The regulation of appetite by gut hormones

A thesis submitted for the degree of
Doctor of Philosophy, Imperial College London

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2015

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Abstract

Food intake is essential to life, and thus the drive to eat is a priority. Hunger and satiety are governed by homeostatic and hedonic pathways. The homeostatic control of food intake is primarily mediated by nuclei of the hypothalamus and brainstem, while non-homeostatic control is predominantly afforded by the mesocortical and mesolimbic pathways. Hedonic drive to eat may override homeostatic control, leading to increased food intake. The increasing intake of calorie-dense, highly palatable food has contributed to escalating levels of obesity, which now represents a major public health burden. Thus, the development of appetite-reducing agents to combat the obesity epidemic is a priority.

Non-specific appetite inhibitors often result in side-effects such as alterations in blood pressure, locomotor activity and disrupted eating patterns. If an appetite-reducing agent is observed to be acting specifically, it may represent a better target for the development of anti-obesity drugs. The anorectic gut hormones, peptide YY (PYY) and glucagon-like-peptide-1 (GLP-1), reduce food intake by peripheral mechanisms, and also have effects on central homeostatic and hedonic pathways. However, exogenous administration of these peptides results in nausea in humans and aversion in rodents at higher doses. This project investigated the effects of peripheral administration of GLP-1 and PYY on food intake, cardiovascular parameters and behaviour in rats.

Feeding studies in fasted animals identified 1.5 nmol/kg as the minimally effective anorectic dose of PYY, while conditioned taste aversion (CTA) was present from doses of 2.5 nmol/kg PYY. Peripheral administration of 300 nmol/kg PYY significantly decreased food intake and led to significant changes in blood pressure. This dose also produced a trend for increased latency to feeding, and decreased activity. In c-Fos studies, peripheral administration of 300 nmol/kg PYY increased neuronal activation in several nuclei of the mesocorticolimbic pathways, and the area postrema (AP). Signalling in these pathways may mediate the aversive properties of PYY, while the AP may detect concurrent alterations in cardiovascular parameters.

Feeding studies in fasted animals identified 10 nmol/kg as the minimally effective anorectic dose of GLP-1. Food intake was significantly reduced by 300 nmol/kg GLP-1, including decreased intake in the first feeding bout and a trend for increased latency to feeding. The same dose significantly depressed ambulatory activity and increased heart rate. A CTA was not established following peripheral GLP-1 administration at any dose tested, though patterns in activity and feeding would suggest that aversion was present at high doses. A dose of 300 nmol/kg GLP-1 increased neuronal activation in several areas important in the acquisition of aversion, including the central nucleus of the amygdala (CeA), nucleus of the solitary tract (NTS) and the mesocorticolimbic system.

Activation of brain regions by high doses of PYY and GLP-1 correspond to neuronal activation by administration of LiCl. However, 32 mg/kg LiCl increased activation to a far greater degree, suggesting a distinction between substances that reduce food intake purely by aversion, and those that have endogenous homeostatic functions.

The effects of GLP-1 and PYY on appetite and aversion are complex, but likely represent separate systems that are activated differentially by different circulating levels of these hormones. By collaborating with the Mathematical Department, Imperial College London, we hope to develop a mathematical model that distinguishes between specific satiety and aversive behaviours. Further work is now required to determine the utility of such modelling in detecting specific appetite inhibitors and reducing animal use.

Copyright Declaration

Declaration of originality

I hereby declare that all work presented in this thesis is the sole work of Eleanor Joan Spreckley. Information described within the text relating to the published work of others has been acknowledged and appropriately referenced.

Declaration of contributors

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

Chapter 2:

In vivo feeding and energy expenditure studies were carried out with assistance from Dr Amin Alamshah.

Telemetry implantation surgery was carried out with assistance from Dr Elina Akalestou.

Chapters 3 & 4:

Radioimmunoassays were carried out with the assistance of Dr Amin Alamshah and under the supervision of Professor Mohammad A. Ghatei (Division of Diabetes, Endocrinology and Metabolism, Imperial College London).

In vivo feeding and energy expenditure studies were carried out with assistance from Dr Amin Alamshah and Aina Fernandez-Rodriguez.

Assistance with transcardial perfusions was provided by Dr Katherine Banks, Dr James Kinsey-Jones and Dr Amin Alamshah.

Blinded counting of c-Fos staining was performed by Aina Fernandez-Rodriguez.

Acknowledgements

Firstly, I would like to express my sincere thanks to my supervisor, Dr Kevin Murphy, for his support, guidance and patience throughout my PhD.

I am also grateful to Sir Professor Stephen Bloom for the opportunity to study within the Section of Investigative Medicine.

I am extremely grateful for the help I have received from many members of the department. I am particularly thankful to Dr Amin Alamshah, Dr Katherine Banks and Dr Elina Akalestou for their time, expertise and friendship.

I am thankful to the CBS staff for their help over the last 3 years.

I would like to thank the NC3Rs for funding my research.

Last but not least, I would like to thank my family for their encouragement and unwavering confidence.

Abbreviations

5-HT	5-hydroxytryptamine
AAV	Adeno-associated virus
ABC	Avidin-biotin-complex
ACTH	Adrenocorticotrophic hormone
AgRP	Agouti-related peptide
AHA	Anterior hypothalamic area
AP	Area postrema
ARC	Arcuate nucleus
AUC	Area under the curve
BBB	Blood brain barrier
BLA	Basolateral amygdala
BLOOM	Behavioural Modification and Lorcaserin for Overweight and Obesity Management
BLOOM-DM	Behavioural Modification and Lorcaserin for Obesity and Overweight Management in Diabetes Mellitus
BP	Blood pressure
BSA	Bovine serum albumin
BSS	Behavioural satiety sequence
CART	Cocaine and amphetamine-regulated transcript
CCK	Cholecystokinin
CeA	Central amygdala
Cl-	Chloride ion
CLAMS	Computed laboratory animal monitoring system
CNS	Central nervous system
CPP	Conditioned place preference
CPu	Caudate putamen
CSF	Cerebrospinal fluid
CTA	Conditioned taste aversion
DAB	Diaminobenzidine tetrahydrochloride
DIO	Diet-induced obese
DMN	Dorsomedial nucleus
DMX	Dorsal motor nucleus of the vagus
DPPIV	Dipeptidyl peptidase IV

DREADD	Designer receptors exclusively activated by designer drugs
DVC	Dorsal vagal complex
EEC	Enteroendocrine cell
EMEA	European Medicines Agency
ERK 1-2	Extracellular signal-regulated kinase 1-2
Ex-4	Exendin-4
FACS	Fluorescence activated cell sorting
FDA	Food and Drug Administration
FFA	Free fatty acid
fMRI	functional magnetic resonance imaging
GABA	γ-aminobutyric acid
$GABA_A$	γ-aminobutyric acid receptor subtype A
$GABA_B$	γ-aminobutyric acid receptor subtype B
GI	Granular insular cortex
GIP	Gastric inhibitory peptide
GIT	Gastrointestinal tract
GLP-1	Glucagon-like peptide 1
GLP-2	Glucagon-like peptide 2
GOAT	Ghrelin O-acyltransferase
GPCR	G-protein coupled receptors
HPLC	High pressure liquid chromatography
HR	Heart rate
ICV	Intracerebroventricular
IP	Intraperitoneal
LC	Locus coeruleus
LH	Lateral hypothalamic area
LHb	Lateral habenula
LPN	Lateral parabrachial nucleus
LS	Lateral septum
LiCl	Lithium chloride
MeA	Medial amygdala
MO	Medial orbital cortex
MSH	Melanocyte stimulating hormone
mRNA	Messenger ribonucleic acid
NAc	Nucleus accumbens
NAcbSh	Nucleus accumbens shell

NO	Nitric oxide
NPY	Neuropeptide Y
PBN	Parabrachial nucleus
PBP	Parabrachial pigmented nucleus
PEG	Polyethylene glycol
PFC	Prefrontal cortex
POMC	Pro-opio melanocortin
PP	Pancreatic peptide
PrL	Prelimbic cortex
PVN	Paraventricular nucleus
PYY	Peptide tyrosine tyrosine
RER	Respiratory exchange rate
SO	Supraoptic nucleus
STAT	Signal transducer and activator of transcription
UCP1	Uncoupling protein 1
VLH	Ventrolateral hypothalamus
VMN	Ventromedial nucleus
VCO_2	Volume of carbon dioxide produced
VO_2	Volume of oxygen consumed
VP	Ventral pallidum
VTA	Ventral tegmental area
WT	Wild type

Table of contents

Table of figures

Chapter I: General Introduction

1.1 Obesity

The body deals with energy imbalance by either the loss, or gain, of adipose tissue. Correcting the balance between energy intake and expenditure should be simple, by increasing or decreasing calorie intake. However, in 2014 over 1.9 billion adults were estimated to be overweight worldwide. This included 600 million individuals with a BMI of 30 kg/m^2 or higher, classifying them as clinically obese. In most countries, overweight and obesity are now linked to more deaths than malnutrition. Obesity is thus epidemic and a major public health burden (World Health Organization, 2015).

Excess calorie intake is stored as adipose tissue, for use in times of scarce food, such as those experienced by our ancestors (Ulijaszek, 2002). However, the current easy availability of calorie-dense highly-palatable food, particularly in the West, has led to increases in bodyweight. When fat accumulates in abnormal proportions, it can have adverse effects on health. Obesity is associated with the development of numerous comorbidities, including cardiovascular disease, type II diabetes mellitus, stroke and some cancers. In addition, it affects factors that may reduce quality of life, such as self-esteem and mental health disorders. The majority of the UK population is overweight or obese. Thus, treatment of the related diseases presents a major public health and economic challenge to the National Health Service, which, it has been proposed, may become bankrupt as a result (Public Health England, 2015). Moreover, obesity affects countries of all income brackets, and hence poses a threat to many health systems worldwide. Therefore, updated health policies to promote healthy living, and the development of safe and effective pharmacological agents to combat the rise in obesity, are essential (Wang et al., 2011).

1.1.1 Current treatment options

Strategies for tackling obesity include lifestyle changes, pharmacotherapy and bariatric surgery. Of these, bariatric surgery produces the largest and most sustained weight loss, of around 20-35% (O'Brien et al., 2006). Gastric banding and sleeve gastrectomy reduce the available volume of the stomach, while gastric bypass reroutes small intestine by attaching the jejunum to a smaller pouch of stomach, bypassing the duodenum entirely (U.S. National Library of Medicine, 2013). In addition to weight loss, bariatric surgery produces a concomitant amelioration of disordered glucose homeostasis and improvement of cardiovascular function (Maggard et al., 2005). In contrast, diet and lifestyle changes result in much more modest changes in body weight and are difficult to adhere to. Previously recommended exclusively for those with life-threatening obesity, bariatric surgery has now been advocated for use in obese patients with type II diabetes in the UK (NICE, 2014). However, the invasive nature, associated risks and sheer number of patients that now qualify suggests that this is an impractical approach that is beyond the scope of the NHS's resources to use to effectively combat the obesity epidemic.

The development of effective anti-obesity drugs would enable treatment of those for whom bariatric surgery is unavailable, or too high a risk. However, in the past, the design of such agents has been fraught with safety issues. In 1973, fenfluramine was approved by the Food and Drug Administration (FDA) for the short term treatment of

obesity. This drug increased extracellular 5-hydroxytyptamine (5-HT), which reduced appetite through its interaction with central 5-HT_{2C} receptors (Khorassani et al., 2015). However, in 1997 fenfluramine and its derivatives were voluntarily withdrawn from the U.S. market, owing to reports of pulmonary hypertension and heart valve disease due to excess circulating 5-HT (Food and Drug Administration, 1997). Sibutramine, another monoamine modulator, inhibits reuptake of norepinephrine and 5-HT, and enjoyed a modest run as a successful weight loss drug following its approval by the FDA in 1997. However, patients taking sibutramine had, on average, a higher mean arterial pressure, and it was withdrawn following a long term safety study investigating its cardiovascular effects (Food and Drug Administration, 2010). Rimonabant, an inverse agonist at the cannabinoid receptor, CB1, was approved as a weight-loss agent for obese or overweight patients with associated risk-factors in Europe in 2006. Patients taking rimonabant for 1 year achieved, on average, a 4.7 kg greater weight loss than those taking placebo (Christensen et al., 2007). However, rimonabant increased the incidence of serious psychiatric adverse events, including depression and anxiety, and was subsequently withdrawn from the market (EMEA, 2008).

For a weight-loss agent to be approved, the FDA and European Medicines Agency (EMEA) recommend a placebo-adjusted weight loss of 5% following 12 months of treatment. Current pharmacological therapies lack this standard of efficacy. Orlistat, a gastric and pancreatic lipase inhibitor, which prevents the breakdown of triglycerides to absorbable monoglycerides and fatty acids, is currently the only licensed anti-obesity drug in the UK. Although Orlistat is relatively safe, only 60% of patients achieve the recommended weight loss after one year, and compliance issues arise due to unpleasant gastrointestinal side effects if dietary fats are not avoided, such as flatulence and steatorrhoea (Hutton and Fergusson, 2004). Due to its comparative benign safety profile, Orlistat is now available as an over the counter medication by GlaxoSmithKline at half the prescribed dose, under the name Alli® (GlaxoSmithKline, 2015).

The range of appetite-reducing medication remained poor until the FDA approved two new agents in 2012; lorcaserin (Belviq®), and a phentermine/topiramate combination (Qsymia®). Lorcaserin hydrochloride is an orally administered, selective agonist of the 5-HT_{2C} receptor. This receptor is highly enriched in central areas controlling food intake (Roth et al., 1998). The Behavioural Modification and Lorcaserin for Overweight and Obesity Management (BLOOM) study was a placebo-controlled, randomized trial that demonstrated its effectiveness and safety profile (Smith et al., 2010). After 1 year, 47.5% of patients treated with lorcaserin had lost 5% or more of their body weight, versus 20.3% in the control group. This was further confirmed by the Behavioural Modification and Lorcaserin for Obesity and Overweight Management in Diabetes Mellitus (BLOOM-DM) trial, which highlighted the additional benefits of lorcaserin in diabetic patients (O'Neil et al., 2012). Although lorcaserin largely circumvents the cardiotoxic effects of nonselective 5-HT agonists, phase III trials revealed increased rates of upper respiratory tract infections, nasopharyngitis, nausea and headaches. However, these relatively benign side-effects were deemed tolerable due to lorcaserin's relative effectiveness as a weight-loss agent, albeit still not satisfying the 5% reduction advocated by the FDA (Smith et al., 2010).

Combination therapies have been of major interest of late as they allow lower doses of multiple agents to drive weight loss without risking the side effects of larger doses, and may avoid tachyphylaxis. Qsymia® is a fixed dose of immediate-release phentermine and delayed-release topiramate. Phentermine is an amphetamine derivative that stimulates the production of noradrenalin, leading to decreased hunger and promoting the utilization of body fat as fuel. The satiety-inducing effects of topiramate, discovered serendipitously during its original use as an anti-epileptic, are not fully understood, but may be due in part to its effects at central γ-aminobutyric acid (GABA) receptors (Richard et al., 2000, Richard et al., 2002, Picard et al., 2000). The EQUIP and CONQUER clinical trials demonstrated that the majority of patients taking Qsymia® achieved a weight-loss of greater than 5% (Garvey et al., 2012, Gadde et al., 2011). Nevertheless, serious unwanted effects appeared in some groups of patients, and as a result the drug is contraindicated in patients with depression, cardiac, or cerebrovascular disease. So far, neither Belviq® nor Qsymia® has been authorized for use in Europe, with relatively low efficacy and unwanted side effects, respectively, standing in the way of their approval.

A pharmacological candidate that may reach the standards of, and be approved by, the EMEA in the near future, is Saxenda® (liraglutide). Liraglutide is a long acting glucagon-like peptide 1 (GLP-1) agonist that results in glucose-stimulated insulin release and delayed gastric emptying, and which was previously approved at a lower dose for the treatment of diabetes. A clinical trial demonstrated an average weight loss of 4.5% in obese patients without diabetes. However, Saxenda® has been associated with potentially serious side effects, including pancreatitis, renal impairment and gallbladder disease, and has been associated with thyroid C-cell tumours in rodents (Bjerre Knudsen et al., 2010, Lee et al., 2011). As a result, if patients do not achieve at least 4% weight loss by 16 weeks, the risks are considered to outweigh the benefits (since it is unlikely that they will respond sufficiently to improve their health), and treatment should be discontinued (Food and Drug Administration, 2014).

1.2 Energy Homeostasis

Food intake is essential to life. Maintaining energy homeostasis is a complex process involving activation and inhibition of feelings of hunger and satiety. These states are governed by both the central and peripheral nervous systems and endocrine signalling, that together form homeostatic and hedonic pathways. Signals from peripheral organs, including the gastrointestinal tract (GIT) and adipose tissue, are integrated centrally, and provide information on nutritional stores and intake. Brain regions involved in the central regulation of energy intake include the hypothalamus, the mesocorticolimbic system and the caudal brainstem. The modern day availability in the developed world of highly palatable, energy dense food has been hypothesised to result in greater hedonic eating in humans, and consequently a general increase in overweight and obesity.

1.2.1 The hypothalamus

The hypothalamus is located at the base of the brain, inferior to the thalamus and proximate to the median eminence, an area with an incomplete blood brain barrier (BBB) and therefore in contact with circulating factors

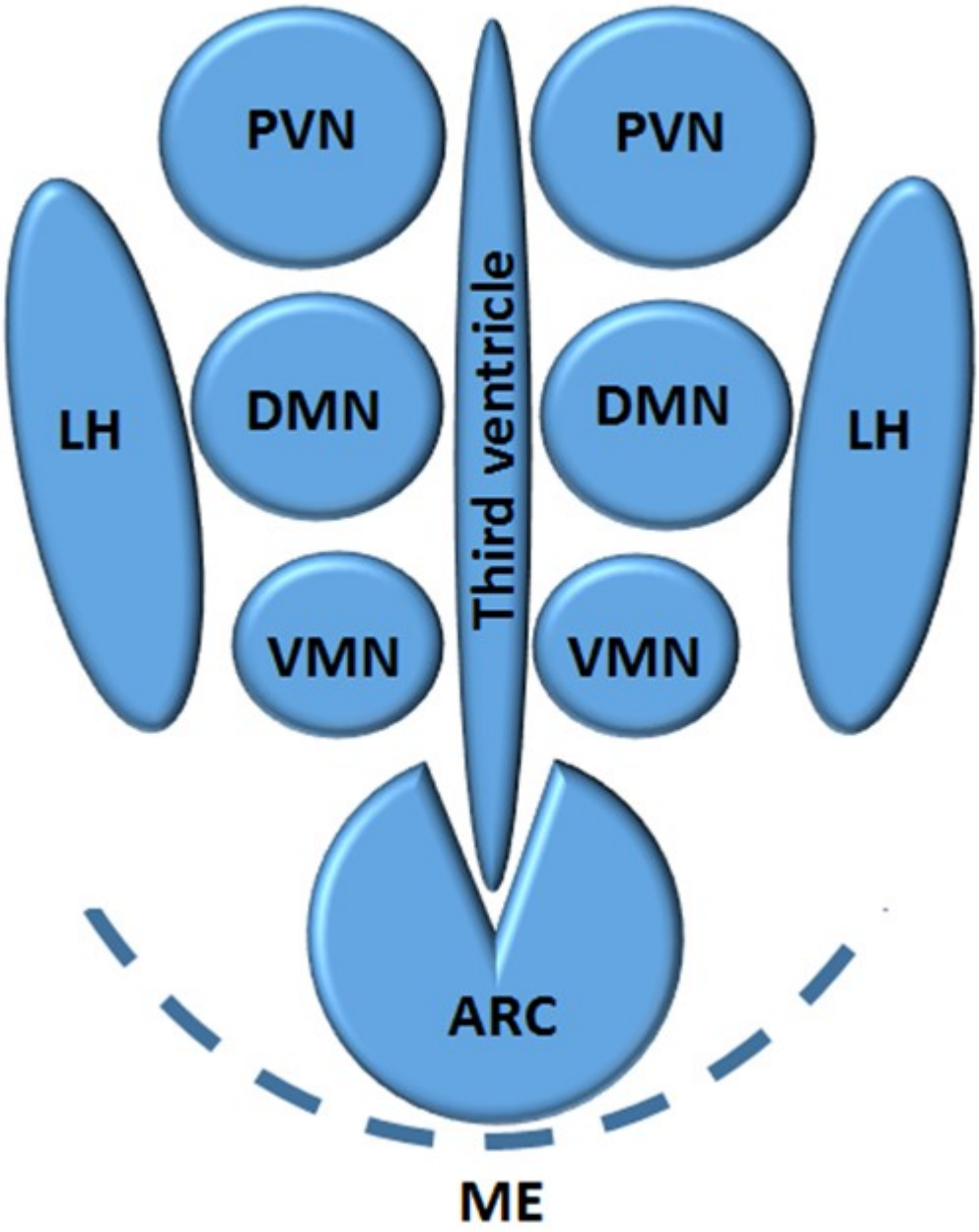


Figure 1.1 A schematic diagram of the hypothalamus in the coronal plane, displaying the relative locations of nuclei important in energy homeostasis.

PVN, paraventricular nucleus; DMN, dorsomedial nucleus; VMN, ventromedial nucleus; ARC, arcuate nucleus; ME, median eminence. Adapted from (Druce and Bloom, 2006).

(Schwartz, 2000). The hypothalamus is the major brain region involved in the control of energy homeostasis, and is divided into distinct nuclei that co-ordinate orexigenic and anorexigenic signals and have extensive intra- and extra-hypothalamic projections (Molina, 2013). These nuclei include the arcuate nucleus (ARC), dorsomedial nucleus (DMN), ventromedial nucleus (VMN), paraventricular nucleus (PVN) and lateral hypothalamic area (LH) (Fig 1.1). Within the ARC are two sets of neurons that have distinct effects on appetite. The activation of pro-

opiomelanocortin (POMC) and cocaine- and amphetamine-regulated transcript (CART) co-expressing neurons inhibits food intake, whilst activation of neuropeptide Y (NPY) and agouti-related peptide (AgRP) co-expressing neurons stimulates appetite (Fig 1.4).

1.3 Non-homeostatic control of food intake

Feeding is essential for life, and hence the brain has evolved to ensure that the drive to find and eat food is a priority. Evolutionary models of energy homeostasis suggest that humans are genetically programmed to defend themselves better against weight loss than against weight gain, in order to retain reproductive efficiency in a hostile environment (Leibel, 2008). The 'thrifty gene' hypothesis proposes that genes that allow a person to lay down fat more rapidly during times of calorie abundance, in order to preserve energy reserves for periods of fasting, will confer survival and thus an evolutionary advantage (Neel, 1962). More recently, the role of non-homeostatic control of food intake has been investigated. The rewarding nature of food, particularly in the current obesogenic environment of the developed world, appears sufficiently strong to drive eating behaviour at times when adequate energy intake has already occurred. This overriding of homeostatic control by hedonic mechanisms has been proposed to contribute to the current obesity epidemic (Yu et al., 2015).

1.3.1 The mesocorticolimbic system

The aforementioned hypothalamic and brainstem regions are the primary locations for homeostatic control of food intake. The mesolimbic structures are thought to guide food intake based on reward value, as these areas have functions in emotional and cognitive signalling. The mesocorticolimbic system has extensive projections to and from the hypothalamus (Fig 1.2). Hence, it is able to integrate information on metabolic status with cortical functions governing food reward evaluation, which are also affected by factors such as emotion and stress. The reward system of the midbrain regulates 'wanting'; the motivation to consume food, and 'liking'; the pleasure associated with eating palatable food, both of which are subconscious processes (Avena et al., 2008). These pathways underlie the 'food addiction' hypothesis, as the same pathways that govern food reward are activated by addictive drugs (Avena et al., 2008). The mesocortical and mesolimbic pathways are two of the four major dopaminergic pathways in the brain, though they are often considered collectively as the mesocorticolimbic pathway due to their closely related functions in hedonic signalling. The mesolimbic pathway projects from the ventral tegmental area (VTA), to several midbrain and forebrain areas. The mesocortical pathway projects from the VTA to frontal cortical areas (Fig 1.2).

1.3.1.1 Dopamine

Dopamine is a catecholamine and an important neurotransmitter within the mesocorticolimbic system. It is synthesised from L-phenylalanine and tyrosine by the brain and the adrenal medulla. Peripheral dopamine acts as a paracrine signal. Central dopaminergic signalling is essential for motor control, sexual arousal, decision making, reward and motivation (Siragy et al., 1989, Mezey et al., 1996, Beaulieu and Gainetdinov, 2011).

There are five dopamine receptors, D_1-D_5, which are GPCRs with an extracellular binding domain (Bunzow et al., 1988). Dopamine receptors are separated into two subtypes; stimulatory (D_1 and D_5) or inhibitory ($D_{2, 3}$ and $_4$). Stimulatory, or D_1-like, receptors stimulate the activity of adenylyl cyclase via $G_{\alpha s}$, which leads to the production of cAMP and the phosphorylation of specific proteins in signal transduction pathways. Conversely, inhibitory, or D_2-like, receptors inhibit adenylyl cyclase via the $G_{\alpha i}$ subunit (Cools and Van Rossum, 1976).

Dopamine receptors exist both pre- and post-synaptically. Presynaptic receptors are involved in negative feedback signalling, typically inhibiting further neurotransmitter release. D_1 and D_2 are the most abundantly expressed, and can be found throughout the amygdala, hippocampus and pituitary, but only D_2 is expressed in the midbrain (Meador-Woodruff and Mansour, 1991, Levey et al., 1993). D_3 is expressed only in the caudate putamen (CPu) and nucleus accumbens (NAcb), while D_4, the receptor with the highest affinity for dopamine, is limited to the frontal and parietal cortices, thalamus, striatum and hippocampus (Landwehrmeyer et al., 1993, Ariano et al., 1997). D_5 is also located in the hypothalamus, thalamus and hippocampus (Meador-Woodruff et al., 1992).

1.3.1.2 GABA and glutamate

GABA is the primary inhibitory neurotransmitter in the mesocorticolimbic system, and signals via ionotropic ($GABA_A$) and metabotropic ($GABA_B$) receptors. The activation of GABA receptors results in the formation of inhibitory post-synaptic potentials, therefore reducing the likelihood of an action potential in the target neuron. $GABA_A$ receptors are ligand-gated ion channels that when activated cause an influx of chloride ions (Cl^-) into the cell, which reduces the membrane potential. $GABA_B$ receptors are coupled to Gi, and therefore inhibit the production of adenylyl cyclase. Addictive drugs, such as opiates and benzodiazepines, inhibit GABA signalling, which disinhibits dopamine neurons and increases their firing (Macdonald and Barker, 1978, Johnson and North, 1992).

Glutamate is an excitatory neurotransmitter that signals via ionotropic and metabotropic receptors. Ionotropic receptors are ligand-gated ion channels; when glutamate binds, a cation influx causes an excitatory postsynaptic potential to be generated, which increases the likelihood of an action potential (Hollmann et al., 1989). Metabotropic glutamate receptors are GPCRs which are coupled to G_i, G_s or G_q, and are able to induce either an excitatory or an inhibitory postsynaptic potential in the target neuron.

Drugs of addiction induce hedonia by affecting dopaminergic signalling. Addiction is believed to represent a pathological dysfunction of neural reward signalling pathways, including those originating in the VTA (Hyman et al., 2006). The co-administration of intra-VTA glutamate receptor antagonists prevents the development of a cocaine-induced conditioned place preference (CPP), indicating attenuation of cocaine's rewarding effects (Harris and Aston-Jones, 2003). Furthermore, the expression of glutamate receptors in the VTA increases following repeated administration of cocaine, morphine or alcohol (Ortiz et al., 1995, Fitzgerald et al., 1996, Lu et al., 2003).

1.3.1.3 The ventral tegmental area

The VTA is located in the ventral midbrain and is the anatomical origin of dopaminergic signalling within the mesocorticolimbic system. Neurons within the VTA are approximately 60% dopaminergic, 30% GABAergic and 2-3% glutamatergic (Bayer and Pickel, 1991, Hur and Zaborszky, 2005). The VTA is composed of four distinct nuclei, of which the parabrachial pigmented nucleus (PBP) contains the highest number of dopaminergic neurons.

The VTA receives extensive afferent input from many brain areas, but is most densely innervated by projections from the prefrontal cortex (PFC), lateral septum (LS), NAcb shell, ventral pallidum (VP), lateral habenula, lateral preoptic area and the LH (Fig 1.2) (Geisler and Zahm, 2005, Phillipson, 1979). The VTA sends efferent projections to the NAcb, around 80% of which are dopaminergic (Fallon, 1981, Klitenick et al., 1992). Efferent projections also extend to the PFC, LS, VP, amygdala, hippocampus, and to the locus coeruleus (LC) and the parabrachial nucleus (PBN) of the brainstem (Fig 1.2) (Taylor et al., 2014).

The primary role of the VTA is to detect rewarding stimuli and elicit a behavioural response via its projections to other mesocorticolimbic areas, therefore producing positive reinforcement (Fields et al., 2007). The reward calculation of this region is demonstrated by studies in which rodents exhibit self-administering behaviour of rewarding substances directly into the VTA, such as ethanol, cocaine and opioids (Roberts and Koob, 1982, Gatto et al., 1994, Bozarth and Wise, 1981). Ablation of dopaminergic signalling in the VTA prevents this behaviour (Roberts and Koob, 1982, Rodd et al., 2004). The ability of animals to gauge reward enables them to distinguish the likeliness of one situation resulting in food compared with another. Midbrain dopamine neurons are rapidly able to adapt to reward-predicting stimuli. The intensity of neuronal firing increases with the rewarding value, or calorie content, of food, and is sensitive over a large range of rewarding stimuli (Tobler et al., 2005).

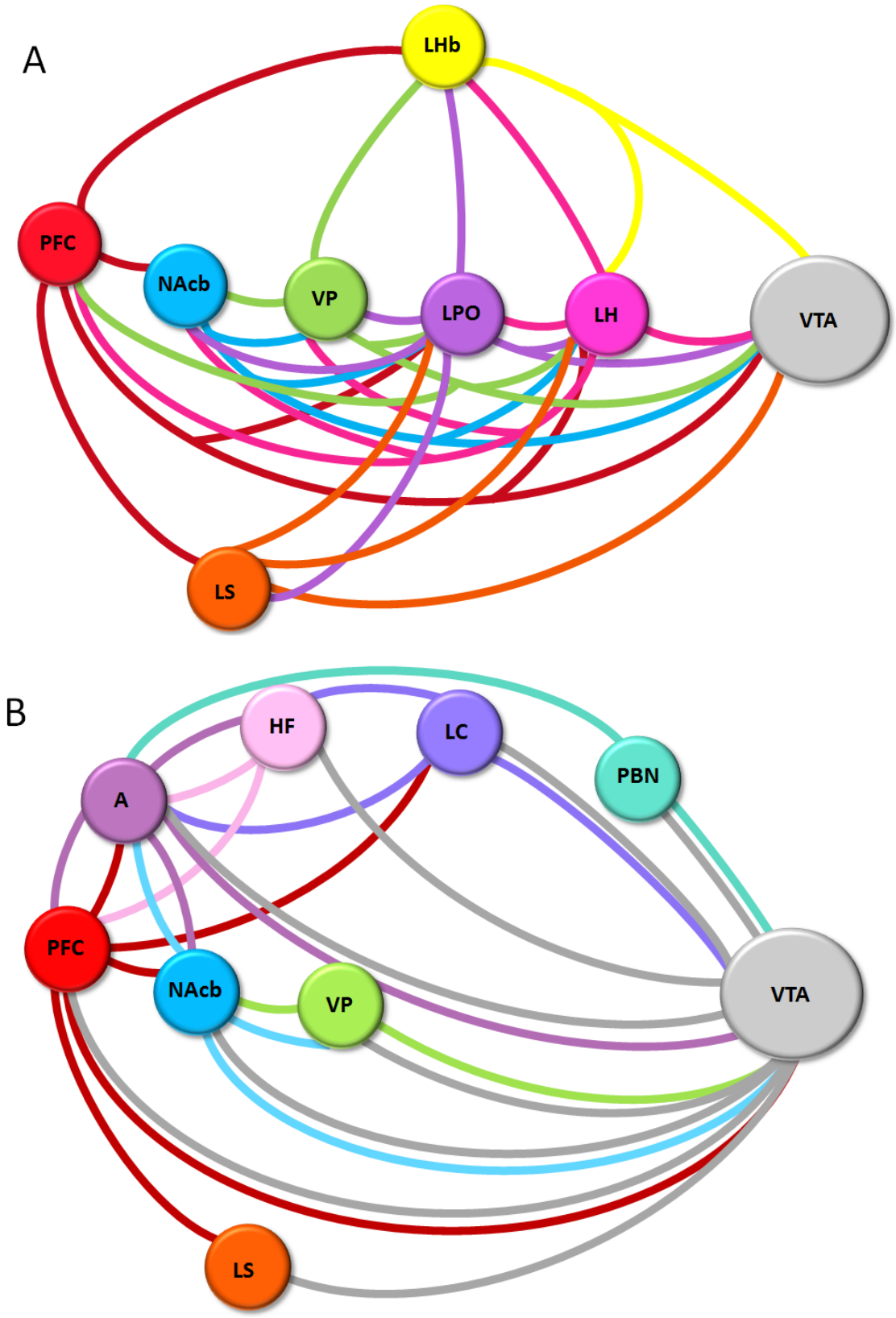


Figure 1.2 Schematic diagram showing afferent (A) and efferent (B) projections of the VTA and their targets.

LHb, lateral habenula; PFC, prefrontal cortex; NAcb, nucleus accumbens; VP, ventral pallidum; LPO, lateral preoptic area; LH, lateral hypothalamus; VTA, ventral tegmental area; LS, lateral septum; PBN, parabrachial nucleus; LC, locus coeruleus; HF, hippocampal formation; A, amygdala. Adapted from Geisler and Zahm, 2005 (Geisler and Zahm, 2005, Swanson, 1982, Albanese and Minciacchi, 1983, Klitenick et al., 1992).

1.3.1.4 The nucleus accumbens

The VTA projects immediately to the NAcb, a midbrain structure located dorsal to the CPu. The NAcb is split into two main regions; the shell and the core, which display separate neuronal projection patterns, though research suggests that further compartmentalisation may be warranted (Reed et al., 2015). The NAcb shell (NAcbSh) projects to the amygdala, VP, LH, VTA, periaqueductal gray and the substantia nigra. The NAcb core projects to the VTA, substantia nigra and the VP. In addition to projections from the VTA, the NAcb receives input from the medial prefrontal cortex (mPFC), amygdala, hippocampus and the thalamus (Heimer et al., 1991).

Rats will repeatedly self-administer amphetamines into the NAcb, suggesting that neuronal activation within the NAcb induces feelings of hedonia and positive reinforcement (Hoebel et al., 1983). A further study indicated that this effect was mediated by D_2 receptors, specifically in the NAcbSh (Rodd-Henricks et al., 2002). Markers of neuronal activation are upregulated within a subregion of the NAcbSh following withdrawal from cocaine in rats, suggesting neuronal plasticity of this area with a role in addiction (Brenhouse and Stellar, 2006).

The NAcb is an area that encodes aversion as well as reward. Appetitive and aversive stimuli converge here and are processed to produce the appropriate behaviour. The NAcbSh and core contribute differently to goal-oriented behaviour, which may reflect their distinct projection targets. Inactivation of the shell increases responses to a cue that doesn't involve a reward, while inactivation of the core decreases responses to a reward (Ambroggi et al., 2011). This suggests that the NAcb core promotes behavioural responses to stimuli that lead to reward, while the NAcbSh negatively regulates responses to stimuli that do not (Ambroggi et al., 2011). Diminished dopaminergic firing, and the resultant decrease in dopamine, appears to have functional effects within the NAcb core, as a fear-inducing cue decreases dopaminergic signalling in the core, while it is increased in the NAcbSh (Badrinarayan et al., 2012). Aversive taste cues also alter dopaminergic signalling within the shell. Administration of a non-preferred hypotonic salt solution to rats increases signalling the NAcbSh, whereas during sodium depletion, when the salt solution is preferred, signalling decreases (Loriaux et al., 2011).

1.3.1.5 The amygdala

The amygdala is a multinuclear complex located within the temporal lobes of the forebrain, which is subdivided into the basolateral (BLA), central (CeA) and medial (MeA) amygdala. The amygdala has extensive reciprocal projections with the hippocampus and the cortex (Pitkanen et al., 2000). A major function of the amygdala is memory formation of emotionally arousing events, as well as determining the emotional significance of visual, auditory and olfactory signals and a range of other cognitive functions (Adolphs et al., 1994, Hamann et al., 1999, Scott et al., 1997, Zald and Pardo, 1997).

The hippocampus is required for memory formation and storage. The extensive connections between the hippocampus and the amygdala indicate that these pathways are important for providing information on past

rewarding stimuli, which aids future behavioural decisions (Baxter and Murray, 2002). Neurotoxic lesion of the amygdala eliminates the ability of rodents to associate a light bulb flash with reward, while rhesus monkeys with amygdaloidal lesions exhibit abnormal food preferences (Hatfield et al., 1996, Murray et al., 1996).

Conditioned taste aversion (CTA) is a well-defined paradigm for nausea in rodents, which lack the necessary anatomy for an emetic reflex. CTA occurs when ingestion of a novel substance results in transient visceral illness, leading to avoidance of the substance in the future. The amygdala is important for expression of CTA (Gallo et al., 1992, Reilly and Bornovalova, 2005, Lasiter and Glanzman, 1985). Specifically, destruction of the CeA disrupts the acquisition of lithium chloride (LiCl)-induced aversion (Lasiter and Glanzman, 1985). Transient inhibition of protein synthesis within the CeA also blocks CTA formation, indicating that this nuclei is important for long-term aversive memory formation (Lamprecht and Dudai, 1996). The CeA is also a prominent area expressing the GLP-1 receptor, and this population of neurons is postulated to mediate the aversion induced by central GLP-1 administration (Kinzig et al., 2002).

1.3.1.6 The prefrontal cortex

The cerebral cortex constitutes the surface of the cerebrum and is organised into six layers, which are further divided into functionally separate regions (Greig et al., 2013). The cortex mediates higher-order cognitive functions, such as learning and decision making (Rushworth et al., 2011). The PFC, located rostral of the forebrain, is the primary cortical area involved in mesocorticolimbic signalling, and has been extensively investigated for its role in reward-guided decision-making (Rushworth et al., 2011). Studies using functional magnetic resonance imaging (fMRI) have repeatedly shown that signalling in the PFC correlates with the reward value of a choice (Rogers et al., 2004, Carlson et al., 2011).

The prelimbic cortex (PrL) is a subdivision of the medial PFC, which corresponds to the dorsolateral prefrontal cortex in humans, and is thought to be heavily involved in contingency learning and performance (Corbit and Balleine, 2003, Fuster, 1989). Contingency learning involves the acquisition of information regarding the relationship of an action with a reward, and their causal association. Lesions of the PrL desensitize rats to variations in the eventuality of a behaviour resulting in a food reward (Balleine and Dickinson, 1998, Corbit and Balleine, 2003). This suggests that the prelimbic cortex encodes learning of outcome-related information, in order to establish a course of action (Corbit and Balleine, 2003).

The rodent granular insular cortex (GI) is a higher order cortical area involved in mediating gustatory information (Shi and Cassell, 1998). The role of the insular cortex in taste memory recognition is well established, and has been shown to be involved in CTA (Nerad et al., 1996, Bermudez-Rattoni and McGaugh, 1991, Berman et al., 2000). The insular cortex is indispensable for the initial processing of a novel taste stimulus, which occurs via activation of the extracellular signal-regulated kinase 1-2 (ERK1/2), mediated by glutamate and muscarinic receptors (Gallo et al., 1992, Berman et al., 2000). The amygdala is then proposed to associate this new gustatory trace with malaise (Gallo et al., 1992).

1.3.1.7 POMC

Centrally, POMC is expressed in the ARC and NTS, and is also peripherally expressed in the pituitary, skin and immune system (Yeo et al., 2000). POMC is a precursor molecule that undergoes post-translational processing by pro-hormone convertases 1 and 2 in a tissue-specific manner (Fig 1.3). This results in the production of peptides with a wide range of functions, from steriodogenesis to skin pigmentation. POMC knockout mice are obese and hyperphagic, and have increased longitudinal growth and adrenal insufficiency (Yaswen et al., 1999). In the brain, POMC cleavage results in the production of the melanocortins; α-, β- and γ-melanocyte stimulating hormone (MSH), and β-endorphin (Cone, 2005).

The melanocortins possess a homologous sequence of amino acids, His-Phe-Arg-Trp, which is essential for binding with their receptors, the G-protein coupled receptors (GPCRs), MC1R-MC5R. Alpha-MSH is an anorectic peptide that acts via MC3R and MC4R, which are both highly expressed in hypothalamic nuclei. Intracerebroventricular (ICV) administration of α-MSH or other agonists of the MC3R and MC4R reduces food intake in rodents, an effect that can be blocked by the administration of a melanocortin antagonist which potently inhibits the MC4R and has less potent effects at the MC3R (Fan et al., 1997). The MC4R appears to be the important receptor with regards to food intake; MC3R knockout mice exhibit increased adiposity with no increase in food intake, implying that this receptor plays a role in the control of energy expenditure (Chen et al., 2000). MC4R knockout mice are obese and hyperphagic with a decreased energy expenditure, and do not demonstrate an anorectic response to α-MSH. Re-expression of MC4R in the PVN and central amygdala (CeA) attenuates this phenotype, suggesting roles for the MC4R in the control of both food intake and energy expenditure (Huszar et al., 1997, Balthasar et al., 2005). Melanocortin 4 receptors in the PVH are downstream targets of orexigenic ARC AgRP neurons. Inhibition of these MC4R receptors has a fundamental role in stimulating feeding (Garfield et al., 2015). Mutations in the MC4R are the most common cause of monogenic human obesity, which presents with either dominant or recessive forms of inheritance (Farooqi et al., 2000). Truncation or frameshift mutations in the POMC gene prevent translation of the product and have also been associated with human obesity, adrenal insufficiency and hyperphagia. These patients also have red hair and fair skin, due to the lack of melanin, a pigment normally expressed by melanocytes due to the effects of Alpha-MSH, γ-MSH and ACTH, via MC1R (Yaswen et al., 1999, Krude et al., 1998).

Within the ARC, POMC expressing neurons project extensively to areas of the brain with known functions in the control of eating, including the hypothalamic PVN, LH, DMN, and VMN, and the dorsal motor nucleus of the brainstem (Yeo et al., 2000). Optogenetic stimulation of arcuate POMC neurons over 24 hours decreases food intake and body weight, and requires melanocortin receptor signalling (Aponte et al., 2011). Neuronal activation using the 'designer receptors exclusively activated by designer drugs' (DREADD) method has suggested that POMC neurons in the mouse ARC require chronic stimulation to elicit their effects on food intake, while acute stimulation of those in the brainstem immediately inhibits eating. Ablation of ARC POMC neurons resulted in obesity and hyperphagia, while ablation of NTS POMC neurons did not. This suggests that hypothalamic POMC neurons regulate energy

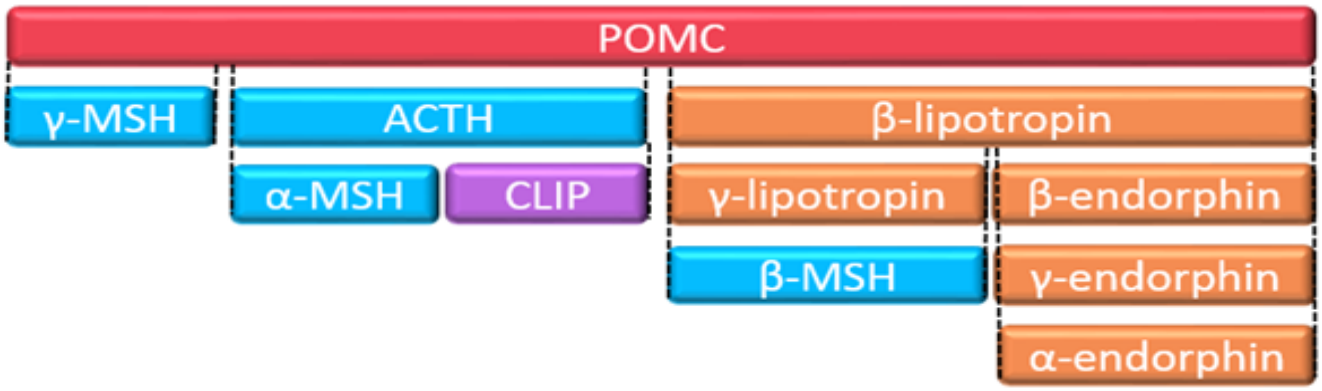


Fig 1.3 Schematic diagram illustrating post-translational processing of pro-opiomelanocortin and its products.

POMC undergoes tissue-specific cleavage into the melanocortins and related peptides. Blue indicates products possessing the His-Phe-Arg-Trp sequence of amino acids. POMC, pro-opiomelanocortin; MSH, melanocyte stimulating hormone; ACTH, adrenocorticotrophic hormone; CLIP, corticotrophin-like intermediate peptide.

homeostasis via long-term adiposity signals, while the NTS may detect short-term satiety signals from the periphery (Zhan et al., 2013).

Due to the widespread expression of MC4R, agents acting at this receptor are often associated with unwanted effects (Mountjoy et al., 2003, Silva et al., 2006, Greenfield et al., 2009). Activation of specific populations of central MC4 receptors increases blood pressure, and obese patients with MC4R mutations thus do not demonstrate the expected obesity-induced hypertension (Greenfield et al., 2009). Re-expression of MC4 receptors in cholinergic neurons restores obesity-induced hypertension in MC4R null mice, suggesting these neurons are responsible for the effects of melanocortins on blood pressure (Sohn et al., 2013). However, a novel, highly selective MC4R agonist has recently been shown to chronically reduce food intake in a nonhuman primate model of obesity, and is currently undergoing clinical trials (Kievit et al., 2013).

1.3.1.8 Neuropeptide Y

Neuropeptide Y is the most potent orexigenic neuropeptide known, and acts via G-protein coupled Y1 and Y5 receptors to mediate its stimulatory effects on food intake. NPY is a member of the pancreatic peptide (PP) hormone family, which also includes PP and peptide YY (PYY) (Tatemoto et al., 1982). The Y1 receptor is expressed in the PVN, ARC and supraoptic nucleus (SO) of the hypothalamus, predominantly on postsynaptic nerve terminals, but is also widely expressed throughout the rest of the central nervous system (CNS) (Kopp et al., 2002). The Y5 receptor is expressed in the PVN, ARC, LH, SO and medial preoptic nucleus of the hypothalamus, as well as other central extra-hypothalamic nuclei (Durkin et al., 2000). Many of these nuclei are positioned close to the third ventricle, and intracerebroventricular administration of selective Y1 and Y5 agonists results in hyperphagia in rats (Cabrele et al., 2000, Mullins et al., 2001). The activity of arcuate POMC neurons is inhibited by factors from NPY/AgRP neurons, including the release of GABA and of NPY, which acts on Y1 receptors to hyperpolarize these neurons and increase food intake (Roseberry et al., 2004). The Y2 receptor is the selective target for the peripherally-derived peptide PYY_{3-}

$_{36}$, and is expressed on most NPY neurons in the ARC, where it is thought to act as an inhibitory autoreceptor, limiting the release of NPY and thus opposing the Y1-mediated inhibition of POMC neurons (Broberger et al., 1997, Roseberry et al., 2004).

Hypothalamic expression of NPY is increased during fasting, and also in the brains of *ob/ob* mice, which lack the adipocytokine leptin and have chronic hunger, suggesting a physiological role in appetite control (Sahu et al., 1990, Schwartz et al., 1996a, Sanacora et al., 1990). Recombinant adeno-associated virus (AAV)- mediated reduction in NPY expression decreases food intake and body weight in rats, indicating that its effects are not only important in fasting, but also in the long term regulation of energy intake (Gardiner et al., 2005). However, there is no appetite or body weight phenotype in germline NPY knockout mice, suggesting developmental compensation occurs (Erickson et al., 1996).

The Y1 and Y5 receptors mediate the orexigenic effects of NPY, while Y2 and Y4 mediate the anorexigenic effects of PYY_{3-36} and pancreatic polypeptide, respectively. Genetic knockdown of the Y1 or Y2 receptors results in hyperphagic mice with increased adiposity. Knockout of the Y5 receptor in mice causes obesity (though only in later life) which appears counter-intuitive given the orexigenic nature of the Y1 and Y5 receptors (Baldock et al., 2007, Kushi et al., 1998, Marsh et al., 1998). Deletion of Y4 produces the opposite effect, resulting in decreased body weight and white adipose mass (Sainsbury et al., 2002). The complex and counter-intuitive phenotypes revealed by Y receptor knockout studies make it difficult to demonstrate the physiological roles of NPY, and highlight the multifaceted nature of this neuronal population in the control of food intake.

Due to their effects on food intake, NPY and its receptors represent attractive pharmacological targets. However, the development of a selective Y1 antagonist, BMS-193885, was hampered by its limited oral bioavailablility, owing to poor intestinal absorption (Antal-Zimanyi et al., 2008), and an orally active Y5 antagonist, MK-0557 that inhibited high fat diet-induced obesity in mice did not cause clinically significant weight loss in humans (Erondu et al., 2006).

1.3.1.9 Agouti-related peptide

NPY neurons co-express the orexigenic peptide AgRP, an inverse agonist at the anorectic melanocortin receptors. AgRP was originally assumed to act primarily by blocking the α-MSH-derived anorectic tone at these receptors, but recent evidence in mice has shown AgRP is able to independently regulate firing activity of neurons in the PVN. AgRP binds to MC4R and opens Kir7.1 inwardly rectifying potassium channels, causing hyperpolarisation independently of inhibition of α-MSH binding (Ghamari-Langroudi et al., 2015). In addition, acute optogenetic stimulation of AgRP neurons in mice can stimulate eating that does not require the interaction of the melanocortin receptors, demonstrating the ability of AgRP to directly initiate feeding behaviour (Aponte et al., 2011). In this study, the magnitude of food seeking behaviour intensified with increasing levels of AgRP neuronal activity, suggesting a dedicated role of this neuronal population in controlling food intake (Aponte et al., 2011). Acute activation of AgRP neurons using DREADD technology induces feeding and reduces energy expenditure in mice. AgRP stimulation also

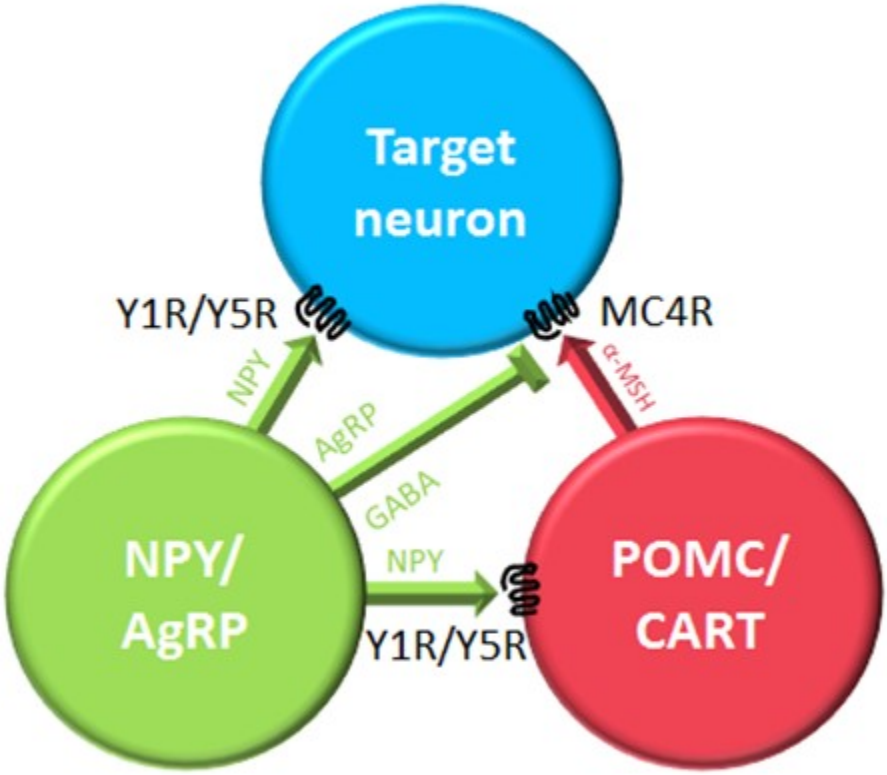


Fig 1.4 A schematic diagram of the two primary arcuate neuronal populations that control food intake.

NPY, neuropeptide Y; AgRP, agouti-related peptide; POMC, pro-opiomelanocortin; CART, cocaine amphetamine regulated transcript; MC4R, melanocortin 4 receptor; Y1R, Y1 receptor; Y5R, Y5 receptor; α-MSH, α-melanocyte stimulating hormone; GABA, γ-aminobutyric acid.

induced fed mice to participate in intense food seeking behaviour, similar to that of a fasted mouse, illustrating potent effects on food motivation and reward (Krashes et al., 2011).

Currently, the release of GABA is believed to underlie some of the MC4R-independent effects of NPY/AgRP neurons on food intake (Wu and Palmiter, 2011). Mice with an AgRP neuron-specific deletion of vesicular GABA transporter are lean and resistant to obesity with an attenuated response to the orexigenic hormone, ghrelin (Tong et al., 2008). In addition, the starvation that results from AgRP neuron ablation can be rescued by the chronic administration of a $GABA_A$ receptor agonist, thus suggesting direct GABAergic neurotransmission inhibits anorexigenic centres in the CNS (Wu et al., 2009).

1.3.1.10 The caudal brainstem

The brainstem is an extra-hypothalamic area involved in the control of food intake. In the absence of hypothalamic and forebrain signalling, neural circuits within the brainstem are sufficient to maintain control of individual meal size (Grill, 2006). The dorsal vagal complex (DVC) is the principal area in the brainstem that plays a role in the negative feedback control of food intake, and is comprised of the nucleus of the solitary tract (NTS), the dorsal motor nucleus of the vagus (DMX) and the area postrema (AP) (Schwartz, 2006). The NTS receives information from the periphery

via gustatory afferents of the vagus nerve regarding food and water content, and gastric distension (Schwartz, 2000). Several factors released from the periphery are able to signal via the vagal nerves, including ghrelin and cholecystokinin (CCK), which are orexigenic and anorexigenic, respectively (Smith et al., 1981, Date et al., 2002). Lesion of the vagal afferents prevents signals from the alimentary tract reaching the central regions that regulate energy homeostasis, and results in increased meal size in rats (Smith et al., 1985, Schwartz et al., 1999). The NTS is in close proximity with the AP, a circumventricular organ with an incomplete BBB, and hence is sensitive to circulating factors. In rats, lesion of the AP results in hypophagia and a reduction in body weight, which may reflect a loss of visceral sensory input (Hyde and Miselis, 1983). POMC expressing neurons are present in the NTS, as is the MC4R, and in conjunction with the DREADD studies discussed above suggest that melanocortin signalling in the brainstem is important in energy homeostasis (Mountjoy et al., 1994).

1.4 Adiposity signals

The periphery is able to communicate information to the CNS regarding the current state of glucose homeostasis and level of available energy reserves. Circulating factors are able to directly signal to nuclei in the hypothalamus via the median eminence. These interactions are crucial for the brain to effectively regulate energy homeostasis. Leptin and insulin are two key long term tonic signals that circulate in levels proportional to fat mass that play key roles in this process.

1.4.1 Leptin

Leptin, coded for by the *ob* gene, is produced by white adipocytes and circulates at levels proportional to adipose tissue (Zhang et al., 1994, Schwartz et al., 1996a). Mice with mutations in the *ob* gene are obese, diabetic and exhibit reduced energy expenditure. This phenotype can be rescued by chronic peripheral administration of leptin (Pelleymounter et al., 1995). Parabiosis studies originally indicated that the *ob* gene encoded a circulating satiety factor, as the obese *ob/ob* mouse lost weight due to leptin produced by a wild type mouse or *db/db* mouse, which lacks the leptin receptor (Coleman, 1973). Leptin acts as a chronic peripheral signal of available energy reserves in the form of fat, and crosses the BBB and binds the leptin receptor, encoded by the *ObRb* gene, to exert its effects. The leptin receptor is expressed on ARC NPY/AgRP and POMC neurons and also in the PVN, DMN and VMN (Baskin et al., 1999, Cheung et al., 1997, Schwartz et al., 1996c). An increase in leptin signalling suppresses food intake and increases energy expenditure (Weigle et al., 1995). This is thought to occur partly due to the increased activity of arcuate POMC neurons and inhibition of NPY/AgRP neurons (Seeley et al., 1997, Wang et al., 1997, Chun and Jo, 2010, Balthasar et al., 2004).

Further evidence that leptin acts as a signalling factor for adiposity came from human studies, indicating a 12 hour fast leads to a drop in leptin levels, while it is released in response to acute and chronic refeeding (Kolaczynski et al., 1996). Due to its ability to alter food intake and role as a chronic signal of energy status, leptin therapy was proposed as a treatment for obesity. However, it was discovered that human obesity is commonly associated with resistance

to leptin, which may arise due to decreased transport into the cerebrospinal fluid (Halaas et al., 1997, Heymsfield et al., 1999). Homozygous frameshift mutations in the leptin gene result in severe early onset obesity in humans, which can be successfully treated with exogenous leptin administration, as in the *ob/ob* mouse (Farooqi et al., 1999). However, congenital leptin deficiency is extremely rare in humans, while mutations in the leptin receptor are even scarcer (Farooqi et al., 2007b). Functional magnetic resonance imaging in patients with congenital leptin deficiency has shown that leptin acts on mesolimbic neural circuits to decrease food reward perception (Farooqi et al., 2007a). A set of Ob-Rb-expressing neurons in the LH also express neurotensin, a peptide involved in the modulation of dopamine signalling. Deletion of the leptin receptor in these neurons results in early onset obesity in mice, resulting from a slight increase in food intake and decreased locomotor activity, indicating the importance of these neurons in body weight homeostasis (Leinninger et al., 2011). In addition, animal studies suggest that leptin suppresses the ability of sucrose to drive dopaminergic neuronal activation, therefore decreasing motivation to work for a reward (Domingos et al., 2011).

1.4.2 Insulin

Insulin, produced by pancreatic β-cells, plays a well characterised role in blood sugar homeostasis by regulating the storage of glucose in the liver, adipose and skeletal muscle. In addition, insulin is proposed to act as a long term adiposity signal, as it circulates at levels proportional to adipose and body mass index, while also reflecting acute changes in energy intake (Polonsky et al., 1988, Bagdade et al., 1967). Circulating insulin crosses the BBB via a saturable transport mechanism and binds central insulin receptors, which are expressed throughout regions involved in energy homeostasis, such as the ARC (Woods et al., 2003, Marks et al., 1990). The central administration of insulin reduces food intake in rodents, and is thought to occur in part via excitation of ARC POMC and inhibition of NPY neurons (Chavez et al., 1995, Qiu et al., 2014). ICV insulin increases the expression of POMC mRNA and prevents fasting-induced increases in arcuate NPY expression (Benoit et al., 2002, Schwartz et al., 1991). Peripheral infusion of low levels of insulin, which do not result in hypoglycaemia, resulted in decreased meal size in normal weight rats (VanderWeele et al., 1982). CNS-specific disruption of the insulin receptor increases food intake in female mice, and results in diet-induced obesity in male and female mice (Bruning et al., 2000). This evidence suggests that insulin plays a role in body weight homeostasis in the brain.

1.5 Gut hormones and enteroendocrine cells

Enteroendocrine cells (EECs) are located throughout the GIT, which represents the largest endocrine organ in the human body. EECs constitute less than 1% of the cell population in the intestinal epithelium, but are an important component of the gut-brain axis (Reimann et al., 2012). EECs are capable of secreting over twenty peptide hormones that influence processes including gut motility, gastric acid secretion and energy intake. It was previously thought that EECs could be separated into discrete families of cells with specific secretory profiles, including gastrin-secreting G-cells, CCK-secreting I-cells and GLP-1- and PYY-secreting L-cells (Rindi et al., 2004). However, recent work has suggested

these families may be less well defined, existing instead as a wide range of cell types that secrete various combinations of peptides (Habib et al., 2012).

EECs sense luminal contents; open-type cells often display a distinct cone-shaped morphology, with one extremity possessing microvilli on apical processes and the other located adjacent to the basal lamina. Microvilli are thus in immediate contact with the luminal contents, sensing of which can lead to the secretion of gut hormones into the nearby blood vessels (Cummings and Overduin, 2007, Engelstoft et al., 2008). Receptors on the apical surface of open-type L-cells directly sense dietary components in the intestinal lumen, and respond to produce the appropriate endocrine response which will influence neuronal signalling in central appetite centres. The macronutrient contents of the intestinal lumen vary considerably with diet, requiring a number of specific chemosensors of a variety of GPCRs to detect the different macronutrients that modulate the secretion of hormones (Fig 1.5). Closed-type EECs react to neural or circulating signals and do not directly interact with ingested nutrients. Furthermore, the peptides released from EECs can act on nearby cells, including neurones, in a paracrine manner. Recent evidence suggests that EECs are able to interact directly with enteric neurones via synapse-like structures termed neuropods (Bohorquez et al., 2011, Bohorquez et al., 2015).

1.5.1 L-cells

Mature EECs that express the preproglucagon gene are commonly defined as L-cells. Preproglucagon is post-translationally processed in a tissue-specific manner, yielding different hormonal products in the pancreas and intestine. L-cells secrete the products of preproglucagon cleavage by prohormone convertase 1, including GLP-1, glucagon-like peptide 2 (GLP-2), glicentin and oxyntomodulin (Fig 1.6) (Petersen et al., 2015, Eissele et al., 1992, Dhanvantari et al., 1996). L-cells co-secrete distinct peptides depending on their location; those in the upper small intestine demonstrate co-localisation with gastric inhibitory peptide (GIP) , while L-cells in the lower small intestine show high levels of co-localisation with PYY and CCK (Habib et al., 2013, Habib et al., 2012). L-cells that co-express GLP-1 and PYY increase in density along the small intestine and then the large intestine. Thus, the contact of ingested nutrients with these L-cells increases along the GIT (Larsson et al., 1975, Eissele et al., 1992).

In a recent study, super-resolution microscopy demonstrated that L-cell secretory vesicles contain primarily GLP-1 or PYY, suggesting that either hormone may be selectively released from a microdomain within a single EEC (Cho et al., 2014). However, how specific combinations of cellular machinery stimulate the differential release of GLP-1 or PYY remains to be elucidated.

The most effective treatment for obesity is bariatric surgery, which results in sustained weight loss. The Roux-en Y-Gastric bypass is the most commonly performed procedure (O'Brien, 2015). The normal postprandial response involves a complex combination of hormones that reflects the ingested and absorbed macronutrients. However, the post-prandial GLP-1 and PYY response in particular, has been reported to be blunted in obese patients (Pedersen-Bjergaard et al., 1996, le Roux et al., 2006a). Interestingly, bariatric surgery patients exhibit increased postprandial levels of GLP-1 and PYY, and reduced levels of ghrelin (Chronaiou et al., 2012). In addition, within days of surgery,

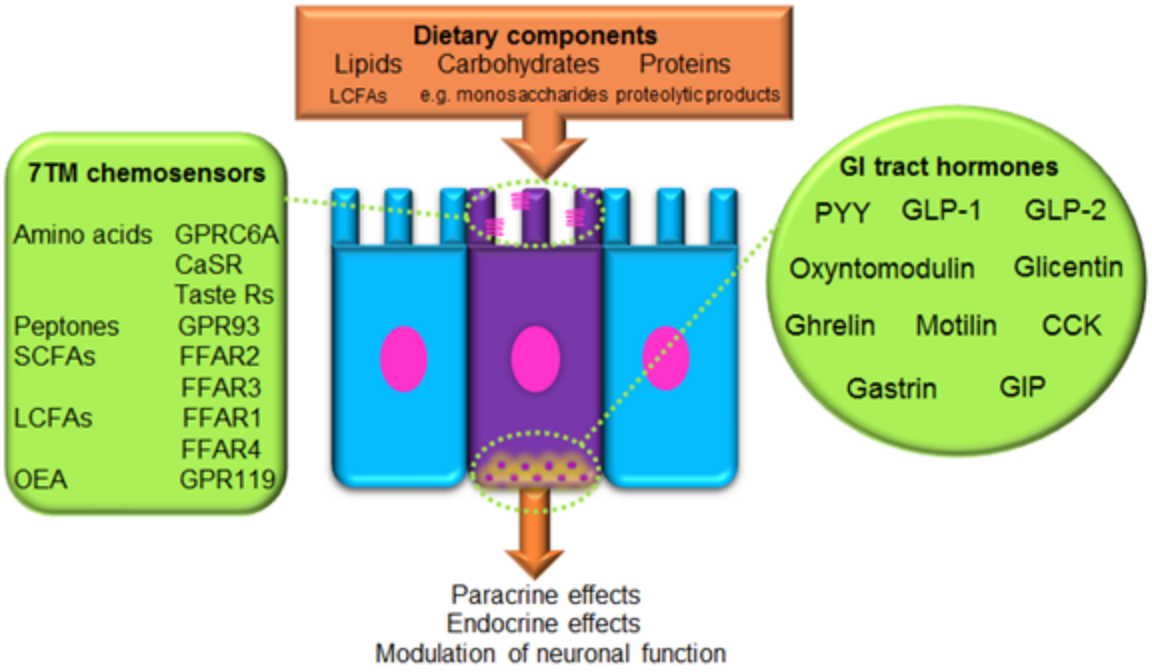


Fig 1.5 Summary of the macronutrient sensing receptors expressed by enteroendocrine cells and the gut peptides they release.

Microvilli of an open-type enteroendocrine cell extend into the gut lumen and directly contact ingested macronutrients. Various GPCRs and transporters located on the apical border sense food components. GI tract hormones are released into the circulation, acting via paracrine, endocrine and neural pathways to modulate food intake. LCFA; long chain fatty acid, 7TM; 7-transmembrane, CaSR; calcium-sensing receptor, GPRC6A; G-protein coupled receptor family C group 6 subtype A, GPR93; G-protein coupled receptor 93, SCFA; short chain fatty acid, FFAR; free fatty acid receptor, OEA; oleoylethanolamide, GPR119; G-protein coupled receptor 119, GI; gastro-intestinal, PYY; peptide YY, GLP; glucagon-like peptide, CCK; cholecystokinin, GIP; gastric inhibitory peptide. Adapted from (Engelstoft et al., 2008).

amelioration of type II diabetes frequently occurs. It has been widely proposed that the altered postprandial levels of these gut peptides may be responsible for some of the metabolic effects of bypass surgery, and EECs are therefore a key area of interest in research into alternatives to surgery. Changes in intestinal morphology, such as villus hyperplasia, have been implicated in the adaptive response to Roux-en Y-Gastric bypass and ileal interposition in rats (Stearns et al., 2010, Kohli et al., 2010).

1.5.2 Ghrelin

Ghrelin is an orexigenic peptide released from X/A-like EECs in the stomach and upper small intestine (Date et al., 2000). Ghrelin is the endogenous ligand for the growth hormone secretagogue receptor, located in the pituitary; where it causes the release of growth hormone, and also acts on hypothalamic NPY/AgRP neurons (Dickson and Luckman, 1997). Dopaminergic D_2 receptors in the shell of the nucleus accumbens also mediate the rewarding effects of food by ghrelin (Skibicka et al., 2013). Ghrelin exists in two major forms: acylated and des-acyl ghrelin. Acylated

ghrelin has an n-octanoyl group attached to the serine at position 3, due to the action of the enzyme ghrelin O-acyltransferase (GOAT), which is essential for its biological activity (Kojima et al., 1999, Yang et al., 2008).

Ghrelin levels rise following fasting and exogenous administration potently stimulates feeding (Cummings et al., 2001, Tschop et al., 2000, Wren et al., 2000). The orexigenic effects of ghrelin are abolished in mice lacking NPY/AgRP (Chen et al., 2004), suggesting this neuronal population mediates the effects of ghrelin on appetite. Genetic deletion of ghrelin results in only minor changes in energy homeostasis (Sun et al., 2003, De Smet et al., 2006). However, deletion of the ghrelin receptor does reduce food intake in mice, and ameliorates diet-induced obesity, suggesting ghrelin signalling is an important physiological regulator of feeding behaviour (Zigman et al., 2005).

1.5.3 Cholecystokinin

Cholecystokinin is an anorexigenic hormone secreted in response to fat and protein from enteroendocrine I-cells, predominantly located in the proximal intestine (Buchan et al., 1978). The actions of CCK on food intake are largely mediated via the CCK1 receptor, expressed by vagal afferent neurons (Moran et al., 1990). The satiating effects of CCK are blocked by vagotomy in rats (Lorenz and Goldman, 1982). In addition, CCK stimulates gall bladder contraction, slows gastric motility and stimulates pancreatic enzyme secretion (Liddle et al., 1985, Brugge et al., 1986). Acute exogenous infusion of CCK reduces food intake in rodents and humans, and antagonism of the CCK1 receptor increases food intake, demonstrating a physiological role in energy homeostasis (Muurahainen et al., 1988, West et al., 1984, Hewson et al., 1988, Beglinger et al., 2001). However, chronic infusion of CCK does not reduce body weight, as feeding patterns adapt to compensate for reduced meal size and hence calorie intake remains unchanged, limiting its potential therapeutic use (West et al., 1987). On the other hand, CCK does appear to increase sensitivity to exogenous leptin and amylin, and may therefore represent a viable component of combination medications in the future (Bhavsar et al., 1998, Matson et al., 2000).

1.5.4 Glucagon-like peptide-1

Peripheral or central administration of GLP-1 suppresses food intake in rodents, and peripheral administration suppresses food intake in humans (Flint et al., 1998, Turton et al., 1996). GLP-1 circulates at basal levels in the fasting state. L-cell-secreted GLP-1 diffuses into the lamina propria and enters the systemic circulation via the hepatic portal vein (Vilsboll et al., 2003). In humans, plasma concentrations rise within 15 minutes of food ingestion, peaking at around 40 minutes following a mixed meal, then reach a plateau (Todd et al., 1998). GLP-1 undergoes enzymatic cleavage in the intestinal endothelium and liver by dipeptidyl peptidase IV (DPPIV), which converts GLP-1 to an inactive form and results in a half-life of approximately 2 minutes (Holst, 2007). GLP-1 mediates its effects via the GLP-1 receptor, though it is unclear precisely how endogenous GLP-1 mediates its anorectic effects. The GLP-1 receptor is expressed centrally in several regions involved in energy homeostasis, including the ARC and PVN of the hypothalamus and the NTS of the brainstem. However, centrally produced GLP-1 is also thought to act as a neuropeptide, and it is difficult to determine whether peripheral administration of GLP-1 may result in

pharmacological activation of central circuits that are normally only responsive to central endogenous GLP-1 (Fig 1.7) (Bullock et al., 1996). Evidence suggests that peripheral administration of liraglutide, a long-acting GLP-1 analog, acts on ARC POMC neurons to drive weight-loss (Secher et al., 2014). GLP-1 administered ICV causes a dose-dependent decrease in food and water intake, and this suppression is abolished by administration of the GLP-1 receptor antagonist, exendin 9-39 (Turton et al., 1996, Navarro et al., 1996, Tang-Christensen et al., 1996). ICV GLP-1 results in c-Fos like immunoreactivity in the PVN and CeA, which is also attenuated by co-administration of exendin 9-39 (Turton et al., 1996). Repeated daily ICV GLP-1 administration decreases body weight in rats, thought to occur due to effects on food intake and increased satiation (Meeran et al., 1999). In addition to roles in homeostatic energy regulation, GLP-1 receptor agonists, such as exendin-4, affect hedonic pathways in the mesocorticolimbic system. The CPP paradigm involves training rodents to pair a stimulus with reward in a certain location. Exendin-4 reduces the preference of a location previously paired with a food reward, and also decreases motivational behaviour for sucrose in rats. These effects are mediated via GLP-1 receptors in the VTA and NAc of the mesolimbic system (Dickson et al., 2012). In humans, fMRI has identified GLP-1 receptors in the insula, CeA, CPu, and orbitofrontal cortex as mediators of exendin-4-induced reduction in food intake (van Bloemendaal et al., 2014). These brain regions are active when human participants are shown food images in the fasted state, and activation decreases following administration of GLP-1 (De Silva et al., 2011).

In the periphery, the GLP-1 receptor is expressed in the heart, kidney, pancreatic islets and the GIT (Bullock et al., 1996). Peripheral GLP-1 may also act via a neural rather than an endocrine route. It may activate receptors near its site of release before it is broken down, such as receptors on sensory afferent fibres of the nodose ganglion, which relays impulses to the hypothalamus and NTS (Holst and Deacon, 2005).

Subdiaphragmatic vagal deafferation attenuates the anorectic effects of intraperitoneally-administered GLP-1, whereas the effects of GLP-1 administered into the vena cava or the hepatic portal vein are not affected. This suggests that intraperitoneal GLP-1 requires abdominal vagal afferent signalling to exert its anorectic effects, whereas circulating GLP-1 acts via an alternate mechanism (Ruttimann et al., 2009). It has been proposed that intraperitoneally administered GLP-1 acts in a paracrine fashion, similar to L-cell-secreted GLP-1, before DPPIV denatures the peptide (Ruttimann et al., 2009, Nakagawa et al., 2004). However, vagal deafferation can result in a number of changes to energy homeostatic and feeding systems, which may alter the background against which GLP-1 acts, and caution must be used in interpreting results from such animal models.

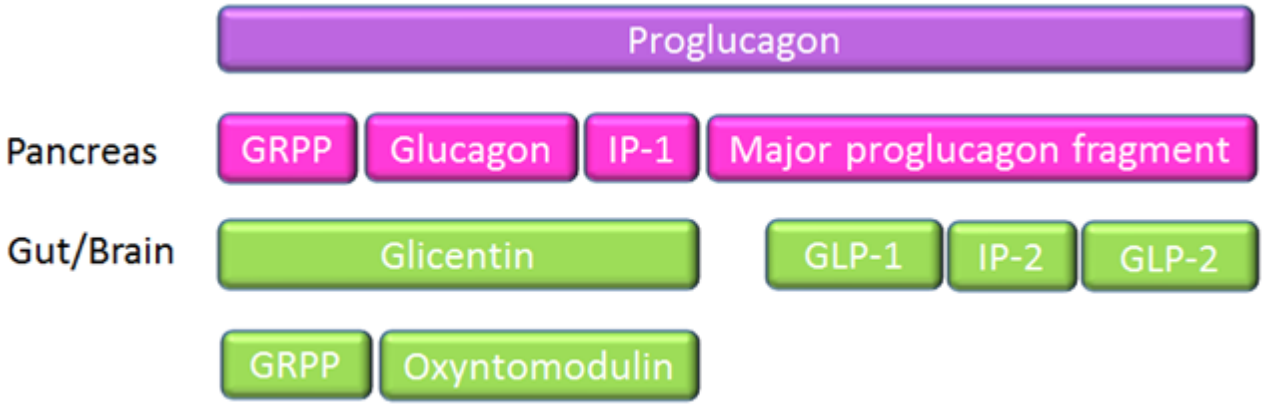


Fig 1.6 Schematic diagram illustrating post-translational processing of preproglucagon in neuroendocrine cells.

GRPP: glicentin-related polypeptide, IP-1: intervening peptide 1, IP-2: intervening peptide 2, GLP-1: glucagon-like peptide-1, GLP-2: glucagon-like peptide-2. Adapted from (Holst, 2007).

GLP-1 also has a well characterised incretin role; peripheral administration in rodents and humans increases glucose-stimulated insulin release (Kreymann et al., 1987, Fridolf et al., 1991). In the presence of glucose, binding of GLP-1 to receptors on pancreatic β-cells causes an increase in intra-cellular calcium, and exocytosis of insulin-containing vesicles (Fig 1.7) (Vilsboll and Holst, 2004). However, due to the relatively small increase and the short lifespan of the peptide, there is some controversy surrounding the importance of the incretin role of GLP-1 (D'Alessio, 2011). Pancreatic knockdown of *Glp1r* in mice impairs glucose tolerance in response to hyperglycaemia, and attenuates insulin secretion in response to exogenous GLP-1. However, a DPPIV inhibitor retained its glucose lowering effects in these mice, suggesting the β-cell GLP-1 receptor is not required for the beneficial effects of such agents on glucose homeostasis (Smith et al., 2014).

GLP-1 also stimulates the ileal brake, i.e. the feedback mechanism initiated by the presence of unabsorbed dietary components in the ileum, which influences proximal gut function to control transit of a meal through the GIT, allowing efficient digestion and uptake of nutrients (Pironi et al., 1993, Nauck et al., 2011). For example, GLP-1 slows the rate of gastric emptying, though this effect is subject to rapid tachyphylaxis following chronic exposure, likely at the level of the vagus nerve. In human subjects given a continuous intravenous infusion of GLP-1, postprandial concentrations of glucose, glucagon and insulin progressively increased at subsequent meals. Therefore, it is possible that part of the glycaemic control afforded by administration of GLP-1 occurs secondary to delayed gastric emptying (Nauck et al., 2011).

1.5.5 Oxyntomodulin

Oxyntomodulin is a dual agonist of the GLP-1 and glucagon receptors, though it has a 10-100 fold lower affinity than their primary endogenous agonists, and its effects appear to combine those of the native ligands (Ghatei et al., 1983, Dakin et al., 2001, Pocai et al., 2009). Central oxyntomodulin induces satiety to the same magnitude as GLP-1, despite a much lower affinity for the GLP-1 receptor (Dakin et al., 2001). Exogenous administration reduces food intake in rodents and humans via the GLP-1 receptor, and also regulates gastric acid and exocrine pancreatic secretion

(Dubrasquet et al., 1982, Baggio et al., 2004, Dakin et al., 2004). Chronic administration of oxyntomodulin before eating caused significant weight loss in overweight and obese human subjects (Wynne et al., 2006). In addition, studies in pair-fed rats suggested that oxyntomodulin-stimulated weight loss may be the result of increased energy expenditure, and subcutaneous administration of oxyntomodulin was confirmed to increase activity-related energy expenditure in humans (Dakin et al., 2004, Wynne et al., 2006). Oxyntomodulin is therefore at the forefront of peptides of interest in the treatment of obesity. Modifying the N-terminus of oxyntomodulin confers resistance to enzymatic degradation by DPPIV, and has shown significant therapeutic potential in terms of improved glycaemic control and appetite suppression (Zhu et al., 2003, Lynch et al., 2014). In addition, modified peptides that activate both GLP-1 and glucagon receptors have also been investigated, with co-agonism resulting in greater body fat reduction compared to GLP-1 receptor agonism alone in DIO mice (Day et al., 2009, Day et al., 2012, Clemmensen et al., 2014).

1.5.6 PYY

Similarly to GLP-1, PYY levels rise approximately 15 minutes postprandially, however they peak later, at around 90 minutes (Batterham and Bloom, 2003). PYY exists in two major circulating forms: the full length peptide, PYY_{1-36}, and a truncated form, PYY_{3-36}, due to the action of DPPIV. Full length PYY acts on receptors Y1, Y2 and Y5, whereas PYY_{3-36} is selective for the Y2 receptor (Fig 1.7) (Grandt et al., 1994b, Guarita et al., 2000). PYY_{3-36} is as an anorectic peptide; acute peripheral administration of PYY_{3-36} to rodents and humans reduces food intake (Batterham et al., 2002, Batterham and Bloom, 2003).

PYY has a half-life of approximately ten minutes in humans, though plasma levels remain increased for up to 6 hours postprandially, due to sustained release (Batterham and Bloom, 2003, Lluis et al., 1989). Intermittent exogenous administration of PYY_{3-36} reduces food intake, body weight and adiposity in rats, and prevents weight gain in diet-induced obese (DIO) rats (Chelikani et al., 2007, Chelikani et al., 2006a). The primary mechanism by which PYY_{3-36} reduces food intake appears to be via Y2 receptors, as its anorectic effect is absent in Y2 receptor knockout mice (Batterham and Bloom, 2003, Batterham et al., 2002). This may be mediated via a direct central effect. Administration of PYY_{3-36} into the ARC inhibits food intake in rats (Batterham et al., 2002), and peripheral administration of PYY_{3-36} activates a number of different brain regions (Batterham et al., 2007, De Silva et al., 2011). Radiolabelled PYY_{3-36} binds strongly to regions in the mesocorticolimbic system, and peripheral administration of PYY_{3-36} activates cortical regions associated with mesocorticolimbic activation (Dumont et al., 1996, Batterham et al., 2007).

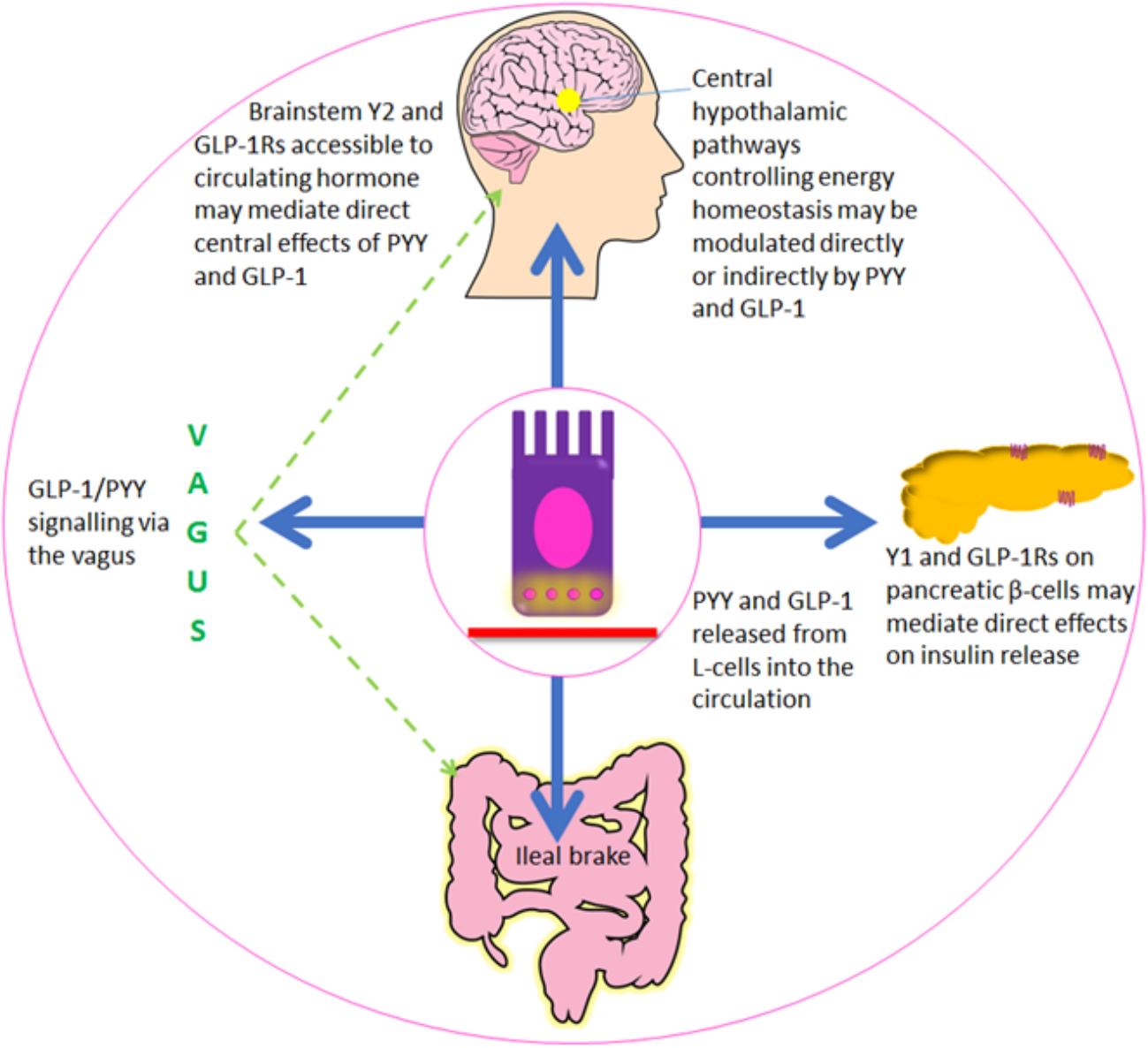


Fig 1.7 A summary of the targets for L-cell secreted PYY and GLP-1 signalling.

Following nutrient ingestion, PYY and GLP-1 diffuse into the lamina propria and enter the circulation. GLP-1 binds its receptors on pancreatic β-cells, leading to insulin secretion (Vilsboll and Holst, 2004). Full-length PYY binds pancreatic Y1 receptors and inhibits glucose-stimulated insulin secretion, while PYY_{3-36} may exert effects on glucose homeostasis via extra-islet Y2 receptors (Boey et al., 2006; Chandarana et al., 2013). Circulating hormones are able to access areas of the hindbrain with an incomplete blood brain barrier, such as the area postrema, which communicates with the nucleus of the solitary tract (Orskov et al., 1996). GLP-1 and PYY_{3-36} may signal via the vagus to central hypothalamic nuclei controlling energy homeostasis, where receptors for these hormones are widely expressed (Batterham and Bloom, 2003; Halatchev and Cone, 2005; Richards et al., 2014; Secher et al., 2014). Adapted from Spreckley and Murphy, 2015 (Spreckley and Murphy, 2015).

However, PYY_{3-36} may also work through peripheral mechanisms. Like GLP-1, PYY_{3-36} stimulates the ileal brake. PYY_{3-36} has been suggested to act via peripheral Y2 receptors on vagal afferent fibres (Fig 1.7). However, there is conflicting evidence as to whether an intact vagus is required for PYY_{3-36} to signal satiety (Halatchev and Cone, 2005). In addition, PYY_{3-36} may induce hypophagia by causing malaise. Infusions of PYY_{3-36} have been dose-dependently associated with CTA in rats and mice, thought in part to be due to inhibition of gastric emptying, and similar effects have been observed in human studies (Chelikani et al., 2006b, Halatchev and Cone, 2005, Degen et al., 2005). The aversive effects of this peptide may thus limit its therapeutic potential as an anti-obesity agent.

1.6 Aversion vs. satiation

Satiation infers a feeling of fullness that brings an end to eating episodes, owing to gastric distension, cognitive and endocrine signals. Satiety is the sense of fullness that persists after eating, and prevents the induction of further eating episodes (Bellisle et al., 2012). Satiation and satiety are consequences of homeostatic signalling. However, reductions in food intake may also reflect feelings of nausea or visceral illness (Deutsch and Gonzalez, 1978). These sensations may involve alterations in hedonic signalling in mesocorticolimbic areas (Ellenbroek et al., 1997, Gallo et al., 1992, Nerad et al., 1996). Physiological levels of peptides involved in satiation, such as $PYY_{3-36,}$ CCK and GLP-1, are not normally associated with nausea, whereas high, pharmacological levels of these agents often are (le Roux et al., 2008, Tella and Rendell, 2015, Greenough et al., 1998). Consequently, it is not clear whether the nausea experienced is an independent effect, or if nausea and satiety exist at opposite ends of the same physiological spectrum (Greenough et al., 1998).

Aversion is the avoidance of a substance that has produced malaise, and in non-emetic species, CTA is a well characterised model to distinguish substances that reduce feeding by satiation or aversion. CTA is a more sensitive paradigm for detecting aversion in rodents than conditioned place aversion, and is able to detect aversion caused by much lower doses of LiCl (Gore-Langton et al., 2015). However, the absence of a significant CTA does not definitively exclude nausea as an explanation for a reduction in eating, as degrees of malaise too small to trigger a CTA may manifest as a suppression of food intake (Deutsch and Gonzalez, 1978). In addition to the endpoint of induction of a CTA, the behaviour of an animal following administration of a substance can be used to detect aversive properties. Originally established to describe the appetite-regulating effects of CCK in rats, the behavioural satiety sequence (BSS) describes the characteristic behaviours that confer the natural process of satiation (Antin et al., 1975). The BSS involves the transition from eating, through grooming and exploration, to resting. Drugs may physiologically induce satiation by accelerating the BSS, or act non-physiologically by interfering with its structure. Behavioural measures typically recorded when assessing the BSS include latency to locating and eating food, eating bout length, drinking, grooming, locomotion, rearing and resting (Rodgers et al., 2010, Tallett et al., 2009).

1.7 Thesis hypothesis and general aims

Work from within this lab and others has established doses of GLP-1 and PYY_{3-36} that reduce food intake in rodents and man (De Silva et al., 2011, le Roux et al., 2008, Chelikani et al., 2006a, Batterham and Bloom, 2003, Batterham et al., 2002, Turton et al., 1996, Kreymann et al., 1987). Furthermore, GLP-1 and PYY_{3-36} are known to cause nausea in humans and CTA in rodents at higher doses, with effects on homeostatic and hedonic pathways (Dickson et al., 2012, De Silva et al., 2011, Kinzig et al., 2002, Seeley et al., 2000, Thiele et al., 1998, Chelikani et al., 2006b, Halatchev and Cone, 2005). This project investigated the effects of GLP-1 and PYY_{3-36} on food intake, other behaviours and physiological parameters, and on aversion, comparing them to other appetite-suppressing agents including leptin, sibutramine and LiCl. Leptin is a physiological anorectic agent that also reduces food intake in rodents and humans, with established actions in homeostatic and hedonic pathways (Leinninger et al., 2011, Hayes et al., 2010). Sibutramine produces robust effects on food intake, which may occur via a combination of physiological and non-physiological mechanisms, and is known to produce cardiovascular side effects (Araujo and Martel, 2012, Jackson et al., 1997, Woolard et al., 2004). LiCl is an emetic agent in humans, and causes aversion in rodents (Rinaman and Dzmura, 2007, Rodriguez et al., 2000). The long-term aim is to establish a mathematical model in collaboration with the Mathematics Department, Imperial College London, capable of distinguishing satiety and satiation from aversion in rodent models from specific data (e.g. food intake patterns, cardiovascular parameters). Establishing a single experimental model to identify specific inhibitors of appetite would significantly reduce animal use in the research community and drug development industry.

1.7.1 Hypotheses

A threshold dose exists at which exogenous GLP-1 and PYY_{3-36} reduce food intake. Central aversive circuits may be activated by further threshold doses, with nausea and aversion representing distinct effects to satiation (Fig 1.8). It will be possible to distinguish between doses of GLP-1 and PYY_{3-36} that specifically influence energy homeostatic pathways to reduce food intake, from those that do so non-specifically, and comparison of effects with agents that have well characterised effects on food intake, such as leptin, LiCl and sibutramine, will assist with this. The combination of CLAMS and telemetry systems will be able to detect these physiological differences.

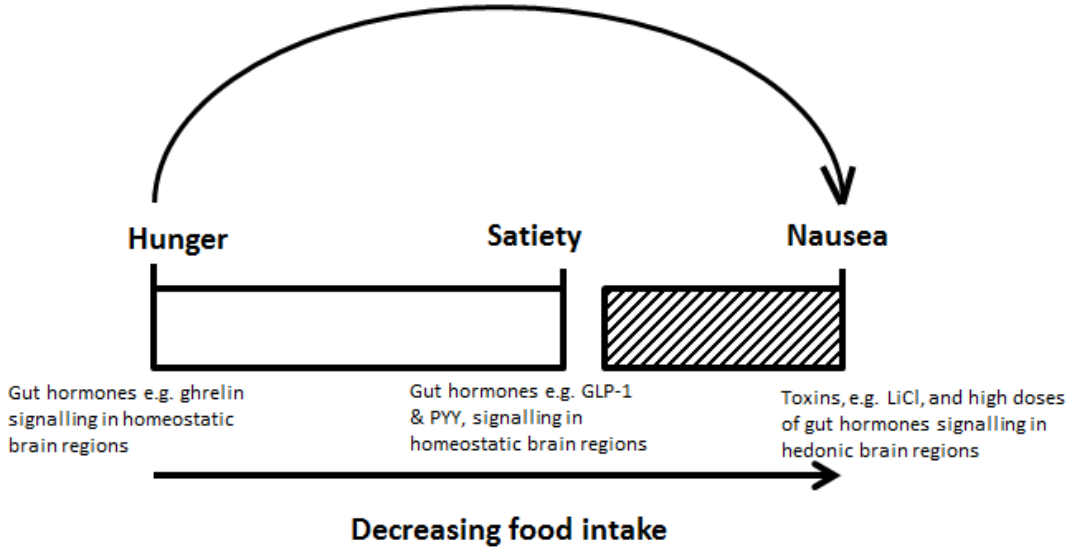


Fig 1.8 A schematic diagram of the continuum of hunger to nausea being investigated, and the role of gut hormones in this process.
Threshold doses of PYY and GLP-1 exist that induce satiation without causing feelings of malaise. Further threshold doses exist at which central aversive circuits are activated. Toxic substances, such as LiCl activate central aversive circuits independently of satiation.

1.7.2 Aims

1. To investigate the effects of peripheral administration of physiological anorectic agents, including GLP-1_{3-37}, PYY_{3-36} and leptin, on food intake, cardiovascular and behavioural parameters in rats.

2. To investigate the effects of peripheral administration of non-physiological appetite-reducing agents, such as LiCl, on food intake, cardiovascular and behavioural parameters in rats.

3. To provide data to establish a mathematical model in collaboration with the Mathematics Department, Imperial College London that can distinguish between physiological and non-physiological anorectic factors.

Chapter II: The effects of leptin and sibutramine on energy homeostasis and cardiovascular parameters, and of lithium chloride on energy homeostasis, in rats.

2.0 Chapter introduction

This chapter will focus on the effects of leptin, sibutramine and LiCl on food intake, energy expenditure and cardiovascular parameters. Leptin reduces food intake in rodents and humans, both endogenously and exogenously, and has established actions in homeostatic and hedonic pathways (Leinninger et al., 2011, Hayes et al., 2010). Sibutramine and LiCl are pharmacological agents that produce robust effects on food intake. LiCl is also a known aversive compound and sibutramine produces cardiovascular side effects in humans and rodents (Araujo and Martel, 2012, Jackson et al., 1997, Woolard et al., 2004, Rinaman and Dzmura, 2007, Rodriguez et al., 2000). Leptin, sibutramine and LiCl will thus provide background information, such as food intake patterns and cardiovascular parameters, on the physiological signatures of endogenous and pharmacological anorectic factors. This data will then be compared with those of various doses of GLP-1 and PYY, and will collectively be used to establish a mathematical model in collaboration with the Mathematics Department, Imperial College London, capable of distinguishing satiety from aversion in rodent models.

2.1 Leptin

2.1.1 Introduction

Leptin is an 167 amino acid protein expressed and secreted by adipocytes, which circulates at levels proportional to fat mass (Zhang et al., 1994). The full length leptin receptor possesses a long carboxyl terminal tail that interacts with specific kinases to promote transcription through the signal transducer and activator of transcription (STAT) pathway (Vaisse et al., 1996). The leptin receptor is expressed in adipose tissue, vascular endothelial cells, heart, kidney, liver, spleen, pancreatic islets, and testis, as well as centrally in hypothalamic and brainstem nuclei (Lee et al., 1996, Emilsson et al., 1997, Sierra-Honigmann et al., 1998, Mizuno et al., 1998, Dhillon et al., 2006).

2.1.2 Effects of leptin on food intake

The ARC has a high level of expression of leptin receptors, which are found both on POMC neurons and NPY/AgRP neurons (Cheung et al., 1997, Mercer et al., 1996). Leptin increases neuronal firing of POMC neurons, and stimulates *POMC* expression and the release of POMC-derived peptides in fasting animals, leading to decreased food intake (Mizuno et al., 1998). Levels of AgRP are increased in leptin-deficient mice, while POMC is decreased, and these effects can be corrected by exogenous leptin administration (Seeley et al., 1997). In rats, a dose of 1 mg/kg exogenous leptin leads to a greater reduction in protein and fat intake than carbohydrate, indicating leptin may affect the palatability of these macronutrients (Wetzler et al., 2004). An additional study found peripheral leptin administration reduced food intake and was associated with decreased levels on NPY mRNA compared with controls, which may underlie increased satiety; this effect was absent in the fatty Zucker rat (Dryden et al., 1999). Zucker rats possess a mutation in the extracellular domain of the Ob leptin receptor expressed on NPY neurons and the choroid plexus, which may be involved in transport of leptin into the CSF (Wang et al., 1996).

Mice carrying homozygous mutations in the *ob* gene do not produce leptin, and are obese as a result (Zhang et al., 1994). Both the peripheral and central administration of recombinant leptin lead to a reduction in the food intake and body weight in *ob/ob* mice (Halaas et al., 1997, Pelleymounter et al., 1995, Campfield et al., 1995). Serum leptin levels correspond to the amount of adipose mass in the body, and hence are proposed to signal the availability of energy stores to the brain, thus potentially limiting weight gain during times of nutritional abundance (Schwartz et al., 1996b). Conversely, leptin levels fall during periods of starvation, which triggers adaptive responses to conserve energy and promote survival, including limiting thyroid hormone-driven thermogenesis and switching off the hypothalamic-pituitary-gonadal axis, delaying ovulation in females. These neuroendocrine changes in response to fasting are attenuated by exogenous administration of leptin (Ahima et al., 1996). A similar phenotype arises in humans with rare homozygous mutations of the *lep* gene, which result in truncated and non-secreted leptin. Though extremely infrequent, frameshift, nonsense and missense mutations in the leptin receptor have also been described in humans, more commonly in consanguineous families. In patients with severe-early onset obesity, congenital leptin deficiency is diagnosed by undetectable serum leptin. These patients exhibit impaired satiety and intense food seeking behaviour, with evidence of hypogonadotropic hypogonadism and absence of puberty. As in mice, human leptin deficiency is treatable by the subcutaneous administration of leptin (Farooqi and O'Rahilly, 2014).

Food seeking in leptin deficiency is indiscriminate of food type, indicating that leptin may be involved in mediating the rewarding properties of food. Functional magnetic resonance imaging studies indicate that in the leptin deficient state, patients gave high ratings to all food images, even after feeding. Following 7 days of exogenous administration, well-liked food images were given a lower score, unless in the fasted state. This was accompanied by changes in neuronal activation; leptin deficient individuals demonstrated higher levels of activation of the nucleus accumbens, caudate nucleus and ventral striatum (Farooqi et al., 2007a).

2.1.3 Effects of leptin on energy expenditure

Leptin treated animals lose more weight than vehicle-treated pair-fed controls, and leptin treatment increases core temperature and metabolic rate, indicating an effect on energy expenditure (Halaas et al., 1995, Pelleymounter et al., 1995). Leptin treatment in leptin-deficient mice leads to an increased energy expenditure, as shown by increased oxygen consumption (VO_2). In addition, a decrease in the ratio of exhaled CO_2:O_2 has been observed, indicating a change in the primary fuel source from carbohydrate to fat, supported by increased serum free fatty acid (FFA) levels (Hwa et al., 1997).

In response to weight loss, obese patients experience a decrease in energy expenditure. This metabolic adaption is prevented by leptin treatment in deficient subjects, and additionally results in higher levels of fat oxidation compared to non-treated controls (Galgani et al., 2010). Leptin also stimulates fatty acid oxidation in skeletal muscle in rodents, via the phosphorylation of AMPK (Minokoshi et al., 2002).

The ARC is often suggested to be the primary site of action of leptin. However, the VMH has appears to be important in mediating the effects of leptin on energy expenditure. Mice lacking the leptin receptor in the VMH are severely

obese and have lower energy expenditure than controls when on a high-fat diet (Bingham et al., 2008, Dhillon et al., 2006). Injection of leptin into the NTS also stimulates energy expenditure, but this effect may be pharmacological rather than physiological; knockdown of leptin receptors in the NTS results in hyperphagia, but no effect on energy expenditure or body temperature (Skibicka and Grill, 2009, Hayes et al., 2010).

It has been suggested that leptin regulates energy expenditure by stimulating the sympathetic nervous system. Thermogenesis in BAT is mediated by sympathetically innervated β_3 adrenergic receptors. Leptin increases expression of uncoupling protein 1 (UCP1), which uncouples oxidative phosphorylation in mitochondria so that the energy produced is used to generate heat (Scarpace and Matheny, 1998). In rats, exogenous administration of leptin increases sympathetic outflow to the adrenal glands and to brown adipose tissue, where it also increases norepinephrine turnover (Haynes et al., 1997a, Collins et al., 1996). A dose of 1 mg/kg murine leptin administered intravenously to rats increases sympathetic nervous activity in brown adipose by 200%. This effect disappears with ganglion blockade, indicating it is mediated via efferent sympathetic nerves (Haynes et al., 1997a). Leptin does not increase sympathetic activation in Zucker obese rats, which possess a mutation in the gene for the leptin receptor, providing convincing evidence that this effect is mediated specifically by the leptin receptor (Haynes et al., 1997a).

However, despite being resistant to the anorectic effects of leptin, diet induced obese (DIO) mice are sensitive to its thermogenic effects in BAT. The effect of leptin on sympathetic activation is delayed, and may represent the saturable transport of leptin into the cerebrospinal fluid. Leptin may then bind to hypothalamic leptin receptors on neurons that project to the PVN, an area important in the regulation of sympathetic outflow (Haynes, 2000). The exact region within the brain that leptin acts upon to mediate its effects on BAT thermogenesis is unclear. This thermogenic effect of leptin in the mouse is abolished by blockade of leptin receptors in the DMH (Enriori et al., 2011). However, lesioning the VMH in rats also blocks the equivalent effect in rats (Satoh et al., 1999). Though the precise brain circuits involved are unclear, these studies support a role for central leptin receptors in sympathoactivation (Haynes et al., 1997a, Satoh et al., 1999).

2.1.4 Effects of leptin on cardiovascular parameters

Obesity increases the risk of hypertension and cardiovascular disease (Dustan, 1983). Increased sympathetic activation by leptin might be expected to increase blood pressure (BP), though several studies have shown contradictory results. Central and chronic peripheral administration of leptin increases heart rate (HR) and BP in rodents (Casto et al., 1998, Shek et al., 1998, Overton et al., 2001). However, acute peripheral administration has minimal effects on cardiovascular parameters (Haynes et al., 1997a, Haynes et al., 1999). This may be due to peripheral leptin driving increased sympathetic activation in nervous fibres which influence metabolic functions but not vascular tone (Haynes et al., 1997a). Leptin also increases renal tubular sodium and water excretion in the rat, which may oppose some of the pressor effects (Jackson and Li, 1997).

The rise in BP associated with hypertension in DIO rats is attenuated by α- and β-adrenergic receptor antagonists, indicating that increased sympathetic activation is an important mediator of obesity-induced hypertension (Carlyle

et al., 2002). Increasing levels of leptin lead to subsequent increases in HR and BP in DIO mice. Despite severe obesity, this effect is absent in *ob/ob* and db/db mice and disappears with administration of a leptin antibody in DIO mice. In addition, humans with loss of function mutations in leptin have normal BP, despite their profound obesity, suggesting increased body weight *per se* is not the sole cause of hypertension in obesity (Simonds et al., 2014). In mice lacking the leptin receptor, selective re-expression of the leptin receptor in the DMH increased HR and BP. The actions of leptin in the DMH may be mediated in part by the melanocortin system. Central administration of α-MSH increases BP and HR in wild-type mice, but not in MC4R KO mice (Simonds et al., 2014). In obese volunteers, BP was acutely increased by the systemic administration of a centrally active melanocortin receptor agonist (Greenfield et al., 2009). In addition, MC4R KO mice maintain a normal BP despite severe obesity (Tallam et al., 2005, Ni et al., 2006). Leptin may be important in linking peripheral obesity with increased sympathetic activity and hypertension, with this effect mediated by central leptin receptors.

2.2 Sibutramine

2.2.1 Introduction

Sibutramine, a β-phenethylamine structurally related to amphetamines, is a centrally-acting serotonin-norepinephrine reuptake inhibitor. Originally designed as an antidepressant, it was successfully used as an adjunct to counteract antipsychotic-induced weight gain in trials (Buckett et al., 1988, Henderson et al., 2005). Sibutramine was subsequently sold as an anorectic agent under the trade names Meridia® and Reductil®, resulting in 5-10% body weight loss at a dose of 5-15 mg/day in most overweight and obese patients when combined with exercise (Araujo and Martel, 2012, Weintraub et al., 1991). However, Sibutramine was withdrawn from the market in 2010 due to reports of cardiovascular complications, which resulted in the conclusion that the benefits of the drug were outweighed by the risks (EMEA, 2010).

2.2.2 Effects of sibutramine on food intake

The parent compound of sibutramine has a half-life of approximately 1.1 h, though the active metabolites have half-lives of 14-16 h (Luscombe et al., 1989). The effects of sibutramine on food intake can be attributed to selective inhibition of presynaptic reuptake of 5-HT, noradrenaline and, to a lesser extent, dopamine, increasing the synaptic concentrations of these neurotransmitters. The action of sibutramine is therefore primarily *dependent* on the neuronal activity of 5HT and noradrenaline neurons; blockade of 5-$HT_{2A/C}$ and α_1/β_1 receptors prevents its anorexigenic effects (Jackson et al., 1997). Sibutramine also has effects on anorexigenic and orexigenic neuropeptides in the ARC, presumably secondary to its effects on classical neurotransmitters. It has been suggested that sibutramine may increase the transport of leptin into the ARC, leading to increased activation of POMC/CART neurons and inhibition of NPY/AgRP neurons, with a resultant increase in α-MSH secretion (Levin and Dunn-Meynell, 2000). Sibutramine appears to reduce the motivation for palatable food, as rats are less likely to work for a reward

after its administration, and reduces binge eating episodes in humans and rodent models (Halford and Harrold, 2012, van der Zwaal et al., 2012).

2.2.3 Effects of sibutramine on energy expenditure

Sibutramine causes a dose-dependent rise in VO_2 and body temperature in rats housed under thermoneutral conditions of 29°C. This likely occurs through the central modulation of sympathetic outflow and activation of β_3-adrenergic receptors by noradrenaline in brown adipose tissue (Connoley et al., 1999). Sibutramine administration induces weight loss without significantly affecting food intake in *ob/ob* mice, an effect which may be due to increased energy expenditure (Day and Bailey, 1998). In humans, an acute dose of sibutramine has a thermogenic effect equivalent to a 3-5% increase in basal metabolic rate (Hansen et al., 1998). Chronic administration may attenuate the decrease in energy expenditure that commonly follows weight loss, allowing patients to maintain a greater degree of weight loss for a longer period (Hansen et al., 1999, Walsh et al., 1999).

2.2.4 Effects of sibutramine on cardiovascular parameters

Sibutramine increases HR and BP, and therefore its use as an anti-obesity agent was limited by contraindications in patients with a history of cardiovascular disease (Abbott Laboratories, 2009). Withdrawal of the drug occurred following a large multicentre trial investigating the effects of sibutramine in subjects with at least one risk factor for cardiovascular disease. The trial found a modest efficacy of only 3% greater weight loss in the treatment versus control group, which was deemed unacceptable by the FDA in light of the cardiovascular risks; sibutramine increased the risk of nonfatal myocardial infarction and nonfatal stroke (James et al., 2010).

Sibutramine has been reported to increase HR and BP in telemetered Sprague-Dawley rats at the optimal doses for inhibition of food intake, with cardiovascular effects evident from doses of 0.9 mg/kg upwards (Woolard et al., 2004). A dose of 1 mg/kg in rats is postulated to relate to a dose of 15 mg/kg in humans. If the sympathomimetic effects of sibutramine can be inhibited, while maintaining the effects of central noradrenergic and serotonergic transmission, then sibutramine could feasibly be administered at a more effective dose (Thomas et al., 2009).

Studies suggest that sibutramine has paradoxical effects on peripheral and central sympathetic regulation, resulting in increased HR and varying effects on BP. Resting BP in obese patients increases slightly following sibutramine treatment while sitting in the upright position, whereas low frequency BP oscillations were decreased during sympathetic stimulation in the supine position (Birkenfeld et al., 2005). The pressor response may be compensated for by a central sympatholytic effect, mediated in part by central α_2-adrenergic receptors that also regulate parasympathetic activity (Heusser et al., 2006, Eisenhofer et al., 1991). Peripheral stimulatory and central inhibitory effects on the sympathetic nervous system may underlie the varying pressor responses to sibutramine in different patient populations (Heusser et al., 2006).

2.3 Lithium chloride

2.3.1 Introduction

Rodents lack the necessary anatomy for an emetic reflex, and hence other paradigms are used to identify nausea and visceral illness in these animals. Conditioned taste aversion (CTA) studies involve associating the substance administered with a novel flavour. Subsequent avoidance of the flavour indicates that the substance causes aversive effects. Pica, an alternate paradigm for visceral illness, involves the ingestion of non-nutritive substances, such as clay, that might bind any toxic material and slow its ingestion (Mitchell et al., 1977).

2.3.2 Effects of LiCl on food intake

Lithium chloride (LiCl) has a well characterised aversive effect in rodents (Rinaman and Dzmura, 2007, Smith, 1978, Rodriguez et al., 2000). Administration of LiCl results in anorexia in rats, the effects of which remain for 3h post administration and are presumed to be at least partly due to nausea (Curtis et al., 1994). LiCl reduces gastric emptying, which may also contribute to hypophagia, thought to be mediated by neuronal populations within the emetic trigger zone of the AP (Bernstein et al., 1992). The AP has an incomplete BBB, and chemosensors here are able to detect toxins and elicit responses that limit further ingestion of potentially harmful substances (Borison, 1989). Lesions of the AP abolish the presence of a CTA in rats, and hence receptors in this area are thought to mediate the feelings of gastrointestinal malaise in response to LiCl (Rabin et al., 1983, Ritter et al., 1980). However, peripheral LiCl administration results in hypophagia in AP lesioned rats, indicating that receptors in additional brain regions are involved in this response, independent of gastric malaise (Curtis et al., 1994). Further studies have suggested that distinct neuronal populations underlie the aversive and hypophagic effects of LiCl; ablation of noradrenergic neurons in the NTS blunts the hypophagia but not the CTA (Rinaman and Dzmura, 2007). Neuronal activation, indicated by c-Fos like immunoreactivity, has been noted in the AP, NTS, and PBN of rats administered LiCl. Hence, vagal input to the NTS and its projections may mediate some of the effects of LiCl on food intake (Olson et al., 1993, Thiele et al., 1998). The administration of LiCl may reduce food intake via homeostatic and non-homeostatic pathways. Blockade of GLP-1 receptors attenuates the aversive effects of LiCl, indicating that these effects may be mediated in part by the endogenous GLP-1 system (Seeley et al., 2000). In addition, ICV infusion of a GLP-1 receptor antagonist reduces LiCl-induced c-Fos expression in several brainstem nuclei (Thiele et al., 1998).

2.3.3 Effects of LiCl on locomotion

There is contradictory evidence concerning the effect of LiCl on locomotion in rodents. When given centrally, LiCl attenuates amphetamine-induced hyperactivity (Smith, 1981). However, peripheral injection of LiCl has been shown to both reduce and increase activity in rats (Segal et al., 1975, Cappeliez and White, 1981). Intraperitoneal administration of LiCl causes a rapid initial increase in circulating levels, which subsequently decrease, and low brain levels, which then increase (Morrison et al., 1971). Initial increases in activity may be due to transient non-specific

effects, including ulceration of the peritoneum and irritation of the injection site (Lerer et al., 1980, Rodriguez et al., 2000). Peripheral injections of LiCl may also cause rearing behaviour in rats, resembling responses to other toxic agents (Smith, 1978).

The work in this chapter will investigate the ability of the CLAMS approach to pick up the well characterised feeding effects of leptin, sibutramine and LiCl. Many standard feeding study paradigms involve manual weighing of food over set time points, which may disturb the natural behaviour of animals. The CLAMS arrangement allows automated recording of food intake over long periods of time, without interfering with the cage setup. Therefore, animals can be injected with anorectic substances and the effects easily monitored in the dark phase, their natural feeding period, to obtain more physiologically relevant data. CLAMS cages monitor locomotion by recording consecutive infra-red beam breaks, allowing concurrent behavioural effects of these drugs to be determined. In addition, CLAMS enables high resolution data recording for food intake and activity, facilitating investigation of any acute effects of test substances.

2.4 Aims and Hypotheses

2.4.1 Hypothesis

The administration of 2 mg/kg leptin and 0.9 mg/kg sibutramine will reduce food intake in rats, which will be detectable using CLAMS metabolic cages, and any simultaneous changes in HR and BP will be detectable using implanted telemetry probes. LiCl will reduce food intake and affect locomotion in rats, which will be detectable using CLAMS metabolic cages.

2.4.2 Aims:

To investigate;

- The effects of leptin on food intake, energy expenditure, HR and BP in freely moving rats.
- The effects of sibutramine on food intake, energy expenditure, HR and BP in freely moving rats.
- The effects of LiCl on food intake and activity in rats.

2.5 Methods

2.5.1 Animals

Male Wistar rats (Charles River, Margate, Kent, UK) weighing 200-220g were housed in individual cages under controlled temperature (21-23°C) and light (12 hours light, 12 hours dark; lights on at 0700) with *ad libitum* access to standard chow (RM1, Special Diet Services Ltd., Witham, Essex, UK) and water, unless stated otherwise. All animal procedures conducted were approved by the British Home Office under the Animals (Scientific Procedures) Act 1986 (Project Licence 70/8068).

Animals were acclimatised to handling and administration protocols prior to all experimental procedures. Animals were randomised by body weight for all studies.

2.5.2 The effect of peripheral leptin administration on food intake, energy expenditure, cardiovascular parameters and body temperature in *ad libitum* fed rats

2.5.3 The effect of peripheral leptin administration on food intake and energy expenditure in *ad libitum* fed rats

A dose of 2 mg/kg has previously been shown to significantly reduce food intake in rats (Sanchez et al., 2008). Rats were administered saline or 2 mg/kg leptin (Sigma, Poole, UK) in a cross over study design (n=8) by intraperitoneal (IP) injection at 1900 hours (early dark phase). Animals had *ad libitum* access to food throughout the study. Studies were carried out in the dark phase in fed animals to investigate alterations in the normal physiological nocturnal eating behaviour. Rats were housed in individual cages in a 16-chamber open-circuit Oxymax comprehensive laboratory animal monitoring system (CLAMS) (Columbus Instruments, OH, USA) for 5 days, with studies carried out on days 2 and 4, and animals being returned to their standard home cages for 2 days between each 5 day experimental period. Standard data recordings depend on the number of active cages in the system, and take place at a maximum rate of once per minute per total number of cages. Studies investigating the BSS commonly study behaviour acutely for 40 minutes post substance administration (Spudeit, 2014, Provensi et al., 2014, Rodgers et al., 2010). Food intake was therefore recorded every 8 minutes for 18 hours, and high resolution data was recorded every minute for 40 minutes post administration. Activity, measured by total infrared beam breaks, was recorded every 8 minutes for 18 hours. Metabolic parameters (VO_2 and VCO_2) were measured by indirect calorimetry and values normalised with respect to body weight. Respiratory exchange ratio (RER) was calculated by CO_2 produced/O_2 consumed to determine the fuel substrate contributing to energy expenditure. VO_2 and VCO_2 were recorded every 8 minutes following administration of the treatments for a period of 18 hours. All animals were acclimatised to the plexiglass cages for 24 hours prior to the experiment to generate stable reference data. Data were recorded using Oxymax v4.94 (Columbus Instruments, OH, USA).

2.5.4 The effect of peripheral administration of leptin on cardiovascular parameters and body temperature in *ad libitum* fed rats

Rats were implanted with telemetry probes (DSI, MN, USA, model HD-S11) anaesthetised under 3% isoflurane (Abbot laboratories, Berkshire, UK) in oxygen with buprenorphine (0.05 mg/kg; Temgesic, RB Pharmaceuticals Ltd., Berkshire, UK) analgesia. The main body of the device was placed in the peritoneal cavity and sutured to the abdominal wall. The aorta was punctured cranial to the iliac bifurcation and the BP catheter advanced upstream into the descending aorta, sealed with tissue adhesive (Vetbond, 3M, MN, USA) and covered with a 3 x 5-mm cellulose fibre patch (DSI, MN, USA). ECG leads were externalised through the muscle layer and anchored under the skin using 4-0 non-absorbable sutures (Ethicon, CA, USA). The abdominal wall was closed using a 4-0 absorbable suture and the skin was closed using a 4-0 non-absorbable suture, which was removed 7 days after surgery (Ethicon, CA, USA). Carprofen (5 mg/kg; Rimadyl, Pfizer, Kent, UK) was administered subcutaneously at the end of surgery and the following day. Oral 2.5% Baytril antibiotic solution (Bayer, PA, USA) was administered via drinking water for 7 days following surgery, animals were monitored closely throughout.

Telemetry probes were activated 1h prior to administration of substances. Data were recorded using Dataquest A.R.T. 4.33 (DSI, MN, USA).

Handling and substance administration are known stressors in rodents, despite sham injections of saline and acclimatisation to restraining procedures. Therefore, an increase in HR and BP is an expected artefact of handling and injection (Azar et al., 2011). Previous studies have observed an increase in HR of 80-100 BPM, and increase in BP of 10-20 mmHg, following handling and subcutaneous injection (Sharp et al., 2002). This change was used to assess correct placement of ECG leads and BP catheters following injection of saline in 12 rats that had undergone surgery; rats that did not exhibit a change in either parameter were excluded from the appropriate data analysis.

2.5.6 The effect of peripheral sibutramine administration on food intake, energy expenditure, cardiovascular parameters and body temperature in *ad libitum* fed rats

2.5.7 The effect of peripheral administration of sibutramine on food intake and energy expenditure in *ad libitum* fed rats

A dose of 0.9 mg/kg sibutramine hydrochloride has previously been identified as the minimal dose required to produce observable effects on HR and BP in rats (Woolard et al., 2004). Therefore, rats were administered vehicle control or 0.9 mg/kg sibutramine hydrochloride (Sigma, Poole, UK) in 20% DMSO/saline in a cross over study design (n=12) by IP injection at 1900 hours (early dark phase). Studies were carried out in the dark phase in fed animals to investigate alterations in the normal physiological nocturnal eating behaviour. Rats were housed in individual CLAMS cages, as described in section 2.5.3. Food intake was recorded every 12 minutes for 18 hours, and high resolution data was recorded every minute for 40 minutes post administration. Activity was recorded every 12 minutes for 18 hours, and high resolution data was recorded every 10 seconds for 40 minutes post administration. VO_2 and VCO_2 were recorded every 12 minutes following administration of the treatments for a period of 18 hours.

2.5.8 The effect of peripheral administration of sibutramine on cardiovascular parameters and body temperature in *ad libitum* fed rats

Rats were implanted with telemetry probes as described in section 2.5.4.

2.5.9 The effect of LiCl on food intake

To investigate the effect of a range of doses of LiCl on food intake, a dose-response study was performed. Rats with *ad libitum* access to food received an IP injection of saline (n=10) or 10, 32, 40, 48 mg/kg LiCl (n=8) during the early light phase. Rats were then returned to their individual home cages with a pre-weighed amount of chow. Food was reweighed 1, 2, 4 and 8 hours post-administration.

2.5.10 The effect of peripheral administration of LiCl on food intake and energy expenditure in fasted rats

As a relatively low dose of LiCl was used, rats were fasted overnight to eliminate any confounding effects of variations in food intake between rats on the effects of LiCl administration. In addition, the daytime is not the normal feeding period for rats, thus fasting overnight accentuates any anorectic effects of LiCl in these animals. Rats were administered saline (n=6) or 16 mg/kg LiCl (n=6; Sigma, Poole, UK) by IP injection at 0700 hours (early light phase). Rats were housed in individual CLAMS cages, as described in section 2.5.3. Food intake was recorded every 12 minutes for 18 hours, and high resolution data was recorded every minute for 40 minutes post administration. Activity was recorded every 12 minutes for 18 hours, and high resolution data was recorded every 10 seconds for 40 minutes post administration. VO_2 and VCO_2 were recorded every 12 minutes following administration of the treatments for a period of 18 hours.

2.5.11 Statistical analysis

All data are expressed as mean ± the standard error of the mean (SEM). High resolution activity data points represent the total infrared beam breaks in one minute. All CLAMS data were analysed by performing area under the curve (AUC) excluding data pre-administration, with a paired (leptin and sibutramine) or unpaired t-test (LiCl). Dose-response studies for LiCl were analysed using one-way ANOVA using Dunnet's multiple comparison post-test. $P<0.05$ was considered statistically significant. All analysis was carried out using Graphpad Prism software (Prism 6.03, GraphPad Software Inc., CA, USA).

2.6 Results

2.6.1 The effect of peripheral leptin administration on food intake and energy expenditure in *ad libitum* fed rats

To investigate the effect of leptin on food intake and energy expenditure, rats were placed in CLAMS metabolic cages as described in section 2.5.3. In addition to food intake data recorded over 18 hours, high resolution data over the first 40 minutes is presented, as this time period is commonly studied when investigating food intake behaviour in the BSS in rodents (Spudeit, 2014, Provensi et al., 2014, Rodgers et al., 2010). IP administration of 2 mg/kg leptin significantly reduced cumulative food intake in the first 40 minutes by 29.09±18.03% (Fig 2.2). This trend remained for the next 18 hours, but was no longer statistically significant. IP leptin had no significant effect on oxygen consumption (VO_2) or carbon dioxide production (VCO_2) in rats during the 18 hours following administration and RER was not affected. Total activity, measured by infra-red beam breaks, was not affected by leptin administration (Fig 2.6).

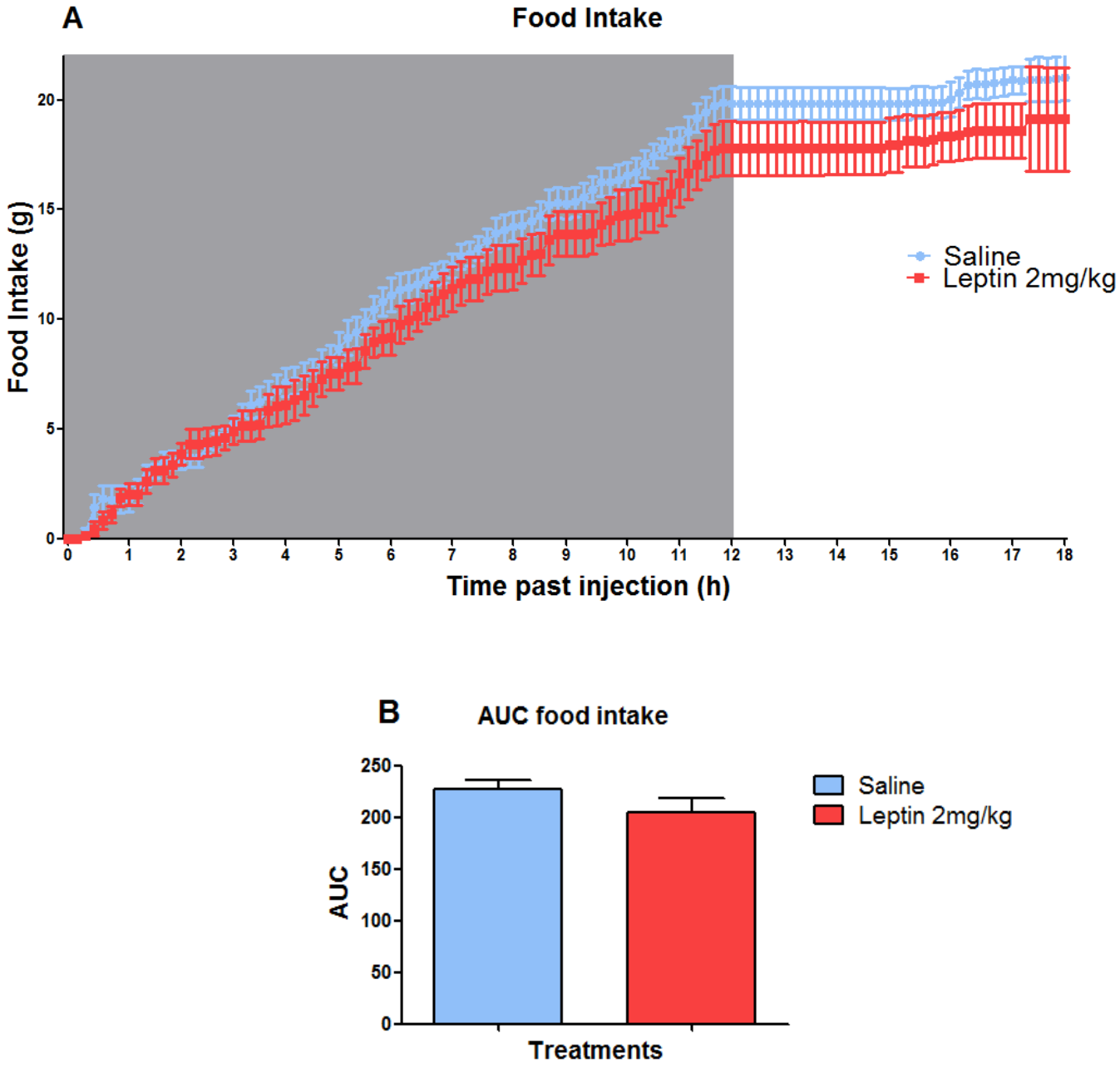


Figure 2.1. The effect of peripheral leptin on food intake in fed rats in the early dark phase.

A The effect of saline or 2 mg/kg leptin on 18 hour cumulative food intake in *ad libitum* fed rats injected in the early dark phase. **B** Area under the curve for graph A; food intake recorded every 8 minutes for 18 hours post injection. N=8 per group. Data presented as mean ± SEM.

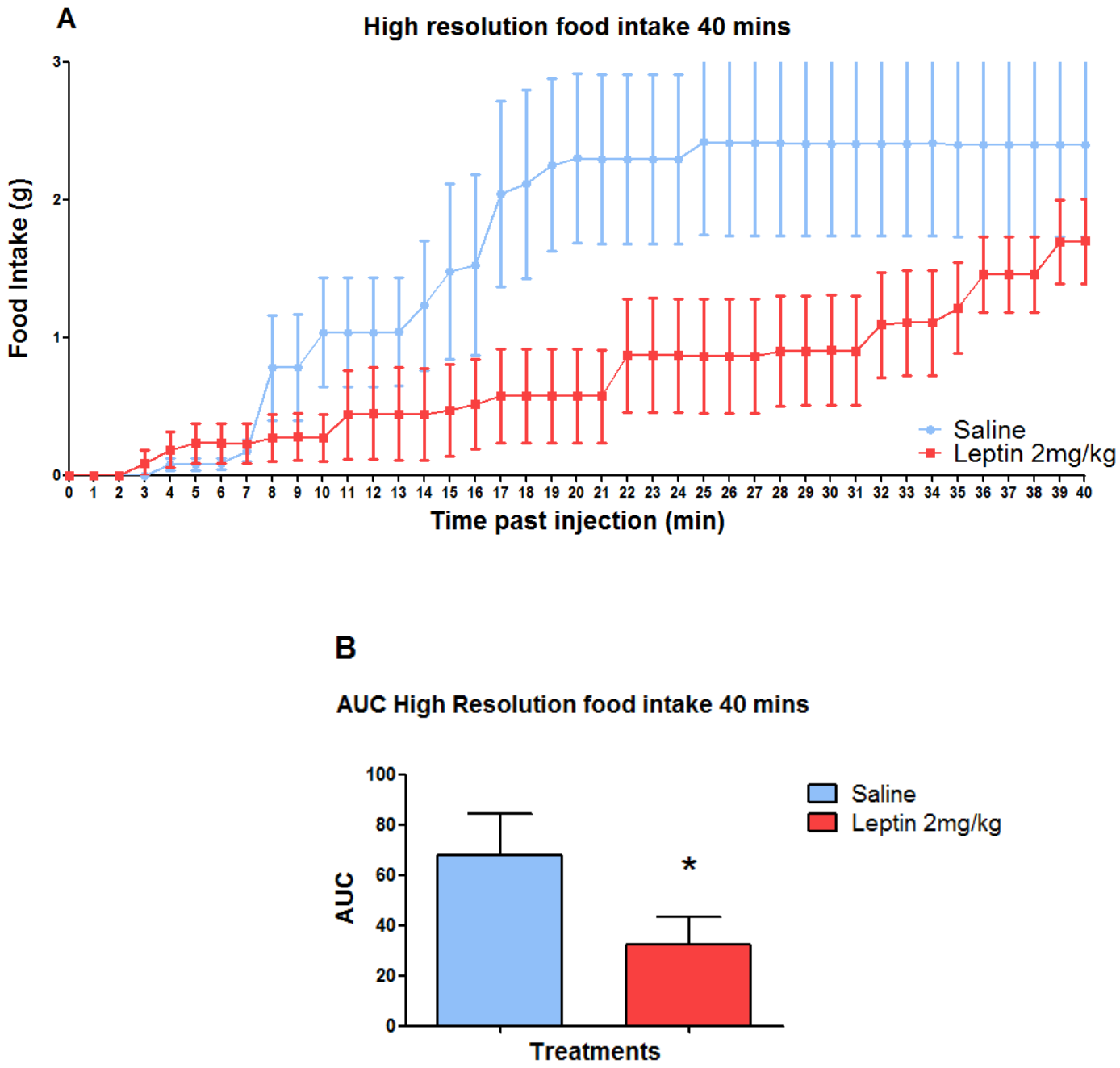


Figure 2.2. The effect of peripheral leptin on food intake in fed rats in the early dark phase.

A The effect of saline or 2 mg/kg leptin on 0-40 minute cumulative food intake in *ad libitum* fed rats injected in the early dark phase; high resolution food intake recorded every minute for 40 mins. **B** Area under the curve for graph A; food intake recorded every minute post injection. N=8 per group. Data presented as mean ± SEM. *p<0.05 vs. saline control using a paired t-test.

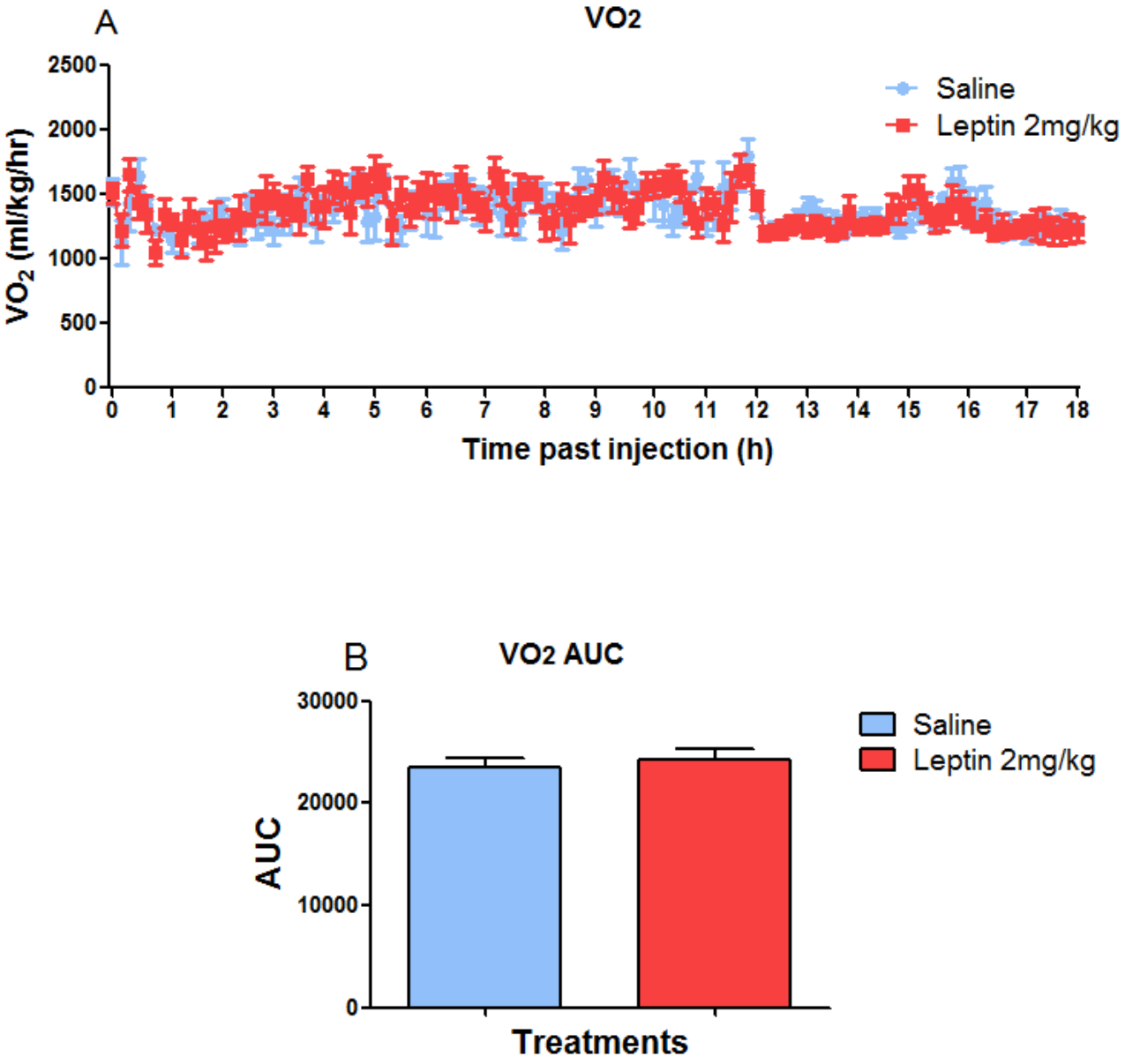


Figure 2.3. The effect of peripheral leptin administration on energy expenditure in fed rats in the early dark phase.

A The effect of saline or 2 mg/kg leptin on VO_2 in ad libitum fed rats in the early dark phase. **B** Area under the curve for A. N=8 per group. Data presented as mean ± SEM.

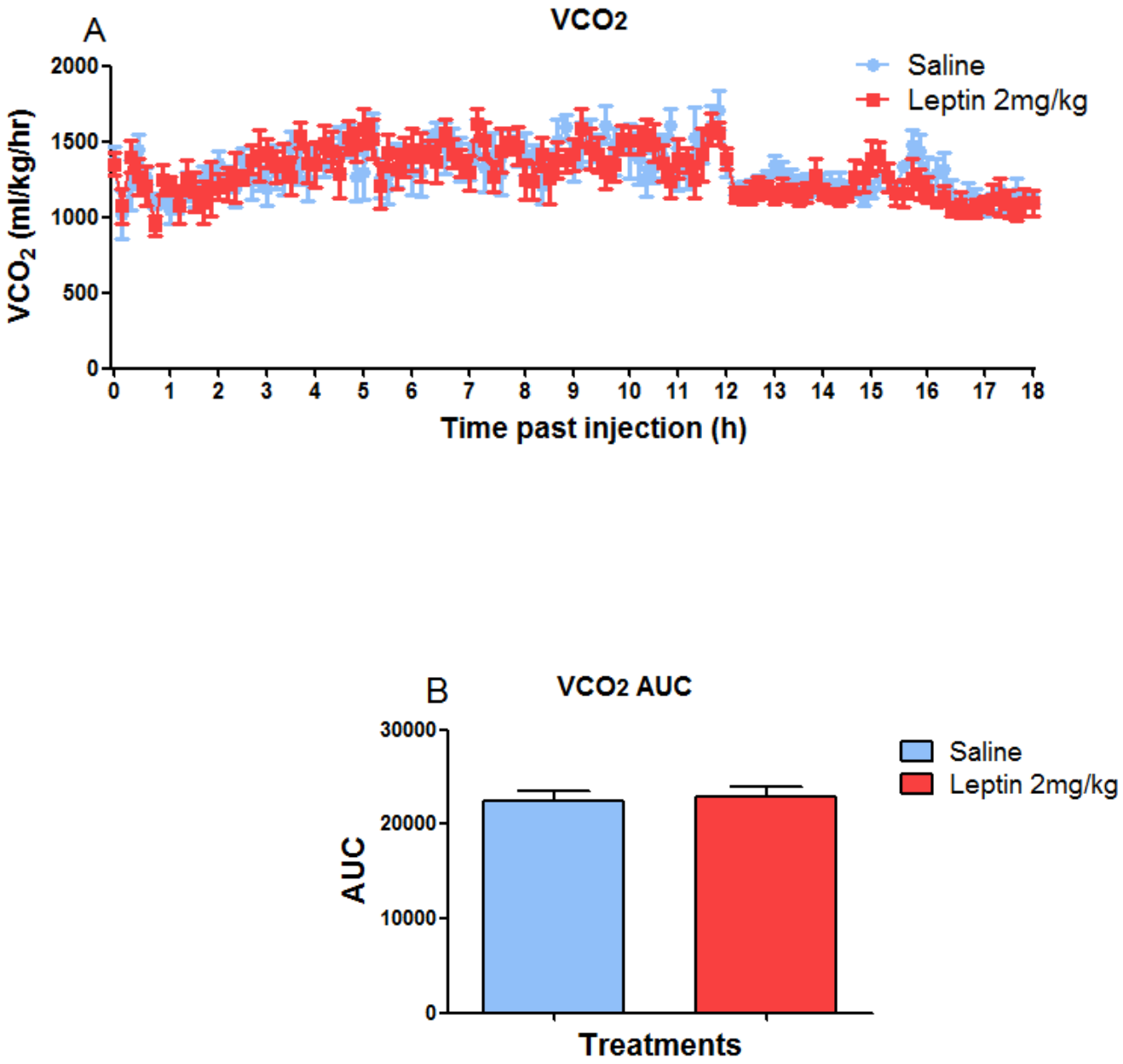


Figure 2.4. The effect of peripheral leptin administration on energy expenditure in fed rats in the early dark phase.

A The effect of saline or 2 mg/kg leptin on VCO_2 in *ad libitum* fed rats in the early dark phase. **B** Area under the curve for A. N=8 per group. Data presented as mean ± SEM.

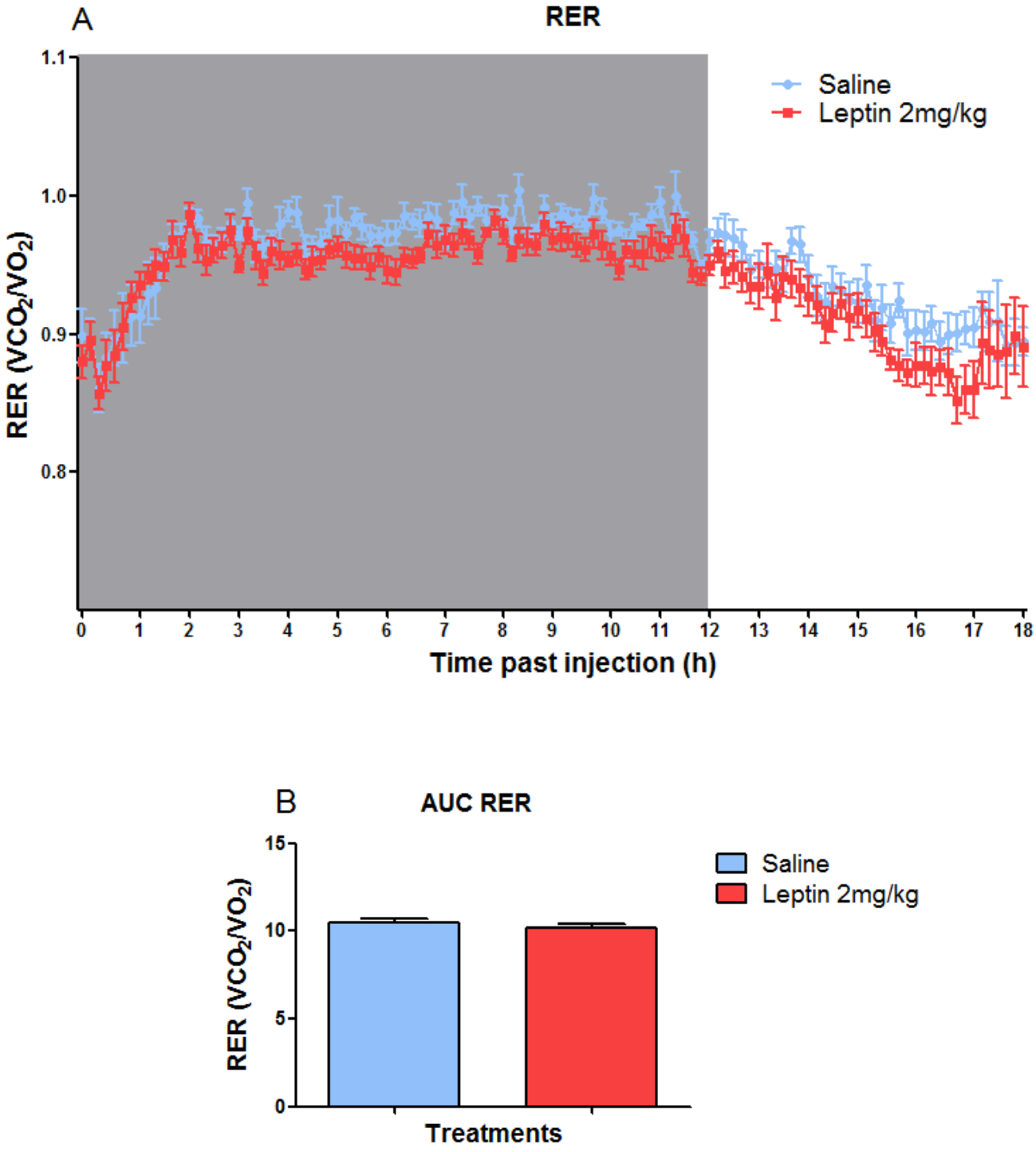


Figure 2.5. The effect of peripheral leptin administration on energy expenditure in fed rats in the early dark phase.

A The effect of saline or 2 mg/kg leptin on RER in *ad libitum* fed rats in the early dark phase. **B** Area under the curve for A. N=8 per group. Data presented as mean ± SEM.

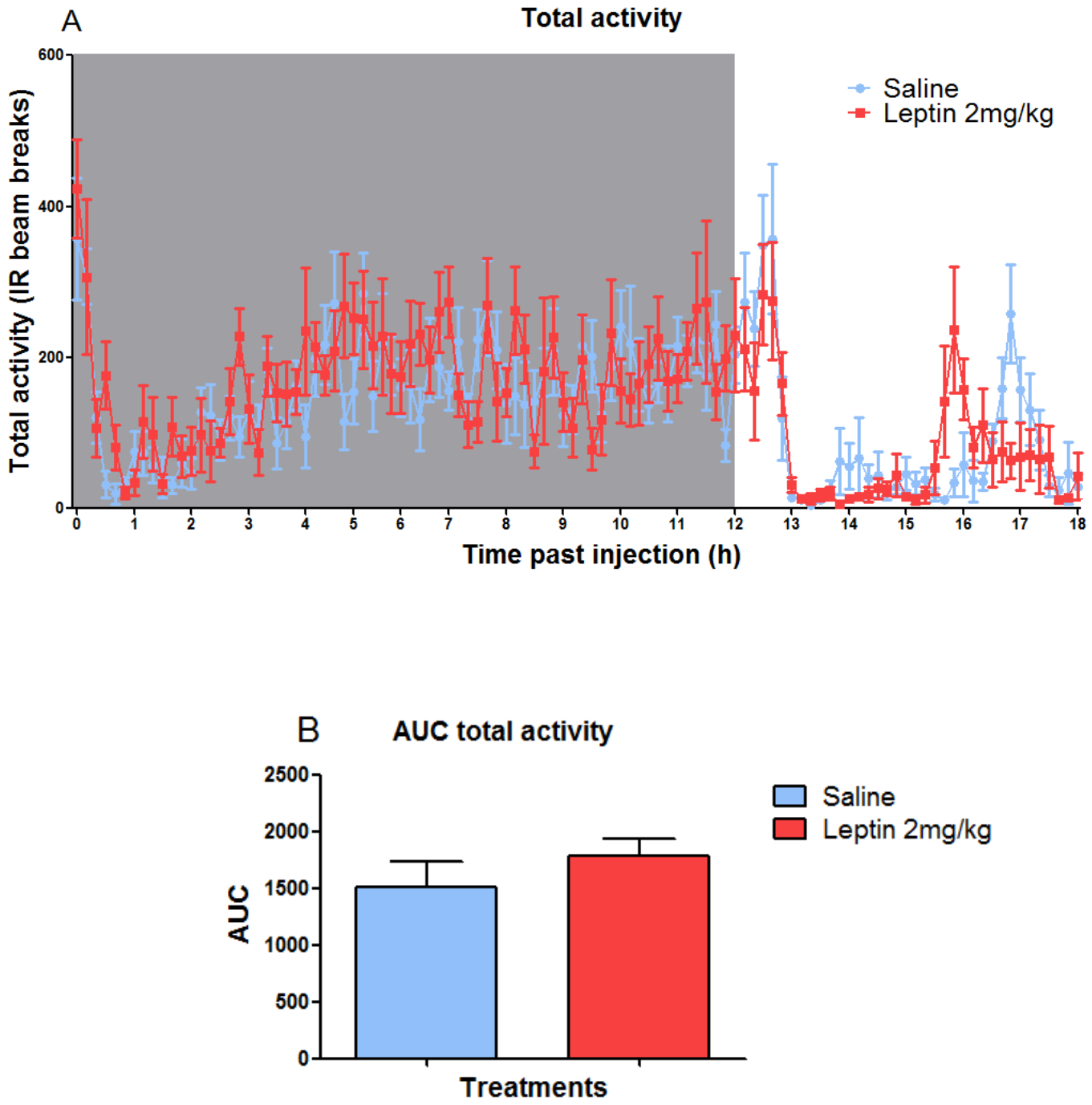


Figure 2.6. The effect of peripheral leptin administration on activity in fed rats in the dark phase.

A The effect of saline or 2 mg/kg leptin on total activity in *ad libitum* fed rats injected in the early dark phase. Infra-red beam breaks recorded every 8 minutes for 11 hours. **B** Area under the curve for A. N=8 per group. Data presented as mean ± SEM.

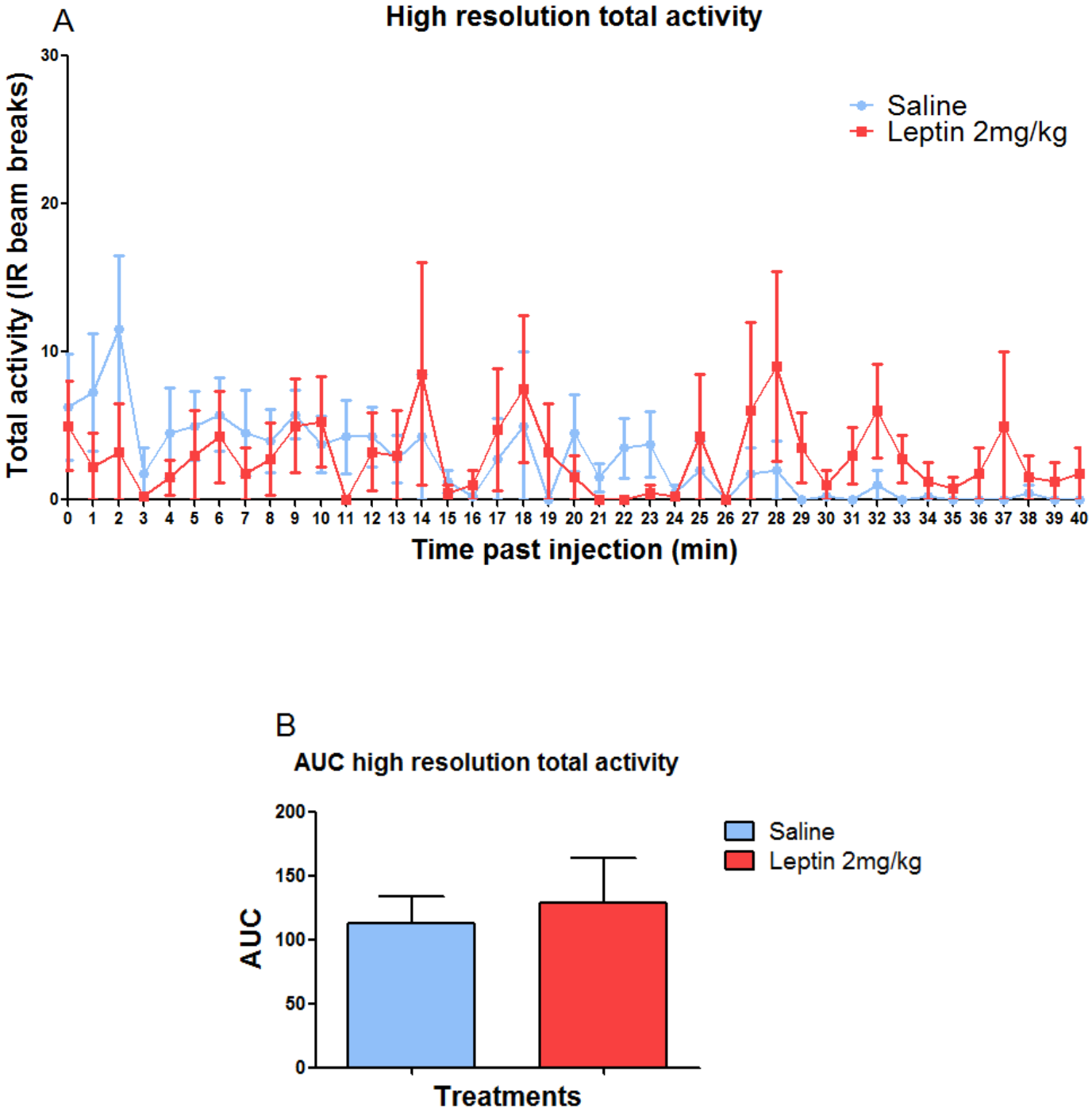
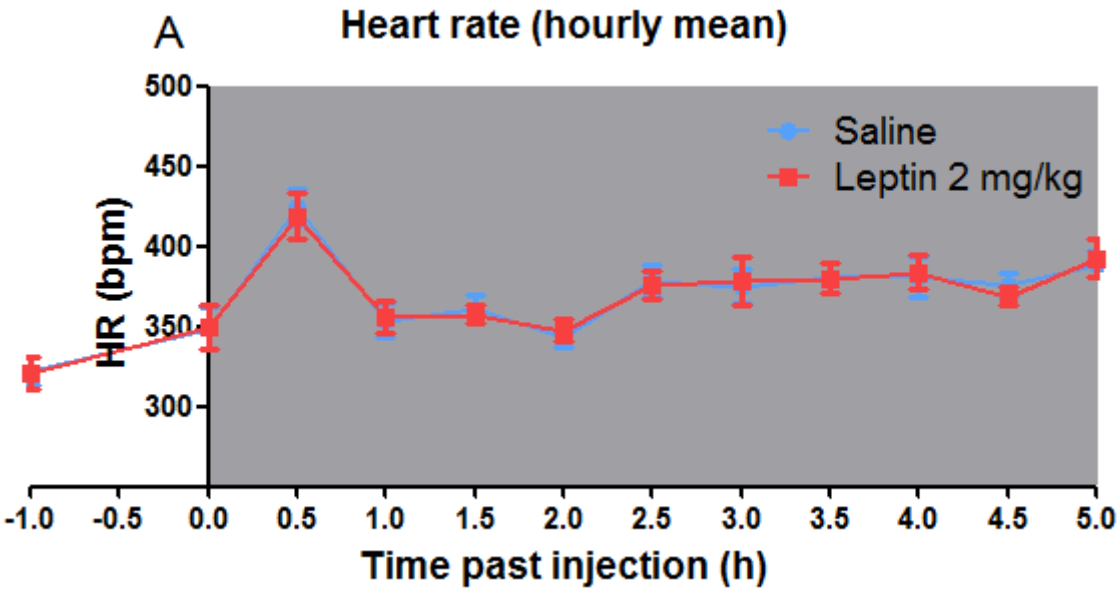


Figure 2.7. The effect of peripheral leptin administration on activity in fed rats in the dark phase.

A The effect of saline or 2 mg/kg leptin on total activity in *ad libitum* fed rats injected in the early dark phase. High resolution data; infra-red beam breaks recorded every 10 seconds. Data presented as total beam breaks per minute. **B** Area under the curve for C. N=8 per group. Data presented as mean ± SEM.

2.6.2 The effect of peripheral administration of leptin on cardiovascular parameters and body temperature in *ad libitum* fed rats

To investigate the effect of leptin on HR, BP and body temperature, rats were implanted with radio-telemetry devices as described in section 2.5.4. Intraperitoneal administration of 2 mg/kg leptin did not significantly affect HR, BP or body temperature in the 5 hours post administration (Figs 2.8-2.9).



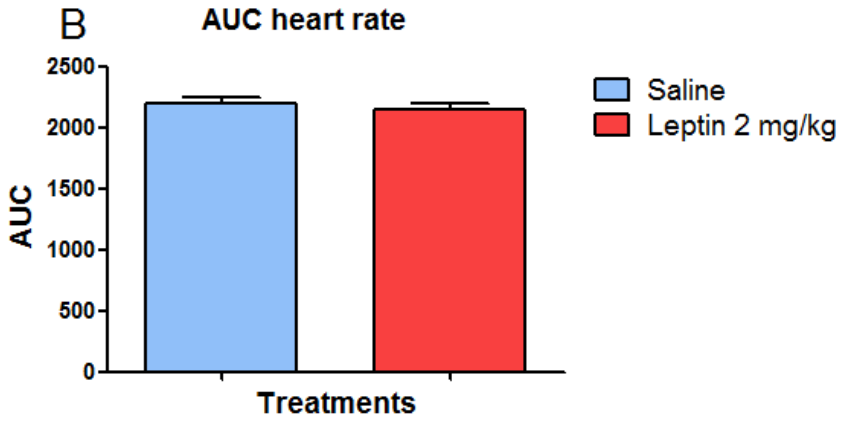


Figure 2.8. The effect of peripheral leptin administration on heart rate in rats.

A The effect of saline or 2 mg/kg leptin on heart rate in freely moving rats injected in the early dark phase. **B** Area under the curve for A. N=7 per group. Data presented as mean ± SEM.

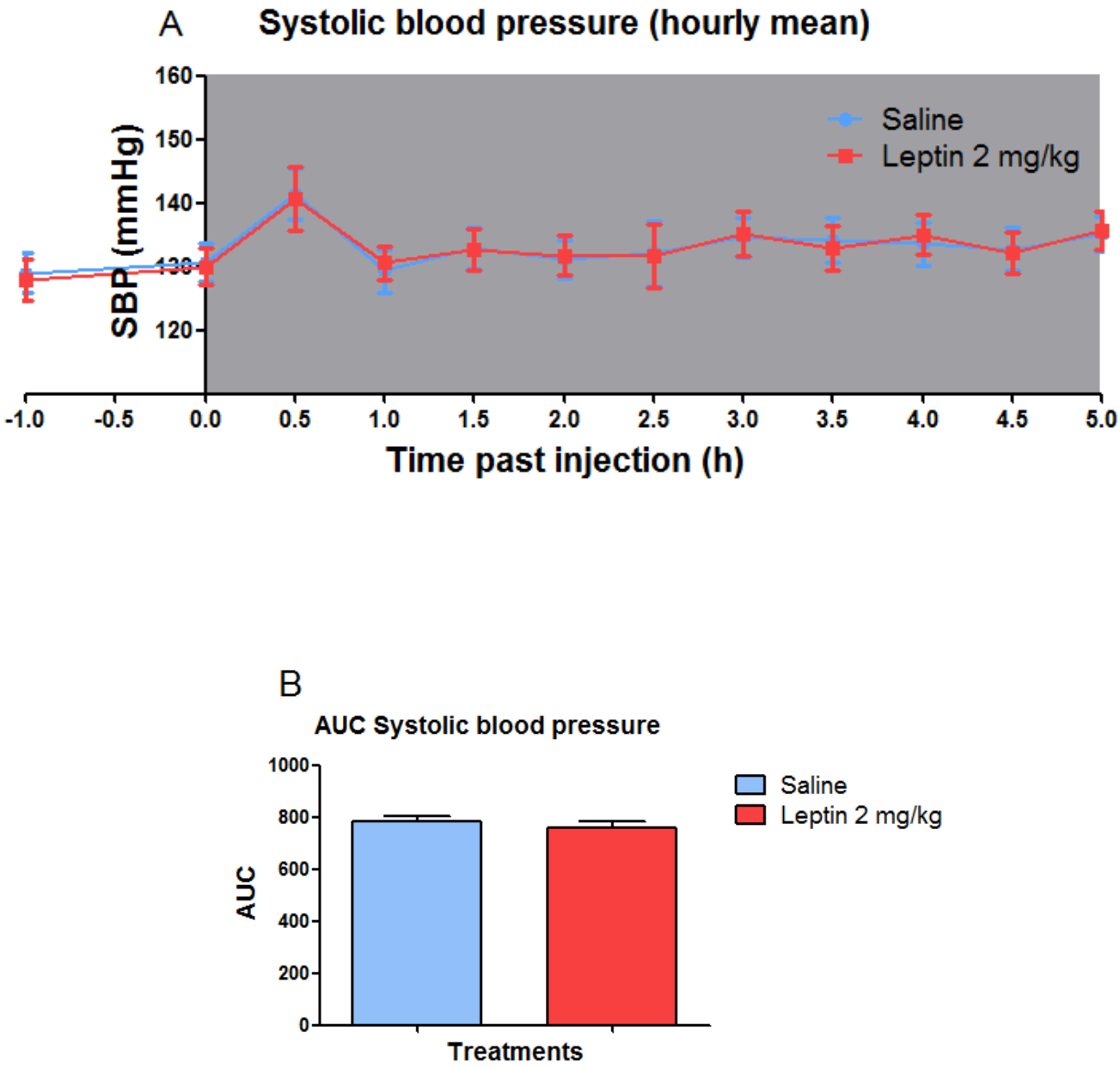
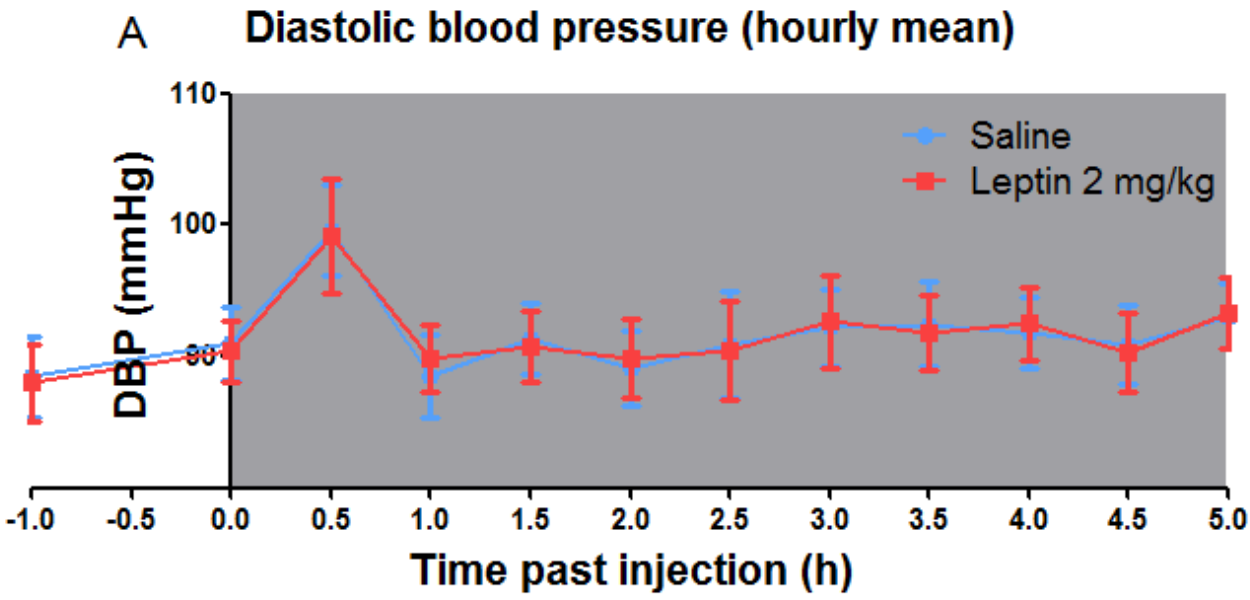


Figure 2.9. The effect of peripheral leptin administration on systolic blood pressure in rats.

A The effect of saline or 2 mg/kg leptin on systolic blood pressure in freely moving rats injected in the early dark phase. **B** Area under the curve for A. N=7 per group. Data presented as mean ± SEM.



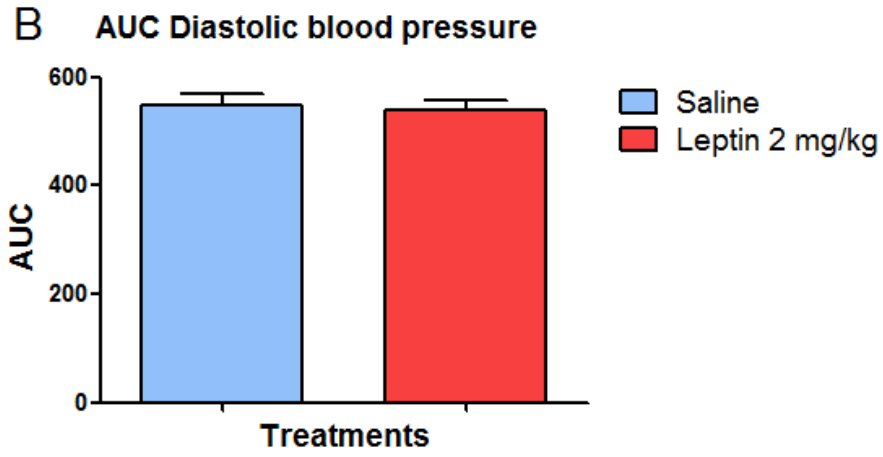
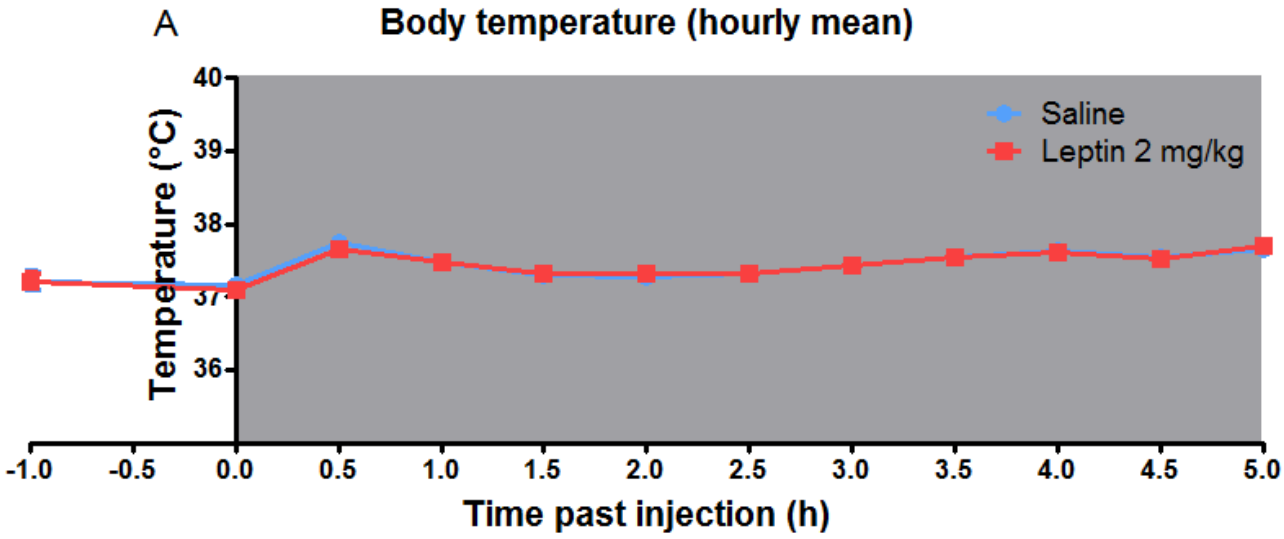


Figure 2.10. The effect of peripheral leptin administration on diastolic blood pressure in rats.

A The effect of saline and 2 mg/kg leptin on diastolic blood pressure in freely moving rats injected in the early dark phase. **B** Area under curve for A. N=7 per group. Data presented as mean ± SEM.



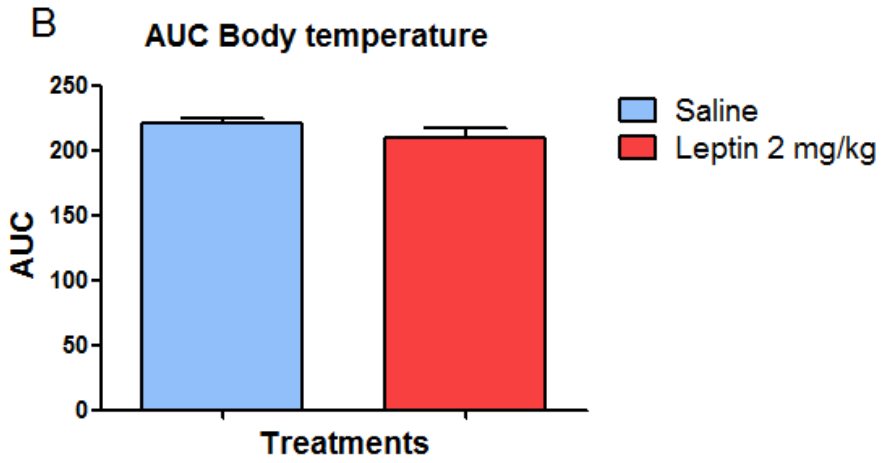


Figure 2.11. The effect of peripheral leptin administration on body temperature in rats.

A The effect of saline or 2 mg/kg leptin on body temperature in freely moving rats injected in the early dark phase. **B** Area under the curve for A. N=8 per group. Data presented as mean ± SEM.

2.6.3 The effect of peripheral administration of sibutramine on food intake and energy expenditure in *ad libitum* fed rats.

To investigate the effect of sibutramine on food intake and energy expenditure, rats were placed in CLAMS metabolic cages as described in section 2.5.3. IP administration of 0.9 mg/kg sibutramine significantly reduced cumulative food intake in the first 40 minutes by 50.98±14.11% compared to vehicle (Fig 2.13). This effect remained significant for the next 18 hours, with a 22.67±4.23% reduction at 18 hours (Fig 2.12). IP sibutramine had no significant effect on oxygen consumption (VO_2), though did significantly decrease carbon dioxide production (VCO_2) and RER during the 18 hours following administration (Figs 2.14-2.16). Total activity, measured by infra-red beam breaks, was significantly reduced by sibutramine administration (Fig 2.17).

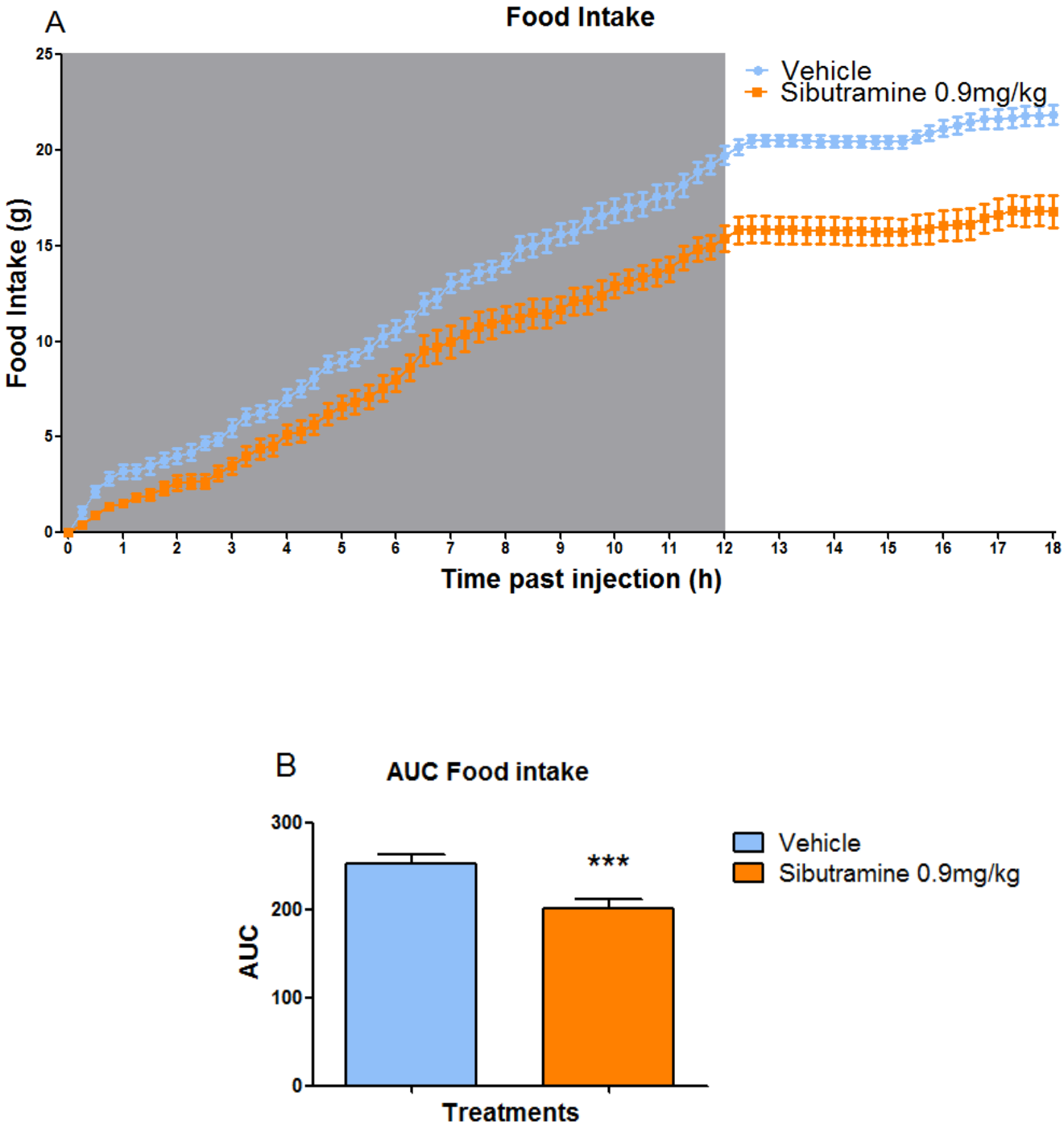


Figure 2.12. The effect of peripheral sibutramine administration on food intake in the early dark phase in fed rats.

A The effect of vehicle or 0.9 mg/kg sibutramine on 18 hour cumulative food intake in ad libitum fed rats injected in the early dark phase. **B** Area under the curve for graph A; food intake recorded every 12 minutes N=12 per group. Data presented as mean ± SEM. ***p<0.001 vs. vehicle control using paired t-test.

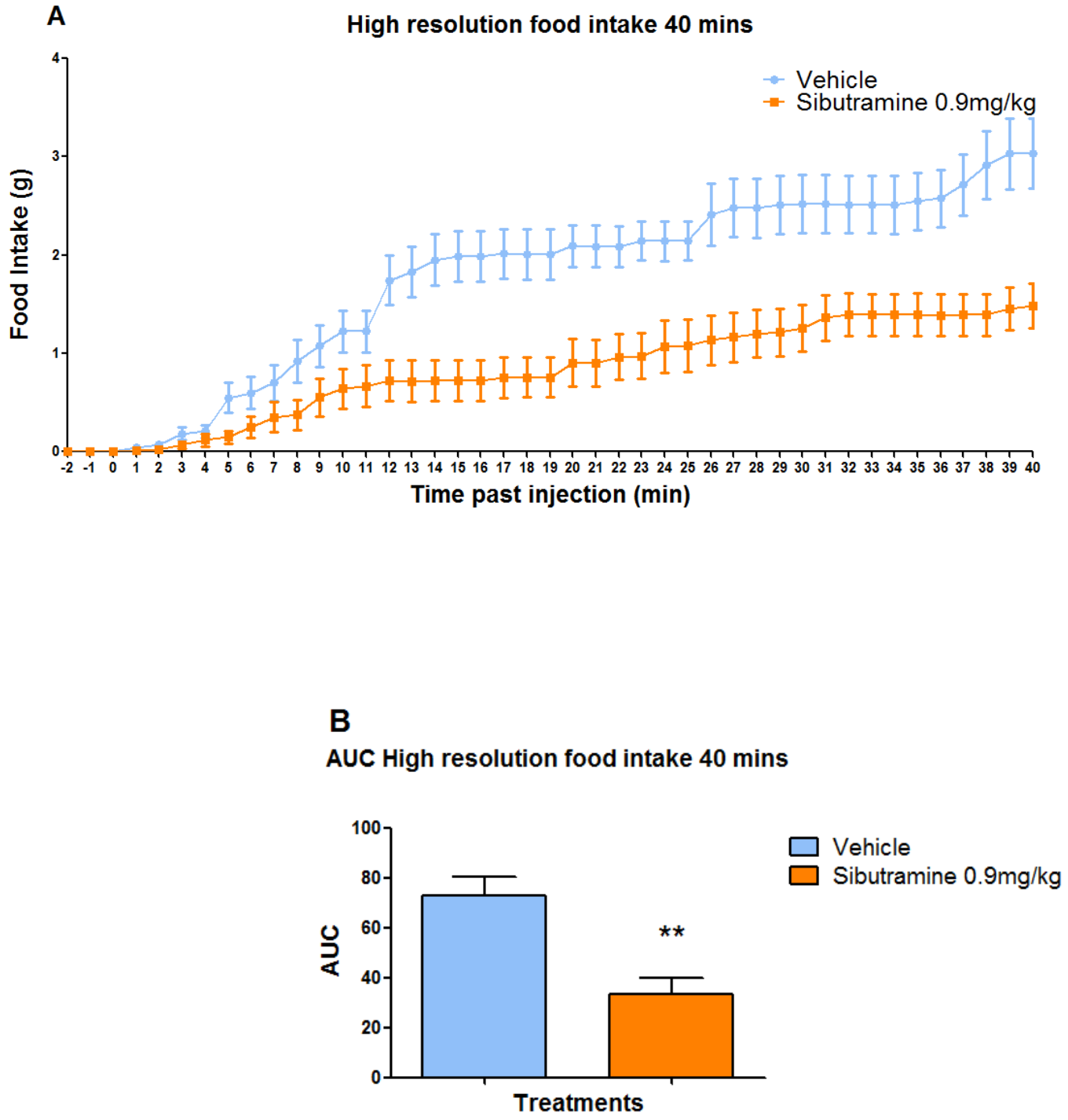


Figure 2.13. The effect of peripheral sibutramine administration on food intake in the early dark phase in fed rats.

A The effect of vehicle or 0.9 mg/kg sibutramine on 0-40 minute cumulative food intake in *ad libitum* fed rats injected in the early dark phase; high resolution food intake recorded every minute for 40 mins. **B** Area under the curve for graph A; food intake recorded every minute. N=12 per group. Data presented as mean ± SEM. **p<0.01 vs. vehicle control using paired t-test

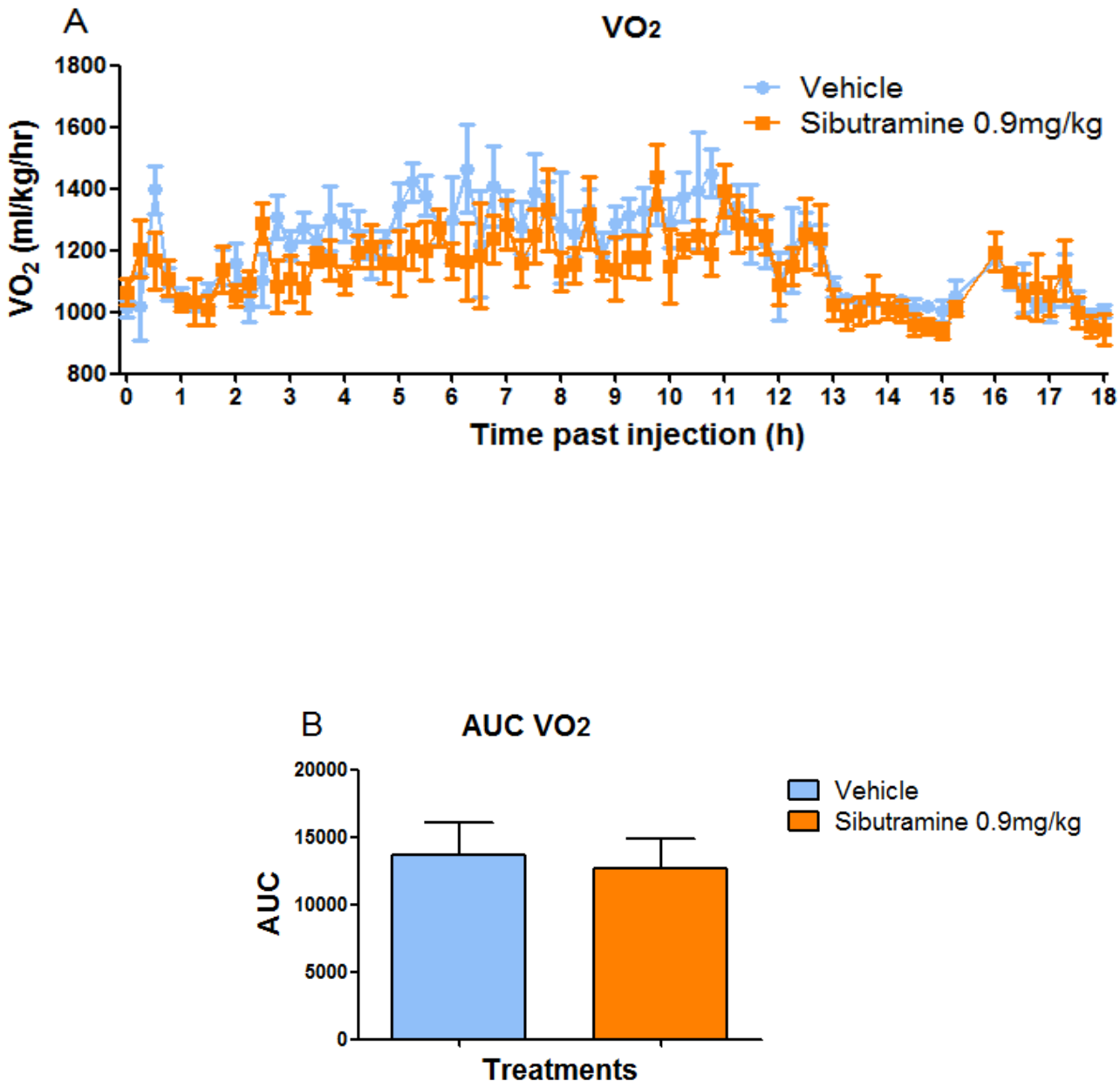


Figure 2.14. The effect of peripheral sibutramine administration on energy expenditure in fed rats in the early dark phase.

A The effect of vehicle or 0.9 mg/kg sibutramine on VO_2 in *ad libitum* fed rats in the early dark phase. **B** Area under the curve for A. N=12 per group. Data presented as mean ± SEM.

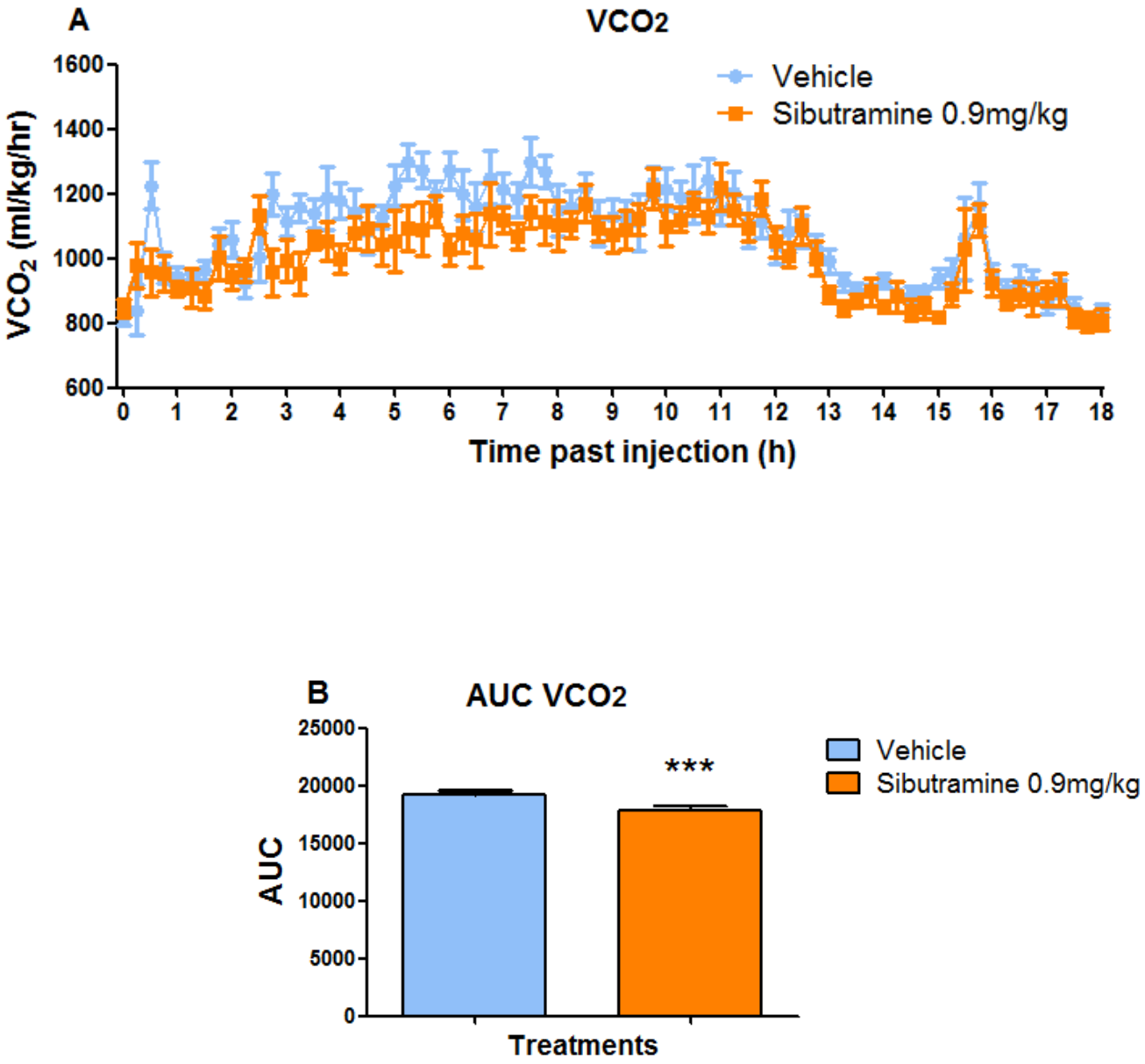


Figure 2.15. The effect of peripheral sibutramine administration on energy expenditure in fed rats in the early dark phase.

A The effect of vehicle or 0.9 mg/kg sibutramine on VCO_2 in *ad libitum* fed rats in the early dark phase. **B** Area under the curve for A & C. N=12 per group. Data presented as mean ± SEM. ***p<0.001 vs. vehicle control using paired t-test.

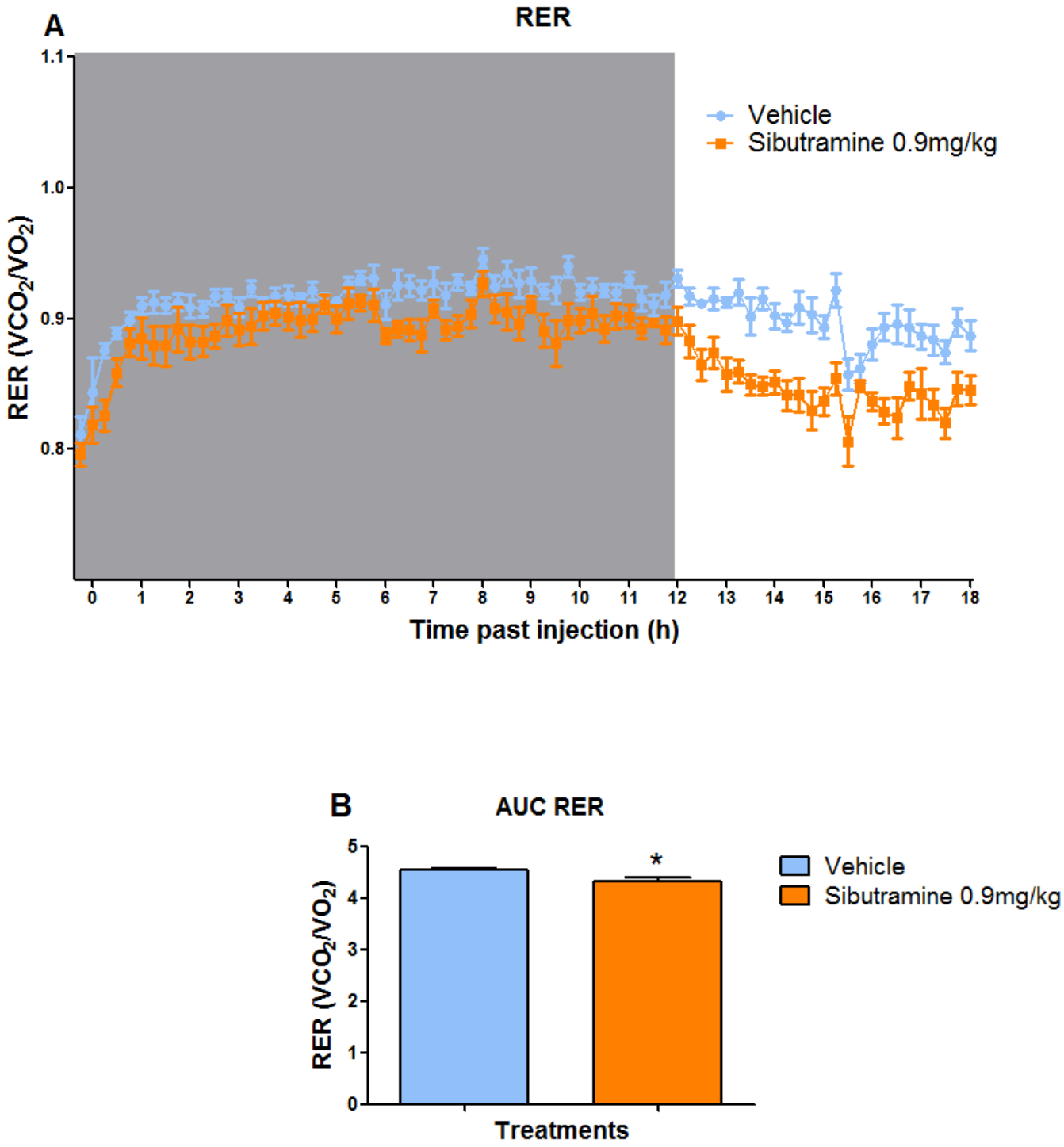


Figure 2.16. The effect of peripheral sibutramine administration on energy expenditure in fed rats in the early dark phase.

A The effect of vehicle or 0.9 mg/kg sibutramine on RER in *ad libitum* fed rats in the early dark phase. **B** Area under the curve for A. N=12 per group. Data presented as mean ± SEM. *p<0.05 vs. vehicle control using paired t-test.

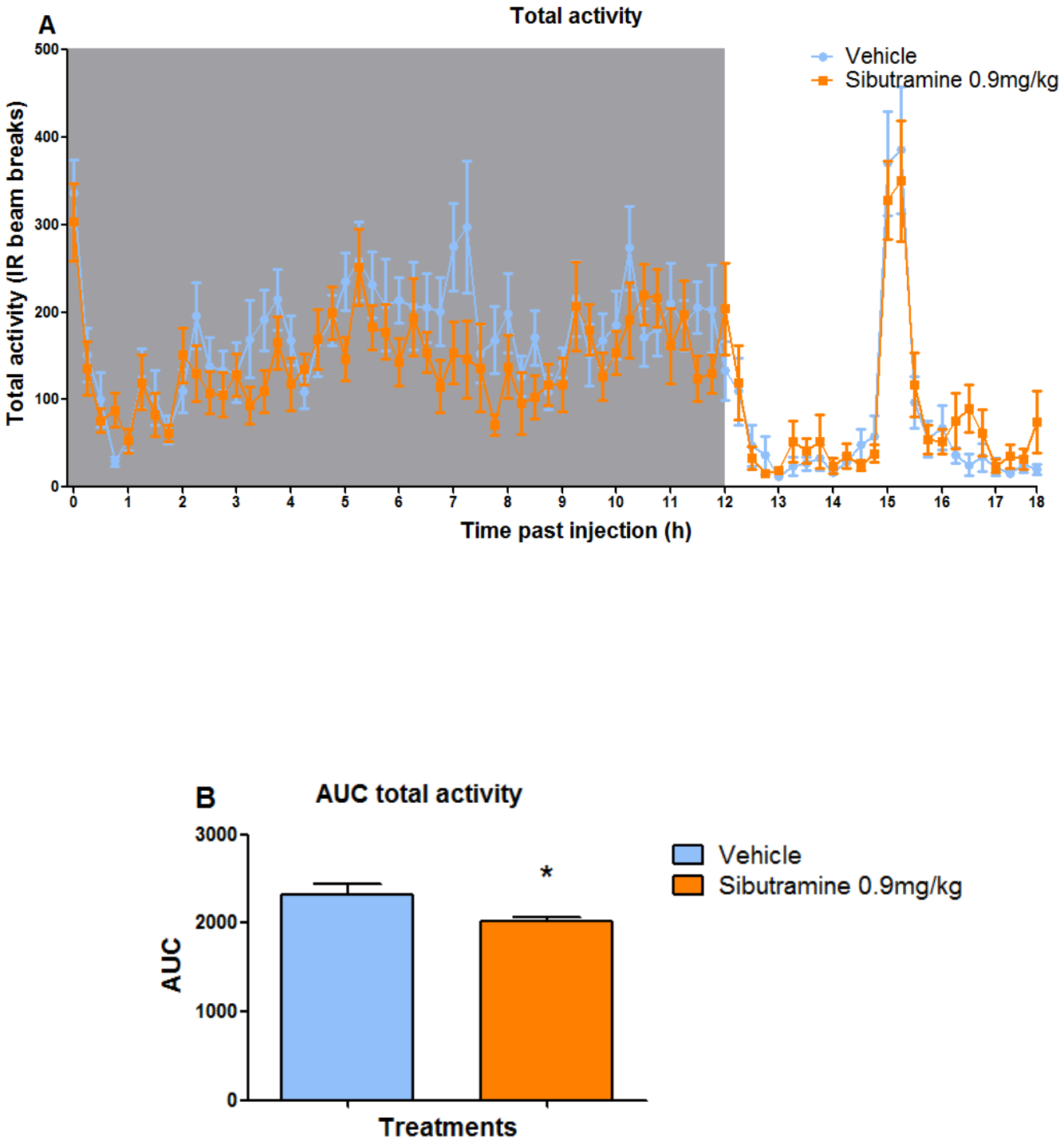


Figure 2.17. The effect of peripheral administration of sibutramine on activity in fed rats in the dark phase.

A The effect of vehicle or 0.9 mg/kg sibutramine on total activity in *ad libitum* fed rats injected in the early dark phase. Infra-red beam breaks recorded every 12 minutes for 18 hours. **B** Area under the curve for A. N=12 per group. Data presented as mean ± SEM. *p<0.05 vs. vehicle control using paired t-test.

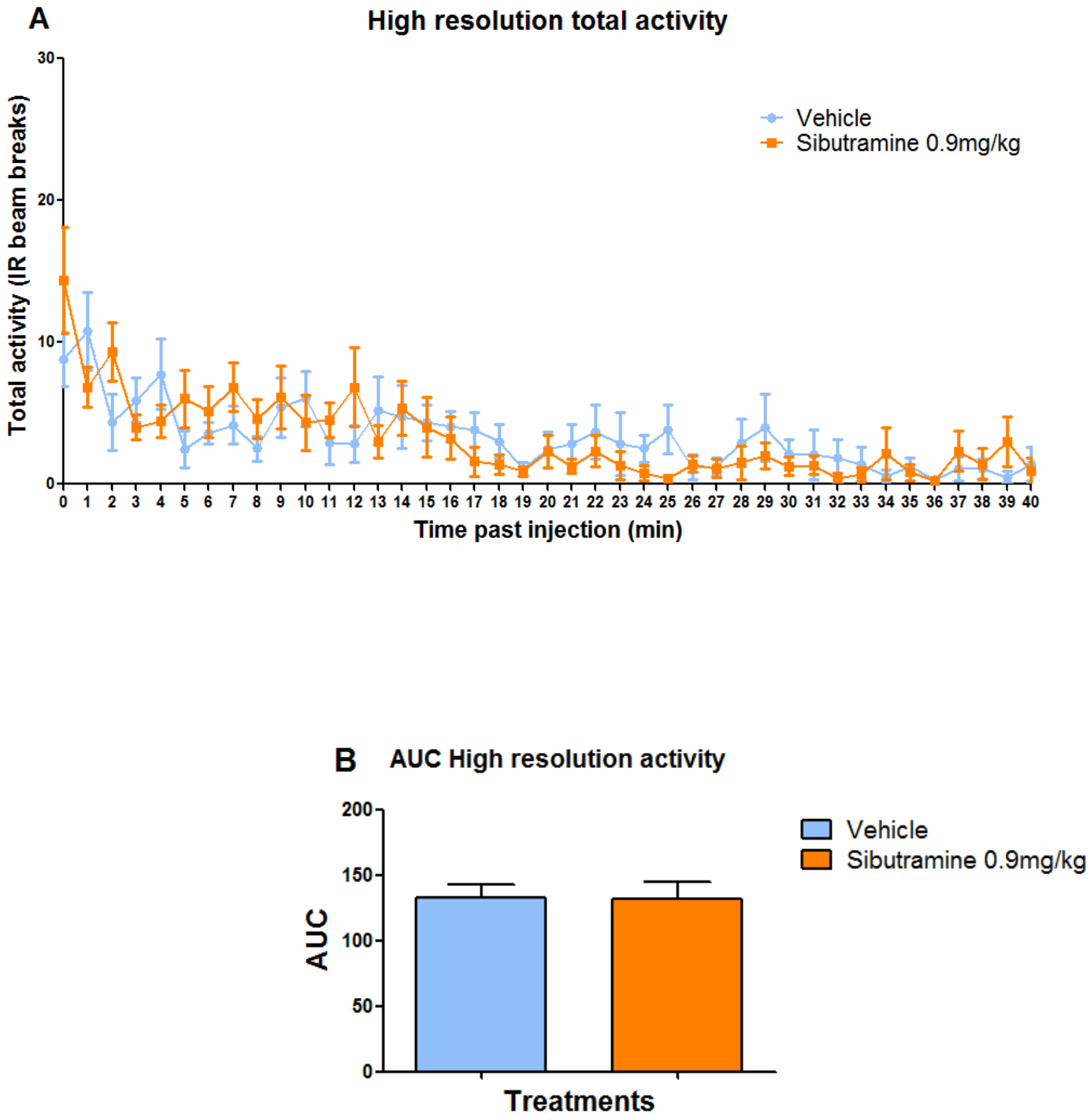


Figure 2.18. The effect of peripheral administration of sibutramine on activity in fed rats in the dark phase.

A The effect of vehicle or 0.9 mg/kg sibutramine on total activity in *ad libitum* fed rats injected in the early dark phase. High resolution data; infra-red beam breaks recorded every 10 seconds. Data presented as total beam breaks per minute. **B** Area under the curve for A. N=12 per group. Data presented as mean ± SEM.

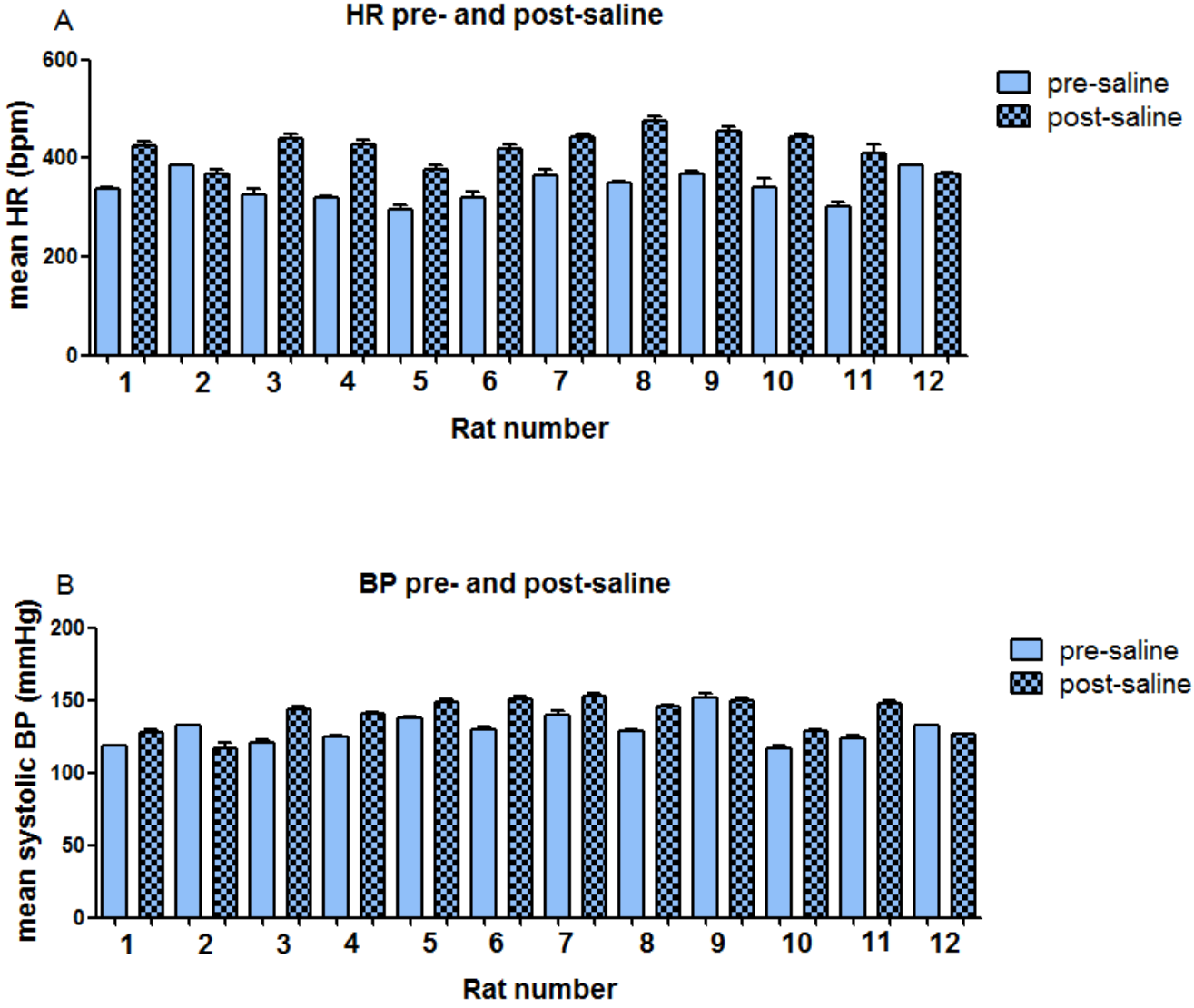


Figure 2.19. A summary of the effect of handling and peripheral administration of saline on heart rate and blood pressure in rats.

A The effect of intraperitoneal injection of saline on heart rate in freely moving rats. **B** The effect of intraperitoneal injection of saline on blood pressure in freely moving rats. N=1 rat. Data presented as mean ± SEM for each rat 30 minutes pre- and post-administration.

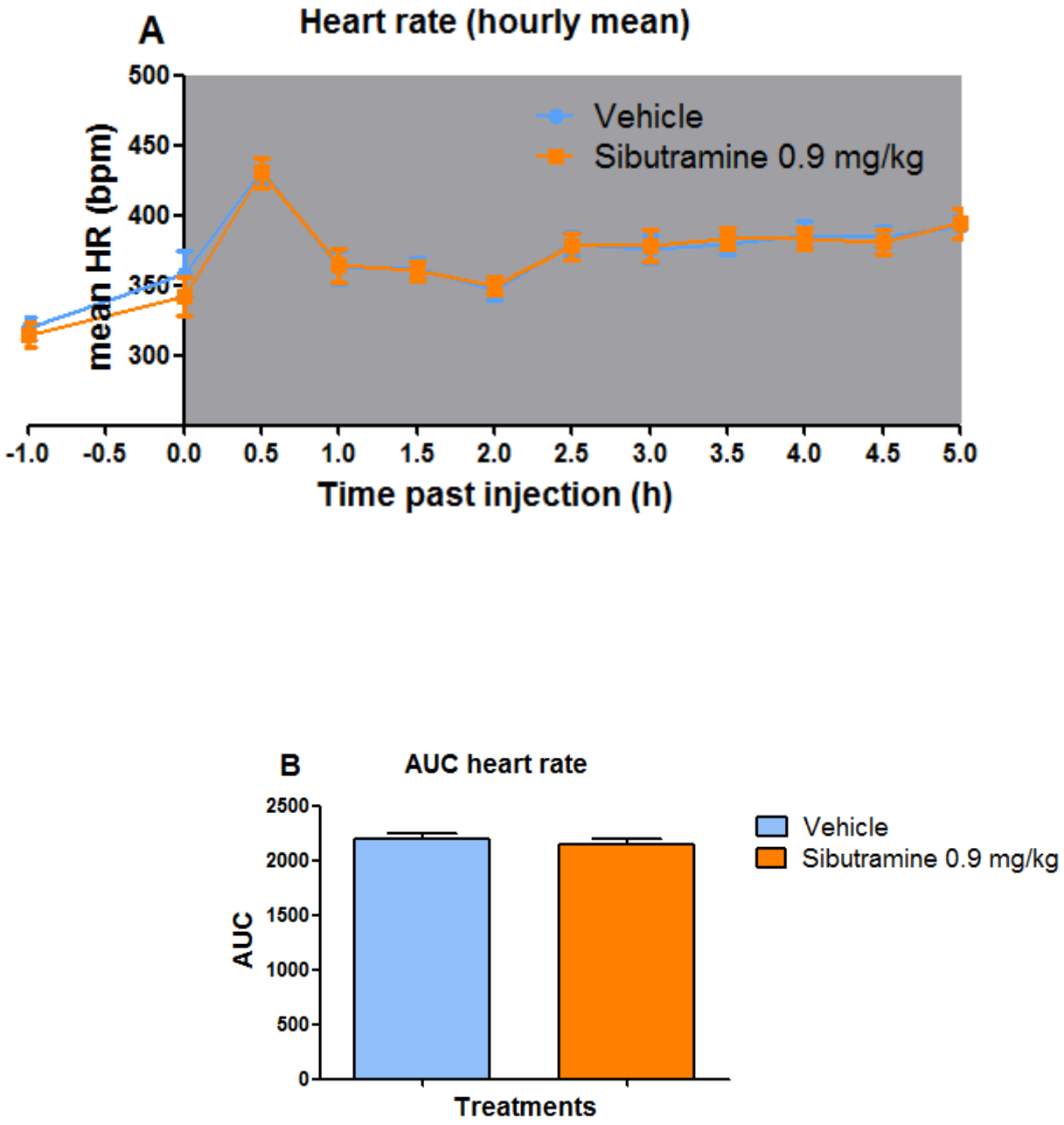
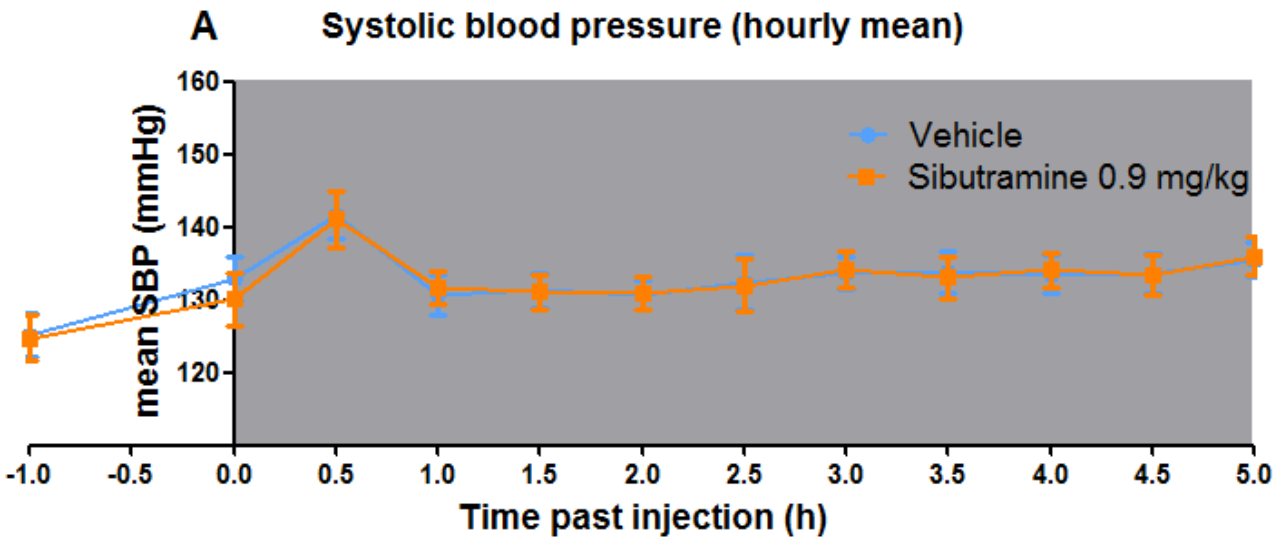


Figure 2.20. The effect of peripheral administration of sibutramine on heart rate in rats.

A The effect of vehicle or 0.9 mg/kg sibutramine on heart rate in freely moving rats injected in the early dark phase. **B** Area under the curve for A. N=10 per group. Data presented as mean ± SEM.



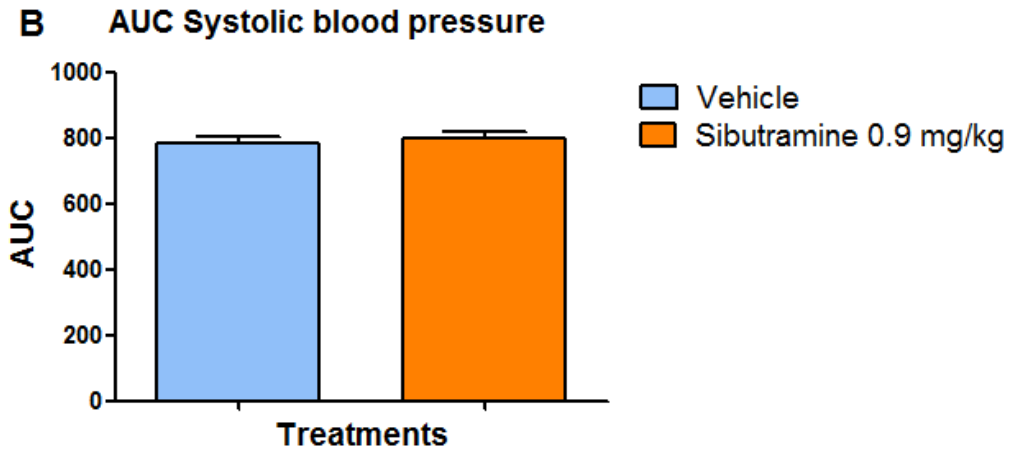


Figure 2.21. The effect of peripheral administration of sibutramine on systolic blood pressure in rats.

A The effect of vehicle or 0.9 mg/kg sibutramine on systolic blood pressure in freely moving rats injected in the early dark phase. **B** Area under the curve for A. N=9 per group. Data presented as mean ± SEM.

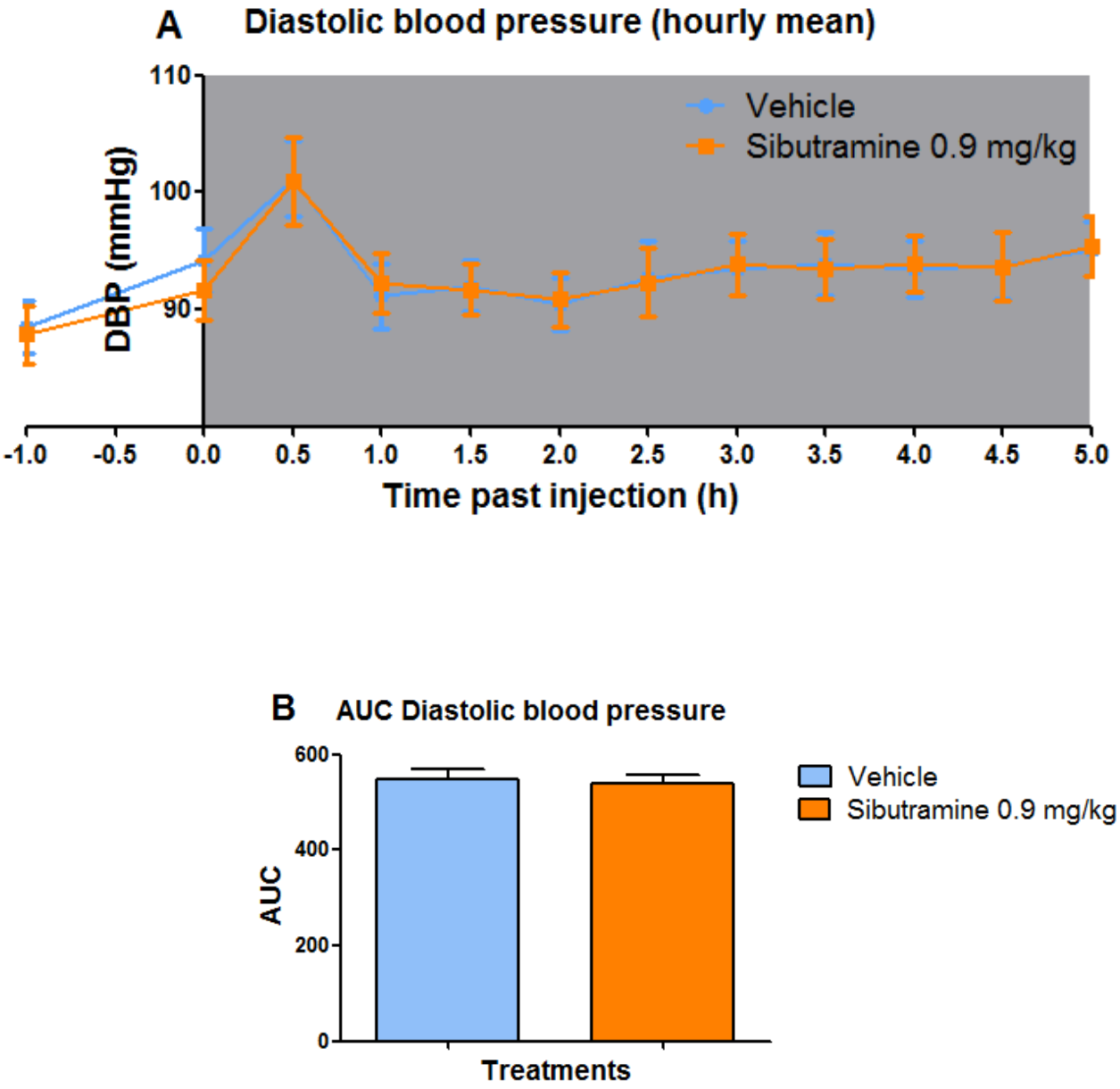


Figure 2.22. The effect of peripheral administration of sibutramine on diastolic blood pressure in rats.

A The effect of vehicle or 0.9 mg/kg sibutramine on diastolic blood pressure in freely moving rats injected in the early dark phase. **B** Area under the curve for A. N=9 per group. Data presented as mean ± SEM.

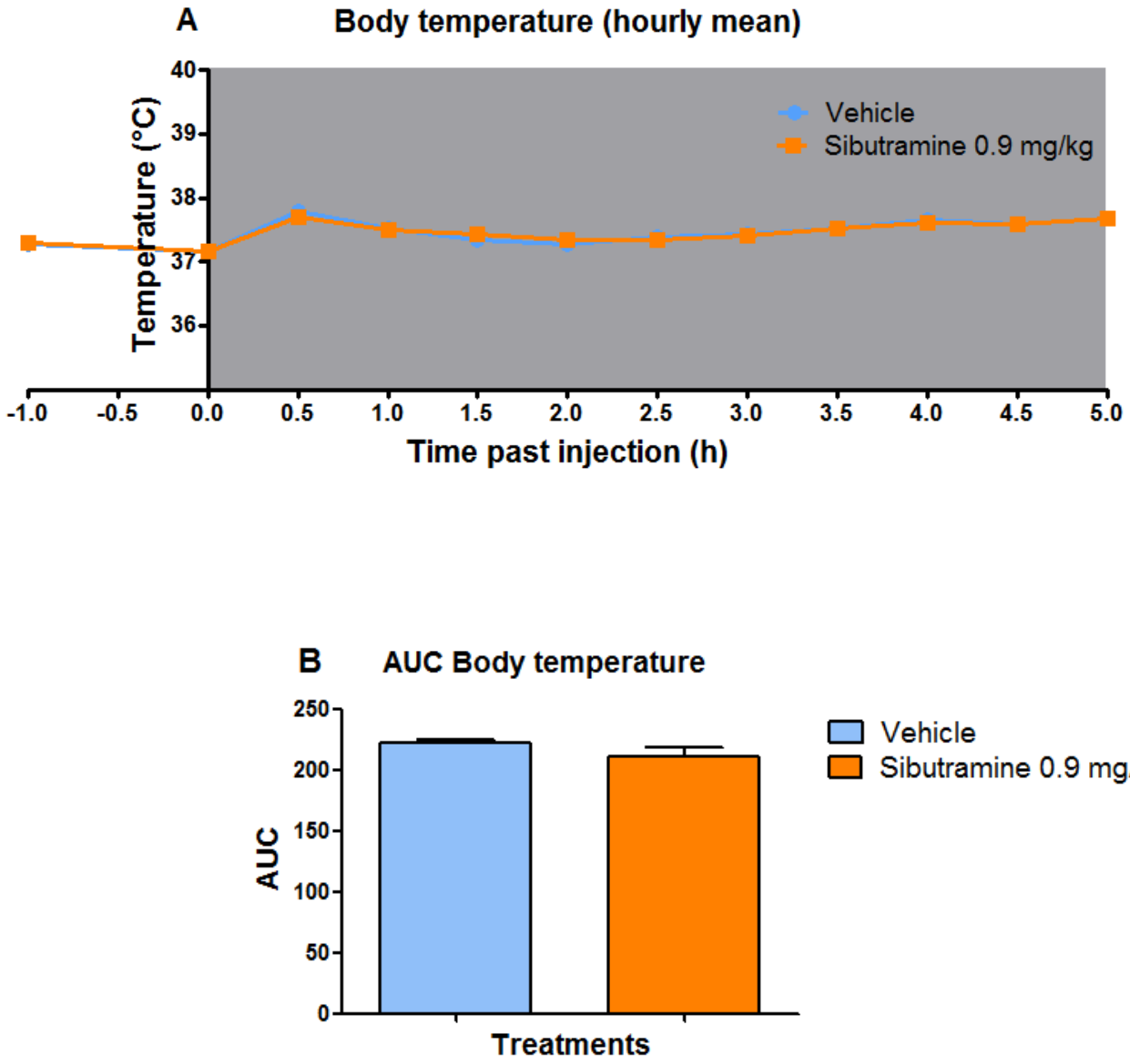


Figure 2.23. The effect of peripheral administration of sibutramine on body temperature in rats.

A The effect of vehicle or 0.9 mg/kg sibutramine on body temperature in freely moving rats injected in the early dark phase. **B** Area under the curve for A. N=12 per group. Data presented as mean ± SEM.

2.6.4 The effect of peripheral administration of LiCl on food intake in fasted rats.

IP administration of a range of doses of LiCl reduced food intake compared to saline control. Doses of 40 and 48 mg/kg LiCl significantly reduced food intake 1-8 hours following administration (Figs 2.24 A-D). A dose of 32 mg/kg LiCl significantly reduced food intake 4-8 hours following administration (Figs 2.24 C-D).

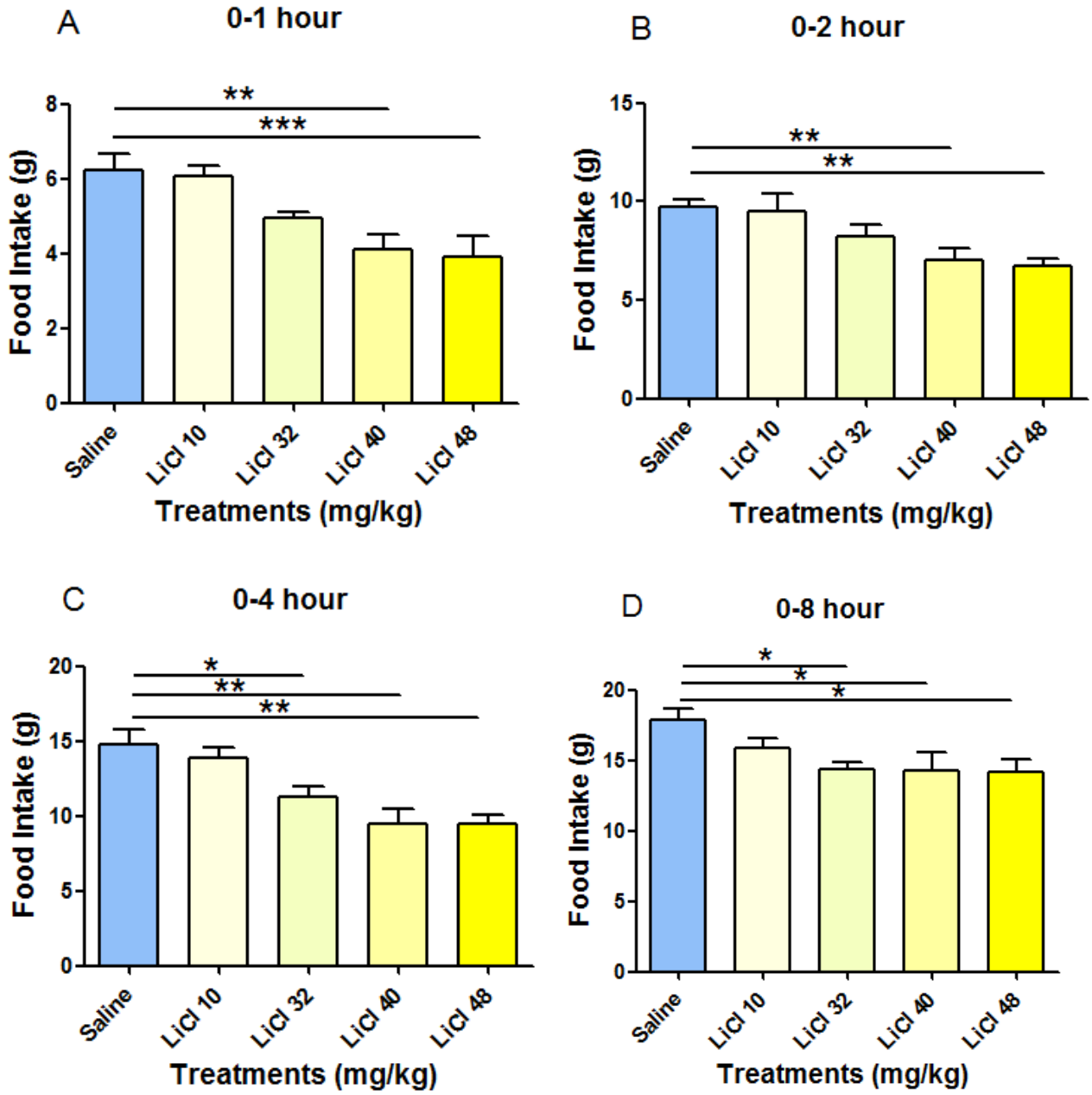


Figure 2.24. The dose response effect of LiCl on food intake in rats.

The effect of saline or LiCl (10, 32, 40, 48 mg/kg) on food intake in fasted rats during 0-1 (**A**), 0-2 (**B**), 0-4 (**C**) and 0-8 (**D**) hour following administration, injected in the light phase. Data presented as mean ± SEM. n=12-13. *p<0.05, **p<0.01, ***p<0.001 vs. saline control, using 1-way ANOVA.

2.6.5 The effect of peripheral administration of LiCl on food intake and energy expenditure in fasted rats.

We were interested in using a dose of LiCl that suppressed food intake by approximately 15-20% compared to control, to allow comparison with subsequent gut hormone studies. Based on the dose response above, we chose to investigate the effects of an intermediate dose of 16 mg/kg on food intake and energy expenditure in the CLAMS. To investigate the effect of LiCl on food intake and energy expenditure, rats were placed in CLAMS metabolic cages as described in section 2.5.3, and fasted overnight. IP administration of 16 mg/kg LiCl significantly reduced cumulative food intake in the first 40 minutes by 21.87±9.12% (Fig 2.26). IP LiCl had no significant effect on VO_2, VCO_2 or RER during the 0-18 hours following administration, though RER was significantly decreased at 0-3h (Fig 2.29 C). Activity, measured by infra-red beam breaks, was not significantly affected by LiCl administration, though there was a trend for increased activity in the first 40 mins and a trend for decreased locomotion in the 18 hours post administration (Fig 2.30-2.31).

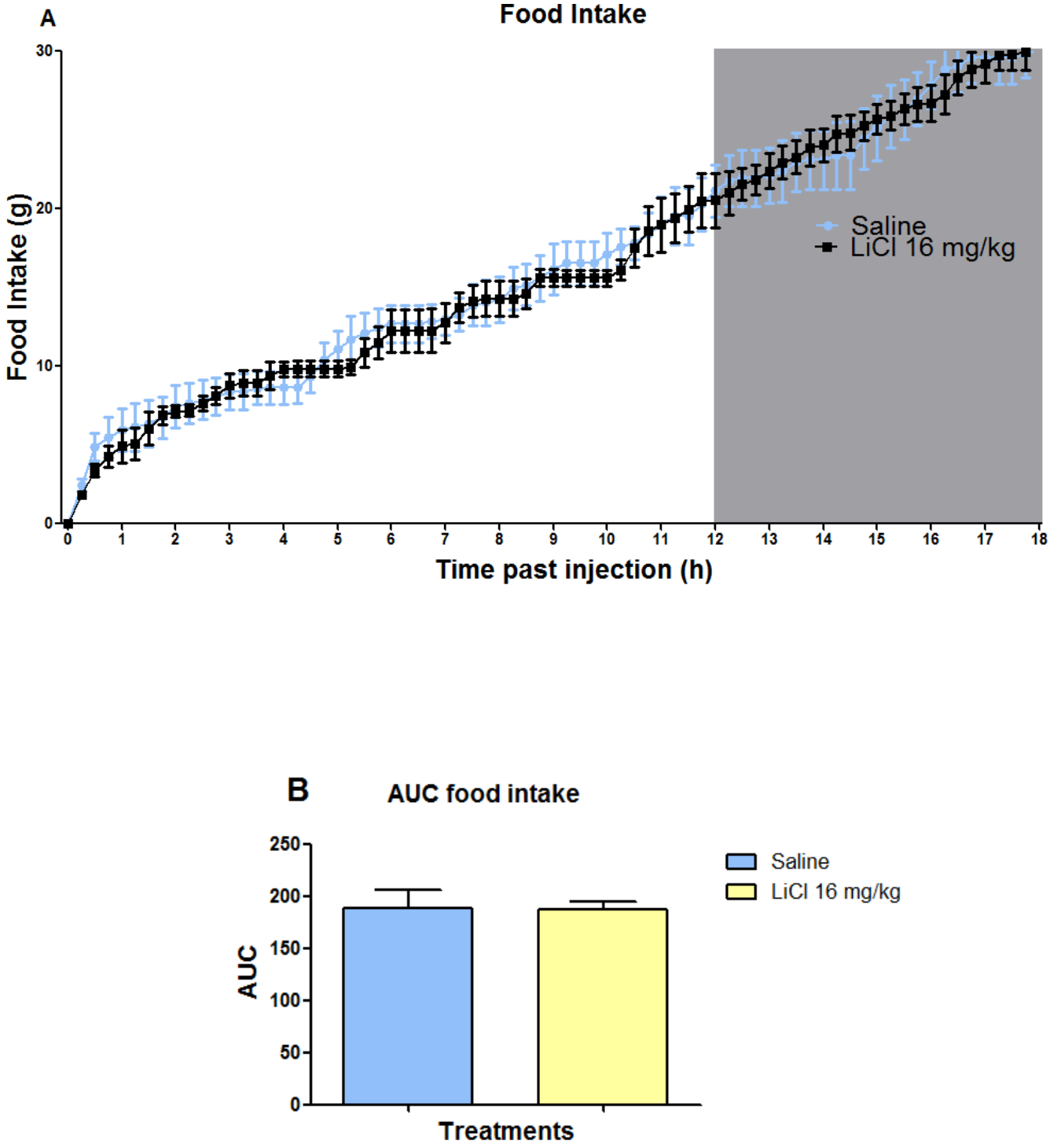
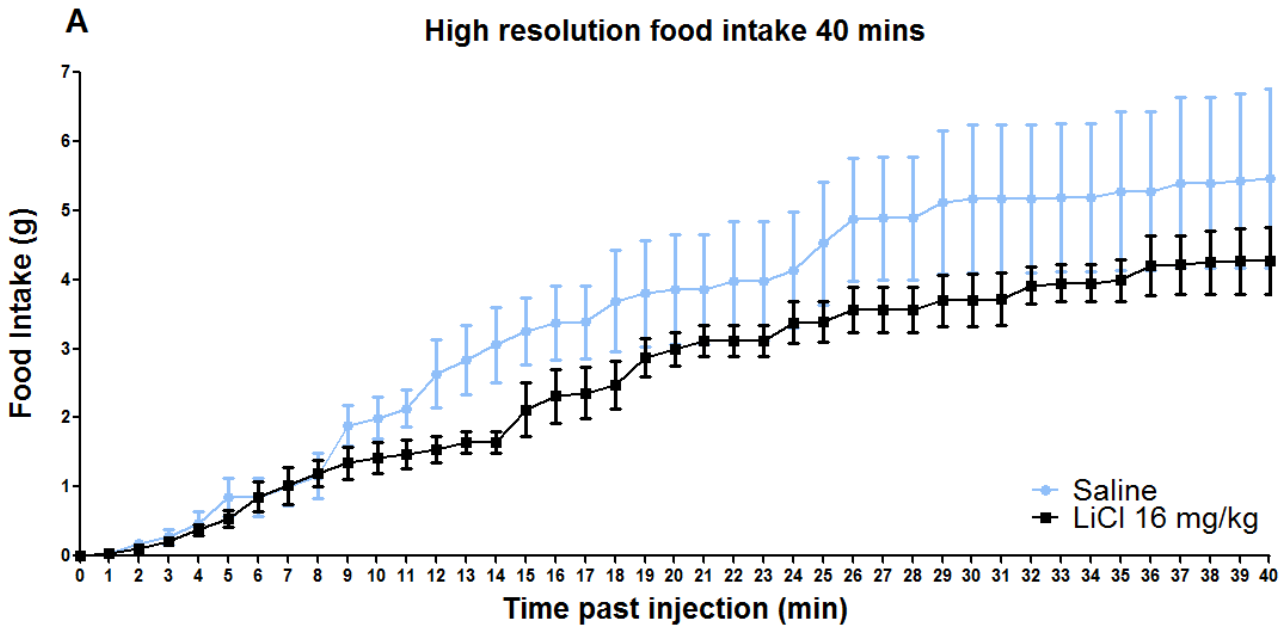


Figure 2.25. The effect of peripheral administration of LiCl on food intake in the light phase in fasted rats.

A The effect of saline or 16 mg/kg LiCl on 14 hour cumulative food intake in fasted rats injected in the early light phase. **B** Area under the curve for graph A; food intake recorded every 12 minutes for 14 hours post injection. N=6 per group. Data presented as mean ± SEM.



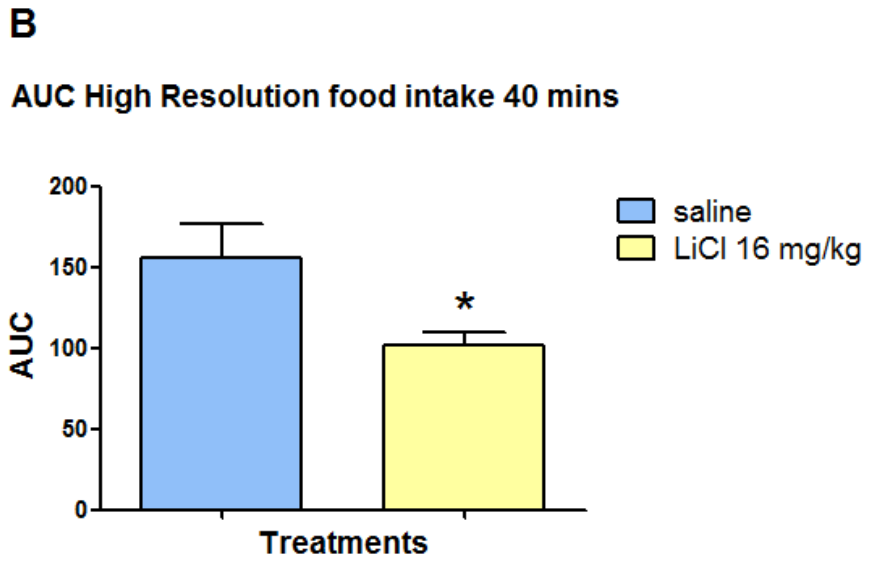


Figure 2.26. The effect of peripheral administration of LiCl on food intake in the light phase in fasted rats.

A The effect of saline or 16 mg/kg LiCl on 0-40 minute cumulative food intake in fasted rats injected in the early light phase; high resolution food intake recorded every minute for 40 mins. **B** Area under the curve for graph A; food intake recorded every minute. N=6 per group. Data presented as mean ± SEM. *p<0.05, vs. saline control using an unpaired t-test.

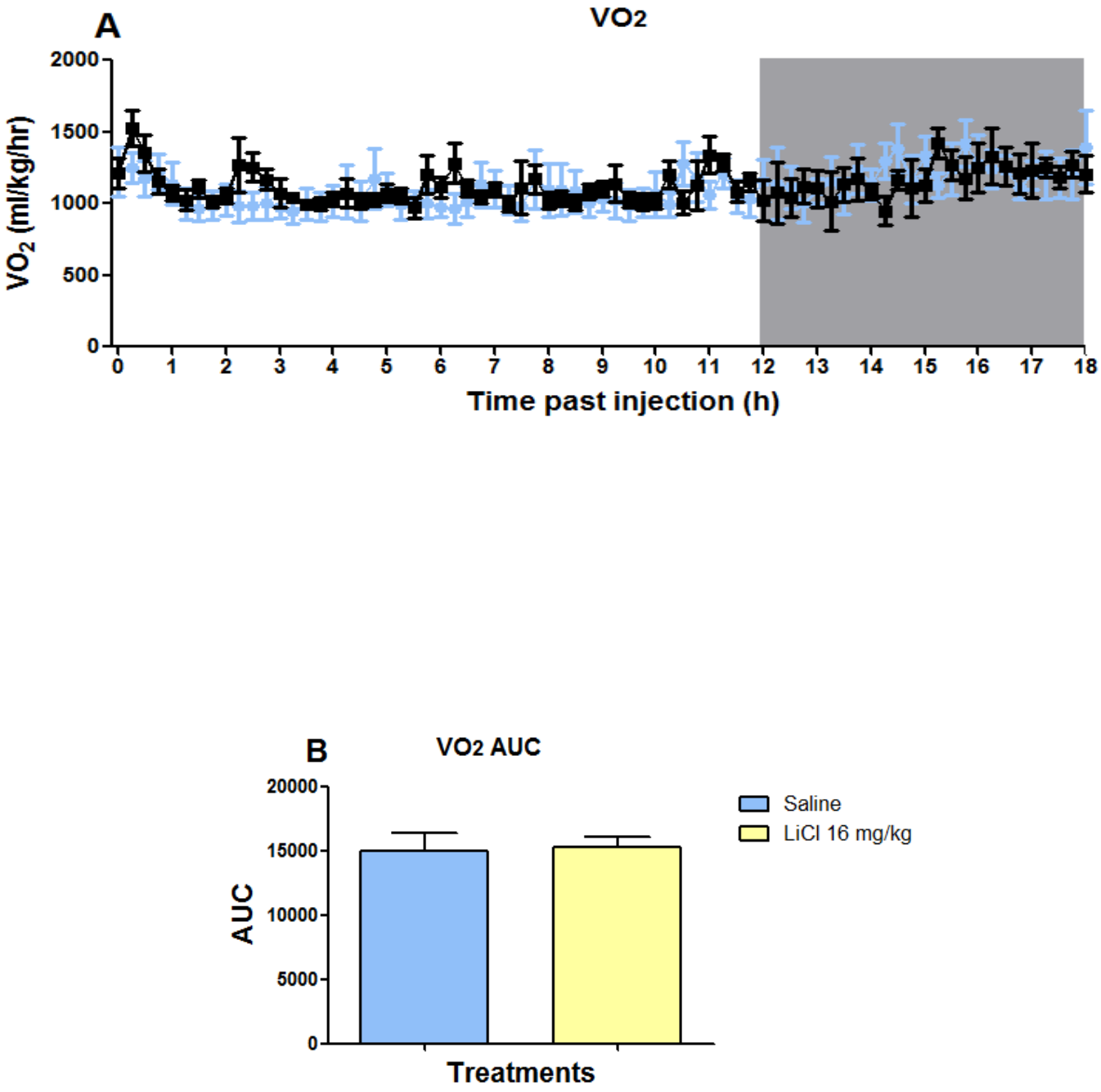


Figure 2.27. The effect of peripheral administration of LiCl on energy expenditure in fasted rats in the early dark phase.

A The effect of saline or 16 mg/kg LiCl on VO_2 in fasted rats in the early light phase. **B** Area under the curve for A. N=6 per group. Data presented as mean ± SEM.

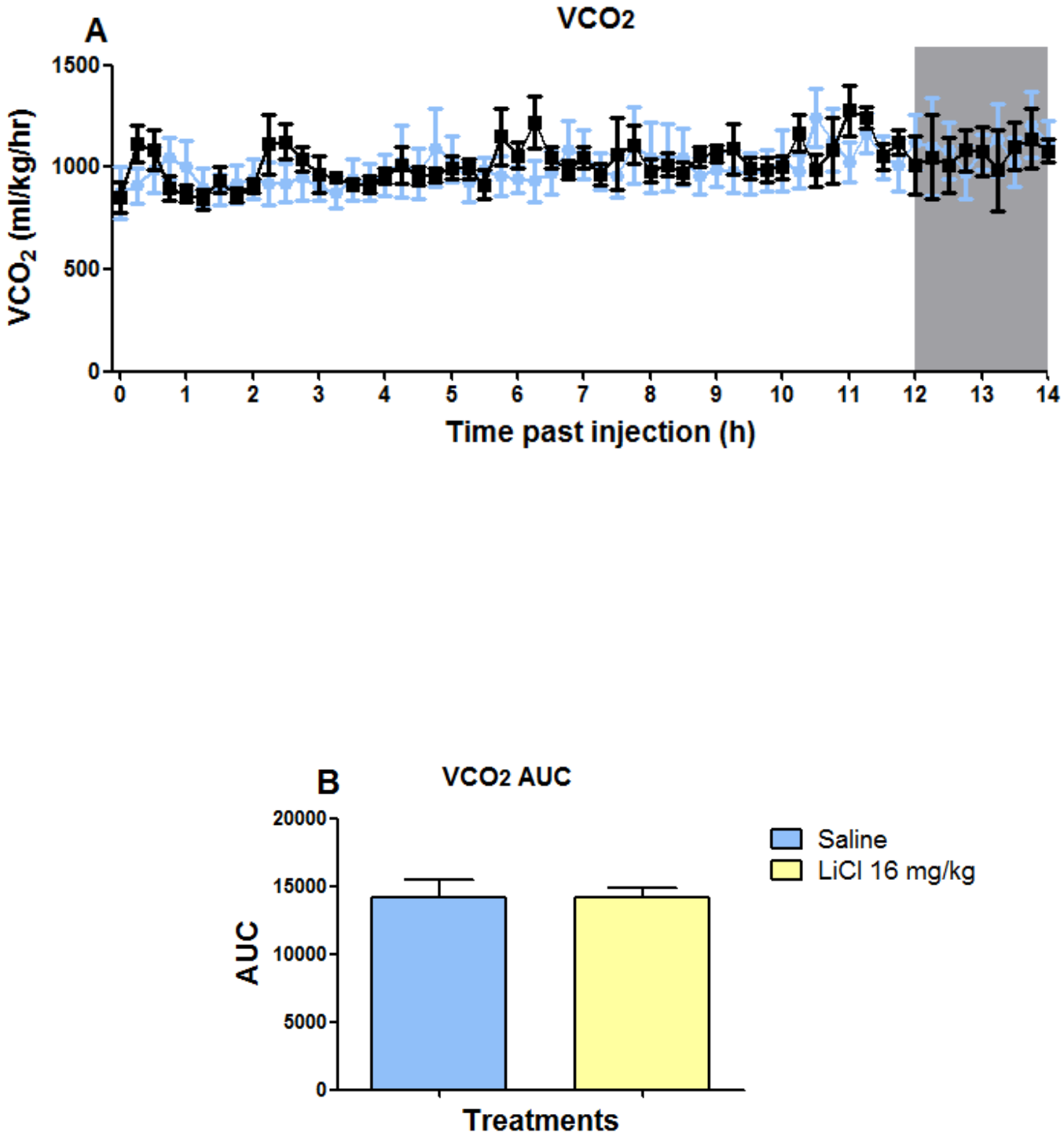


Figure 2.28. The effect of peripheral administration of LiCl on energy expenditure in fasted rats in the early dark phase.

A The effect of saline or 16 mg/kg LiCl on VCO_2 in fasted rats in the early light phase. **B** Area under the curve for A. N=6 per group. Data presented as mean ± SEM.

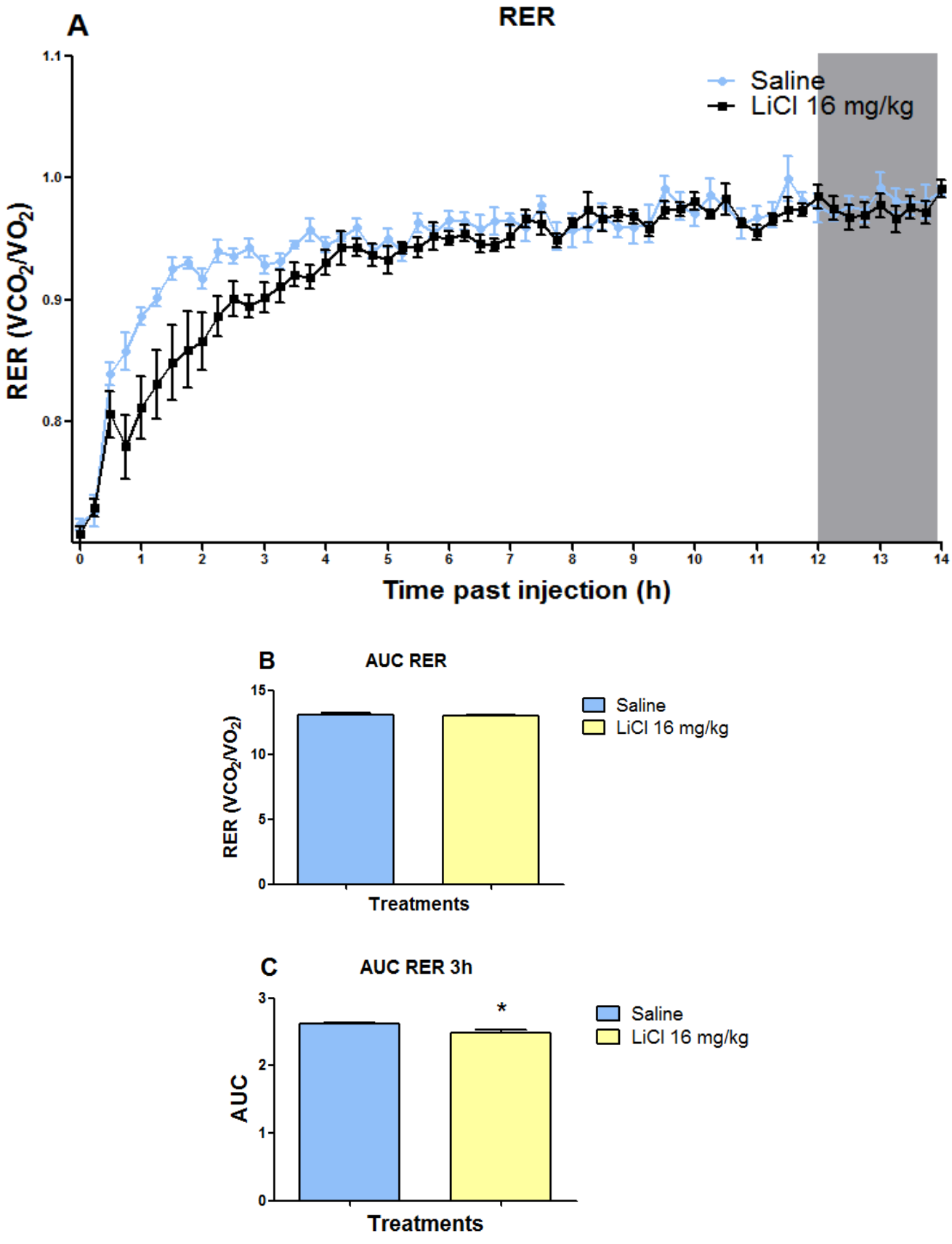


Figure 2.29. The effect of peripheral administration of LiCl on energy expenditure in fasted rats in the early dark phase.

A The effect of saline or 16 mg/kg LiCl on RER in fasted rats in the early light phase. **B** Area under the curve for A (18h). **C** Area under the curve for A (0-3h). N=6 per group. Data presented as mean ± SEM. *p<0.05 vs. saline control using an unpaired t-test.

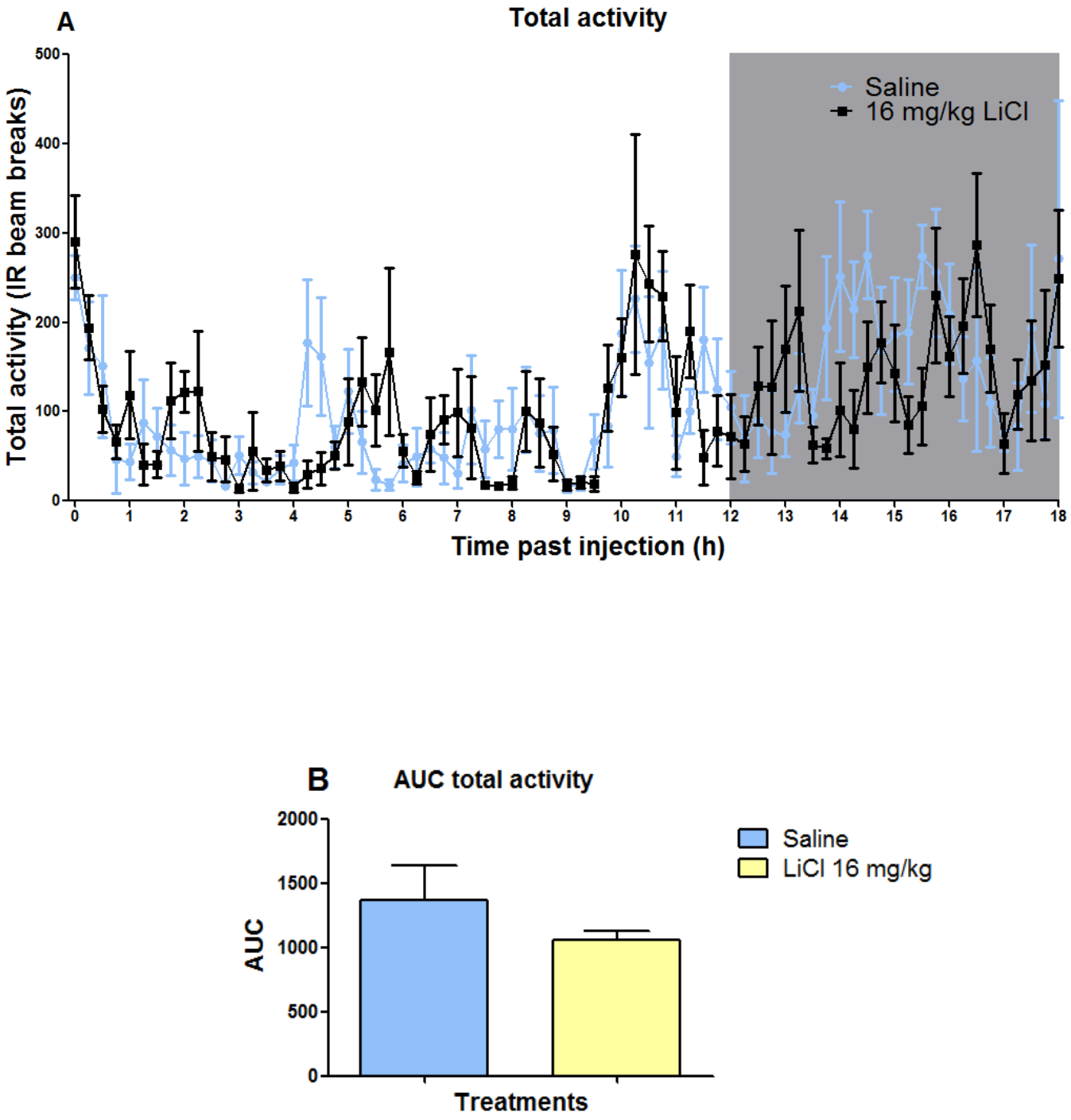


Figure 2.30. The effect of peripheral administration of LiCl on activity in fed rats in the light phase.

A The effect of saline or 16 mg/kg LiCl on total activity in fasted rats injected in the early light phase. Infra-red beam breaks recorded every 12 minutes for 14 hours. **B** Area under the curve for A. Data presented as total beam breaks per minute. N=6 per group. Data presented as mean ± SEM.

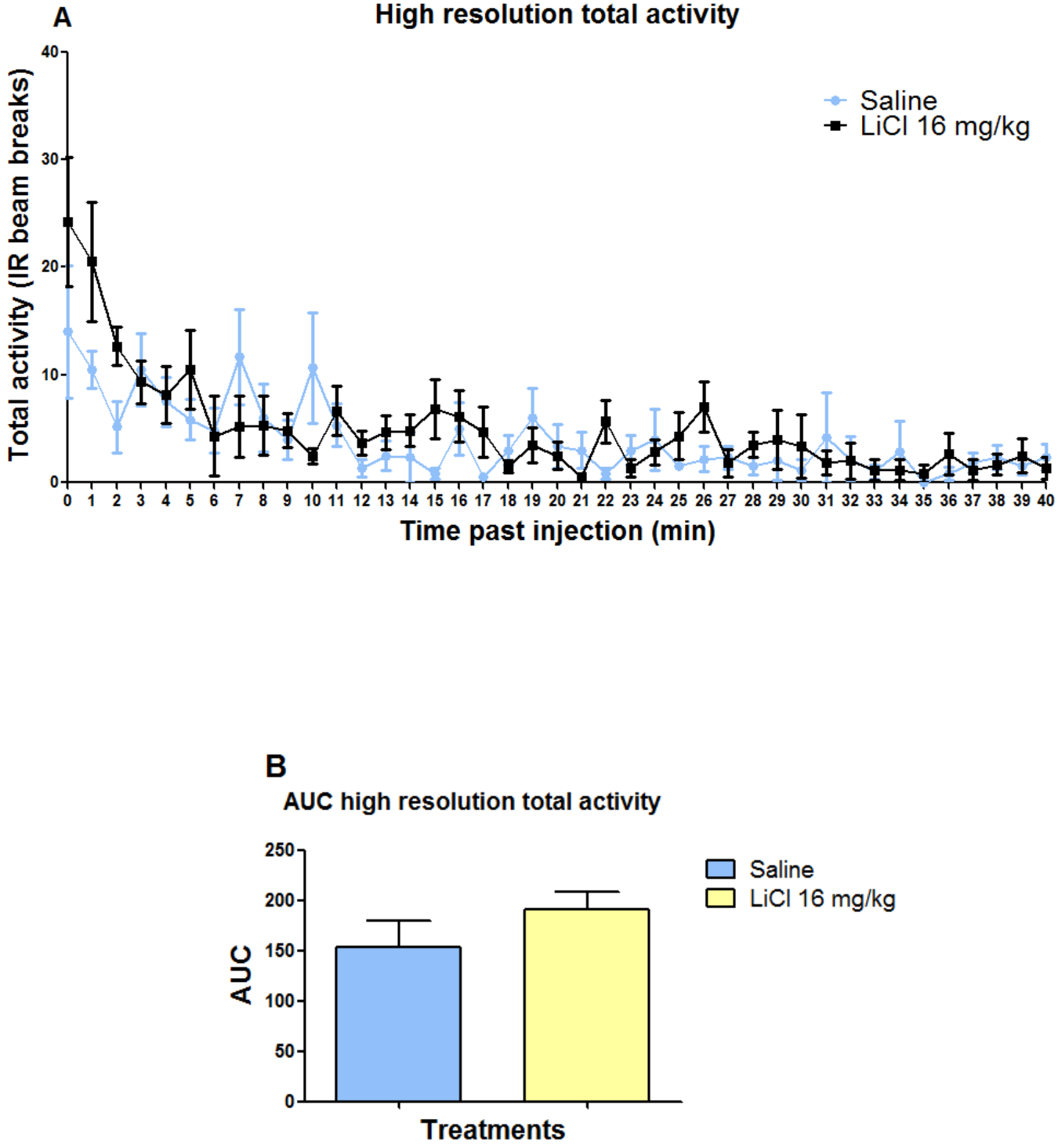


Figure 2.31. The effect of peripheral administration of LiCl on activity in fed rats in the light phase.

A The effect of saline or 16 mg/kg LiCl on total activity in fasted rats injected in the early light phase. High resolution data; infra-red beam breaks recorded every 10 seconds; data presented as total breaks per minute. **B** Area under the curve for A. Data presented as total beam breaks per minute. N=6 per group. Data presented as mean ± SEM.

2.7 Discussion

Leptin, sibutramine and LiCl have well characterised effects on food intake, though they cause their anorectic effects through different mechanisms (Pelleymounter et al., 1995, Farooqi et al., 2007a, Halford and Harrold, 2012, Curtis et al., 1994). The work in this chapter investigated the effects of leptin and sibutramine on food intake, energy expenditure and cardiovascular parameters in rats, and the effects of LiCl on food intake and activity, with the eventual aim of comparing these effects to those obtained for the gut hormones GLP-1 and PYY_{3-36} using the same monitoring system.

High resolution data revealed that 2 mg/kg leptin significantly reduced food intake compared to saline, equivalent to a 29.09±18.03% reduction, in fed rats in the first 40 minutes following administration in the early dark phase (Fig 2.2). This trend remained for 18h, but was no longer significant. In the leptin group, total food intake was reduced by 10.07±6.39% at the end of the dark phase, and 11.04±6.67% after 18h, compared to saline (Fig 2.1). After the initial anorectic effect, it appears that there is no additional effect until 4h after injection, where there is a clearer separation of food intake between the two groups (Fig 2.1). A delayed anorectic effect is common following peripheral leptin administration. A study using lower doses of 300 and 600 µg/kg leptin, observed a significant reduction in food intake at 2-4h post administration, however, higher resolution data is not shown, making discrete effects in the first hour hard to detect (Dryden et al., 1999). Similarly, IV leptin administered to *ob/ob* mice appears to induce robust effects on food intake from 1-2h, (Campfield et al., 1995). Leptin moves into the cerebrospinal fluid via saturable transport, which may explain a more robust effect several hours after administration (Banks et al., 1996). Alternatively, the early and late effects of leptin may be mediated via different mechanisms. The expression of anorexigenic peptides, such as POMC and CART, is increased by leptin, which may contribute to the later effects on food intake, while earlier effects may be due to the inhibited action of ghrelin (Klok et al., 2007).

No significant difference in VO_2, VCO_2, RER, activity, or body temperature was observed following leptin administration, indicating there was no change in energy expenditure or fuel substrate following the peripheral administration of leptin (Fig 2.3-2.5). However, there was a trend for a decrease in RER from 3-18h in the leptin treated group. These results are in contrast to similar studies in *ob/ob* mice, in which leptin increased energy expenditure and lead to a change in fuel source from carbohydrate to fat (Hwa et al., 1997, Pelleymounter et al., 1995). However, RER drops with reduced food intake, and the effect on RER observed may simply reflect the lower food intake in this group. In another study, intravenous infusion of leptin increased energy expenditure in rats; it is possible that serum leptin levels simply were not high enough to significantly affect sympathetic nervous activity in our study (Haynes et al., 1997a). Leptin attenuates the reduction in energy expenditure seen following weight loss in humans and rodents (Galgani et al., 2010, Halaas et al., 1997). In the current study, it is possible that administration of leptin attenuated the decrease in RER caused by leptin-treated rats eating less than controls. In order to accurately investigate energy expenditure, studies must be carried out in fasted animals to avoid the primary effects of food intake on RER.

The effects of leptin on HR and BP are contentious. Studies in obese animals and humans have indicated that leptin may couple increases in body weight to increases in BP (Simonds et al., 2014). Microinjections of leptin into the VMH and DMH evoke a significant increase in mean arterial pressure in rats, suggesting that neurons in the hypothalamus mediate the pressor effects of leptin (Marsh et al., 2003). This may involve leptin-sensitive, CART-containing neurons that directly innervate sympathetic preganglionic neurons (Elias et al., 1998). However, a 3h peripheral leptin infusion did not affect HR or BP in rats, despite activation of the sympathetic nervous system (Haynes et al., 1997a). In the current study, 2 mg/kg leptin did not affect HR or BP in lean rats (Fig 2.8-2.9). Chronic exposure to hyperleptinaemia, as is experienced in obesity, may be necessary to observe the expected pressor effect of renal sympathoactivation (Haynes et al., 1997b).

Sibutramine is a well-characterised anorectic agent in humans and rodents (Grignaschi et al., 1999, Jackson et al., 1997, Weintraub et al., 1991). When administered orally, 10 mg/kg sibutramine reduces nocturnal food intake in rats by 50% (Connoley et al., 1999). High resolution data revealed that 0.9 mg/kg sibutramine significantly reduced food intake in rats compared to vehicle, equivalent to a 50.98±14.11% reduction, in the first 40 minutes following administration in the early dark phase (Fig 2.13). This effect remained significant for 18h following administration, when cumulative food intake was reduced by 22.67±4.23% (Fig 2.12). A similar study achieved comparable results; 1mg/kg IP sibutramine reduced intake of palatable diet by approximately 45% after 2h, however, food intake was not documented after this time point (Pratt and Connolly, 2010).

Peripheral sibutramine significantly reduced VCO_2 and RER in the 18h following administration, suggesting a change in primary fuel source from carbohydrate to fat, which compliments the large reduction in food intake observed. A previous study found that sibutramine peripherally administered at 1 mg/kg had no effect on locomotion in rats, but that central administration modestly inhibited activity levels (Pratt and Connolly, 2010). Additional studies have shown a sibutramine-induced increase in locomotion, but only with higher doses than those used in the present study (Golozoubova et al., 2006). High resolution data showed no difference in locomotion in the 40 minutes following administration; however, there was a significant decrease in total activity over 18h (Fig 2.17). Decreased locomotion may be indicative of decreased food seeking, as described in a study using similar peripheral doses that decreased food intake and increased resting in rats (Tallett et al., 2009). The behavioural satiety sequence (BSS) describes the normal structure of feeding behaviour and satiety in rodents; where consumption of food is followed by grooming and resting. The BSS is used to discriminate between drugs that induce satiety by physiological or non-physiological means in rodents. Drugs that increase synaptic 5-HT, such as sibutramine, preserve the BSS but advance the onset of resting (Halford et al., 1998). The marked effects on food intake in this study in the absence of additional side effects, suggest that 0.9 mg/kg sibutramine may reduce food intake relatively specifically by influencing central energy homeostasis pathways.

In previous studies, an oral dose of 10 mg/kg sibutramine increased HR and BP 2-8h post administration in rats (Thomas et al., 2009). In humans, a dose of 60 mg/kg increased HR and BP at 1, 2 and 6h after administration (King and Devaney, 1988). In addition, the recommended daily dose of sibutramine in obese patients, of 15-20 mg (much

lower than the dose used in the current study) chronically leads to a higher number of cardiovascular events than in untreated controls, and may thus be expected to have an effect on cardiovascular parameters (Food and Drug Administration, 2010). In the current study, sibutramine did not produce any effects on BP or HR, despite profound effects on food intake. This is at odds with a similar study, in which cardiovascular effects were evident from a dose of 0.9 mg/kg sibutramine, though only in the first ten minutes following administration (Woolard et al., 2004). There was no significant effect on HR in the current study in the first ten minutes. In the study by Woolard *et al*., rats were housed individually in normal cages. Though rats were acclimatised to handling in our study, rats were housed in CLAMS cages, which may have increased basal HR and BP due to stress, thereby masking any subtle cardiovascular effects of sibutramine at this low dose (Gardiner and Bennett, 1977). In particular, the rises in BP and HR observed following injection may have masked a specific early rise caused by sibutramine.

Rodents lack an emetic reflex, and thus do not vomit in response to known aversive substances, such as LiCl (Horn et al., 2013). However, LiCl is known to reduce food intake in rats, an effect presumed to be due to reduced gastric emptying and visceral illness. This may be mediated by chemoreceptors in the emetic trigger zone of the AP and GLP-1 receptors in the NTS (Bernstein et al., 1992, Curtis et al., 1994, Seeley et al., 2000). In our dose-response studies, 40 and 48 mg/kg LiCl significantly reduced food intake at 1, 2, 4 and 8 hours post administration compared to saline, and 32 mg/kg significantly reduced food intake at 2 and 4 hours. In a previous study, reductions in food intake were observed at 0.5h, starting at a dose of 11.25 mg/kg. However, after 1h, the minimal dose for an effect on food intake was 45 mg/kg, and 90 mg/kg at 6h (Deutsch and Gonzalez, 1978). It's unclear why our results and those of these studies differ, though it may reflect, for example, differences between sub-strains of rat, or in the conditions in which they were housed.

As there was no effect of 10 mg/kg LiCl on food intake in the current study, we next investigated the effect of 16 mg/kg LiCl on fasted rats in the CLAMS environment, as this automated setup allows for high resolution monitoring that may pick up more subtle differences in food intake than standard manual feeding studies. Standard recordings did not demonstrate a significant effect of 16 mg/kg LiCl injected at the beginning of the early light phase in fed animals in the 18h post administration (Fig 2.25). However, high resolution data indicated a significant decrease in cumulative food intake, equivalent to a 21.87±9.12% reduction, 40 minutes post administration (2.26). No effect on VO_2, VCO_2, or RER was observed 18h post administration. However, RER was significantly reduced in the 3h post administration in the LiCl treated group, reflecting a primary fuel source of fat for energy expenditure, which may be due to the decreased food intake in these animals (Fig 2.29). There was no significant effect of 16 mg/kg LiCl on activity in our study. However, there was a trend for increased activity in the first 40 minutes post administration, which may reflect initial irritation of the injection site, and for decreased cumulative activity after 18h (Figs 2.30-2.31) (Rodriguez et al., 2000). Previous studies have shown that LiCl disrupts the normal BSS, involving widespread changes in the duration and number of eating episodes (Ishii et al., 2004). Grooming is a normal transition behaviour between eating and resting in rodents; LiCl increased resting and decreased grooming, indicating that LiCl-induced anorexia is not dependent on satiation (Blundell et al., 1985).

LiCl is commonly used as a positive control in CTA studies. A commonly used dose for initiating CTA in rats is 127 mg/kg (Nolan et al., 1997, Davies and Wellman, 1990, Thiele et al., 1997). However, studies have suggested that the minimal threshold dose to produce a CTA in rats is much lower, at around 25 mg/kg (Riley and Tuck, 1985, Benoit et al., 2003). Due to the unpleasant side-effects of LiCl, it seemed appropriate to use a relatively low dose, though one that still produced robust effects on food intake and aversion. Though 16 mg/kg significantly reduced food intake in the first 40 minutes, a dose was required that would produce more sustained effects. Therefore, in subsequent studies a dose of 32 mg/kg was used to cause non-specific reductions in food intake in rats.

No significant differences in HR or BP were observed following administration of leptin or sibutramine in these studies. Previous studies have produced conflicting evidence on the effect of leptin on cardiovascular parameters (Haynes et al., 1997a, Marsh et al., 2003). In the case of sibutramine, the lack of effect may have been due to the use of a relatively low dose used, which was the lowest dose capable of affecting HR and BP in another study (Woolard et al., 2004). However, following handling and injection, HR and BP was increased in all studies, indicating the effectiveness of the telemetry implants to pick up larger changes in the cardiovascular parameters measured (Sharp et al., 2002). Data recorded from two individual rats indicated improper probe placement, and cardiovascular data from these animals was therefore excluded from the data analysis. It therefore seems that the telemetry system is capable of detecting changes in HR and BP, and that the CLAMS is capable of detecting relatively small changes in food intake caused by administration of anorectic agents in subsequent studies.

The studies in this chapter aimed to look at the ability of the CLAMS system to pick up changes in food intake and activity, and the ability of the telemetry system to pick up changes in cardiovascular parameters. Overall, initial studies indicate that the CLAMS system is able to provide useful information under both high and low resolution data recording conditions, while the telemetry aspect may lack the sensitivity needed to detect acute changes. In addition, these studies have generated background data for the development of a novel mathematical model to test the specificity of appetite-reducing agents. Following these studies on various anorectic factors that reduce food intake via different mechanisms, I proceeded to characterise the effects of the anorectic gut hormones GLP-1 and PYY_{3-36} on a variety of physiological parameters.

Chapter III: The effect of PYY on energy homeostasis and cardiovascular parameters

3.0 PYY

3.1 Introduction

PYY is released postprandially at levels that generally reflect the quantity of calories ingested (Pedersen-Bjergaard et al., 1996, Adrian et al., 1985). The full length peptide, PYY_{1-36}, is a relatively non-selective agonist of NPY receptors, with highest affinity for NPY receptors Y1 and Y5, and increases food intake via these receptors following central administration in rodents. PYY_{1-36} is processed to form the truncated form, PYY_{3-36}, by DPPIV. This processing confers selectivity for the Y2 receptor, which mediates the anorectic effects of PYY_{3-36} (Keire et al., 2000). PYY_{3-36} is thought to represent 40-55% of total circulating PYY in humans (Grandt et al., 1994a). Recent work in the pig has revealed that PYY_{3-36} has a half-life of around 4 minutes, before it is further hydrolysed into PYY_{3-34}, thus rendering it inactive at the Y2 receptor (Torang et al., 2015). The work described in this chapter will focus on the effects of PYY_{3-36} (referred to hereafter as PYY) on food intake, energy homeostasis and cardiovascular parameters.

3.1.1 Effects of PYY on food intake

ICV administration of PYY increases food intake in mice, an effect which is presumed to be due to pharmacological activation of the Y5 receptor. In contrast, peripheral PYY decreases food intake in rodents via the Y2 receptor, suggesting central and peripheral PYY access different Y receptor populations in the brain to alter food intake (Kanatani et al., 2000, Batterham et al., 2002). It has been postulated that ICV administration results in widespread receptor activation throughout the brain (including Y5R), whereas during peripheral administration, PYY crosses the BBB in specific regions to gain access to Y2 receptors in, for example, the ARC (Kanatani et al., 2000, Keire et al., 2000). In addition, cytoplasmic processes, termed neuropods, have recently been identified on PYY-secreting EECs of the small intestine and colon (Bohorquez et al., 2011). Neuropods provide a potential route for direct contact of EECs with the enteric nervous system. This newly discovered neuroepithelial circuit raises the possibility that, in addition to paracrine transmission, EECs are able to transmit precise sensory signals regarding nutrient intake to peripheral neurons, and in turn receive direct modulatory feedback from these neurons to influence hormone secretion (Bohorquez et al., 2015).

The Y2 receptor is located on almost all ARC NPY neurons, and is reported to act as a presynaptic inhibitory autoreceptor, activation of which inhibits NPY release (Broberger et al., 1997). ARC NPY neurons confer tonic GABA-mediated inhibition to POMC neurons. PYY may act in part by inhibiting NPY neurons, thus disinhibiting POMC neurons, leading to increased α-MSH release. Mice deficient for the Y2R lack an anorectic response to exogenous PYY (Batterham et al., 2002). In addition, specific pharmacological antagonism of the Y2R in the ARC attenuates the effects of exogenous PYY, which has thus been suggested to be the primary target of peripheral PYY (Abbott et al., 2005b). Peripheral PYY administration decreases hypothalamic NPY expression and release, while increasing POMC neuronal activation, with the resulting release of α-MSH (Batterham et al., 2002).

Though the melanocortin pathway appears to be involved in endogenous PYY signalling to an extent, melanocortin-independent pathways are also postulated to play a role, as peripheral PYY is able to attenuate feeding in MC4R knockout, agouti and POMC knockout mice (Halatchev et al., 2004, Challis et al., 2004, Martin et al., 2004). Some effects of PYY may be mediated via peripheral neurons. The Y2 receptor is synthesized in the nodose ganglion and transported to vagal nerve terminals in close proximity to the site of PYY release in the gut (Abbott et al., 2005a, Zhang et al., 1997). Following vagotomy and brainstem-hypothalamic transection in rats, 10 nmol/kg PYY did not alter food intake compared to saline. In addition, these procedures attenuated the c-Fos like immunoreactivity observed in the ARC following peripheral administration of PYY. Overall, this suggests that gastric vagal afferents mediate the effects of circulating PYY on central circuits in rats (Abbott et al., 2005a). Conversely, vagotomised mice have been reported to exhibit an anorexigenic response to PYY of a longer duration, suggesting that in mice, vagal tone modulates the period of satiation, but that the vagus is not necessary for the anorectic actions of PYY (Halatchev and Cone, 2005).

Continuous PYY administration via osmotic mini pump in rodents reduces food intake, but this effect lasts only for the first 3-4 days only, and after this tachyphylaxis develops, perhaps due to receptor desensitization (Morley and Flood, 1987, Pittner et al., 2004). In contrast, periodic infusion is able to reduce food intake and body weight in rodents without causing tachyphylaxis (Chelikani et al., 2007, Reidelberger et al., 2008). Chronic intermittent PYY administration is able to reduce food intake and body weight in DIO rats, as well as having beneficial effects on glucose tolerance and insulin sensitivity (Vrang et al., 2006).

In mice, 30 nmol/kg PYY elicits a 25% reduction in food intake. At higher doses of 100 nmol/kg and above, food intake is attenuated by approximately 30%. This suggests that maximal food intake inhibition occurs between 30 and 100 nmol/kg PYY, with no additional effects on satiety despite a wide dose range (Neary et al., 2005). However, the normal physiological postprandial response involves the modulation of the release of a cocktail of hormones, released at different levels and time intervals. It is highly likely that the effects of these hormones interact to influence satiety, and thus it can be difficult to determine the physiological role of individual hormones from simple administration studies. Coadministration of peripheral GLP-1 and PYY at low doses, which do not individually affect food intake, significantly reduced food intake in lean and obese rodents (Neary et al., 2005). IP PYY also reduces food intake synergistically in combination with Ex-4 in mice, with the anorexigenic effects persisting for up to 8h (Talsania et al., 2005).

IV infusions of PYY reduce food intake and body weight in monkeys, but only at supraphysiological doses (Koegler et al., 2005). In healthy humans, IV PYY reduced food intake at a buffet meal by 36%, and also reduced food intake in obese subjects (Batterham et al., 2002, Batterham et al., 2003). Obese individuals have reduced plasma levels of PYY, and an attenuated postprandial PYY response, requiring greater calorie ingestion to raise circulating PYY levels to that of normal-weight subjects. This suggests that obese subjects may experience reduced PYY-driven satiety following a meal, perhaps contributing to increased meal sizes and perpetuating further weight gain (le Roux et al., 2006b).

Thus, obesity does not result in resistance to PYY, as it does to leptin and insulin. Low plasma PYY is thought to occur as a result of decreased release, rather than decreased synthesis, and is likely to be a consequence, rather than a cause, of obesity, which then propagates further weight gain (le Roux et al., 2006b). PYY has therefore been postulated to have clinical utility in the treatment of human obesity.

3.1.2 Effects of PYY on energy expenditure

PYY has been suggested to have beneficial effects on fuel partitioning, involving the mobilization of fat stores in preference to other energy stores. The dark cycle respiratory quotient is transiently decreased following peripheral PYY administration in obese mice, implying increased utilization of fat as a primary fuel source during this period (Adams et al., 2006). A further study in WT mice found that energy expenditure was not directly affected, but that respiratory quotient was again decreased by PYY. This effect remained throughout chronic treatment, even when the anorectic effects had declined (van den Hoek et al., 2007).

In a human study, infusions of PYY lead to a near-significant increase in energy expenditure, and decreased respiratory quotient, indicating increased fat oxidation in these subjects (Sloth et al., 2007). However, over half of participants in this study were not able to complete the study, due to nausea and abdominal discomfort. The exact mechanisms that underlie PYY-related changes in energy expenditure remain to be elucidated.

PYY reduces locomotion in *ad libitum* and HFD-fed rats, but does not alter locomotion in food-restricted rats. In the former, food intake was reduced by PYY administration, while in the latter it was not affected. This suggests that the altered activity levels mirror decreases in food intake, which may reflect decreased food-seeking due to increased satiation (Nordheim and Hofbauer, 2004). Conversely, central PYY administration transiently increases locomotion in rats, postulated to be due to pharmacological activation of Y1 and Y5 receptors (Pfluger et al., 2011). PYY also increased body temperature in food-restricted rats, which have high central levels of basal NPY tone, but not in *ad libitum* fed rats (Nordheim and Hofbauer, 2004), and transgenic mice overexpressing PYY are protected against DIO and exhibit increased body temperature, indicative of a role for PYY in thermogenesis (Boey et al., 2008).

3.1.3 Effects of PYY on cardiovascular parameters

Peripheral PYY administration increases mean arterial pressure and HR in food-restricted rats, while it has no effect on rats fed a HFD; it results in an intermediate response in *ad libitum* fed animals. As previously mentioned, PYY did not affect locomotion in food-restricted rats, and thus the observed increases in HR and BP were not secondary to changes in activity (Nordheim and Hofbauer, 2004). Hypothalamic NPY release is increased by fasting, and it has been suggested the effects of PYY on HR and BP depend on endogenous NPY levels, which in turn depend on nutritional state (Nordheim and Hofbauer, 2004). Central infusion of NPY in anaesthetised rats induces bradycardia and decreases systolic and diastolic BP, via activation of Y1 receptors (Tseng et al., 1989, Cheng et al., 2012). The

pressor response evoked by central PYY is not affected by administration of an α1-adrenoceptor antagonist, suggesting sympathoexcitation is not directly involved (Martin, 2005).

In fasted humans, HR was significantly increased during a 90 minute infusion of a range of doses of PYY, and remained increased for 30 minutes post administration. There was no difference between the HR of subjects administered PYY or placebo following an *ad libitum* meal, again indicating that PYY elicits cardiovascular effects only when basal NPY levels are high, as is the case during food restriction (Sloth et al., 2007). However, an alternative study in humans found a small but significant decrease in HR, and increased BP when PYY was administered at doses resulting in elevated circulating levels similar to those found during diarrheal disease, such as tropical sprue (Playford et al., 1992).

3.1.4 Effects of PYY on aversion and neuronal activation

Peripheral PYY increased the proportion of ARC POMC neurons that expressed c-Fos immunoreactivity 2.6-fold in mice (Batterham et al., 2002). Further studies in mice confirmed this effect in the ARC, and also found increased c-Fos in the PVN, LHA, NTS and AP (Halatchev and Cone, 2005, Hurtado et al., 2013). The pattern of neuronal activation in the brainstem is similar to that induced by peripheral administration of LiCl in rats (Schafe et al., 1995). It is therefore possible that the anorectic effects of PYY are due in part to a transient aversive response, which negatively modulates the palatability of food. In rats, IV infusions of PYY dose-dependently produce a CTA (Chelikani et al., 2006b). In mice, peripheral doses of 3.7 and 12.4 nmol/kg also produce a CTA (Halatchev and Cone, 2005). PYY evokes a potent emetic response in dogs, which is postulated to be mediated by receptors in the AP (Harding and McDonald, 1989).

Studies using fMRI in humans have suggested that administration of PYY may promote the control of food intake by hedonic rather than homeostatic regulatory systems. During the fasted state, changes in hypothalamic neuronal activation govern eating behaviour. However, infusion of PYY at levels that mimic those seen postprandially leads to increased brain activation in the OFC, ventral striatum, anterior cingulate, insula and VTA. These mesocorticolimbic and higher cortical brain regions have roles in determining the reward value of food and hedonic behaviour (Batterham et al., 2007).

In humans, IV PYY administered at physiological circulating levels attenuates food intake and does not induce feelings of malaise. However, following infusions of supraphysiological doses of PYY, sensations of nausea are increased, with no additional effect on food intake (le Roux et al., 2008). This suggests the existence of two threshold doses of PYY; the first reducing food intake physiologically without causing nausea, while the second causes nausea with no further effects on food intake. It also suggests that discrete circuits may mediate satiety and nausea. In this study, nausea was transient and lasted for around 30 minutes. Food intake remained suppressed after nausea levels had returned to baseline, indicating the anorectic effects of PYY are independent of effects on aversion (le Roux et al., 2008). In another study using infusions of PYY at a slightly higher dose, over half of subjects discontinued the protocol due to the severity of adverse effects, including nausea, abdominal discomfort and hot flushes. The

remaining subjects, though not experiencing nausea, reported reduced subjective ratings of general well-being (Sloth et al., 2007).

3.2 Aims and hypothesis

3.2.1 Hypothesis

The administration of PYY and LiCl will result in concurrent changes in food intake, HR and BP in rats, detectable using CLAMS metabolic cages and implanted telemetry probes. Higher doses of PYY will induce a CTA in rats, and will result in neuronal activation in brain regions with roles in the homeostatic and the non-homeostatic control of energy intake.

3.2.2 Aims:

To investigate the effects of peripheral bolus IP injection of PYY on:

- Food intake, energy expenditure, HR and BP in freely moving rats.
- Aversion in rats.
- c-Fos like immunoreactivity in the rat brain.

3.3 Methods

3.3.1 Animals

Male Wistar rats (Charles River, Margate, Kent, UK) weighing 200-220g were housed in individual cages under controlled temperature (21-23°C) and light (12 hours light, 12 hours dark; lights on at 0700) with *ad libitum* access to standard chow (RM1, Special Diet Services Ltd., Witham, Essex, UK) and water, unless stated otherwise. All animal procedures conducted were approved by the British Home Office under the Animals (Scientific Procedures) Act 1986 (Project Licence 70/8068).
Animals were acclimatised to handling and administration protocols prior to all experimental procedures. Animals were randomised by body weight for all studies.

3.3.2 The effect of peripheral administration of 50 and 300 nmol/kg PYY on food intake in fasted rats

To investigate the effects of high doses of PYY, which may activate non-homeostatic pathways, we sought a dose of PYY that would maximally reduce food intake. Previous studies in mice have shown doses of 30, 100, 150 and 300 nmol/kg PYY reduce 1h food intake by 24, 31, 30, and 28%, respectively (Neary et al., 2005). A feeding study was performed to investigate the effect of high doses of PYY on food intake in rats. Rats were fasted in the early dark phase overnight (to exclude variations in endogenous postprandial PYY release) and received an IP injection of saline, 50 or 300 nmol/kg PYY_{3-36} (Bachem, Merseyside, UK) (n=12-14), during the early light phase the following morning. Rats were then returned to their individual home cages with a pre-weighed amount of chow. Food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

3.3.3 The effect of peripheral administration of 0.75, 7.5, 25 nmol/kg PYY on food intake in fasted rats

To investigate the effect of a range of doses of PYY on food intake, a dose-response study was performed. We sought a dose of PYY that reduced food intake by approximately 15-20%, as we hypothesised that any anorectic effects of this magnitude were more likely to be mediated by homeostatic pathways. In a previous study, 7.5 nmol/kg PYY reduced cumulative food intake at 1h by 25% (Abbott et al., 2005b). Rats were fasted in the early dark phase overnight and received an IP injection of saline or 0.75, 7.5, 25 nmol/kg PYY_{3-36} (Bachem, Merseyside, UK) (n=38, cross-over design), during the early light phase the following morning. Rats were then returned to their individual home cages with a pre-weighed amount of chow. Food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

3.3.4 The effect of peripheral administration of 2.5 nmol/kg PYY on food intake in fasted rats

A dose of 2.5 nmol/kg PYY had previously been shown to significantly reduce food intake by 21% in rats (Abbott et al., 2005b). As part of our studies to identify a low dose of PYY that reduced food intake by approximately 15-20%, the effect of 2.5 nmol/kg PYY on food intake was investigated. Rats were fasted in the early dark phase overnight (to exclude variations in endogenous postprandial PYY release) and received an IP injection of saline or 2.5 nmol/kg PYY_{3-36} (Bachem, Merseyside, UK) (n=19-20), during the early light phase the following morning. Rats were then returned to their individual home cages with a pre-weighed amount of chow. Food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

3.3.5 The effect of peripheral administration of 1.5 nmol/kg PYY on food intake in fasted rats

As 2.5 nmol/kg significantly reduced food intake, while 0.75 nmol/kg PYY failed to produce a significant effect, the effects of 1.5 nmol/kg PYY were subsequently investigated. Rats were fasted in the early dark phase overnight and received an IP injection of saline or 1.5 nmol/kg PYY_{3-36} (Bachem, Merseyside, UK) (n=19), during the early light phase the following morning. Rats were then returned to their individual home cages with a pre-weighed amount of chow. Food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

3.3.6 The effect of IP administration of PYY on plasma PYY levels in rats

Plasma samples were assayed to investigate the levels of circulating PYY, following peripheral administration of saline, 1.5, 2.5 or 7.5 nmol/kg PYY in fasted rats (n=8-12). Rats were fasted over night to exclude variations in endogenous postprandial PYY release. Controls included fasted rats administered saline, fasted rats not administered saline, and rats *ad libitum* fed for 20 or 30 minutes following an overnight fast, to determine the normal physiological levels released after eating (n=8-12). Blood was collected from fed and fasted controls at 20 or 30 minutes, to allow digestion of food and release of peptides into the circulation in the fed groups. Blood was collected from rats treated with saline, 1.5, 2.5 or 7.5 nmol/kg PYY at 10 minutes, and 2.5 or 7.5 nmol/kg PYY at 20 minutes due detect the expected rapid increases in circulating peptide levels following IP injection.

Plasma PYY release was measured using an established specific and sensitive in-house radioimmunoassay (Adrian et al., 1985). The PYY antibody was raised against synthetic porcine PYY coupled to BSA in rabbits. The antibody fully cross-reacts with full length (PYY_{1-36}) and truncated (PYY_{3-36}) forms of PYY, and does not cross-react with any other known gut peptides. ^{125}I-PYY was prepared by Professor Mohammad Ghatei, using the Iodogen method (Wood et al., 1981) and purified by high pressure liquid chromatography (HPLC). The assay was performed in 700µl of pH7.3 phosphate buffer (0.06M; Appendix 1) containing 0.3% bovine serum albumin (BSA; Sigma, Poole, UK) (200µl/100ml). The standard curve composed of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl of synthetic PYY at a concentration of 0.5pmol/ml. One hundred µl of plasma sample was used and all were assayed in duplicate. The assay was incubated for three days at 4°C. Free and antibody-bound PYY were separated by immunoprecipitation using sheep anti-rabbit antibody (Pharmacia Diagnostics, Uppsala, Sweden). One hundred µl secondary antibody was added, and following a one hour incubation at room temperature, 500µl 0.01% Triton X-100 (Sigma, UK) and 100µl

of 10% polyethylene glycol (PEG) (Sigma, Poole, UK) were added. Tubes were centrifuged immediately at 1500 g at 4°C for 30 minutes. The supernatant, containing unbound-PYY, was separated by aspiration. Free and bound radioactivity were then counted using a gamma scintillation counter (LB2111 Multi Crystal Gamma Counter, Berthold Technologies, Bad Wildbad, Germany) for 179 seconds. Peptide concentrations in each plasma sample were calculated using a non-linear plot (Prism version 6.03, GraphPad Software Inc., CA, USA).

3.3.7 The effect of peripheral PYY administration on food intake, energy expenditure and cardiovascular parameters in *ad libitum* fed rats

Data from the abovementioned feeding studies resulted in the selection of doses of 1.5, 7.5 and 300 nmol/kg PYY for use in subsequent CLAMS and telemetry studies. In addition LiCl was used as a positive control for aversion at a dose of 32 mg/kg that, in the studies described in chapter II, elicited an anorectic effect of a similar magnitude to the 300 nmol/kg dose of PYY.

3.3.8 The effect of peripheral PYY administration on food intake and energy expenditure in *ad libitum* fed rats

Rats were administered saline, 1.5, 7.5, 300 nmol/kg PYY, or 32 mg/kg (n=12) by IP injection in a cross-over study design at 1900 hours (early dark phase), conducted as previously described in section 2.5.3. A dose of 32 mg/kg LiCl was selected as previous studies have indicated that the minimal dose of LiCl to cause aversion is approximately 25 mg/kg, and 32 mg/kg produced a robust reduction in food intake (Fig 2.24, section 2.6.4), and was thus deemed adequate to act as a positive control for aversion (Riley and Tuck, 1985).
Food intake was recorded every 12 minutes for 18 hours. Activity, measured by total infrared beam breaks, was recorded every 12 minutes for 18 hours, and high resolution data was recorded every 10 seconds for 40 minutes post administration, presented as mean activity per minute. Metabolic parameters (VO_2 and VCO_2) were measured by indirect calorimetry and values normalised with respect to body weight. RER was calculated by determining the ratio between CO_2 produced/O_2 consumed to determine the fuel substrate contributing to energy expenditure. VO_2 and VCO_2 were recorded every 12 minutes following administration of the treatments for a period of 18 hours.

3.3.9 The effect of peripheral administration of PYY on cardiovascular parameters and body temperature in *ad libitum* fed rats

Rats were implanted with telemetry probes, as previously described in chapter II, section 2.5.4.

3.3.10 The effect of peripheral administration of PYY on conditioned taste aversion in rats

To investigate whether the peripheral administration of PYY is associated with feelings of visceral illness, a CTA protocol was used.

The experimental protocol was adapted from an established method for analysis of CTA using a single-bottle method (Lachey et al., 2005). During the first week, adult male Wistar rats were trained to consume their daily fluid intake within a one hour period; water access was restricted at all other times. Within the training period, any animal failing to consume a minimum of 40ml/kg on two consecutive days, or showing clinical signs of dehydration, was excluded from the study.

In the first study, treatments tested included; saline, 2.5, 7.5 nmol/kg PYY (n=14-15), and 127mg/kg LiCl (n=5) as a positive control.

In the second study, treatments tested included; saline, 1.5 nmol/kg PYY (n=14-15), and 48 mg/kg LiCl (n=5) as a positive control. These doses were selected to investigate the lowest dose of PYY that would cause a significant CTA. Though 127 mg/kg is commonly used in CTA protocols, previous studies have found the threshold dose of LiCl to cause aversion to be around 25 mg/kg (Riley and Tuck, 1985). It is important to cause as little suffering as possible when performing *in vivo* studies, thus the LiCl dose was lowered in subsequent studies. However, I chose a higher dose than the threshold to avoid having to repeat the study, as this would require a new cohort of animals. Therefore, 48 mg/kg was selected, as it had robustly reduced food intake in previous studies (Fig 2.24, section 2.6.4), and was thus deemed to be adequately aversive.

During the test week, animals were introduced to a novel flavour; Grape Kool-Aid (Northfield, IL, USA) diluted according to manufacturer's instructions, during the one hour fluid access period in place of 70ml water on days 1 and 3. Kool-Aid access was immediately followed by an IP injection of their allocated treatment, randomised by body weight. Animals had access to water during the fluid access period on days 2 and 4, and had *ad libitum* access to food at all times. On day 5, all animals received Kool-Aid, without any subsequent injection. Fluid intake was then compared between groups.

3.3.11 The effect of peripheral administration of PYY on c-Fos-like immunoreactivity in nuclei of the mesocorticolimbic pathways of fasted rats

3.3.12 Tissue collection

Rats were fasted overnight (to exclude variations in endogenous PYY release and subsequent neuronal activation) and received an IP injection of saline, 2.5, 25 or 300 nmol/kg PYY (n=4-6) during the early light phase. These doses were selected as they represented the three levels of food intake inhibition (~15, 30 and 40%) determined from earlier feeding studies. Ninety minutes post-administration, rats were deeply anaesthetised with an IP injection of 2.5ml pentobarbital (Euthatal, Merial Animal Health Ltd. Harlow, UK). Once pedal reflexes had ceased, rats were transcardially perfused with 0.01M PBS (Appendix 1) to flush blood from the vasculature and were subsequently perfused with 4% formaldehyde (Appendix 1) to fix the brain tissue. Brains were dissected and stored in 4% formaldehyde solution for 24h at 4°C, and then transferred to

40% (w/v) sucrose solution (Appendix 1) for 7 days to dehydrate them for cryopreservation. Brains were then snap frozen in isopentane chilled to -80°C and stored at -80°C until sectioning.
Coronal sections of the whole brain were sliced to 40µm, using a freezing sled microtome (Shandon Southern Products, Ltd., Runcorn, Cheshire, U.K.). Free-floating sections were stored in antifreeze (Appendix 1) at -20°C.

3.3.13 c-Fos immunohistochemistry

Free-floating sections were washed in PBS to remove anti-freeze. Sections were then incubated in methanol with 0.6% hydrogen peroxide for 15 minutes to block endogenous peroxidase activity, sections were then incubated for two hours in blocking solution (Appendix 1) at room temperature to minimise non-specific binding. Sections were incubated overnight in 1:20,000 dilution of anti-rabbit c-Fos primary antibody (Merck Chemicals Ltd. Nottingham, UK) and subsequently incubated in a 1:400 dilution of secondary antibody (biotinylated goat anti-rabbit ImmunoglobinG; Vector Laboratories, Peterborough, UK; Appendix 1) for two hours, followed by incubation in a 1:200 dilution of avidin-biotin-complex (ABC) solution for one hour. The sections were submerged in a 1% diaminobenzedine tetrahydrochloride (DAB) solution (Appendix 1)for approximately 4 minutes for staining. Sections were washed in 0.01M PBS (Appendix 1) between each incubation step. Sections were mounted onto poly-L-lysine-coated slides and left to dry overnight. They were then dehydrated in ethanol, delipidated in xylene and had cover-slips affixed with DPX mounting medium.
Cell bodies positive for c-Fos-like immunoreactivity in bilaterally matched sections were counted by an individual blinded to the treatment groups, using a light microscope (Nikon, Eclipse 50i). Hypothalamic and brainstem nuclei examined included; SO, VLH, AHA, PVN, VMH, ARC, DMN, NTS, AP and dorsal motor nucleus of the vagus (10N). Mesocorticolimbic areas examined included; VTA, CeA, MO, PrL, GI, CPu and NAcbSh. All nuclei were defined in relation to anatomical landmarks according to the rat brain atlas of Paxinos and Watson (Paxinos, 2007).

3.3.14 The effect of peripheral administration of LiCl on c-Fos-like immunoreactivity in brainstem nuclei of fasted rats

3.3.15 Tissue collection

Rats were fasted overnight (to exclude neuronal activation resulting from eating) and received an IP injection of saline or 32 mg/kg LiCl (n=2-6) during the early light phase. Brains were perfused and fixed as described in section 3.3.12.

3.3.16 c-Fos immunohistochemistry

Free-floating brainstem sections, including the MO, PrL, GI, NAcbSh, CPu, NTS, AP and 10N, were stained as described in section 3.3.13.

3.3.17 Statistical analysis

All data except IHC are expressed as mean ± SEM. Dose-response studies for 0.75, 7.5 and 25 nmol/kg PYY were analysed using Repeated Measures one-way ANOVA and Dunnet's multiple comparison *post-hoc* test. Feeding studies for 1.5 and 2.5 nmol/kg PYY were analysed using an unpaired t-test. Feeding studies for 50 and 300 nmol/kg PYY were analysed using one-way ANOVA and Dunnet's multiple comparison *post-hoc* test. Plasma PYY levels, and CTA data were analysed using one-way ANOVA using Tukey and Dunnet's multiple comparison *post-hoc* tests, respectively.

All CLAMS and telemetry data were analysed by performing area under the curve (AUC) with a one-way ANOVA and Tukey's *post-hoc* test, excluding data pre-administration. High resolution activity data points represent the total infrared beam breaks in one minute. Telemetry data are presented as mean HR and BP in 30 minute intervals.

IHC c-Fos data are presented as the median, inter-quartile and total range. PYY IHC data were analysed using Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison *post-hoc* test. LiCl IHC data were analysed using a Mann Whitney U test. All analyses were carried out using Graphpad Prism software (Prism 6.03, GraphPad Software Inc., CA, USA). $P<0.05$ was considered statistically significant.

3.4 Results

3.4.1 The effect of peripheral administration of 50 and 300 nmol/kg PYY on food intake in fasted rats.

IP administration of 50 and 300 nmol/kg PYY significantly reduced food intake 0-1 hours following administration ($p<0.05$ and $p<0.001$, respectively; Fig 3.1A). A dose of 300 nmol/kg PYY significantly reduced cumulative food intake 0-2 and 0-4h following administration ($p<0.001$ and $p<0.01$, respectively, Fig 3.1 B-C). Doses of 50 and 300 nmol/kg PYY significantly reduced food intake at 0-8h ($p<0.05$; Fig 3.1D).

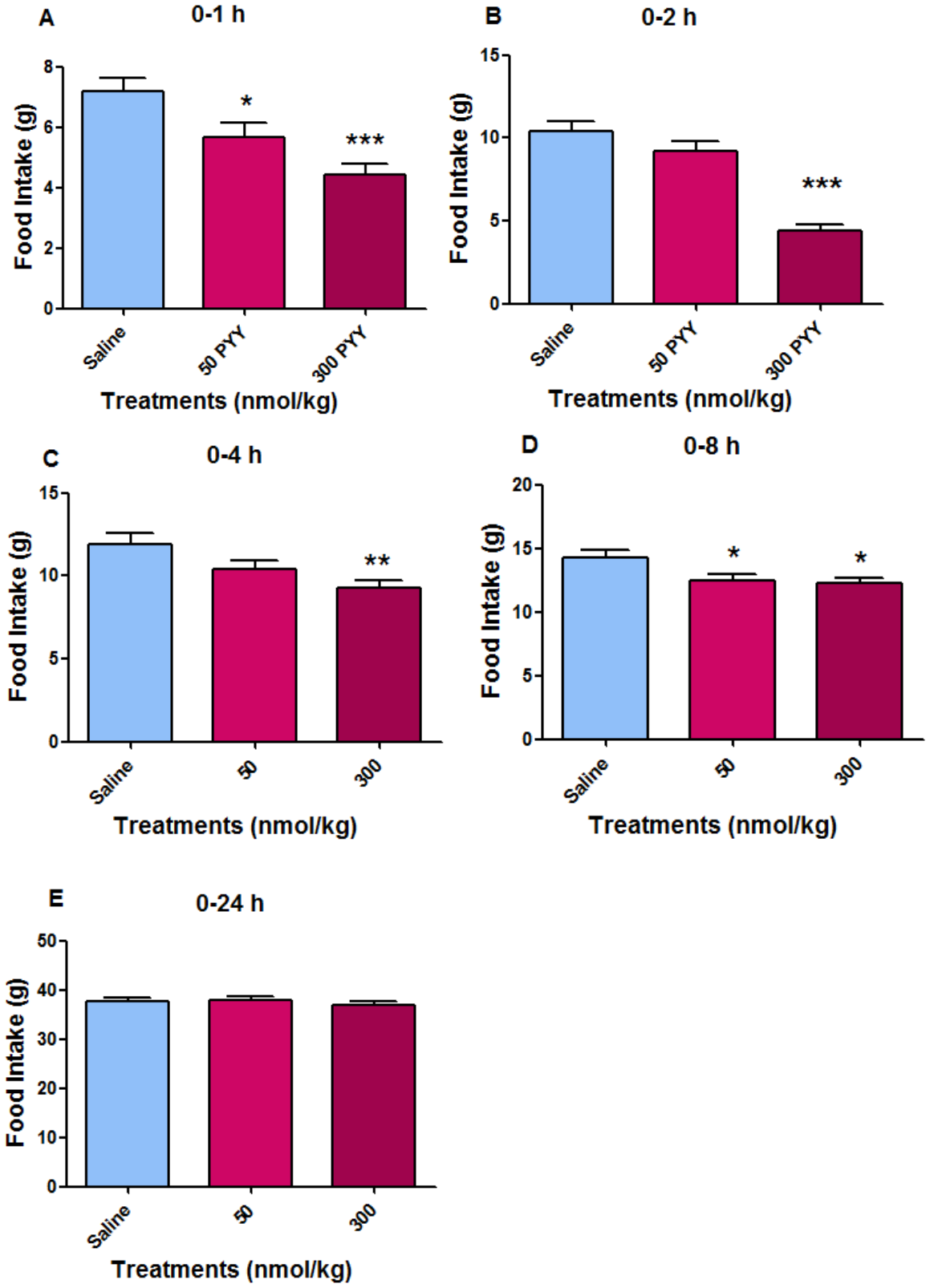


Figure 3.1. The effect of IP administration of 50 and 300 nmol/kg PYY on food intake in fasted rats during the early light phase.

The effect of intraperitoneal administration of saline or 50 or 300 nmol/kg PYY on food intake in overnight fasted rats during the early light phase at 0-1 (A), 0-2 (B), 0-4 (C), 0-8 (D) and 0-24 (E) hours post administration. Data presented as mean ± SEM. n= 12-13 per group. *p<0.05, **p<0.01, ***p<0.001 vs. saline control, using 1-way ANOVA.

3.4.2 The effect of peripheral administration of 0.75, 7.5 and 25 nmol/kg PYY on food intake in fasted rats.

IP administration of a range of doses of PYY reduced food intake compared to saline control. Doses of 7.5 and 25 nmol/kg PYY significantly reduced food intake 0-1 hours following administration ($p<0.01$ and $p<0.001$, respectively; Fig 3.2A). A dose of 25 nmol/kg PYY significantly reduced cumulative food intake 0-2 hours following administration ($p<0.001$; Fig 3.2B).

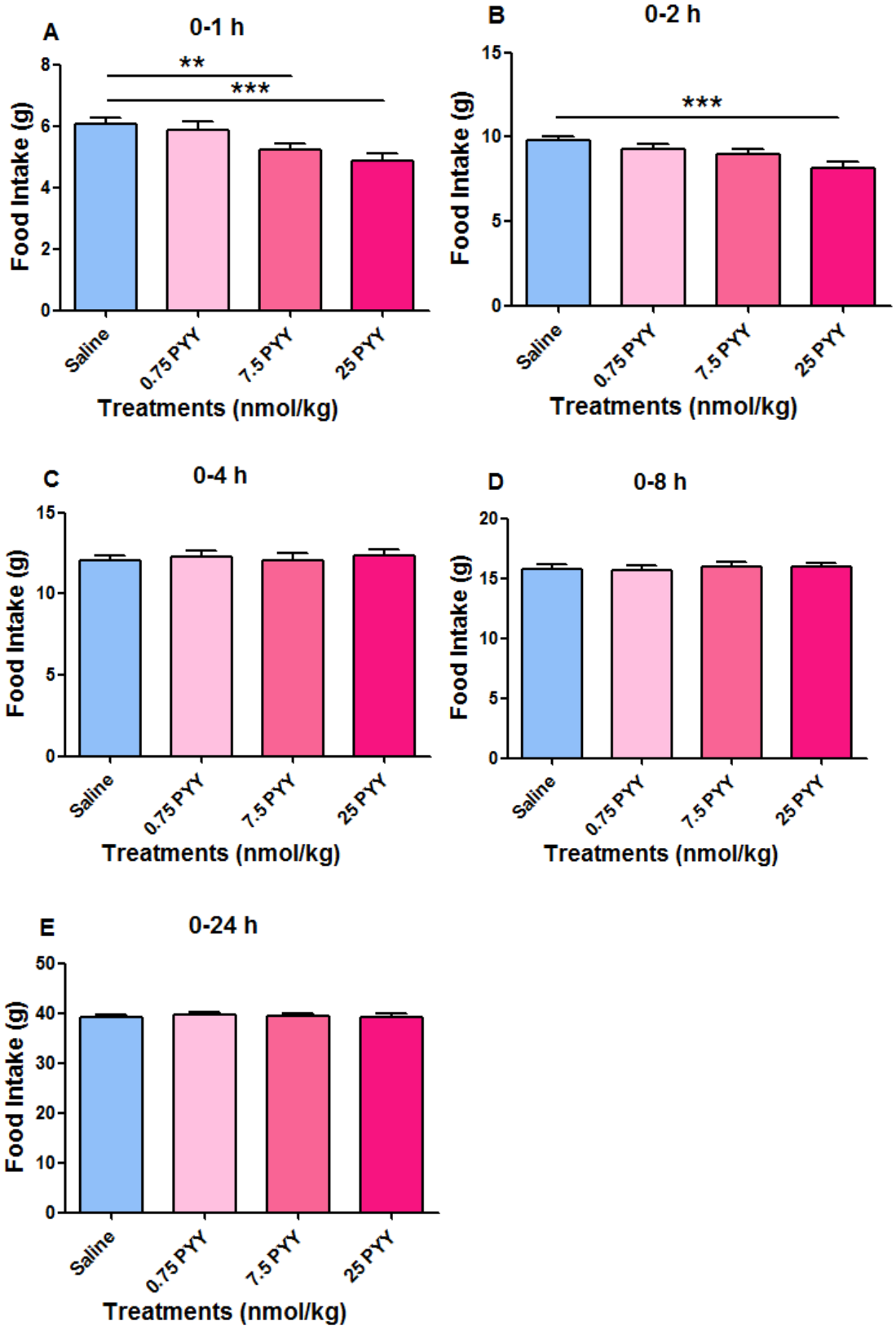


Figure 3.2. The effect of IP administration of PYY on food intake in fasted rats during the early light phase.

The effect of intraperitoneal administration of saline or 0.75, 7.5, 25 nmol/kg PYY on food intake in overnight fasted rats during the early light phase at 0-1 (A), 0-2 (B), 0-4 (C), 0-8 (D) and 0-24 (E) hours post administration. Data presented as mean ± SEM. n= 38 per group. **p<0.01, ***p<0.001 vs. saline control, using Repeated Measures 1-way ANOVA.

3.4.3 The effect of peripheral administration of 2.5 nmol/kg PYY on food intake in fasted rats.

IP administration of 2.5 nmol/kg PYY significantly reduced food intake at 0-1h ($p<0.05$; Fig 3.3A).

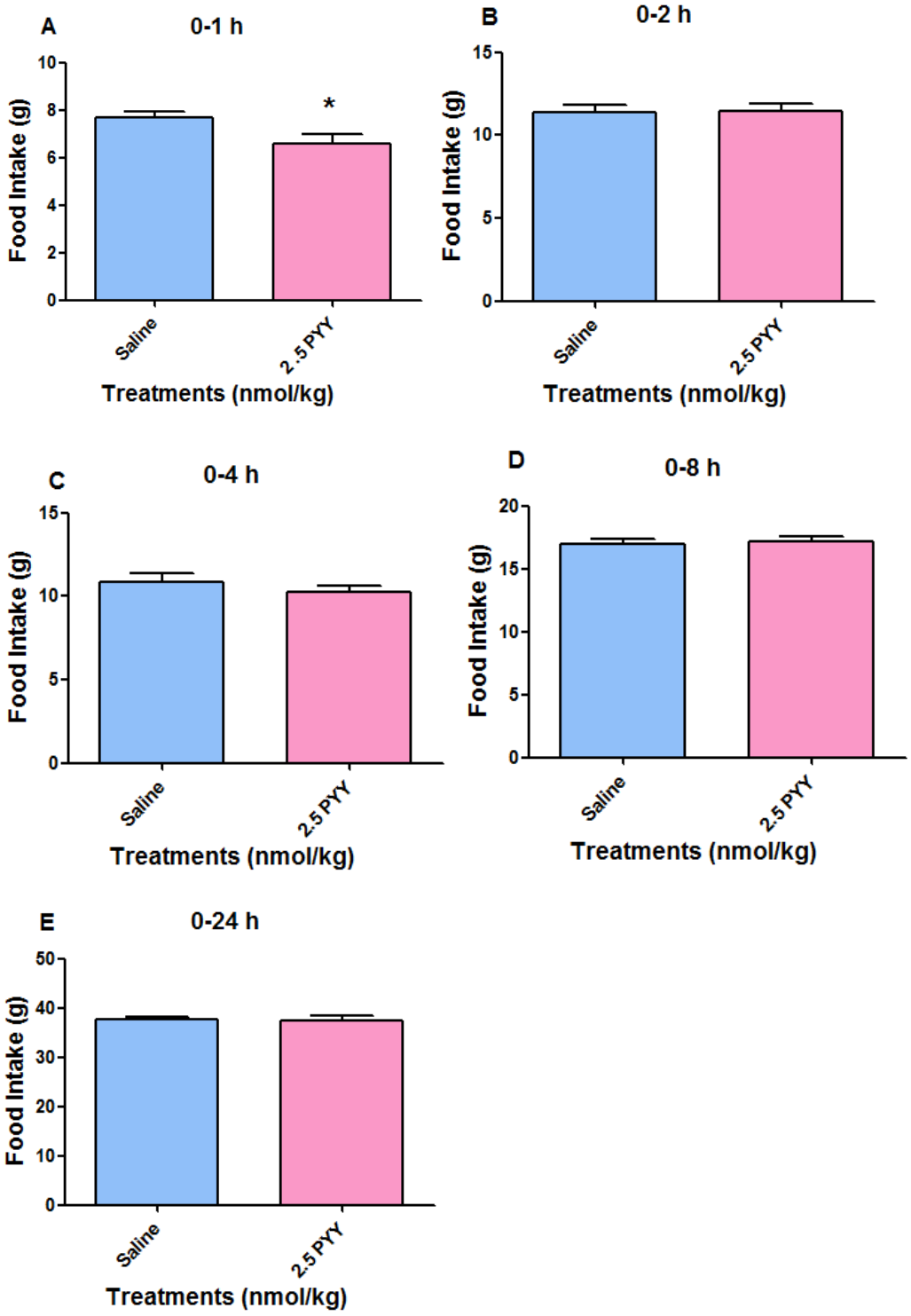


Figure 3.3. The effect of IP administration of 2.5 nmol/kg PYY on food intake in fasted rats during the early light phase.

The effect of intraperitoneal administration of saline or 2.5 nmol/kg PYY on food intake in overnight fasted rats during the early light phase at 0-1 (A), 0-2 (B), 0-4 (C), 0-8 (D) and 0-24 (E) hours post administration. Data presented as mean ± SEM. n= 19 per group. *$p<0.05$, using an unpaired t-test.

3.4.4 The effect of peripheral administration of 1.5 nmol/kg PYY on food intake in fasted rats.

IP administration of 1.5 nmol/kg PYY significantly reduced food intake at 0-1h ($p<0.05$; Fig 3.4A).

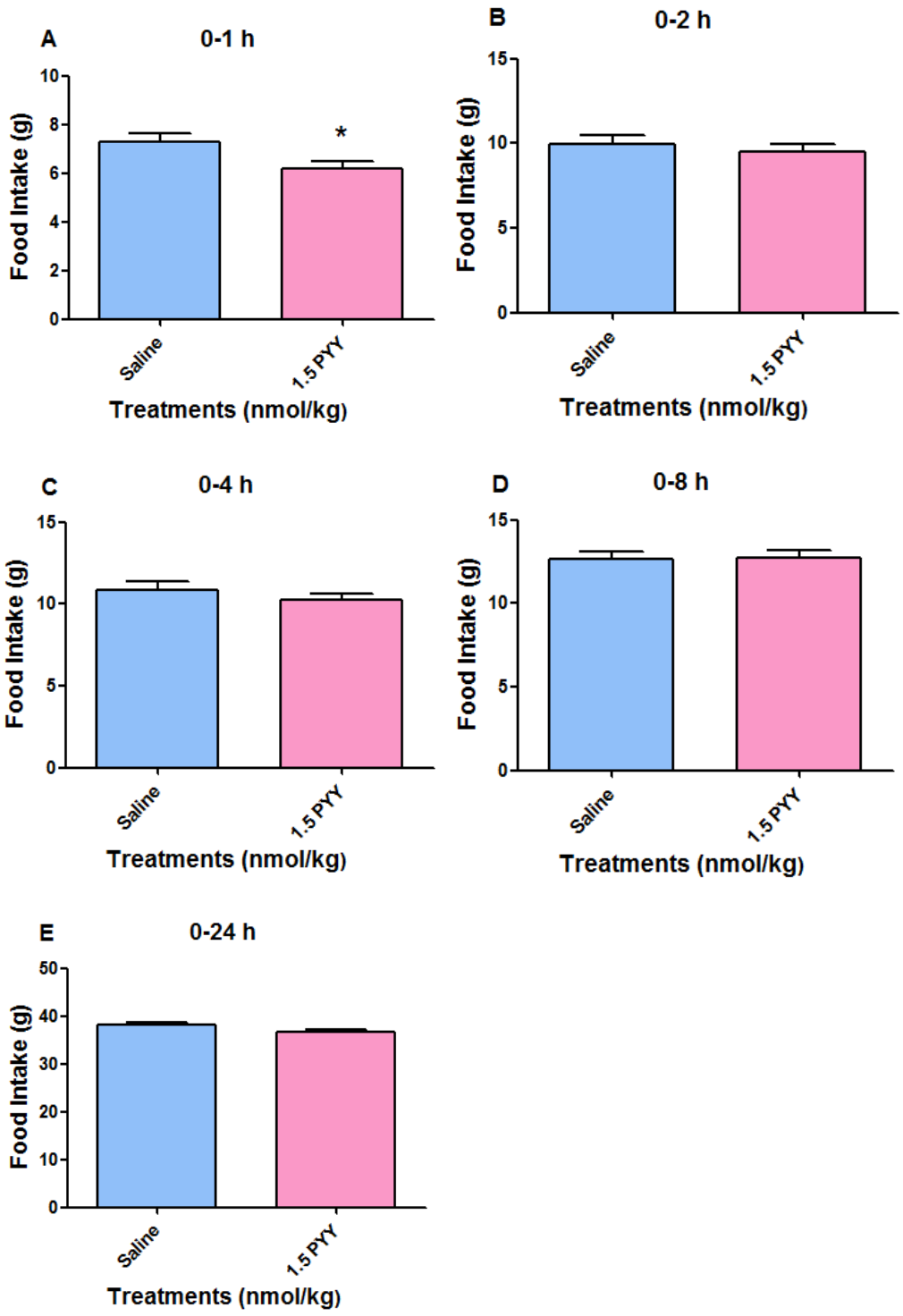


Figure 3.4. The effect of IP administration of 1.5 nmol/kg PYY on food intake in fasted rats during the early light phase.

The effect of intraperitoneal administration of saline or 1.5 nmol/kg PYY on food intake in overnight fasted rats during the early light phase at 0-1 (A), 0-2 (B), 0-4 (C), 0-8 (D) 0-24 (E) hours post administration. Data presented as mean ± SEM. N = 19-20 per group. *$p<0.05$, using an unpaired t-test.

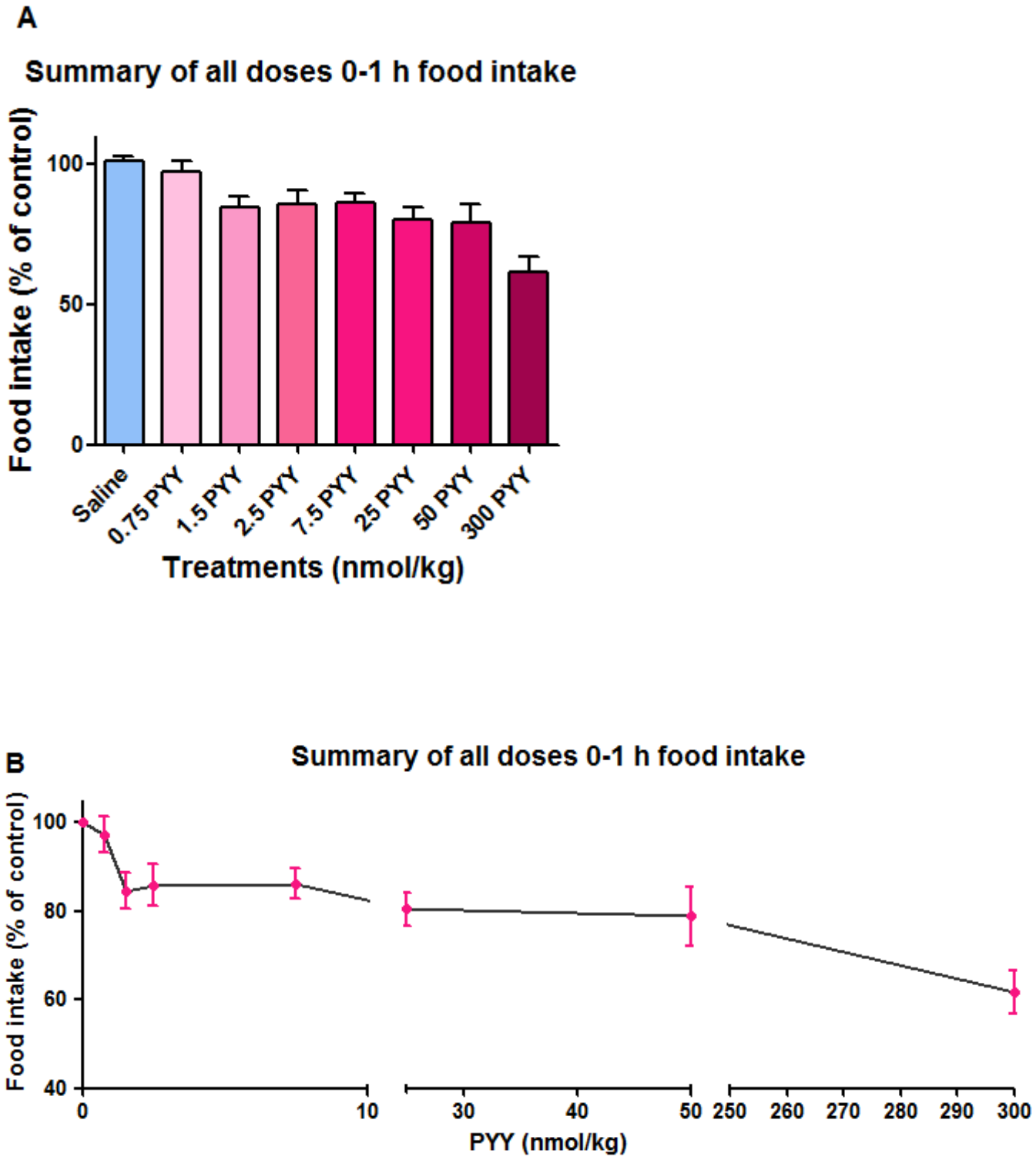


Figure 3.5. Summary of the effects of IP administration of 0.75, 1.5, 2.5, 7.5, 25, 50 and 300 nmol/kg PYY at 0-1h on food intake in fasted rats during the early light phase.

A & B The effect of intraperitoneal administration of saline or 0.75, 1.5, 2.5, 7.5, 25, 50 and 300 nmol/kg PYY at 0-1h, presented as % of control. N = 90 (saline), 19 (1.5, 2.5 nmol/kg), 38 (0.75, 7.5, 25 nmol/kg PYY), 12-13 (50, 300 nmol/kg PYY).

3.4.5 The effect of IP administration of PYY on plasma PYY levels in rats

An RIA was performed to assess the circulating levels of PYY following peripheral administration in comparison to physiological levels. Circulating PYY was significantly higher in rats that had been fed for 30 minutes compared to fasted controls ($p<0.01$) and rats fed for 20 minutes ($p<0.05$). IP administration of 1.5 nmol/kg PYY significantly elevated plasma PYY levels in rats at 10 minutes following administration compared to fed (blood collected at 20 min, $p<0.001$), fasted (blood collected at 20 min, $p<0.001$), fed (30 min, $p<0.001$), fasted (30 min, $p<0.001$), saline (10 min, $p<0.001$) and saline (20 min, $p<0.001$). Similarly, doses of 2.5 and 7.5 nmol/kg PYY significantly increased plasma PYY levels at 10 and 20 minutes following administration compared to all controls ($p<0.001$; Fig 3.6).

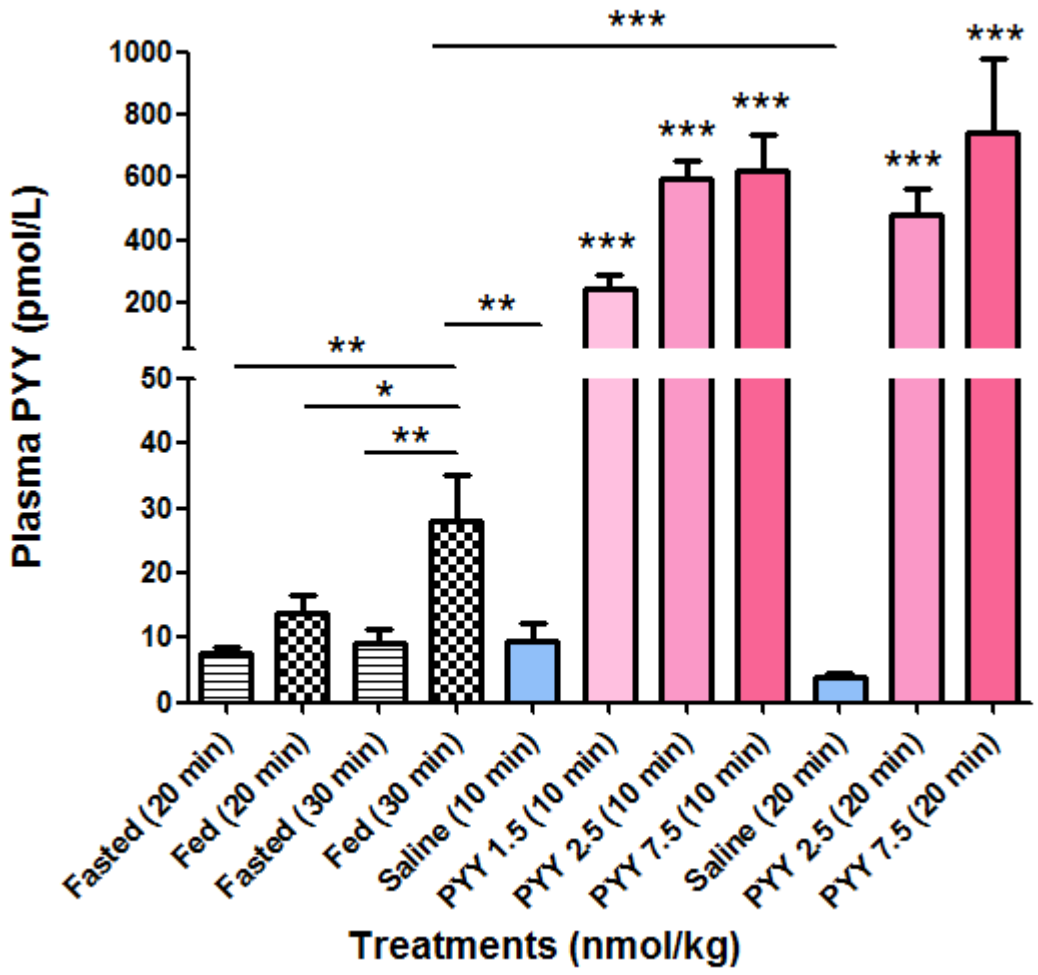


Figure 3.6 The effect of IP administration of PYY on circulating PYY in fasted rats in the early light phase.

The effect of IP administration of saline, 1.5, 2.5 or 7.5 mmol/kg PYY on circulating PYY in overnight fasted male rats at 10 and 20 minutes following administration. Data presented as mean ± SEM. N = 8-12. *$p<0.05$, **$p<0.01$, ***$p<0.001$, using 1-way ANOVA. Pink bars significant vs. fed (20 min), fasted (20 min), fed (30 min), fasted (30 min), saline (10 min) and saline (20 min) controls ($p<0.001$).

3.4.6 The effect of peripheral PYY administration on food intake and energy expenditure in *ad libitum* fed rats

To investigate the effect of PYY on food intake and energy expenditure, rats were placed in CLAMS metabolic cages as described in section 3.3.7. A dose of 300 nmol/kg PYY significantly reduced cumulative 12h and 18h food intake vs. saline control by 7.96±2.80% and 6.45±2.14%, respectively ($p<0.05$; Fig 3.7), and 32 mg/kg LiCl significantly reduced 18h cumulative food intake by 8.34±2.26%. IP administration of 32 mg/kg LiCl significantly reduced cumulative food intake vs. saline control in the first 40 minutes by 11.54±12.50% ($p<0.05$; Fig 3.8). Investigation of the BSS in rodents has previously shown that increased latency to feeding and decreased food consumption in the first and second feeding bouts corresponds to disruption of the normal behavioural sequence, and thus indicates visceral illness induced by administration of a substance (Spudeit, 2014, Provensi et al., 2014, Rodgers et al., 2010). Administration of 32 mg/kg LiCl significantly increased the latency to first feeding bout vs. saline, 1.5 nmol/kg PYY and 7.5 nmol/kg PYY ($p<0.001$, $p<0.05$, $p<0.01$, respectively; Fig 3.9E). There was also a trend for decreased food intake in the first feeding and second bouts following administration of 300 nmol/kg PYY and 32 mg/kg LiCl (Fig 3.9 A&C).

IP PYY had no significant effect on oxygen consumption (VO_2; Fig 3.10) or carbon dioxide production (VCO_2; Fig 3.11) in rats during the 18 hours following administration. RER was significantly reduced 2h following administration of 32 mg/kg LiCl vs. saline and 1.5 nmol/kg PYY ($p<0.05$; Fig 3.12). Activity was not significantly altered following administration of PYY at any dose (Figs 3.13 and 3.14).

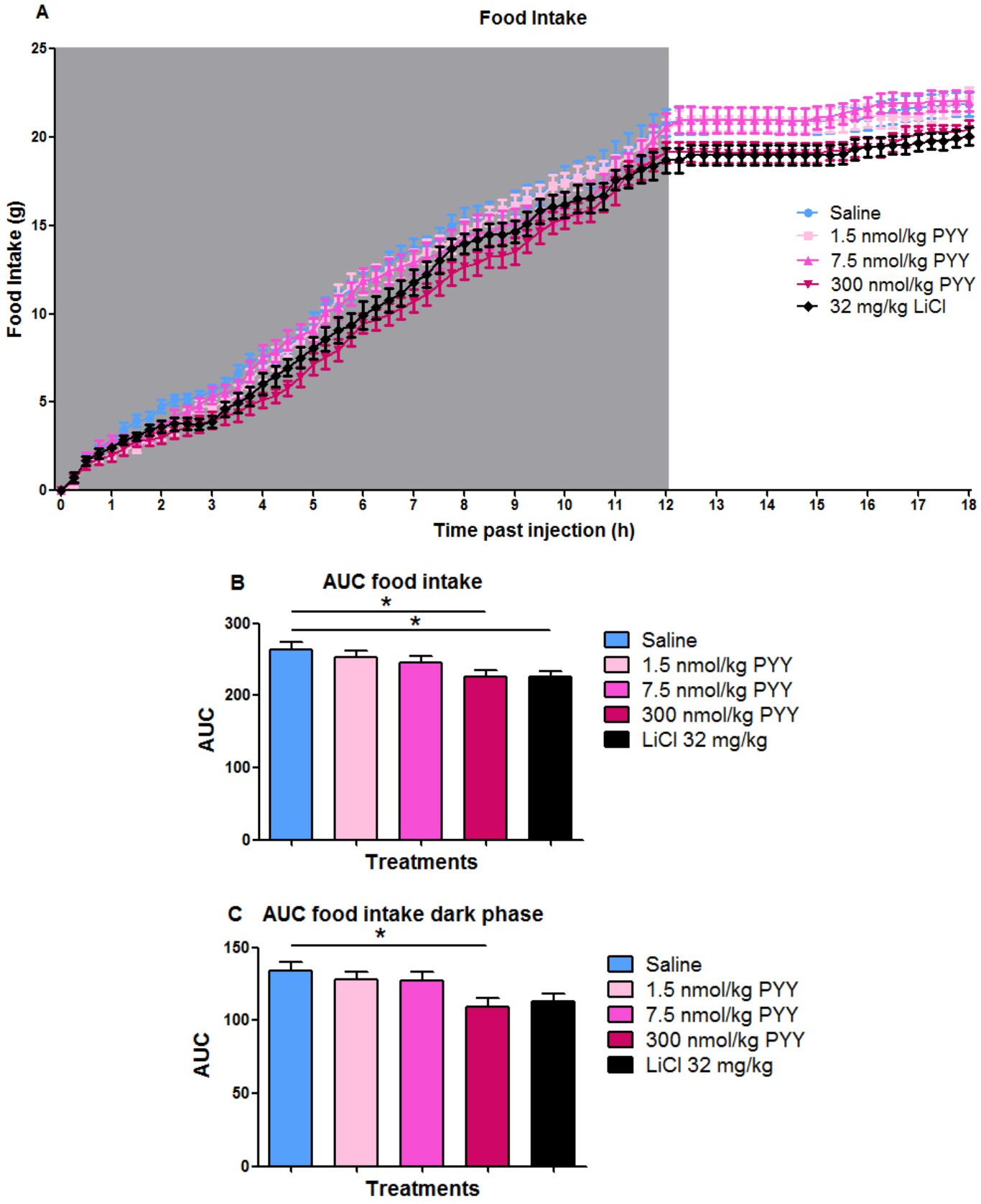


Figure 3.7. The effect of peripheral PYY administration on food intake in fed rats in the early dark phase.

A The effect of saline and 1.5, 7.5, 300 nmol/kg PYY or 32 mg/kg LiCl on 18h cumulative food intake in *ad libitum* fed rats injected in the early dark phase; food intake recorded every 12 minutes for 18 hours post injection. B Area under the curve for graph A. C Area under the curve for 12h cumulative food intake (dark phase only). N=12 per group. Data presented as mean ± SEM. *p<0.05, using 1-way ANOVA.

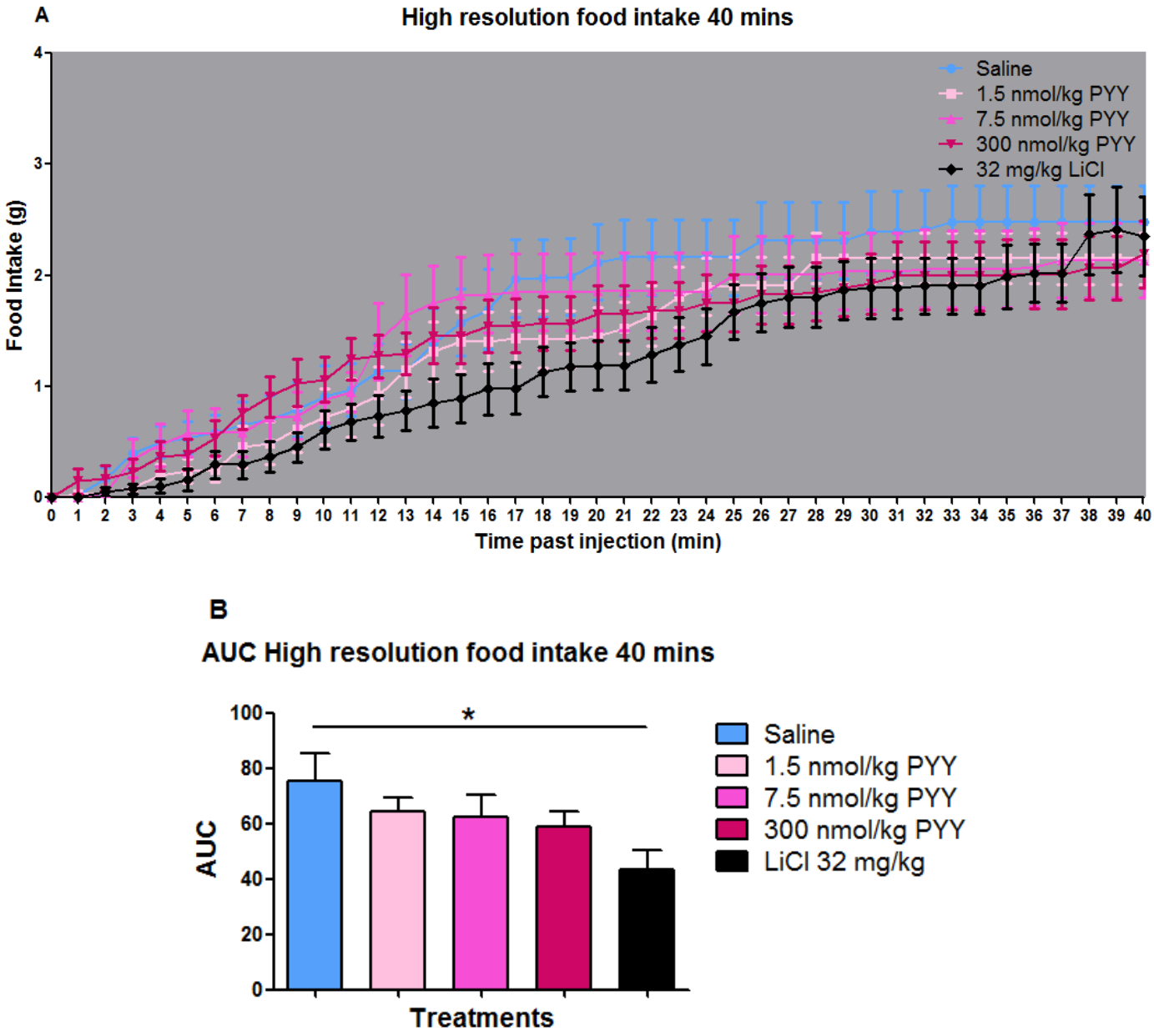


Figure 3.8. The effect of peripheral PYY administration on food intake in fed rats in the early dark phase.

A The effect of saline and 1.5, 7.5 and 300 nmol/kg PYY or 32 mg/kg LiCl on 0-40 minute cumulative food intake in *ad libitum* fed rats injected in the early dark phase; high resolution food intake recorded every minute for 40 mins. B Area under the curve for graph A. N=12 per group. Data presented as mean ± SEM. *p<0.05, using 1-way ANOVA.

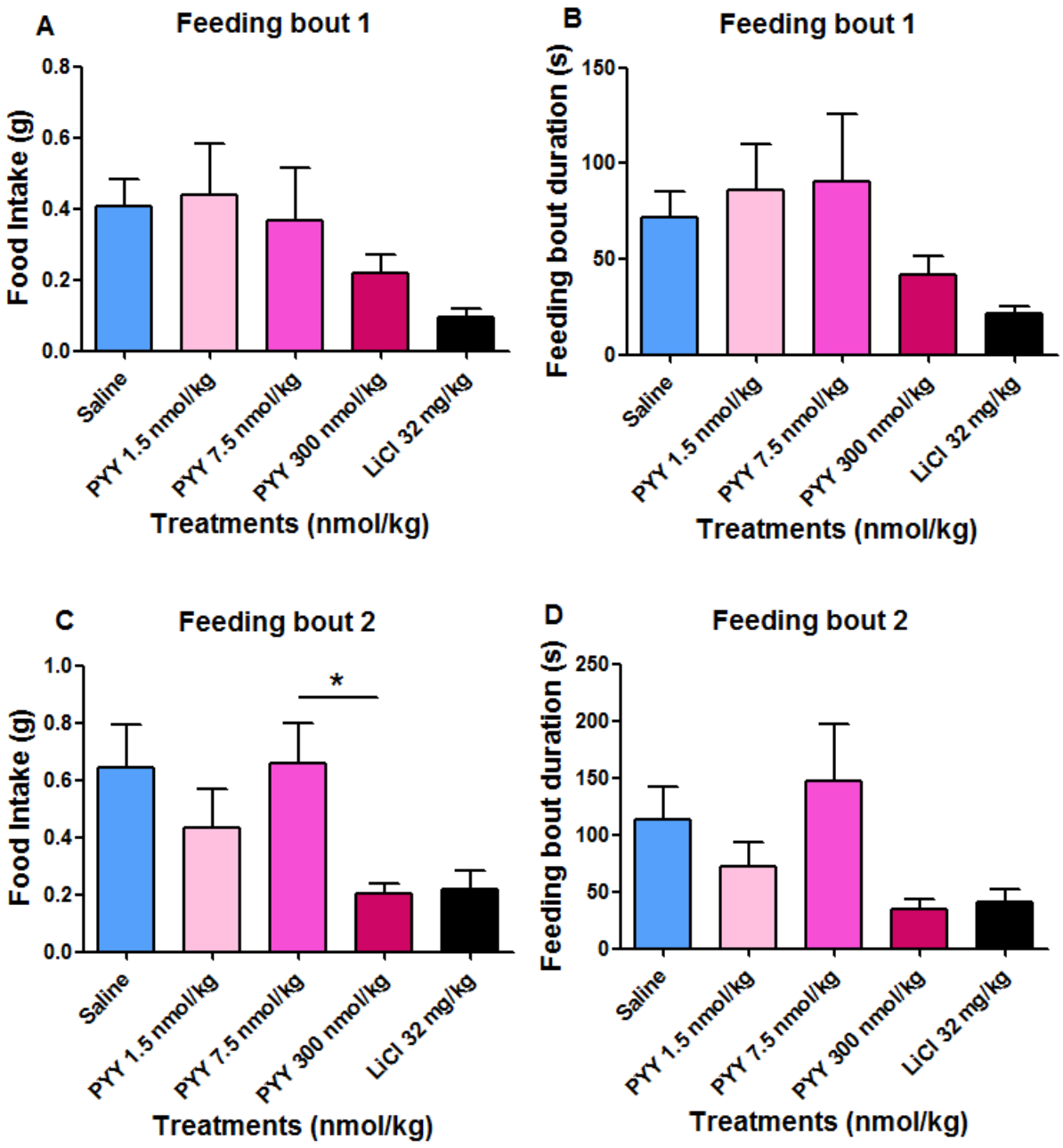


Figure 3.9. The effect of peripheral PYY administration on food intake in first and second feeding bouts in the early dark phase.

The effect of saline and 1.5, 7.5, 300 nmol/kg PYY or 32 mg/kg LiCl on food intake in first (A) and second (C) feeding bouts, duration of first (B) and second (D) feeding bouts and latency to feeding (E). Data presented as mean ± SEM. N= 10-12 per group. *p<0.05, **p<0.01, ***p<0.001, using 1-way ANOVA.

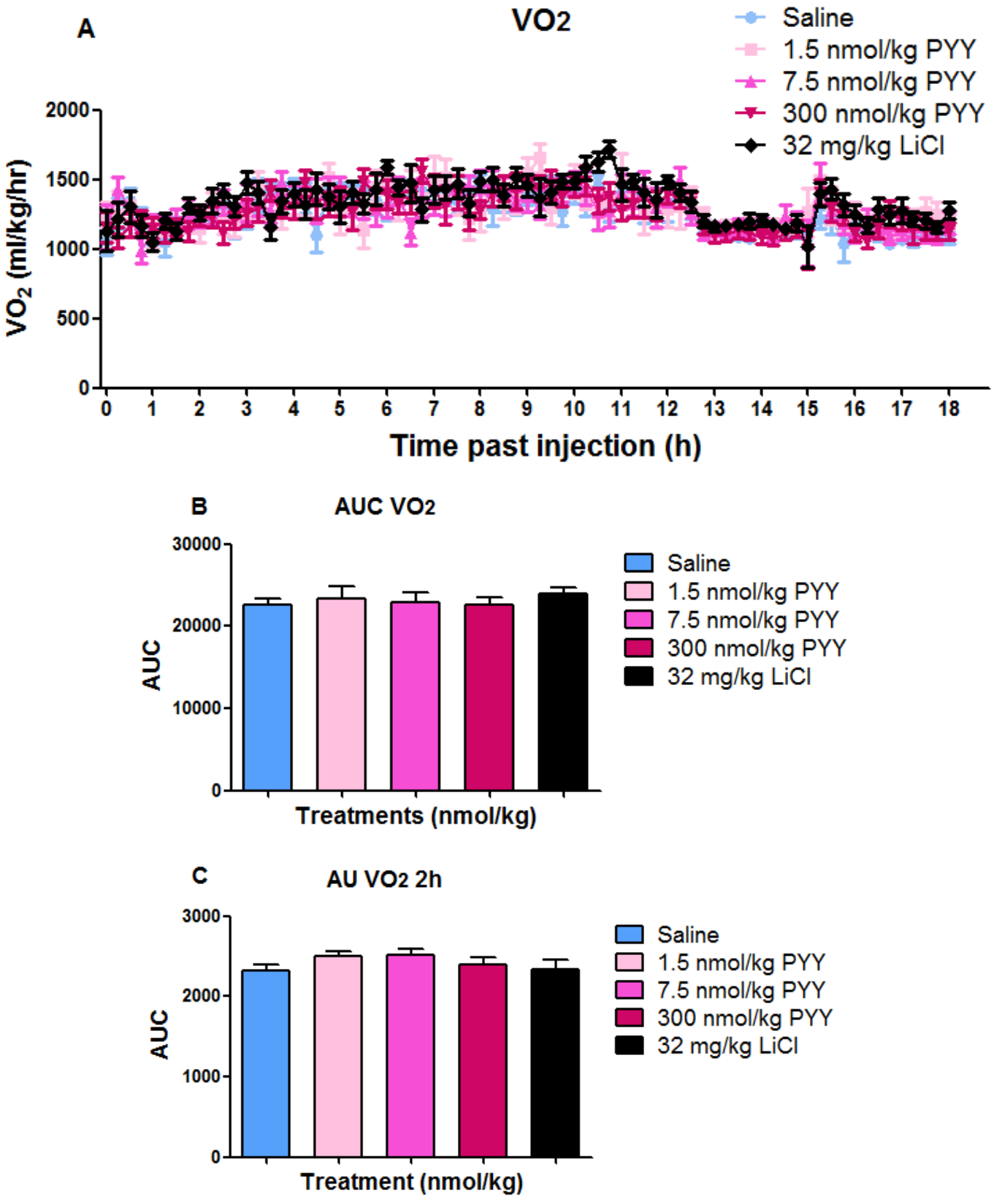


Figure 3.10. The effect of peripheral PYY administration on energy expenditure in fed rats in the early dark phase.

A The effect of saline or 1.5, 7.5, 300 nmol/kg PYY or 32 mg/kg LiCl on VO_2 in *ad libitum* fed rats in the early dark phase. B Area under the curve for A. C Area under the curve for A; 0-2h. N=10 per group. Data presented as mean ± SEM.

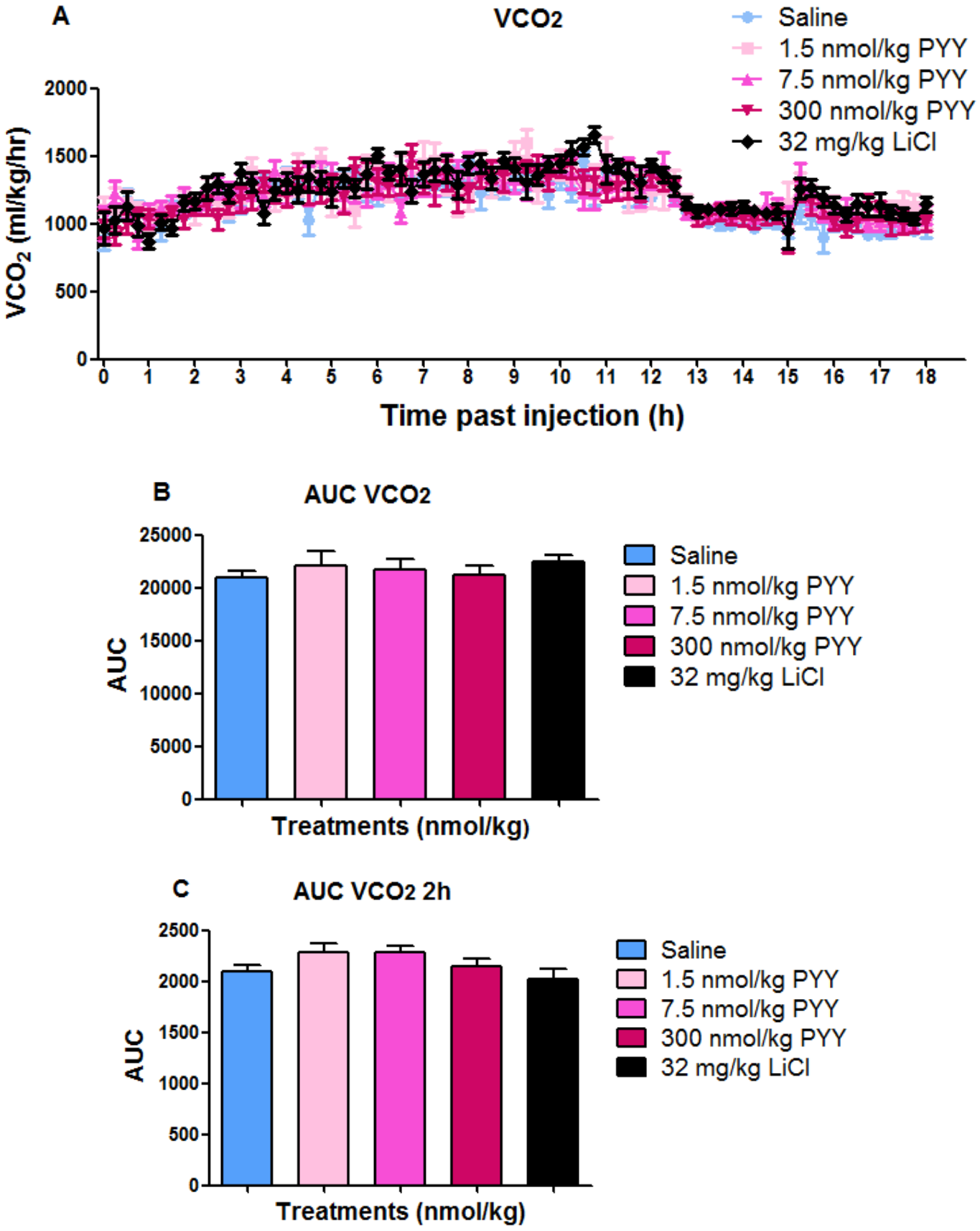


Figure 3.11. The effect of peripheral PYY administration on energy expenditure in fed rats in the early dark phase.

A The effect of saline and 1.5, 7.5, 300 nmol/kg PYY or 32 mg/kg LiCl on VCO_2 in *ad libitum* fed rats in the early dark phase. B Area under the curve for A. C Area under the curve for A; 0-2h. N=10 per group. Data presented as mean ± SEM.

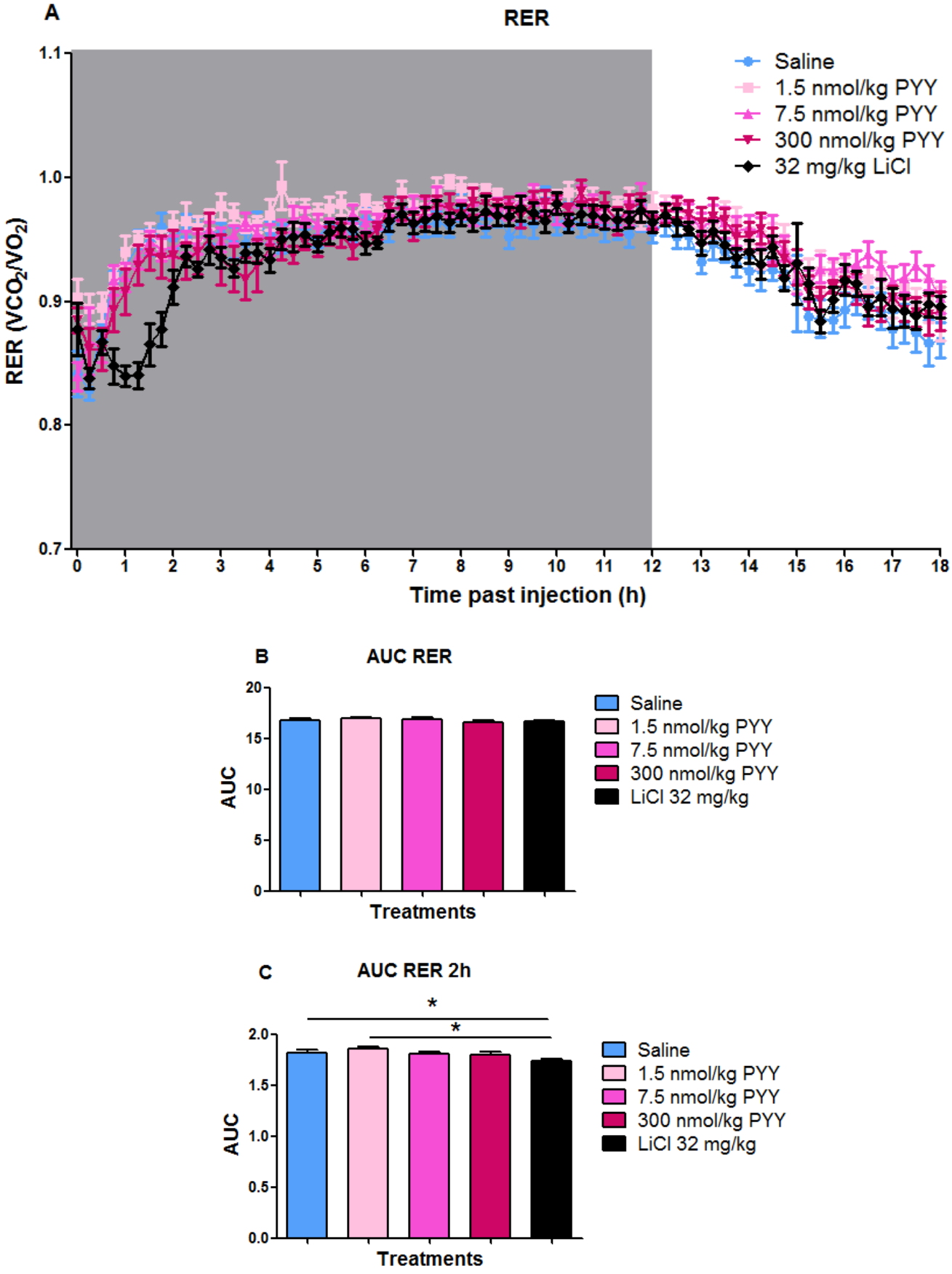
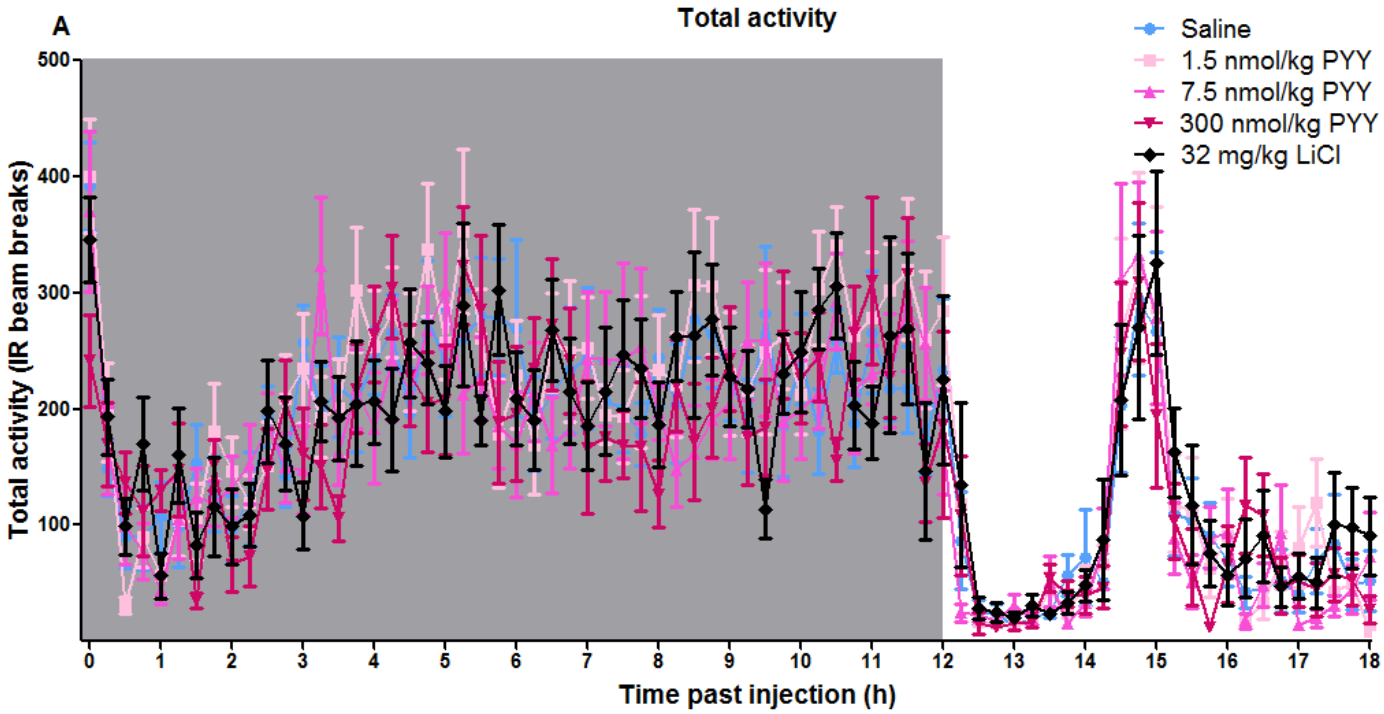


Figure 3.12. The effect of peripheral PYY administration on energy expenditure in fed rats in the early dark phase.

A The effect of saline and 1.5, 7.5, 300 nmol/kg PYY or 32 mg/kg LiCl on RER in *ad libitum* fed rats in the early dark phase. B Area under the curve for A. C Area under the curve for A; 0-2h. N=11 per group. Data presented as mean ± SEM. *p<0.05, using 1-way ANOVA.



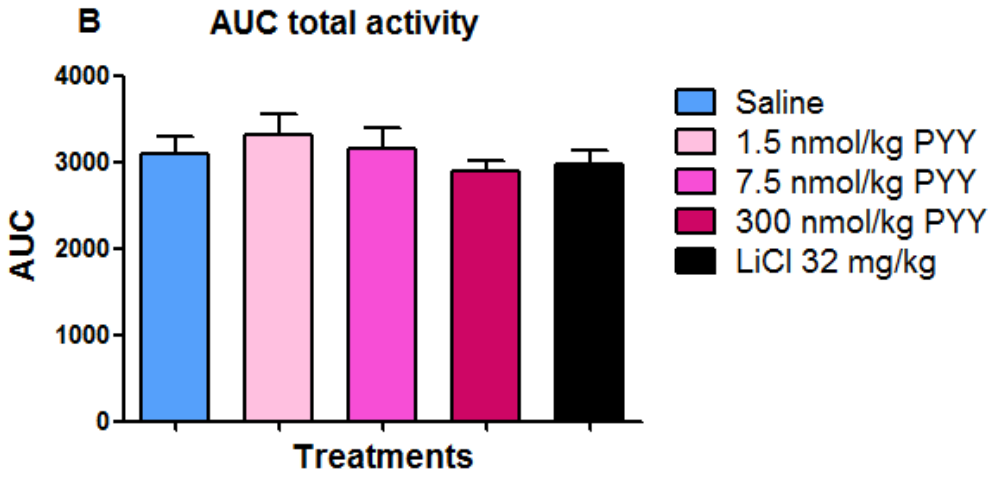


Figure 3.13. The effect of peripheral PYY administration on activity in fed rats in the dark phase.

A The effect of saline and 1.5, 7.5, 300 nmol/kg PYY or 32 mg/kg LiCl on total activity in *ad libitum* fed rats injected in the early dark phase. Infra-red beam breaks recorded every 12 minutes for 18 hours. B Area under the curve for A. N=12 per group. Data presented as mean ± SEM.

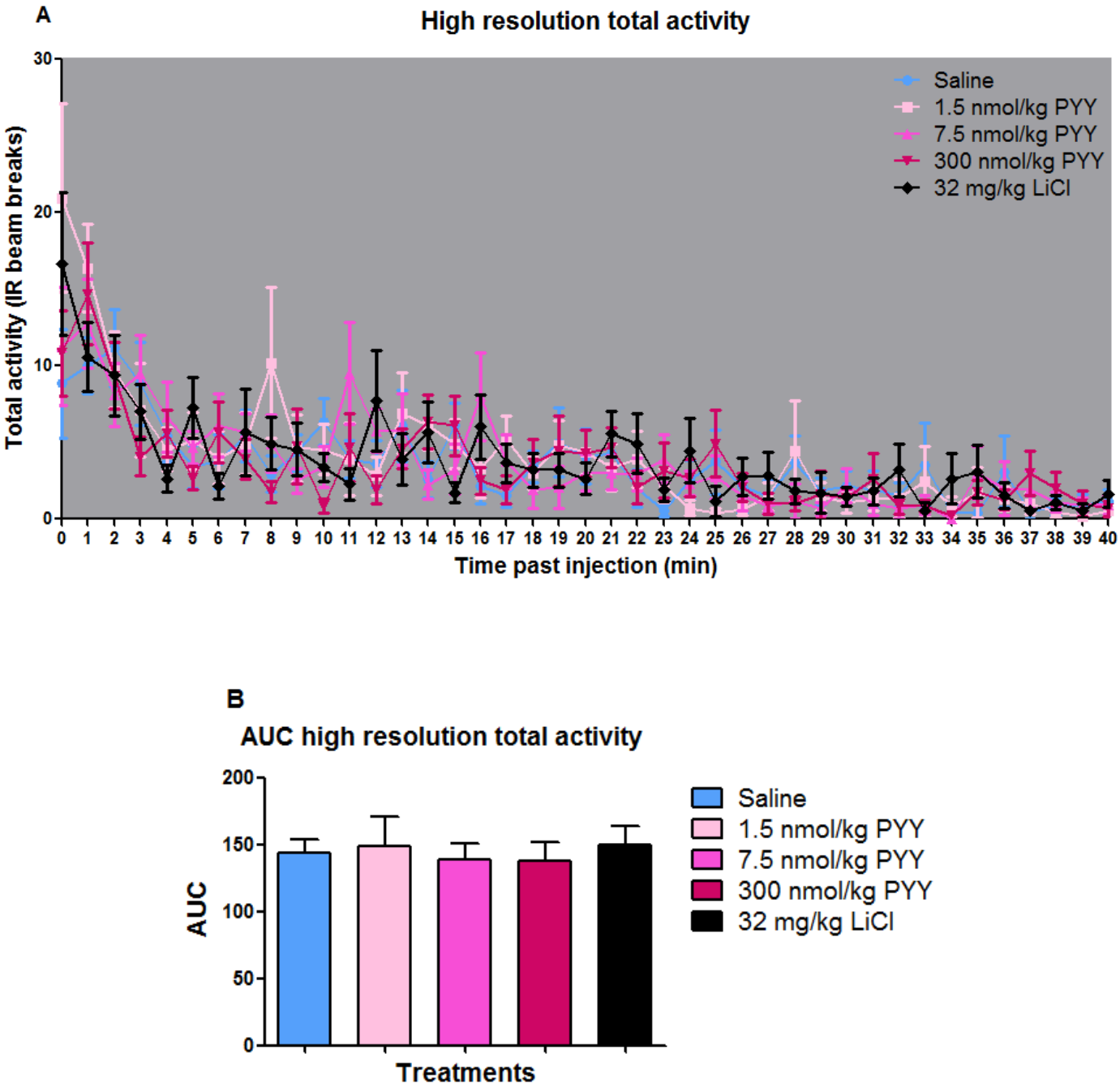


Figure 3.14. The effect of peripheral PYY administration on activity in fed rats in the dark phase.

A The effect of saline and 1.5, 7.5, 300 nmol/kg PYY or 32 mg/kg LiCl on total activity in *ad libitum* fed rats injected in the early dark phase. High resolution data; infra-red beam breaks recorded every 10 seconds, presented as mean per minute. B Area under the curve for A. N=12 per group. Data presented as mean ± SEM.

3.4.7 The effect of peripheral administration of PYY on cardiovascular parameters and body temperature in *ad libitum* fed rats

To investigate the effect of PYY on HR, BP and body temperature, rats were implanted with radio-telemetry devices as described in section 3.3.9. HR was significantly decreased 1h following administration of LiCl vs. saline, 7.5 nmol/kg PYY and 300 nmol/kg PYY ($p<0.05$; Fig 3.15). There was a trend for decreased HR following IP administration of 32 mg/kg LiCl in the 5 hours following injection.

Administration of 32 mg/kg LiCl significantly increased systolic BP 1h after administration vs. saline and 1.5 nmol/kg PYY ($p<0.05$), and significantly increased systolic BP vs. saline in the 5h following administration ($p<0.05$; Fig 3.16).

Diastolic BP was significantly increased by 32 mg/kg LiCl vs. saline, 1.5 and 7.5 nmol/kg PYY in the first hour ($p<0.01$, $p<0.05$, $p<0.01$, respectively). A dose of 300 nmol/kg PYY significantly increased diastolic BP vs. saline, 1.5 and 7.5 nmol/kg PYY ($p<0.05$, $p<0.05$ and $p<0.01$). There was a trend for increased diastolic BP following administration of 300 nmol/kg PYY and 32 mg/kg LiCl (Fig 3.17) 5h post injection. Body temperature was not significantly affected, though it tended to be lower in the group administered 32 mg/kg LiCl 2h following administration (Fig 3.18).

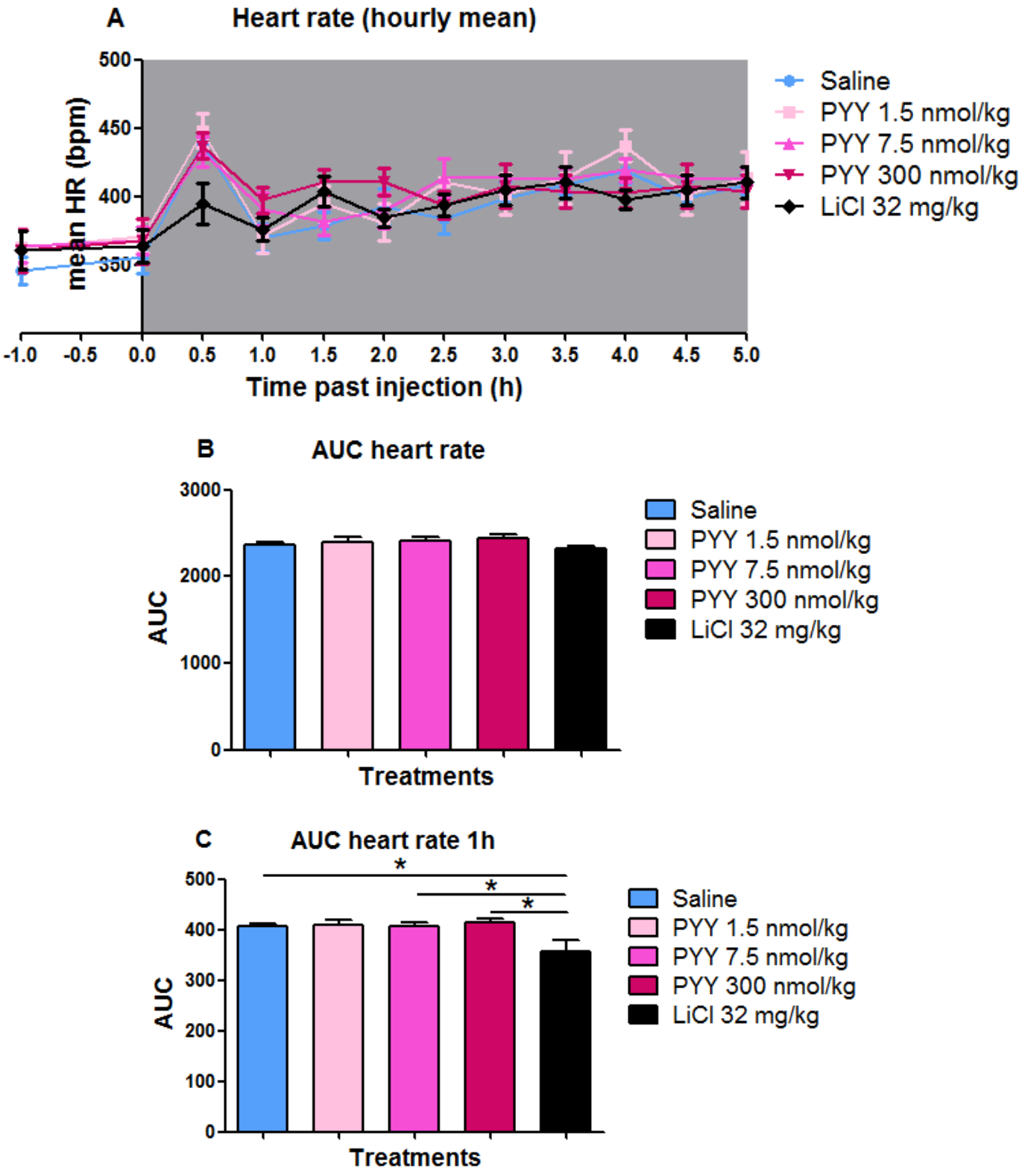


Figure 3.15. The effect of peripheral PYY administration on heart rate in rats.

A The effect of saline and 1.5, 7.5, 300 nmol/kg PYY or 32 mg/kg LiCl on heart rate in freely moving rats injected in the early dark phase. **B** Area under the curve for A. **C** Area under the curve for A (0-1h). N=8-10 per group. Data presented as mean ± SEM. *p<0.05, using 1-way ANOVA.

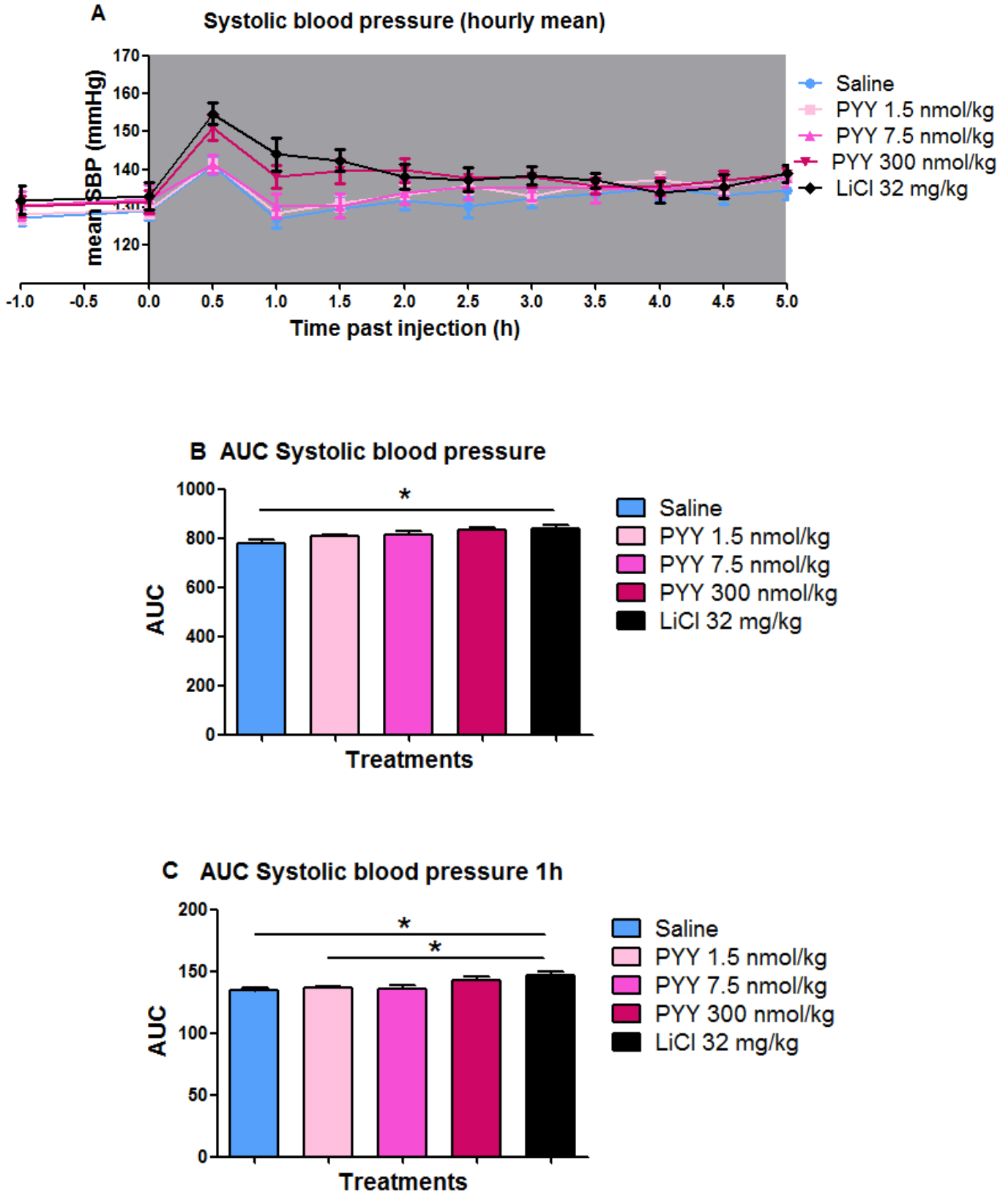


Figure 3.16. The effect of peripheral PYY administration on systolic blood pressure in rats.

A The effect of saline and1.5, 7.5, 300 nmol/kg PYY or 32 mg/kg LiCl on systolic blood pressure in freely moving rats injected in the early dark phase. **B** Area under the curve for A. **C** Area under the curve for A (0-1h). N=9 per group. Data presented as mean ± SEM. *p<0.05, using 1-way ANOVA.

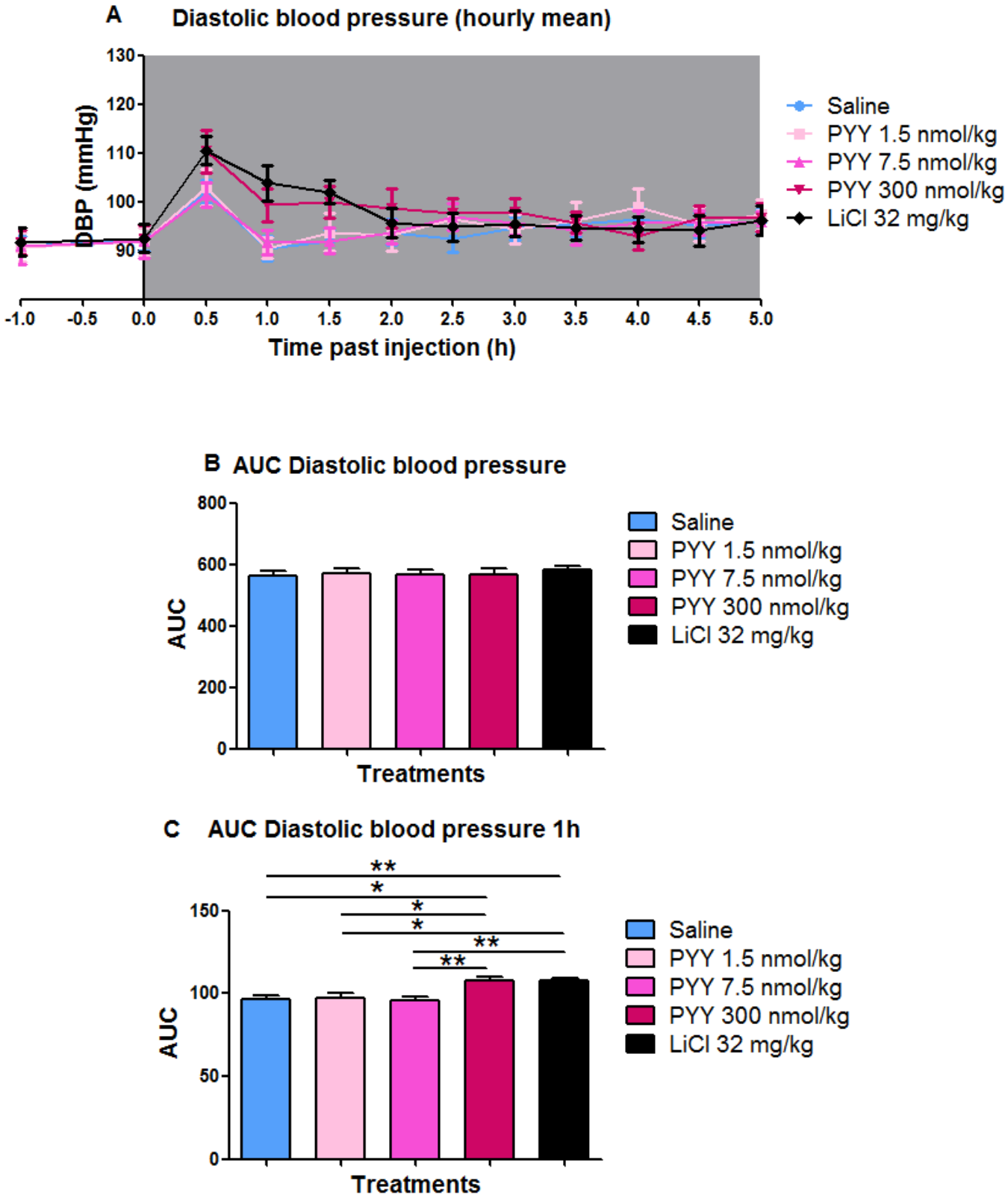


Figure 3.17. The effect of peripheral PYY administration on diastolic blood pressure in rats.

A The effect of saline and 1.5, 7.5, 300 nmol/kg PYY or 32 mg/kg LiCl on diastolic blood pressure in freely moving rats injected in the early dark phase. **B** Area under the curve for A. **C** Area under the curve for A (0-1h). N=9 per group. Data presented as mean ± SEM. *p<0.05, **p<0.01, using 1-way ANOVA.

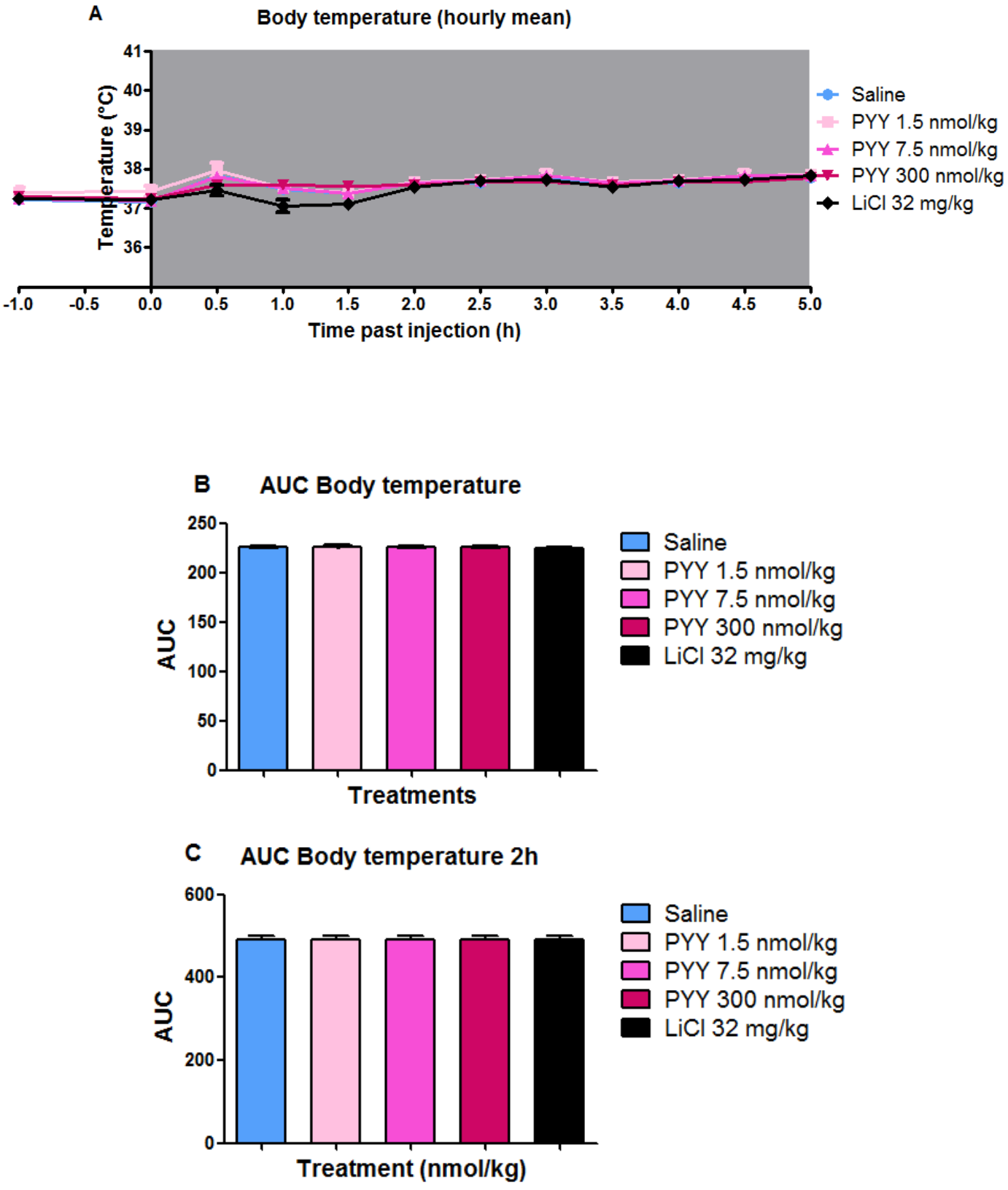


Figure 3.18. The effect of peripheral PYY administration on body temperature in rats.

A The effect of saline and 1.5, 7.5, 300 nmol/kg PYY or 32 mg/kg LiCl on body temperature in freely moving rats injected in the early dark phase. **B** Area under the curve for A. **C** Area under the curve for A (0-2h). N=12 per group. Data presented as mean ± SEM.

3.4.8 The effect of peripheral administration of PYY on conditioned taste aversion in rats

Rats were subjected to a one-bottle CTA protocol as described in section 3.3.10. When administered peripherally, doses of 2.5 and 7.5 nmol/kg PYY produced a significant CTA vs. saline control ($p<0.05$; Fig 3.19A). Administration of 1.5 nmol/kg PYY did not result in significant CTA (Fig 3.19B). LiCl induced a significant taste aversion at both 48 and127 mg/kg ($p<0.001$).

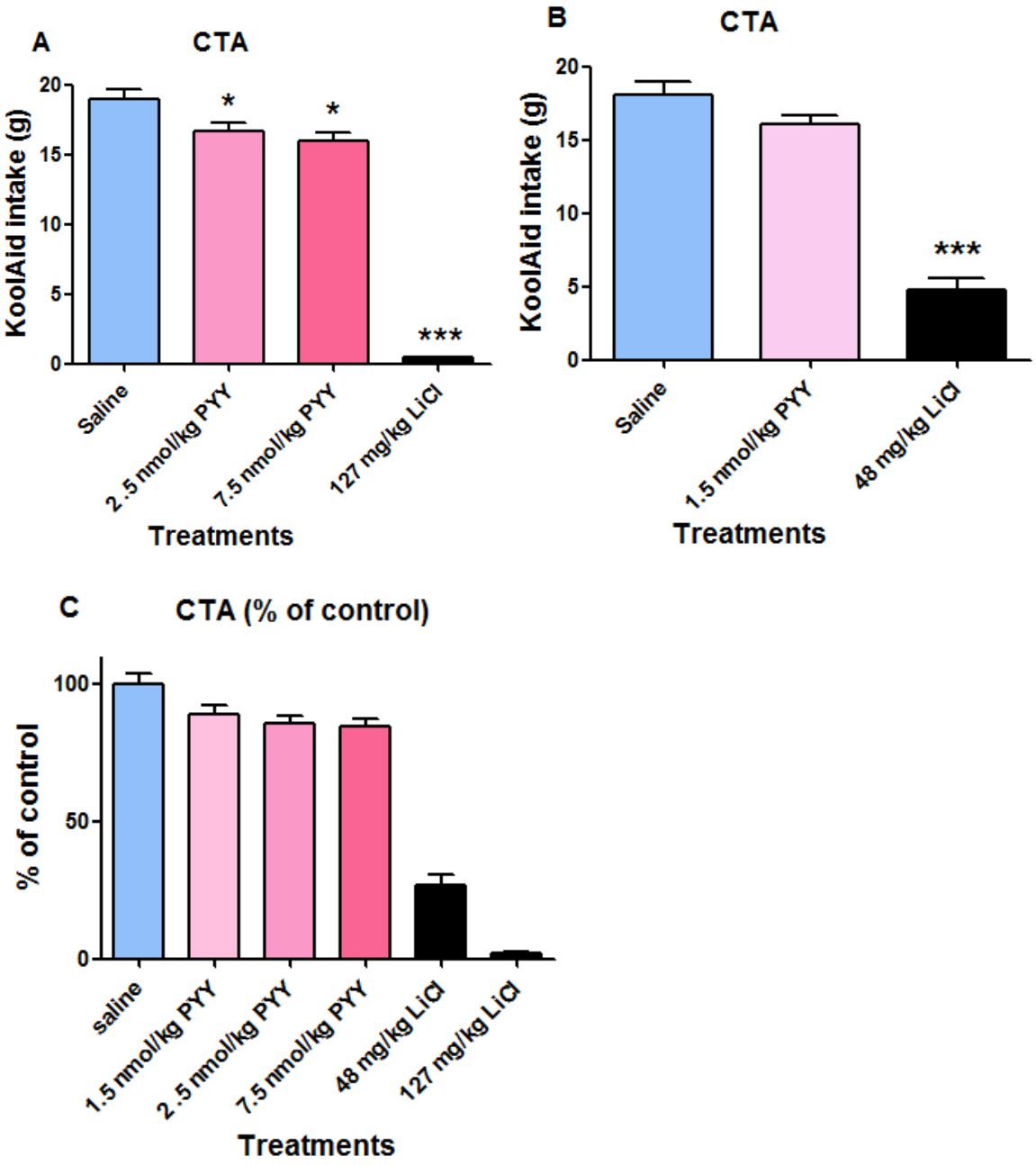


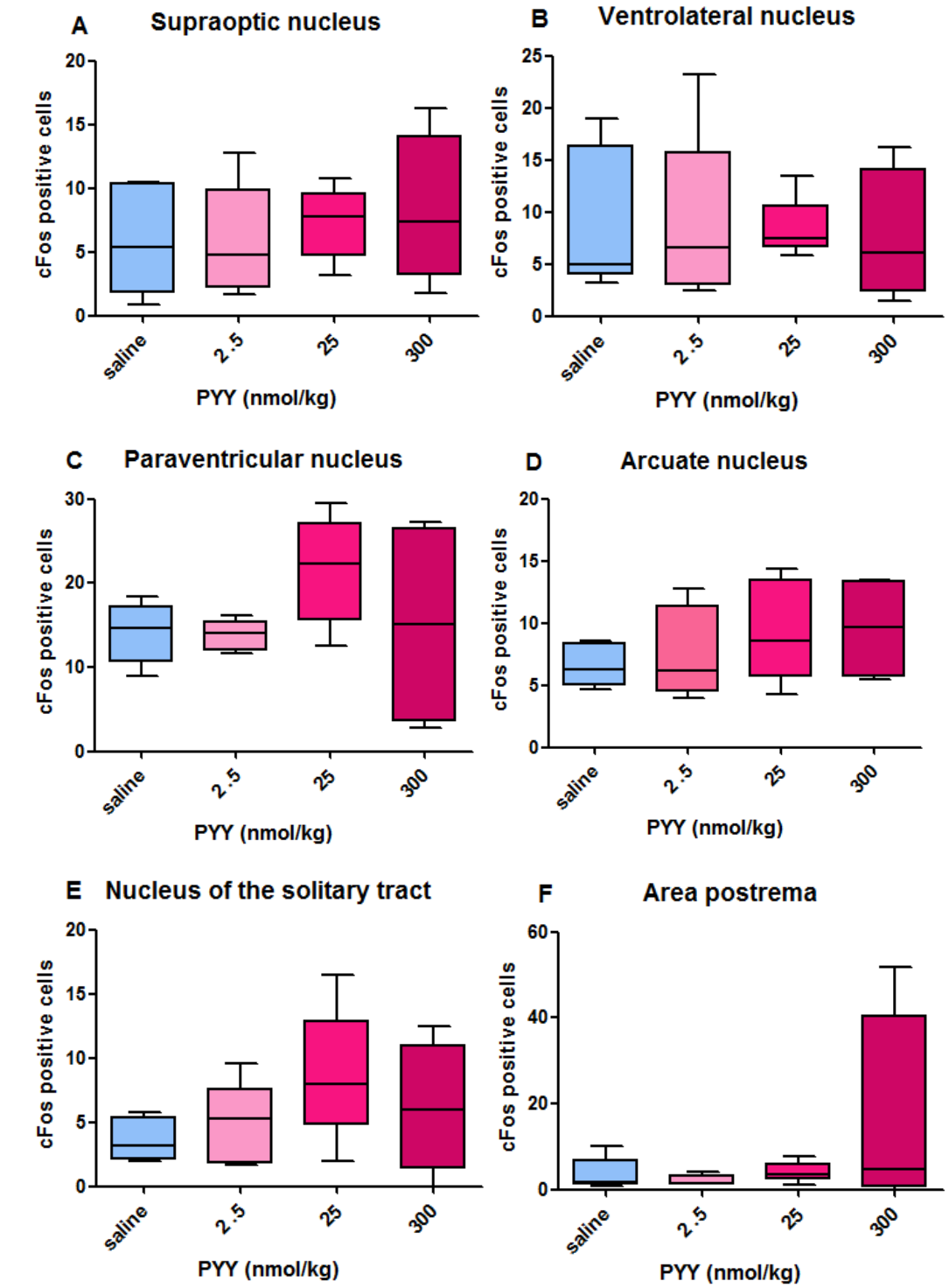
Figure 3.19. The effect of peripheral PYY administration on aversion in rats.

A The effect of saline, 127 mg/kg LiCl or 2.5 and 7.5 nmol/kg PYY on conditioned taste aversion in rats. **B** The effect of saline, 48 mg/kg LiCl or 1.5 nmol/kg PYY on conditioned taste aversion in rats. **C** A summary of the effects of 1.5, 2.5, 7.5 nmol/kg PYY and 48 and 127 mg/kg LiCl on conditioned taste aversion in rats, expressed as % of control. Data presented as mean ± SEM. *$p<0.05$, ***$p<0.001$ vs. saline control, using 1-way ANOVA. N= 14-15 per saline and all doses of PYY groups, 127 mg/kg LiCl n=5, 48 mg/kg LiCl n=13.

3.4.9 The effect of peripheral administration of PYY on c-Fos-like immunoreactivity in the hypothalamus and brainstem of fasted rats

To assess neuronal activation in regions with roles in energy homeostasis, PYY or saline were administered to rats and c-Fos like immunoreactivity was counted. Rats were transcardially perfused 90 minutes after administration as described in section 3.3.12, and brain sections stained as described in 3.3.13.

Peripheral administration of 2.5, 25 or 300 nmol/kg PYY did not significantly increase c-Fos-like immunoreactivity in any hypothalamic or brainstem nuclei investigated, though there was a trend for increased neuronal activation in the AP following administration of 300 nmol/kg PYY (Fig 3.20F).



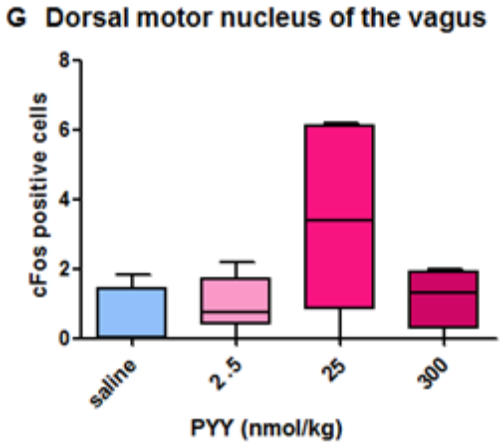


Figure 3.20. The effect of peripheral PYY administration on neuronal activation in the hypothalamic and brainstem nuclei of fasted rats.

The effect of saline and 2.5, 25, 300 nmol/kg PYY on c-Fos like immunoreactivity in the (**A**) supraoptic nucleus (**B**) ventrolateral nucleus (**C**) paraventricular nucleus (**D**) arcuate nucleus (**E**) nucleus of the solitary tract (**F**) area postrema and (**G**) dorsal motor nucleus of the vagus. Data presented as median, inter-quartile and total range. N=4-6.

3.4.10 The effect of peripheral administration of PYY on c-Fos-like immunoreactivity in nuclei of the mesocorticolimbic system in fasted rats

To assess neuronal activation in regions with roles in reward and aversion, saline or PYY were administered to rats and c-Fos like immunoreactivity was counted. Rats were transcardially perfused 90 minutes after administration as described in section 3.3.12, and brain sections stained as described in 3.3.13.

Peripheral administration of 300 nmol/kg PYY significantly increased c-Fos-like immunoreactivity in the PrL compared to saline and 2.5 nmol/kg PYY ($p<0.05$ and $p<0.01$, respectively; Fig 3.21D). c-Fos-like immunoreactivity was significantly increased in the NAcbSh by 300 nmol/kg PYY vs. saline ($p<0.05$; Fig 3.21F). There was a trend for increased c-Fos like immunoreactivity in the VTA, CeA, MO and GI with increasing doses of PYY.

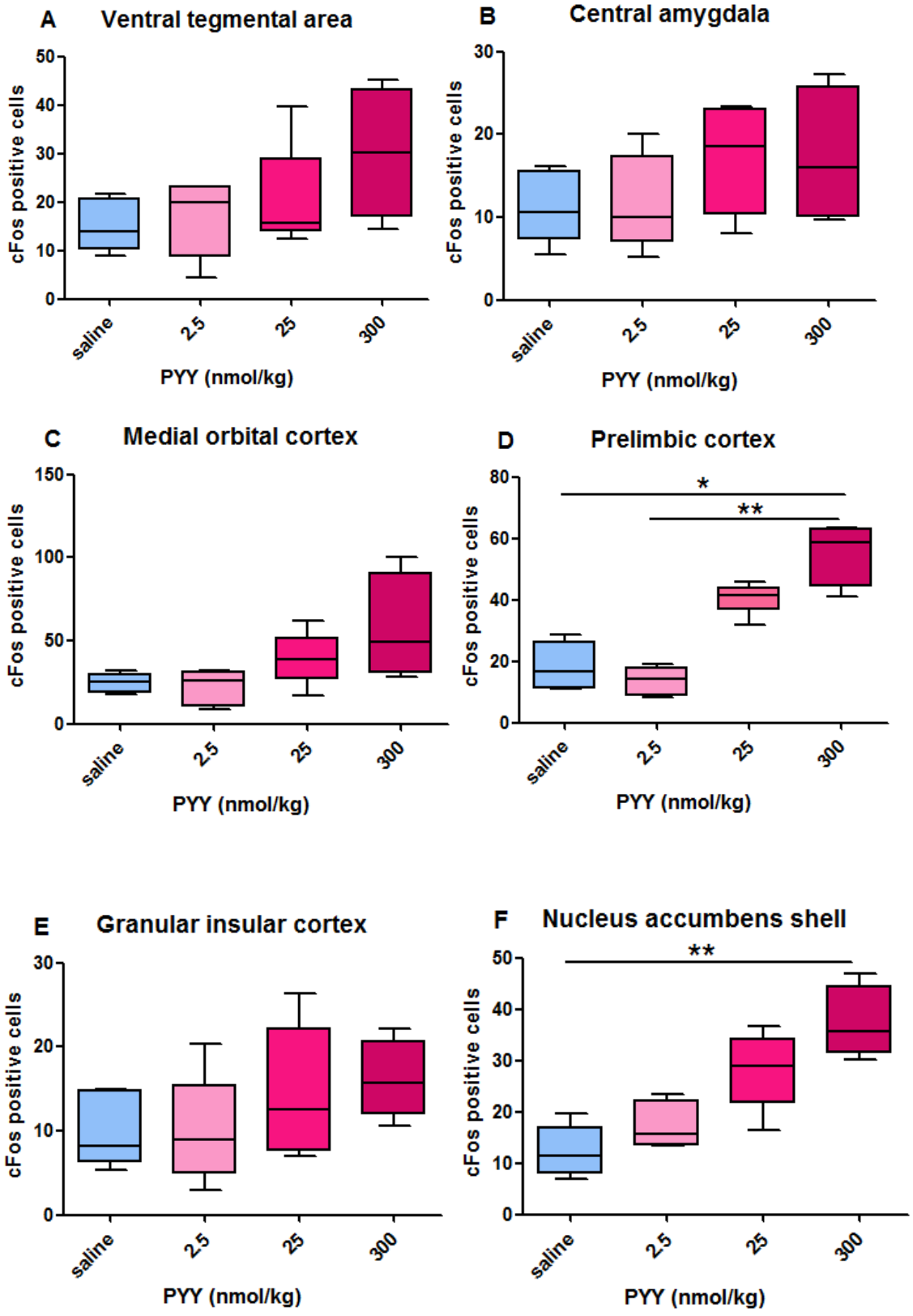


Figure 3.21. The effect of peripheral PYY administration on neuronal activation in the mesocorticolimbic nuclei of fasted rats.

The effect of saline and 2.5, 25, 300 nmol/kg PYY on c-Fos like immunoreactivity in the (**A**) ventral tegmental area (**B**) central amygdala (**C**) medial orbital cortex (**D**) prelimbic cortex (**E**) granular insular cortex and (**F**) nucleus accumbens shell. Data presented as median, inter-quartile and total range. N=4-6. *p<0.05, **p<0.01, using Kruskal-Wallis 1-way ANOVA.

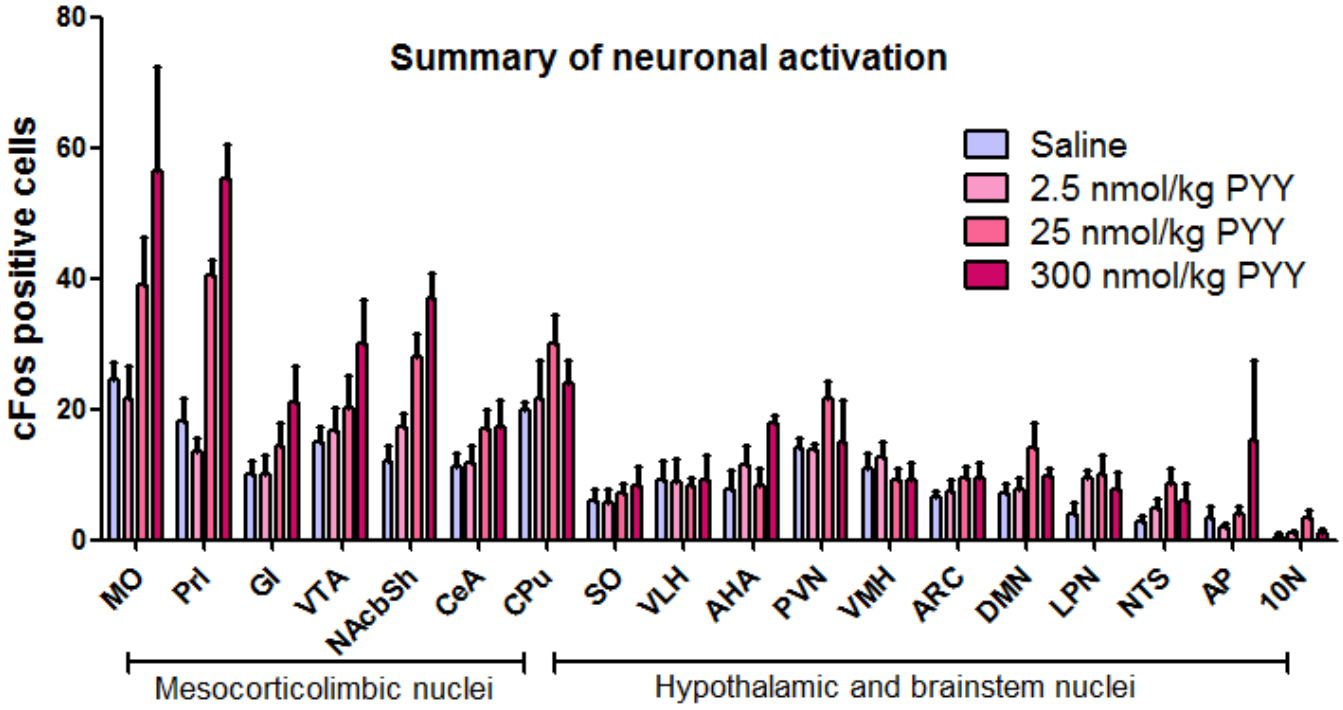


Figure 3.22. Summary of neuronal activation indicated by c-Fos like immunoreactivity in mesocorticolimbic, hypothalamic and brainstem nuclei of the rat brain, following peripheral administration of 2.5, 25, 300 nmol/kg PYY or saline.

MO, medial orbital cortex; PrL, prelimbic cortex; GI, granular insular cortex; VTA, ventral tegmental area; NAcbSh, nucleus accumbens shell; CeA, central amygdala; CPu, caudate putamen; SO, supra-optic nucleus of the hypothalamus; VLH, ventrolateral hypothalamus; AHA, anterior hypothalamic area; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus; ARC, arcuate nucleus; DMN, dorsomedial nucleus of the hypothalamus; LPN, lateral parabrachial nucleus; NTS, nucleus of the solitary tract; AP, area postrema; 10N, dorsal motor nucleus of the vagus.

3.4.11 The effect of peripheral administration of LiCl on c-Fos-like immunoreactivity in brainstem nuclei of fasted rats

Saline or 32 mg/kg LiCl were administered to rats and c-Fos like immunoreactivity was counted in the NTS, AP and 10N.

Peripheral administration of 32 mg/kg LiCl significantly increased c-Fos-like immunoreactivity in the NTS, AP and 10N compared to saline ($p<0.05$; Fig 3.23 A-C).

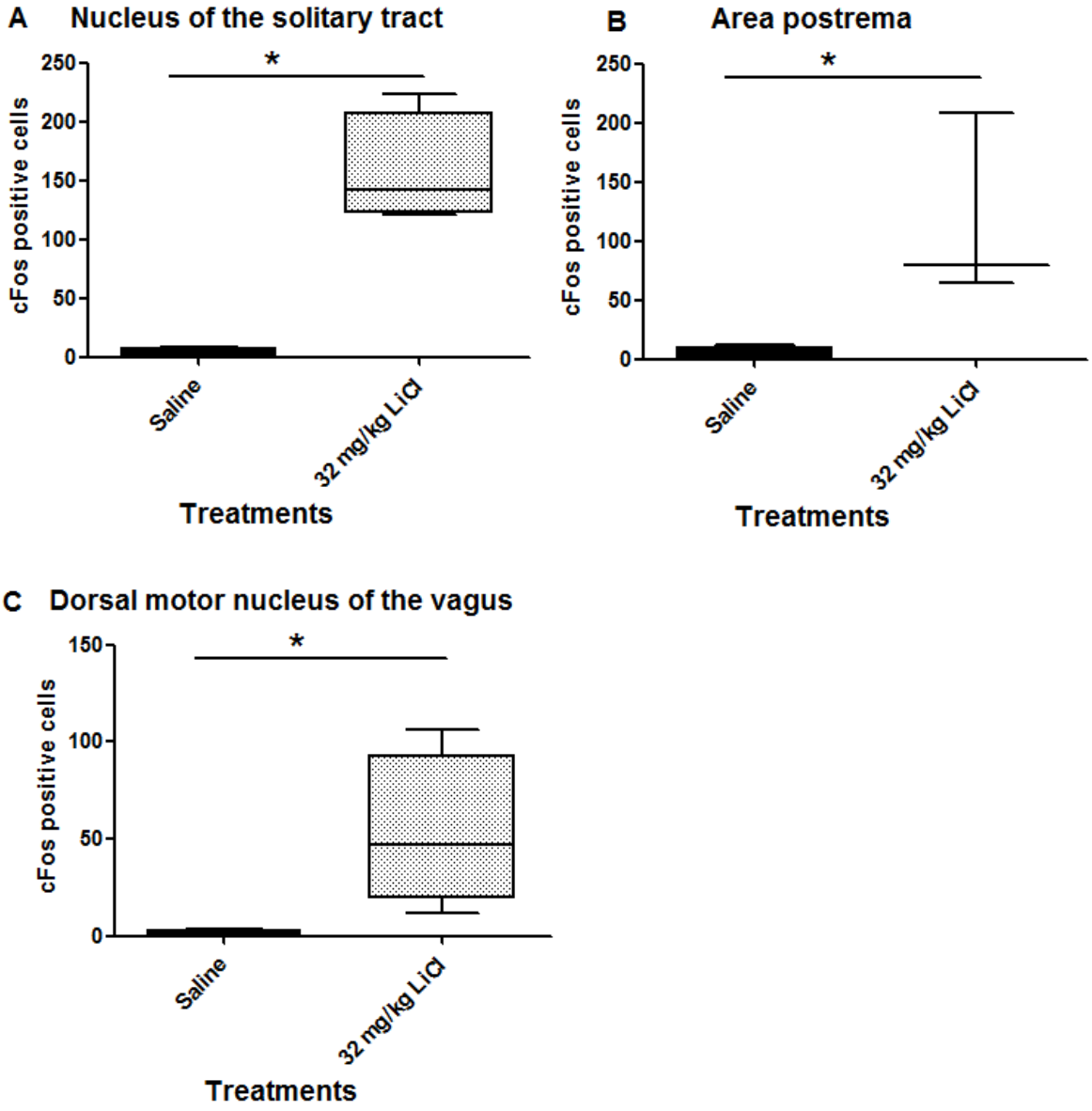


Figure 3.23. The effect of peripheral LiCl administration on neuronal activation in the brainstem nuclei of rats.

The effect of saline and 32 mg/kg LiCl on c-Fos like immunoreactivity in the (**A**) nucleus of the solitary tract (**B**) area postrema and (**C**) dorsal motor nucleus of the vagus. Data presented as median, inter-quartile and total range. N=3-6. *$p<0.05$, using Mann-Whitney U test.

3.4.12 The effect of peripheral administration of LiCl on c-Fos-like immunoreactivity in mesocorticolimbic pathways of fasted rats

Saline or 32 mg/kg LiCl were administered to rats and c-Fos like immunoreactivity was counted in the MO, PrL, GI, NAcbSh, and CPu. Peripheral administration of 32 mg/kg LiCl significantly increased c-Fos-like immunoreactivity in the MO, PrL, GI and CPu compared to saline ($p<0.05$; Fig 3.24 A-C & E).

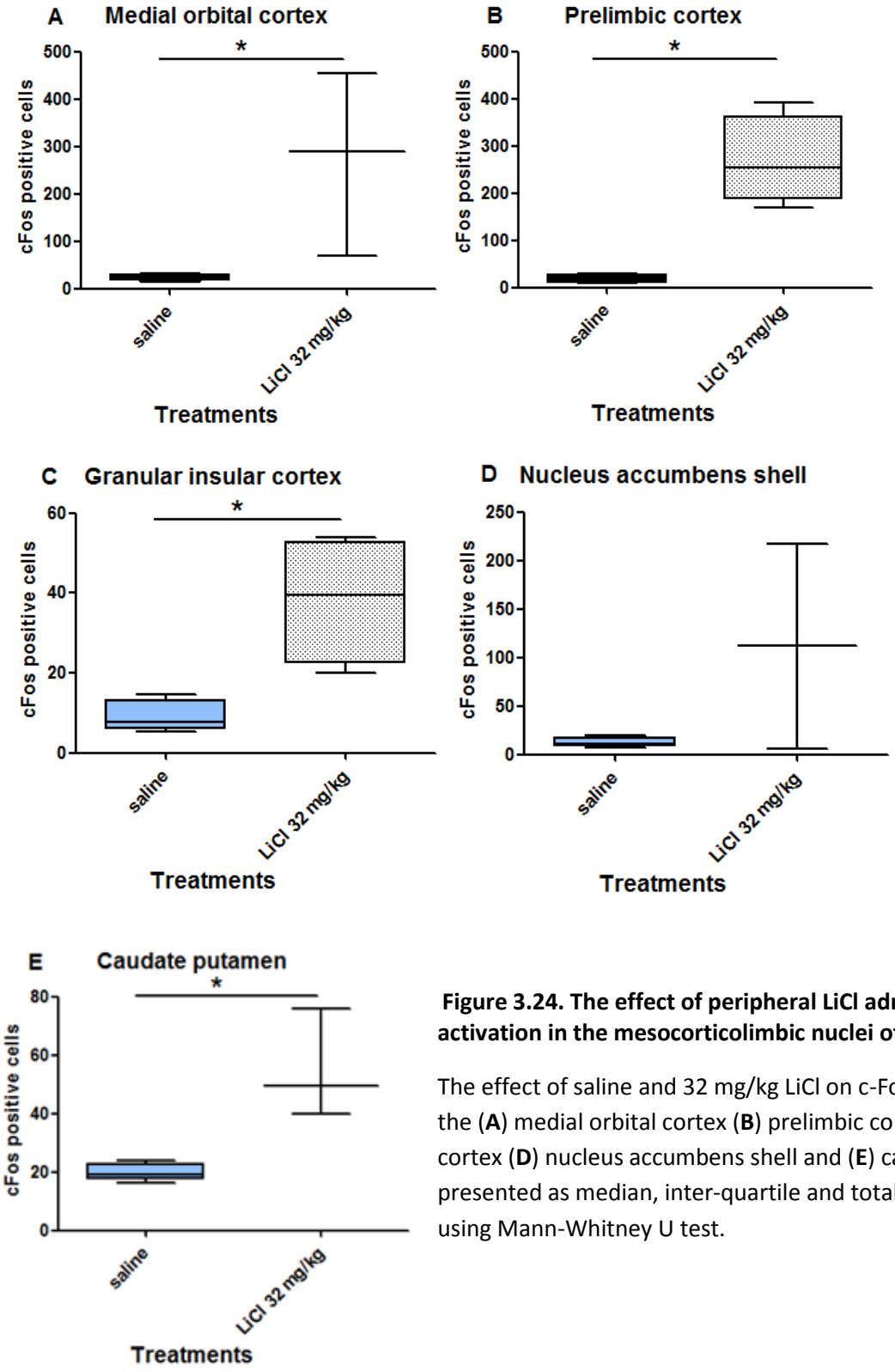


Figure 3.24. The effect of peripheral LiCl administration on neuronal activation in the mesocorticolimbic nuclei of rats.

The effect of saline and 32 mg/kg LiCl on c-Fos like immunoreactivity in the (**A**) medial orbital cortex (**B**) prelimbic cortex (**C**) granular insular cortex (**D**) nucleus accumbens shell and (**E**) caudate putamen. Data presented as median, inter-quartile and total range. N=2-6. *p<0.05, using Mann-Whitney U test.

3.5 Discussion

Previous studies have found that intermittent administration of PYY reduces body weight in rodents (Reidelberger et al., 2008, Chelikani et al., 2007). High doses of PYY significantly reduce food intake and cause CTA in rats and mice, while supraphysiological doses cause severe nausea in humans (Halatchev and Cone, 2005, Chelikani et al., 2006b, Sloth et al., 2007). Thus, whether PYY reduces food intake via homeostatic or aversive mechanisms, and whether it has clinical utility as a weight loss agent, is unclear. The work described in this chapter investigated the effects of PYY on food intake, energy expenditure and cardiovascular parameters in rats, using the same monitoring system as previously described for leptin, sibutramine, LiCl and GLP-1. In addition, the effects of peripheral PYY and LiCl on aversion and neuronal activation in rats were investigated.

To explore the effects of PYY on food intake and aversion, I investigated the effects of a range of doses on food intake in rats, commencing with 50 and 300 nmol/kg PYY. A dose of 300 nmol/kg PYY significantly reduced food intake at 0-1, 0-2, 0-4 and 0-8h post administration, whereas 50 nmol/kg PYY significantly reduced food intake at 0-1, and 0-8h, suggesting compensatory feeding at 0-2 and 0-4h (Fig 3.1). In the first hour, 50 nmol/kg reduced food intake by 22%. A previous study in mice found optimal food intake inhibition using 100 nmol/kg PYY that was sustained for 8h post administration, with higher doses having no additional effects (Neary et al., 2005). In the current study, 300 nmol/kg PYY reduced food intake by 39%, and thus appears to have a more potent effect on food intake in rats than the same dose in mice, which reduced food intake by approximately 30% (Neary et al., 2005).

Subsequently, the effects of lower doses of PYY on food intake were investigated. A previous study in rats determined that 2.5 and 7.5 nmol/kg PYY significantly reduced food intake in the first hour by 21 and 25%, respectively, with 0.75 nmol/kg having no significant effect (Abbott et al., 2005b). In addition, a study in mice found an approximate reduction in food intake of 27% following administration of 30 nmol/kg PYY (Neary et al., 2005). Similarly, no effect was found with 0.75 nmol/kg in the current study, whereas 7.5 and 25 nmol/kg PYY reduced food intake by 14 and 20%, respectively (Fig 3.2). The reasons for the somewhat weaker effects on food intake are unclear. In a subsequent study, 2.5 nmol/kg also reduced food intake by 14%, indicating similar levels of satiety as were induced by administration of 7.5 nmol/kg.

In the abovementioned study, Abbott *et al* found an effect on food intake at 2.5 nmol/kg, but not 0.75 nmol/kg. I therefore next investigated whether 1.5 nmol/kg PYY would elicit an anorectic effect. At this dose, food intake was reduced by 16% in the first hour, similar to the reduction induced by 2.5 and 7.5 nmol/kg (Fig 3.3). As shown in summary Figures 3.5A and B, the doses studied did not appear to cause a simple dose-response effect on food intake. There was no effect on food intake at 0.75 nmol/kg PYY. However, doses of 1.5, 2.5 and 7.5 reduced food intake comparably, by around 15%. Doses of 25 and 50 nmol/kg PYY reduced food intake by 20-22%, and 300 nmol/kg reduced food intake by 39%. Though the levels of food intake inhibition are slightly lower, these results are in-keeping with feeding studies from other groups, in which PYY appears to elicit discrete effects on food intake at

particular threshold doses, rather than producing a standard dose-response effect (Abbott et al., 2005b, Neary et al., 2005).

Plasma samples were assayed using an RIA to determine the circulating levels of PYY, following peripheral administration of 1.5, 2.5 and 7.5 nmol/kg PYY. Fasted rats were used to exclude variations in endogenous postprandial PYY release. Levels of PYY were significantly increased in the group that had access to food for 30 minutes, compared to fasted controls (Fig 3.6). All doses of PYY resulted in significantly increased levels of circulating PYY compared to all controls, confirming that 1.5, 2.5 and 7.5 nmol/kg PYY result in pharmacological, rather than physiological, circulating levels of PYY. However, pharmacological levels may still act via physiological circuits. It is also unknown whether the levels measured represent the appetite-regulating form of PYY. The assay used detects total PYY, and does not differentiate between PYY of various lengths. A recent study has found that PYY is rapidly metabolized to PYY_{3-34} in the pig. The specific effects of PYY_{3-34} are not known. This raises the possibility that PYY has a much shorter half-life, and thus is more potent, than current data suggests (Torang et al., 2015).

CLAMS studies were used to detect any changes in the physiology or behaviour of rats administered PYY at various doses. IP administration of 300 nmol/kg PYY significantly reduced cumulative food intake by 7.96±2.80% and 6.45±2.14% at 12h (corresponding to the dark phase) and 18h post administration, respectively. High resolution data revealed there was also a trend for decreased food intake with increasing doses of PYY in the 40 minutes post administration. LiCl was used a positive control for non-specific reductions in food intake and aversion. A dose of 32 mg/kg LiCl was selected, as previous studies indicated that this dose reduced food intake at a similar magnitude to 300 nmol/kg PYY in the first hour (Fig 2.24, section 2.64), and has previously been shown to induce CTA in rats (Riley and Tuck, 1985). LiCl significantly reduced cumulative food intake at 40 minutes post administration by 11.54±12.50%, compared to saline control, and at 18h by 8.34±2.26% (Figs 3.8 and 3.7). As mentioned above, 1.5 and 7.5 nmol/kg PYY significantly reduced food intake in earlier studies, consistent with results from other groups (Abbott et al., 2005b). There was a trend for decreased food intake in the first and second feeding bouts following administration of 300 nmol/kg PYY and 32 mg/kg LiCl (Fig 3.9 A&C). Administration of 32 mg/kg LiCl significantly increased the latency to feeding compared to saline, 1.5 and 7.5 nmol/kg PYY. There was also a trend for increased latency to feeding following administration of 300 nmol/kg PYY. Increased latency to feeding may represent disruption of the BSS, and thus aversion. The lack of a significant effect at the lower doses of PYY on food intake in the CLAMS studies likely reflects the constraints of the maximal n-numbers that could be used in the CLAMS-telemetry setup. In addition, previous studies were carried out in fasted animals in the light phase. The natural feeding period for rodents is in the dark phase, and they do not tend to eat large quantities in the day. Therefore, in the CLAMS studies, rats were fed *ad libitum*, and food intake was measured in the subsequent dark phase, to investigate the effects of PYY on normal physiological feeding patterns. Consequently, it is possible that PYY administration failed to have a significant effect at lower doses due to increased endogenous PYY, as has been postulated to be the case in other studies using fed animals (Tschop et al., 2004, Batterham et al., 2004), or simply because food intake was lower in these animals, and thus the baseline against which to show anorectic effects was

lower. However, it is reassuring that the high resolution food intake data suggests that the percentage food intake reduction at 40 minutes is similar to the suppression observed at 1 hour in fasted animals.

Following initial controversy surrounding the anorectic effects of PYY, in which several laboratories were unable to replicate effects on food intake, it has been established that mild environmental stress may blunt the anorectic effects of gut hormones by an unknown mechanism (Tschop et al., 2004, Batterham et al., 2002). It is therefore imperative that animals are acclimated to handling and the injection procedure prior to investigations. In addition, the satiating effects of PYY are attenuated in rats subject to the relatively mild stress of being returned to a clean cage following administration, compared to those maintained in home cages that had not been cleaned 7 days prior (Abbott et al., 2006). In our studies, rats were housed in CLAMS cages for 5 consecutive days, and returned to their standard home cages for 2 days, with cleaning occurring in between. Although attempts at acclimatization were made in these studies, 24h may not have been long enough, and it is possible that moving animals between novel environments influenced the feeding response to PYY administration.

The BSS describes feeding behaviour as a discontinuous process that involves eating, grooming, sniffing, exploration and finally, resting. Disruption of the structural integrity of the BSS can involve increased resting at the expense of decreased grooming, for example, grooming does not act as a transitory behaviour following LiCl administration (Ishii et al., 2004). Previous studies have found conflicting results regarding activity levels following PYY administration. The normal BSS, including locomotion, was not disrupted following IV infusions of PYY, leading the authors to conclude that PYY acted via homeostatic mechanisms (Scott et al., 2005). Conversely, activity was slightly increased in studies using bolus PYY administration (Vrang et al., 2006). Studies investigating the effects of amphetamine have concluded that increased locomotion is not compatible with physiological satiation (Blundell et al., 1985, Halford et al., 1998). In the current studies, locomotion was not significantly affected by PYY or LiCl, though there was a trend of increased activity following administration of 1.5 nmol/kg PYY, and decreased activity following administration of 300 nmol/kg PYY and 32 mg/kg LiCl, in the 18h following administration (Fig 3.13). A decrease in activity may reflect reduced food-seeking due to satiety or aversion, and corresponds to the increased latency to first feeding bout. Though cameras were not used to detect specific changes in grooming, sniffing, scratching and drinking, the decrease in activity suggests these behaviours were attenuated, as they would be registered as movement by the infra-red beams. A general decrease in activity could therefore be taken as indication that the normal structure of the BSS was altered by 300 nmol/kg PYY and LiCl. Decreased activity was reflected in the body temperature data, revealing a non-significant increase following 1.5 nmol/kg PYY and non-significant decrease following 32 mg/kg LiCl. Transgenic mice overexpressing PYY have higher basal body temperatures, suggesting increased thermogenesis and energy expenditure. However, acute administration is likely insufficient to induce this effect (Boey et al., 2008). Previous studies have reported conflicting effects of LiCl on activity. Peripheral administration of LiCl is acutely associated with spastic movement in the rat, involving abdomen dragging due to irritation of the injection site (Kosten and Contreras, 1989). These non-specific effects could plausibly be the cause of initial increases in activity, with a later decrease in activity indicative of a disrupted BSS due to aversion (Segal et al., 1975, Cappeliez and White,

1981, Lerer et al., 1980, Rodriguez et al., 2000). However, in my studies, at the dose administered, no gross effects on behaviour, such as abdomen dragging, were observed.

VO_2 and VCO_2 were not significantly affected, though there was a trend for increased VO_2, and thus energy expenditure, following administration of LiCl. RER was significantly decreased following administration of 32 mg/kg LiCl compared to saline, indicating increased use of fat as the primary fuel source for energy expenditure (Fig 3.12). This likely reflects the decreased food consumption, and therefore diminished availability of carbohydrates, in these rats. Unlike humans, rodents switch to utilising fat before their carbohydrate stores are exhausted, often reflecting acute reductions in food intake. In the above studies, PYY did not affect energy expenditure. However, previous studies in mice have observed beneficial effects on fuel partitioning following chronic PYY administration, without direct effects on activity or energy expenditure (Adams et al., 2006, van den Hoek et al., 2007). This has been suggested to increase the mobilization of fat during periods of negative energy balance when carbohydrate is not available, thus preventing muscle wastage due to oxidation of protein (Neary and Batterham, 2009).

To investigate the effect of PYY on HR and BP, rats were implanted with radio-telemetry devices as described in section 3.3.9. An increase in HR and BP is an expected artefact of handling and injection. This was used to assess correct placement of ECG leads and BP catheters following injection of saline; rats that did not exhibit a change in either parameter were excluded from the appropriate data analysis (Fig 2.19). IP administration of 32 mg/kg LiCl significant reduced HR in the first hour following injection, and resulted in a non-significant reduction in HR over 5 hours. Activity was not significantly affected during this time, and thus these changes cannot be attributed to increased locomotion. This is consistent with previous studies which have also found a deceleration of HR following LiCl administration (Wilkin et al., 1982). LiCl lowers HR by decreasing norepinephrine release and increasing reuptake at sympathetic nerve terminals (Kosten and Contreras, 1989). In the first hour, 32 mg/kg LiCl significantly increased systolic BP compared to saline and 1.5 nmol/kg PYY. Though non-significant, there was a clear trend for increased systolic BP following administration of 300 nmol/kg PYY, while LiCl significantly increased systolic BP in the 5h following administration (Fig 3.16). Diastolic BP was significantly increased in the first hour following administration of LiCl and 300 nmol/kg PYY (Fig 3.17). Over the following 5 hours, diastolic BP was not significantly affected, but tended to be increased following administration of LiCl and 300 nmol/kg PYY. Peripheral LiCl has previously been found to decrease BP in rats (Cheng et al., 1986). Despite PYY having no clear effect on HR, these results are consistent with those from other studies, in which peripheral PYY increased BP and HR in food-restricted rats, but had a lesser effect on *ad libitum* fed animals (Nordheim and Hofbauer, 2004). In addition, PYY infusions increased HR in fasted humans, but not after an *ad libitum* meal (Sloth et al., 2007). This difference is thought to occur due to variations in NPY levels, which are increased during fasting, and decreased in the fed state. Central NPY decreases HR and BP, thus the effects of PYY administration on cardiovascular parameters may be dependent on endogenous NPY levels (Tseng et al., 1989). Though not food-restricted *per se*, rats ate less following administration of 300 nmol/kg PYY, which may have contributed to increased effects on BP in these animals. In addition, the above study by Nordheim and Hofbauer used a much lower dose, approximately 75 nmol/kg PYY, than the maximal PYY dose used in

the current study. Therefore, higher doses of PYY may elicit more powerful effects on cardiovascular parameters, which may in turn contribute to sensations of visceral illness and malaise.

A CTA protocol was used to investigate the effects of peripheral administration of PYY on aversion in rats. LiCl was used as a positive control, and produced a significant CTA at 127 mg/kg. This was reduced to 48 mg/kg LiCl in a subsequent CTA study, which produced an equally significant aversive effect. Doses of 2.5 and 7.5 nmol/kg PYY produced a significant CTA in rats, suggesting that malaise was induced by PYY, causing the rats to avoid Kool-Aid consumption (Fig 3.19A). A dose of 1.5 nmol/kg PYY did not produce a CTA, though there was a trend for decreased Kool-Aid intake. In human studies, nausea and abdominal discomfort are a common symptom of PYY infusion (Sloth et al., 2007, le Roux et al., 2008). Supraphysiological doses of PYY cause aversive side effects with no additional reduction in food intake (le Roux et al., 2008). In humans, it appears that the therapeutic window to achieve a reduction in food intake without causing intolerable side effects is narrow (Sloth et al., 2007). The lowest dose I tested that had an effect on food intake in rats was 1.5 nmol/kg PYY, with no significant effect detected after administration of 0.75 nmol/kg. It is possible that an anorectic dose exists between 0.75 and 1.5 nmol/kg PYY, which does not induce aversion in rats. If PYY is to be used as a treatment for obesity, routes of administration may be required to produce relatively stable physiological levels, while avoiding tachyphylaxis, perhaps via an intermittent infusion from a subcutaneous mini pump (Pittner et al., 2004, Chelikani et al., 2007, Chelikani et al., 2006a).

To assess neuronal patterns of activation in response to peripheral administration of 2.5, 7.5 and 300 nmol/kg PYY, c-Fos IHC was performed. In the hypothalamic and brainstem nuclei investigated, PYY did not significantly alter c-Fos like immunoreactivity at any dose tested. However, there was a trend for increased neuronal activation in the PVN and NTS, following administration of 25 nmol/kg PYY vs. saline (Figs 3.20 C&E). Higher n-numbers may be needed to adequately detect the variations in brain signalling in homeostatic pathways. Previous studies in mice found increased neuronal activation in the ARC, PVN, LHA, NTS and AP (Hurtado et al., 2013, Halatchev and Cone, 2005). In addition, Batterham *et al* found peripheral PYY administration led to the activation of a small percentage (~12%) of POMC neurons in the murine ARC (Batterham et al., 2002). Thus, PYY may act via the hypothalamic pathways to some extent.

However, it is interesting that there were much more evident effects of PYY in the mesocorticolimbic system. It has also been postulated that PYY exerts some of its effects by reducing the palatability of food and causing aversion. Neuronal activation of the AP and intermediate NTS is known to be induced by aversive substances, such as LiCl (Schafe et al., 1995). To confirm this, c-Fos IHC was performed following peripheral administration of 32 mg/kg LiCl. LiCl significantly increased c-Fos like immunoreactivity in the brainstem nuclei investigated, including the NTS, AP and 10N (Fig 3.23 A-C). The AP is also thought to mediate the emetic response to PYY in dogs (Harding and McDonald, 1989). Though there was no significant change in the NTS or AP in the current PYY IHC studies, 300 nmol/kg PYY did appear to evoke c-Fos like immunoreactivity in these areas (Fig 3.20 E&F). Toxins that act through the blood stream require an intact AP to exert a CTA, mediated by the chemoreceptor trigger zone (Kosten and Contreras, 1989). As PYY is thought to signal through several mechanisms, including vagal afferents, it is likely that

other brain regions contribute to the aversive response, such as decreased reward perceptibility mediated by the mesocorticolimbic pathways. However, it is interesting that a dose of LiCl that produced a similar reduction in food intake (see Fig 2.24, chapter II) to 300nmol/kg PYY resulted in a much larger effect on neuronal activation in the brainstem. PYY at 300 nmol/kg also likely results in a lower aversive effect than LiCl at 32 mg/kg, though this was not assessed. This suggests some independence of the effects of PYY on food intake and known aversive pathways. Additionally, administration of 32 mg/kg LiCl significantly increased c-Fos like immunoreactivity in the MO, PrL, GI and CPu, illustrating robust activation of the reward pathways even at a low dose. Neuroanatomical studies have previously shown the insular cortex to be important in aversion learning due to its role in processing gustatory information, with lesions of this area attenuating CTA acquisition (Braun, 1982). Unfortunately, due to issues with tissue preservation, we were not able to investigate the activation of hypothalamic areas following administration of LiCl in the current study. However, previous studies have found increased c-Fos like immunoreactivity in the PVN, SON and CeA; areas thought to be involved in the transmission of visceral information that contributes to the formation of a CTA (Parkinson et al., 2009, Sakai and Yamamoto, 1999, Rinaman and Dzmura, 2007, Olszewski et al., 2000).

Though not significantly affected, there was a clear trend for decreased HR, while systolic and diastolic BP were significantly increased following administration of 300 nmol/kg PYY, which was not apparent at the lower doses (Figs 3.15-3.17). This alteration in cardiovascular function may contribute to feelings of visceral illness in rodents. Increased neuronal activation in the AP, and increased BP were both elicited by administration of 300 nmol/kg PYY. LiCl also significantly increased BP and decreased HR. The AP has strong neural links with areas controlling autonomic output, and has well characterised functions in nausea and vomiting (Shapiro and Miselis, 1985, Miller and Leslie, 1994). Lesions of the AP disrupt the development of conditioned responses and cause bradycardia in rats (Contreras et al., 1984). Changes in autonomic nervous system activity have been implicated in the acquisition and expression of CTA, particularly when LiCl is the unconditioned stimulus. Rats are unable to rid themselves of ingested toxins, but have a strong reaction to novel tastants that enables them to detect potentially harmful substances. Changes in autonomic nervous system activity may act as a visceral warning in this instance (Kosten and Contreras, 1989).

Though originally thought to act primarily via the melanocortin system, PYY retains its anorectic effects in mice that lack POMC or the MC4R (Halatchev et al., 2004, Challis et al., 2004). Therefore, it has been proposed that PYY elicits some of its effects via alterations in hedonic signalling pathways in the mesocorticolimbic system. Of the mesocorticolimbic nuclei investigated in the current study, 300 nmol/kg PYY significantly increased c-Fos like immunoreactivity in the PrL, compared to saline and 2.5 nmol/kg PYY. In the NAcbSh, c-Fos like immunoreactivity was also increased by administration of 300 nmol/kg PYY. In addition, there was a non-significant trend for increased neuronal activation in the VTA, CeA and MO. The increase in neuronal activation seen in the frontal cortex and VTA mirrors the changes that have been recorded in humans using fMRI. Peripheral infusion of PYY to fasted humans increases signalling in the mesocorticolimbic brain regions, which may lead to decreased reward perception of food (Batterham et al., 2007). Y2R mRNA has been identified throughout the hypothalamic areas investigated here, in

addition to the AP and NTS of the brainstem of the rat (Parker and Herzog, 1999). Thus, neuronal activation in these homeostatic regions may represent direct activation of the Y2R by circulating PYY. Of the mesocorticolimbic regions investigated, Y2R mRNA has been detected in the VTA and CeA (Parker and Herzog, 1999). It is possible that PYY directly activated neurons within these nuclei, which project to the MO, PrL, GI and NAcbSh, causing further downstream stimulation.

The underlying anorectic mechanisms of PYY are not completely understood. Due to the dense connections between the homeostatic and hedonic brain regions, it is highly likely that endogenous PYY drives synergistic changes in both systems. PYY may induce feelings of satiety and fullness by activation of Y2 receptors on POMC neurons and vagal afferents, while simultaneously decreasing the rewarding value of food via the mesocorticolimbic system (Batterham et al., 2002, Batterham et al., 2007). PYY may also induce feelings of malaise during periods of over-eating or diarrhoeal disease, when PYY secretions are increasingly augmented, activating nuclei in the AP and NTS (Halatchev and Cone, 2005, Adrian et al., 1985, Playford et al., 1992). The data in this chapter support an anorectic effect of PYY at a range of doses, whilst inducing CTA from 2.5 nmol/kg. Doses below this may reduce food intake via physiological pathways, while higher doses induce malaise, activating brain regions involved in hedonic signalling and nausea.

The data in this chapter highlight the ability of PYY to reduce food intake at low doses in a home cage setting where it is possible to pick up 10-15% reductions in food intake in fasted animals, while it is difficult to detect effects of the same doses in the CLAMS system in fed animals. However, CLAMS has provided useful data regarding high resolution food intake in the first 40 minutes, and information on meal patterns, in particular latency to feeding. The novel concept of combining food intake data with meal patterns, behavioural data and telemetry recordings, may provide insight into the impact of gut hormones on the continuum of the hunger-satiety-nausea systems. Having characterised the effects of PYY on food intake and other physiological parameters in rats, I proceeded to characterise the effects of GLP-1 on the same biological systems.

Chapter IV: The effect of GLP-1 and exendin-4 on energy homeostasis and cardiovascular parameters.

4.0 GLP-1

4.1 Introduction

GLP-1 is produced peripherally by intestinal L-cells, and centrally by a subset of neurons expressing preproglucagon in the brainstem (Han et al., 1986, Mojsov et al., 1990). It is thought that the peripheral and central GLP-1 systems are distinct, and represent discrete signalling mechanisms, though there is some overlap in function.

Peripheral GLP-1 is secreted post-prandially, and has a well characterised role as an incretin hormone, driving increased glucose-stimulated insulin secretion, inhibition of glucagon secretion and deceleration of gastric emptying (Holst et al., 1987, Kreymann et al., 1987). This has resulted in the development and approval of GLP-1 analogs to treat type 2 diabetes mellitus, including exenatide, liraglutide and albiglutide. These molecules are structurally based on GLP-1, but have modifications that confer some protection from the enzymatic actions of DPPIV and, in some cases, promote binding to albumin and consequently impair renal clearance, and they therefore exhibit longer half-lives (Finan et al., 2015). Peripheral GLP-1 may also regulate food intake, though it is unclear whether this effect is purely pharmacological rather than physiological. Peripheral or central administration of exogenous GLP-1 or GLP-1 receptor agonists decreases food intake and body weight in animals, and peripheral administration suppresses food intake in humans (Turton et al., 1996, Rodriquez de Fonseca et al., 2000, Amori et al., 2007, Tang-Christensen et al., 1996). Central GLP-1 is also postulated to play roles in the central regulation of energy homeostasis, though the precise circuits involved require elucidation.

The GLP-1 receptor is abundantly expressed in the CNS, gut and pancreas, and moderately expressed in the lung, kidney and heart (Campbell and Drucker, 2013). Peripheral GLP-1 may signal to the brain via sensory afferent vagal neurons; there is also data suggesting it may act directly on specific brain regions in the brainstem and the hypothalamus, though it is unclear whether this is only an effect of exogenous rather than endogenous GLP-1. It is possible that the effects of peripherally administered GLP-1 on food intake reflect a pharmacological activation of neuronal pathways normally only physiologically activated by central GLP-1 neurons. Central GLP-1-producing neurons project to a number of brain areas which are involved in food intake and which express the GLP-1 receptor, including the ARC, CeA, PVN and VTA (Goke et al., 1995, Han et al., 1986, Merchenthaler et al., 1999).

The following chapter will investigate the effects of GLP-1 on food intake, energy expenditure, HR and BP to determine whether a combination of CLAMS and telemetry is able to detect physiological and behavioural changes following administration of a range of doses. The effects of exogenous GLP-1 on aversion, and the brain regions that it activates, will also be investigated.

4.1.1 Effects of GLP-1 on food intake

ICV administration of GLP-1 induces hypophagia in food restricted rats and reduces water intake. These effects are blocked by antagonism of the GLP-1 receptor by exendin-(9-39) (Tang-Christensen et al., 1996, Thiele et al., 1997,

Turton et al., 1996). However, though mice with targeted deletion of the GLP-1 receptor are glucose intolerant, they exhibit normal body weight and feeding behaviour on a normal chow diet. The lack of a body weight phenotype may signify that GLP-1 does not play an important role in energy homeostasis. Conversely, this may reflect developmental compensation, as is thought to occur in the NPY KO mouse, which also lacks a body weight phenotype (Erickson et al., 1996). Interestingly, when fed a HFD, GLP-1 receptor KO mice have a lower body weight than WT mice. Congenital disruption of the GLP-1 receptor may therefore confer resistance to diet-induced obesity via a compensatory mechanism, possibly involving other anorectic peptides and their receptors (Ali et al., 2011). Some of the body weight lowering effects of bariatric surgery, particularly the Roux-en Y gastric bypass, have been attributed to increased basal levels of GLP-1. GLP-1 may also play a role in the amelioration of diabetes in these patients (Chronaiou et al., 2012). In rats, changes in L-cell morphology, such as villus hyperplasia, are thought to contribute to increased secretion of gut hormones following Roux-en-Y gastric bypass and ileal interposition (Kohli et al., 2010, Stearns et al., 2010).

Chronic peripheral administration of long-acting GLP-1 receptor agonists, such as liraglutide and exendin-4 (Ex-4), reduces food intake and body weight in rodents and humans (Rodriquez de Fonseca et al., 2000, Amori et al., 2007). In rodents, liraglutide administration was associated with a lowered reward value of palatable foods, and prevented weight-loss induced decreases in energy expenditure (Raun et al., 2007). The FDA has recently approved liraglutide, under the name Saxenda, as a weight loss agent at a maximum dose of 3 mg, which may lead the way for the next generation of GLP-1-based obesity drugs (FDA, 2014). Leptin interacts with ObRb receptors on GLP-1-producing neuronal populations in the NTS of the hindbrain, supporting a possible interaction between leptin and central GLP-1 (Goldstone et al., 1997). Treatment of DIO mice with Ex-4 restores responsiveness to leptin to a greater degree than diet alone, allowing further leptin treatment to drive additional weight loss when switched to a lower fat diet (Muller et al., 2012). Peripheral administration of low doses of Ex-4 and PYY additively reduces food intake in mice without inducing aversion (Talsania et al., 2005). GLP-1 and its analogs are therefore well placed for combination therapies with other anorectic hormones that might have additive or synergistic effects on weight loss (Finan et al., 2015).

Peripheral infusions of GLP-1 into the hepato-portal vein reduce food intake in rats. IP administration of 10 nmol/kg GLP-1 also reduces meal size in rats. Subdiaphragmatic deafferation abolishes the effect of IP, but not IV GLP-1, suggesting the anorectic effects of IP GLP-1 require vagal signalling (Ruttimann et al., 2009). Further studies in rats found that the anorectic effects of IP GLP-1 are abolished following transection of the brainstem-hypothalamic pathway (Abbott et al., 2005a). This indicates the importance of the vagal-brainstem-hypothalamic pathway in the satiating effects of circulating GLP-1.

Peripheral GLP-1 activates the ileal brake, involving activation of GLP-1 receptors on enteric neurons, and initiation of vagal afferent and efferent signalling, to slow gastrointestinal motility and aid the digestion of nutrients. Reduced gastric emptying and slowed gastric motility may limit meal size and absorption of glucose, therefore contributing to the satiating and incretin effects of GLP-1 (Willms et al., 1996). However, the slowing of gastric emptying by chronic

exogenous GLP-1 is subject to rapid tachyphylaxis, which leads to attenuation of postprandial glucose control (Nauck et al., 2011).

4.1.2 Effects of GLP-1 on energy expenditure

Consumption of a HFD results in diet-induced thermogenesis and a consequent increase in energy expenditure. Down-regulation of preproglucagon expression in the NTS results in hyperphagia and exacerbates HFD-induced obesity in rats, suggesting a role for endogenous GLP-1 in long term energy homeostasis (Barrera et al., 2011). However, HFD-induced diabetes is associated with increased expression of central GLP-1, insulin resistance and increased fat mass. In diabetic mice, chronic blockade of central GLP-1 receptors by exendin-9 results in increased energy intake without an increase in fat mass, suggesting GLP-1 signalling may mediate HFD-induced changes in energy expenditure. In this case, blockade of GLP-1 signalling results in increased VO_2 and VCO_2, increased thermogenesis, and in addition, increased locomotion (Knauf et al., 2008). The differences between the above studies by Barerra and Knauf are thought to occur due to variations in the animal models used and dietary composition (Barrera et al., 2011).

Glp-1 -/- mice exhibit lower energy expenditure, indicated by VO_2, than wild type (WT) controls when fed a HFD at room temperature (but not at thermoneutral temperatures of 31°C), supporting a role for GLP-1 signalling in diet-induced thermogenesis (Heppner et al., 2015). Sitagliptin, a competitive inhibitor of DPPIV, treats hyperglycaemia by increasing serum GLP-1 levels. Sitagliptin reduces adiposity and body weight in WT mice, and also increases energy expenditure. These effects are thought to be mediated by increased GLP-1, as they are absent in GLP-1 receptor KO mice (Goldsmith et al., 2015). However, *Glp-1-/-* mice exhibit a normal thermogenic response to cold exposure, indicating that these animals are capable of increasing thermogenesis in response to specific circumstances (Lockie et al., 2012).

Activation of BAT, a highly metabolically active tissue, contributes to energy expenditure due to its capacity for uncoupled mitochondrial respiration. Animals lacking BAT have decreased energy expenditure and are predisposed to obesity (Lowell et al., 1993). Small amounts of BAT are present in adult humans. Agents that increase energy expenditure through BAT activation represent possible targets for the development of obesity agents. However, compelling evidence that GLP-1R agonists acutely increase thermogenesis in human subjects is lacking (Campbell and Drucker, 2013, Bradley et al., 2012, Harder et al., 2004, Tan et al., 2013).

In rodents, central GLP-1 increased BAT thermogenesis by increasing sympathetic activity, though IP administration had no effect in this study (Lockie et al., 2012). However, IV GLP-1 has been reported to increase VO_2 and body temperature in rats. These effects were attenuated by cervical spinal transection, but were unaffected by decerebration, suggesting they were mediated by the brainstem (Osaka et al., 2005). Conversely, injection of liraglutide specifically into the VMH causes weight loss independent of food intake. This appears to occur via stimulation of BAT thermogenesis, and the browning of white adipocytes, with effects persisting for 24h post injection, and is dependent on decreased hypothalamic expression of AMPK, as pharmacological activation of AMPK

prevented intra-VMH liraglutide-induced weight loss (Beiroa et al., 2014). Furthermore, this study indicated that obese patients with T2DM exhibited chronically increased energy expenditure when treated with liraglutide for one year (Beiroa et al., 2014).

4.1.3 Effects of GLP-1 on cardiovascular parameters

GLP-1 has complex, species-dependent effects on HR and BP. *Glp-1* receptor mRNA has been detected in the mouse, rat, dog and human heart (Bullock et al., 1996, Ban et al., 2008, Wei and Mojsov, 1996). *Glp-1 -/-* mice exhibit reduced resting HR and elevated diastolic BP. At 5 months these mice possess thicker ventricular walls, and display impaired left ventricular contractility in response to insulin. Abnormalities in contractility and wall thickness suggest a role for GLP-1 signalling in normal cardiovascular development (Gros et al., 2003). An IV bolus dose of GLP-1 or Ex-4 increased systolic and diastolic BP and HR in rats independent of catecholamines. These effects were relatively short-lived, with BP and HR returning to basal levels 25 minutes after administration (Barragan et al., 1994). These effects appear to require GLP-1 receptor signalling and the vagus, as the effects were blocked by exendin-(9-39) and were absent in vagotomised rats (Barragan et al., 1996, Barragan et al., 1999).

Peripherally or centrally administered Ex-4 increased BP and HR in telemetered rats. This was associated with c-Fos expression in autonomic control centres of the brain, including medullary catecholaminergic neurons projecting to sympathetic preganglionic neurons (Yamamoto et al., 2002). GLP-1 immunoreactivity is found in the caudal NTS and dorsal and ventral parts of the medullary reticular nucleus of the rat brain- areas receiving vagal afferent signals from the cardiovascular system (Jin et al., 1988). The tachycardic effects of Ex-4 are suppressed by blockade of β-adrenoceptors and are completely absent in adrenalectomized rats, suggesting they are mediated via the sympathetic nervous system (Gardiner et al., 2006, Usdin et al., 1993). The vasopressin system may also be involved, as administration of the vasopressin receptor antagonist B-mercapto blocks the pressor effect of ICV GLP-1 in rodents (Isbil-Buyukcoskun and Gulec, 2004).

Conversely, GLP-1 concurrently increases nitric oxide (NO) production in endothelial cells, and as such would be expected to have antihypertensive effects. Such is the case in *db/db* mice treated chronically with Ex-4, in which the development of salt-sensitive hypertension is prevented, though it is unclear whether these effects are secondary to changes in body weight (Hirata et al., 2009). Similarly, continuous chronic infusion of Ex-4 reversed glucocorticoid-induced increases in BP in rats, independent of changes in body weight (Laugero et al., 2009). Hence, acute activation of the GLP-1 receptor may increase BP, while sustained activation may reduce or prevent increases in BP in rodents.

Human studies using GLP-1 analogs also present a complex range of effects. In one study, intravenous infusion of GLP-1 had no effect on HR, BP, or indices of cardiac sympathetic and parasympathetic activity (Bharucha et al., 2008). Acute subcutaneous abdominal injection of GLP-1 was reported to transiently increase HR and BP for 1h following administration in human subjects. However, clinical trials investigating the anti-diabetic potential of GLP-1 receptor agonists have reported decreases in systolic BP. Overweight patients reported improvements in BP when

treated twice weekly with exenatide, with those achieving the most weight loss experiencing the greatest reductions in BP (Buse et al., 2010, Blonde et al., 2006). Therefore, chronic GLP-1 treatment may have cardioprotective effects in overweight and obese subjects, though these seem likely to be secondary to effects on weight loss (Edwards et al., 1998).

4.1.4 Effects of GLP-1 on aversion and neuronal activation

The administration of LiCl induces nausea and vomiting in emetic species. LiCl induces pica and CTA in rodents (non-emetic species), indicative of feelings of visceral illness, which encompasses the nausea and anorexia typical of ingestion of toxins (Mitchell et al., 1977, Garcia et al., 1974). Central administration of GLP-1 also potently reduces food intake and induces CTA in rats, raising the possibility that GLP-1-induced anorexia occurs as a secondary response to visceral illness (Tang-Christensen et al., 1996, Turton et al., 1996, Thiele et al., 1997). The reduction in food intake following administration of GLP-1 analogs in humans is associated with gastrointestinal side effects, such as nausea and vomiting (Amori et al., 2007), though these effects appear to reduce after several weeks of treatment. Reduced gastric emptying may underlie some of the feelings of visceral illness that LiCl and GLP-1 can produce (Willms et al., 1996).

Administration of GLP-1 and LiCl activate similar neuronal populations in the brain. IV GLP-1 induces c-Fos like immunoreactivity in the rat PVN and CeA, and this effect is attenuated by co-administration of exendin-(9-39) (Turton et al., 1996). Additional studies in the rat identified neuronal activation in the AP, NTS and LPN following peripheral infusion, and in the ARC following central infusion (Thiele et al., 1998, Van Dijk et al., 1996). Peripheral LiCl also induces c-Fos like immunoreactivity in the AP, NTS, and LPN of the rat; interestingly, this effect is also attenuated by central administration of a GLP-1 receptor antagonist. In addition, the aversive properties of LiCl are inhibited by antagonism of the GLP-1 receptor (Seeley et al., 2000, Rinaman, 1999). Hence, a sub-set of GLP-1-producing neurons in the hindbrain are postulated to mediate the aversive effects of LiCl, and its consequent non-homeostatic effects on food intake. Administration of GLP-1 directly into the CeA, which is implicated in aversive learning, is capable of producing a CTA independent of an effect on food intake (Lasiter and Glanzman, 1985, Kinzig et al., 2002). However, direct injection of GLP-1 into the PVN reduces food intake in rodents without causing CTA, indicating hypothalamic circuits may mediate the specific homeostatic influence of GLP-1 on energy intake (Turton et al., 1996, Goldstone et al., 1997). In addition, fourth ventricular administration of GLP-1 reduces food intake without CTA, suggesting activation of brainstem GLP-1 receptors may reduce food intake independently of malaise. This is consistent with the rostral-caudal flow of cerebrospinal fluid (CSF), as GLP-1 administered to the fourth ventricle would access brainstem GLP-1 receptors, but not those in the CeA.

Central GLP-1 may therefore integrate the functions of the homeostatic and non-homeostatic pathways, with the diverse responses a result of projections to specific effector nuclei (Seeley et al., 2000, Kinzig et al., 2002). However, ICV injections result in diffusion into nearby areas, simultaneously activating multiple circuits, and thus the present

difficulties with determining the specific sites of action for particular biological effects (Kinzig et al., 2002, Thiele et al., 1998).

4.2 Aims and Hypothesis

4.2.1 Hypotheses

The administration of GLP-1 or Ex-4 will result in concurrent changes in food intake, HR and BP, detectable using the CLAMS metabolic cages and implanted telemetry probes. Higher doses of GLP-1 will induce CTA in rats, and will result in neuronal activation in brain regions with roles in the homeostatic and non-homeostatic control of energy intake.

4.2.2 Aims:

To investigate the effects of peripheral bolus IP injection of GLP-1 on:

▪ Food intake, energy expenditure, HR and BP in freely moving rats.

▪ Aversion in rats.

▪ c-Fos like immunoreactivity in the rat brain.

4.3 Methods

4.3.1 Animals

Male Wistar rats (Charles River, Margate, Kent, UK) weighing 200-220g were housed in individual cages under controlled temperature (21-23°C) and light (12 hours light, 12 hours dark; lights on at 0700) with *ad libitum* access to standard chow (RM1, Special Diet Services Ltd., Witham, Essex, UK) and water, unless stated otherwise. All animal procedures conducted were approved by the British Home Office under the Animals (Scientific Procedures) Act 1986 (Project Licence 70/8068).
Animals were acclimatised to handling and administration protocols prior to all experimental procedures. Animals were randomised by body weight for all studies.

4.3.2 The effect of peripheral administration of GLP-1 and Exendin-4 on food intake in fasted rats.

I hypothesised that the anorectic effects of high doses of GLP-1 were more likely to be mediated by non-homeostatic, aversive mechanisms. I therefore sought to establish a dose of GLP-1 that reduced food intake by approximately 40-50%. A previous study found a ~20% reduction in food intake in the first hour following a single IP injection of 100 nmol/kg GLP-1 in fasted rats (Abbott et al., 2005a). A feeding study was performed to investigate the effect of the higher dose of 300 nmol/kg GLP-1 on food intake. In addition, to investigate whether Ex-4 acted in a comparable manner to GLP-1, a dose of Ex-4 was required that would reduce food intake by approximately 15-20%. Previous data from within the department indicated that a dose of 0.3 nmol/kg Ex-4 might be suitable. Rats were fasted in the early dark phase overnight (to exclude variations in endogenous postprandial GLP-1 release) and received an IP injection of saline, 300 nmol/kg GLP-1 or 0.3 nmol/kg Ex-4 (Bachem, Merseyside, UK) (n=14), during the early light phase the following morning. Rats were then returned to their individual home cages with a pre-weighed amount of chow. Food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

4.3.2 The effect of peripheral administration of GLP-1 on food intake in fasted rats

It was hypothesised the minor anorectic effects of low doses of GLP-1 were more likely to be mediated by homeostatic pathways rather than aversive mechanisms. I therefore sought to establish a low dose of GLP-1 that reduced food intake by approximately 15-20%. Previous studies found that IP administration of 10 nmol/kg GLP-1 reduced the first spontaneous meal size in rats (Ruttimann et al., 2009). To investigate the effect of a range of doses of GLP-1 on food intake, a dose-response study was performed. Rats were fasted in the early dark phase overnight (to exclude variations in endogenous postprandial GLP-1 release) and received an IP injection of saline or 5, 10, or 30 mg/kg GLP-$1_{7\text{-}37}$ (Bachem, Merseyside, UK) (n=36; cross-over design), during the early light phase the following morning. Rats were then returned to their individual home cages with a pre-weighed amount of chow, and food was reweighed 1, 2, 4, 8 and 24 hours post-administration.

4.3.3 The effect of IP administration of GLP-1 on plasma GLP-1 levels in rats

Plasma samples were assayed to investigate the levels of circulating GLP-1 achieved following peripheral administration of saline, 10 or 30 nmol/kg GLP-1 (n=8-12) in fasted rats (to minimise the effect of variations in endogenous GLP-1 levels). Controls included fasted rats administered saline, fasted rats not administered saline, and rats fed *ad libitum* for 20 or 30 minutes following an overnight fast, to determine the normal physiological postprandial levels of circulating GLP-1 (n=8-12). Blood was collected from fed and fasted controls at 20 or 30 minutes, to allow digestion of food and release of peptides into the circulation. Blood was collected from rats treated with saline, 10 or 30 nmol/kg GLP-1 at 10 or 20 minutes to detect the predicted rapid increases in circulating peptide levels following IP injection.

Plasma GLP-1 levels were measured using an established specific and sensitive in-house radioimmunoassay (Kreymann et al., 1987). The GLP-1 antibody was raised against GLP-1 coupled to BSA in rabbits. The antibody fully cross-reacts with all amidated forms of GLP-1. It does not cross-react with the glycine extended forms ($GLP\text{-}1_{1\text{-}37}$ and $GLP\text{-}1_{7\text{-}37}$) or any other gut peptides. ^{125}I-GLP-1 was prepared by Professor Mohammad Ghatei, using the Iodogen method (Wood et al., 1981) and purified by HPLC. The assay was performed in 700µl of pH8 veronal buffer (Appendix 1) containing 0.3% BSA and 0.2% TWEEN20 (Sigma, Poole, UK) (200µl 1:10 TWEEN/100ml). The standard curve was composed of 1, 2, 3, 5, 10, 15, 20, 30, 50 and 100µl of GLP-1 at a concentration of 0.125pmol/ml. One hundred µl of plasma sample was used and all samples were assayed in duplicate. The assay was incubated for four days at 4°C. Free and antibody-bound GLP-1 were separated using the charcoal absorption technique (Appendix 1). Two hundred and fifty µl of Dextran coated charcoal (Merck; Appendix 1) was added to each tube. Samples were centrifuged immediately at 1500 g at 4°C for 20 minutes. The supernatant, containing bound-GLP-1, was separated by aspiration. Free and bound radioactivity were then counted using a gamma scintillation counter (LB2111 Multi Crystal Gamma Counter, Berthold Technologies, Bad Wildbad, Germany) for 179 seconds. Peptide concentrations in each plasma sample were calculated using a non-linear plot (Prism version 6.03, GraphPad Software Inc., CA, USA).

4.3.4 The effect of peripheral GLP-1 administration on food intake, energy expenditure and cardiovascular parameters in *ad libitum* fed rats

Data from the abovementioned feeding studies resulted in the selection of doses of 30, 100 and 300 nmol/kg GLP-1, and 0.3 nmol/kg Ex-4, to use in subsequent CLAMS and telemetry studies.

4.3.5 The effect of peripheral GLP-1 administration on food intake and energy expenditure in *ad libitum* fed rats

Ad libitum fed rats were administered saline, 30, 100, 300 nmol/kg GLP-1, or 0.3 nmol/kg Ex-4 (n=12) by IP injections in a cross-over study design at 1900 hours (early dark phase), conducted as described in section 2.5.3. Food intake was recorded every 12 minutes for 18 hours. Activity, measured by total infrared beam breaks, was recorded every

12 minutes for 18 hours, and high resolution data was recorded every 10 seconds for 40 minutes post administration, and presented as mean activity per minute. VO_2 and VCO_2 were recorded every 12 minutes following administration of the treatments for a period of 18 hours.

4.3.6 The effect of peripheral administration of GLP-1 on cardiovascular parameters and body temperature in *ad libitum* fed rats

Rats were implanted with telemetry probes as previously described in chapter II, section 2.5.4.

4.3.7 The effect of peripheral administration of GLP-1 on conditioned taste aversion in rats

To investigate whether the peripheral administration of GLP-1 is associated with feelings of visceral illness, a CTA protocol was used as described in section 3.3.10.
In the first study, treatments tested included; saline, 10, 30 nmol/kg GLP-1 (n=13-15), and 127mg/kg LiCl (n=5) as a positive control.
In the second study, treatments tested included; saline, 100, 300 nmol/kg GLP-1 (n=13-15), and 48 mg/kg LiCl (n=5) as a positive control.

4.3.8 The effect of peripheral administration of GLP-1 on c-Fos-like immunoreactivity in nuclei of the mesocorticolimbic pathways in fasted rats

4.3.9 Tissue collection

Rats were fasted overnight to avoid variations in neuronal activation due to differences in acute food intake, and received an IP injection of saline, or 10, 30, 100 nmol/kg GLP-1 (n=4-6) in the early light phase. Brains were perfused as described in section 3.3.12.

4.3.10 c-Fos immunohistochemistry

Free-floating sections were stained as described in section 3.3.13. Hypothalamic and brainstem nuclei examined included the SO, ventrolateral hypothalamus (VLH), anterior hypothalamic area (AHA), PVN, VMN, ARC, DMN, lateral parabrachial nucleus (LPN) and NTS. Mesocorticolimbic areas examined included the VTA, CeA, medial orbital cortex (MO), PrL, GI, CPu. All nuclei were defined in relation to anatomical landmarks according to the rat brain atlas of Paxinos and Watson (Paxinos, 2007).

4.3.11 Statistical analysis

All feeding study, CLAMS and telemetry data are expressed as mean ± SEM. Dose-response studies for 5, 10, 30 nmol/kg GLP-1 were analysed using Repeated Measures one-way ANOVA and Dunnet's multiple comparison *post-hoc* test. Feeding studies for 300 nmol/kg and 0.3 nmol/kg Ex-4 were analysed using one-way ANOVA and Dunnet's multiple comparison *post-hoc* test. Plasma GLP-1 levels, and CTA data were analysed using one-way ANOVA using Tukey and Dunnet's multiple comparison *post-hoc* tests, respectively.

All CLAMS and telemetry data were analysed by performing AUC excluding data pre-administration, with a one-way ANOVA and Tukey's *post-hoc* test. High resolution activity data points represent the total infrared beam breaks in one minute. Telemetry data are presented as mean HR and BP in 30 minute intervals.

IHC c-Fos data are presented as the median, inter-quartile and total range, and analysed using Kruskal-Wallis one-way ANOVA with Dunn's multiple comparison *post-hoc* test. All analyses were carried out using Graphpad Prism software (Prism 6.03, GraphPad Software Inc., CA, USA). $P<0.05$ was considered statistically significant.

4.4 Results

4.4.1 The effect of peripheral administration of GLP-1 and Exendin-4 on food intake in fasted rats.

IP administration of 300 nmol/kg GLP-1 significantly reduced cumulative food intake compared to saline control at 0-1h ($p<0.001$; Fig 4.1A) and 0-2h ($p<0.05$; Fig 4.1B). A dose of 0.3 nmol/kg Ex-4 was used; a lower dose than doses of GLP-1 analogs recommended for use in humans, such as saxenda (3 mg/ total body weight daily, equivalent to approximately 115 umol/kg) and exenatide (5-10 ug/ total body weight twice daily, equivalent to approximately 0.9 umol/kg). In rats, 0.3 nmol/kg Ex-4 significantly reduced food intake 0-1 hours following administration ($p<0.05$; Fig 4.1A).

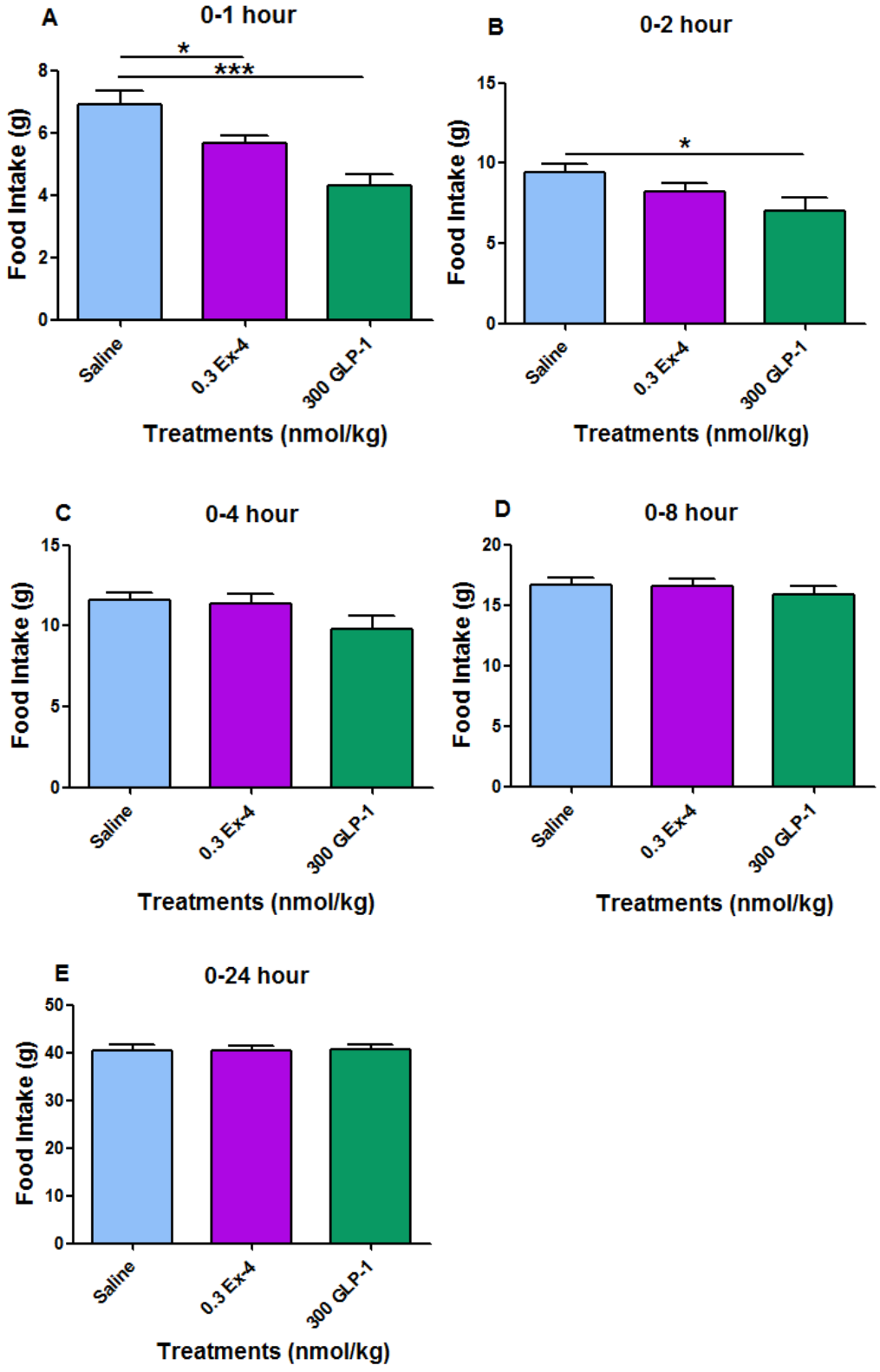


Figure 4.1. The effect of IP administration of GLP-1 and Exendin-4 on food intake in fasted rats during the early light phase.

The effect of intraperitoneal administration of saline, 300 nmol/kg GLP-1, or 0.3 nmol/kg exendin-4 on food intake in overnight fasted rats during the early light phase at 0-1 (A), 0-2 (B), 0-4 (C), 0-8 (D), 0-24 (E) hours post administration. Data presented as mean ± SEM. N = 14 per group. *p<0.05, ***p<0.001 vs saline control, using 1-way ANOVA.

4.4.2 The effect of peripheral administration of GLP-1 on food intake in fasted rats.

IP administration of a range of doses of GLP-1 reduced food intake compared to saline control. Doses of 10 and 30 nmol/kg significantly reduced food intake 0-1 hours following administration ($p<0.05$ and $p<0.001$, respectively; Fig 4.2 A). A dose of 30 nmol/kg GLP-1 also significantly reduced cumulative food intake at 0-2h and 0-8h following administration ($p<0.05$; Fig 4.2 B&D).

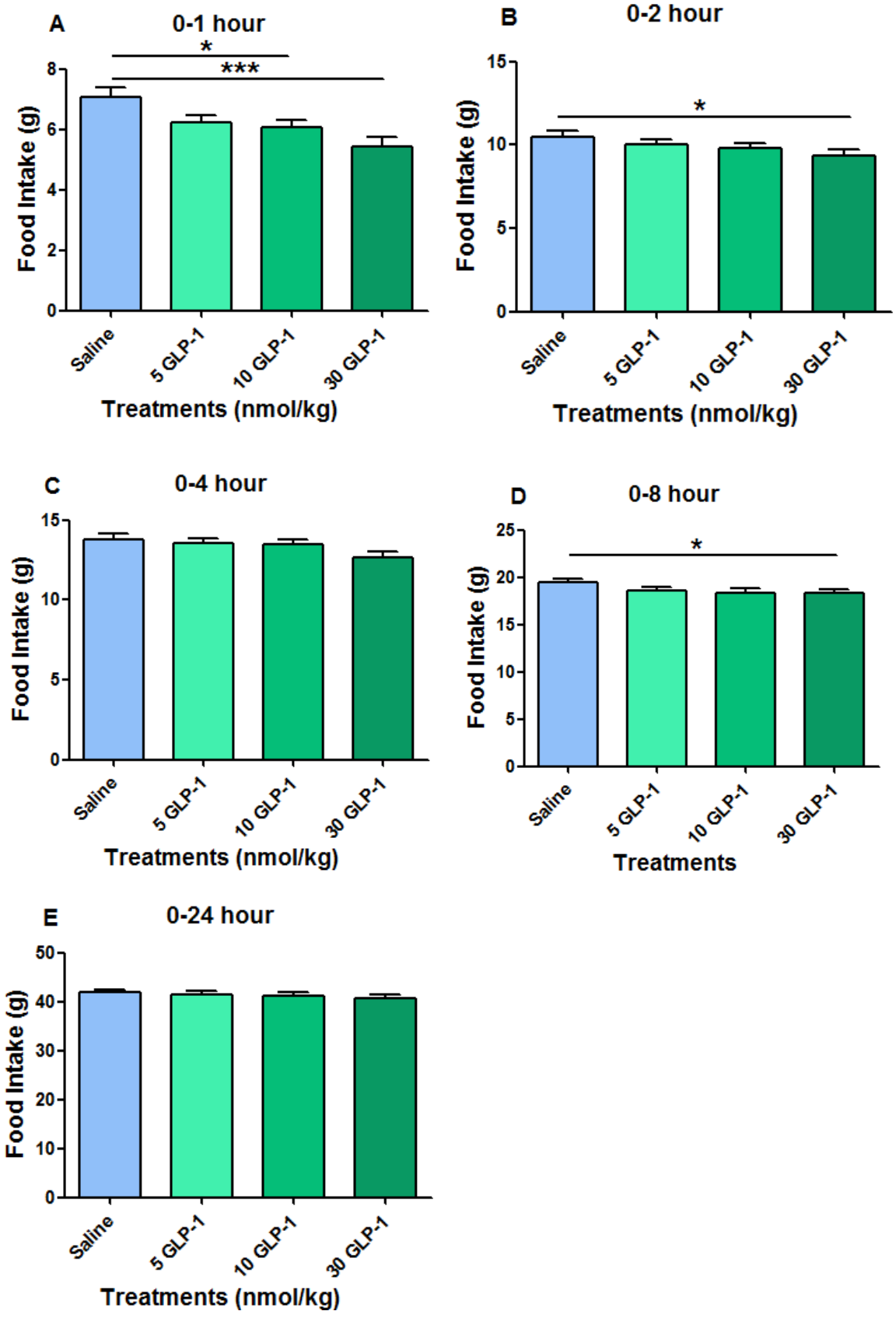


Figure 4.2. The effect of IP administration of 5, 10, 30 nmol/kg GLP-1 on food intake in fasted rats during the early light phase.

The effect of intraperitoneal administration of saline or 5, 10, 30 nmol/kg GLP-1 on food intake in overnight fasted rats during the early light phase at 0-1 (A), 0-2 (B), 0-4 (C), 0-8 (D) and 0-24 (E) hours post administration. Data presented as mean ± SEM. n= 36 per group. *p<0.05, ***p<0.001 vs. saline control, using 1-way ANOVA.

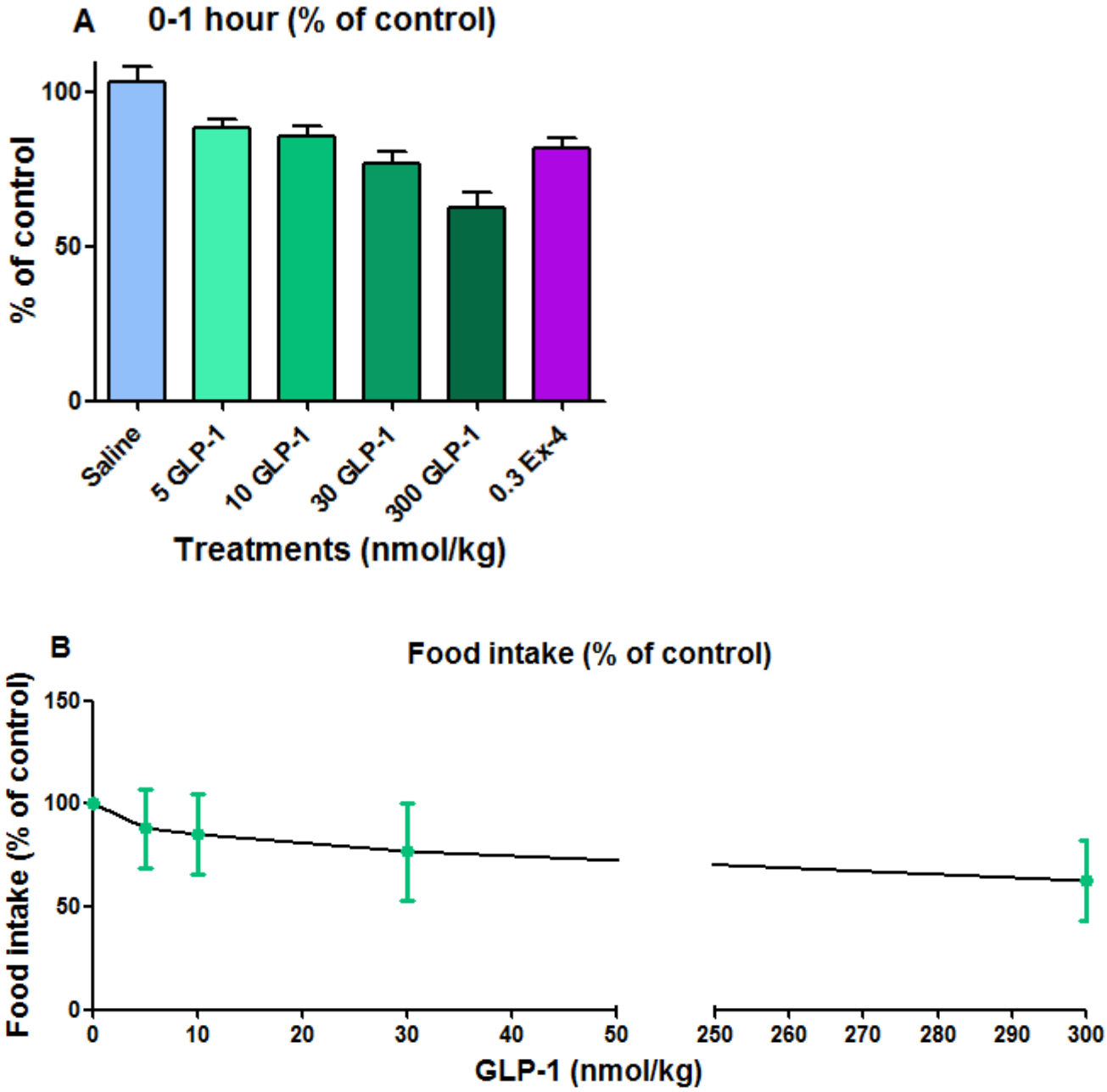


Figure 4.3. Summary of the effects of IP administration of 5, 10, 30, 300 nmol/kg GLP-1 and 0.3 nmol/kg Exendin-4 on food intake in fasted rats during the early light phase.

A summary of the effects of 5, 10, 30 and 300 nmol/kg GLP-1 (**B**) and 0.3 nmol/kg Exendin-4 (A) at 0-1 hours post administration, expressed as % of control. Data presented as mean ± SEM. N = 50 (saline), 14 (5, 10, 30 nmol/kg GLP-1), 36 (300 nmol/kg GLP-1 and Ex-4).

4.4.3 The effect of IP administration of GLP-1 on plasma GLP-1 levels in rats

Circulating GLP-1 was significantly increased in rats fed for 30 mins compared to all fasted rats ($p<0.001$), and in rats fed for 20 min vs rats administered saline and killed at 20 minutes ($p<0.05$). Circulating GLP-1 was significantly increased in rats fed for 30 minutes compared to fasted rats ($p<0.001$). Intraperitoneal administration of 10 nmol/kg GLP-1 significantly elevated the plasma GLP-1 levels in rats at both 10 minutes and 20 minutes following administration compared to fed (blood collected at 20 min), fasted (blood collected at 20 min), fed (30 min), fasted (30 min), saline (10 min) and saline (20 min) ($p<0.001$ for all). Similarly, a higher dose of 30 nmol/kg GLP-1 significantly increased plasma GLP-1 levels at 10 and 20 minutes following administration compared to all controls ($p<0.001$; Fig 4.4).

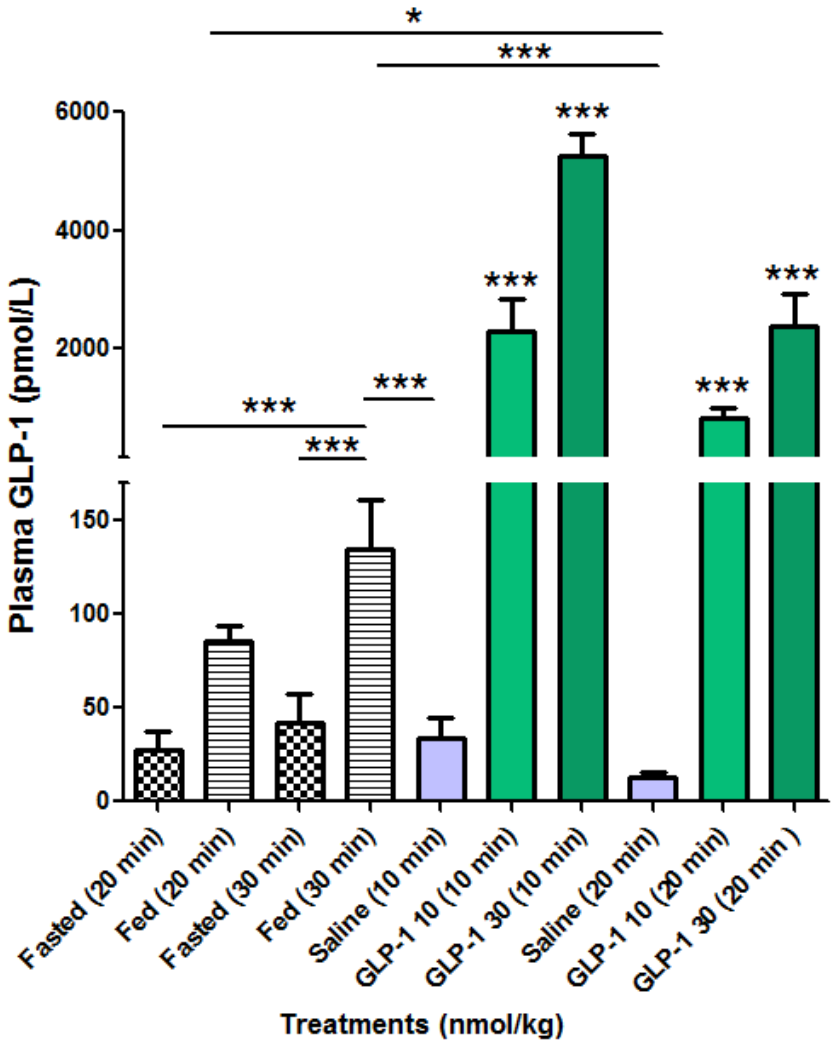


Figure 4.4. The effect of IP administration of GLP-1 on circulating GLP-1 in fasted rats in the early light phase.

The effect of IP administration of saline, 10 or 30 mmol/kg GLP-1 on circulating GLP-1 in overnight fasted male rats at 10 and 20 minutes following administration. Data presented as mean ± SEM. N = 8-12. *p<0.5, ***p<0.001, using 1-way ANOVA. Green bars significant ($p<0.001$) vs. fed (20 min), fasted (20 min), fed (30 min), fasted (30 min), saline (10 min) and saline (20 min) controls.

4.4.4 The effect of peripheral GLP-1 administration on food intake and energy expenditure in *ad libitum* fed rats

To investigate the co-ordinated effects of GLP-1 on food intake and energy expenditure, rats were placed in CLAMS metabolic cages as described in section 4.3.5. A dose of 300 nmol/kg GLP-1 significantly reduced cumulative food intake vs. saline control by 6.36±3.72% in the dark phase (12h following administration; $p<0.05$; Fig 4.5C). Intraperitoneal administration of 300 nmol/kg GLP-1 significantly reduced cumulative food intake vs. saline control in the first 40 minutes by 18.48±13.51% ($p<0.01$; Fig 4.6). A dose of 300 nmol/kg GLP-1 and 0.3 nmol/kg Ex-4 significantly reduced food intake in the first feeding bout vs. saline control ($p<0.05$ and $p<0.01$, respectively; Fig 4.7A), there was also a trend for increased latency to first feeding bout with increasing doses of GLP-1.

A dose of 300 nmol/kg GLP-1 significantly decreased VO_2 in the first hour vs. saline, 30 nmol/kg GLP-1 and 0.3 nmol/kg Ex-4 ($p<0.05$ for all; Fig 4.8), and VCO_2 in the first hour vs. saline, 100 nmol/kg GLP-1 and 0.4 nmol/kg Ex-4 ($p<0.01$, $p<0.01$, $p<0.05$, respectively; Fig 4.9). Intraperitoneal GLP-1 had no significant effect on VO_2 (Fig 4.8) or VCO_2 (Fig 4.9) in rats during the 18 hours following administration. RER was significantly reduced 2h following administration of 300 nmol/kg GLP-1 ($p<0.05$; Fig 4.10). Total activity was significantly reduced in the first 40 mins following administration of 300 nmol/kg GLP-1 ($p<0.001$; Fig 4.12).

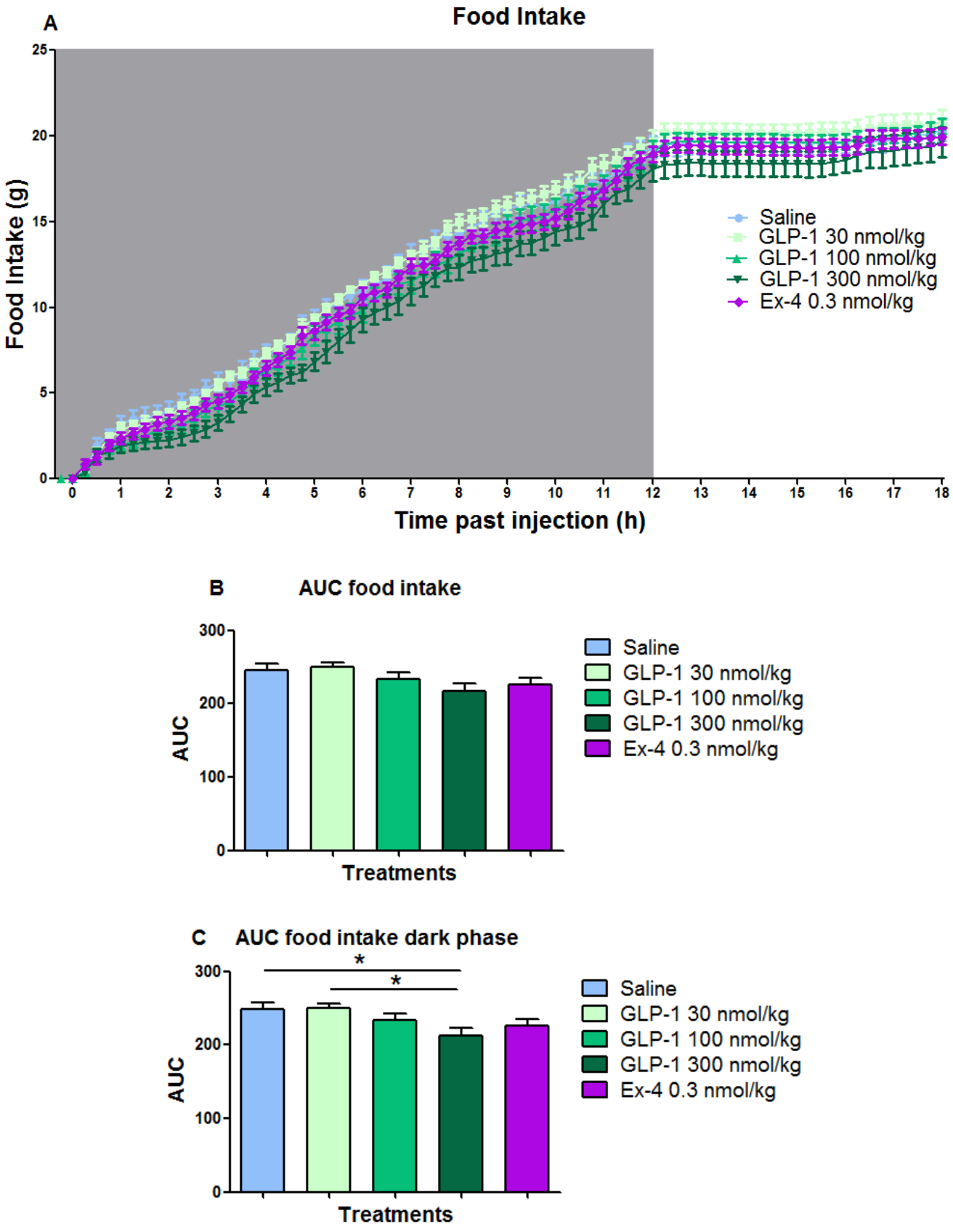


Figure 4.5. The effect of peripheral GLP-1 administration on food intake in fed rats in the early dark phase.

A The effect of saline and 30, 100, 300 nmol/kg GLP-1 or 0.3 nmol/kg exendin-4 on 18h cumulative food intake in *ad libitum* fed rats injected in the early dark phase; food intake recorded every 12 minutes for 18 hours post injection. **B** Area under the curve for graph A. **C** Area under the curve for 12h cumulative food intake (dark phase only). N=12 per group. Data presented as mean ± SEM. **p<0.01, using 1-way ANOVA.

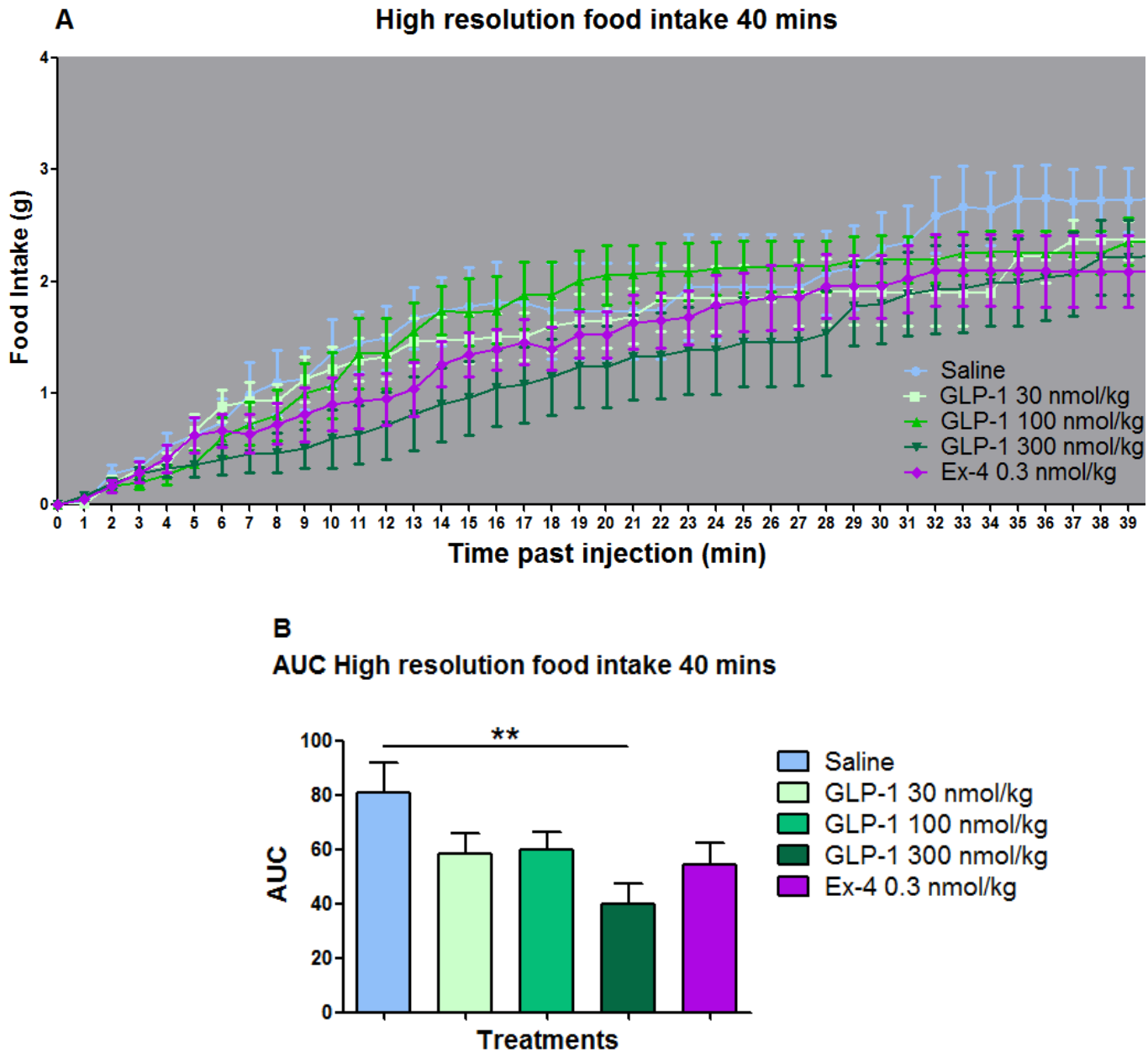


Figure 4.6. The effect of peripheral GLP-1 administration on food intake in fed rats in the early dark phase.

A The effect of saline and 30, 100, 300 nmol/kg GLP-1 or 0.3 nmol/kg exendin-4 on 0-40 minute cumulative food intake in *ad libitum* fed rats injected in the early dark phase; high resolution food intake recorded every minute for 40 mins. **B** Area under the curve for graph A. N=12 per group. Data presented as mean ± SEM. **p<0.01, using 1-way ANOVA.

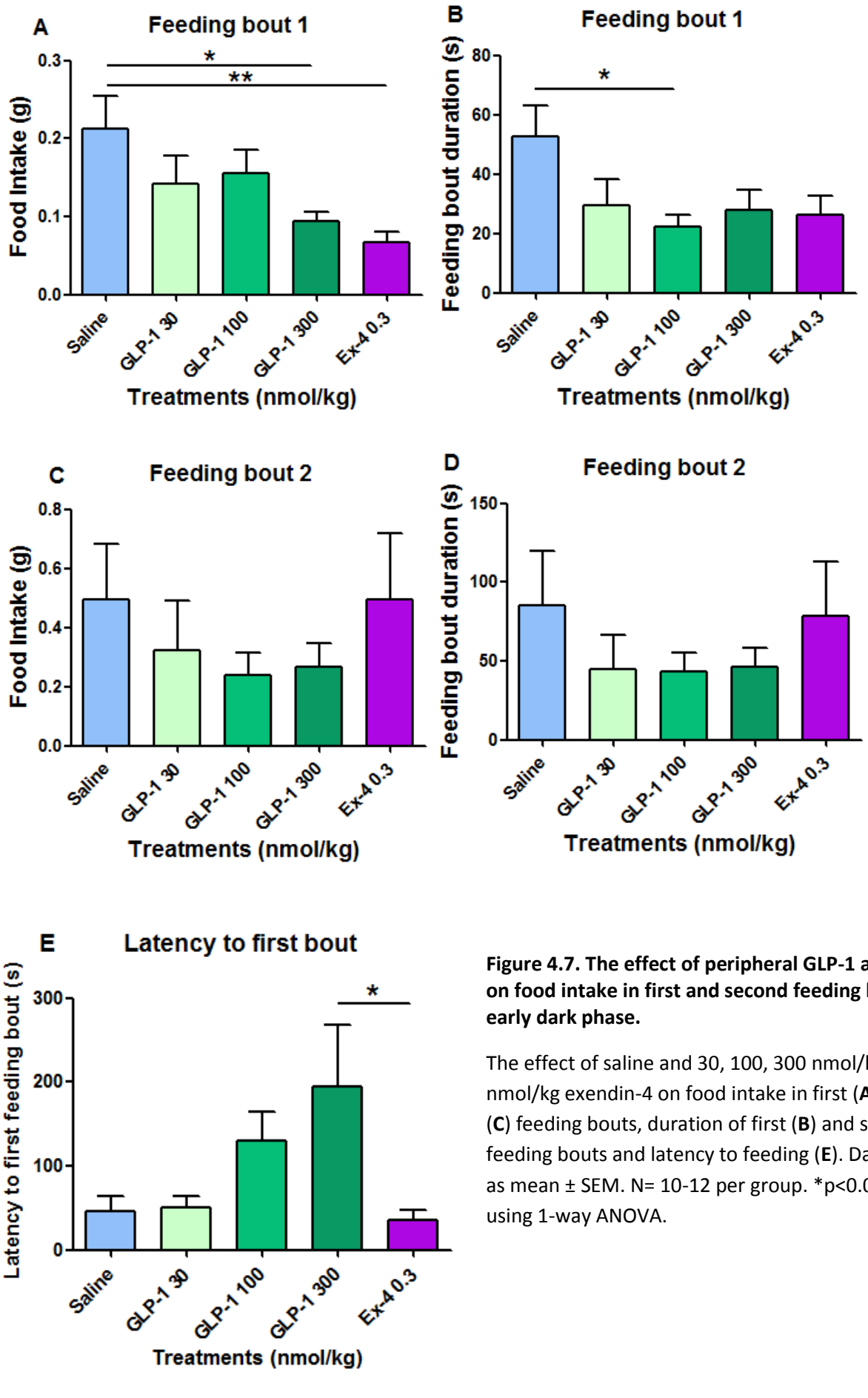


Figure 4.7. The effect of peripheral GLP-1 administration on food intake in first and second feeding bouts in the early dark phase.

The effect of saline and 30, 100, 300 nmol/kg GLP-1 or 0.3 nmol/kg exendin-4 on food intake in first (**A**) and second (**C**) feeding bouts, duration of first (**B**) and second (**D**) feeding bouts and latency to feeding (**E**). Data presented as mean ± SEM. N= 10-12 per group. *p<0.05, **p<0.01, using 1-way ANOVA.

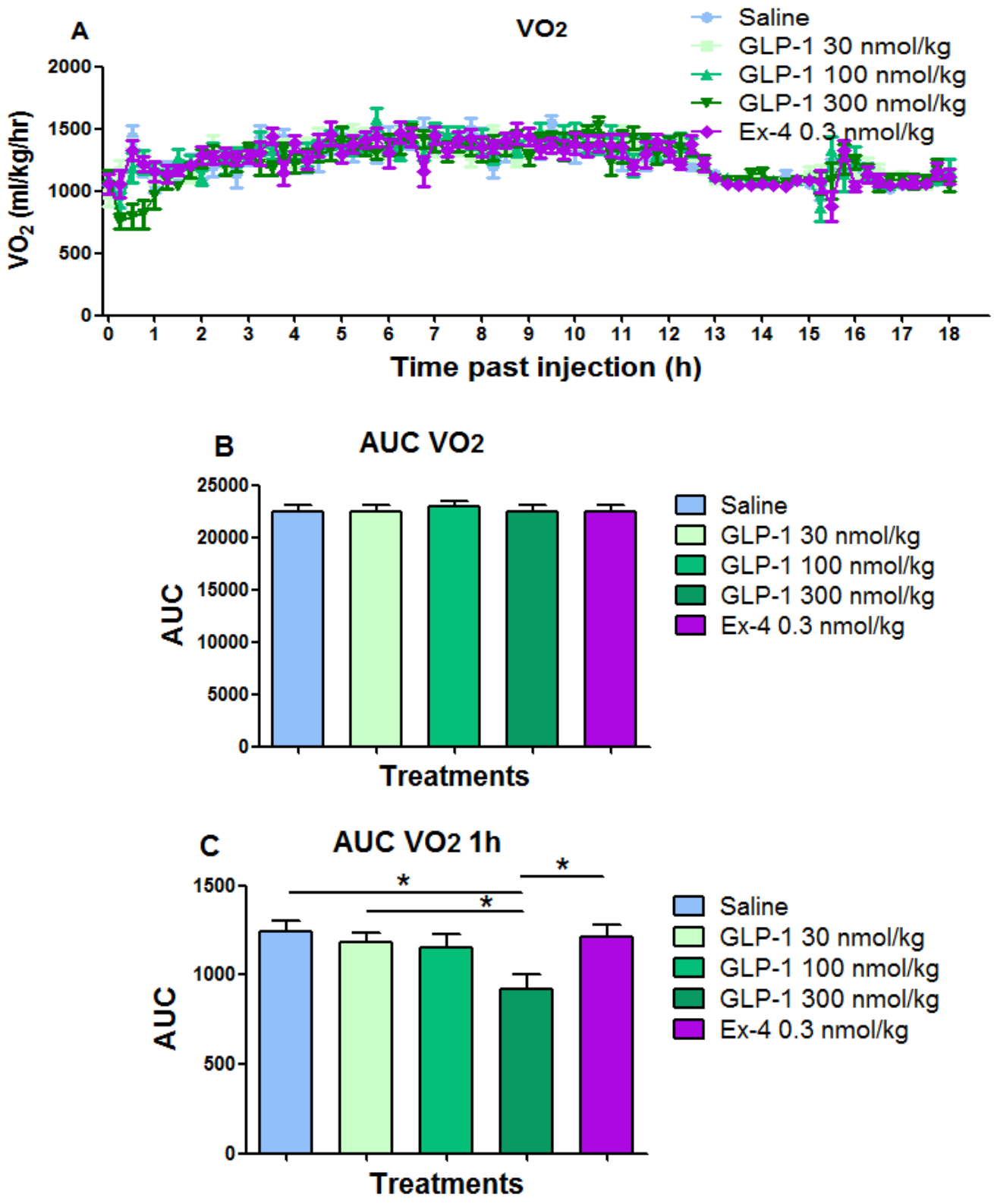


Figure 4.8. The effect of peripheral GLP-1 administration on energy expenditure in fed rats in the early dark phase.

A The effect of saline and 30, 100, 300 nmol/kg GLP-1 or 0.3 nmol/kg exendin-4 on VO_2 in *ad libitum* fed rats in the early dark phase. **B** Area under the curve for A. **C** Area under the curve for A; 0-1h. N=10-12 per group. Data presented as mean ± SEM. *p<0.05, using 1-way ANOVA.

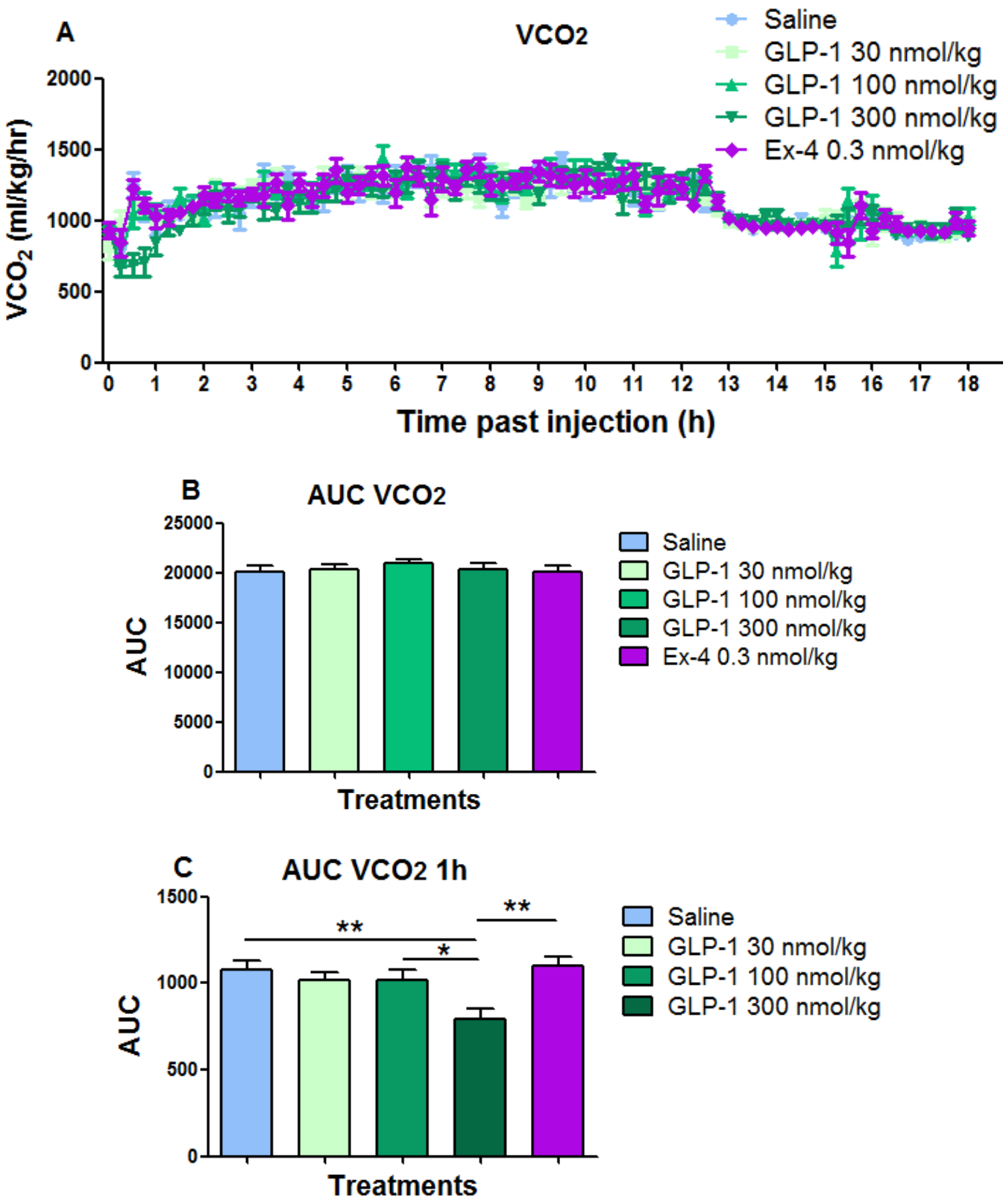


Figure 4.9. The effect of peripheral GLP-1 administration on energy expenditure in fed rats in the early dark phase.

A The effect of saline and 30, 100, 300 nmol/kg GLP-1 or 0.3 nmol/kg exendin-4 on VCO_2 in *ad libitum* fed rats in the early dark phase. **B** Area under the curve for A. **C** Area under the curve for A; 0-1h. N=10-12 per group. Data presented as mean ± SEM. *p<0.05, **p<0.01, using 1-way ANOVA.

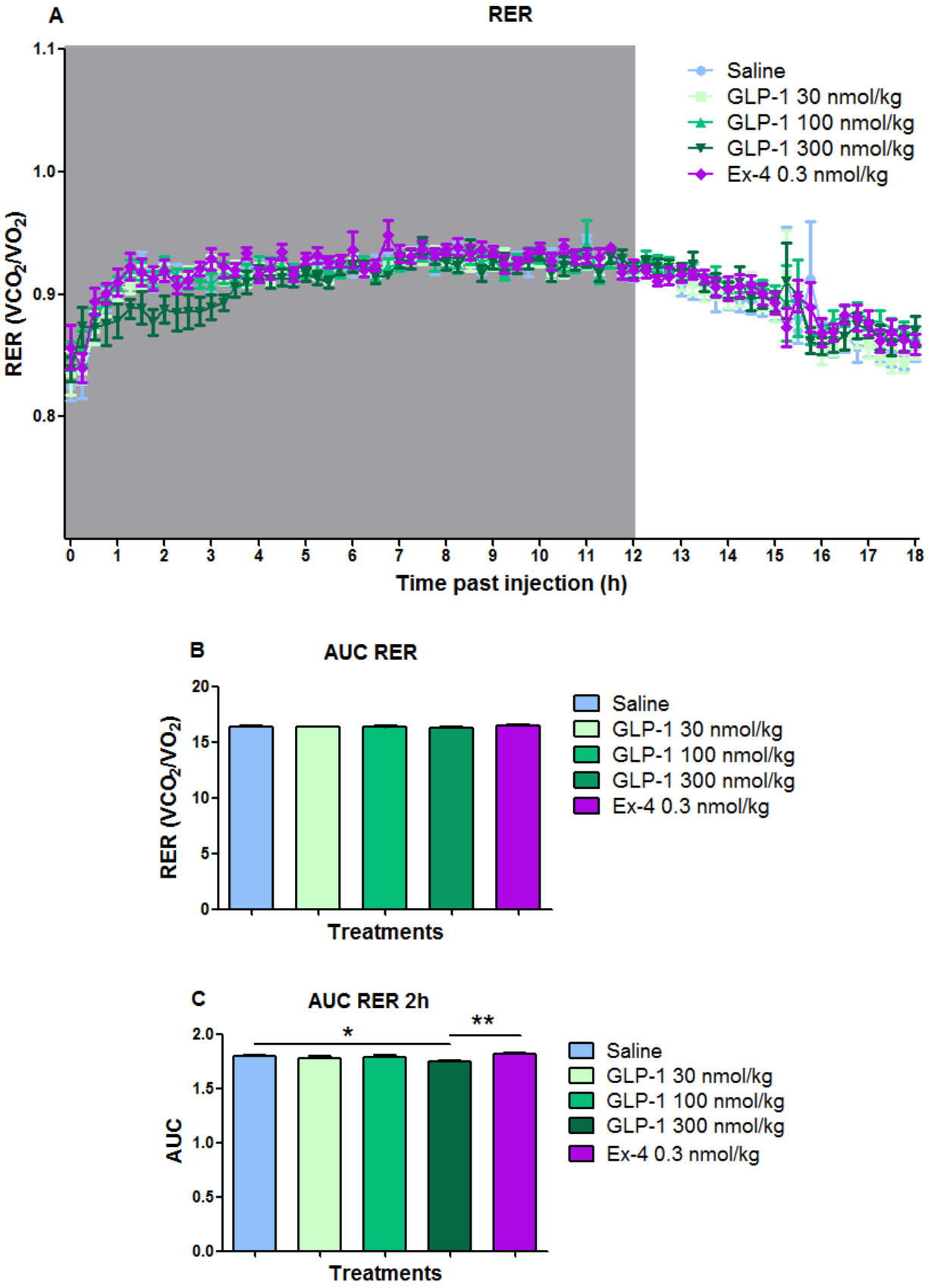


Figure 4.10. The effect of peripheral GLP-1 administration on respiratory exchange rate in fed rats in the early dark phase.

A The effect of saline and 30, 100, 300 nmol/kg GLP-1 or 0.3 nmol/kg exendin-4 on RER in *ad libitum* fed rats in the early dark phase. **B** Area under the curve for A. **C** Area under the curve for A; 0-2h. N=10-12 per group. Data presented as mean ± SEM. *p<0.05, **p<0.01, using 1-way ANOVA.

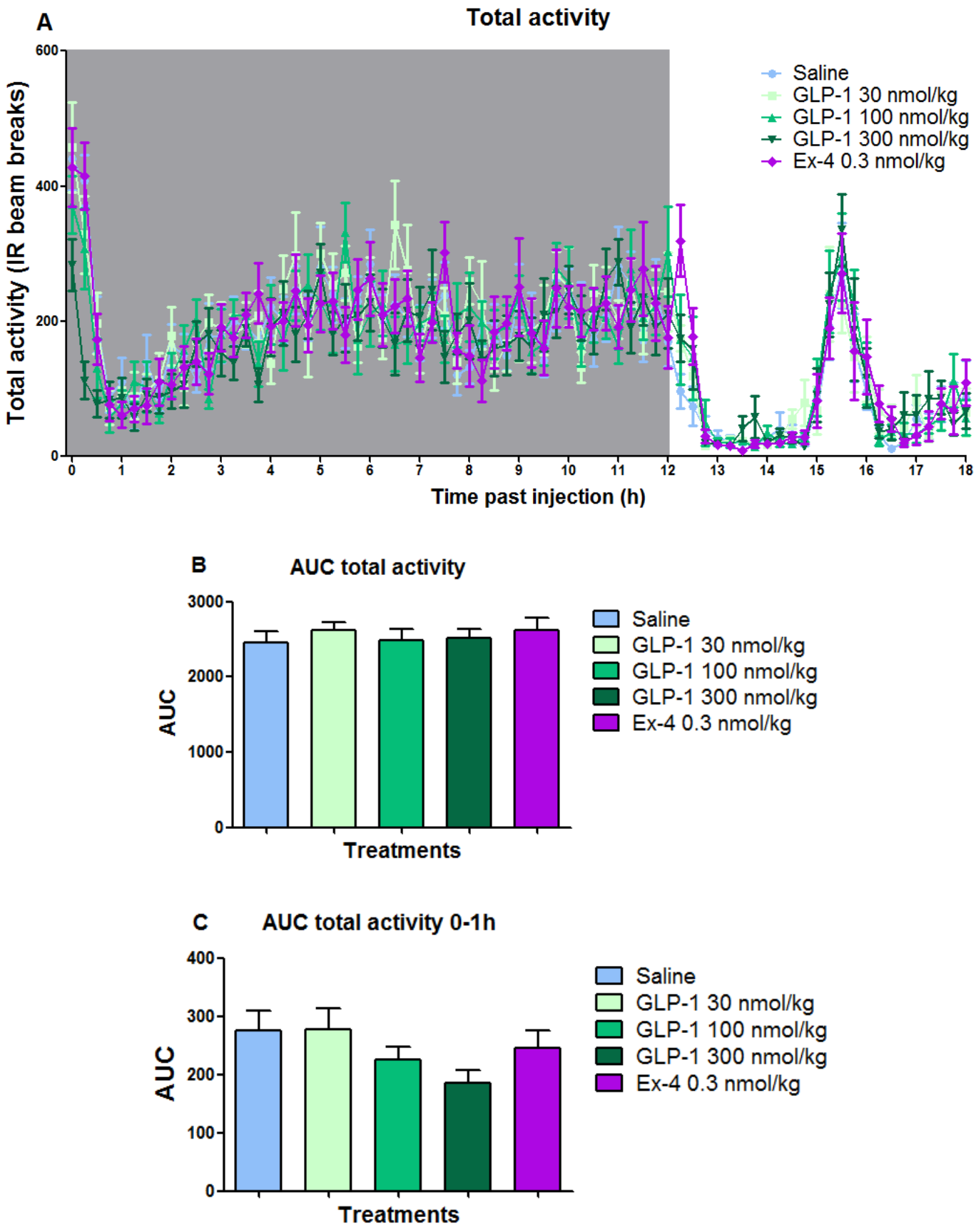


Figure 4.11. The effect of peripheral GLP-1 administration on activity in fed rats in the dark phase.

A The effect of saline and 30, 100, 300 nmol/kg GLP-1 or 0.3 nmol/kg exendin-4 on total activity in *ad libitum* fed rats injected in the early dark phase. Infra-red beam breaks recorded every 12 minutes for 18 hours. **B** Area under the curve for A. **C** Area under the curve for A (0-1h). N=12 per group. Data presented as mean ± SEM.

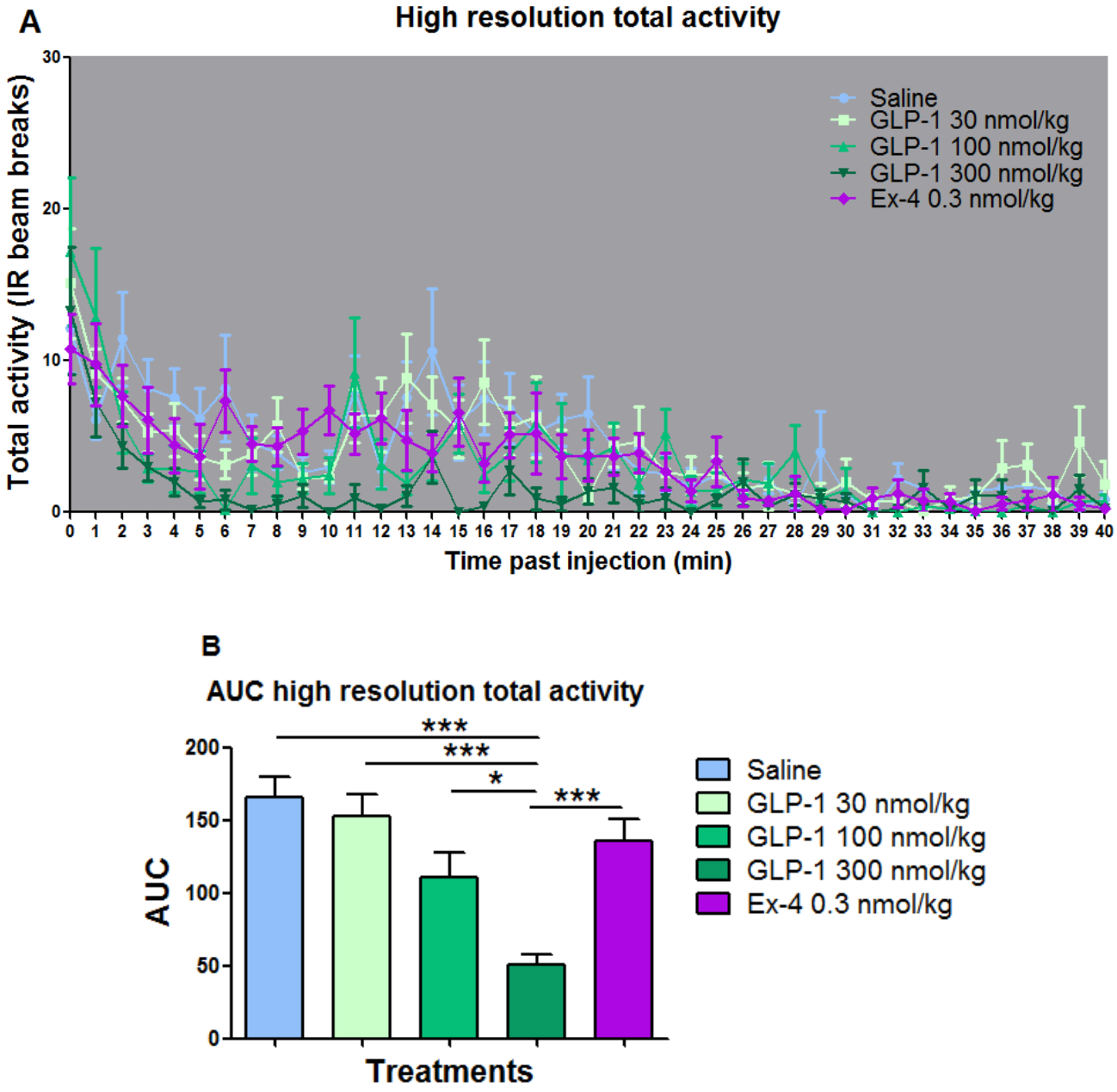


Figure 4.12. The effect of peripheral GLP-1 administration on activity in fed rats in the dark phase.

A The effect of saline and 30, 100, 300 nmol/kg GLP-1 or 0.3 nmol/kg exendin-4 on total activity in *ad libitum* fed rats injected in the early dark phase. High resolution data; infra-red beam breaks recorded every 10 seconds. **B** Area under the curve for A. N=12 per group. Data presented as mean ± SEM. *p<0.05, ***p<0.001, using 1-way ANOVA.

4.4.5 The effect of peripheral administration of GLP-1 on cardiovascular parameters and body temperature in *ad libitum* fed rats

To investigate the effect of GLP-1 on HR, BP and body temperature, rats were implanted with radio-telemetry devices as described in section 4.3.6. Intraperitoneal administration of 300 nmol/kg GLP-1 significantly increased HR in the 5 hours following injection ($p<0.05$; Fig 4.13). There was a trend for an increase in systolic BP following administration of 300 nmol/kg GLP-1 (Fig 4.14). Body temperature was not significantly affected, though it tended to be lower in the group administered 300 nmol/g GLP-1 (Fig 4.16).

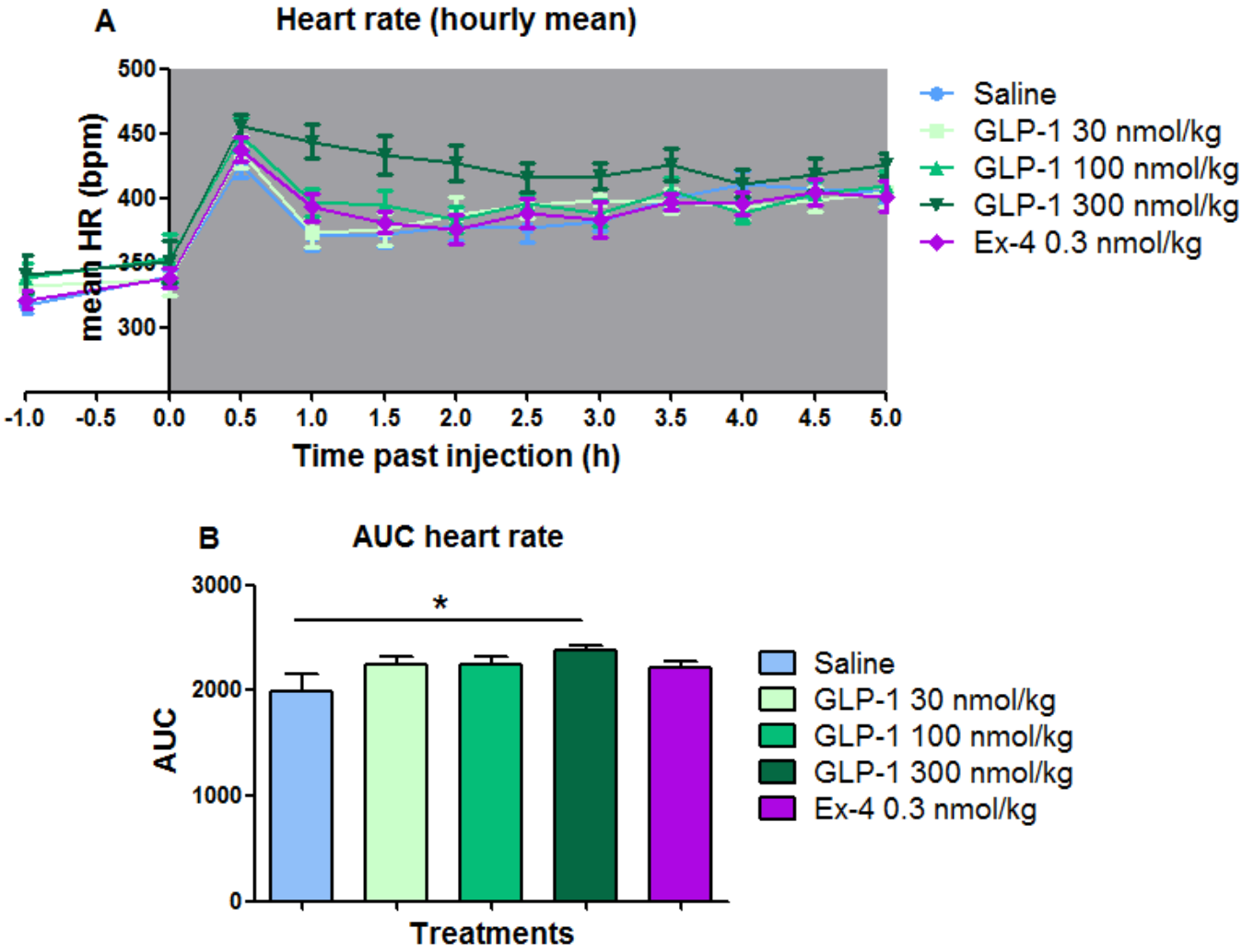
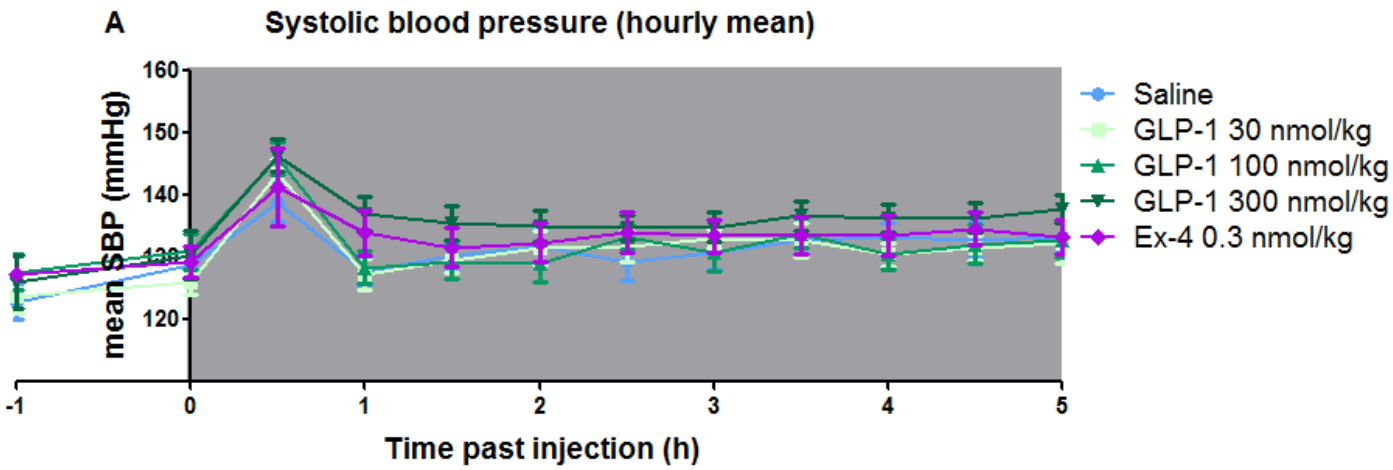


Figure 4.13. The effect of peripheral GLP-1 administration on heart rate in rats.

A The effect of saline and 30, 100, 300 nmol/kg GLP-1 or 0.3 nmol/kg exendin-4 on heart rate in freely moving rats injected in the early dark phase. **B** Area under the curve for A. N=10 per group. Data presented as mean ± SEM. *p<0.05, using 1-way ANOVA.



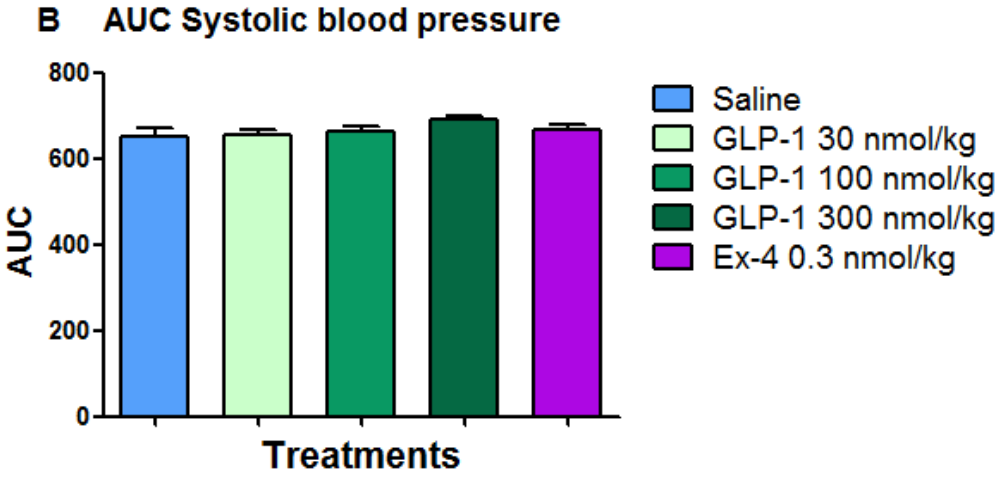


Figure 4.14. The effect of peripheral GLP-1 administration on systolic blood pressure in rats.

A The effect of saline and 30, 100, 300 nmol/kg GLP-1 or 0.3 nmol/kg exendin-4 on systolic blood pressure in freely moving rats injected in the early dark phase. **B** Area under the curve for A. N=9 per group. Data presented as mean ± SEM.

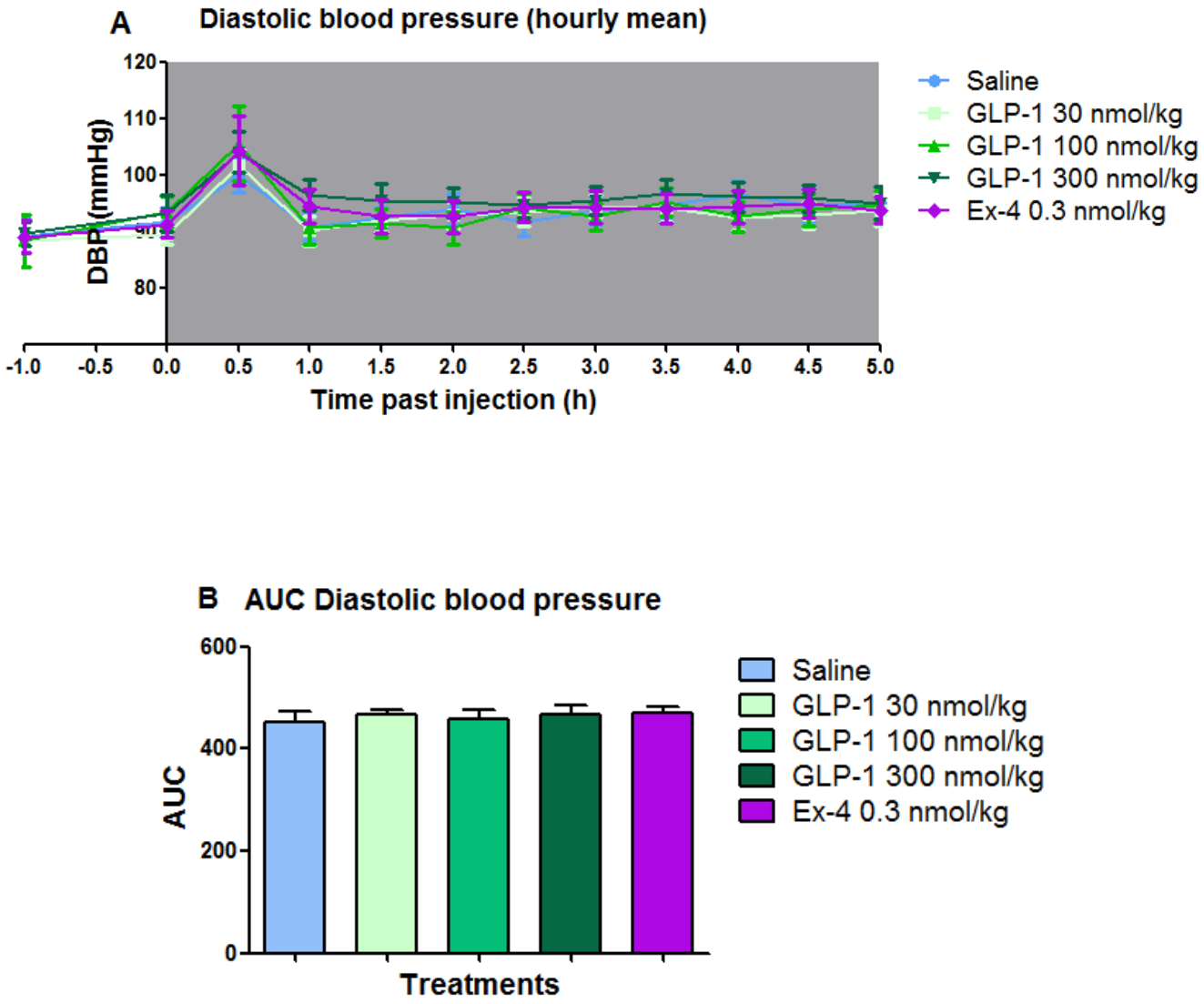


Figure 4.15. The effect of peripheral GLP-1 administration on diastolic blood pressure in rats.

A The effect of saline and 30, 100, 300 nmol/kg GLP-1 or 0.3 nmol/kg exendin-4 on diastolic blood pressure in freely moving rats injected in the early dark phase. **B** Area under the curve for A. N=9 per group. Data presented as mean ± SEM.

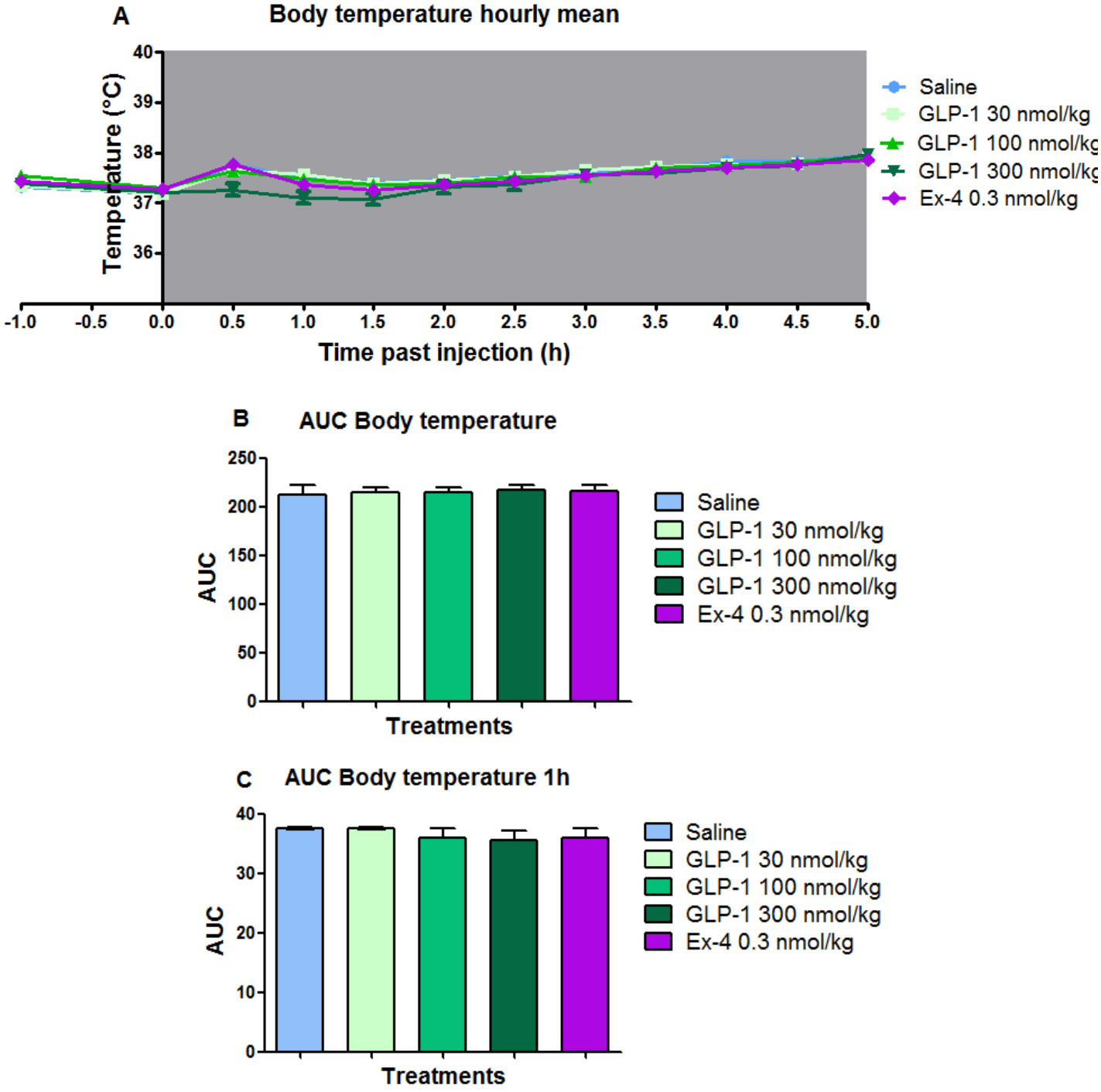


Figure 4.16. The effect of peripheral GLP-1 administration on body temperature in rats.

A The effect of saline and 30, 100, 300 nmol/kg GLP-1 or 0.3 nmol/kg exendin-4 on body temperature in freely moving rats injected in the early dark phase. **B** Area under the curve for A. **C** Area under the curve for A 0-1 h only. N=12 per group. Data presented as mean ± SEM.

4.4.6 The effect of peripheral administration of GLP-1 on conditioned taste aversion in rats

Rats were subjected to a one-bottle conditioned taste aversion protocol as described in section 4.3.7. When administered peripherally, doses of 10, 30, 100 and 300 nmol/kg GLP-1 did not produce a significant CTA vs. saline control. However, there was a trend for decreased Kool-Aid consumption in groups administered doses of 30 nmol/kg GLP-1 and above (Fig 4.17). Lithium chloride induced a significant taste aversion at 48 and 127 mg/kg.

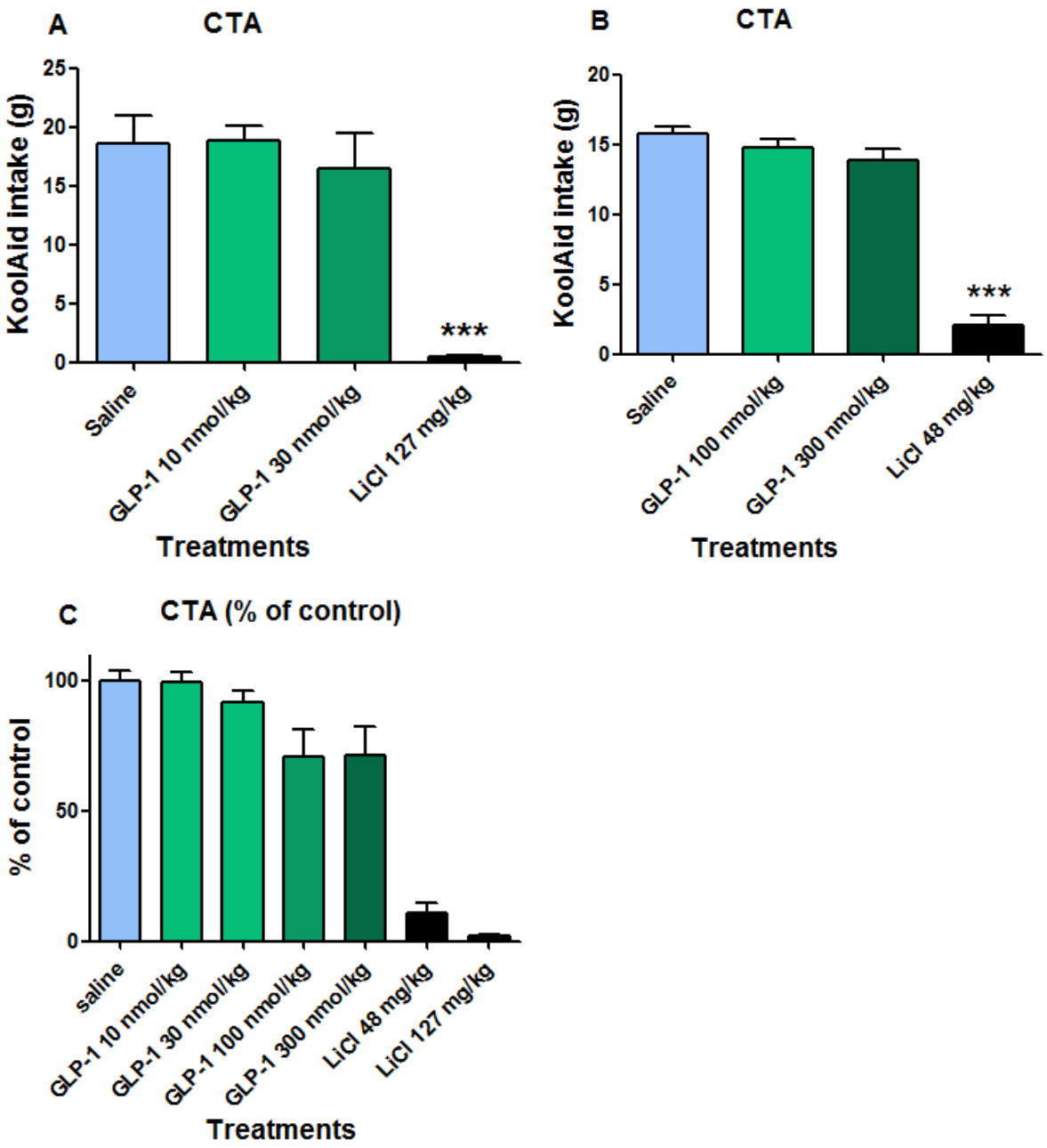


Figure 4.17. The effect of peripheral GLP-1 administration on aversion in rats.

A The effect of saline, 127 mg/kg LiCl or 10 and 30 nmol/kg GLP-1 on conditioned taste aversion in rats. **B** The effect of saline, 48 mg/kg LiCl or 100 and 300 nmol/kg GLP-1 on conditioned taste aversion in rats. **C** A summary of the effects of 10, 30, 100 and 300 nmol/kg GLP-1, 48 and 127 mg/kg LiCl on conditioned taste aversion in rats, expressed as % of control. Data presented as mean ± SEM. ***p<0.001, vs. saline control using 1-way ANOVA. N= 13-15 per saline and GLP-1 groups, n=5 per LiCl group.

4.4.7 The effect of peripheral administration of GLP-1 on c-Fos-like immunoreactivity in the hypothalamus and brainstem of fasted rats

To assess neuronal activation in regions with roles in energy homeostasis, GLP-1 or saline was administered to rats and c-Fos like immunoreactivity was counted. Rats were transcardially perfused 90 minutes after administration as described in section 3.3.12, and brain sections stained as described in 3.3.13.

Peripheral administration of 30 nmol/kg GLP-1 significantly increased c-Fos-like immunoreactivity in the VLA vs. saline ($p<0.05$; Fig 4.18B). A dose of 300 nmol/kg GLP-1 significantly increased c-Fos-like immunoreactivity in the PVN vs. saline ($p<0.01$; Fig 4.18C). Doses of 30 and 300 nmol/kg GLP-1 significantly increased c-Fos-like immunoreactivity in the DMN vs. 10 nmol/kg GLP-1 ($p<0.05$; Fig 4.18E). A dose of 300 nmol/kg GLP-1 significantly increased c-Fos-like immunoreactivity in the LPN vs. saline and 10 nmol/kg GLP-1 ($p<0.05$; Fig 4.18F). c-Fos-like immunoreactivity was significantly increased in the NTS by 300 nmol/kg GLP-1 vs. 10 nmol/kg GLP-1 ($p<0.05$; Fig 4.18G).

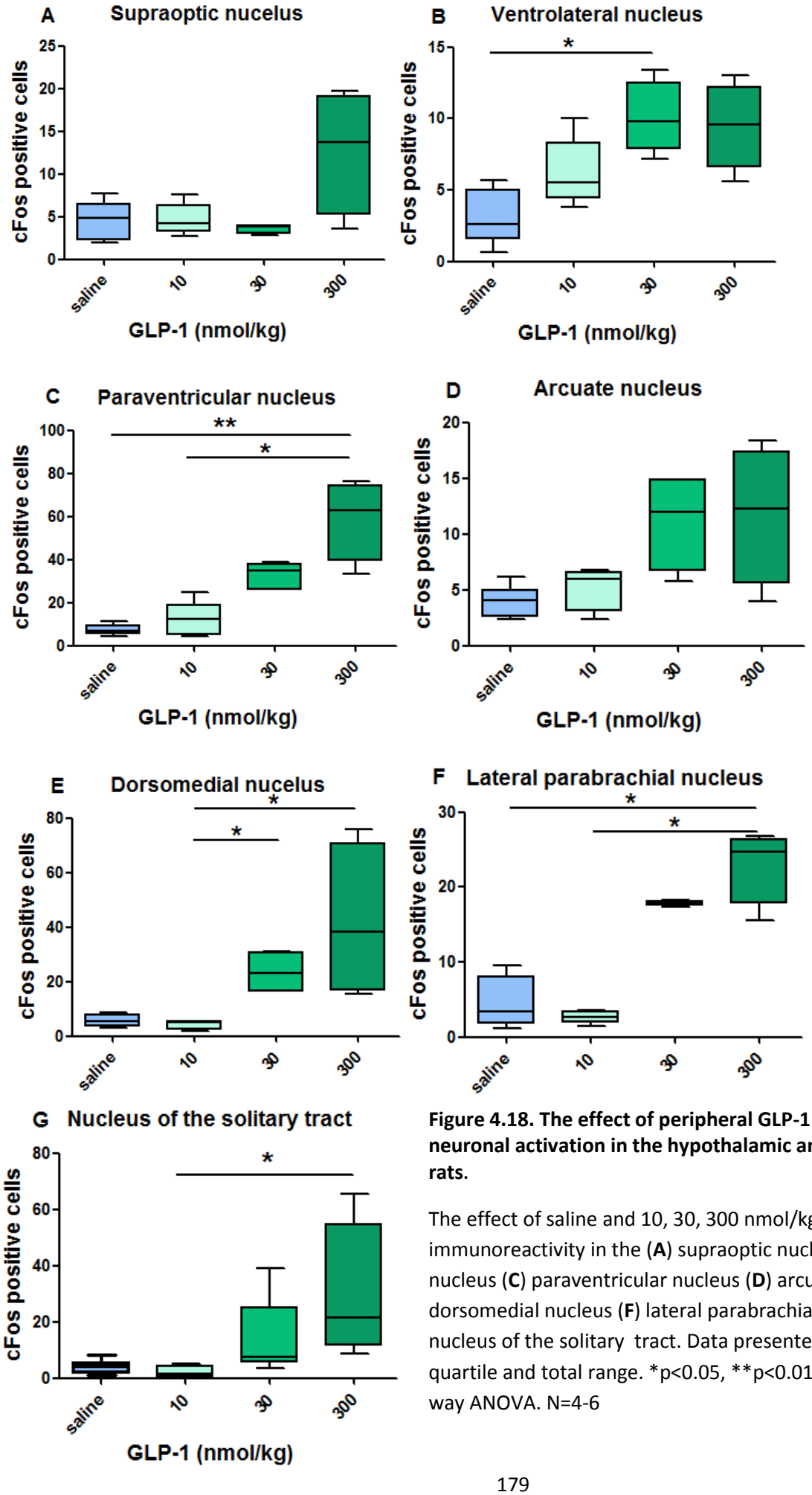


Figure 4.18. The effect of peripheral GLP-1 administration on neuronal activation in the hypothalamic and brainstem nuclei of rats.

The effect of saline and 10, 30, 300 nmol/kg GLP-1 on c-Fos like immunoreactivity in the (**A**) supraoptic nucleus (**B**) ventrolateral nucleus (**C**) paraventricular nucleus (**D**) arcuate nucleus (**E**) dorsomedial nucleus (**F**) lateral parabrachial nucleus and (**G**) nucleus of the solitary tract. Data presented as median, inter-quartile and total range. *p<0.05, **p<0.01, using Kruskal-Wallis 1-way ANOVA. N=4-6

4.4.8 The effect of peripheral administration of GLP-1 on c-Fos-like immunoreactivity in the mesocorticolimbic system of fasted rats

To assess neuronal activation in regions with roles in reward and aversion, GLP-1 or saline was administered to rats and c-Fos like immunoreactivity was counted. Rats were transcardially perfused 90 minutes after administration as described in section 3.2.8.1, and brain sections stained as described in 3.2.8.2.

Peripheral administration of 30 nmol/kg GLP-1 significantly increased c-Fos-like immunoreactivity in the VTA vs. saline ($p<0.05$; Fig 4.19A). c-Fos-like immunoreactivity was significantly increased in the CeA by 30 and 300 nmol/kg GLP-1 vs. saline ($p<0.05$; Fig 4.19B). A dose of 300 nmol/kg GLP-1 significantly increased c-Fos-like immunoreactivity in the MO, PrL and CPu vs. saline ($p<0.05$, $p<0.05$ and $p<0.01$, respectively; Fig 4.19 C-D,F). Doses of 30 and 300 nmol/kg GLP-1 significantly increased c-Fos-like immunoreactivity in the GI vs. saline ($p<0.01$ and $p<0.05$, respectively; Fig 4.19E).

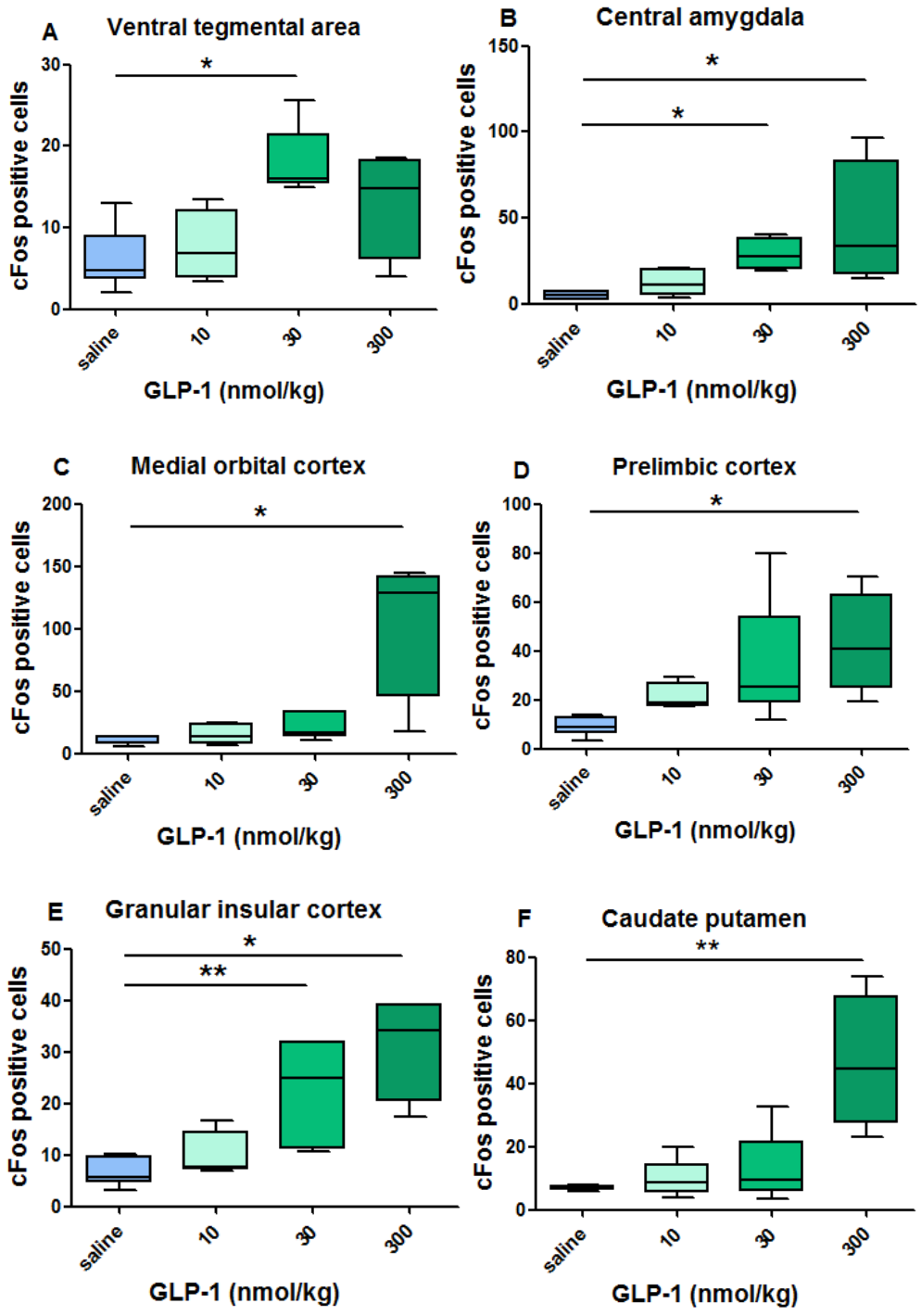


Figure 4.19. The effect of peripheral GLP-1 administration on neuronal activation in the mesocorticolimbic nuclei of rats.

The effect of saline and 10, 30, 300 nmol/kg GLP-1 on c-Fos like immunoreactivity in the (**A**) ventral tegmental area (**B**) central amygdala (**C**) medial orbital cortex (**D**) prelimbic cortex (**E**) granular insular cortex (**F**) caudate putamen (**G**) Nucleus accumbens shell. Data presented as median, inter-quartile and total range. N=4-6. *p<0.05, **p<0.01, using Kruskal-Wallis 1-way ANOVA.

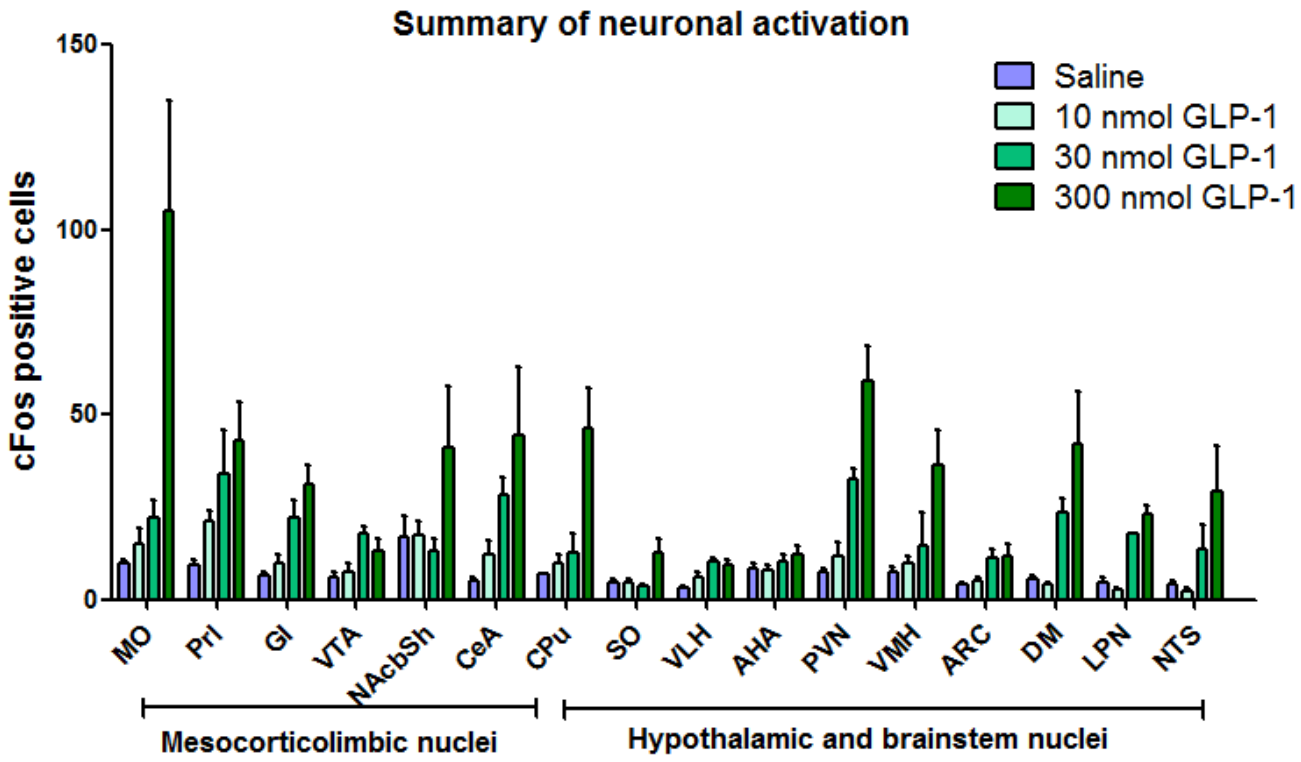


Figure 4.20. Summary of neuronal activation indicated by c-Fos like immunoreactivity in mesocorticolimbic, hypothalamic and brainstem nuclei of the rat brain, following peripheral administration of saline or 10, 30, 300 nmol/kg GLP-1.

MO, medial orbital cortex; PrL, prelimbic cortex; GI, granular insular cortex; VTA, ventral tegmental area; NAcbSh, nucleus accumbens shell; CeA, central amygdala; CPu, caudate putamen; SO, supra-optic nucleus of the hypothalamus; AHA, anterior hypothalamic, area; PVN, paraventricular nucleus; VMH, ventromedial hypothalamus; ARC, arcuate nucleus; DMN, dorsomedial nucleus; LPN, lateral parabrachial nucleus; NTS, nucleus of the solitary tract.

4.5 Discussion

GLP-1 has diverse roles in glucose homeostasis, food intake, energy expenditure, and cardiovascular function (Barragan et al., 1994, Edwards et al., 1998, Goldsmith et al., 2015). Endogenous and exogenous agonism of the GLP-1 receptor reduces food intake in rodents and humans (Secher et al., 2014, Turton et al., 1996). Central GLP-1 has been repeatedly shown to induce CTA in rodents, while intravenous administration can cause nausea and vomiting in humans (Tang-Christensen et al., 1996, Thiele et al., 1997, Amori et al., 2007). The aversive properties of LiCl, a known emetic compound in humans, may be mediated by a specific neuronal population expressing preproglucagon (Seeley et al., 2000). It is possible that GLP-1 receptors located in nuclei with homeostatic roles in food intake mediate the anorectic roles of GLP-1, while a subset of neurons in the brainstem with projections to mesocorticolimbic regions, mediate the aversive properties (Lachey et al., 2005, Kinzig et al., 2002). The work described in this chapter investigated the effects of GLP-1 on food intake, energy expenditure and cardiovascular parameters in rats; with the eventual aim of comparing these effects with those discussed in the previous chapters for leptin, sibutramine, LiCl, and PYY_{3-36}, using the same monitoring system. In addition, the effects of peripheral GLP-1 on aversion and neuronal activation in rats were investigated.

Initial dose-response studies were performed to investigate doses of GLP-1 that reduce food intake in rats. I aimed to establish a dose of GLP-1 that reduced food intake by approximately 15-20%, as I hypothesised that anorectic effects of this magnitude were likely to be mediated by homeostatic pathways. Previous studies found that 10 nmol/kg GLP-1 administered IP reduced first spontaneous meal size in rats (Ruttimann et al., 2009). In additional studies, 10 nmol/kg IP GLP-1 decreased food intake in the first hour by ~10%, 30 nmol/kg by ~20% and 100 nmol/kg significantly reduced food intake by ~25% in the first hour (Dakin et al., 2004). Further studies from the same group found 40 nmol/kg IP GLP-1 also resulted in a ~25% decrease in food intake in the first hour, indicating the potentially similar levels of appetite suppression of doses from 40-100 nmol/kg (Abbott et al., 2006). In the first hour of the current studies, a dose of 10 nmol/kg GLP-1 significantly reduced food intake by 14.36±3.27%, and 30 nmol/kg GLP-1 significantly reduced food intake by 23.19±4.02%. This anorectic effect remained significant at 0-2h, and 0-8h. In addition, a higher dose was required that reduced food intake more powerfully, by around 40-50%, to investigate the effects of levels that were more likely to activate non-homeostatic pathways. Previous studies found a ~20% reduction in food intake in the first hour following a single IP injection of 100 nmol/kg GLP-1 (Abbott et al., 2005a). A dose of 300 nmol/kg GLP-1 significantly reduced food intake at 0-1h by 37.58±5.20%, and this anorectic effect remained significant until 0-2h. Previous data from within the department indicated that 3.0 nmol/kg Ex-4 reduced food intake by 90% compared to saline in rats. In our studies, a much lower dose of 0.3 nmol/kg Ex-4 reduced food intake by 18.07±3.22% at 0-1h. From this data, doses of 30 and 300 nmol/kg were selected for subsequent CLAMS and telemetry studies. In addition, an intermediate dose of 100 nmol/kg GLP-1, and 0.3 nmol/kg Ex-4 were also selected for further investigation.

Plasma samples were assayed using an RIA to determine the circulating levels of GLP-1 following peripheral administration of 10 and 30 nmol/kg GLP-1. Fasted rats were used to exclude variations in endogenous postprandial

GLP-1 release. Both doses resulted in significantly increased levels of circulating GLP-1 compared to all controls, confirming that 10 and 30 nmol/kg GLP-1 result in pharmacological, rather than physiological, circulating levels of GLP-1.

CLAMS studies were subsequently used to detect any changes in the physiology or behaviour in rats administered GLP-1 at various doses. IP administration of 300 nmol/kg GLP-1 significantly reduced cumulative food intake by 6.36±3.72% compared to saline treated rats in the 12h following administration, corresponding to the dark phase. High resolution data revealed that 300 nmol/kg GLP-1 significantly reduced cumulative food intake by 18.48±1.51%, compared to saline, 40 minutes following administration. Doses of 300 nmol/kg GLP-1 and 0.3 nmol/kg Ex-4 significantly reduced chow intake in the first feeding bout compared to saline control, and there was also a trend for increased latency to first feeding bout with increasing doses of GLP-1. Previous studies using 10 nmol/kg GLP-1 administered IP reduced meal size in rats, though without affecting cumulative food intake (Ruttimann et al., 2009). Administration of GLP-1 or GLP-1 receptor agonists has previously been shown to reduce eating bout frequency and meal size (Wright and Rodgers, 2014, Chelikani et al., 2005). The lack of a significant effect of the lower doses of GLP-1 on food intake may reflect the constraints of maximal n-numbers in the CLAMS-telemetry setup, as there are a limited number of cages available. In addition, previous studies using IP GLP-1 have indicated that mild environmental stress may blunt the anorectic effects of gut hormones by an unknown mechanism. The satiating effects of GLP-1 are attenuated in rats returned to a clean cage following administration, compared to those maintained in home cages that had not been cleaned 7 days prior to administration (Abbott et al., 2006). In our studies, rats were housed in CLAMS cages for 5 consecutive days, and returned to their standard home cages for 2 days. Although attempts at acclimatization were made in these studies, 24h may not be long enough, and it is possible that moving animals between novel environments impacts on the feeding response to GLP-1 administration.

There was a trend for reduced activity 40 minutes following administration of all doses of GLP-1. Total activity was significantly reduced in this time period following administration of 300 nmol/kg GLP-1. Agonism of the GLP-1 receptor has previously been shown to attenuate amphetamine-induced increases in activity (Erreger et al., 2012). In addition, GLP-1 receptor agonism by Ex-4 decreases locomotion at doses that reduce food intake and increase total resting time (Mack et al., 2006). A narrow dose range reduces food intake without affecting ambulation in rats and mice. It is therefore feasible that additional GLP-1 receptor populations are recruited at higher doses and result in decreased locomotion (Mack et al., 2006). Peripheral administration of 300 nmol/kg GLP-1 significantly reduced VO_2 and VCO_2 in the first hour following administration. In addition, RER was significantly reduced 2h following administration of 300 nmol/kg GLP-1, indicating increased use of fat as the primary fuel source for energy expenditure, and presumably reflecting the decreased food intake and activity in these rats. Unlike humans, who tend to exhaust their glycogen stores before switching to metabolise fats, rodents appear to modulate their fuel source in response to even relatively small and short term changes in food intake (Friedman, 1998, Le Magnen and Devos, 1982). Telemetry studies indicated that body temperature was not significantly affected by any of the treatments, though there was a trend for decreased body temperature in rats that were administered 300 nmol/kg GLP-1, compared to all other treatments, which may reflect the decreased levels of total activity in this group.

To investigate the effect of GLP-1 on HR and BP, rats were implanted with radio-telemetry devices as described in section 4.3.6. An increase in HR and BP is an expected artefact of handling and injection. This was used to assess correct placement of ECG leads and BP catheters following injection of saline; rats that did not exhibit a change in either parameter were excluded from the appropriate data analysis (Fig 2.19). Intraperitoneal administration of 300 nmol/kg GLP-1 significantly increased HR in the 5 hours following injection (Fig 4.13). The animals in this group were less physically active, and thus these changes cannot be attributed to increased locomotion. There was also a trend for increased systolic BP following administration of 300 nmol/kg GLP-1, though diastolic BP was not affected (Figs 4.14-4.15). Peripheral Ex-4 has previously been shown to increase HR and BP in rats. This effect can be blocked by exendin-(9-39), indicating that peripheral agonism of the GLP-1 receptor is able to alter these cardiovascular parameters (Yamamoto et al., 2002, Barragan et al., 1996).

It is possible that the alterations in cardiovascular function caused by high doses of GLP-1 result in lowered activity, and that they make the animal feel unwell. However, when a CTA protocol was used to investigate the effects of peripheral administration of GLP-1 on aversion in rats, doses of 10, 30, 100 or 300 nmol/kg GLP-1 did not produce a significant CTA (Fig 4.17). A CTA was produced by the positive controls, 127 and 42 mg/kg LiCl. In addition, there was a trend for decreased Kool-Aid consumption in the GLP-1 treated groups, which may be a symptom of low levels of aversion. Previous studies have indicated that marginal aversion may be present following agonism of the GLP-1 receptor, indicated by decreased levels of activity in rats (Wright and Rodgers, 2014). GLP-1 agonists may decrease food intake at a narrow range that do not induce aversion, as low doses of exenatide are able to decrease food intake without inducing CTA or effects on locomotion, while higher doses induce a significant CTA in rats (Mack et al., 2006, Talsania et al., 2005, Liang et al., 2013). However, some doses may still produce low levels of aversion without causing the overt illness detected by a CTA protocol (Mack et al., 2006). Studies in rats administered Ex-4 have also indicated a dose-dependent decrease in non-ingestive behaviours, such as rearing, grooming, sniffing and locomotion, and increased resting. This behavioural profile is not dissimilar to that induced by LiCl, suggesting that agonism of the GLP-1 receptor can result in both anorexia and malaise (Wright and Rodgers, 2014, Ishii et al., 2004). This data suggests that, under circumstances where potentially aversive substances do not manifest in a significant CTA, locomotive and behavioural data may be useful as an alternative method for detecting LiCl-like behavioural signatures, indicative of malaise. In this case, the CLAMS is a particularly useful tool as it allows high resolution recording of activity data, in addition to yielding feeding bout data, such as length and frequency of meals.

To assess neuronal patterns of activation in response to peripheral GLP-1, c-Fos immunohistochemistry was performed following administration of 10, 30 and 300 nmol/kg GLP-1. In the hypothalamic and brainstem nuclei investigated, 10 nmol/kg GLP-1 did not significantly alter c-Fos like immunoreactivity compared to saline. Peripheral administration of 30 nmol/kg significantly increased c-Fos like immunoreactivity in the VLA and DMN, compared to saline and 10 nmol/kg GLP-1, respectively. Peripheral administration of 300 nmol/kg GLP-1 significantly increased c-Fos like immunoreactivity in the PVN, DMN, LPN and NTS. There was also a trend for a dose-dependent increase in neuronal activation in the ARC, though this was not significant, which may reflect the relatively low n-numbers used in this study. These results are similar to those of previous studies, which found GLP-1 induced c-Fos like

immunoreactivity in the PVN, LPN, ARC, AP and NTS. GLP-1 immunoreactivity has previously been detected in the NTS. This nucleus receives vagal afferent innervation from the cardiovascular system (Jin et al., 1988). In telemetry studies, 300 nmol/kg resulted in increased HR and a trend for increased BP; these changes may serve as a peripheral warning of malaise that is sensed by the NTS. Interestingly, no evidence of neuronal activation was found in the AP at any dose of GLP-1, or saline, unlike other central and peripheral studies that have found neuronal activation in the AP (Zuger et al., 2013, Turton et al., 1996, Van Dijk et al., 1996). The reasons for this difference is not apparent, but they may reflect different strains of animal, or perhaps different housing and husbandry conditions.

In the mesocorticolimbic nuclei investigated, 10 nmol/kg GLP-1 did not significantly alter c-Fos like immunoreactivity. Peripheral administration of 30 nmol/kg GLP-1 increased c-Fos like immunoreactivity in the VTA, CeA and GI, and 300 nmol/kg GLP-1 significantly increased c-Fos like immunoreactivity in the CeA, MO, PrL, GI, and CPu, compared to saline. There was also an increase in neuronal activation in the NAcbSh at the highest dose of GLP-1. Again, these data reflect previous studies that have shown GLP-1 induced activation spanning a number of areas in the mesocorticolimbic system (Van Dijk et al., 1996, Turton et al., 1996). This is particularly interesting in the CeA, an area known to process aversion, as injection of GLP-1 directly into the CeA is able to induce CTA without affecting food intake (Kinzig et al., 2002). This data correlates with human studies, in which participants were administered GLP-1 to investigate neuronal activation using fMRI. In these subjects, subsequent energy intake was reduced (independent of nausea), which was associated with a reduction in brain activity in areas involved in reward signalling, including the amygdala, CPu, NAcb, GI and OFC (De Silva et al., 2011). There may appear to be a discrepancy between increased c-Fos staining in our study, and decreased brain activity in the previously mentioned human study. However, this highlights a disadvantage of c-Fos like immunoreactivity: that it is unable to distinguish between inhibitory or stimulatory signalling. Thus, it is unclear whether increases in c-Fos like immunoreactivity are a result of GABAergic or glutamatergic signalling in the brain.

The levels of neuronal activation in the MO, NAcbSh, CPu and CeA in response to 300 nmol/kg GLP-1, appear in accord with the greater effect on food intake following the administration of 300 nmol/kg GLP-1 in the CLAMS studies, compared to other doses tested. In addition, 300 nmol/kg GLP-1 markedly depressed locomotor activity, implying possible sensations of visceral illness. This dose also significantly increased HR, indicating disruption of the normal physiological state by GLP-1, which may contribute to feelings of malaise. Though differences in neuronal activation at the lower doses are discrete, food intake was attenuated by doses as low as 10 nmol/kg GLP-1 in earlier studies. Administration of GLP-1 directly into the CeA has previously been shown to induce CTA independently of effects on food intake (Kinzig et al., 2002). Some areas, including the MO, CPu, and CeA, show very little activation at low doses of GLP-1, but significant activation at 300 nmol/kg GLP-1. These regions of the mesocorticolimbic system may solely mediate the nausea and aversion that is present at the high dose of GLP-1, rather than homeostatic control of food intake. It has been suggested that GLP-1 producing neurons in the brainstem mediate the aversive properties of LiCl (Seeley et al., 2000, Kinzig et al., 2002). These neurons project to nuclei in the mesocorticolimbic systems. The data presented in this chapter support the notion that GLP-1 receptors in the mesocorticolimbic system, particularly the MO, CPu and CeA, are recruited at higher doses, and are thus responsible for mediating the

more powerful anorectic actions of GLP-1, which occur secondary to aversion and reduce the rewarding value of food.

CLAMS was able to provide useful information on food intake, in addition to meal patterns, such as feeding bout length, duration, and latency to feeding, emphasizing the utility of this system in detecting highly structured data that is suitable for use in a novel mathematical model. In addition, CLAMS was able to detect effects on behavioural parameters that imply aversion is present following GLP-1 administration. This effect was not detectable using a standard CTA paradigm, and may represent a novel method for investigating peripheral GLP-1-induced aversion. The novel concept of combining food intake data with meal patterns, behavioural data and telemetry recordings may provide insight into the impact of gut hormones on the continuum of the hunger-satiety-nausea systems. Having characterised the effects of PYY on food intake and other physiological parameters in rats, I proceeded to characterise the effects of GLP-1 on the same biological systems.

Chapter V: General Discussion

5. General discussion

In vivo protocols are helpful to establish whether effects on food intake are due to agents acting specifically in the physiological energy homeostatic pathways, or by non-specifically affecting eating behaviour, such as through toxicity or malaise. Non-specific appetite-inhibitors may result in side effects such as alterations in BP, locomotor activity and disrupted eating patterns. If an appetite-reducing agent is observed to be acting specifically, it likely represents a better target for the development of anti-obesity drugs. This project investigated the effects of peripheral administration of GLP-1 and PYY on food intake, cardiovascular parameters and behaviour in rats, in comparison to the effects of leptin, sibutramine and LiCl on the same parameters. These agents are known to have effects on appetite, thus the current study was not a dosing experiment, but an investigation into the continuum of hunger, satiety and nausea (Fig 1.8, Chapter 1) and the effects of varying doses of gut hormones on these aspects. The eventual aim is to use this data to establish a novel mathematical model that distinguishes between physiological and non-physiological anorectic factors that will facilitate the rapid development of new pharmacological agents, whilst simultaneously reducing animal use across industry and academia.

Investigations into the effects of PYY in fasted rats in home cages were able to detect effects on food intake at low doses, and were comparable to results found by others (Neary et al., 2005, Abbot et al., 2005b). In addition, a standard CTA protocol was able to significantly detect aversion. However, when PYY was administered at similar doses in the CLAMS setting, a significant effect on food intake was only evident at very high doses. At this dose, reductions in food intake were similar to those induced by administration of LiCl, which may indicate that, rather than satiation, feelings of visceral illness were the primary reasons behind reductions in food intake. Performing feeding studies in fasted animals may lead to more substantial reductions in food intake than those in fed animals, due to the increased basal levels of hunger which exacerbates effects on feeding in these animals. Additionally, it is possible that the effects of stress, due to the CLAMS environment and having undergone invasive telemetry surgery, masked the effects of lower doses of PYY to an extent that they were only detectable when overt aversion was present. Due to the additive effects of stress and increased basal levels of anorectic hormones in fed animals, it may be advantageous to carry out studies in fasted animals to see potential effects at the lower doses in the CLAMS setting. However, there is also the possibility that lower doses of PYY may be unable to counter the hunger of fasted animals, which thus may similarly not show a significant response.

Alternatively, the lack of significant effects at the lower doses studied may well reflect the lower n numbers used and the low sensitivity of the statistical analysis utilized in this thesis. Throughout the CLAMS data presented, distinct effects of the lower doses of PYY on food intake can be seen, particularly in the high resolution data (Fig 3.8). However, when converted to AUC and analysed using ANOVA, the advantage of high resolution is lost due to low sensitivity levels. This rudimentary analysis was useful for the purposes of this thesis in demonstrating the cumulative effects of substance administration at set time points. However, continuous data requires more sophisticated analysis to examine how the changes in outcomes vary over time between treatment groups. Therefore an external statistician was consulted, who was able to examine the size of the time by treatment

interaction using multilevel regression analysis. Using these methods, significant differences in food intake are detectable even at the lower doses studied, including a highly significant interaction between treatment and time for all groups ($p<0.001$, data not shown). Furthermore, multilevel regression methods were able to detect a significant effect of PYY on activity, suggesting that, similar to high doses of GLP-1, high doses of PYY decrease activity levels in rodents. Overall, preliminary analysis suggested that the CLAMS system was not sufficiently sensitive to pick up the effects of PYY on behavioural and physiological parameters. However, more appropriate statistical analysis has since highlighted the utility of this data in detecting subtle differences of effects of varying doses of gut hormones on the nausea and satiety pathways.

The c-Fos IHC data was expected to show activation of the hypothalamic nuclei, in particular the ARC, as has been found by others (Batterham et al., 2002, Halatchev and Cone, 2005). However, in the current study, PYY only significantly increased levels of neuronal activation in the mesocorticolimbic pathways, and also the AP, following administration of 300nmol/kg PYY. It is possible that signalling in these pathways mediates the aversive properties of PYY, decreasing palatability of food, while signalling in the AP may reflect peripheral changes, such as alterations in cardiovascular parameters that were apparent at high doses. Though interesting that high doses cause such a clear increase in neuronal activation in the mesocorticolimbic areas, it is unclear why lower doses that had been shown to cause CTA did not result in changes in activation, and indeed why hypothalamic signals failed to be detected. It may be useful in future to look at c-Fos expression over a series of time points, to see whether the sequence of changes in neuronal activation could be better detected. In addition, it is worth noting that c-Fos expression represents a mixture of the effects of environmental cues in the time period before culling, which included overnight fasting, handling and injection. It is thus possible that any discrete effects of the lower doses were masked by the effects of, for example, stress.

The CLAMS system proved more useful in investigating the effects of GLP-1 on food intake and behavioural parameters in rats. GLP-1 significantly reduced food intake, decreasing intake in the first feeding bout and driving a trend for increased latency to feeding. Though no CTA was established following peripheral GLP-1 administration, activity and feeding patterns seen in the CLAMS studies at high doses suggest that aversion was present. Though limited by insensitive analysis methods, reductions in food intake were more obvious in the CLAMS setting, particularly for high resolution data, than those for PYY. This was perhaps unexpected, due to the shorter half-life of GLP-1 than PYY, but may represent a reduced influence of stress on the effects of this peptide, making it more suitable for the experimental conditions used.

Overall, the combination of CLAMS and telemetry proved successful in detecting the effects of GLP-1 at high doses on physiological and behavioural parameters, but was not as useful in investigating the behavioural and activity effects of PYY. This model was not able to distinguish between the effects of lower doses of the peptides, which had previously been shown to reduce food intake to varying degrees, and may show that with the n numbers used that these systems are only effective for detecting results at the nausea extreme of the satiety continuum, with stress masking the effects of physiological satiation that would usually be present after administration of intermediate

doses. The results in this thesis suggest that there are threshold doses at which GLP-1 and PYY reduce food intake, potentially involving predominantly homeostatic brain circuits. Increasing doses past a further threshold may then activate central aversive circuits, reducing food intake non-specifically. Large bolus doses of GLP-1 and PYY may additionally act in a similar fashion to LiCl, bypassing homeostatic pathways, with nausea and aversion representing distinct effects to satiation (Fig 5.1).

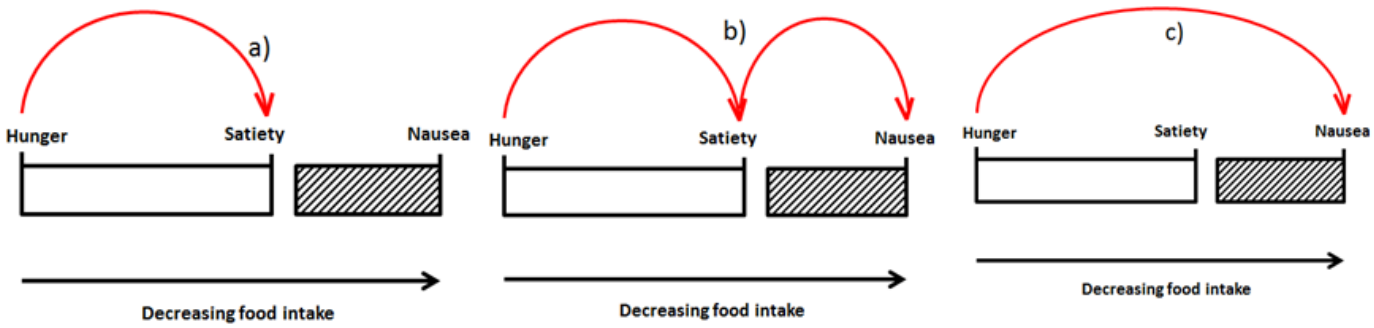


Fig 5.1 A schematic diagram of the continuum of hunger to nausea, with nausea representing an additional independent pathway. A threshold dose exists at which GLP-1 and PYY reduce food intake via predominantly homeostatic pathways (a), increasing this dose activates aversive pathways and food intake is reduced via homeostatic and hedonic mechanisms (b), a distinct pathway exists that is activated by, for example, high bolus doses of anorectic gut hormones and toxic substances (c).

In IHC c-Fos studies, a dose of GLP-1 increased neuronal activation in several areas important in the acquisition of aversion, including the CeA, NTS and mesocorticolimbic system. Activation of mesocorticolimbic brain regions by high doses of PYY and GLP-1 correspond to activation by LiCl. However, LiCl increased activation to a far greater degree, suggesting a distinction between substances that reduce food intake purely by aversion and those that have endogenous homeostatic functions. GLP-1 also induced changes in neuronal activation throughout several hypothalamic nuclei, which may also indicate that the effects of GLP-1 were less affected by stress than those for PYY.

GLP-1 significantly increased c-Fos in the lateral PBN, which receives information from the brainstem and transmits to the hypothalamus. Previous work has identified calcitonin-gene related peptide (CGRP)-expressing neurons in the external lateral subdivision of the PBN as being instrumental in the anorexigenic effects of aversive substances, such as LiCl (Carter et al., 2013). It is therefore interesting that increased signalling was detected in this area in the current study. The PBN may have acted as an important intermediary signal pathway between the brainstem and hypothalamus, as signals were detected in both brain regions, with hypothalamic signalling potentially contributing to the reductions in food intake detected. Furthermore, optogenetic stimulation of CGRP neurons in the external lateral subdivision of the PBN has been shown to induce CTA in the absence of aversive substances, while silencing of these neurons attenuates CTA (Carter et al., 2015). GLP-1 induced significant activation of this area, which corresponds to behavioural CLAMS data at this dose indicating that aversion was present, even though it was not

detectable using the CTA paradigm. As PYY induced a significant CTA, it may have been expected to intensely stimulate the neurons of the PBN. Though PYY increased activation of the PBN, this did not reach significance, and may represent the confounding factor of low n-numbers in these studies. However, I was able to detect significant effects of PYY in mesocortical regions. It is possible that these results demonstrate a disparity between PYY and GLP-1 and the neuronal populations that high doses stimulate in order to cause their aversive effects, with GLP-1 working via similar pathways to LiCl. It is interesting that blocking this PBN circuit does not completely block the effects on food intake of aversive substances, perhaps suggesting the presence of other aversive or non-specific circuits (Carter et al., 2013).

Hypothalamic AgRP neurons are activated or inhibited during periods of energy deficit and excess, respectively (Aponte et al., 2011). Activation of these neurons results in intense food seeking behaviour. During energy deficit, the 'positive valence' of food can potentiate the motivation and performance of behaviour in animals, such as lever pressing, to obtain a reward in response to a cue. Alternatively, 'negative valence' can condition behaviour (Yiin et al., 2005). The valence of a subset of neurons can be distinguished based on whether their stimulation or inhibition corresponds to a preferred cue in an animal in the absence of food (Betley et al., 2015). Studies in animals have indicated that stimulation of neurons in the lateral hypothalamus is associated with positive valence. In addition, starvation-sensitive AgRP neurons have been associated with negative valence, with mice avoiding activation of these neurons. This corresponds to the unpleasant feelings associated with energy deficit in humans, which can be reduced by eating (Betley et al., 2015). The distinction in valence between lateral hypothalamic neurons and AgRP neurons may represent mechanistic differences between homeostatic and hedonic control of food intake. During periods of homeostatic deficit, behaviour is regulated by a combination of positive and negative valence signals, which are modulated by gut hormones acting via both pathways. It is possible that physiological doses of gut hormones, such as GLP-1 and PYY, act via positive valence signals, resulting in decreased food intake associated with positive feelings of satiation (Fig 5.1a). Conversely, high bolus doses of GLP-1 and PYY, and toxic substances, such as LiCl, may act primarily via negative valence signals, leading to decreases in food intake associated with feelings of visceral illness, such as those detected in the current study (Fig 5.1a and b).

To accurately calculate basal metabolic rate, animals must be fasted (which they were not in this study, as food intake was the primary outcome) to exclude the thermic effects of food on energy expenditure. In addition, a multi-channel system was used, which is only capable of sampling gas at a maximum of once per cage per cycle, and is thus not ideal for measuring the acute impact of the compounds tested on energy metabolism (Speakman, 2013). Furthermore, to accurately assess basal metabolic rate, rodents should ideally be housed under thermoneutral temperatures. It has been suggested that animals maintained at normal temperatures are subject to perpetual cold stress and are thus a poor reflection of the situation in humans (Karp, 2012). In spite of this, CLAMS has proven useful for the primary objectives of recording food intake and activity. CLAMS allows high resolution recording of these parameters, so that acute effects of bolus injections of anorectic agents can be studied. CLAMS has proven particularly useful in cases where an overt CTA is not produced, such as following peripheral GLP-1 administration, as depressed ambulation combined with effects on feeding bout size, duration and latency to feeding, imply

disruptions to the BSS and suggest that a certain level of aversion is present. Generating such data may provide an alternative to the CTA paradigm, which involves a lengthy and laborious protocol for the researcher, administration of a toxic and aversive substance, and restricted water access for animals.

Radio-telemetry allows parameters such as HR and BP to be recorded remotely, thus reducing the amount of stress and discomfort caused to animals by taking repeated measurements. Promoting the wellbeing of animals is an important factor when acquiring reliable results, particularly when such data is readily affected by stress. Telemetry also provides the ability to use the same animals in multiple studies, and as they act as their own controls, reduces the total number required. The use of telemetry has been reported to reduce animal use by 60-70% in single studies (Kramer and Kinter, 2003). However, implantation of such devices requires surgical skill and expensive resources. In addition, adding to the animal's mass by probe implantation can in itself cause discomfort; though body weight and behaviour commonly return to normal 5-7 days after surgery (Braga and Prabhakar, 2009). Telemetry provided useful information regarding LiCl and high doses of PYY and GLP-1. However, it failed to detect changes in cardiovascular parameters following administration of lower doses, even following agents known to have cardiovascular effects, such as sibutramine and leptin (Casto et al., 1998, Shek et al., 1998, Overton et al., 2001, Woolard et al., 2004).

Overall, omitting the telemetry surgery would greatly reduce the stress experienced by the animals and may thus improve the quality of CLAMS data collection. Telemetry was not able to detect changes in the physiological parameters of rats in the current studies when it may have been expected, and thus does not add significant value in determining the specificity of the actions of GLP-1 and PYY in the continuum of hunger, satiety and aversion. The CLAMS system proved effective in detecting physiological and behavioural changes following administration of anorectic substances, and thus is likely to represent a useful tool for providing data for the mathematical model, particularly when data is analysed using multilevel regression methods. Further improvements may be made by altering the study design to include shorter periods spent in the CLAMS cages, which contain no bedding or environmental enrichment, and thus are not ideal for animal welfare. Thus future studies investigating the effects of gut hormones on satiety and nausea might be done in rodents that do not have telemetry devices implanted and provide equally valuable data.

If improvements to the experimental design can be achieved that improve the sensitivity of the CLAMS system to detect changes at lower doses, it would be interesting to investigate the effects of combinations of anorectic peptides. Combinations of PYY with either GLP-1 or exendin-4 have been found to have additive effects; they reduce food intake at lower doses when combined than when alone, and these effects persist for longer than with single peptide administration (Neary et al., 2005, Talsania et al., 2005). Again it would be appealing to investigate the neuronal pathways activated by combinations of lower doses of peptides, which more closely mimics the cocktail of hormones released postprandially; one might expect increased activation of homeostatic compared to hedonic regions.

This project has aimed to address the “three Rs”; replacement, reduction and refinement, that have been developed as a framework for humane animal research, and in particular to reduce animal use in subsequent studies. By contributing to the establishment of a mathematical model to distinguish between physiological and non-physiological anorectic factors, this project has aimed to reduce future animal use by decreasing early stage studies within the department, and the findings have the potential to be used across the drug development industry and academia. If such a model can be established, it may be used in place of CTA protocols, which can be stressful to animals and which require relatively large n numbers to demonstrate mild aversive effects. In addition, the telemetry and cross-over design elements of this project have directly addressed reduction and refinement, by using the minimum number of animals and recording cardiovascular parameters with the use of remotely activated probes. We have also used a lower dose of LiCl as a positive control in our CTA studies than is commonly used, which will also contribute to refinement by minimising suffering and distress caused to these animals, and hopefully to animals in other studies if adopted beyond the department.

Understanding the regulation food intake is essential to determine the aetiology of obesity and to identify possible treatments. The novel aspects of this thesis lie in the attempt to develop a system that can investigate the continuum of the hunger-satiety-nausea systems. In addition, this data will feed into the development of a novel mathematical model that can test the specificity of appetite reducing agents. Though there is limited modelling of energy homeostasis in the literature, there are examples for beta cell function in the regulation of blood glucose, and of leptin and adipose tissue (Mari, 2002, Pattaranit and van den Berg, 2008). By collaborating with the Department of Mathematics, Imperial College London, we hope to develop similar models that distinguish between specific satiety and aversive behaviours, and which can incorporate and shed light on different elements of energy homeostasis. Patterns of statistical association between physiological parameters will be detected, taking time-dependent modulations into account. Linear and nonlinear latent variable regression methods will be used; such multivariate models allow for simultaneous detection of co-regulated factors. It is likely that the more sensitive empirical methods used in mathematical modelling are capable of distinguishing statistical associations that were not apparent in my initial analyses. Due to the ability of CLAMS to provide high resolution information regarding activity and feeding patterns, as dicussed above, it is possible that telemetry will not be required to further develop the model.

Consequently, further *in vivo* studies using specific and non-specific appetite-inhibitors will be required to test the model. Preliminary analysis of the data I have generated by collaborators in the Department of Mathematics suggests that feeding data obtained from the CLAMS is highly structured and thus appropriate for mathematical modelling, highlighting the power of using a multidisciplinary collaboration to answer important questions in the regulation of energy homeostasis and metabolism. Further work is now required to determine the utility of such modelling in reducing animal use.

The results of my studies suggest that the effects of GLP-1 and PYY on appetite and aversion are complex, but likely represent separate systems that are activated differentially by different circulating levels of these hormones. Further

work is now required to characterise the precise neuronal circuitries involved. Generation of novel stochastic models that predict how the internal metabolic state affects feeding behaviour will allow better prediction of physiological responses, and may aid the design of pharmacological interventions for obesity, while reducing animal numbers in preclinical work to discover novel anti-obesity therapies.

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Appendix 1: Solutions

Antifreeze
30% (v/v) ethylene glycol and 20% (v/v) glycerol in 0.01M PBS.

Avidin-biotin complex (ABC) solution
0.5% (v/v) avidin and biotin from the Vectastain Elite avidin-biotin-complex kit (Vector Laboratories, UK).

Blocking Solution
3% (v/v) goat serum and 0.25% (v/v) Triton X in 0.01M PBS.

3,3'-Diaminobenzidine (DAB) solution (50mg/ml)
1g $(NH_2)_2C_6H_3C_6H_3(NH_2)_2$ (DAB) (Sigma, Aldrich, UK) and 20ml GDW. Stir in the dark to dissolve, filter through 11µm pore diameter filter paper. Store at -20°C.

Dextran coated charcoal solution
2.4g charcoal and 0.24g dextran dissolved in 100ml phosphate buffer with gelatine.

Formaldehyde solution (4%)
100ml 37% HCHO (formaldehyde) solution (VWR, UK) to 900ml of 1xPBS. Store at 4°C.

H_20_2 (0.6%)
0.6% H_20_2 (v/v) in methanol.

Phosphate buffer (0.06M)
48g di-sodium hydrogen phosphate ($Na_2HPO_4.2H_20$), 4.14g potassium dihydrogen phosphate (KH_2PO_4), 18.61g EDTA ($(HO_2CCH_2)_2NCH_2CH_2N(CH_2CO_2H)_2$), 2.5g sodium azide ($NaN_3$) were dissolved in 5L of cooled boiled GDW, pH 7.29, stored at 4°C.

Phosphate buffer with gelatine
Phosphate buffer (0.06M) with 12.5g gelatine.

Phosphate buffered saline (PBS) (1M)
87g Sodium chloride (NaCl; VWR, UK), 14.1g di-sodium hydrogen phosphate (Na_2HPO_4, VWR, UK) and 2.72g potassium dihydrogen phosphate (KH_2PO_4,VWR, UK) in 1L GDW.

Phosphate buffered saline (PBS) (0.01M)
1:100 dilution of 1M PBS.

Secondary antibody diluent
0.3% BSA (w/v) in 0.25% (v/v) 10% (w/v) Triton-X 100 solution in GDW.

Sucrose solution (40%)
400g sucrose (Tate & Lyle, UK) dissolved in 1L GDW.

Tris/HCl solution (0.1M) (pH 7.6)
12.1g Trizma base in 1L GDW, pH 7.6 with HCl.

Veronal Buffer
5.15g sodium barbitone ($C_8H_{11}N_2NaO_3$) and 0.15g sodium azide (NaN_3) dissolved in 0.5L boiled GDW.