: 689.3 ± 24.7 g; OX2: 571.8 ± 21.1 ; pair-fed group: 567.3 ± 2.3 g; $p < 0.001$ vehicle vs both OX2 and pair-fed to OX2) (Figure 6.1). Over the 22 days of the study, the OX2 group ate 17% less food than the vehicle group.

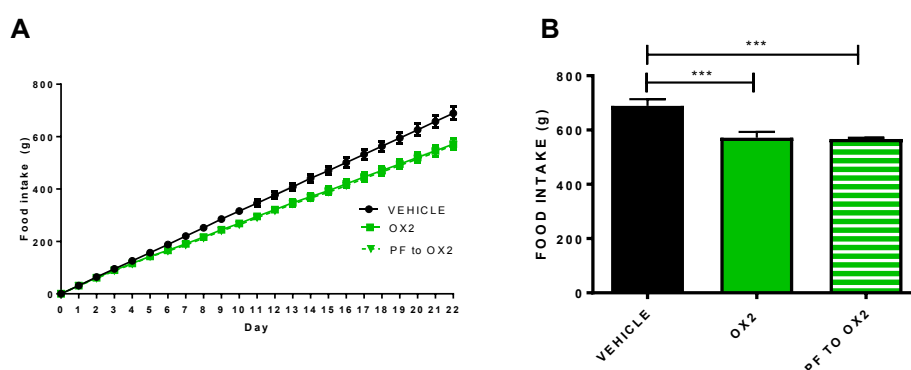


Figure 6.1 Effect of repeated injections of OX2 on food intake in rats.

Cumulative daily food intake (g) (A) and total food intake (g) (B) in rats receiving vehicle, OX2 (40nmol/kg), or pair-fed to the OX2 group for 22 days. N=9. Data presented as mean $\pm$ SEM. Final food intake analysed by one-way ANOVA with post-hoc Tukey's Multiple Comparison Test. *** $p < 0.001$ vs vehicle.

All groups entered the study with the same average body weight (353g). During the study, both the vehicle and pair-fed groups gained weight from baseline while the OX2 group lost weight (average change in body weight from baseline, mean $\pm$ SEM (g), vehicle: 91 ± 7.4 g; OX2: -33 ± 8.7 g; pair-fed group: 63 ± 3.3 g). From baseline, the vehicle group gained 26% body weight, the pair-fed group gained 18% body weight and the OX2 group lost 9% of body weight. The change in body weight from baseline was significantly different in both the OX2 and pair-fed groups when compared to vehicle ($p < 0.0001$ and $p < 0.05$ respectively); moreover, the body weight change between the OX2 and pair-fed groups was significantly different ($p < 0.0001$). As the pair-fed and OX2 groups ate the same amount, but the OX2 group lost weight compared to the pair-fed group, this suggests increased energy expenditure in the OX2 group (Figure 6.2).

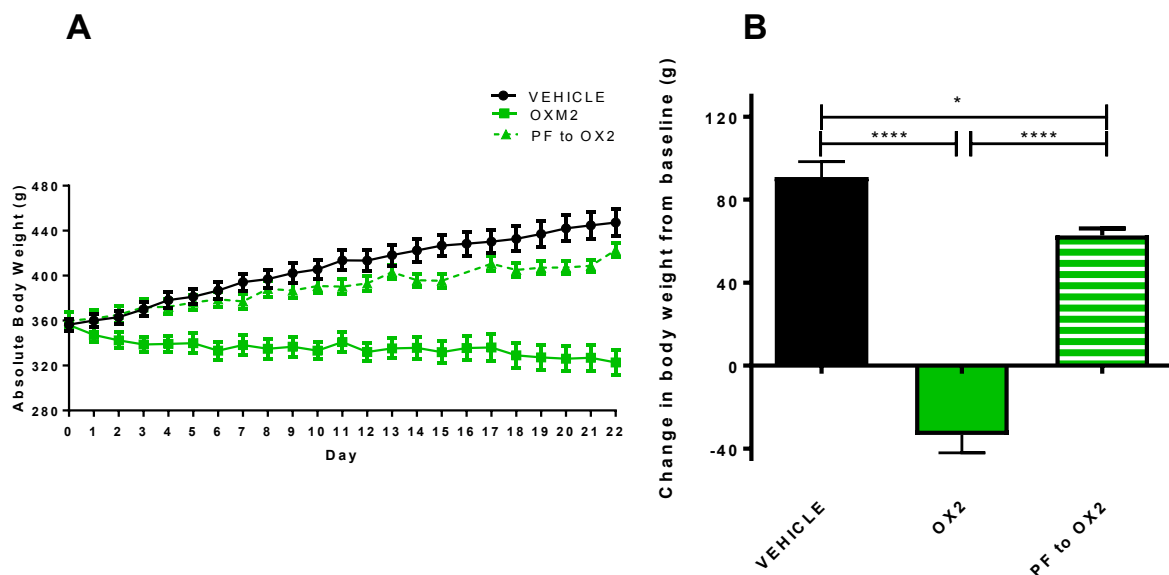


Figure 6.2 Effect of repeated injections of OX2 on body weight in rats.

Daily absolute body weight (A) and final body weight change from baseline (B) in rats receiving daily injections of vehicle, OX2 (40nmol/kg) or pair-fed to the OX2 group for 22 days. Baseline body weight in all groups was 353g. N=9. Data presented as mean $\pm$ SEM. Final body weight change analysed by one-way ANOVA with post-hoc Tukey's Multiple Comparison Test. * $p < 0.05$, **** $p < 0.0001$.

6.4.2.2 EFFECT OF CHRONIC OX2 ON GROWTH AND TISSUE WEIGHT IN RATS

OX2 affected the overall growth of rats (ordinary 2-way ANOVA, effect of treatment, $F(2,42)=27.6$, $p < 0.0001$; effect of tissue, $F(1,42)=16404$, $p < 0.0001$, interaction $F(2,42)=16.4$, $p < 0.0001$). At the end of the study, the OX2 rats were significantly smaller than those in the other groups (average final nose-to-base-of-tail length, mean $\pm$ SEM (cm), vehicle: 24 ± 0.3 cm; OX2: 22 ± 0.3 cm; pair-fed: $24 \pm$

0.1cm); OX2 vs vehicle and pair-fed group, $p<0.0001$ in both instances) (Figure 6.3). There was a contemporaneous non-significant reduction in skeletal growth in the OX2 group, as exemplified by reduced tibial length (average tibia length, mean $\pm$ SEM (cm), vehicle: 4.6 ± 0.1 cm; OX2: 4.3 ± 0.1 cm; pair-fed: 4.5 ± 0.1 cm) (Figure 6.3).

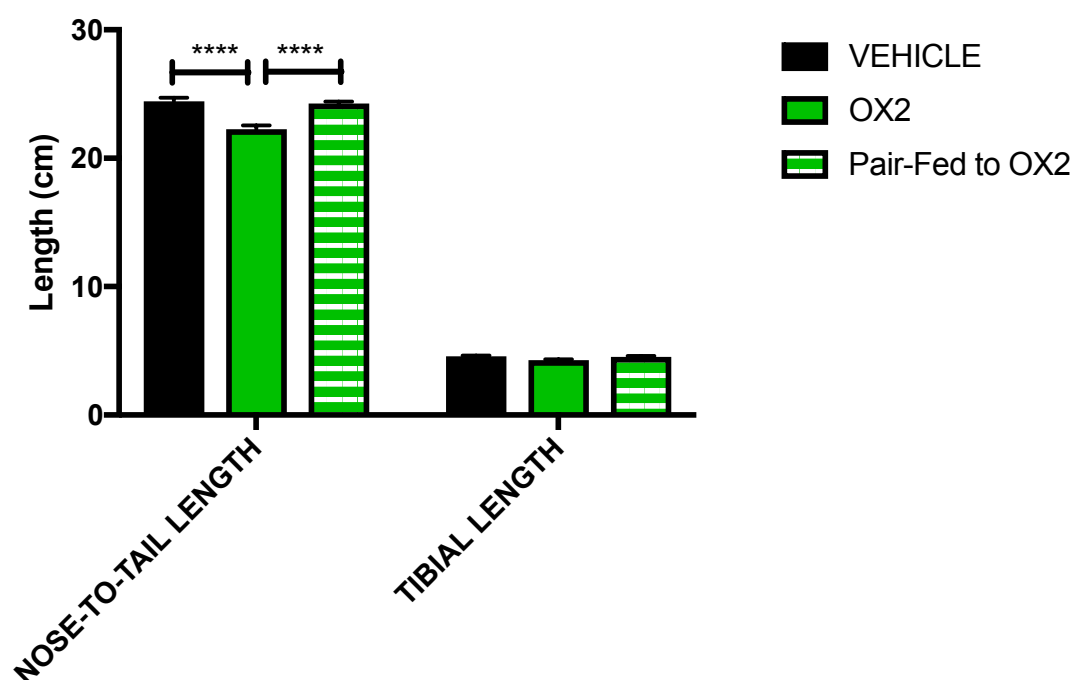


Figure 6.3 Effect of repeated injections of OX2 on growth in rats.

Nose-to-tail length and tibial length (cm) and in rats receiving daily injections of vehicle, OX2 (40nmol/kg) or pair-fed to OX2 group for 22 days. Animals were fasted from 1600 on the day before culling. N=8-9. Data presented as mean $\pm$ SEM. Statistical analysis undertaken by ordinary two-way ANOVA with post-hoc Tukey's Multiple Comparison Test. **** $p<0.0001$.

OX2 also affected organ weight (ordinary 2-way ANOVA, effect of treatment, $F(2,94)=17.5$, $p<0.0001$; effect of tissue, $F(3,94) = 182.7$, $p<0.0001$; interaction $F(6, 94)= 7.5$, $p<0.0001$), with many organs smaller in the OX2 group than in the control group at the end of treatment (Table 6-1).

TISSUE	TISSUE MASS (mean $\pm$ SEM g) [mass as % compared to vehicle]			STATISTICAL ANALYSIS (two-way ANOVA with post-hoc Tukey's multiple comparison test)
	VEHICLE	OX2 (40nmol/kg)	PAIR-FED	
RIGHT KIDNEY	1.6 ± 0.1 [100]	1.3 ± 0.1 [81]	1.5 ± 0.1 [94]	NS
EPIDIDYMAL WAT	3.3 ± 0.2 [100]	1.7 ± 0.1 [52]	2.9 ± 0.3 [88]	Vehicle vs OX2 $p<0.0001$ Vehicle vs Pair-fed $p<0.05$ Pair-fed vs OX2 $p<0.0001$

GASTROCNEMIUS	0.8 ± 0.1 [100]	0.6 ± 0.1 [75]	0.7 ± 0.1 [87.5]	NS
INTRASCAPULAR BAT	0.7 ± 0.1 [100]	0.7 ± 0.1 [100]	0.6 ± 0.1 [86]	NS

Table 6-1 Effect of chronic OX2 on final weight of selected tissues in rats.

Epididymal white adipose (eWAT) depot was significantly reduced in the OX2 group compared to both the vehicle and pair-fed groups, and in the pair-fed group compared to vehicle (average eWAT weight, mean ± SEM (g), vehicle: 3.3 ± 0.2g; OX2: 1.7 ± 0.1g; pair-fed: 2.9 ± 0.3g; vehicle vs OX2, $p<0.0001$; pair-fed vs OX2, $p<0.0001$; vehicle vs pair-fed, $p<0.05$) (Figure 6.4). There was a tendency for gastrocnemius muscle weight to be lower in the OX2 group compared to vehicle and pair-fed groups (average muscle weight, mean ± SEM (g), vehicle: 0.8 ± 0.1g; OX2: 0.6 ± 0.1g; pair-fed: 0.7 ± 0.1g;) (Figure 6.4). Kidney weight was also reduced, though non-significantly, by treatment with OX2 (average kidney weight, mean ± SEM (g), vehicle: 1.6 ± 0.1g; OX2: 1.3 ± 0.1g; pair-fed: 1.5 ± 0.1g) (Figure 6.4). Total brown adipose tissue mass did not vary significantly between groups (average BAT weight, mean ± SEM (g), vehicle: 0.7 ± 0.1g; OX2: 0.7 ± 0.1g; pair-fed 0.6 ± 0.1g) (Figure 6.4) (Table 6-1).

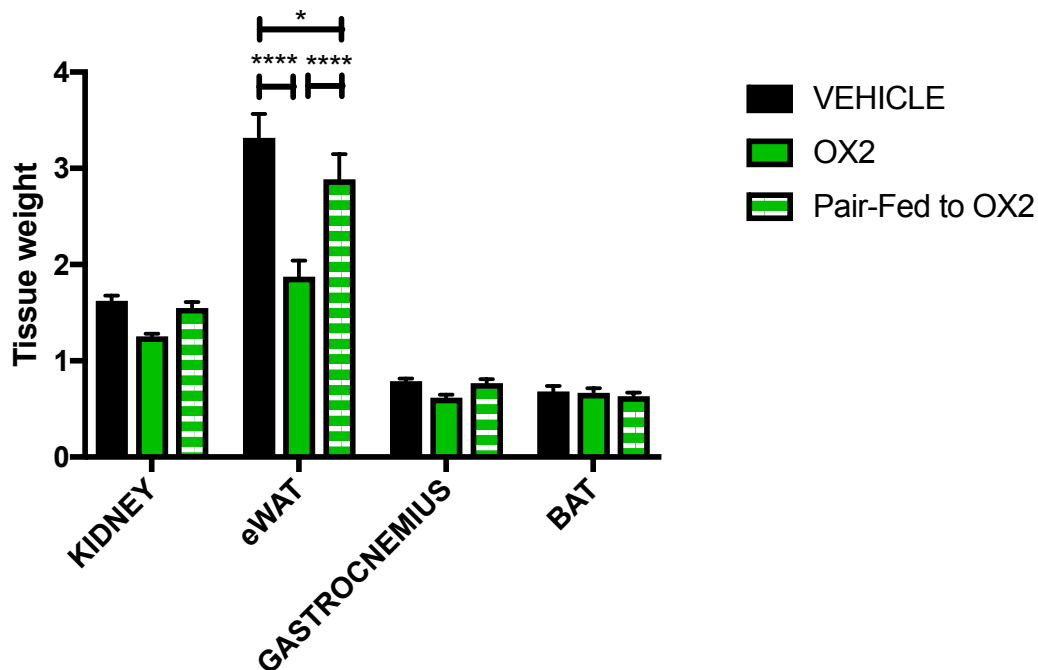


Figure 6.4 Effect of repeated injections of OX2 on tissue weights in rats.

Weight of kidney, epididymal WAT depot, gastrocnemius muscle and BAT pad in rats receiving daily injections of vehicle, OX2 (40nmol/kg) or pair-fed to the OX2 group for 22 days. Baseline body weight in all groups was 353g. All animals were fasted from 1600 the day before culling. N=9. Data presented as mean ± SEM. Tissue weight analysed by two-way ANOVA with post-hoc Tukey's Multiple Comparison Test. * $p<0.05$, **** $p<0.0001$.

6.4.2.3 EFFECT OF CHRONIC OX2 ON BODY COMPOSITION IN RATS

OX2 had a significant effect on body composition (ordinary two-way ANOVA, effect of treatment, $F(2,46)=13.0$, $p<0.0001$; effect of body component, $F(1,46)=2.0$, $p=0.2$; interaction $F(2,46) = 0.4$, $p=0.6$). OX2 reduced body fat (average amount of glycerol per carcass, mean ± SEM (g), vehicle: 3.8

$\pm 0.3\text{g}$; OX2: $2.3 \pm 0.3\text{g}$; pair-fed $3.6 \pm 0.4\text{g}$; when converted to triglycerides, mean $\pm$ SEM(g), vehicle: $36.5 \pm 3.3\text{g}$; OX2: $21.9 \pm 2.9\text{g}$; pair-fed $34.8 \pm 3.4\text{g}$; vehicle vs OX2, $p < 0.001$; pair-fed vs OX2, $p < 0.01$) (Figure 6.5). This is a 40% reduction in total body triglycerides in the OX2 group compared to the vehicle group, compared to only a 5% reduction in total body triglycerides when the pair-fed and vehicle groups are compared. However, when total triglyceride content was expressed as a percentage of the average body weight of each group, there was no significant difference between any of the groups (triglyceride content as a percentage of total body weight, mean % $\pm$ SEM, vehicle: $8.1 \pm 0.7\%$; OX2: $6.7 \pm 0.7\%$; pair-fed: $7.7 \pm 1.0\%$).

OX2 also caused a loss of body protein when compared to both the vehicle and pair-fed groups (average amount of protein per carcass, mean $\pm$ SEM(g), vehicle: $38.5 \pm 2.1\text{g}$; OX2: $27.9 \pm 1.8\text{g}$; pair-fed, $36.2 \pm 2.3\text{g}$; vehicle vs OX2, $p < 0.05$) (Figure 6.5). This was equivalent to a reduction in total body protein of 28% in the OX2 group, but only 6% in the pair-fed group, when compared to the vehicle group. When protein content was expressed as a percentage of the average body weight, there was no significant difference between groups (protein content as a percentage of total body weight, mean % $\pm$ SEM, vehicle: $8.7 \pm 0.5\%$; OX2: $8.7 \pm 0.5\%$; pair-fed, $8.2 \pm 0.6\%$).

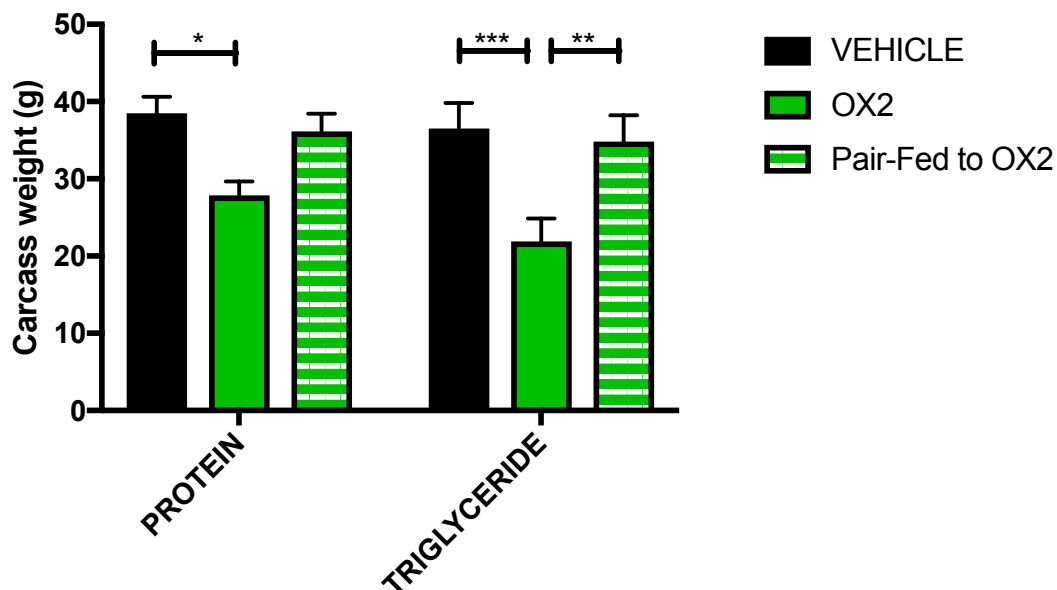


Figure 6.5 Effect of repeated injections of OX2 on body composition in rats.

Weight of carcass due to triglycerides and protein in rats receiving daily injections of vehicle, OX2 (40nmol/kg) or pair-fed to the OX2 group for 22 days. Baseline body weight in all groups was 353g. N=9. Data presented as mean $\pm$ SEM. Body composition analysed by ordinary two-way ANOVA with post-hoc Tukey's Multiple Comparison Test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.4.3 EFFECT OF CHRONIC OX2 ON TISSUE METABOLIC PROCESSES

The effects of OX2 on different tissue-specific metabolic processes are summarised in the following table (Table 6-2). Animals were fasted from 1600 the day before culling. The results shall be further expounded below.

Table 6-2 Effect of chronic administration of OX2 on tissue-specific metabolic processes.

TISSUE	PROCESS	Process mean $\pm$ SEM		
		VEHICLE	OX2 (40nmol/kg)	PAIR-FED
Plasma	Glycerol $\mu\text{mol/l}$	242 $\pm$ 16	201 $\pm$ 23	317 $\pm$ 47
	Triglyceride mmol/l	1.1 $\pm$ 0.1	0.8 $\pm$ 0.1	1.7 $\pm$ 0.2
	β -Hydroxybutyrate mM	0.5 $\pm$ 0.05	1.2 $\pm$ 1.27	0.6 $\pm$ 0.05
	L-Amino Acid nmol/ μl	3.3 $\pm$ 0.1	2.2 $\pm$ 0.1	3.1 $\pm$ 0.1
	Urea (mmol/l)	7.3 $\pm$ 0.4	8.7 $\pm$ 0.6	7.6 $\pm$ 0.6
	Glucose (mmol/l)	4.3 $\pm$ 0.2	4.1 $\pm$ 0.2	4.0 $\pm$ 0.2
	IGF-1 ($\mu\text{g/ml}$)	22.8 $\pm$ 0.3	16.9 $\pm$ 0.7	22.3 $\pm$ 0.4
	fT4 (pmol/l)	6.1 $\pm$ 0.2	5.0 $\pm$ 0.3	6.7 $\pm$ 0.3
	fT3 (pmol/l)	5.1 $\pm$ 0.2	4.7 $\pm$ 0.1	4.8 $\pm$ 0.1
Serum	CTX (ng/ml)	57.1 $\pm$ 8.2	39.9 $\pm$ 4.5	48.8 $\pm$ 5.8
	P1NP (ng/ml)	103 $\pm$ 14.5	20.6 $\pm$ 2.0	89.6 $\pm$ 17.6
eWAT	HSL mRNA (fold change)	1.1 $\pm$ 0.1	2.8 $\pm$ 0.4	1.6 $\pm$ 0.3
Skeletal muscle	MURF-1 mRNA (fold change)	1.0 $\pm$ 0.2	2.3 $\pm$ 0.5	0.9 $\pm$ 0.1
	Atrogin 1 mRNA (fold change)	1.1 $\pm$ 0.3	2.1 $\pm$ 0.4	0.5 $\pm$ 0.1
Liver	ASS mRNA (fold change)	1.0 $\pm$ 0.2	3.0 $\pm$ 0.3	1.1 $\pm$ 0.1
	CPS mRNA (fold change)	1.0 $\pm$ 0.2	1.4 $\pm$ 0.3	0.7 $\pm$ 0.1
	FGF21 mRNA (fold change)	1.0 $\pm$ 0.1	7.6 $\pm$ 1.6	0.5 $\pm$ 0.1
	G6Pase mRNA (fold change)	1.0 $\pm$ 0.2	1.5 $\pm$ 0.4	1.6 $\pm$ 0.3
	PEPCK mRNA (fold change)	1.0 $\pm$ 0.1	1.0 $\pm$ 0.2	1.2 $\pm$ 0.2
Urine	Volume (ml)	17 $\pm$ 3	26 $\pm$ 6	15 $\pm$ 2
	Urea concentration (mmol/l)	491 $\pm$ 73	450 $\pm$ 76	571 $\pm$ 58
	Total urea excretion (mmol)	6.8 $\pm$ 0.5	9.3 $\pm$ 1.0	8.1 $\pm$ 0.6
BAT	UCP-1 mRNA (fold change)	1.0 $\pm$ 0.3	0.6 $\pm$ 0.1	0.3 $\pm$ 0.1

6.4.3.1 EFFECT OF CHRONIC OX2 ON LIPID METABOLISM

Different treatments affected different plasma makers of lipid metabolism (ordinary two-way ANOVA, effect of treatment $F(2,63)=2.7$, $p=0.07$; effect of blood test $F(2,63)=39.1$, $p<0.0001$, interaction $F(4,63)=8.6$, $p<0.0001$). Plasma ketone levels were significantly elevated in the OX2 group compared to both the vehicle and pair-fed groups (average plasma β -hydroxybutyrate levels, mean $\pm$ SEM (mmol/l), vehicle: 0.5 ± 0.05 mmol/l; OX2: 1.2 ± 0.27 ; pair-fed: 0.6 ± 0.05 ; $p<0.001$ for OX2 vs both vehicle and pair-fed). Pair-feeding increased products of lipolysis with a significant elevation in plasma triglycerides, and a tendency to increased plasma glycerol when compared to both the vehicle and OX2 groups (average plasma triglyceride level, mean $\pm$ SEM (mmol/l), vehicle: 1.1 ± 0.1 mmol/l; OX2: 0.8 ± 0.1 mmol/l; pair-fed, 1.7 ± 0.2 mmol/l; vehicle vs pair-fed, $p<0.01$; OX2 vs pair-fed, $p<0.0001$; average plasma glycerol level, mean $\pm$ SEM (mmol/l), vehicle: 0.2 ± 0.02 mmol/l; OX2: 0.2 ± 0.02 mmol/l; pair-fed: 0.3 ± 0.05 mmol/l) (Figure 6.6A).

Hormone-sensitive lipase (HSL) is a lipase which enables the breakdown of triacylglycerol in adipose tissue to glycerol and free fatty acids. OX2 caused an increase in hormone-sensitive lipase (HSL) mRNA in eWAT depots, which was not seen in the pair-fed group (average HSL mRNA level, mean $\pm$ SEM, vehicle: 1.1 ± 0.1 ; OX2: 2.8 ± 0.4 ; pair-fed, 1.6 ± 0.3 ; vehicle vs OX2, $p<0.01$; pair-fed vs OX2, $p<0.05$) (Figure 6.6B).

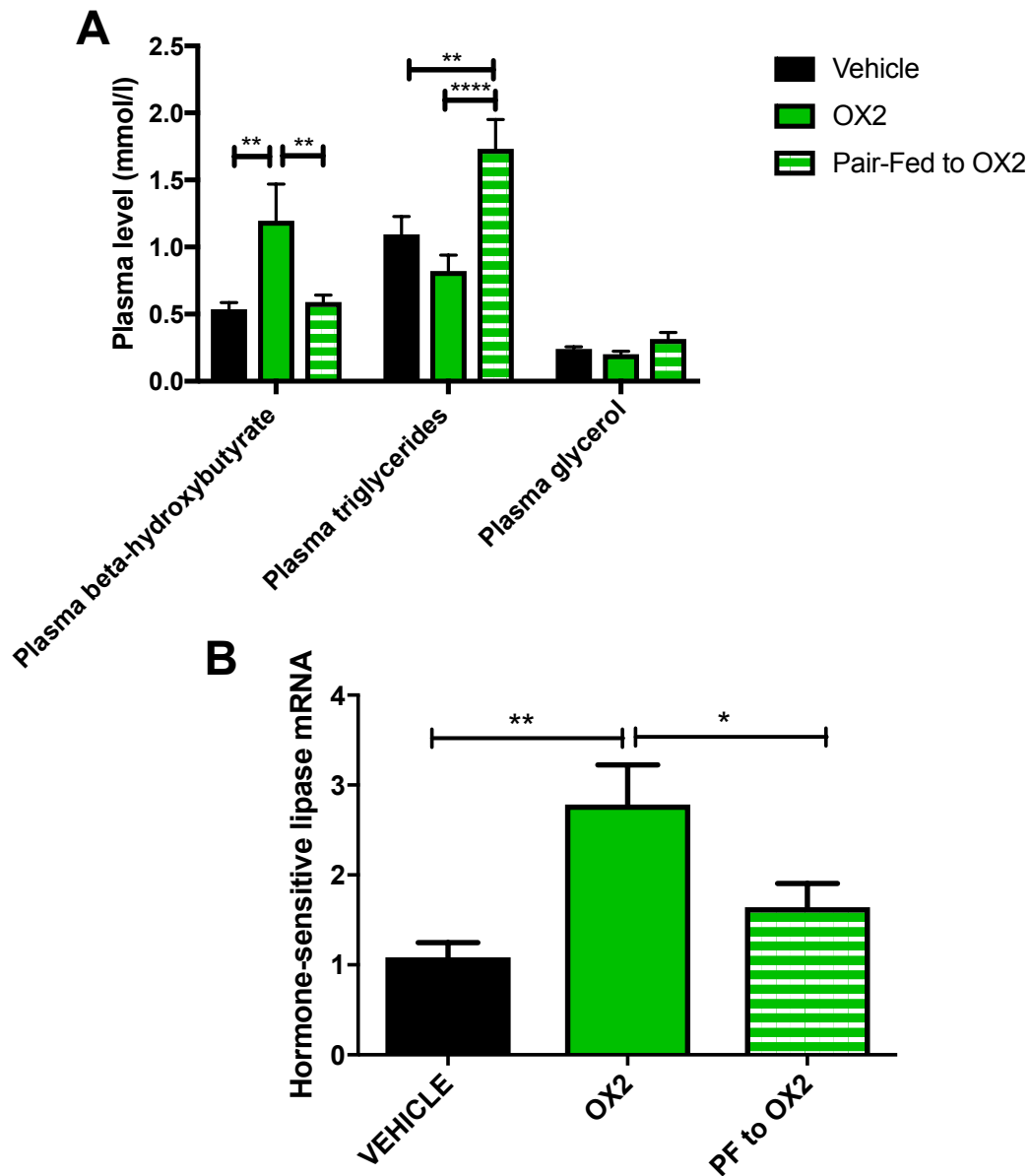


Figure 6.6 Effect of repeated injections of OX2 on lipid metabolism in rats.

Plasma markers of lipid metabolism (A) and hormone-sensitive lipase mRNA (B) in rats receiving daily injections of vehicle, OX2 (40nmol/kg) or pair-fed to the OX2 group for 22 days. Animals were fasted from 1600 the day prior to culling. All mRNA expression normalised to the housekeeping gene 18s. N=8. Data presented as mean $\pm$ SEM. All statistical analysis of plasma markers undertaken using an ordinary two-way ANOVA with post-hoc Tukey's Multiple Comparison Test; analysis of hormone sensitive lipase undertaken using a one-way ANOVA with post-hoc Tukey's Multiple Comparison test. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

6.4.3.2 EFFECT OF CHRONIC OX2 ON PROTEIN METABOLISM

OX2 caused a significant reduction in plasma L-amino-acids, leading to a notable hypoaminoacidaemia (average plasma L-amino acid levels, mean $\pm$ SEM (nmol/ μ l), vehicle: 3.3 ± 0.1 nmol/ μ l; OX2: 2.2 ± 0.1 nmol/ μ l; pair-fed: 3.1 ± 0.1 nmol/ μ l; $p < 0.001$ for OX2 vs both vehicle and pair-fed groups) (Figure 6.7). OX2 also affected muscle proteolysis pathways (ordinary two-way ANOVA, effect of treatment $F(2,44)=14.2$, $p < 0.0001$, effect of gene $F(1,44)=3.8$ $p=0.05$, interaction $F(2,44)=0.6$, $p=0.6$). OX2 caused a significant increase markers of the ubiquitin ligase pathway, with

an increase in MuRF1 mRNA, one muscle-specific E3 ubiquitin ligase (average MuRF1 mRNA levels, mean $\pm$ SEM, vehicle: 1.0 ± 0.2 ; OX2: 2.3 ± 0.5 ; pair-fed: 0.9 ± 0.1 ; vehicle vs OX2, $p < 0.05$; pair-fed vs OX2, $p < 0.001$), and an elevation in Atrogin-1 mRNA, another muscle-specific E3 ubiquitin ligase (average Atrogin-1 mRNA levels, mean $\pm$ SEM, vehicle: 1.1 ± 0.3 ; OX2: 2.1 ± 0.4 ; pair-feeding: 0.5 ± 0.1 ; vehicle vs OX2, pair-fed vs OX2, $p < 0.05$) (Figure 6.8).

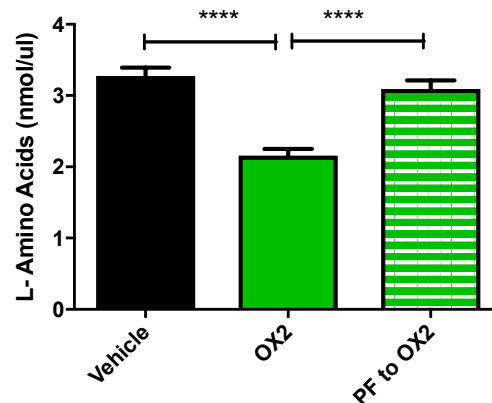


Figure 6.7 Effect of repeated injections of OX2 on plasma L-amino acids in rats.

Plasma L-amino acids in rats receiving daily injections of vehicle, OX2 (40nmol/kg) or pair-fed to the OX2 group for 22 days. Animals were fasted from 1600 on the day before culling. N=8. Data presented as mean $\pm$ SEM. Statistical analysis undertaken by one-way ANOVA with post-hoc Tukey's Multiple Comparison Test. **** $p < 0.0001$.

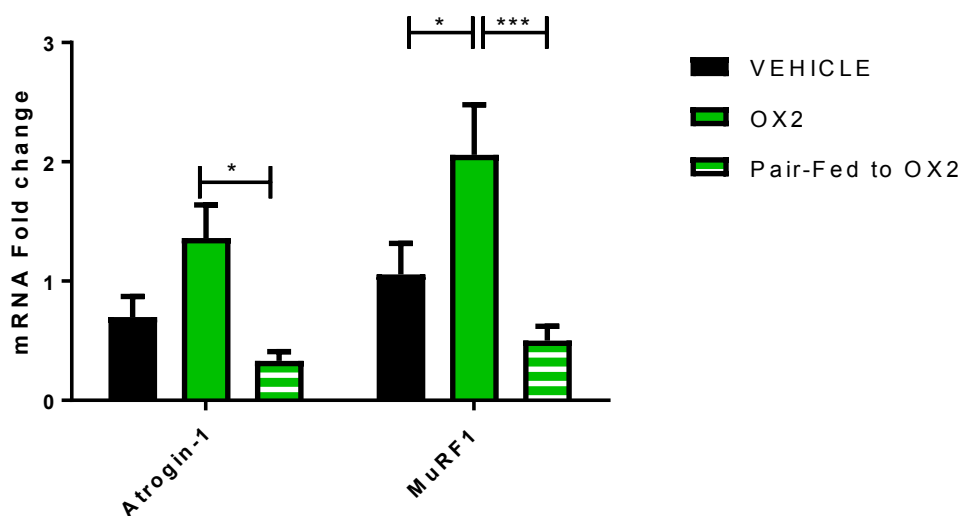


Figure 6.8 Effect of repeated injections of OX2 on muscle-specific E3 ubiquitin ligases.

Gastrocnemius muscle mRNA levels of MuRF-1 and Atrogin-1 in rats receiving daily injections of vehicle, OX2 (40nmol/kg) or pair-fed to the OX2 group for 22 days. Animals were fasted from 1600 on the day before culling. All mRNA expression was normalised to the housekeeping gene 18s. N=8. Data presented as mean $\pm$ SEM. Statistical analysis undertaken by ordinary two-way ANOVA with post-hoc Tukey's Multiple Comparison Test. * $p < 0.05$, *** $p < 0.001$.

Congruent with this enhanced muscle breakdown, OX2 also affect urea cycle activity (ordinary 2-way ANOVA, effect of treatment $F(2,46)=19.3$, $p < 0.0001$; effect of enzyme $F(1,46)=12.8$, $p < 0.001$;

interaction $F(2,46)=6.8$, $p<0.01$). There was increased ureagenesis in the OX2 group, with significantly increased hepatic mRNA expression of argininosuccinate synthetase and a tendency to increased expression of carbamoyl phosphate synthetase (average argininosuccinate synthetase mRNA levels, mean $\pm$ SEM, vehicle: 1.0 ± 0.2 ; OX2: 3.0 ± 0.3 ; pair-fed: 1.1 ± 0.1 , vehicle vs OX2, $p<0.0001$; pair-fed vs OX2, $p<0.0001$; average carbamoyl phosphate synthetase mRNA levels, mean $\pm$ SEM, vehicle: 1.0 ± 0.2 ; OX2: 1.4 ± 0.3 ; pair-fed: 0.7 ± 0.1 , pair-fed vs OX2, $p=0.1$) (Figure 6.9).

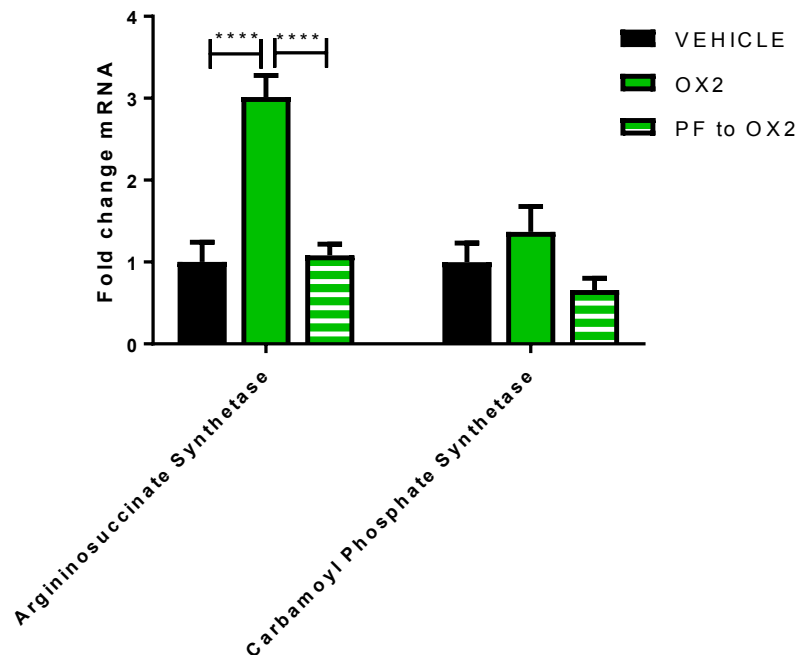


Figure 6.9 Effect of repeated injections of OX2 on urea cycle enzymes.

Hepatic mRNA levels of argininosuccinate synthetase and carbamoyl phosphate synthetase in rats receiving daily injections of vehicle, OX2 (40nmol/kg) or pair-fed to the OX2 group for 22 days. Animals were fasted from 1600 on the day before culling. All mRNA expression was normalised to the housekeeping gene 18s. N=8. Data presented as mean $\pm$ SEM. Statistical analysis undertaken by ordinary 2-way ANOVA with post-hoc Tukey's Multiple Comparison Test. **** $p<0.0001$.

Data from the renal metabolic cages showed changes in urea excretion following OX2 administration. There was no significant difference in plasma or urine urea concentration in the different groups (average plasma urea concentration, mean $\pm$ SEM (mmol/l), vehicle: 7.3 ± 0.4 mmol/l; OX2: 8.7 ± 0.6 mmol/l; pair-fed: 7.6 ± 0.6 mmol/l; average urinary urea concentration, mean $\pm$ SEM (mmol/l), vehicle: 491 ± 73 mmol/l; OX2: 450 ± 76 mmol/l; pair-fed: 571 ± 58 mmol/l) (Figure 6.10A and B). However, though statistically non-significant, there was an increase in urine output in the OX2 group during the 16 hours in the renal metabolic cages (average urine output, mean $\pm$ SEM (ml), vehicle: 17 ± 3 ml; OX2: 26 ± 6 ml; pair-fed: 15 ± 2 ml) (Figure 6.10C), and therefore combined with the urinary urea levels, there was a tendency for increased urea excretion in the OX2

group during the 16 hours in the renal metabolic cage (average urea excretion, mean $\pm$ SEM (mmol), vehicle: 6.8 ± 0.5 mmol; OX2: 9.3 ± 1.0 mmol; pair-fed: 8.1 ± 0.6 mmol; vehicle vs OX2 $p=0.06$) (Figure 6.10D).

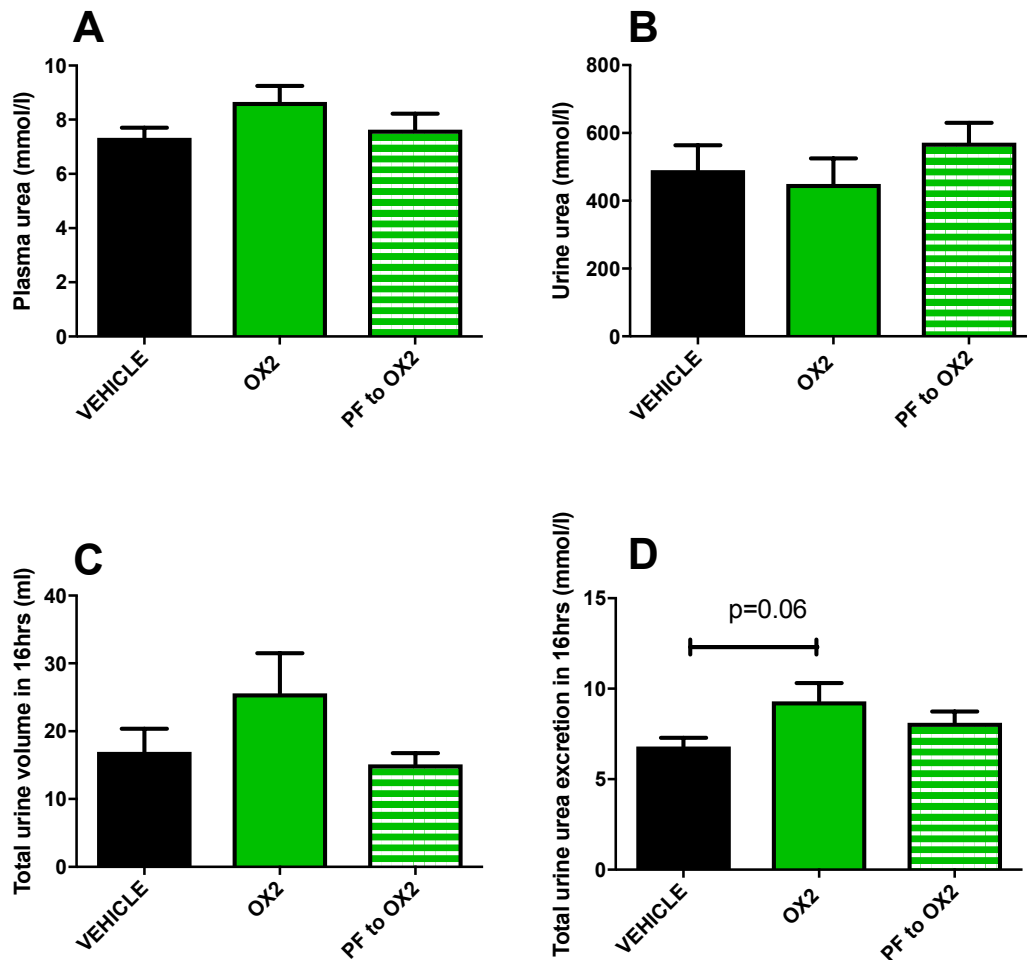


Figure 6.10 Effect of repeated injections of OX2 on urea levels in rats.

Plasma urea concentration (A), urine urea concentration (B), total urine volume (C) and total urinary urea excretion (D) in rats who spent 16 hours in renal metabolic cages after 21 days of daily injections of vehicle, OX2 (40nmol/kg) or pair-fed to the OX2 group for 22 days. Animals were fasted from 1600 on the day prior to culling. N=8. Data presented as mean $\pm$ SEM. Statistical analysis undertaken by one-way ANOVA with post-hoc Tukey's Multiple Comparison Test.

Hepatic expression of FGF21 mRNA, a marker of protein restriction, was also elevated in the OX2 group (average hepatic FGF21 mRNA levels, mean $\pm$ SEM, vehicle: 1.0 ± 0.1 ; OX2: 7.6 ± 1.6 ; pair-fed: 0.5 ± 0.1 ; $p<0.001$ for OX2 vs both vehicle and pair-fed groups) (Figure 6.11).

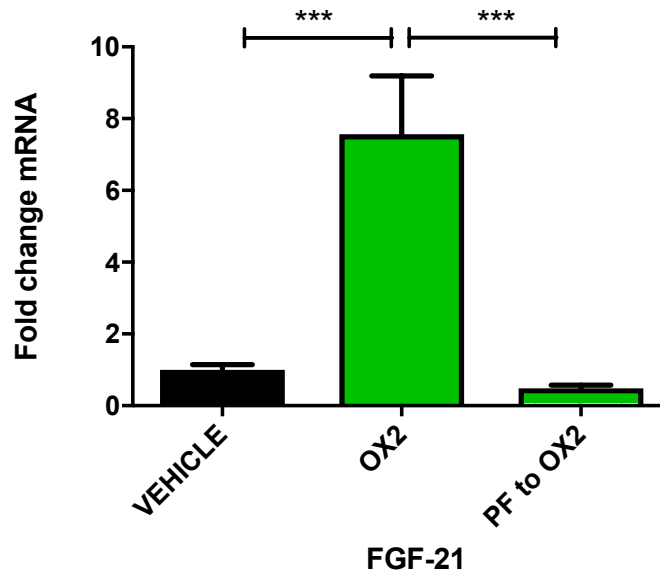


Figure 6.11 Effect of repeated injections of OX2 on hepatic FGF21 mRNA levels rats.

Rats received daily injections of vehicle, OX2 (40nmol/kg) or were pair-fed to the OX2 group for 22 days. Animals were fasted from 1600 on the day prior to culling. All mRNA expression was normalised to the housekeeping gene 18s. N=8. Data presented as mean $\pm$ SEM. Statistical analysis undertaken by one-way ANOVA with post-hoc Tukey's Multiple Comparison Test. *** $p < 0.001$.

6.4.3.3 EFFECT OF CHRONIC OX2 ON GROWTH HORMONE AND THYROID AXES IN RATS

Plasma IGF-1 levels, which are a marker of the growth hormone axis, were significantly suppressed by OX2 (average IGF-1 level, mean $\pm$ SEM ($\mu\text{g/ml}$), vehicle: $22.8 \pm 0.3\mu\text{g/ml}$; OX2: $16.9 \pm 0.7\mu\text{g/ml}$; pair-fed: $22.3 \pm 0.4\mu\text{g/ml}$; $p < 0.0001$ for OX2 vs both vehicle and pair-fed groups) (Figure 6.12A). The thyroid axis was also affected by OX2 treatment (ordinary 2-way ANOVA, effect of treatment $F(2,41)=9.4$, $p < 0.001$; effect of thyroid hormone, $F(1,41)=36.1$, $p < 0.0001$; interaction $F(2,41)=5.8$, $p < 0.01$). There was suppression in free T4 levels in the OX2 group (average fT4 level, mean $\pm$ SEM(pmol/l), vehicle: $6.1 \pm 0.2\text{pmol/l}$; OX2: $5.0 \pm 0.3\text{pmol/l}$; pair-fed: $6.7 \pm 0.3\text{pmol/l}$; vehicle vs OX2, $p < 0.01$; pair-fed vs OX2, $p < 0.0001$); but there was no significant difference in plasma triiodothyronine (fT3) levels between groups (average plasma fT3, mean $\pm$ SEM (pmol/l), vehicle: $5.1 \pm 0.2\text{pmol/l}$; OX2: $4.7 \pm 0.1\text{pmol/l}$; pair-fed: $4.8 \pm 0.1\text{pmol/l}$) (Figure 6.12B).

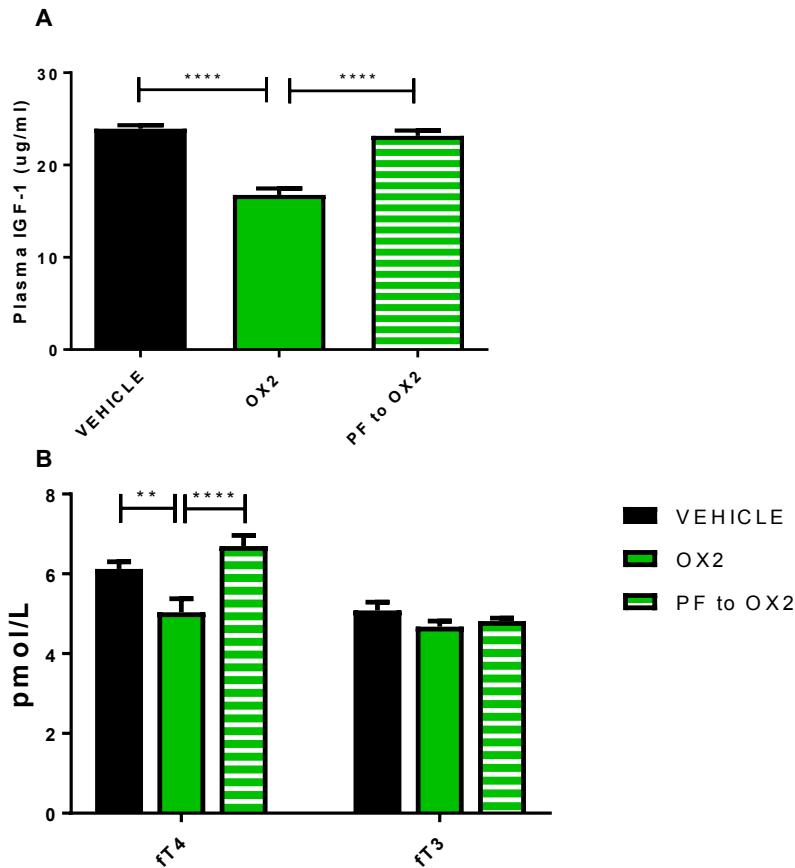


Figure 6.12 Effect of repeated injections of OX2 on the growth hormone and thyroid axes in rats.

Plasma IGF-1 (A), free thyroxine and free tri-iodothyronine (B) levels in rats who received daily injections of vehicle, OX2 (40nmol/kg) or were pair-fed to the OX2 group for 22 days. Animals were fasted from 1600 on the day prior to culling. N=8. Data presented as mean $\pm$ SEM. Statistical analysis of IGF-1 levels undertaken by one-way ANOVA with post-hoc Tukey's Multiple Comparison Test, while thyroid hormone levels were assessed using ordinary two-way ANOVA with post-hoc Tukey's Multiple Comparison Test. ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

6.4.3.4 EFFECT OF CHRONIC OX2 ON BROWN ADIPOSE UCP-1 LEVELS IN RATS

BAT mRNA levels of UCP-1 were significantly suppressed in the pair-fed group, and there was a tendency for it to be lower in the OX2 group compared to vehicle (average BAT UCP-1 mRNA levels, mean $\pm$ SEM, vehicle: 1.0 ± 0.3 ; OX2: 0.6 ± 0.1 ; pair-fed: 0.3 ± 0.1 ; vehicle vs pair-fed, $p < 0.05$; vehicle vs OX2 $p = 0.17$) (Figure 6.13).

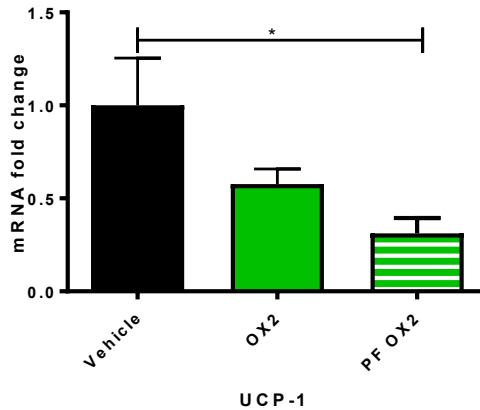


Figure 6.13 Effect of repeated injections of OX2 on interscapular BAT UCP-1 mRNA levels.

BAT UCP-1 mRNA levels in rats who received daily injections of vehicle, OX2 (40nmol/kg) or were pair-fed to the OX2 group for 22 days. Animals were fasted from 1600 on the day prior to culling. All mRNA expression was normalised to the housekeeping gene 18s. N=8. Data presented as mean ± SEM. Statistical analysis undertaken by one-way ANOVA with post-hoc Tukey's Multiple Comparison Test * $p < 0.05$.

6.4.3.5 EFFECT OF CHRONIC OX2 ON BONE METABOLISM IN RATS

Bone metabolism was reduced by OX2 (ordinary 2-way ANOVA, effect of treatment $F(2,39)=14.1$, $p < 0.0001$; effect of bone marker $F(1,39)=7.5$, $p < 0.01$; interaction $F(2,39)=6.7$, $p < 0.01$). There was significant suppression in the bone-formation marker P1NP by OX2 (average plasma P1NP levels, mean ± SEM (ng/ml), vehicle: 103 ± 14.5 ng/ml; OX2: 20.6 ± 2.0 ng/ml; pair-fed: 89.6 ± 17.6 ng/ml; vehicle vs OX2, $p < 0.0001$; PF to OX2 vs OX2, $p < 0.0001$). There was also a slight tendency to reduction in CTX, a marker of bone resorption, in the OX2 group (average plasma CTX levels, mean ± SEM (ng/ml), vehicle: 57.1 ± 8.2 ng/ml; OX2: 39.9 ± 4.5 ng/ml; pair-fed: 48.8 ± 5.8 ng/ml) (Figure 6.14).

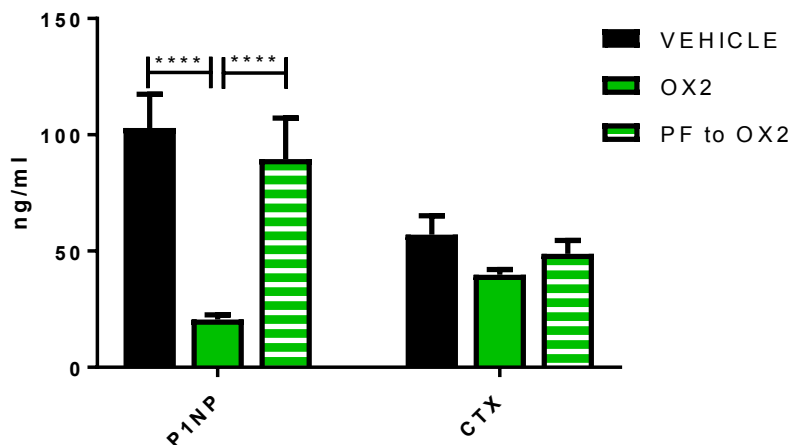


Figure 6.14 Effect of repeated injections of OX2 on bone metabolism in rats.

Serum P1NP and CTX levels in rats who received daily injections of vehicle, OX2 (40nmol/kg) or were pair-fed to the OX2 group for 22 days. Animals were fasted from 1600 on the day prior to culling. N=8. Data presented as mean ± SEM. Statistical analysis undertaken by ordinary two-way ANOVA with post-hoc Tukey's Multiple Comparison Test **** $p < 0.0001$.

6.4.3.6 EFFECT OF CHRONIC OX2 ON BLOOD GLUCOSE AND GLUCONEOGENESIS

There is no significant difference in final tail-capillary blood glucose in any of the groups (average blood glucose level, mean $\pm$ SEM (mmol/l), vehicle: 4.3 ± 0.2 mmol/l; OX2: 4.1 ± 0.2 mmol/l; pair-fed: 4.0 ± 0.2 mmol/l) (Figure 6.15).

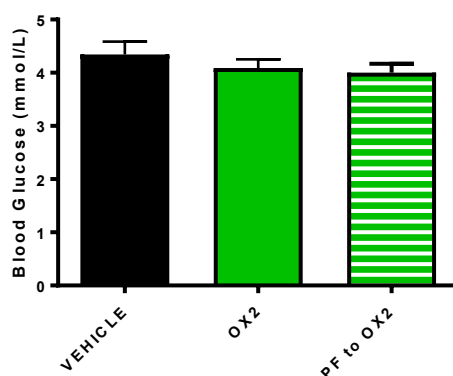


Figure 6.15 Effect of repeated injections of OX2 on blood glucose levels in rats.

Capillary glucose levels taken in rats who had been fasted from 1600 the previous day, having received daily injections of vehicle, OX2 (40nmol/kg) or pair-fed to the OX2 group for 22 days. N=8. Data presented as mean $\pm$ SEM. Statistical analysis undertaken by one-way ANOVA with post-hoc Tukey's Multiple Comparison Test.

Neither OX2 nor pair-feeding had any significant effect on the expression of hepatic mRNA expression of the gluconeogenic enzymes glucose-6-phosphatase (G6Pase) or phosphoenolpyruvate carboxykinase (PEPCK) (ordinary two-way ANOVA, effect of treatment $F(2,46)=1.3$, $p=0.3$; effect of enzyme $F(1,46)=2.1$, $p=0.2$; interaction $F(2,46)=0.6$, $p=0.6$) (average G6Pase mRNA levels, mean $\pm$ SEM, vehicle: 1.0 ± 0.2 ; OX2: 1.5 ± 0.4 ; pair-fed: 1.6 ± 0.3 ; average PEPCK mRNA levels, mean $\pm$ SEM, vehicle: 1.0 ± 0.1 ; OX2: 1.0 ± 0.2 ; pair-fed: 1.2 ± 0.2) (Figure 6.16).

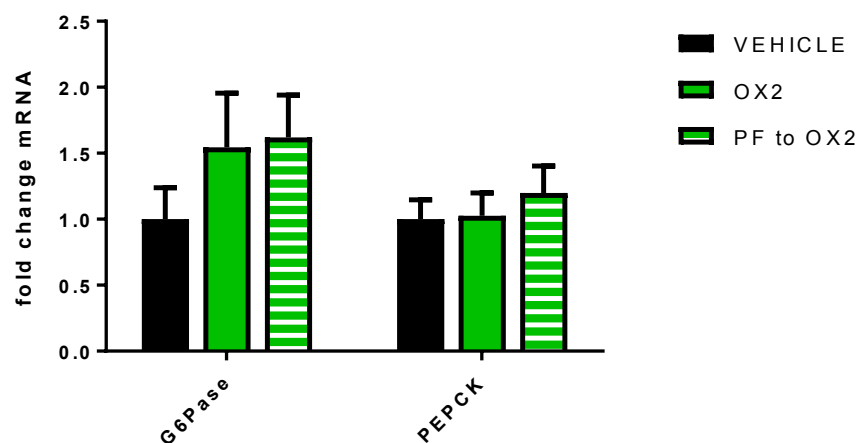


Figure 6.16 Effect of repeated injections of OX2 on hepatic gluconeogenic enzymes in rats.

Hepatic expression of G6Pase and PEPCK mRNA in rats who received daily injections of vehicle, OX2 (40nmol/kg) or were pair-fed to the OX2 group for 22 days. Animals were fasted from 1600 on the day prior to culling. All mRNA expression was normalised to the housekeeping gene 18s. N=8. Data presented as mean $\pm$ SEM. Statistical analyses undertaken by ordinary two-way ANOVA with post-hoc Tukey's Multiple Comparison Test.

6.5 DISCUSSION

The work in this chapter investigated the effect of chronic administration of the oxyntomodulin analogue OX2 on body composition and metabolic processes in rats.

Chronic administration of OX2 in rats caused a mild anorectic effect, but profound weight loss. The loss of weight was greater than that seen in the pair-fed group, confirming that OX2 has an energy expenditure effect (Figure 6.2). Moreover, neither of the anorectic or energy expenditure effects attenuated with repeated drug administration. This supports the concept of using oxyntomodulin analogues as long-term anti-obesity therapy. Despite the glucagon activity of OX2, there was no difference in blood glucose levels between the groups at the end of the study (Figure 6.15). This agrees with other studies administering chronic OXM analogues (Pocai et al., 2009, Liu et al., 2010, Day et al., 2009, Henderson et al., 2016), and may be attributed to either the enhanced weight loss in the OX2 group, or the contemporaneous GLP-1 activity in OXM. This is also reassuring in a potential treatment for obesity, as type 2 diabetes coexists in many obese patients, and no viable treatment for obesity would be acceptable that could also cause a deterioration in glucose homeostasis. Any oxyntomodulin analogues that enter clinical trials will nevertheless have to be demonstrated to be safe as well as efficacious in diabetic subjects.

Previous studies into the effect of OXM and its analogues on body composition have been limited. Dakin *et al.* (Dakin et al., 2002) showed that 7 days treatment with ICV OXM causes a reduction in eWAT and BAT pad mass in rats, but did not look at any other aspects of body composition. Several studies have shown oxyntomodulin and its analogues reduce both lean and fat mass in mice (Pocai et al., 2009, Kosinski et al., 2012, Bianchi et al., 2013, Day et al., 2009, Clemmensen et al., 2014). However, none of these studies looked at which specific tissues are affected, nor determined if these effects are due to the anorectic or energy-expenditure effect of the drug.

In agreement with the studies in mice described above (Pocai et al., 2009, Kosinski et al., 2012, Bianchi et al., 2013, Clemmensen et al., 2014), the first study in this chapter shows that chronic treatment with an oxyntomodulin analogue causes a reduction in both lean and fat mass. The composition analysis suggests fat mass is 40% lower after treatment with OX2, and total body protein is 28% lower, when compared to vehicle (Figure 6.5). This correlates well with the reduction in the epididymal WAT pad (48% reduction in the OX2 group compared to vehicle), and in the weight of the gastrocnemius muscle (25% reduction in the OX2 group compared to vehicle), as exemplars of fat and protein depots (Figure 6.4). However the relative percentage of total body weight due to protein and fat did not change between any of the groups. Though an ideal anti-obesity treatment would show a reduction in fat but not lean mass, a similar pattern of results was seen in the initial

studies with liraglutide in rats, where both fat and lean mass were reduced, but the proportion of body weight due to fat and lean mass did not change (Larsen et al., 2001). Interestingly, in man, sustained treatment with liraglutide on body composition causes a predominant loss of fat mass; though there is an absolute loss of lean mass too, this is much smaller, such that the proportion of body weight due to lean tissue may actually increase (Rondanelli et al., 2016, Perna et al., 2016, Li et al., 2014). Caution must therefore be taken in assuming that the effects of oxyntomodulin on the body composition of rats will be mirrored in man.

This is the first study which shows that OXM causes a reduction in overall rat growth, as demonstrated by the reduced nose-to-tail length in the OX2 group (Figure 6.3). It is also the first study to show that the reduction in lean mass from an oxyntomodulin analogue was caused by a reduction in size of many different tissues, with bone and kidney being affected as well as muscle and fat (Figure 6.4). This reduction in growth and tissue size was not seen in the pair-fed group. This suggests that growth was reduced consequent to increased energy expenditure prompted by OX2, rather than due to reduced food intake.

Rats continue to grow from birth throughout adulthood, though after infancy the growth rate slows (Pullen, 1976). Cellular growth and tissue development are highly energy-expensive processes. Although the exact energetic cost of growth is difficult to ascertain, one estimate is that 53.4kJ and 52.9kJ of energy are required per gram of fat and protein deposition respectively (Pullar and Webster, 1977). Therefore, the OX2 group may restrict their growth to redirect energy which would be used on growth to supply the metabolic processes that are upregulated by OX2.

The inhibitory effect on growth is supported by the noted suppression of the growth hormone and thyroid axes in the OX2 group: both pathways facilitate growth. Down-regulation in the growth hormone axis and the thyroid axis are also seen in starvation to preserve limited energy stores, as exemplified by patients with anorexia nervosa (Douyon and Schteingart, 2002). OX2 also caused down regulation of tissue-specific metabolic pathways, such as bone formation (Figure 6.14), and brown adipose UCP-1 levels (in agreement with the findings in Chapter 4 – Figure 4.10) (Figure 6.13); this would conserve energy stores. Similar suppression in multiple metabolic pathways, notably UCP-1 levels and the thyroid axis, is seen in mice treated with the chemical uncoupler dinitrophenol, to compensate for the increased energy expenditure caused by this compound (Goldgof et al., 2014).

Interestingly, though OX2 caused a reduction in the size of most tissues measured, it did not change overall BAT mass (Figure 6.4). Maximal BAT mass is obtained in young animals, and it does not continue to increase as rats age, unlike most other tissues (Horan et al., 1988). Suppression of BAT

activity, as measured by UCP-1 levels, would reduce energy consumption; but there is no tissue growth to suppress to save energy. Dakin *et al.* did find a reduction in BAT mass in rats given twice daily IP injections of OXM, when compared to a pair-fed group (Dakin *et al.*, 2004); they attributed this to a loss of adiposity, which may infer fatty infiltration in their BAT pads not seen in the current experiment.

The suppression of growth in rats caused by OX2 has implications for the use of oxyntomodulin as an anti-obesity therapy in humans. Unlike the rodents used in these studies, growth ceases in humans in early adulthood. Cellular turnover continues to replace old and damaged cells, but there is rarely organ growth. In adult humans, obesity reflects accumulation of lipid within adipocytes, rather than an increase in cell number (Salans *et al.*, 1973). Therefore, in humans, OXM analogues may not suppress overall growth to conserve energy supplies, but they may affect tissue metabolic pathways. There may also be greater fat burning in man, to fuel the energy processes upregulated by oxyntomodulin, rather than redirecting energy supplies from tissue growth. It will be necessary to see in clinical trials how oxyntomodulin analogues affect cellular turnover and replacement, as well as the effects on specific tissue pathways such as bone turnover and the growth hormone axis.

It is important to note that, although OX2 did suppress multiple metabolic pathways, many were only slightly affected. Where possible, multiple pathways were compared by two-way ANOVA to mitigate for the statistical effects of multiple comparisons, but this was not always possible due to the different analytes, necessitating the use of one-way ANOVA for many comparisons. However, this does mean that the suppression of some of these pathways may not be biologically relevant. It will therefore be important to investigate the physiological and biological consequences of oxyntomodulin administration on many different metabolic processes in man.

The studies in this thesis demonstrated increased breakdown of lipid stores, with a reduction in WAT depot size (Figure 6.4) and overall carcass lipid content (Figure 6.5), as well as increased ketogenesis (Figure 6.6). HSL mRNA, a lipase which enables the breakdown of triacylglycerol in adipose tissue, was also increased. Moreover, this was not found in the pair-fed group, showing that this breakdown in lipids is not due to reduced food intake. Despite the increase in lipolysis, plasma glycerol and triglyceride levels were not increased by OX2 (Figure 6.6). A similar suppression in triglycerides has been shown with oxyntomodulin analogues in mice (Pocai *et al.*, 2009, Day *et al.*, 2009, Clemmensen *et al.*, 2014). This may reflect enhanced uptake of released glycerol and triglycerides from plasma in to tissues to fuel the metabolic pathways upregulated by OXM. Further studies could look at the effect of oxyntomodulin on flux through the lipid pathway. Interestingly, pair-feeding caused an elevation in both plasma glycerol and triglycerides. This may reflect an acute increase in lipolysis due

to prolonged fasting prior to the cull, the pair-fed group having adapted to restricted access to food such that they already consumed their daily chow before they were officially fasted (in study observation).

Protein stores were also broken down in the OX2 group, resulting in reduced carcass protein (Figure 6.5) and smaller muscle mass (Figure 6.4); there was also an increase in enzymes of the E3 ubiquitin ligase pathway (which is responsible for muscle breakdown) (Figure 6.8) (Finn and Dice, 2006). The hypoaminoacidaemia in the OX2 group suggested increased hepatic uptake of amino acids, while increased activity in the urea cycle is required for the removal of the amine group produced by amino acid metabolism. Furthermore, hepatic expression of FGF21, a marker of protein deficiency (Laeger et al., 2014), was increased by chronic OX2 administration (Figure 6.11).

The changes in lipid metabolism reflect those seen in previous studies of oxyntomodulin and its analogues. Kosinski *et al.* showed that continuous administration of OXM for 14 days reduced fat mass in mice (Kosinski et al., 2012), Dakin *et al.* showed 7 days of OXM reduced WAT pad mass in rats (Dakin et al., 2004), while several studies have shown prolonged treatment with OXM analogues reduces fat mass in mice (Clemmensen et al., 2014, Pocai et al., 2009, Bianchi et al., 2013). They also caused a suppression in plasma triglyceride level and an increase in plasma β -hydroxybutyrate (Pocai et al., 2009, Clemmensen et al., 2014). Similar changes in lipid metabolism can be seen following chronic administration of either glucagon or GLP-1. Repeated administration of either hormone causes a reduction in body lipid content in rats (Salter, 1960, Larsen et al., 2001), as well as a reduction in plasma-free fatty acids (Fitzpatrick et al., 1977, Shalev et al., 1997). An increase in plasma ketones is seen with glucagon administration (Ubukata et al., 1996, Longuet et al., 2008). The studies in this chapter, however, cannot determine if the changes in lipid metabolism seen in this chapter were due to the direct effect of oxyntomodulin on adipocytes, or are through indirect pathways. Both GLP-1 and glucagon receptors are found on adipocytes (Merida et al., 1993), and *in vitro* incubation of adipocytes with both GLP-1 and glucagon can cause lipolysis (Vaughan and Steinberg, 1963, Sancho et al., 2005, Ruizgrande et al., 1992). However, when administered acutely in man, neither GLP-1 or glucagon causes lipolysis (Bertin et al., 2001, Gravholt et al., 2001, Seghieri et al., 2013). It is possible that the chronic changes in lipid metabolism are due to the need to release stored fuel to support the anorectic and energy-expenditure effects of OXM and its analogues, rather than being a receptor-specific effect. There is potential for future studies to look at the direct effect of oxyntomodulin on adipose tissue, and which receptor is responsible for these effects, as well as whether there are acute demonstrable effects of oxyntomodulin on lipid pathways *in vivo*.

Activation of the glucagon receptors is most likely responsible for the changes in protein metabolism seen in the OX2 group, as the same changes are seen with chronic glucagon exposure. Patients with slow-growing, glucagon-secreting pancreatic tumours (glucagonomas) show a profound reduction in plasma amino acids (Barazzoni et al., 1999, Almdal et al., 1990, Klein et al., 1992), as well as increased whole-body protein breakdown (Barazzoni et al., 1999) and urea synthesis (Almdal and Vilstrup, 1988). Several studies have shown the same changes can be recapitulated in healthy volunteers receiving an infusion of glucagon (Liljenquist et al., 1981, Hamberg and Vilstrup, 1994, Vilstrup et al., 1990, Fitzpatrick et al., 1977). In contrast, there is little evidence for any effect of GLP-1 on protein metabolism. One study showed an overall reduction in whole-body protein breakdown in man following an acute infusion of GLP-1 (Shalev et al., 1997), while a chronic study using the GLP-1 receptor agonist liraglutide in man showed an increase in skeletal muscle index (a measure of fat-free mass in legs and arms) (Perna et al., 2016); however there are no published studies on the effect of GLP-1 on plasma amino acids, or urea cycle enzymes.

Glucagon directly enhances protein metabolism through actions in the liver: it increases uptake of amino acids into hepatocytes (Tews et al., 1975, Cariappa and Kilberg, 1990, Fehlmann et al., 1979, Flakoll et al., 1994); it upregulates transaminases which are required for amino acids to be converted to pyruvate for gluconeogenesis (Noda et al., 1988, Brown and Civen, 1968); and it increases expression and activity of urea cycle enzymes (Lin et al., 1982, Gebhardt and Mecke, 1979, Ulbright and Snodgrass, 1993) as well as ureagenesis (Ayuso-Parrilla et al., 1976). Glucagon also reduces protein synthesis within the liver (Ayuso-Parrilla et al., 1976, Preedy and Garlick, 1985). Extra-hepatically, glucagon enhances urea excretion via the kidney, and therefore disposal of amino acids, both by direct actions of the nephron and indirectly through increased circulating levels of cAMP (Bankir et al., 2016).

In contrast to the direct action of glucagon on the liver, there is little evidence for a direct effect of glucagon on skeletal muscles. The glucagon receptor is found in only very small quantities on skeletal muscle (Hansen et al., 1995), and *in vitro* incubation of the epitrochlearis muscle with glucagon failed to cause any significant release of amino acids (Garber et al., 1976). Glucagon infusion in rats does reduce protein synthesis in both the liver and skeletal muscle, but the effect in the liver occurs almost immediately, whereas it takes several hours for there to be an effect in muscle (Preedy and Garlick, 1985). In man, an infusion of glucagon at a level which causes profound hypoaminoacidaemia, failed to increase the release of 3-methylhistidine (a marker of skeletal muscle breakdown) (Fitzpatrick et al., 1977), while five days of IM glucagon did not increase skeletal muscle protease activity in rats (Mayer et al., 1980). Together, these studies suggest that the reduction in

body protein prompted by glucagon is secondary to its hepatic effects; amino acids taken up from the plasma then have to be replaced by skeletal muscle breakdown. Therefore the loss of skeletal muscle mass, and increase in the ubiquitin-ligase pathway within the OX2 group, may be a secondary to an increase in amino acid uptake and metabolism within the liver, rather than a direct effect of OX2 on muscle.

The effect of OX2, and therefore by inference oxyntomodulin, on protein metabolism has implications if OXM analogues are to be used as a treatment for obesity. If the breakdown of skeletal muscle occurs secondarily to the increased turnover in the liver, supplementing the diet of subjects with additional protein may prevent the loss of muscle mass. If oxyntomodulin has a direct effect on skeletal muscle, then this side-effect may preclude the use of oxyntomodulin analogues as pharmacotherapy for obesity. Future studies should examine the effect of different diets on the efficacy of oxyntomodulin analogues, as well as looking at the direct effects of oxyntomodulin on skeletal muscle.

Both adipose tissue and skeletal muscle are the long-term energy stores in the body. Increased breakdown of both tissues occurs when extra fuel is required. The resultant products can be used to generate fuel through gluconeogenesis (amino acids are broken down to pyruvate while glycerol is converted to oxaloacetate, both of which can then enter the gluconeogenic pathway) or through β -oxidation (free fatty acids). Therefore the increase in both lipid and protein breakdown prompted by chronic administration of OX2 may reflect a need to release stored energy to support the metabolic processes upregulated by OX2. Surprisingly, there was no increase in gluconeogenic enzymes in the OX2 group (Figure 6.15), which would be expected if the lipid and protein metabolism was to support gluconeogenesis. Similarly no change in gluconeogenic enzymes was seen in mice after one month of treatment with an oxyntomodulin analogue by Day *et al.* (Day *et al.*, 2009). However, changes in gluconeogenic enzyme mRNA expression do not necessarily reflect increased flux through the gluconeogenic pathway (Chung *et al.*, 2015, Samuel *et al.*, 2009); further studies could look at gluconeogenic enzyme activity or *in vivo* flux through these pathways in response to oxyntomodulin administration.

However, there is an alternative explanation for the changes in metabolic pathways, especially changes in protein metabolism. Oxyntomodulin may directly upregulate these pathways. *In vitro* data shows that glucagon directly upregulates many of the processes involved in protein metabolism, including the uptake of amino acids into the liver and synthesis of urea, in part through directly increasing transcription of these enzymes, as described above. Importantly, protein breakdown and metabolism is a highly energy-consumptive process. The ubiquitin-proteasome

pathway, which breaks down skeletal muscle, contains 6 separate ATPases which act in sequence to allow the degradation of an ubiquitinated protein; in total, 50-160 ATP molecules can be required to break down a single protein (Bar-Nun and Glickman, 2012, Peth et al., 2013). Additionally, after amino acids are taken up into the liver for deamination, the amino groups must be converted to urea for excretion to prevent nitrogen toxicity. Ureagenesis requires 4 ATP molecules for synthesis of each urea molecule. Transcription of the enzymes required to undertake these processes also requires energy. Therefore, oxyntomodulin may increase energy expenditure by upregulating protein metabolism, rather than protein metabolism being increased to fuel other upregulated metabolic processes. This possibility will be explored in Chapter 7 of this thesis.

6.6 CONCLUSIONS

Chronic administration of the oxyntomodulin analogue OX2 caused a significant reduction in body weight and food intake in rats, with no adverse effect on plasma glucose. It also caused a significant reduction in body fat content. Moreover, neither the anorectic nor energy expenditure effects reduced with recurrent administration of the drug. These findings all support the use of oxyntomodulin analogues as anti-obesity therapies. However, OX2 also affected rat growth: treated animals are smaller, and had reduced tissue and bone weight. They also showed an overall reduction in body protein. Though OXM analogues are unlikely to affect growth in adult humans, the effect on multiple metabolic pathways, and in particular protein metabolism, needs to be explored further.

Chapter 7

Oxyntomodulin And Protein

Metabolism

7.1 INTRODUCTION

As seen in Chapter 6, chronic OX2 administration increases protein metabolism. This is the first study to show that OXM or its analogues affect protein turnover. As also discussed in chapter 6, protein metabolism consumes a lot of energy, through the ubiquitination and breakdown of proteins, the synthesis of urea, and the transcription of those enzymes required for these processes. Therefore, if OX2 directly upregulates these processes, this may contribute to the energy expenditure effects of OX2.

Chronic administration of OX2 has an anorectic effect as well as an energy expenditure effect. It is impossible to determine from the studies in Chapter 6 whether the changes in protein metabolism are directly caused by OX2, or whether the increased breakdown of both fat and protein provide fuel to the, as yet undetermined, metabolic processes that OX2 does upregulate and which increase energy expenditure. These two hypotheses are illustrated below:

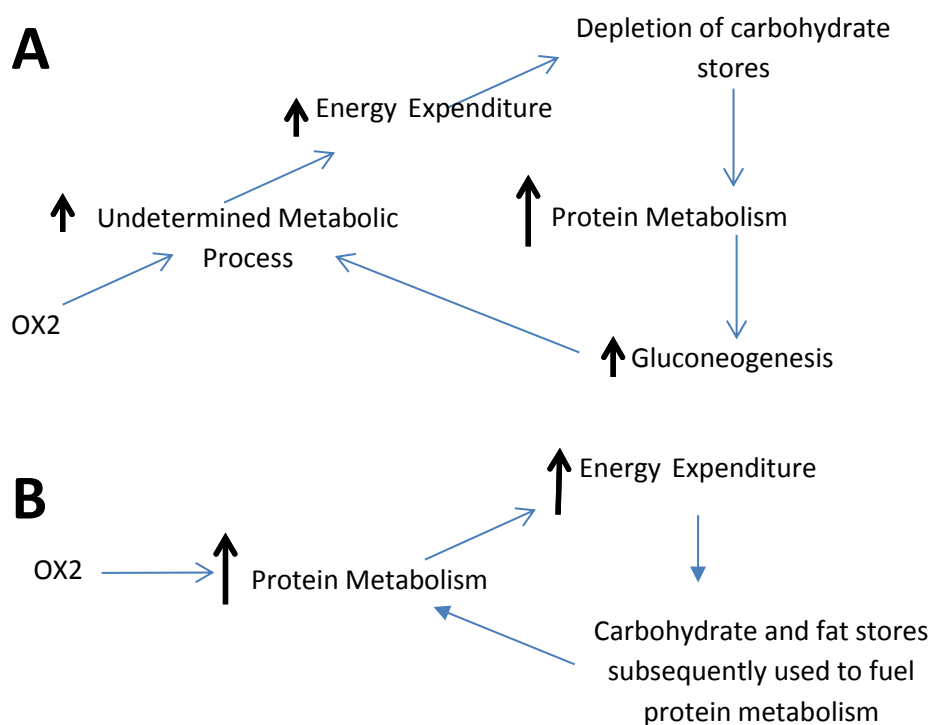


Figure 7.1 Representation of possible mechanisms by which OX2 increases energy expenditure and affects protein and fat metabolism.

In (A), fat and protein are broken down to allow gluconeogenesis to fuel an as yet undetermined metabolic process, after carbohydrate stores have all been used up. In (B), OX2 directly upregulates protein metabolism, and carbohydrate/fat stores are then used up to fuel this.

To investigate these possibilities, studies in this chapter will look at the acute effects of OX2 on protein metabolism. As seen in Chapter 2, OX2 increases energy expenditure within 2 hours of

administration, with a peak in oxygen consumption reached by 4 hours; enhanced oxygen consumption persists for 12 hours after administration (Figure 7.2).

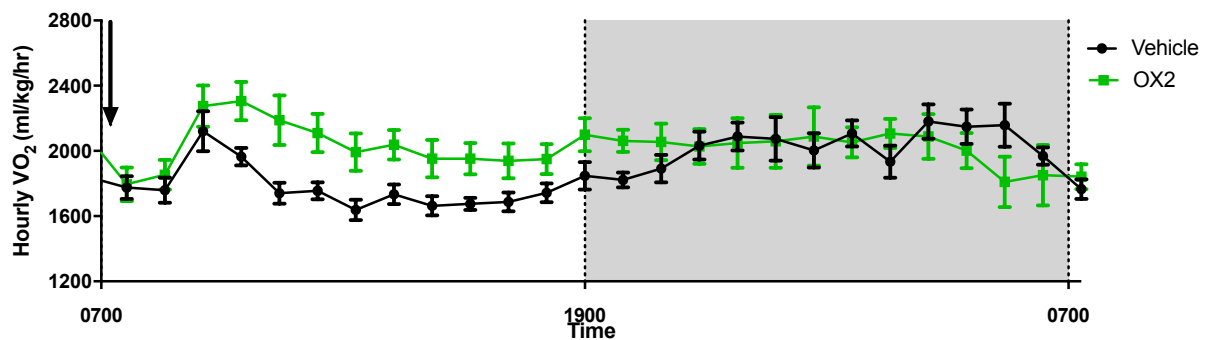


Figure 7.2 Effect of acute administration of OX2 on oxygen consumption in rats.
For study details please see 2.3.4.1, page 52

Therefore, whatever processes caused the initial acute energy expenditure effect of OX2 must be upregulated within 4 hours of OX2 administration. The rapid reduction in RER that occurred after an injection of OX2 (Figure 2.8) supported the idea that there was an acute increase in protein and/or fat metabolism. The chapter will therefore first determine if OX2 changes protein metabolism over the same time-course as it affects energy expenditure.

Contemporaneous changes in protein metabolism and energy expenditure would suggest, but cannot prove, that protein metabolism causes the increase in energy expenditure. Therefore, the second aim of this chapter is to determine if OX2 directly upregulates protein metabolism *in vivo*, or whether it is only secondary to the need to fuel other metabolic processes.

7.2 CHAPTER SUMMARY AND AIMS

This chapter will

- Look at acute changes in protein metabolism after administration of the oxyntomodulin analogue OX2, to determine if this occurs over the same time course as an increase in energy expenditure.
- Determine whether these changes in metabolism are a primary effect of OX2 or secondary to the need for fuel of another underlying enhanced metabolic process as carbohydrate resources are exhausted.

7.3 METHODS

7.3.1 PEPTIDES

As described in previous chapters, the oxyntomodulin analogue OX2 was designed by Professor S Bloom (Imperial College, London) and synthesized by Insight Biotechnology Limited (Wembley, UK). In all studies in this chapter, OX2 was administered in a long-acting formulation as previously described 2.3.1.

7.3.2 *IN VIVO* STUDIES

All animal procedures undertaken were approved by the British Home Office Animal (Scientific Procedures) Act 1986 (Project Licence 70/7236).

7.3.2.1 ANIMAL HUSBANDRY

Adult male Wistar rats (Charles River, Margate, UK) were single-housed in individual cages under controlled temperature environment (21-23°C) with a 12:12 hour light-dark cycle (lights on at 0700) conditions. All animals had *ad libitum* access to water and RM1 diet (Special Diet Services, Witham, UK). Prior to all experiments, animals were extensively handled and acclimatised to IP or SC injections to limit the non-specific stress effects of handling during any study.

7.3.3 ACUTE EFFECT OF OX2 ON PLASMA MARKERS OF FAT AND PROTEIN METABOLISM IN *AD LIBITUM* FED RATS

This study was designed to see if changes in protein and fat metabolism in response to a single dose of OX2 (as demonstrated by plasma amino acid and β -hydroxybutyrate levels) take place over a similar time course to the changes in energy expenditure that were seen in CLAMS metabolic cages following a single dose of OX2.

16 rats were randomised to two groups of equal mean body weight (average body weight 515g, range 467-597g). All animals were *ad libitum* fed on standard chow. At 0900 they received a single subcutaneous injection of either vehicle or OX2 (40nmol/kg). Food weight was measured at baseline and again at 2, 6 and 24 hours. Blood was taken at each of these time points from tail-vein venesection for immediate assessment of blood glucose (Contour blood glucose monitor, Bayer, Leverkusen, Germany). A further 200 μ l of blood was collected into lithium heparin Microvette® tubes (Starstedt, Numbrecht, Germany). The blood was centrifuged at 10,000rpm for 8 minutes and the plasma stored at -80°C. The plasma was subsequently analysed for L-amino acid concentration using a colorimetric test following the manufacturer's instructions (MAK002, Sigma-Aldrich), and for beta-hydroxybutyrate using a colorimetric test following the manufacturer's instructions (CAY700190, Cayman Chemical).

7.3.4 ACUTE EFFECT OF OX2 ON MOLECULAR MARKERS OF PROTEIN METABOLISM IN *AD LIBITUM* FED RATS

This study was designed to augment the results obtained from the previous study (7.3.3) by looking at acute changes in molecular markers of the protein metabolism in response to a single dose of OX2.

16 rats were randomised to two groups of equal mean body weight (average body weight 538g, range 475-627g). All animals were *ad libitum* fed on standard RM1 chow. Between 0900 and 1100, rats received a subcutaneous injection of either vehicle or OX2 (40nmol/kg). Body weight and food weight were measured at baseline. Exactly 4 hours after the initial injection, the animal was culled using CO₂ asphyxiation and cervical dislocation. Food intake over the 4 hours was also noted. Just prior to culling, blood was taken from tail-vein venesection for a glucose measurement (Contour blood glucose meter, Bayer, Leverkusen, Germany). After cervical dislocation, blood was obtained from cardiac puncture into EDTA tubes (BD Vacutainer, Franklin Lakes, USA) containing 4µl of protease inhibitor (Sigma Aldrich). The blood was centrifuged at 6000rpm for 10 minutes and the plasma stored at -80°C. Brown adipose, liver, kidney, white adipose and skeletal muscle samples were dissected and snap-frozen in liquid nitrogen. These samples were stored at -80°C.

Plasma samples were subsequently assayed for L-amino acid levels as previously described (7.3.3). Liver samples underwent mRNA extraction and quantitative PCR as described previously (see paragraph 4.3.4). All primers were purchased from ThermoFischer Scientific (Waltham, USA). The following TaqMan[®] gene-expression primers sets were used: argininosuccinate synthetase Rn00565808_g1; carbamoyl phosphate synthetase Rn00567109_m1; glucose-6-phosphatase Rn00689876_m1; phosphoenolpyruvate carboxykinase (PEPCK) Rn01529014_m1; these were compared to the endogenous control 18s.

7.3.5 ACUTE EFFECT OF OX2 ON URINARY UREA IN *AD LIBITUM* FED RATS

When protein is degraded, the amine group is converted to urea for excretion; urinary urea is therefore a marker of protein metabolism. This study investigated the effect of acute administration of OX2 on urea excretion in rats.

16 rats were randomised to two groups of equal mean body weight (average body weight 556g, range 506-606g). At 1600 all animals were placed within renal metabolic cages with free access to water and powdered RM1 diet. At 16:30, animals received a subcutaneous injection of either vehicle or OX2 (40nmol/kg). The animals were returned to their home cages at 0800 the following morning. Water consumption, food intake, urine volume and faecal mass were recorded. Urine samples were

taken and centrifuged at 2000rpm for 10 minutes, and the supernatant was then stored at -80°C. Urine urea concentration was subsequently determined by an enzymatic method run on Abbott Architect autoanalyser.

7.3.6 EFFECT OF SUPPLEMENTARY DEXTROSE ON THE CHANGES IN METABOLIC PROCESSES INDUCED BY OX2 IN *AD LIBITUM* FED RATS.

If OX2 increases protein metabolism because carbohydrate stores are exhausted and there is a need for a gluconeogenic substrate, then supplementation of rats with extra glucose should limit the changes in protein metabolism as there would be less requirement for gluconeogenesis. This hypothesis was investigated in this study, where animals given vehicle or OX2 were supplemented with IP dextrose, and the effect on a variety of metabolic processes was determined.

32 rats were randomised to 4 groups of equal mean body weight (average body weight 387g, range 344-423g). All animals had free access to standard laboratory chow. Between 0900 and 1100 all animals received a single subcutaneous injection of either vehicle or OX2 (40nmol/kg). At the same time, half of those animals given vehicle and half of those receiving OX2 were given an intraperitoneal dose of 20% dextrose (10ml/kg); the other animals received the same volume injection of saline. The same injections were repeated 2 hours later. 4 hours after the initial injections, the animals were culled using CO₂ asphyxiation and cervical dislocation. Immediately prior to CO₂ asphyxiation, blood glucose was measured following tail-vein venesection using a Contour blood glucose monitor (Bayer, Leverkusen, Germany). After cervical dislocation, blood was obtained from cardiac puncture into EDTA tubes (BD Vacutainer, Franklin Lakes, USA) containing 4µl of protease inhibitor (Sigma Aldrich). The blood was centrifuged at 6000rpm for 10 minutes and the plasma stored at -80°C. Liver, white adipose and skeletal muscle samples were dissected and stored at -80°C.

Plasma samples were subsequently assayed for L-amino acid levels as previously described (7.3.3). Liver samples underwent mRNA extraction and quantitative PCR for argininosuccinate synthetase, carbamoyl phosphate synthetase, glucose-6-phosphatase and phosphoenolpyruvate carboxykinase mRNA. The primers used were as above. Hepatic glycogen was also determined using a colourimetric assay as per the manufacturer's instructions (ab65620, Abcam, Cambridge). Plasma insulin was measured using an ultra-sensitive rat insulin ELISA kit (catalogue number 90060, Crystal Chem inc, Downers Grove, USA), under the manufacturer's high-range assay protocol.

7.4 RESULTS

7.4.1 KEY RESULTS FOR CHAPTER 7

- Single dose OX2 acutely affects protein metabolism, over the same time-course as it increases energy expenditure, with:
 - Reduction in plasma amino acids.
 - Increased hepatic expression of urea cycle enzyme mRNA.
 - OX2 does not increase ketogenesis within 4 hours.
 - OX2 acutely increases plasma glucose but does not change hepatic expression of gluconeogenic enzyme mRNA.
 - The changes in protein metabolism are not suppressed by supplementary glucose administration, suggesting these effects are directly caused by OX2 rather than secondary to inadequate carbohydrate/fuel stores.
-

7.4.2 ACUTE EFFECT OF OX2 ON PLASMA MARKERS OF FAT AND PROTEIN METABOLISM IN *AD LIBITUM* FED RATS

After an acute injection of OX2 in fed rats, there were significant effects of OX2, time and an interaction between OX2 and time on plasma L-amino acid concentration (simple repeated measures 2-way ANOVA, effect of OX2, $F(1,40)=85.8$, $p<0.0001$; effect of time: $F(2,40)=36.0$, $p<0.0001$; interaction, $F(2,40)=10.9$, $p<0.001$). OX2 significantly suppressed plasma L-amino acids at 2 and 6 hours; levels returned to those of control animals by 24 hours (average plasma L amino-acid level, mean $\pm$ SEM nmol/ μ l, 2hr vehicle: 2.5 ± 0.1 nmol/ μ l; OX2: 1.8 ± 0.1 nmol/ μ l, $p<0.0001$; 6hr vehicle: 2.7 ± 0.1 nmol/ μ l; OX2: 2.0 ± 0.1 nmol/ μ l, $p<0.0001$; 24hr vehicle: 2.8 ± 0.1 nmol/ μ l; OX2: 2.6 ± 0.1 nmol/ μ l) (Figure 7.3A and Table 7-1).

There was a significant effect of time but not of OX2 on plasma ketone levels, and no interaction between OX2 and time (simple repeated measures 2-way ANOVA, effect of OX2, $F(1,40)=0.03$, $p=0.9$; effect of time, $F(2,40)=5.8$, $p<0.01$; interaction, $F(2,40)=1.8$, $p=0.2$). Ketone levels tended to increase over time, though mainly in the OX2 group, where ketone levels started lower than in the vehicle group (average plasma beta-hydroxybutyrate concentration mean $\pm$ SEM mM, 2hr vehicle: 0.27 ± 0.1 mM; OX2: 0.23 ± 0.1 mM; 6hr vehicle: 0.25 ± 0.1 mM; OX2: 0.27 ± 0.1 mM; 24hr vehicle: 0.29 ± 0.1 mM; OX2: 0.32 ± 0.1 mM) (Figure 7.3B).

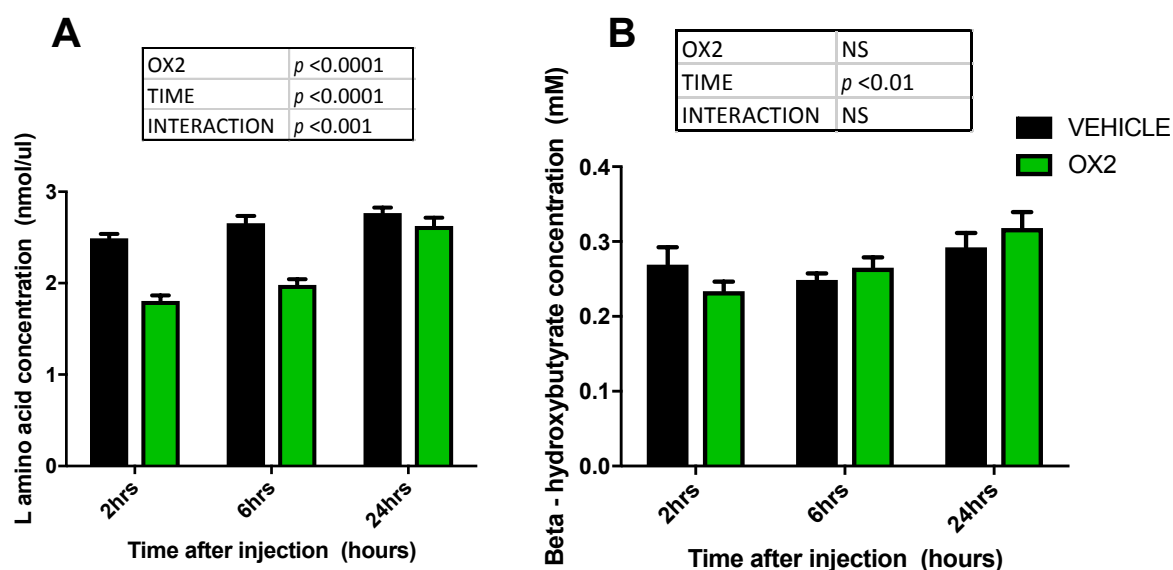


Figure 7.3 Effect of OX2 on plasma L-amino acid and β -hydroxybutyrate levels in fed rats over 24 hours.

Fed male Wistar rats received injections of either vehicle or OX2 (40nmol/kg) at 0900. Blood samples were taken at 2, 6 and 24 hours and assessed for L amino acid concentration (A) and plasma β hydroxybutyrate levels (B). Animals had free access to standard chow throughout. Data presented as mean $\pm$ SEM. $n=8$. Statistical analysis undertaken with repeated measures 2-way ANOVA.

TIME (hours after injection)	Plasma L-amino acid levels mean $\pm$ SEM nmol/ μ l		STATISTICAL ANALYSIS 2-Way ANOVA (post-hoc Sidak's multiple comparison test)
	VEHICLE	OX2 (40nmol/kg)	
2	2.5 $\pm$ 0.1	1.8 $\pm$ 0.1	$p < 0.0001$
6	2.7 $\pm$ 0.1	2.0 $\pm$ 0.1	$p < 0.0001$
24	2.8 $\pm$ 0.1	2.6 $\pm$ 0.1	NS

Table 7-1 Acute effect of OX2 on plasma L-amino acids over 24 hours

After an acute injection, there was a significant effect of OX2 but not time on blood sugar levels in fed animals; there was no interaction between OX2 and time (ordinary 2-way ANOVA, effect of OX2, $F(1,24)=16.7$, $p < 0.001$; effect of time, $F(2,24)=2.5$, $p=0.1$; interaction, $F(2,24)=1.1$, $p=0.4$). OX2 caused a rise in blood glucose levels, particularly at 2 hours after injection, and levels remained above vehicle throughout the 24 hours (average BM, mean $\pm$ SEM mmol/L, 2hr vehicle: 5.8 ± 0.1 mmol/L; OX2: 7.4 ± 0.5 mmol/L; 6hr vehicle: 5.8 ± 0.1 mmol/L; OX2: 6.7 ± 0.2 mmol/L; 24hr vehicle: 5.5 ± 0.5 mmol/L; OX2: 6.3 ± 0.3 mmol/L) (Figure 7.4A and Table 7-2).

There was a significant effect of time but not OX2 on food intake over each time period, and there was an interaction between OX2 and time (ordinary 2-way ANOVA, effect of OX2, $F(1,42)=0.0004$, $p=1$; effect of time, $F(2, 42)=560$, $p < 0.0001$; interaction $F(2,42)=0.8$, $p=0.5$). However, there was a

tendency over each time-period for the OX2 group to eat more than the vehicle group (average food intake, mean $\pm$ SEM(g), 0-2hrs vehicle: 1.7 ± 0.5 g; OX2: 2.4 ± 0.7 g; 2-6hrs vehicle: 1.9 ± 0.5 g; OX2: 2.3 ± 0.5 g; 6-24hrs vehicle: 25.1 ± 0.8 g; OX2: 23.9 ± 1.3 g) (Figure 7.4B).

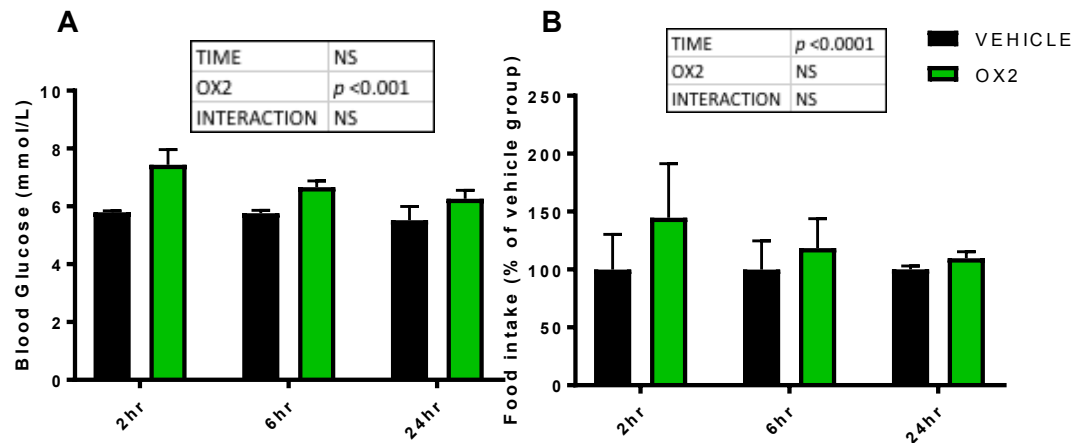


Figure 7.4 Effect of OX2 on blood glucose and food intake in fed rats over 24 hours.

Fed male Wistar rats received injections of either vehicle or OX2 (40nmol/kg) at 0900. Blood samples were taken at 2, 6 and 24 hours and assessed for blood glucose levels (A) using a Contour Blood Glucose meter; over the same time intervals (B) food weight was measured – expressed as % of the vehicle group intake. Animals had free access to standard chow throughout. Data of blood glucose presented as mean $\pm$ SEM while data of food intake presented as percentage compared to vehicle. $n=8$. Statistical analysis was undertaken with ordinary 2-way ANOVA on the raw data.

TIME (hours after injection)	Plasma glucose levels mean $\pm$ SEM mmol/L		STATISTICAL ANALYSIS 2-way ANOVA (post-hoc Sidak's multiple comparson test)
	VEHICLE	OX2 (40nmol/kg)	
2	5.8 ± 0.1	7.4 ± 0.5	$p < 0.01$
6	5.8 ± 0.1	6.7 ± 0.2	NS
24	5.5 ± 0.5	6.3 ± 0.3	NS

Table 7-2 Effect of OX2 on blood glucose levels over 24 hours.

7.4.3 ACUTE EFFECT OF OX2 ON METABOLIC MARKERS OF PROTEIN AND FAT METABOLISM

4 hours after an injection of OX2, there was a trend to increased food intake in the OX2 group (mean food intake $\pm$ SEM (g), vehicle: 2.3 ± 0.7 g; OX2: 4.2 ± 0.6 g, $p=0.08$) (Figure 7.5A). At the same time, there was a significant elevation in blood glucose levels in the OX2 group (average blood glucose level, mean $\pm$ SEM (mmol/L), vehicle: 4.9 ± 0.2 mmol/L; OX2: 5.7 ± 0.3 mmol/L, $p < 0.05$) (Figure 7.5B).

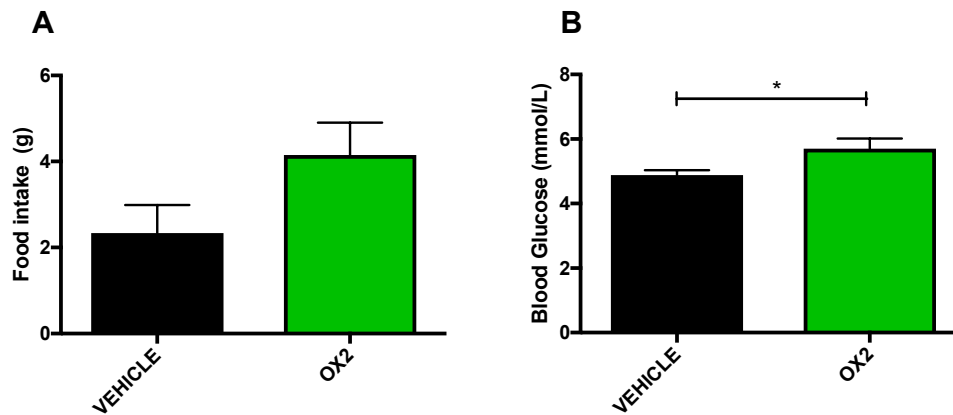


Figure 7.5 Effect of OX2 at 4 hours on food intake and blood glucose in fed rats.

Male Wistar rats were randomised to two groups of equal baseline body weight. Between 9 and 11am rats received injections of either vehicle or OX2 (40nmol/kg). Animals had free access to standard chow throughout. 4 hours after injection food intake (A) and blood glucose levels (B) were measured. Data presented as mean ± SEM. n=8. Statistical analysis was undertaken using unpaired t-tests. * $p < 0.05$ vs vehicle.

4 hours after injection of OX2, there was a significant reduction in plasma L-amino acids (average plasma L-amino acid concentration, mean ± SEM (nmol/μl), vehicle: 2.9 ± 0.09 nmol/μl; OX2: 2.2 ± 0.06 nmol/μl, $p < 0.0001$). In contrast, there was no significant difference in plasma ketone levels (average beta-hydroxybutyrate concentration, mean ± SEM (mM), vehicle: 0.2 ± 0.01 mM; OX2: 0.2 ± 0.02 mM) (Figure 7.6A and B).

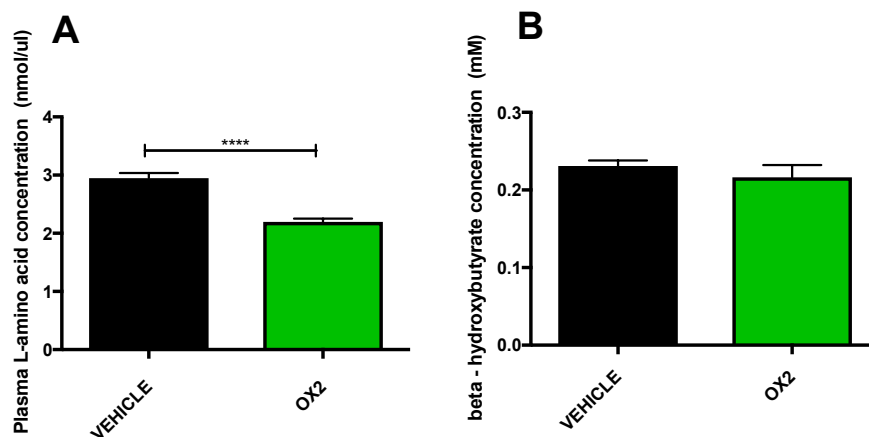


Figure 7.6 Effect of OX2 on plasma L-amino acid and ketone levels 4 hours after injection.

Male Wistar rats were randomised to two groups of equal baseline body weight. Between 9 and 11am rats received injections of either vehicle or OX2 (40nmol/kg). Animals had free access to standard chow throughout. 4 hours after injection blood was taken and plasma L-amino acid concentration (A) and plasma ketone levels (B) were determined. Data presented as mean ± SEM. n=8. Statistical analysis was undertaken using unpaired t-tests. **** $p < 0.0001$ vs vehicle.

OX2 significantly affected hepatic expression of urea cycle enzymes, but not gluconeogenic enzymes (ordinary 2-way ANOVA, effect of treatment $F(1,50)=73.9$, $p < 0.0001$; effect of enzyme $F(3,50)=20.6$, $p < 0.0001$; interaction $F(3,50)=20.6$, $p < 0.0001$). Both argininosuccinate synthetase and carbamoyl

phosphate synthetase, two enzymes in the urea cycle, were significantly elevated within 4 hours of administration of OX2 (argininosuccinate synthetase average mRNA fold change, mean $\pm$ SEM, vehicle: 1.0 ± 0.1 ; OX2: 11.4 ± 2.1 ; $p < 0.0001$; carbamoyl phosphate synthetase average mRNA fold change, mean $\pm$ SEM, vehicle: 1.0 ± 0.1 ; OX2: 8.3 ± 1.5 , $p < 0.0001$). There was no significant elevation in either of the gluconeogenic enzymes glucose-6-phosphatase or PEPCK (glucose-6-phosphatase average mRNA fold change, mean $\pm$ SEM, vehicle: 1.0 ± 0.2 ; OX2: 1.5 ± 0.4 , $p = \text{NS}$; PEPCK average mRNA fold change, mean $\pm$ SEM, vehicle: 1.0 ± 0.3 ; OX2: 1.6 ± 0.3 , $p = \text{NS}$) (Figure 7.7).

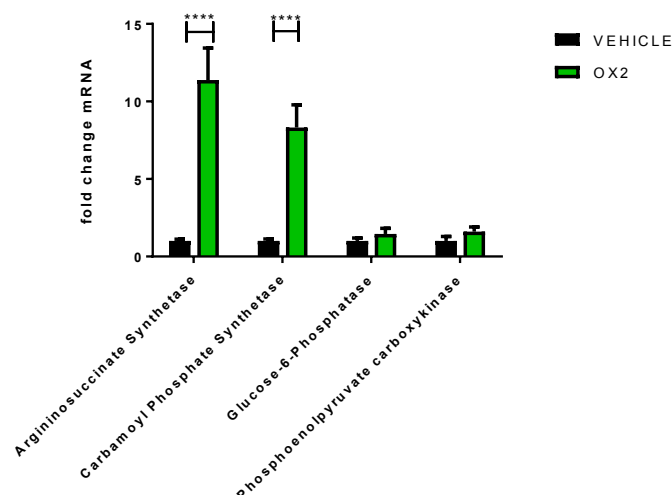


Figure 7.7 Effect of OX2 on mRNA levels of urea cycle and gluconeogenic enzymes 4 hours after injection.

Male Wistar rats were randomised to two groups of equal baseline body weight. Between 9 and 11am rats received injections of either vehicle or OX2 (40nmol/kg). 4 hours after injection all animals were culled and liver samples taken for mRNA extraction and qPCR. Levels of the urea cycle enzymes argininosuccinate synthetase and carbamoyl phosphate synthetase, and the gluconeogenic enzymes glucose-6-phosphatase and phosphoenolpyruvate carboxykinase were assessed. Animals had free access to standard chow throughout. All mRNA expression was normalised to the housekeeping gene 18s. Data presented as mean mRNA fold change $\pm$ SEM. $n=8$. Statistical analysis undertaken using ordinary 2-way ANOVA with post-hoc Tukey's Multiple Comparison Tests. **** $p < 0.0001$ vs vehicle.

	VEHICLE	OX2	
FOOD INTAKE	REFERENCE	INCREASED	Figure 7.5
PLASMA AMINO ACIDS	REFERENCE	SUPPRESSED	Figure 7.6
PLASMA KETONES	REFERENCE	NO EFFECT	Figure 7.6
PLASMA GLUCOSE	REFERENCE	INCREASED	Figure 7.5
UREA CYCLE ENZYMES	REFERENCE	INCREASED	Figure 7.7
GLUCONEOGENIC ENZYMES	REFERENCE	NO EFFECT	Figure 7.7

Table 7-3 Summary of metabolic changes which occur in rats 4 hours after a single dose of vehicle or OX2.

7.4.4 ACUTE EFFECT OF OX2 ON URINARY UREA EXCRETION IN RATS

Over the 16 hours in the renal metabolic cages, total urine output in the OX2 group was increased when compared to the vehicle group, though not significantly (average urine output, mean $\pm$ SEM

(ml), vehicle: 24 ± 4 ml; OX2 group: 37 ± 5 ml) ($p=0.057$). There was also a non-significant increase in water intake in the OX2 group (average water consumption, mean $\pm$ SEM (ml), vehicle group: 23 ± 3 ml; OX2 group: 27 ± 6 ml) (Figure 7.8A and B).

There was a tendency to reduced urinary urea concentration in the OX2 group (average urinary urea concentration, mean $\pm$ SEM (nmol/ μ l), vehicle group: 378 ± 34 nmol/ μ l; OX2 group: 331 ± 49 nmol/ μ l); however, when combined with the increased urinary volume, there was an increased total urea excretion in the OX2 group compared to the vehicle group (average total urea excretion, mean $\pm$ SEM (nmol/16 hours); vehicle group 8 ± 1 nmol/16 hours; OX2 group 13 ± 3 nmol/16 hours, $p=0.05$) (Figure 7.9A and B).

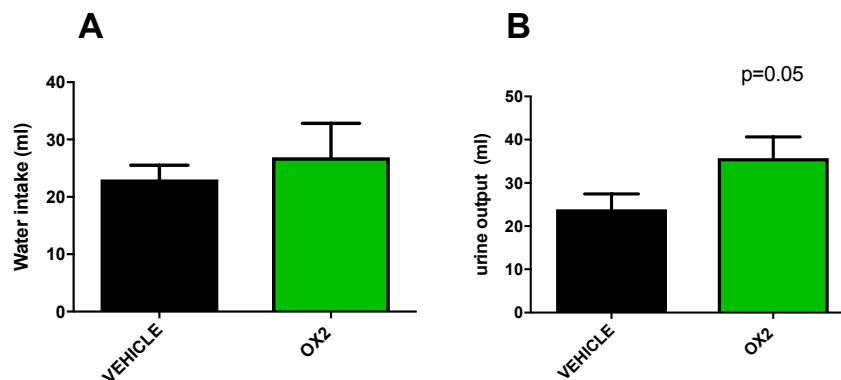


Figure 7.8 Effect of OX2 on fluid balance in rats over 16 hours.

Water intake (A) and urine output (B) over 16 hours in rats who had a single subcutaneous injection of vehicle or OX2 (40nmol/kg). Animals had free access to laboratory chow throughout. Data shown is mean $\pm$ SEM. $n=8$. Statistical analysis of water intake done with an unpaired t-test; of urine output with a Mann-Whitney U test.

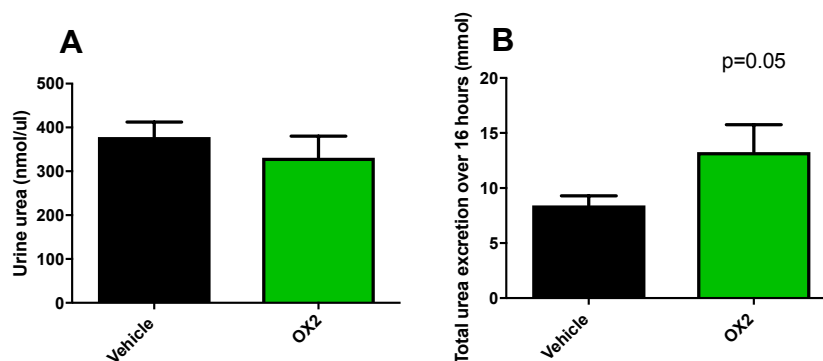


Figure 7.9 Effect of OX2 on urea excretion in rats over 16 hours.

Urine urea concentration (A) and total urea output (B) over 16 hours in male Wistar rats who had a single subcutaneous injection of vehicle or OX2 (40nmol/kg). Animals had free access to laboratory chow throughout. Data shown is mean $\pm$ SEM. $n=6-7$. Statistical analysis undertaken using unpaired t-tests.

7.4.5 EFFECT OF DEXTROSE SUPPLEMENTATION ON THE CHANGES IN PROTEIN METABOLISM CAUSED BY OX2

Over the 4 hours after the injections of OX2 and dextrose, in animals with free access to laboratory chow, there was no effect of OX2 or dextrose on food intake, and there was no interaction between OX2 and dextrose (ordinary 2-way ANOVA, effect of dextrose, $F(1,25)=2.3$, $p=0.1$; effect of OX2, $F(1,25)=3.5$, $p=0.07$; interaction (1,25)=1.9, $p=0.2$), though there was a tendency for the animals given OX2 to eat more (average food intake, mean $\pm$ SEM(g), vehicle/saline: 1.7 ± 0.6 g; OX2/saline: 3.4 ± 0.5 g; vehicle/dextrose: 1.7 ± 0.4 g; OX2/dextrose: 1.9 ± 0.5 g) (Figure 7.10A).

There was no significant effect of either OX2 or dextrose on blood glucose levels, nor was there an interaction between OX2 and dextrose (ordinary 2-way ANOVA, effect of dextrose, $F(1,25)=0.9$, $p=0.3$; effect of OX2, $F(1,25)=1.9$, $p=0.2$; interaction, $F(1,25)=0.2$, $p=0.7$) (average blood glucose, mean $\pm$ SEM (mmol/l), vehicle/saline: 5.4 ± 0.2 mmol/l; OX2/saline: 5.7 ± 0.3 mmol/l; vehicle/dextrose: 5.0 ± 0.3 mmol/l; OX2/dextrose: 5.6 ± 0.3 mmol/l) (Figure 7.10B).

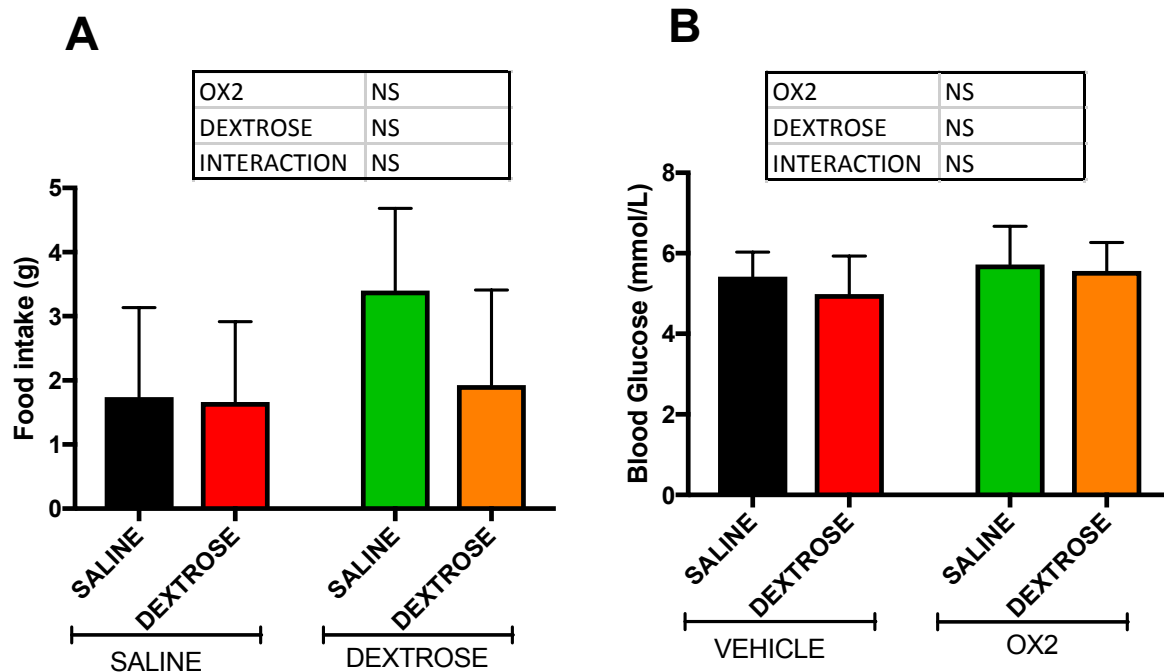


Figure 7.10 Effect of combined OX2 and dextrose on blood glucose level and food intake in fed rats 4 hours after injection.

Male Wistar rats were randomised to four groups of equal baseline body weight. Between 9 and 11am rats received SC injections of either vehicle or OX2 (40nmol/kg). Half of the vehicle rats and half the OX2 rats also received an IP injection of 20% dextrose (10ml/kg); the remaining rats had an IP injection of saline at the same volume. The IP injection was repeated 2 hours later. 4 hours after the SC injection blood glucose (A) and food intake (B) were measured. Animals had free access to standard laboratory chow throughout. Data presented as mean $\pm$ SEM. $n=8$. Statistical analysis was undertaken using ordinary 2-way ANOVA.

There was a significant effect of OX2 but not dextrose on hepatic glycogen content 4 hours after injection, and there was no interaction between OX2 and dextrose (ordinary 2-way ANOVA, effect of dextrose, $F(1, 28)=2.1$, $p=0.2$; effect of OX2, $F(1,28)=76.8$, $p<0.0001$; interaction, $F(1,28)=0.2$, $p=0.6$).

OX2 reduced hepatic glycogen content – no glycogen was detectable in any of the OX2/saline animals, but low levels were found in the OX2/dextrose animals (average hepatic glycogen content $\pm$ SEM mg/g tissue, vehicle/saline: 27 ± 2 mg/g; OX2/saline: 0 ± 0 mg/g; vehicle/dextrose: 30 ± 3 mg/g; OX2/dextrose: 6 ± 5 mg/g) (Figure 7.11A).

Similarly, OX2 but not dextrose had a significant effect on plasma L-amino acid levels, and there was no interaction between OX2 and dextrose (ordinary 2-way ANOVA, effect of dextrose, $F(1,28)=0.3$, $p=0.6$; effect of OX2, $F(1,28)=23.2$, $p<0.0001$; interaction, $F(1,28)=0.04$, $p=0.8$). As seen in the previous studies, OX2 reduced plasma L-amino acid levels (average plasma L-amino acid level, mean $\pm$ SEM (nmol/ μ l), vehicle/saline: 2.1 ± 0.1 nmol/ μ l; OX2/saline: 1.7 ± 0.1 nmol/ μ l; vehicle/dextrose: 2.1 ± 0.1 nmol/ μ l; OX2/dextrose: 1.6 ± 0.1 nmol/ μ l) (Figure 7.11B).

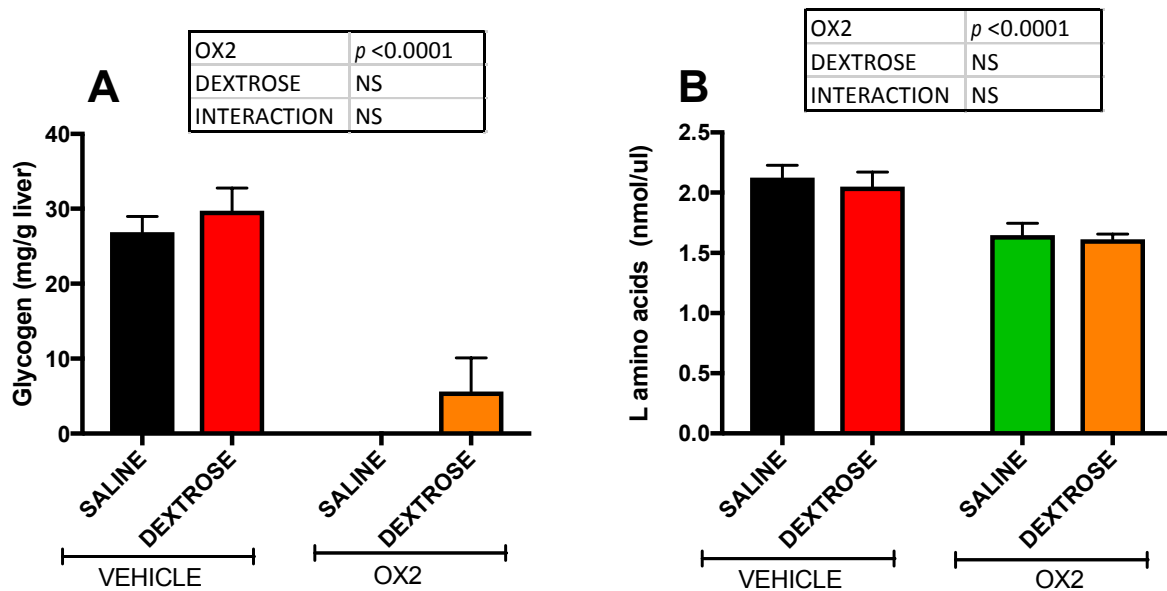


Figure 7.11 Effect of combined OX2 and dextrose on hepatic glycogen and plasma L-amino acid levels in fed rats 4 hours after injection.

Male Wistar rats were randomised to four groups of equal baseline body weight. Between 9 and 11am rats received SC injections of either vehicle or OX2 (40nmol/kg). Half of the vehicle rats and half the OX2 rats also received an IP injection of 20% dextrose (10ml/kg); the remaining rats had an IP injection of saline at the same volume. The IP injection was repeated 2 hours later. Animals had free access to standard laboratory chow throughout. 4 hours after the SC injection, animals were culled. Liver samples were analysed for glycogen content (A) and plasma L-amino acids (B) were determined. Data presented as mean $\pm$ SEM. $n=8$. Statistical analysis was undertaken using ordinary 2-way ANOVA.

There was an effect of OX2, an effect of dextrose, and an interaction between OX2 and dextrose on urea cycle enzyme mRNA levels (2-way ANOVA of argininosuccinate synthetase (ASS) mRNA, effect of dextrose $F(1,26)=5.7$, $p<0.05$; effect of OX2, $F(1,26)=13.1$, $p<0.01$; interaction, $F(1,26)=5.0$, $p<0.05$; 2-way ANOVA of carbamoyl phosphate synthetase mRNA, effect of dextrose, $F(1,23)=13.5$, $p<0.01$; effect of OX2, $F(1,23)=29.5$, $p<0.0001$; interaction, $F(1,23)=9.9$, $p<0.01$). OX2 elevated mRNA levels

of both enzymes, but the elevation was greater in those animals also given dextrose (argininosuccinate synthetase mRNA levels, average fold change compared to control group $\pm$ SEM, vehicle/saline: 1.0 ± 0.1 ; OX2/saline: 2.1 ± 0.5 ; vehicle/dextrose: 1.1 ± 0.3 ; OX2/dextrose: 3.9 ± 0.9) (carbamoyl phosphate synthetase mRNA levels, average fold change control group $\pm$ SEM, vehicle/saline: 1.0 ± 0.2 ; OX2/saline: 1.5 ± 0.1 ; vehicle/dextrose: 1.3 ± 0.2 ; OX2/dextrose: 3.2 ± 0.4) (Figure 7.12 and Table 7-4).

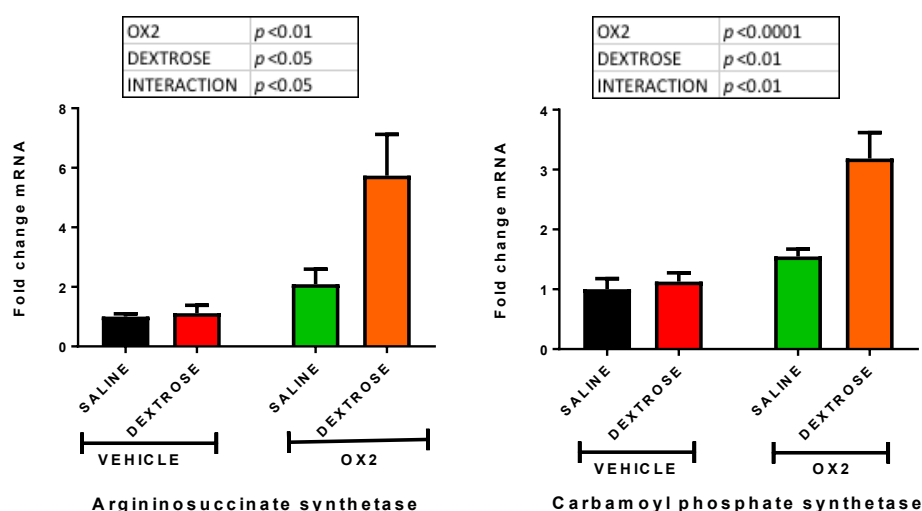


Figure 7.12 Effect of combined OX2 and dextrose on hepatic expression of urea cycle enzymes in fed rats 4 hours after injection.

Male Wistar rats were randomised to four groups of equal baseline body weight. Between 9 and 11am rats received SC injections of either vehicle or OX2 (40nmol/kg). Half of the vehicle rats and half the OX2 rats also received an IP injection of 20% dextrose (10ml/kg); the remaining rats had an IP injection of saline at the same volume. The IP injection was repeated 2 hours later. Animals had free access to standard laboratory chow throughout. 4 hours after the SC injection, animals were culled. Liver samples were processed for urea cycle enzyme mRNA (A) argininosuccinate synthetase (ASS) and (B) carbamoyl phosphate synthetase (CPS). All mRNA expression normalised to the housekeeping gene 18s. Data presented as mean mRNA fold change $\pm$ SEM. $n=8$. Statistical analysis was undertaken using ordinary 2-way ANOVA.

ENZYME	mRNA FOLD CHANGE $\pm$ SEM			
	IP SALINE		IP DEXTROSE	
	VEHICLE	OX2	VEHICLE	OX2
ASS	1.0 ± 0.1^c	2.1 ± 0.5^b	1.1 ± 0.3^a	3.9 ± 0.9^{abc}
CPS	1.0 ± 0.2^f	1.5 ± 0.1^d	1.3 ± 0.2^e	3.2 ± 0.4^{def}

Table 7-4 Effect of combined OX2 and dextrose on levels of hepatic urea cycle enzymes 4 hours after injection.

Statistical analysis was undertaken using ordinary 2-way ANOVA, with post-hoc Sidak's multiple comparison test. Significant differences exist between groups with the same superscript letter.

When gluconeogenic enzyme expression was analysed, there was no significant effect of OX2 or dextrose, nor an interaction between these factors on hepatic glucose-6-phosphatase mRNA levels

(2-way ANOVA of glucose-6-phosphatase (G6Pase) mRNA, effect of dextrose, $F(1,28)=0.01$, $p=0.9$; effect of OX2, $F(1,28)=0.6$, $p=0.5$; interaction, $F(1,28)=0.03$, $p=0.9$) (G6Pase mRNA levels, average fold change compared to control group $\pm$ SEM, vehicle/saline: 1.0 ± 0.4 ; OX2/saline: 0.8 ± 0.3 ; vehicle/dextrose: 1.0 ± 0.3 ; OX2/dextrose: 0.7 ± 0.2) (Figure 7.13A). OX2 did have a significant effect on levels of phosphoenolpyruvate carboxykinase (PEPCK) mRNA levels, though dextrose did not, nor was there any interaction (2-way ANOVA of phosphoenolpyruvate carboxykinase (PEPCK) mRNA, effect of dextrose, $F(1,28)=0.4$, $p=0.5$; effect of OX2, $F(1,28)=4.6$, $p<0.05$; interaction, $F(1,28)=0.08$, $p=0.8$). OX2 caused an increase in PEPCK levels (PEPCK mRNA levels, average fold change control group $\pm$ SEM, vehicle/saline: 1.0 ± 0.3 ; OX2/saline: 1.9 ± 0.7 ; vehicle/dextrose: 0.6 ± 0.1 ; OX2/dextrose: 1.7 ± 0.5) (Figure 7.13B).

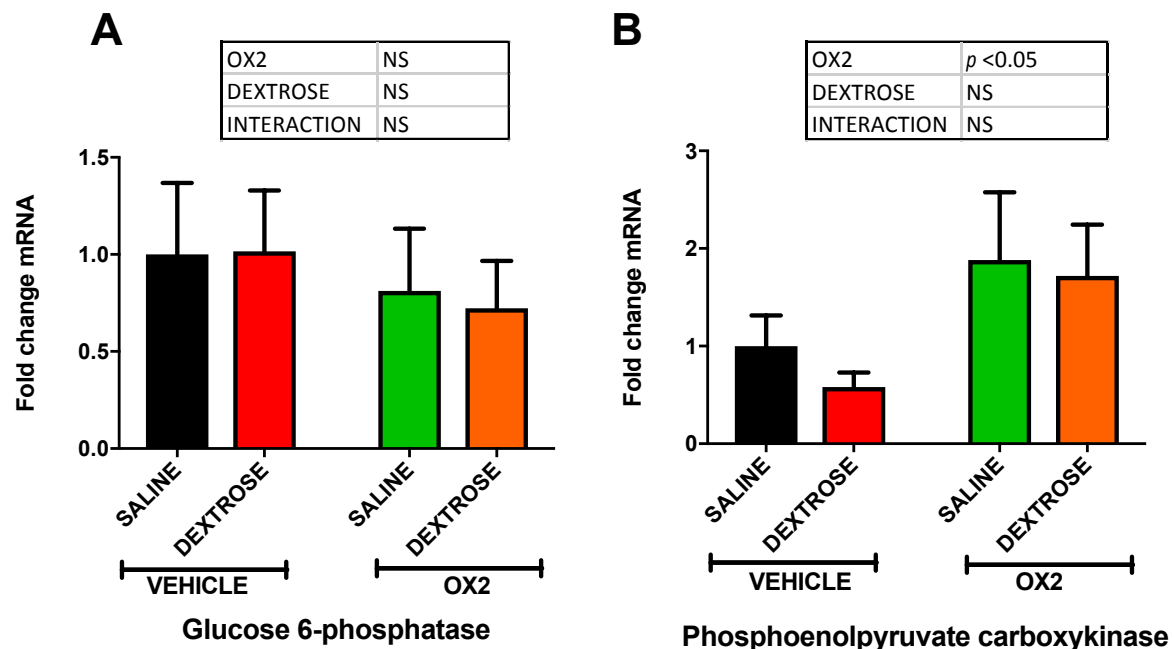


Figure 7.13 Effect of combined OX2 and dextrose on hepatic expression of gluconeogenic enzymes in fed rats 4 hours after injection.

Male Wistar rats were randomised to four groups of equal baseline body weight. Between 9 and 11am rats received SC injections of either vehicle or OX2 (40nmol/kg). Half of the vehicle rats and half the OX2 rats also received an IP injection of 20% dextrose (10ml/kg); the remaining rats had an IP injection of saline at the same volume. The IP injection was repeated 2 hours later. Animals had free access to standard laboratory chow throughout. 4 hours after the SC injection, animals were culled. Liver samples were processed for gluconeogenic enzyme mRNA (A) glucose 6-phosphatase (G6Pase) and (B) phosphoenolpyruvate carboxykinase (PEPCK). All mRNA expression normalised to the housekeeping gene 18s. Data presented as mean mRNA fold change $\pm$ SEM. $n=8$. Statistical analysis was undertaken using ordinary 2-way ANOVA.

There was no effect of either dextrose or OX2 on plasma insulin levels, nor was there any interaction between dextrose and OX2 (ordinary 2-way ANOVA, effect of dextrose, $F(1,25)=1.5$, $p=0.2$; effect of OX2, $F(1,25)=0.7$, $p=0.4$; interaction, $F(1,25)=0.003$, $p=1.0$) (average plasma insulin levels, mean $\pm$ SEM (ng/ml); vehicle/saline: 4.5 ± 1.0 ng/ml; OX2/saline: 5.5 ± 1.8 ng/ml; vehicle/dextrose: 3.2 ± 0.5 ng/ml; OX2/dextrose: 4.1 ± 0.7 ng/ml) (Figure 7.14).

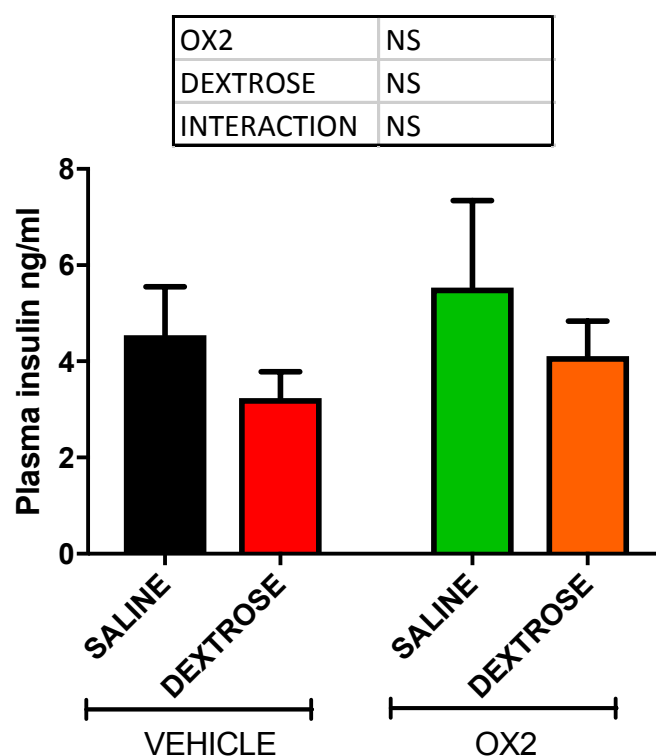


Figure 7.14 Effect of combined OX2 and dextrose on plasma insulin levels in fed rats 4 hours after injection.

Male Wistar rats were randomised to four groups of equal baseline body weight. Between 9 and 11am rats received SC injections of either vehicle or OX2 (40nmol/kg). Half of the vehicle rats and half the OX2 rats also received an IP injection of 20% dextrose (10ml/kg); the remaining rats had an IP injection of saline at the same volume. The IP injection was repeated 2 hours later. Animals had free access to standard laboratory chow throughout. 4 hours after the SC injection, animals were culled. Plasma samples were collected and analysed for insulin levels. Data presented as mean $\pm$ SEM. n=8. Statistical analysis was undertaken using ordinary 2-way ANOVA.

7.5 DISCUSSION

The first aim of this chapter was to determine if changes in protein metabolism caused by OX2 occur over the same time course as changes in energy expenditure. The studies in chapter 2 of this thesis showed that oxygen consumption increased straight after an injection of OX2, remained high for 12 hours, but normalised by 24 hours. In this chapter, plasma amino acids decreased from 2 to 6 hours (Figure 7.3, Figure 7.6 and Figure 7.11), reflecting an increased hepatic uptake of amino acids; levels normalised by 24 hours (Figure 7.3). These changes therefore occur over the same time course as the change in energy expenditure. There was also an increase in urea cycle enzymes within 4 hours of OX2 administration, enzymes involved in deamination of the amino-acids that enter the liver (Figure 7.7). Furthermore, urinary urea excretion increases over the 16 hours after OX2 administration, suggesting proteins have been degraded and excreted over this time period (Figure 7.8 and Figure 7.9). Together, these results show a change in protein metabolism in parallel to the change in energy expenditure.

The second aim of this chapter was to further investigate whether OX2 directly increase protein metabolism, or it is a secondary effect of requirement for carbon skeletons for gluconeogenesis. While protein metabolism changes acutely after an injection of OX2, fat metabolism does not. Lipolysis produces fatty acids which undergo β oxidation to ketones; but plasma ketones were not increased by acute administration of OX2 (Figure 7.3 and Figure 7.6). This contrasts with 2 previous studies which have shown an acute rise in plasma ketones after an injection of oxyntomodulin or a related analogue in mice (Du *et al.*, 2012, Pocai *et al.*, 2009). However, in the study by Du *et al.* the mice were fasting rather than fed, so the OXM injection may have augmented the natural response to fasting i.e. lipolysis leading to ketogenesis and gluconeogenesis; the feeding state of animals in the study by Pocai is not known. This contrasts with the studies in this chapter, where the rats were fed throughout the experiments, so there would be no fasting response. Moreover, when gluconeogenesis is required, both protein and fat stores are mobilised; it would be unusual for only protein to be broken down. Therefore the results in this chapter suggest a direct upregulation of protein metabolism independent of a need for a gluconeogenic source.

Additionally, the data about whether OXM acutely increase gluconeogenesis is conflicting. Pocai *et al.* found an increase in expression of several gluconeogenic enzymes including PEPCK after administration of a glucagon/GLP-1 co-agonist, whereas Day *et al.* found no increase in gluconeogenic enzymes in mice given a glucagon/GLP-1 coagonist (Day *et al.*, 2009). In this chapter, OX2 did significantly elevate PEPCK mRNA levels in the combined dextrose/OX2 study (Figure 7.13), but had no effect on PEPCK when a single dose was given to investigate the acute effects of OX2 on the molecular markers of fat and protein metabolism, despite otherwise-identical experimental

conditions (Figure 7.7). Furthermore, in neither study did OX2 significantly alter levels of glucose-6-phosphatase mRNA. However, it is recognised that expression of gluconeogenic enzyme mRNA does not necessarily correlate with passage through the gluconeogenic pathways. Therefore future *in vivo* isotopic studies would be necessary to confirm that oxyntomodulin does not change gluconeogenesis acutely (Chung et al., 2015, Samuel et al., 2009). Studies in this chapter do show that OX2 acutely increases blood glucose levels (Figure 7.4 and Figure 7.5), and further studies should determine if this is due only to glycogenolysis or a combination of glycogenolysis and gluconeogenesis.

The OX2/dextrose study further supports the direct effect of OX2 on protein metabolism. OX2 significantly reduced hepatic glycogen stores; however, while glycogen stores were completely exhausted in the saline/OX2 group, they remained detectable in the dextrose/OX2 group (Figure 7.11). This suggests that the added dextrose was used as a fuel source rather than glycogen in the dextrose/OX2 group; and as glycogen was still present, gluconeogenesis was unlikely to have been stimulated (McDaniel, 1975). If the dextrose injection had caused a surge in insulin release, this may have prevented glycogen breakdown and necessitated gluconeogenesis; however, there was no difference in insulin levels between any of the groups (Figure 7.14).

Despite the residual glycogen stores, plasma amino acid levels were equally suppressed in the dextrose/OX2 group as the saline/OX2 group (Figure 7.11). This suggests hepatic uptake of amino acids was equally elevated in both groups. Urea cycle enzymes were also increased in both groups given OX2; however, they were increased to a greater level in the OX2/dextrose group when compared to the OX2/saline group. Rather than dextrose suppressing protein metabolism, it may actually have increased it. A possible explanation for this is that if OXM directly increases transcription of urea cycle enzymes, supplying extra glucose via the IP-injection will further enable further upregulation of urea cycle enzymes as gene transcription and subsequent translation is an energy-consuming process.

Taken together, the findings in this chapter suggest that OX2 has a direct effect on hepatic protein metabolism, rather than the changes being secondary to a need to fuel gluconeogenesis. This was supported by the CLAMS studies in Chapter 2 (see 2.3.4.1): there was suppression of RER almost immediately after an injection of OX2, suggesting an acute change in metabolic pathways (Figure 2.8). To further investigate this, *in vitro* studies could determine the direct effect of oxyntomodulin on protein metabolism in the liver, including the effect on hepatic uptake of amino acids, activity of transaminases and urea synthesis within hepatocytes.

Though OX2 appears to change protein metabolism over the same time-course as it increases energy expenditure, this cannot confirm that the change in protein metabolism causes the change in energy expenditure. It is not possible *in vivo* to block hepatic uptake of amino acids, or urea enzyme synthesis, to determine if OXM still causes an increase in energy expenditure when these processes are inhibited. However, if oxyntomodulin analogues are to be used clinically to treat obesity, it is essential to know if protein metabolism is affected. Therefore further studies should look at acute changes in protein metabolism (using, for example, radiolabelled protein tracers) and changes in energy expenditure in man. Demonstrating this effect in man and rodents would support the idea that protein metabolism is essential for the energy expenditure effects of oxyntomodulin analogues.

7.6 CONCLUSIONS

The studies in this chapter show that OX2 causes changes in protein metabolism over the same time course as it changes energy expenditure. They also suggest that OXM directly stimulates protein metabolism. However, they cannot confirm that the changes in protein metabolism are responsible for the energy expenditure effects of OXM.

Chapter 8

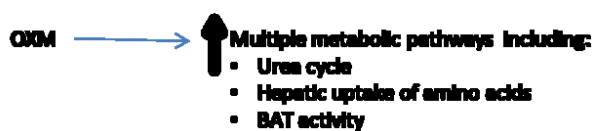
General Discussion

8.1 GENERAL DISCUSSION

Researchers have been interested in the potential of the gut hormone oxyntomodulin to treat obesity since it was shown to cause significant weight loss in overweight subjects in the early 2000s (Wynne et al., 2005). This weight loss is caused by a combination of reduced food intake and increased energy expenditure. Usually, increased energy expenditure is associated with hyperphagia, while a reduction in food intake suppresses metabolism; these compensatory mechanisms limit achievable weight loss. Therefore coupling an anorectic effect with increase energy expenditure is ideal for obesity treatments.

The aim of this thesis was to understand how oxyntomodulin increases energy expenditure. Using the oxyntomodulin analogue OX2, I have provided the first direct evidence that OXM increases energy expenditure via the glucagon receptor. While I have shown that oxyntomodulin does not increase BAT-mediated thermogenesis, I have shown that oxyntomodulin can affect metabolic pathways as diverse as bone turnover and the IGF-1 axis, and that it can affect growth in multiple tissues in rats. I have also shown that the sympathetic nervous system is important for the energy expenditure effects of oxyntomodulin. Moreover, this thesis contains the first studies to look at the effect of oxyntomodulin on protein metabolism.

ACUTELY:



CHRONICALLY:

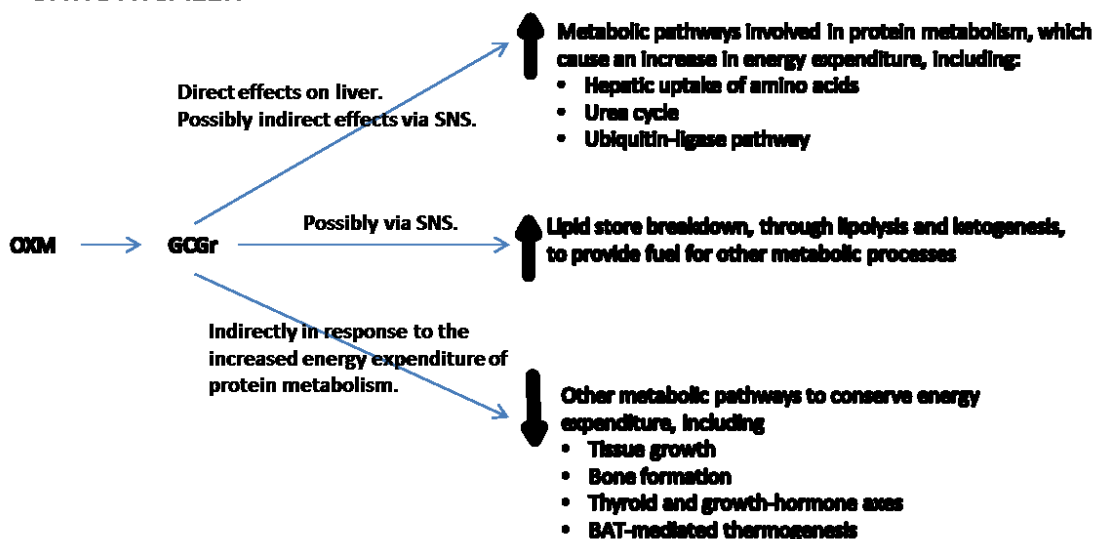


Figure 8.1 Mechanisms by which OXM increase energy expenditure as suggested by this thesis

Many studies have looked at the pathways by which oxyntomodulin reduces food intake (Cohen et al., 2003, Baggio et al., 2000, Dakin et al., 2001, Patterson et al., 2009). However, little is known about the way in which oxyntomodulin increases energy expenditure. This imbalance in knowledge may be, in part, because oxyntomodulin has a very short half-life. While it is relatively straightforward to measure acute changes in food intake following administration of the native hormone (Dakin et al., 2004, Baggio et al., 2004, Dakin et al., 2002), acute changes in energy expenditure are difficult to ascertain because of limitations in the equipment. Metabolic cages are often not readily available, and even when they can be used, they only measure oxygen consumption for 1 out of every 20 minutes for each animal; this means that any transient increases in oxygen consumption following an injection may be missed (Speakman, 2013).

There may be a more philosophical reason why studies have focussed on food intake rather than energy expenditure. When we think about obesity, the tendency is to assume that it will be solved by removing or reducing something. After all, an overweight body needs to 'lose' weight. It seems more intuitive to think about obesity treatment as being an analogous process of removal, whether of food or appetite. Patients are also more willing to diet/eat less, than increase their physical activity. The notion that something might need to be increased in order to reduce body weight is psychologically counter-intuitive and this may explain in part why many researchers have gravitated towards the input reduction end of the problem.

Because of the native hormone's short half-life, I used the oxyntomodulin analogue OX2 to investigate the energy expenditure effects of OXM. The first studies in this thesis confirmed that OX2 is a dual-agonist at the GLP-1 and GCG receptors. Though OX2 is more potent at both receptors than native OXM, OX2 has a similar ratio of potency at each receptor to OXM, validating its use to investigate the physiology of OXM. OX2 also has the advantage, as shown in Chapter 2, of being able to complex with zinc to form a subcutaneous depot, which enables a longer action *in vivo*.

In the CLAMS metabolic studies in Chapter 2, I demonstrated directly that OX2 acutely increases energy expenditure in rats, as measured by increased oxygen consumption. This was supported by the results of a pair-feeding study, with greater weight loss in the OX2 group than the pair-fed group; over the three days, the OX2 group lost 1.9% body weight, and the pair-fed group gained 1.7% body weight. These studies also gave a protocol for OX2 administration which would allow an investigation into the underlying mechanisms of this energy expenditure.

The studies in Chapter 3 of this thesis provide the first direct confirmation that OXM increases energy expenditure via activation of the glucagon receptor. This was shown by the preservation of the energy expenditure effect of OX2 when co-administered with the GLP-1 receptor blocker Ex9-39, while the same effect was lost when the glucagon receptor activity was eliminated by using the OX2-Glu3 peptide. This confirms what has been hypothesised in other studies in the literature (Kosinski et al., 2012, Price et al., 2015b). It is also supported by studies in the literature since the 1950s which show that administration of glucagon increases energy expenditure (Davidson et al., 1957, Salter et al., 1957), and more recent studies which find no increase in energy expenditure after administration of GLP-1 or its analogues (Tan et al., 2013, Larsen et al., 2001, Baggio et al., 2000).

The studies in CLAMS metabolic cages did not show any increase in physical activity in rats following OX2 administration. This supported the results of Liu *et al.* where rats given the oxyntomodulin analogue OX6421 did not increase their movement despite an increase in energy expenditure (Liu et al., 2010). This contrasts, however, with the findings of Lynch *et al.*, who found an increase in nocturnal movement in mice after administration of a glucagon/GLP-1 dual agonist (Lynch et al., 2014), and the findings of Wynne *et al.* who found that native oxyntomodulin increased physical-activity-related energy expenditure in humans subjects self-administering oxyntomodulin in a 'free living' environment. There are no explanations in either of these papers as to why administration of oxyntomodulin would increase physical activity. Voluntary physical activity requires a motivational component, and there have been no studies that investigate effects of oxyntomodulin on this. To further determine the impact of oxyntomodulin on voluntary physical activity, studies exploring modification in behaviour could be undertaken. However, the data from chapters 2 and 3, with the majority of published literature, suggest that OXM increases energy expenditure through changes in metabolic processes.

In the remaining chapters of this thesis, I looked at which underlying metabolic processes are changed by OX2, and whether these processes may affect energy expenditure in rats. As described in the General Introduction, many different processes contribute to the energy expended by an animal. This thesis took a systematic approach to examining these processes.

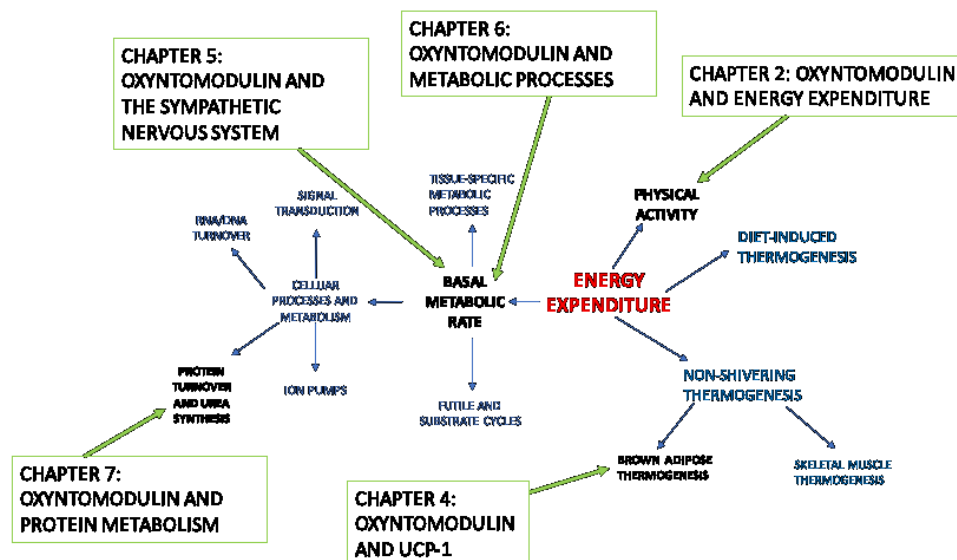


Figure 8.2 Metabolic processes involved in energy expenditure investigated in this thesis

Pharmacologically-stimulated brown adipose thermogenesis is an attractive mechanism for anti-obesity treatments (Kusminski et al., 2016). Targeted, chronic BAT activation in man by recurrent cold exposure is not associated with any adverse side effects, nor with down-regulation of its thermogenic effects (Lee et al., 2014). These are ideal features of an anti-obesity therapy. BAT-mediated thermogenesis was therefore the first mechanism I studied. OX2 did cause an acute increase in UCP-1, Dio2, ATGL and PPARGC1a mRNA, all markers of BAT activity, following a single injection, suggesting that oxyntomodulin could increase energy expenditure via BAT activation. However, even though I showed that chronic administration of OX2 can cause a sustained elevation in energy expenditure, with raised oxygen consumption in rats after 8 days continuous OX2 administration, this treatment was associated with suppression rather than elevation in BAT activity; UCP-1 levels were suppressed, and BAT temperature did not remain elevated. Therefore other mechanisms are more likely to be responsible for the chronic effects of OX2 on energy expenditure.

It is notable that, despite the significant interest in BAT-mediated thermogenesis, to date there are no successful treatments for obesity which activate BAT. Mirabegron, a β_3 -specific agonist used to treat over-active bladders, has potential as an obesity treatment, since it increases BAT activity as measured by the uptake of (18)F-FDG on PET/CT, as well as increasing metabolic rate (Cypess et al., 2015). However, it was also associated with an increase in heart rate and blood pressure. Moreover, as shown by the β_3 group in the study in Chapter 4, BAT activation is often accompanied by a compensatory hyperphagia which negates the weight-loss effect of any increase in energy expenditure. Human volunteers report an increased desire to eat and lower satiety following chronic BAT stimulation through cold exposure, with no subsequent reduction in body weight (Lee et al.,

2014). Therefore drugs which activate BAT may not be successful in the treatment of obesity unless also combined with an anorectic agent.

Induction of brite adipose tissue is another suggested target for anti-obesity pharmacotherapy. The study in chapter 4, however, showed no evidence for brite adipose induction following the chronic administration of OX2. Moreover, the $\beta 3$ group showed that, even though induction of brite adipose is a theoretically attractive target for obesity treatment, it is unlikely to make a significant clinical difference. UCP-1 levels in the $\beta 3$ group were elevated by almost 200x compared to vehicle; however, there was no weight loss in the $\beta 3$ group. This may be due to the compensatory hyperphagia noted above, but may also be because the exceedingly low baseline levels of brite adipose mean that, despite the statistically-significant increase in UCP-1 in WAT in the $\beta 3$ group, the actual mass of brite adipose is too low to have a measurable effect on thermogenesis. This is supported by the work of Shabalina *et al.* who estimated that, even after cold acclimation, the thermogenic capacity of brite adipose would be only 10-37% of that of BAT (Shabalina et al., 2013).

Research into non-shivering thermogenesis has focused on the role of brown and brite adipose. However, there are other potential mediators of NST e.g. skeletal muscle induced thermogenesis through sarcolipin, or the recently discovered PM20D1, a secreted enzyme which uncouples respiration in myoblast and osteosarcoma cell lines *in vitro*. Alternative sources of NST may be more important than BAT in man (Cypess et al., 2012). Alternative NST pathways were not investigated in this thesis, and therefore any future studies in this arena should look at whether OXM analogues have any effect on these other mediators of NST.

The studies in chapter 4 did show that OX2 increases sympathetic nervous system activity, as measured by plasma norepinephrine levels. Because SNS activation does increase energy expenditure, the experiments in chapter 5 were designed to see if SNS activation was responsible for the energy expenditure effects of oxyntomodulin. These studies confirmed previous studies that show the SNS controls basal metabolic rate (Villanueva et al., 2002, Lin, 1979, Depocas et al., 1984). They also showed that, at least part of the energy expenditure effect of OXM occurs via the SNS: global blockade of the SNS with 6-OHDA did reduce the relative increase in oxygen consumption caused by OX2. However, one of the most important roles of the SNS is to regulate blood pressure and heart rate, and therefore these may also be affected by OXM administration. Previous treatments for obesity using SNS activators have all been withdrawn due to related side effects. As recently as 2010, the centrally acting sympathomimetic sibutramine was withdrawn from the market because of undesirable side effects such as hypertension (James et al., 2010). No cardiovascular parameters were measured in the studies herein, but it should be noted that Wynne *et al.* found no

significant change in blood pressure or pulse following administration of native OX2 to man (Wynne et al., 2006, Wynne et al., 2005). Nevertheless, any future studies using oxyntomodulin analogues in man should look at parameters of sympathetic nervous system activity as well as cardiovascular parameters to ensure the drug can be given safely.

In Chapter 6, I investigated the effects of chronic administration of OX2, since chronic administration will be required if oxyntomodulin analogues are to be used as an anti-obesity treatment. I observed many effects of chronic OX2 which are beneficial features for a treatment for obesity: energy expenditure remained elevated with an associated anorectic effect; there was a significant loss of body fat; and there was no deterioration in glucose homeostasis. However, the studies in this chapter showed that OXM can suppress multiple metabolic pathways as diverse as bone turnover and the thyroid axis. Rats given OX2 also showed significantly smaller organ weights and a loss of protein mass, as well as increased urinary nitrogen loss. These changes have important implications for the use of oxyntomodulin analogues to treat obesity. One must be cautious at assuming the direct translation of effects from rodent in to man, as, particularly in relation to growth in adults, the physiology of rats and humans is different. Changes in organ weight are unlikely to occur in man but the effect of oxyntomodulin on other metabolic processes in man needs to be explored if analogues are to be used successfully as an anti-obesity therapy.

Interestingly, the same effects on organ weight and protein metabolism had previously been demonstrated in rats given daily injections of glucagon for 22 days (Salter, 1960). These metabolic effects of oxyntomodulin could therefore be mediated via the glucagon receptor. The effect of oxyntomodulin on protein metabolism was investigated in this thesis since protein metabolism is highly energy-consuming, and therefore may explain the increase in energy expenditure seen with oxyntomodulin. Chapter 7 explored the acute changes in protein metabolism seen with OX2, and showed an increased uptake in amino acids from the plasma, and an increase in ureagenesis, over the same time course as energy expenditure changes. This supports the idea that the increase in energy expenditure caused by oxyntomodulin happens by way of changes in protein metabolism. Importantly, the changes in protein metabolism seen in rodents after glucagon administration are also seen in humans, so it is possible that the changes seen with OX2 may be directly translatable into the clinical use of oxyntomodulin analogues to treat obesity. This means researchers should look for acute changes in protein metabolism following OXM administration in man to determine if there are likely to be significant changes in body protein levels with OXM treatment.

This thesis is the first work to look at the effect of oxyntomodulin on several metabolic processes, most notably protein metabolism. However, as seen from the diagram above, there are many other

metabolic processes, each that contributes to energy expenditure, which remain unexplored. The challenges in determining what metabolic processes are affected by a particular physiological stimulus are highlighted by research into the effects of the thyroid axis on metabolism.

The role of the thyroid gland in energy expenditure was first described by Magnus-Levy in 1895 (Kim, 2008, Lindholm and Laurberg, 2011). Since then, the thyroid axis has been shown to affect metabolic processes as diverse as cardiovascular function, glucose metabolism and energy homeostasis. Furthermore, each of these processes is affected by the thyroid in several different ways: energy homeostasis is affected by changes in ion pumps and futile cycles, in mitochondrial biogenesis and uncoupling thermogenesis in BAT (Vaitkus et al., 2015, Martinez-Sanchez et al., 2014, Mullur et al., 2014). Yet we still do not know which of these processes is physiologically or pharmacologically relevant for energy expenditure (Kim, 2008). Appreciating the pleiotropic effects of thyroxine, however, helps to understand the multiple side effects of hyperthyroidism, and indeed why it is not used as a treatment for obesity (Kaptein et al., 2009). Therefore, understanding the different metabolic processes that can be affected by OXM is important, even if the physiological relevance of each process is not fully determined.

Though glucagon has been known to increase energy expenditure since the 1950s, relatively few studies have looked in to the underlying mechanism. This probably arises from the lack of clinical use for chronic glucagon administration, and the rarity of glucagonomas. Indeed, glucagon has, for many years, been perceived as a hormone of predominantly negative consequences, viewed by some as responsible for the pathophysiological development of diabetes (Unger and Cherrington, 2012). However, if gut hormone analogues which are co-agonists at the glucagon receptor plus the GLP-1, the GIP receptor and even the thyroid receptor, are to enter clinical use, then understanding the metabolic effects of chronic glucagon receptor stimulation will be essential (Finan et al., 2015, Finan et al., 2016).

This thesis has provided the first evidence for the effect of oxyntomodulin on multiple metabolic processes. It is work which provides a platform from which further studies can be undertaken to enhance our understanding of the effects of oxyntomodulin, both physiologically and pharmacologically. As many of these effects are mediated via the glucagon receptor, the information garnered herein is pertinent not only for the use of oxyntomodulin analogues, but all drugs which have activity at the glucagon receptor, many of which may have a role in the future in tackling the obesity epidemic.

Chapter 9

Appendices

Peptide sequence information contained within the appendices is restricted, confidential and legally protected.

It remains the intellectual property of Imperial College London.

9.1 APPENDIX A: PEPTIDE SEQUENCES

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Figure 9.1 Amino-acid sequences of peptides used in this thesis

Detailed above are the amino acid sequences of native glucagon, GLP-1 (7-36) and oxyntomodulin, and the oxyntomodulin-based analogues OX2 and OX2-Glu3. Yellow amino acids are common to all peptides, green amino acids are those found specifically in glucagon, blue amino acids are those found specifically in GLP-1, orange amino acids represent the octapeptide tail which is unique to oxyntomodulin, while purple amino acids are the modifications made to the analogues to enhance activity and prolong half-life. OX2-Glu3 is a comparator peptide with a single amino-acid residue change (Gln 3 to Glu 3), and used in Chapter 3 of this thesis.

9.2 APPENDIX B: DOSE FINDING FOR EX9-39

Preliminary studies were taken to determine the dose of Ex9-39 to deliver via the osmotic mini-pumps. The anorectic effect of OXM is predominantly through the GLP-1 receptors. Therefore the first step was to determine dose of the GLP-1 agonist Ex4 which was more potent at suppressing food intake than OX2, suggesting greater activation of the GLP-1 receptor by Ex4. The second stage found a dose of Ex9-39 which, after 3 days administration via a subcutaneous osmotic mini-pump, suppressed the anorectic effect of the previously determined Ex4.

9.2.1 DETERMINING THE DOSE OF EX4

Previous work in our laboratory had shown a single subcutaneous dose of exendin 4 (0.5nmol/kg) to inhibit food intake in rats over 24 hours. A comparison of the effects of a single dose of Ex-4 (0.5nmol/kg) and a single dose of OX2 (40nmol/kg) on food intake in rats was undertaken.

18 male Wistar rats were randomised to three groups by body weight. They underwent an overnight fast, before receiving a single SC dose of vehicle, OX2 (40nmol/kg), or Ex4 (0.5nmol/kg). Pre-weighed food was instantly returned to the rats' cages. Food was then reweighed at 24 hours.

There was no significant difference in food intake in the vehicle and OX2 groups; Ex4 suppressed food intake significantly compared to both the vehicle and OX2 groups (average food intake, mean $\pm$ SEM (g): vehicle: 41.1 ± 1.4 g; OX2: 39.0 ± 0.9 g; pair-fed, 33.3 ± 1.2 g; vehicle vs Ex4, $p < 0.001$; OX2 vs Ex4, $p < 0.05$).

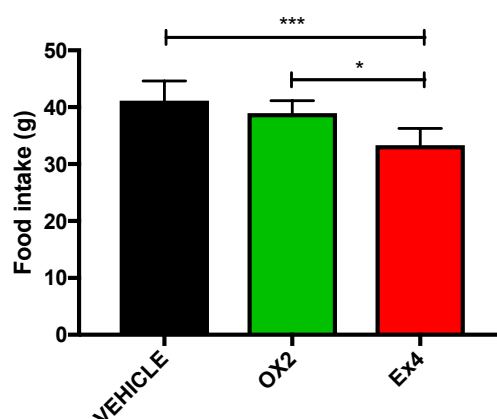


Figure 9.2 Comparison of the effect of OX2 and Ex4 on food intake in rats.

Cumulative food intake over 24 hours in rats given a single SC dose of vehicle, OX2 (40nmol/kg) or Ex4 (0.5nmol/kg) after an overnight fast. N=6. Data presented as mean $\pm$ SEM. Statistical analysis undertaken using one-way ANOVA with post-hoc Tukey's multiple comparison test. * $p < 0.05$, *** $p < 0.001$.

9.2.2 DETERMINING THE DOSE OF EX9-39

Further to the study described above (9.2.1), a study was undertaken to determine if Ex9-39 administered at 100nmol/kg/hr via a subcutaneous osmotic mini-pump would block the effect of Ex4 (0.5nmol/kg) three days after insertion of the mini-pump.

18 male Wistar rats were randomised to 3 groups by body weight. On day 0 of the study, all animals had a subcutaneous mini-osmotic pump inserted into the intra-scapular region (Alzet, Model 1003D, Durect Corporation, Cupertino, USA). In two groups, these pumps delivered saline, and in one group, they delivered Ex9-39 (100nmol/kg/hour). The animals were then returned to their home cages. They were then fasted overnight from day 1 to day 2. At 8am on day 2, the Ex9-39 group and one of the saline groups had a single SC injection of Ex4 (0.5nmol/kg); the final group had a single SC injection of saline. Pre-weighed food was instantly returned to the animals, and food intake measured at 1, 2, 4, 8 and 24 hours.

Food intake was significantly suppressed in the saline/Ex4 group compared to the saline/saline group throughout the 24 hours. Food intake was entirely normalised in the EX9-39/Ex4 group (2 way repeated measures ANOVA, effect of time $F(5,75)=745$, $p<0.0001$; effect of treatment $F(2,15)=29.2$, $p<0.0001$; interaction $F(10,75)=18.1$, $p<0.001$; subjects(matching) $F(15,75)=4.5$, $p<0.001$; post-hoc comparisons by Tukey's multiple comparison test show $p<0.0001$ for saline/saline vs saline/Ex4 and for Ex9-39/Ex4 vs saline/Ex4).

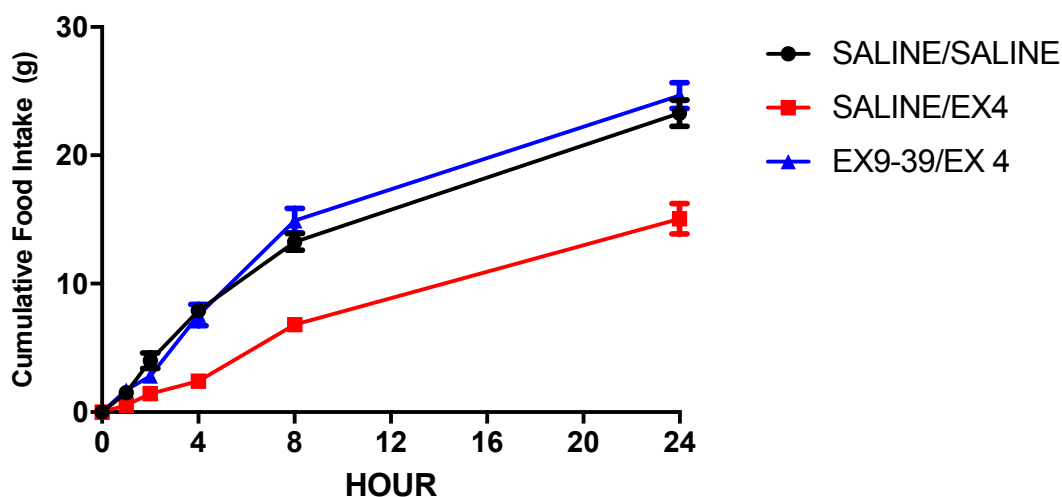


Figure 9.3 Effect of EX9-39 and Ex4 on food intake in rats

Cumulative food intake over 24 hours in rats after injections of either saline or Ex4 (0.5nmol/kg). 2 days previously the rats had subcutaneous osmotic mini-pumps inserted delivering either saline or EX9-39(100nmol/kg/hr).

9.2.3 CONCLUSION

This pilot study showed that Ex9-39 is stable for at least 3 days in a subcutaneous osmotic mini-pump. Ex9-39 delivered at 100nmol/kg/hr blocked the anorectic effect of the GLP-1 agonist Ex4 (0.5nmol/kg). This dose of Ex4 is more potent *in vivo* at suppressing food intake than the oxyntomodulin analogue OX2 (40nmol/kg). Therefore, Ex9-39 (100nmol/kg/hr) is sufficient to block the GLP-1 receptor for at least 3 days while determining the role of the GLP-1 receptor in the energy expenditure effects of OX2.

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