The role of the preproglucagon neurons in cardiovascular control, energy homeostasis and glucose homeostasis

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Declaration of originality

I, Daniel Reece Cook, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, the source of the information will be clearly identified in the thesis

Abstract

Glucagon-like peptide-1 (GLP-1) is produced in the gut and central nervous system. Microinjection of GLP-1 analogues into specific brain regions causes hypophagia, induces insulin release and tachycardia. GLP-1 is produced within the brain by preproglucagon (PPG) neurons, situated in the caudal brainstem. PPG neurons project to nuclei involved in cardiovascular control, glucose homeostasis, and food intake.

I hypothesised that PPG neurons are the endogenous source of GLP-1 in the brain, and their activation should reproduce the effects seen upon injection of exogenous GLP-1 into the brain. I demonstrated that selective chemogenetic activation of PPG neurons increased heart rate, but, their inhibition did not reduce it. These results demonstrate that PPG neurons can modulate heart rate but are not doing so under resting conditions. Similarly, I showed that PPG neuron activation acutely but not chronically decreases food intake.

To further understand under which circumstances PPG neurons are activated to control food intake, c-Fos- immunoreactivity (C-Fos-IR) was used to detect neuronal activity in response to stimuli. There were few C-Fos-IR positive PPG neurons with normal feeding, but intake of highly palatable Ensure (Vanilla milkshake) substantially increased the number of C-Fos-IR positive PPG neurons suggesting that they are activated when consuming a high calorific and/or a high-volume meal. This suggests that PPG neurons induce satiety only in response to large or palatable meals. Neither chemogenetic activation nor ablation of the PPG neurons affected glucose tolerance, arguing that PPG neurons do not modulate blood glucose levels.

The results presented here provide evidence for the physiological role of PPG neurons in satiety, implicating them as a possible target for weight loss drugs. Additionally, PPG neurons are capable of raising heart rate, however further studies must define under which conditions PPG neurons induce tachycardia. As these results mimic the effects of exogenous GLP-1, it seems likely that PPG neurons induce satiety and tachycardia through the release of GLP-1.

Impact Statement

Glucagon-Like Peptide-1 (GLP-1) is produced in the gut and in the brain. GLP-1 has been identified to strongly induce insulin release and regulate appetite. The ability of GLP-1 to induce insulin release has identified GLP-1 analogues to be used for the treatment of type 2 diabetes since 2009 in the UK. As GLP-1 has been further identified to reduce food intake and promote weight loss, GLP-1 analogues have begun to be used as treatment for obesity in the U.S. Unfortunately, GLP-1 analogues have been demonstrated to induce tachycardia consistently across a multitude of species. As obesity is commonly associated with comorbidities, specifically cardiovascular diseases such as myocardial infarction and stroke, treatment with a drug that induces tachycardia increases the risk of cardiovascular complications, in those that are already susceptible. Therefore, it is important to understand the mechanism in which the tachycardia is induced by GLP-1 analogues, in an effort to avoid the side effect, while maintaining the insulinotropic and anorexigenic effects of GLP-1.

Working under Dr. S.Trapp, with the support of the British Heart Foundation, the work presented in this thesis focussed primarily on the GLP-1 produced in the brain. In this study it was demonstrated that the GLP-1 producing neurons in the brain were the primary source of GLP-1 in the central nervous system. Although the GLP-1 neurons were generally considered the source of brain GLP-1, it had not previously been demonstrated. This discovery implicated the GLP-1 neurons to have a role in control food intake, control of blood glucose and control of the cardiovascular system, as the application of GLP-1 in the brain has been shown to have affected these 3 processes. Expanding on these ideas, the GLP-1 neurons did, in fact, have the capacity to reduce food intake and increase heart rate, but they did not alter blood glucose. The further

clarification of the GLP-1 neurons in this role has expanded the knowledge on how appetite is regulated and has increased the understanding of how GLP-1 induces tachycardia.

The data produced in this thesis may also be used medically. Based on the discoveries in the lab and evidence presented by other studies, it may be possible to retain the anorexigenic and blood glucose lowering effect of GLP-1, without inducing tachycardia. It appears that the tachycardia induced by GLP-1 analogues is due to the GLP-1 in the brain, and the anorexigenic and blood glucose effects can be mediated through action outside the CNS. Therefore, production of a GLP-1 analogue that does not enter the CNS will produce the blood glucose lowering and food intake reducing effect without the side effect of tachycardia. These data represents the possibility for a new obesity and type 2 diabetes GLP-1 based treatment, that would reduce the side effects of current GLP-1 analogues, allowing safer treatment.

Contents

Figures & Tables

1. Introductions

2. Methods

3. Role of the preproglucagon neurons in metabolic control

4. Tachycardia: GLP-1 and the preproglucagon neurons

5. In vivo activation of the preproglucagon neurons

6. Summary and perspective

Figure 6.1 Hypothesised pathway of peripheral and central GLP-1 to induce hypophagia and tachycardia

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Abbreviations

Neuroanatomical Abbreviations

AP: Area postrema
ARC: Arcuate nucleus
BNST: Bed nucleus stria terminalis
CAA: Central autonomic area
CNS: Central nervous system
CVO: Circumventricular organ
DMH: Dorsomedial hypothalamus
DMNX: Dorsal motor nucleus of the vagus
IML: Intermediolateral nucleus
IRT: Intermediate reticular nucleus
NAc: Nucleus accumbens
NAm: Nucleus ambiguus
NTS: Nucleus tractus solitarius
PBN: Parabrachial nucleus
PVN: Paraventricular nucleus of the hypothalamus
RVLML Rostral ventral lateral medulla
VMH: Ventromedial hypothalamus
VTA: Ventral tegmental area

Miscellaneous

AAV: Adeno-associated virus
Ach: Acetylcholine
AChR: Acetylcholine receptor
aCSF: Artificial cerebrospinal fluid
ATP Adenosine triphosphate
AUC: Area under the curve
AV: Atrio-ventricular
BBB: Blood brain barrier
BPM: Beats per minute
BSA: Bovine serum albumin
cAMP: Cyclic adenosine monophosphate
CCK: Cholecystokinin
CNO: Clozapine *N*-Oxide
CNS: Central nervous system
CRE: Cre-recombinase
DPP-IV: Dipeptidylpeptidase-IV
DREADD: Designer Receptor Exclusively Activated by Designer Drugs
DTA: Diphtheria toxin subunit A
eGFP: Enhanced green fluorescent protein
Ex-4: Exendin-4
Ex-9: Exendin-9
GLP-1: Glucagon-like peptide-1 (7-36) amide
GLP-1R: GLP-1 receptor
GRPP: Glicentin related polypeptide

GOI: Gene of interest
IP: Intervening peptide
i.c.v.: Intracerebroventricular
i.p.: Intraperitoneal
i.v.: Intravenous
MAP: Mean arterial blood pressure
MPF: Major proglucagon fragments
NA: Noradrenaline
NPY: Neuropeptide-Y
PKA: Protein Kinase-A
PPG: Preproglucagon
PC: Prohormone convertase
SA: Sino-atrial
RFP: red fluorescent protein
YFP: Yellow fluorescent protein

1. Introduction

Heart disease and diabetes are among the top causes of death in developed countries according to the World Health Organisation (WHO) (World Health Organisation). The rise in both diseases can be majorly attributed to the rise in obesity, which is occurring across most developed countries. Obesity is associated with a multitude of life-threatening comorbidities including cardiovascular disease, type 2 diabetes and stroke (Mccowen and Blackburn, 1980). Obesity is not only life threatening, it is also life-restricting, with many of the afflicted suffering from a reduced quality of life. Obesity is estimated to be responsible for 35.8 million Disability Adjusted Life Years (DALYs), therefore it is crucial to understand the causes and mechanisms of obesity, and its co-morbidities in an attempt to reduce the impact of obesity (World Health Organisation).

Obesity is diagnosed when the body mass index (BMI) of a person has exceeded 30 (NHS). Obesity is defined as abnormal or excessive fat accumulation that may impair health (World Health Organisation). The general cause of obesity is considered to be an energy imbalance, where calories that are ingested, heavily exceed those that are expended. Therefore, obesity can be tackled from two fronts: reducing the energy consumed or increasing the energy expended i.e. by decreasing food intake or increasing exercise.

Food intake (appetite regulation) is a complex process governed by neuronal and hormonal mechanisms. In this thesis, I focus on the appetite regulatory gut hormone glucagon-like peptide-1 (GLP-1). GLP-1 is a potent insulinotropic (induces insulin release), and a regulator of food intake. The insulinotropic properties mark GLP-1 analogues as ideal for treatment of type II diabetes. More recently, the appetite regulatory properties of GLP-1 have been recognised, as the GLP-1 analogue

liraglutide (marketed as Saxenda) has been approved by the FDA for treatment of weight management in the U.S (Saxenda, Novo Nordisk; Mehta, Marso and Neeland, 2017). As the clinical use of GLP-1 analogues increases, there has also been an increase in the number of studies addressing how exactly GLP-1 works. In animal studies, GLP-1 has been demonstrated to induce tachycardia and hypertension. As GLP-1 is used for the treatment of the obese, tachycardia and hypertension increases the risk of cardiovascular disease, in those who are already susceptible. Therefore, it is important to understand all the effects of GLP-1, to correctly balance the risk and benefits, when used in the treatment of diabetes and obesity. In this thesis, I will expand on the current knowledge of the roles of GLP-1, in regard to glucose homeostasis, appetite regulation and the cardiovascular system.

1.1 Glucagon-like Peptide-1

1.1.1 Post-translational processing of proglucagon to produce GLP-1

Glucagon-Like Peptide-1 (GLP-1) is produced from the glucagon gene. The glucagon gene is transcribed into the preproglucagon mRNA, which is translated into the proglucagon prohormone. The proglucagon peptide is then post-translationally processed, to produce the peptides outlined in Fig 1.1. In the intestinal L-Cells and in the CNS proglucagon is processed by prohormone convertase-1/3 (PC-1/3) to produce GLP-1, GLP-2, oxyntomodulin, glicentin-related pancreatic polypeptide (GRPP) and intervening peptide-2 (IP-2). In the pancreatic α-cells, the proglucagon protein is post-translationally cleaved by PC-2 to produce glucagon, GRPP, IP-1, and major proglucagon fragments (MPF) (Druckers, Mojsov, and Habener, 1986). This thesis will focus on GLP-1, but it is important to note that GLP-2, oxyntomodulin, GRPP, and IP-2 are produced in tandem with GLP-1, and possibly released together.

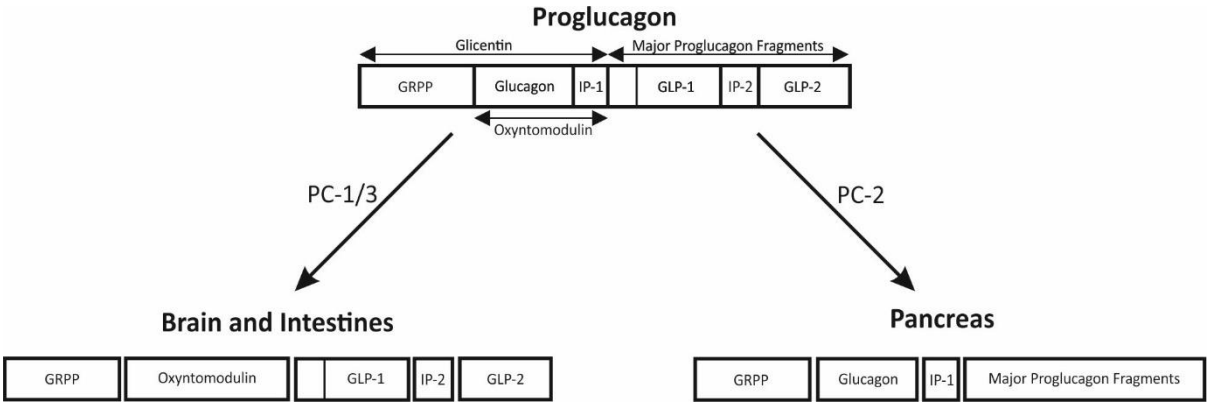


Figure 1.1 Post-translational processing of proglucagon: The prohormone proglucagon is post translationally processed dependent on the tissue in which it is expressed. The brain and the intestinal L-cells proglucagon is cleaved in glicentin related pancreatic polypeptide (GRPP), oxyntomodulin, glucagon-like peptide-1 (GLP-1), intervening peptide-2 (IP-2) and glucagon-like peptide-2 (GLP-2) by prohormone convertase-1/3 (PC-1/3). In the pancreatic α-cells the proglucagon prohormone is cleaved into GRPP, glucagon, intervening peptide-1 (IP-1) and major proglucagon fragments (MPF) by prohormone convertase-2 (PC-2).

1.1.2 Peripheral release of Glucagon-like peptide-1

GLP-1 is released from L-cells in the gastrointestinal mucosa, in response to luminal sugars, amino acids and fatty acids (Holst, 2007; Reimann *et al.*, 2008). Once released GLP-1 diffuses across the basal lamina, into the lamina propria, where it enters the bloodstream through the capillaries (Dailey, 2013). Active GLP-1 (7-36) is quickly metabolised by dipeptidyl peptidase-IV (DPP-IV), cleaving two N-terminal amino acids generating GLP-1 (9-36), the inactive form of GLP-1 (Baggio and Drucker, 2007). DPP-IV is widely expressed across a multitude of tissues and cell types including the kidneys, lungs, adrenal gland, liver, intestines, spleen, testis, pancreas, CNS, lymphocytes, and macrophages (Baggio and Drucker, 2007). DPP-IV is also expressed on the surface of the endothelial cells lining blood vessels that interact with the intestinal mucosa where GLP-1 is secreted from the L-cells (Baggio and Drucker, 2007). This results in more than half of the GLP-1 that enters the bloodstream being

inactivated (Hansen *et al.*, 1999). As a consequence of wide DPP-IV expression, GLP-1 has a short half-life, between 1-2 minutes (Deacon *et al.*, 1996).

Due to the short half-life of GLP-1, the GLP-1 receptor (GLP-1R) agonist, Exendin-4 (Ex-4), is often used to study the effects of GLP-1. Exendin-4 (Ex-4) was discovered in the saliva of the Gila monster and is not inactivated by DPP-IV (Egan, Clocquet and Elahi, 2002). Consequently, Ex-4 remains active in the bloodstream for significantly longer, allowing for the more comprehensive study of the effects of GLP-1R activation. Peripheral GLP-1 is defined here as GLP-1 outside the central nervous system (CNS). Studies using exogenous GLP-1, Ex-4, and GLP-1R antagonist (Exendin-9) have demonstrated that peripheral GLP-1 acts to reduce gastric motility, suppress appetite and lower blood glucose (Shah and Vella, 2013; Nadkarni, Chepurny, and Holz, 2014).

1.1.3 Central Glucagon-Like Peptide-1

Although GLP-1 was initially discovered in the gut, it was soon found to be expressed in the CNS (Samson *et al.*, 1979; Druckers and Asa, 1988; Jin *et al.*, 1988). The preproglucagon (PPG) neurons are a population of neurons that are defined by the production of glucagon products. The PPG neurons were identified initially in rats using immunohistochemistry for GLP-1/GLP-2 or in situ hybridisation (Jin *et al.*, 1988; Merchenthaler, Lane and Shughrue, 1999; Vrang and Larsen, 2010). More recently, the PPG neurons were characterised in mice through the expression of YFP under the glucagon promoter (Reimann *et al.*, 2008; Hisadome *et al.*, 2010; Llewellyn-Smith *et al.*, 2011, 2013, 2015). As PPG is under the control of the glucagon promoter, YFP is produced in the PPG neurons, allowing their identification by YFP fluorescence. The PPG neurons are believed to produce GLP-1 to be used as a neurotransmitter and are possibly the source of brain GLP-1. The cell bodies of PPG neurons reside in the

caudal nucleus tractus solitarius (NTS); the intermediate reticular nucleus (IRT); and along the midline, ventral to the hypoglossal nucleus (Fig 1.2) (Merchenthaler, Lane and Shughrue, 1999; Hisadome *et al.*, 2010; Vrang and Larsen, 2010; Llewellyn-Smith *et al.*, 2011, 2013) There is also a population of PPG neurons in the olfactory bulb and in the caudal spinal cord (Theibaud et al., 2016) . The olfactory bulb PPG neurons appear to be granule cells or short axon cells and are therefore believed to be interneurons (Merchenthaler, Lane and Shughrue, 1999). The PPG neurons in the spinal cord do not send ascending axons to the brain, as the PPG neurons reside in the lumbar enlargement of the spinal cord and there was no co-localisation between fluorogold and YFP when fluorogold was injected into the thoracic IML (Llewellyn-Smith *et al.*, 2015). The lack of ascending PPG neuron projections in the spinal cord, suggests that GLP-1 produced in the spinal cord primarily act within the spinal cord and outside the CNS. The PPG neurons that reside in the brainstem are focussed on primarily in this thesis. The PPG neurons in the brainstem, project widely across brainstem, midbrain, forebrain and spinal cord, to areas associated with glucose homeostasis, energy homeostasis, food reward behaviour, and cardiovascular control. The projection pattern is depicted in Fig 1.3 and will be described in detail throughout the thesis, where necessary. The PPG neurons appear to be conserved across mammals, as they have been demonstrated to exist in mice, rats, macaques and humans (Merchenthaler, Lane and Shughrue, 1999; Llewellyn-Smith *et al.*, 2011, 2013; Vrang and Grove, 2011; Zheng and Rinaman, 2014; Heppner *et al.*, 2015). The PPG neurons have been identified to be glutamatergic and identified not to be cholinergic or catecholaminergic (Zheng *et al.*, 2014; Cork *et al.*, 2015). Therefore, it is possible that GLP-1/ other PPG products are used as co-transmitter in tandem with glutamate release.

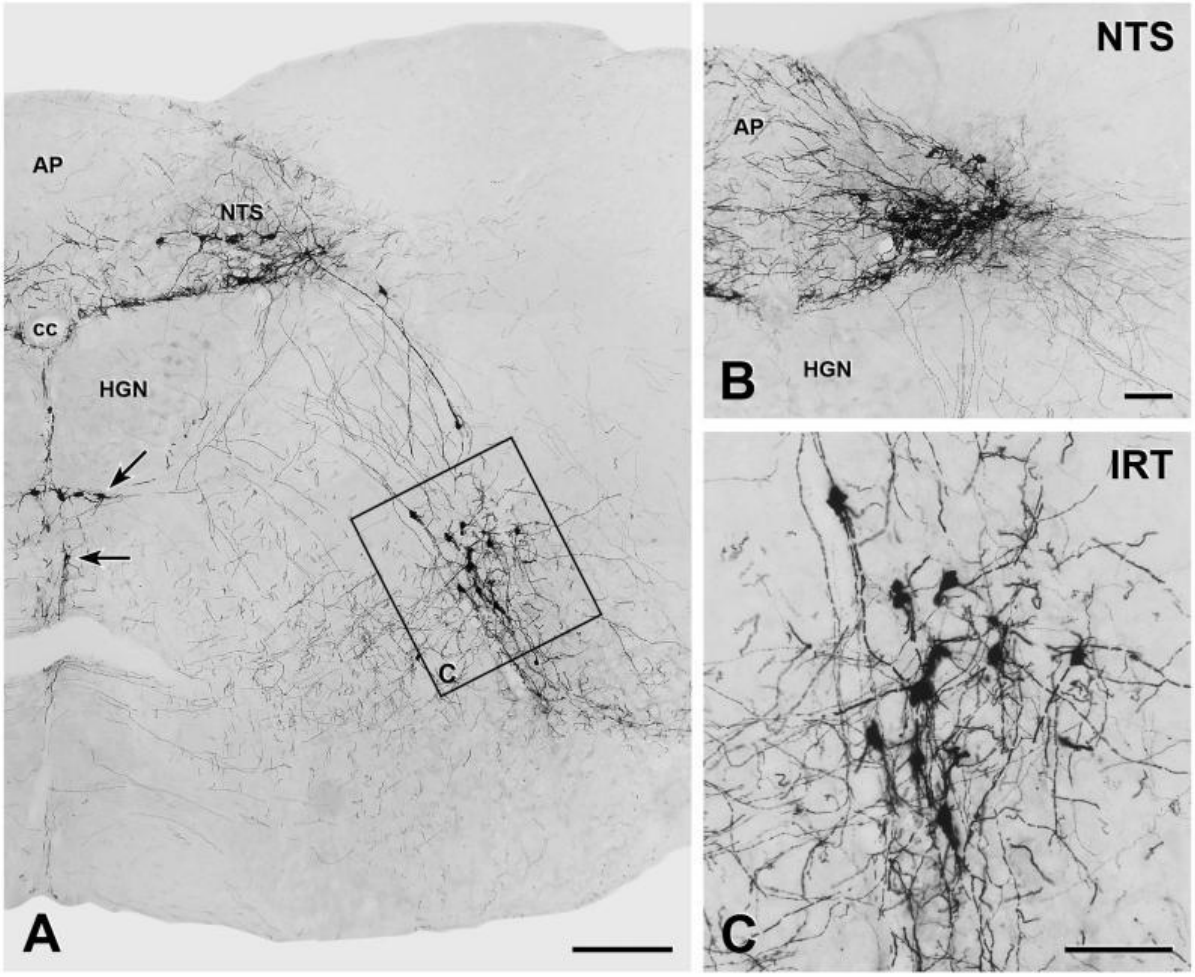


Figure 1.2 The preproglucagon neurons: The preproglucagon (PPG) neurons are defined by a set of neurons that produce glucagon products. The PPG neurons were visualised here in the Glu-YFP mice using immunoperoxidase staining by Llewelyn-Smith et al (2011). The PPG neurons cell bodies reside in the brainstem, more specifically the intermediate reticular nucleus (IRT) (C) and the caudal nucleus tractus solitarius (NTS) (B). A small population of PPG neurons are found in the midline of the brainstem, ventral to the central canal (CC) and hypoglossal nucleus (HGN, indicated by arrow). From here the PPG neurons project widely across the brainstem, midbrain and forebrain. This figure was reproduced from a figure presented by Llewellyn-Smith et al (2011). **SB_A= 250µm, $SB_{b,c}$=100µm**) **AP: area postrema, CC: central canal, IRT: intermediate reticular nucleus, HGN: hypoglossal nucleus, NTS: nucleus tractus solitarius**

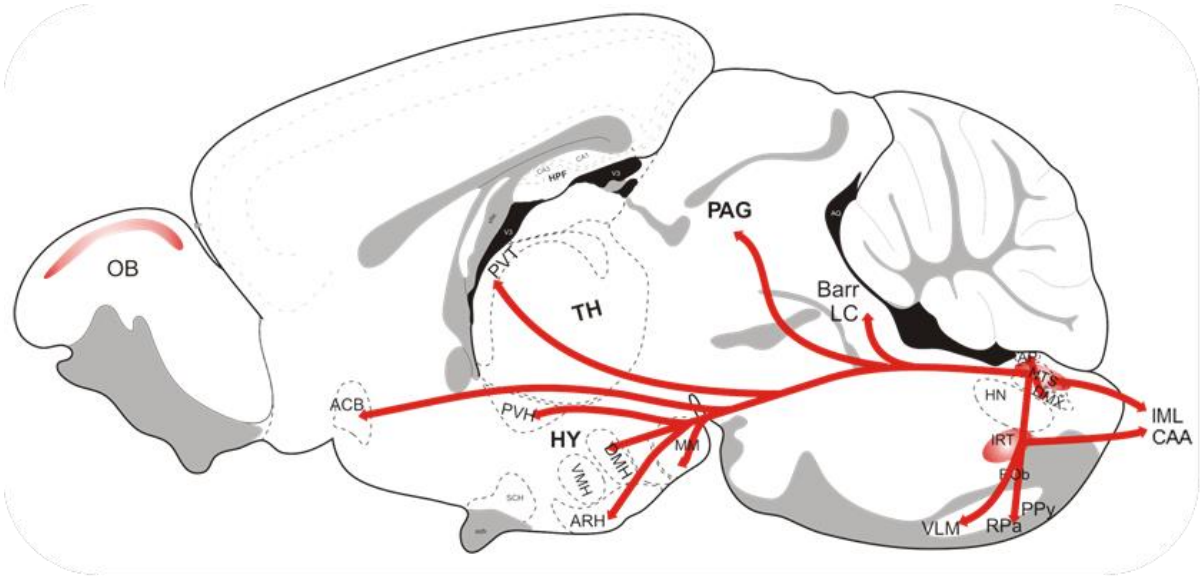


Figure 1.3 Projection pattern of the preproglucagon neurons: The preproglucagon neuron reside in the NTS , and project widely across the CNS terminating in nuclei within the spinal cord, brainstem, midbrain and forebrain. There is a population of PPG neurons expressed in the OB, but these are believed to be interneurons and do not project outside the OB. **Intermediolateral Column (IML), Central Autonomic Area (CAA), ventral lateral medulla (VLM), olfactory bulb (OB), arcuate nucleus (ARH), dorsal medial hypothalmus (DMH), paraventricular nucleus of the hypothalamus (PVH), paraventricular nucleus of the thalamus (PVT), nuclear accumbens (ACB), periaqueductal grey (PAG), locus coeruleus (LC), Nucleus tractus solitarius (NTS), area postrema (AP), dorsal motor nucleus of the vagus (DMNX), intermediate reticular nucleus (IRT), Raphe pallidus (RPa)**

Additionally, the GLP-1 receptor (GLP-1R) is expressed widely across the brain. Discovered initially in rats using in situ hybridization, it was more recently demonstrated in mouse using a transgenic mouse expressing RFP in the GLP-1R neurons. GLP-1R was discovered to be expressed throughout the entire rostral-caudal length of the CNS, with the exceptions of the cerebellum and cerebral cortex (Merchenthaler, Lane and Shughrue, 1999; Cork *et al.*, 2015; Heppner *et al.*, 2015). Specific nuclei expressing the GLP-1R will be described throughout this thesis, where necessary.

1.1.4 The source of central GLP-1

As the GLP-1R is widely expressed across the CNS, it would make sense that GLP-1 acts directly within the brain. However, the source of central GLP-1 (GLP-1 within the CNS) is currently unknown and a divisive topic within the field. It is debated whether GLP-1 released from the L-cells acts directly in the CNS or whether the PPG neurons are the source of central GLP-1. Peripheral GLP-1 would need to cross the blood-brain barrier (BBB) to induce an effect in the CNS, however, it is disputed whether GLP-1 can cross the BBB. It has been demonstrated that I^{125}-labelled GLP-1 and albumin-labelled GLP-1 do not cross the blood-brain barrier, only binding to circumventricular organs, the area postrema and the subfornical organ (areas with an incomplete blood-brain barrier) (Orskov *et al.*, 1996). However, conjugating a protein or element of a protein can impede transport into the brain, therefore it is unknown whether GLP-1 in these studies cannot enter the CNS due to the conjugated element. A contradicting study has suggested that I^{125} labelled [Ser^8]GLP-1 readily crosses the blood-brain barrier, without the use of a saturable transporter (Kastin, Akerstrom and Pan, 2002). Based on the evidence presented it is difficult to conclude whether GLP-1 can or cannot cross the BBB, however whether GLP-1 can cross the BBB may be irrelevant as it is believed that the short half-life of GLP-1 prevents peripherally released GLP-1 from reaching the brain in a high enough concentration to exert an effect, therefore peripheral GLP-1 cannot act on the GLP-1R expressed in the CNS. It is important to note that the concentration of GLP-1 required to enter the CNS to induce an effect is unknown, and therefore peripheral may enter the brain in a high enough concentration, however, GLP-1 was detected in the hepatic portal vein after meal intake in rats, but was not detected in the vena cava, suggesting that GLP-1

released in response to a meal is entirely degraded before it reaches the brain, and therefore would not induce a central effect (Punjabi *et al.*, 2014).

Based on the evidence presented it appears more likely that the PPG neurons are the source of central GLP-1.

1.2 GLP-1 regulates glucose homeostasis

1.2.1 Peripheral GLP- 1 regulates glucose homeostasis

Peripheral GLP-1 is a well-known potent insulinotropic agent, meaning it promotes insulin release from pancreatic β-cells. Blood glucose is controlled primarily by the pancreas. In the pancreas, β-cells release insulin in response to elevated blood glucose levels. Insulin acts to decrease glucose in the blood by increasing glucose uptake by muscle, increasing glycogenesis in the liver, and inhibiting glucagon secretion (Wilcox, 2005). Pancreatic β-cells express the GLP-1R, allowing GLP-1 to promote insulin release. GLP-1 binding to GLP-1R expressed on the β-cells stimulates plasma membrane-bound adenylate cyclase producing cAMP, cAMP then activates protein kinase A (PKA). PKA inhibits K_{ATP} channels. This depolarises the membrane, causing an influx of Ca^{2+} through voltage-dependent calcium channels and initiates insulin release. PKA also acts on the endoplasmic reticulum to increase the release of Ca^{2+} from the endoplasmic reticulum stores to increase insulin release (Doyle and Egan, 2007). Although GLP-1 can strongly induce insulin relasease, the ability for GLP-1 to increase insulin release is glucose-dependent. When extracellular glucose is in fasting range, the insulinotropic effect of GLP-1 is attenuated (Meloni *et al.*, 2013). Finally GLP-1 also increases insulin gene transcription, insulin mRNA translation, proinsulin biosynthesis in β-cell, and induces β-cell proliferation, thus increasing β-cell

mass, further emphasising the role for peripheral GLP-1 in control of blood glucose (Buteau *et al.*, 2003; Doyle and Egan, 2007). The ability of GLP-1 to modulate the islets of the pancreas demonstrate a mechanism for GLP-1 agonists to treat type II diabetes. The ability of GLP-1 to induce insulin secretion, defines GLP-1 as an incretin (INtestine seCRETtion Insulin), with the incretin effect defined as an enhanced insulin secretion upon oral glucose consumption compared to intravenous glucose infusion.

1.2.2 Does central GLP-1 regulate glucose homeostasis?

Additional to direct sensing of blood glucose by the pancreatic islets, the central nervous system can modulate the release of insulin and glucagon from the pancreas to control blood glucose. The pancreas is innervated by the sympathetic and the parasympathetic branches of the autonomic nervous system (Rodriguez-Diaz *et al.*, 2012). The parasympathetic nerve endings release acetylcholine (ACh) in the pancreas acting on the β-cell to release insulin and increase β-cell mass, by increasing β-cell proliferation. Sympathetic nerve endings release noradrenaline which acts directly on pancreatic α-cells to increase the release of glucagon, increasing blood glucose. In general, the parasympathetic innervation acts to reduce blood glucose and sympathetic innervation acts to increase blood glucose (Rodriguez-Diaz *et al.*, 2012).

The effect of GLP-1 on the pancreas enhancing insulin release is well established, however, the effect GLP-1 has within the CNS on glucose homeostasis is less understood. As the CNS can modulate the pancreas, it is possible that central GLP-1 could have a role in regulation of blood glucose. In support of this intracerebroventricular injection (i.c.v) of GLP-1 transiently reduces blood glucose and increases insulin secretion (Knauf *et al.*, 2005). This effect was attenuated by lesioning the hypothalamic arcuate nucleus (ARC). Microinjection of GLP- 1 into the ARC also

reduced glucose production and increased glucose uptake. Both are evidence suggesting a role for the ARC in GLP-1 control in blood glucose (Sandoval *et al.*, 2008). However, another study demonstrated that central GLP-1R is not necessary for the role of central liraglutide (a long-acting GLP-1R agonist) in glucose homeostasis, as knock down (KD) of the central GLP-1Rs (90% reduction) did not attenuate the glucose lowering effect of liraglutide (Sisley *et al.*, 2014). Additionally, i.c.v infusion of the neuropeptide Y (NPY) increased insulin (Wisialowski *et al.*, 2000). NPY is a protein produced by the NPY/POMC neurons in the ARC nucleus. Microinjection of GLP-1 into the ARC attenuated fasting-induced mRNA expression of NPY, suggesting an indirect mechanism in which central GLP-1 can control blood glucose (Seo, Ju, and Chung, 2008)**.** In this case, this would act to increase blood glucose, further complicating the effects of central GLP-1 on blood glucose. Overall, it is difficult to conclude a role for central GLP-1 in the regulation of glucose homeostasis. It seems likely that if there is a role for central GLP-1 in glucose homeostasis that the ARC is required.

1.3 Regulation of food intake by GLP-1

1.3.1 Peripheral GLP-1 induced hypophagia

GLP-1 has been demonstrated to regulate appetite. The GLP-1 analogue Ex-4 reduced food intake when injected into the peritoneum (i.p), and into the bloodstream (i.v) (Larsen *et al.*, 2001; Chelikani, 2005; Williams, Baskin and Schwartz, 2006). Additionally, Exendin-9 (GLP-1R antagonist) increased food intake when injected i.p, suggesting a physiological role for peripheral GLP-1 in the regulation of appetite (Williams, Baskin and Schwartz, 2009). The most convincing evidence that Ex-4 acts in the peripheral system to induce hypophagia is that hypophagia induced by i.p

injection of Ex-4 is attenuated by i.p injection of Ex-9, but is not attenuated by i.c.v Ex-9, therefore Ex-9 must act on the GLP-1Rs expressed outside the CNS to induce hypophgia (Williams, Baskin, and Schwartz, 2009). As food intake and energy homeostasis are controlled in the brain, this would suggest that GLP-1 modulates the nuclei associated with food intake either indirectly. Nuclei in the brain that have been implicated to have a role in the regulation of energy homeostasis include the ARC, PVN, DMH, PBN, and NTS. As all these nuclei express the GLP-1R, it is possible that GLP-1 modulates these nuclei in the CNS directly. However, as stated earlier the amount of GLP-1 that enters the brain is considered to be insufficient to activate the neuronal GLP-1Rs and appears to act peripherally. If peripheral GLP-1 acts in the periphery to induce hypophagia it is possible that the afferent vagus nerve is the mediator of this effect. In support of this, vagal afferents have been demonstrated to express the GLP-1 receptor (GLP-1R) in the nodose ganglia and the vagal nerve terminals in the peripheral system, plus intraportal and intravenous GLP-1 has been demonstrated to increase firing rates in vagal afferents (Vahl *et al.*, 2007; Paul Richards *et al.*, 2014; Krieger *et al.*, 2016). Additionally, severing/chemically lesioning the vagus below the diaphragm (bilateral sub-diaphragmatic vagotomy) attenuates the anorexic effect of GLP-1, however lesioning of the vagus removes vagal efferents, as well as the vagal afferents, and therefore could be removing the descending motor action to reduce food intake (Nakabayashi and Niijima, 1996). However, 50% knock down (KD) of the GLP-1Rs expressed in the vagal afferents by injecting shRNA targeting the GLP-1R increased food intake, suggesting that the GLP-1R expressed on the vagus are required to modulate food intake (Krieger *et al.*, 2016). This data demonstrates that GLP-1 can modulate vagal activity and the vagus nerve is at least partially responsible for peripheral GLP-1 induced hypophagia. Conversely, KD of the

GLP-1R in the vagus by crossing a Phox2B-CRE mouse with a floxed GLP-1R mouse (reducing PPG mRNA levels in the nodose ganglion) did not attenuate the anorexic effect of systemic liraglutide (Sisley *et al.*, 2014). Though since liraglutide can cross the BBB and is not degraded by DPP-IV, the anorexic effect induced might be due to central GLP-1R activation and therefore this study cannot conclude that the vagus has no role in hypophagia induced by peripheral GLP-1. Additionally, i.p injection of a albumin conjugated GLP-1R agonist that is believed to be BBB impermeable (CJC-1311)d id not induce hypophagia suggesting that GLP-1 needs to act centrally to induce an affect (Kim *et al.*, 2003), although data suggesting that CJC-1131 does not cross the BBB is suggestive but not conclusive (Baggio et al., 2008).

Based on the evidence presented it seems peripheral GLP-1 exhibits its effect on food intake through vagal afferents, where the vagus nerve relays the signal into the CNS, to reduce food intake. It is likely that the vagus is activated by peripheral GLP-1, which in turn terminates in the NTS activating an unknown population in the CNS to induce hypophagia. It is hypothesised that the unknown population activated is the PPG neurons. However, I would expect peripheral GLP-1 induced hypophagia to be attenuated by central injection of Ex-9, as the PPG neurons are expected to release GLP-1 to induce hypophagia (Fig 1.4).

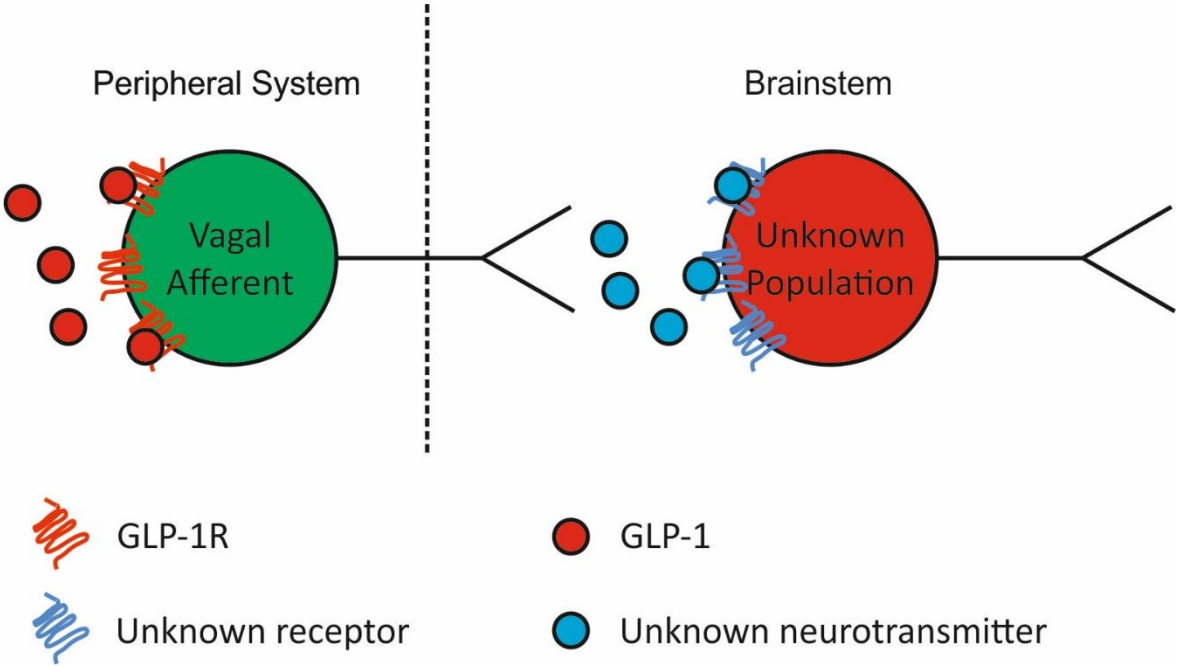


Figure 1.4 Possible mechanism for peripheral GLP-1 to induce hypophagia: Current data suggests that the vagus may be required for peripheral GLP-1 to induce hypophagia. It is possible that GLP-1 released from the L-Cells enters the blood stream and activates the GLP-1R expressed on the vagal afferents. The vagal afferents send ascending projections to the brainstem, where the signal could be relayed into the CNS, where pathways in the brain will act to reduce food intake. The population of neurons in which the vagal afferent will activate/inhibit to induce hypophagia is currently unknown. However, it is possible that the PPG neurons in the NTS are the next step in the pathway to induce hypophagia by acting in the CNS.

1.3.2 Central GLP-1 induced hypophagia

Numerous studies have shown that exogenous GLP-1, or GLP-1 analogues, reduced food intake when applied directly to the CNS (Tang-christensen *et al.*, 1996; Turton *et al.*, 1996; Gutzwiller *et al.*, 1999; Chelikani, 2005; Scott and Moran, 2007; Burmeister *et al.*, 2017). Turton et al (1996) were the first to demonstrate this with i.c.v injection of GLP-1 in rats (Turton *et al.*, 1996). Building on this, microinjections of GLP-1 and GLP-1R agonists have demonstrated the role of specific brain nuclei in the central GLP-1 anorexigenic response. The hindbrain NTS, and PBN and the hypothalamic paraventricular nucleus (PVN), dorsal medial hypothalamus (DMH), and ARC have all been identified to induce hypophagia in response to microinjection of GLP-1 (Schick

et al., 2003; Hayes, Bradley and Grill, 2009; Alhadeff, Rupprecht and Hayes, 2012; Dossat *et al.*, 2012; Alhadeff *et al.*, 2014; Katsurada *et al.*, 2014; Secher *et al.*, 2014). This hypophagic response to GLP-1 injection into these nuclei suggested a role for GLP-1 in the regulation of homeostatic food intake (food intake concerned with the regulation of energy). Although these studies demonstrated that exogenous central GLP-1 can induce hypophagia, they did not provide evidence that endogenous central GLP-1 has a role in the regulation of homeostatic food intake. To demonstrate a role for endogenous GLP-1 in the regulation of food intake Turton et al (1996) injected Ex-9 i.c.v in satiated rats and elicited increased food intake (Turton *et al.*, 1996). This response to Ex-9 demonstrated that endogenous GLP-1 was present in the brain and activated the GLP-1R to reduce food intake, and Ex-9 blocked this response. Later studies in rats showed that microinjection of Ex-9 into the PVN also increased food intake (Burmeister et al,. 2017)**.** By injecting antagonists of the GLP-1R into the brain and inducing hyperphagia these studies demonstrated that GLP-1R activation occurs natively in the brain to reduce food intake.

As well as regulation of homeostatic food intake, GLP-1 was demonstrated to have role in the regulation of hedonic food intake (food intake associated with reward behaviour). Microinjection of GLP-1R agonists into the mesolimbic ventral tegmental area (VTA) reduced intake of highly palatable diet (Mietlicki-Baase., 2013) and microinjection of GLP-1 in the nucleus accumbens (NA) reduced food intake over 2 hours (Dossat et al., 2011). As these areas are highly associated with food reward behaviour, and GLP-1 induced reduction in intake of highly palatable diet, this data suggests a role for central GLP-1 in the regulation of hedonic food intake. Furthermore, the responses in the NAc has been demonstrated to be physiologically relevant with

microinjection of Ex-9 into the NAc core increased intake of highly palatable diet (Dossat *et al.*, 2011; Katsurada *et al.*, 2014).

Based on this evidence it is clear the GLP-1 has a role in the brain regarding regulation of food intake, all the aforementioned studies were performed in rats however the case I less clear in mice. In mice microinjection of Ex-4 into the PVN and the ARC induces hypophagia in mice, demonstrating that central GLP-1R activation is sufficient to induce hypophagia in mice (McMahon and Wellman, 1998; Secher *et al.*, 2014)**.** However, global transgenic knockout (KO) of the GLP-1R did not affect food intake, and knockdown of central GLP-1R did not affect body weight and food intake, in contrast to the results in rats (Scrocchi *et al.*, 1996; Sisley *et al.*, 2014). Interestingly, this data suggests that central GLP-1 is not physiologically relevant in mouse, however as the global KO of the GLP-1R is present during development, the lack of effect due to the GLP-1 KO may be compensated during development or it could represent a species-specific effect.

It seems GLP-1 can directly act in both the peripheral system and CNS to regulate appetite. It is currently unknown whether these two systems are linked, or they both act independently to induce hypophagia through two distinct mechanisms. Further study is required to distinguish the mechanisms by which peripheral and central GLP-1 induced hypophagia.

1.4 GLP-1 regulates the cardiovascular system

1.4.1 Peripheral GLP-1 and GLP-1R agonist induce tachycardia

Tachycardia and hypertension defined as an abnormal increase in heart rate and blood pressure (respectively) under resting conditions are two cardiovascular

challenges that increase the risk of cardiovascular diseases. Exogenous GLP-1 has been demonstrated to induce tachycardia in mice, rats, and humans and to induce hypertension in rats only (Barragan, Rodriguez and Blazquez, 1994; Yamamoto *et al.*, 2002; Hayes, Skibicka, and Grill, 2008; Griffioen *et al.*, 2011; Kang and Jung, 2016). Furthermore, the metabolite of GLP-1 (7-36), GLP-1 (9-36), does not induce tachycardia, therefore the intact protein is necessary to induce tachycardia (Barragan *et al.*, 1996; Barragán *et al.*, 1999).

The mechanism by which GLP-1 induces tachycardia is currently unknown. Most likely it involves modulation of the sympathetic and parasympathetic nervous system, two branches of the autonomic nervous system used to regulate the bodies unconscious systems. The sympathetic nervous system is generally considered to be the mediator of the “flight or fight” response, whereas the parasympathetic nervous is considered to be “rest and digest” acting to regulate the body at rest. It is important to note that although the sympathetic nervous system is generally considered to be involved at times of high stress, it is active during resting conditions and acts to oppose parasympathetic activity. In regulation of heart rate, the NTS receives sensory input from baroreceptors, chemoreceptors, and hypothalamic nuclei, relaying the information onto the parasympathetic preganglionic neurons in the nucleus ambiguus (NAm), the dorsal vagal motor nucleus (DMNX) and the sympathetic preganglionic neurons (SPN) in the intermediolateral column (IML) and the central autonomic area (CAA) of the spinal cord. The connections to these sympathetic neurons might be direct, or via presympathetic neurons in the paraventricular nucleus of the hypothalamus (PVN) or the rostral ventrolateral medulla (RVLM). Additionally, the NTS relays the information to the GABAergic neurons in the caudal ventrolateral medulla

(CVLM). The CVLM sends these inhibitory inputs into the presympathetic neurons in the RVLM, inhibiting sympathetic outflow in the RVLM. The parasympathetic nuclei project to the ganglia, which then project to the sino-atrial (SA) node and the atrioventricular (AV) node, releasing acetylcholine, acting to decrease heart rate. The sympathetic nuclei project to the ganglia in the sympathetic chain. The post-ganglionic sympathetic neurons then innervate the the heart, in particular, the SA node, and release noradrenaline to increase heart rate (Fig1.4) (Purves, Augustine, and Fitzpatrick, 2001)**.**

Hypothalamic nuclei have also been demonstrated to regulate the cardiovascular system, through the autonomic nervous system. Both the ARC and PVN have been demonstrated to influence sympathetic outflow through the projections to the PVN, NTS, RVLM and the spinal preganglionic IML and through humoral factors (Ferguson, Latchford, and Samson, 2009; Rahmouni, 2014)

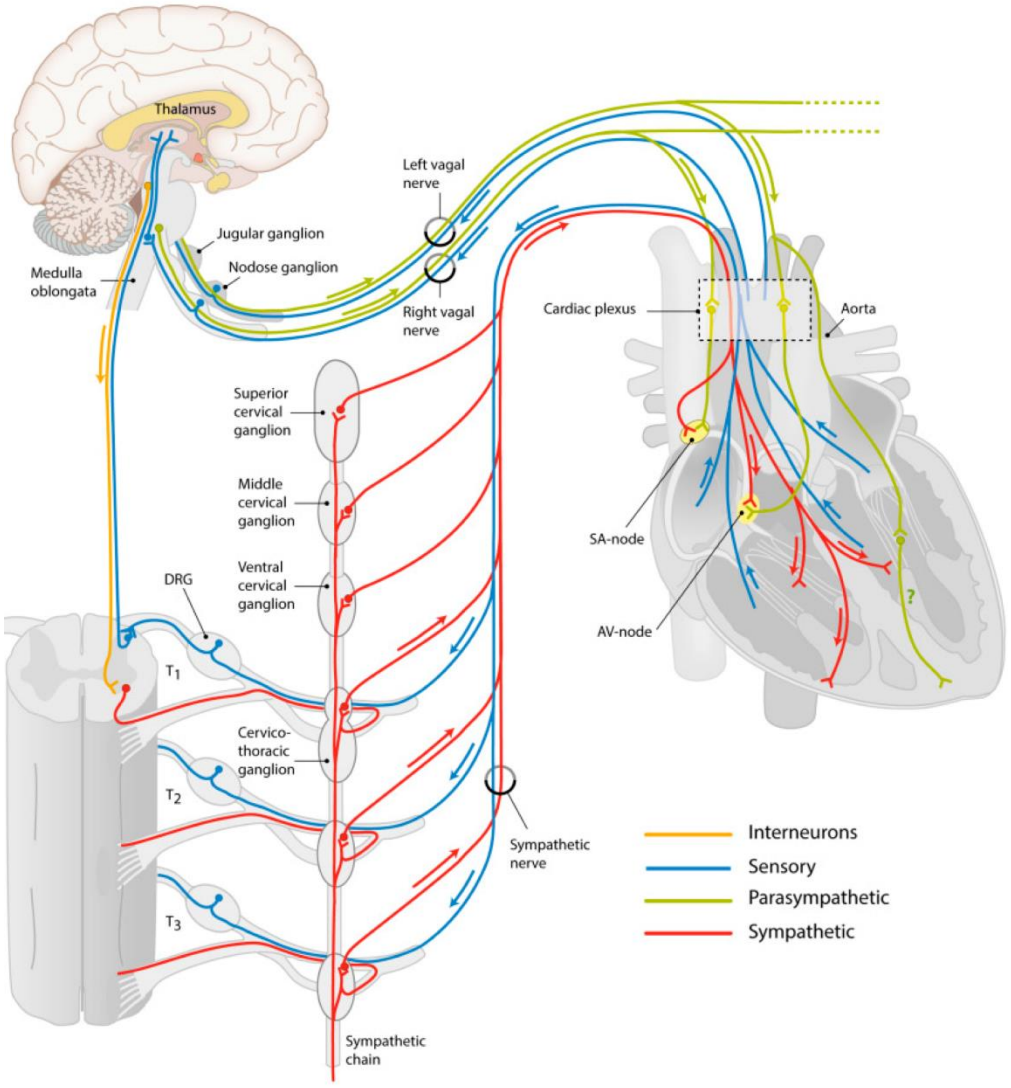


Figure 1.4: Autonomic regulation of the cardiovascular system: Autonomic control of the heart is regulated within the brain. In response to a stressor the nucleus tractus solitarius (NTS) in the brain stem a the hypothalamic arcuate nucleus (ARC) receives sensory input from the baroreceptors (measuring pressure changes in the tension of the arterial wall), and the chemoreceptors (measuring hypoxia and hypercapnia) both located in the carotid sinus and aortic arch. The ARC and NTS relay the sensory information onto the parasympathetic preganglionic neuronal population in the nucleus ambiguus (NA), dorsal vagus motor nucleus (DMNX) and to the presympathetic neurons in the rostral ventral lateral medulla (RVLM) and the spinal intermediolateral medulla (IML) and central autonomic area (CAA). The ARC and NTS also project to higher hypothalamic centres which can regulate the cardiovascular system through humoral factor. The parasympathetic nuclei then project to ganglia, located near the heart, release acetylcholine (ACh) to stimulate the postganglionic pareuronsasympathetic neurons. The post-ganglionic parasympathetic neurons project to the sino-atrial (SA) and atrio-ventricular (AV) node and release ACh, stimulating the muscarinic ACh receptors, acting to reduce heart rate. Sympathetic preganglionic neurons project to the ganglia, located in the sympathetic chain ganglia near the spine, act by releasing ACh, stimulating the nicotinic ACh receptors on the sympathetic post-ganglionic neurons. The sympathetic post-ganglionic neurons innervate the SA node and the blood vessels of arteries, where release of noradrenaline acts to increase heart rate, via activating β1-adrenergic receptors, and increase blood pressure, by acting on the α-adrenergic receptors in the blood vessels to constrict the blood vessels **(This figure was reproduced from Anna M.D Vegh et al. 2016)**.

Peripheral GLP-1 can possibly induce tachycardia through modulation of the autonomic nervous system or direct action on the heart. As the GLP-1R is expressed in the cardiac atrium, there is a possible mechanism by which GLP-1 can influence the heart directly (Paul Richards *et al.*, 2014; Wallner *et al.*, 2015). Despite this, GLP-1 and GLP-1R agonists did not increase heart rate or increase SA node firing rate in perfused mouse hearts *ex vivo* (Baggio *et al.*, 2017). This data suggests that heart rate is not regulated by the GLP-1R expressed on the heart, and therefore most likely is regulated by the autonomic nervous system.

This suggests that GLP-1 modulates the autonomic nervous system to induce tachycardia. Although GLP-1 most likely doesn't reach the brain in a high enough concentration to exert an effect, the vagus nerve has been demonstrated to express the GLP-1R in the nodose ganglia, and GLP-1 increases firing rate of the vagus afferents (Nakabayashi and Niijima, 1996; Kakei *et al.*, 2002). This suggests a direct mechanism for peripheral GLP-1 to modulate vagal signals entering the brain, where the signal can be relayed into the CNS to modulate autonomic control of the heart.

1.4.2 Central GLP-1 induces changes in the cardiovascular system

It seems likely that GLP-1 induces tachycardia through activation of the GLP-1R expressed in the autonomic control nuclei in the central nervous system. In support of this, injection of GLP-1 and Ex-4 into the ventricular system of the brain (i.c.v), induces hypertension in rats and tachycardia in mice and rats and GLP-1 induced tachycardia (i.v) and hypertension was blocked by i.c.v injection of the GLP-1R antagonist, Exendin-9 in rats (Barragán *et al.*, 1999; Yamamoto *et al.*, 2002; Griffeon et al., 2011)**.** This suggests that Ex-4 mediates its cardiovascular response through action on the GLP-1R in the central nervous system. As Ex-4 is not degraded and can cross the

blood-brain barrier, it is probable that Ex-4 injected peripherally crosses the blood-brain barrier and directly activates GLP-1R expressed in the DMNX, RVLM, PVN, and possibly in the IML and CAA, thus inducing tachycardia (Kastin and Akerstrom, 2003).

1.4.3 Species-specific effects of GLP-1 induced tachycardia

The cardiovascular effects induced by GLP-1 and GLP-1R agonists seem variable and differ between species. In humans, systemic infusion of GLP-1 agonist (Exenatide and Liraglutide) modestly increased heart rate and decreased blood pressure mm.Hg in type 2 diabetics (Robinson *et al.*, 2013; Wang et al., 2013; Katout et al.,2014; Sun et al., 2015; Kang and Jung, 2016). Unfortunately, all these studies were done in type 2 diabetics which causes bias within the results. As type 2 diabetes is often associated with obesity, which in turn induces hypertension. As these studies have taken place over a significant time period and GLP-1R agonist are known anorexigenic and weight loss drugs, the reduction in blood pressure could be secondary to the weight loss induced by the GLP-1R agonist. Additionally, another study that acutely infused a bolus of GLP-1, did not observe any change in blood pressure during fasting or after a meal (Bharucha *et al.*, 2008). In rats, systemic infusion and i.c.v infusion of GLP-1 induced tachycardia and hypertension (Barragan, Rodriguez and Blazquez, 1994; Yamamoto *et al.*, 2002; Isbil-Buyukcoskun and Gulec, 2004; Gardiner *et al.*, 2008). In mice, i.c.v infusion of Ex-4 induced tachycardia, with no current report on blood pressure, and in monkeys and calfs, systemic GLP-1 increased heart rate only, with no effect on blood pressure (Edwards, Edwards, and Bloom, 1997; Griffioen *et al.*, 2011). Based on the evidence to date, it appears that the tachycardia caused by GLP-1R agonists is consistent and conserved across species, whereas the Ex-4 induced hypertension appears to be only found in rats. As the hypertension is exclusive to rats,

mice are a better model to study the effects of GLP-1/Ex-4, as it more accurately recapitulates the effects of GLP-1 on the cardiovascular system that are seen in humans, however, the evidence generated from rats should not be ignored.

1.4.4 GLP-1 induced tachycardia: Sympathetic or Parasympathetic?

Tachycardia can be induced either by an increase in sympathetic drive to the heart, a reduction in parasympathetic tone, or a combination of both. Currently, there is conflicting data from several laboratories investigating whether GLP-1 induces tachycardia and hypertension through a reduction in parasympathetic tone or an increase in sympathetic outflow.

Central injection GLP-1 failed to induce tachycardia in vagotomised rats, suggesting that the vagus nerve (a principle effector of parasympathetic tone) is required for Ex-4 to induce tachycardia (Barragán *et al.*, 1999). However, vagotomised rats have the fundamental physiology of the cardiovascular system altered and therefore the may have exhibit an unusual phenotype, that is not expected in healthy animals (Reed and Layman, 1929; Zefirov and Sviatova, 1997). Further support for parasympathetic tone is that Ex-4 injected i.p in conscious mice increased heart rate, and decreased heart rate variability (HRV). HRV is an indicator of parasympathetic tone, where an increase in parasympathetic activity leads to an increase in HRV, and a decrease in parasympathetic tone leads to a decrease in HRV (Griffioen et al., 2011). Therefore, a reduction in HRV in response to GLP-1, suggests a reduction in parasympathetic tone. Gardiner et al also observed a significant reduction in the tachycardia induced by systemic Ex-4 in rats pre-treated with atropine compared to controls. As atropine is a muscarinic acetylcholine receptor antagonist**,** atropine stops parasympathetic tone from influencing the heart. Therefore, as atropine attenuated Ex-4 induced

tachycardia, the data suggests that a decrease in parasympathetic tone contributes to the tachycardia induced by GLP-1R agonists in rats (Gardiner *et al.*, 2006). Although this data suggests a change in parasympathetic tone is responsible for the effects of GLP-1 induced tachycardia and hypertension, there is a substantial amount of data suggesting that the tachycardia and hypertension induced by systemic GLP-1R agonist are due to an increase in sympathetic tone.

Evidence towards an increase in sympathetic outflow includes C-Fos-immunoreactivity (C-Fos-IR) induced by Ex-4 in the PVN, ARC and the C1/A1 catecholaminergic neurons residing in the RVLM (Yamamoto *et al.*, 2002). The PVN, ARC and RVLM have all been demonstrated to increase heart rate, through an increase in sympathetic outflow (Ferguson, Latchford, and Samson, 2009; Rahmouni, 2014). Additionally, i.c.v Ex-4 induced C-Fos-IR in the catecholaminergic neurons in the NTS, Locus Coeruleus (LC), and Area Postrema (AP) (Yamamoto et al., 2003). C-Fos-IR is a marker for neuronal activity, therefore C-Fos-IR in brain nuclei associated with increases in sympathetic drive to the heart, suggest that Ex-4 acts through the PVN, ARC, AP, and LC. However, C-Fos-IR does not detect an inhibitory response within neurons, therefore it would be incorrect to assume no function from the parasympathetic side of the autonomic system based on C-Fos-IR. However, pre-treatment with the α-receptor antagonist, phentolamine, blocked hypertension induced by systemic Ex-4, but it did not attenuate the tachycardia induced by Ex-4 (Gardiner *et al.*, 2010). However, the inability of phentolamine to attenuate GLP-1 induced tachycardia is not surprising as tachycardia is likely caused by activation of the NA β1 receptor, whilst phentolamine is an NA α-receptor antagonist. Propranolol is a β1 adrenoreceptor antagonist, blocking the noradrenergic receptors on cardiomyocytes,

therefore, propranolol blocks sympathetic effects on the heart. Continuous perfusion of propranolol attenuated the tachycardia induced by Ex-4 (i.p) in rats (Gardiner et al,. 2008). Therefore Ex-4 cannot induce tachycardia independent of the sympathetic nervous system in this study.

Based on the evidence presented it is unclear whether GLP-1R agonist induce tachycardia through changes in sympathetic or parasympathetic tone. Additionally majority of the evidence is produced in rats, and the most prominent data in mice produced by Griffieon et al suggested that GLP-1 induces tachycardia through a decrease in parasympathetic tone in mice (Griffieon et al, 2011). As stated earlier the effects of the GLP-1R agonist on the cardiovascular system are species-specific, therefore, it is imperative to understand the pharmacological effects of GLP-1 in each species to completely understand how GLP-1 cardiovascular system.

1.5 Overall Hypothesis:

The evidence presented in this introduction suggests a role for central GLP-1 in cardiovascular control, control of food intake and regulation of glucose homeostasis. Many of the effects of GLP-1 in the brain have been demonstrated by exogenous injection of GLP-1R agonist into the ventricular system of the brain in rodents. As the PPG neurons are possibly the source of GLP-1 in the brain and are believed to use GLP-1 as a neurotransmitter, I hypothesise that the PPG neurons are the endogenous source of the responses demonstrated by exogenous injection of GLP-1. Therefore, the PPG neurons should induce hypophagia, tachycardia and reduce blood glucose upon activation. Currently, the role of the brain GLP-1 neurons (PPG neurons) in the role of cardiovascular control, food intake, and glucose homeostasis is unknown or limited.

In this thesis I aim to:

- Investigate whether the PPG neurons are the source of central GLP-1
- Investigate the role of the PPG neurons in cardiovascular control, food intake and glucose homeostasis
- Clarify the mechanisms by which exendin-4 induces tachycardia in mice
- Investigate under which conditions the PPG neurons are activated *in vivo*

2. Materials and Methods

2.1 Solutions and reagents

2.1.1 Immunohistochemistry

Blocking solution: *0.1M PB containing 1% BSA, 0.1% Triton X-100 and 10% serum (from the same origin species that the secondary antibody was raised in)*

Paraformaldehyde (4% Sigma, 0.1M PB)

Optimal cutting temperature medium, OCT, (Tissue-Tek, Sakura Finetek)

Phosphate buffer: *PB, 0.1 M Na_2PO_4 in water, pH 7.4*

SuperFrost Plus microscope slides (VWR)

Coverslip glasses (VWR)

VectaShield antifade mounting media for fluorescence (Vector Laboratories)

VectaShield antifade mounting media for fluorescence with DAPI (Vector Laboratories)

2.1.2 *In Vitro* assessment of functional hM3Dq expression

High-Mg^{2+}/low-Ca^{2+} aCSF (in mM in water): *2.5 KCl, 200 Sucrose, 28 $NaHCO_3$, 1.25 NaH_2PO_4, 7 Glucose, 7 $MgCl_2$, 0.5 $CaCl_2$; pH 7.4*

Recovery solution (in mM in water): *3 KCl, 118 NaCl, 25 NaHCO3, 1.2 NaH2PO4, 2.5 Glucose, 7 MgCl2, 0.5 CaCl2; pH 7.4*

aCSF (in mM in dH_2O): *3 KCl, 118 NaCl, 25 NaHCO3, 10 Glucose, 1$MgCl_2$, 2 $CaCl_2$; pH 7.*

2.1.3 Antibodies

Primary Antibodies			
Application	**Type**	**Dilution**	**Source**
GCaMP3/YFP	Chicken Anti-GFP	1:1000	#AB13970, lot #623923, Abc
tdRFP/mCherry	Rabbit Anti-dsRed	1:1000	#632496, Clontech
cFOS	Rabbit Anti-Fos	1:1000	#2250S,Cell Signalling Tech

Table 2.1 List of Primary Antibodies

Secondary Antibodies			
Application	**Type**	**Dilution**	**Source**
Chicken IgG	Goat Anti-chicken IgG	1:500	#A-11039, Life Technologies
Rabbit IgG	Sheep Anti-rabbit IgG	1:500	#C2306, Sigma

Table 2.2 List of Secondary Antibodies

2.2 Drugs

2.2.1 *In vivo* glucose homeostasis, food intake, and cardiovascular studies

All drugs for *in vivo* experiments were dissolved in saline (0.9% w/v NaCl) unless stated otherwise.

Drug	Administration	Dose	Source
α-Chloralose/ Urethane	intravenous	50mg/Kg 650mg/kg In 10ml/kg	Sigma
Atenolol	Intraperitoneal	2 mg/kg in 5ml/kg	Sigma
Atropine Sulphate	Intraperitoneal	2mg/kg in 5ml/kg	Sigma
Clozapine-N-Oxide	Intraperitoneal	2 mg/kg in 5 ml/kg	Hello Bio
Cholecystokinin-8	Intraperitoneal	20µg/kg in 5ml/kg	Tocris
Exendin-4	Intraperitoneal	10 µg/kg in 5ml/kg	Tocris
Exendin-9	Intraperitoneal	100µg/kg in 5ml/kg	American Peptides

Glucagon-like peptide-1	Intravenous	100 µg/kg in 5ml/kg	Tocris
Glucose	Intraperitoneal	1g/kg in 5ml/kg	Sigma
Lithium Chloride	Intraperitoneal	128µg/kg in 10ml/kg (dissolved in water)	Sigma
Phenylephrine	Intravenous	1mg/ml	Sigma
Sodium Nitroprusside dihydrate	Intravenous	1mg/ml	Sigma
Urethane	intraperitoneal	1300mg/kg in 6.5ml/kg	Sigma

Table 2.3 Drugs used in vivo food intake and cardiovascular studies

2.2.2 Stereotaxic Surgery

Drug	Administration	Dose	Source
Ketamine (Vetelar)	Intramuscular	50µg/kg	Zoetis
Medatomidine (Dormitor)	Intramuscular	1mg/kg	Orion Pharma
Atipamezol Hydrochloride (Antisedan)	Intramuscular	2.5mg/kg	Zoetis
Buprenorphine	Subcutaneous	0.5mg/kg	

Table 2.4 Drugs used in stereotaxic surgery

2.2.3 Viral Constructs

Viral Constructs	Expression	Use	Source
AAV8-mCherry-Flex-DTA	mCherry or DTA	Ablation	UNC vectorcore
AAV2-FLEX-HM3Dq:mCherry	HM3Dq:mCherry	Activation	UNC vectorcore
AAV2-FLEX-HM4Di:mCherry	HM4Di:mCherry	Inhibition	UNC vectorcore
AAV8-FLEX-eGFP	EGFP	Control	Made in House/VVF/ ZNZ
AAV1/2-FLEX-Perceval	Perceval	Control	Made in House

Table 2.5 Viral Constructs

2.3 Animals

Adult female and male mice were used in these studies. Mice were kept under a 12:12-hour light-dark cycle with ab libitum access to standard chow and water, unless stated otherwise. The ethics committee at University College London (UCL) approved all animal procedure, and all studies at were conducted in accordance with the U.K Animals (Scientific Procedures) Act, 1986.

2.3.1 Transgenic Mice

Transgenic mouse strains were kindly provided by Professors Frank Reimann and Fiona M. Gribble.

Glu-CRE/rosa26-GCaMP3 and Glu-CRE/rosa26-tdRFP mice expressing GCaMP3 and tdRFP respectively were generated by Professor Frank Reimann by crossing Glu-CRE12 (Parker et al,. 2012) with a commercially available ROSA26-lox-stop-lox-GCaMP3 (Jax strain 014538) (Zariwala et al,. 2012) or the ROSA26-lox-stop-lox-tdRFP reporter strain. The Glu12-CRE mouse were created using bacterial artificial chromosome (BAC) based on RP23-343C17 (Children's Hospital Oakland Research Institute, Oakland, CA, USA), inserting iCre, replacing the sequence between the proglucagon start codon in exon 2 and the stop codon in exon 6. The crossing of the Glu-CRE12 mouse with the commercially available Cre dependent reporter strains generated the Glu-CRE-tdRFP and Glu-CRE-GCaMP3 mice.

The Glu-YFP was generated by Professor Frank Reimann in a similar method. Using BAC CH230-36N4 (Children's Hospital Oakland Research Institute, Oakland, CA, USA) and BAC RP23-343C17, encoding YFP-Venus between the start codon of exon 2 and stop codon in exon 6 of the coding region of the preproglucagon gene (F.Reimann et al,. 2008).

Transgenic Mouse	Expression
Glu-YFP	Expresses YFP under the control of the glucagon promoter
Glu-CRE-tdRFP	Expresses CRE under control of the glucagon promoter and a cre-dependent tdRFP in the rosa26 locus
Glu-CRE-GCaMP3	Expresses CRE under control of the glucagon promoter and a cre-dependent GCaMP3 in the rosa26 locus

Table 2.6 Transgenic Mice

2.4 Surgery

2.4.1 Stereotaxic Injection / Brainstem injections

Adult Glu-CRE/tdRFP mice were anaesthetised using isoflurane (1.5-2.5% at 1.5L/min with 1.5L/min O_2) or ketamine hydrochloride (50µg/kg) and medetomidine (1mg/kg, in 45µl H_2O into the quadriceps). Deep anaesthesia was confirmed by the absence of the pedal withdrawal reflex in response to a toe pinch. The skull was fixed into a stereotaxic frame and a heating mat was used to maintain body temperature of the mouse. The nose of the mouse was pushed down to stretch the neck and expose the obex, which would normally be obscured by the cerebellum. Injection needles were pulled on a horizontal puller and negative pressure was used to tip-fill the needle with the virus. An incision was made from the occipital bone to the first vertebra, followed by exposure of the obex by parting the muscle layer. The dura mater was pierced using a 30g needle. The virus was injected bilaterally at 500µm lateral, 100µm rostral, and 350µm ventral from the obex, targeting the NTS (Holt et al,. 2018). Following the injection, muscle and skin were sutured with 6-0 absorbable suture. In ketamine hydrochloride and medetomidine anaesthetised mice, the anaesthetic was reversed with Antisedan (2.5mg/kg). Animals received a subcutaneous (s.c) injection of buprenorphine (0.5mg/kg) and 100µl saline for pain relief and to replace fluid lost during surgery. The mouse was then left to recover in a

34°C heated chamber before return to its home cage (Stereotaxic injections were performed by Dr. James Richards and Dr. Marie Holt).

2.4.2 Anaesthetised cardiovascular recordings

ECG recordings were performed under two different types of anaesthesia. In one paradigm, mice were anaesthetised with urethane (1300mg/kg, i.p, 20% W/V), whilst in the other, they were anaesthetised through isoflurane inhalation (induction 3-4%, maintenance 1.5-2.5% at 1.5L/min with 1.5L/min O_2). Anaesthetic depth was assessed by absence of the paw withdrawal reflex in response to a toe pinch. Core temperature was maintained at 37°C, using a heat mat. An incision was made in the inguinal area of the mouse, following the natural angle of the hind legs. The muscle and connective tissue were gently separated using cotton buds, exposing the femoral artery and femoral vein. The femoral vein and artery were gently separated from the femoral nerve by opening fine-tipped forceps parallel to the artery and the nerve. Care was taken to avoid stimulating the femoral nerve. The fine cotton suture was looped at one end and threaded underneath the femoral vein and artery and cut at the loop end to generate two cotton sutures. The femoral vein and artery were tied together at the proximal end, towards the hind leg to occlude blood flow towards the leg. The femoral artery was tied with the vein to provide support as the artery is significantly less fragile than the femoral vein. A haemostat was used to pull the distal thread pulling the femoral vein and artery taught to occlude further blood flow A a small incision was made in the vein and PE tubing (0.28mm ID, 0.61mm OD, Fisher Scientific), flushed with saline, was inserted into the vein. The distal occlusion was lifted, and the tubing was inserted further in to the vein. The tubing was tied in the vein to ensure the tubing does not fall out of the vein. Mice initially anaesthetised

on isoflurane were now injected intravenously with urethane/α-chloralose (650mg/kg / 50mg/kg in 10ml/kg volume) mix to permanently anaesthetise the mouse and was slowly withdrawn from isoflurane inhalation.

As urethane induces mucous release in the airways, mice were intubated to avoid choking during the experimental procedures. Following cannulation of the femoral vein, an incision was made from the thyroid bone to the sternum. The fat pads in the neck were gently parted using cotton swabs and fine tip forceps to expose the trachealis muscle. The trachealis muscle was separated using fine tip forceps exposing the trachea. A fine cotton thread was threaded under the trachea and a small incision, partially through the trachea was made at a 45° angle. PE tubing was inserted into the trachea towards the lungs, and the suture was tied, holding the intubation tube in place.

2.4.3 Heart rate recordings

ECG probes were manufactured by soldering a two core cable to a 20g syringe needle, to create the probes. To connect to the headstage, the negative and ground wire was soldered to 1mm plugs and the positive wire to a 2mm plug. The ECG cables were connected to an AC preamplifier Headstage (Digitimer, NL100AK) and the signal was passed through an NL104A- AC Preamplifier (Digitimer) and a bandpass filter (NL125/6, Digitimer) to amplify the signal and remove any noise. The signal was recorded through a micro1401 (CED), to convert the signal into a digital output. The ECG probes were placed s/c into the chest of the mouse in the lead I orientation (negative to the right of the heart, positive to the left of the heart and ground below the heart). ECG traces were recorded in Spike7 software.

Heart rate was determined from the ECG in Spike7. In Spike7 peak find was used to identify the S wave of each ECG spike. The frequency of the R wave was averaged over 5 seconds, converted into beats per minutes (1/Freq) and plotted as a graph.

Raw ECG data was exported from Spike7 into Microsoft Excel, including the heart rate output. Baseline heart rate was taken before the start of the experiment by taking the mean heart rate over a 10-minute period. At each timepoint mean heart rate was extracted by averaging the heart rate at timepoint ± 30s to remove the effect of any possible interference in the trace. Baseline heart rate was subtracted from each time point to calculate the change in heart rate (Δheart rate). Statistical significance was tested by a repeated measure ANOVA, followed by Bonferroni's multi comparison test, unless stated otherwise.

2.4.4 Vagal nerve recordings

The carotid artery, jugular vein, and vagus nerve were identified through the incision made for intubation. The vagus nerve was carefully dissected from the carotid artery, opening fine tip forceps between the carotid artery and the vagus nerve, parallel to both. Once separated a bipolar silver recording electrode was placed around the vagus nerve and insulated with dental cement (Coltene Presidents Tube-Light Body). The electrode was connected to an AC preamplifier Headstage (Digitimer, NL100AK) and the signal was passed through an NL104A- AC Preamplifier (Digitimer) and a bandpass filter (NL125/6, Digitimer) to amplify the signal and remove any noise. The signal was recorded in Spike7, where it was rectified and smoothed.

Vagal nerve activity was assessed by smoothing the raw signal with a time constant of 10s. Raw vagal nerve activity was exported into Excel (Microsoft). Baseline vagal

nerve activity was recorded 10-minutes for the start of the experiment and obtained by averaging activity over the 10-minute period. Evoked changes in the mean level of activity were assessed by subtracting mean activity at baseline. Vagal nerve activity at each time point was obtained by averaging the vagal nerve activity at each time point ± 30s to remove the effect of any possible interference in the trace. Statistical significance was tested by a repeated measure ANOVA, followed by Bonferroni multi comparison test, unless stated otherwise.

2.4.5 Blood pressure recordings

The carotid artery was exposed using the incision made for the intubation. The left carotid artery was carefully dissected from the vagus nerve with fine tip forceps, being careful not to stimulate the vagus. Once exposed a loop thread was threaded under the carotid artery and cut under the loop end to create to suture threads. One thread was placed proximally and the other distally from the carotid bifurcation. The proximal thread was tied to occlude blood flow. A haemostat was used to pull the distal thread pulling the carotid artery taut to occlude further blood flow. A small incision was made in the carotid artery and PE tubing (0.28mm ID, 0.61mm, Fisher scientific), flushed with heparinised saline, was inserted into the carotid artery. The haemostatic clamp was released, and the PE tubing was pushed towards the heart and placed in the aortic arch. The suture was tied around the PE tubing and the carotid artery to ensure the tube was not released. The carotid cannulation was fixed to a pressure transducer (NL108D2, Digitimer), where the signal was amplified and processed through a pressure amplifier (NL108A, Digitimer) and Micro 1401 (CED). The blood pressure transducer was precalibrated with a pressure pump before each experiment.

2.5 *In vitro* assessment of functional hM3Dq expression

Glu-CRE-GCaMP3 mice stereotaxically injected with AAV-Flex-hM3Dq:mCherry into the brainstem were anaesthetised with isoflurane and decapitated. The brain stem was quickly removed and placed in ice-cold high-Mg^{2+}/low-Ca^{2+} aCSF. 200µm coronal sections were cut on a vibratome (Campden Instruments) and left in 34°C recovery solution. Recovery solution was bubbled with 95% O_2/5% CO_2 to maintain pH 7.4 and oxygenation. Sections were imaged on a widefield microscope (Zeiss Azioskop) with a 40x water immersion lens (0.8 numerical aperture). Light scattering and background fluorescence were reduced by restricting the field of view through restriction of the field diaphragm. GCaMP3 was excited using an LED light source (CoolLED pE300white, QImaging) for 250ms every 5s. Excitation was filtered at 470±20nm and emission was filtered at 515±17nm (Chroma 59004). Images were captured on a charged-coupled device camera (Q-Click, Qimaging). The camera and the LED light source were controlled in Micro-Manager (Edelstein at al.2014). Sections were continuously superfused with 32°C aCSF at flow rate 3-4ml/min. CNO stocks were dissolved in saline and diluted in aCSF into a final working concentration of 1µM. CNO was superfused over the brain sections for 3 minutes, followed by superfusion of aCSF.

Image stacks from *in vitro* Ca^{2+} recordings were imported into Fiji image analysis software (Schindelin et al. 2012). XY-drift was adjusted for using the StackReg plugin (Thevenaz et al. 1998). The mean pixel intensity was measured from outlined regions of interest (ROIs) and an area representing background fluorescence. Background intensity was subtracted from each ROI and recordings were adjusted for bleaching using a cubic polynomial function (Balkenius et al. 2015). Fluorescence

intensity is reported as the relative change in intensity from baseline, *ΔF/F0*, with *F0* defined as the mean fluorescence intensity five mins prior to the first stimulus and *ΔF* being the fluorescence intensity, *F*, at a given time *n*, *Fn*, minus *F0*. Data were plotted in Origin 9.0 (OriginLab, US) and are presented as individual and mean traces. Responses were quantified by calculating the area under the curve (AUC) over ten mins during the stimulus, starting at the beginning of the stimulus. GCaMP imaging and analysis were performed by Dr. Marie Holt.

2.6 Food intake and glucose studies

All mice used in food intake studies were singly housed several days before the beginning of the experiment to allow the mouse to acclimatise to its new surroundings.

2.6.1 Acute light-phase food intake

Mice were food restricted overnight (16 hours) from 2 hours before dark onset to 2 hours after the light onset. Standard chow weight was measured in the hopper before being returned to the mouse. Standard chow and the hopper were measured together 1 hour and 2 hours after the return of standard chow. Standard chow intake was calculated by subtracting the weight of food plus hopper at each time point from the initial weight of standard chow plus hopper.

2.6.2 Chronic Food intake

Mice were transferred into new cages at the start of the experiment. Initial hopper weight and bodyweight were measured during the transfer to the new cage, hopper weight and bodyweight were recorded at 11am each day (4 hours after light onset).

Daily food intake was calculated by subtracting the hopper weight from the preceding day's hopper weight each day.

2.6.3 Intraperitoneal glucose tolerance tests (IPGTT)

Mice were food restricted overnight (16 hours) from 2 hours preceding the dark phase to 2 hours into the light phase the next day. Blood samples were collected by tail snip and blood glucose was measured using a hand-held glucose meter (Accu-Chek, Roche, UK) at time point 0, 15, 30, 60, 90 and 120 minutes unless stated otherwise. Recordings were taken from each mouse at 20seconds intervals at each time point. Glucose solution (20% W/V, 1g/kg) was injected i.p at time point 0.

2.7 Immunohistochemistry:

2.7.1 Transcardial perfusion

Mice were anaesthetised with urethane 1300mg/kg, 20% w/v, i.p. Anaesthetic depth was assessed by the absence of the pedal withdrawal reflex in response to a paw pinch. An incision was made in the lower abdomen, and the diaphragm was pierced, to expose the heart. A needle was inserted into the left ventricle and clamped in place. The inferior vena cava was severed to allow fluid outflow for the perfused solution. The mouse was perfused, using 50ml syringes, with 4°C 0.1M phosphate buffer (PB), followed by 4% paraformaldehyde (PFA) solution in 0.1M PB at 4°C. The brain was removed and post-fixed in 4% PFA overnight. The brain was transferred into 0.1M PB to wash any remaining PFA and stored at 4°C overnight. The following day the brain was transferred into 30% sucrose solution in 0.1M PB for cryoprotection. Following cryoprotection, the brain sliced coronally separating the brainstem and midbrain. The brainstem sections were mounted onto a pedestal and

embedded in OCT. The brain was sectioned on a cryostat (Bright Instruments, UK) in 30µm coronal sections, into five series, which were kept in 0.1M PB.

2.7.2 Immunostaining procedure

Sections were incubated in blocking solution for 60 minutes prior to the addition of the primary antibody. The blocking solution was removed and replaced with 0.1% primary antibody in blocking solution and incubated overnight at 4°C under gentle agitation. The following day sections were washed 5 x 5min in 0.1PB at room temperature, followed by incubation in 0.2% secondary antibody in blocking solution for 2 hours at room temperature on a shaking platform. Sections were washed again with 5 x 5min in 0.1M PB at room temperature. The sections were then mounted onto microscope slides. A drop of VectaShield mounting media was added and sections were covered with glass coverslips. An upright widefield microscope (Leica) equipped with GFP (Chroma 49002, Excitation 470±20nm, Emission 525±25nm, dichroic mirror: 495nm) and RFP (Chroma 49008, Excitation 560±20nm, Emission 630±37nm, dichroic mirror: 585nm) filter sets, was used to visualise immunofluorescence. Images were recorded with a Retiga 3000 colour camera (QImaging) and Q-Capture Pro Software. Composite images containing several frames were produced using image composite editor (Microsoft). Fiji image analysis software (Schindelin et al.2012) was used to adjust brightness and contrast.

2.8 C-Fos studies

All mice used for C-Fos-IR studies were Glu-YFP mice. The immunostaining protocol for C-Fos-IR deviated from the baseline protocol (Section 2.7.2). Following the mounting of sections on permafrost slides, sections were washed in tap water to remove any crystallised salts, reducing background fluorescence. Following

immunostaining for C-Fos-IR and YFP, total YFP cells and C-Fos-IR positive cells were counted in each section. Numbers of C-Fos-IR positive YFP neurons were expressed as a percentage of total YFP cells in the NTS, IRT, and in Total (including the midline PPG neurons). Statistical significance was tested using a repeated measure and two-way ANOVA followed by a Bonferroni multiple comparison test.

2.8.1 Measuring Ensure intake before C-Fos-IR

Mice were singly housed several days before the onset of the experiment to ensure acclimatisation to the new environment. Mice were trained to consume Ensure, to overcome neophobia, ensuring consistent intake. Mice were food restricted 3 hours before the onset of the dark phase. At the beginning of the dark phase, mice received a water bottle, filled with 20ml of Ensure for 30-minutes. The weight of the Ensure and the bottle was recorded before and after the 30-minute feeding period. Ensure consumed was calculated by subtracting the final weight from the initial weight of the filled bottle. Training was repeated until Ensure intake was consistent for 3 consecutive feeding sessions.

After training mice were randomised and allocated to *no Ensure* and *Ensure* cohorts. Both cohorts were food restricted as before, but only the Ensure fed mice received the Ensure bottle. Ensure intake was measured after 30 minutes and 90 minutes. Following the 90-minute time point, mice were transcardially perfused.

2.8.2 Measuring the effect of CCK on wetmash intake

Wet mash was created by combining powdered normal chow (teklad 2018) mixed with tap water in a ratio 2:3. 5g of wet mash was compacted into a small dish and placed into the home cages. After 1 hour the small dish was replaced with another

5g wet mash small dish and intake was calculated from the first dish. The mass of the second dish was measured 1 hour after it was placed in the cage, therefore intake was obtained after 1 and 2 hours. An additional dish was placed inside the cabinet, but outside the cage, to account for evaporation. Change in mass due to evaporation (typically 5%) was removed from the final intake total. Where evaporation was greater than intake, values were corrected to 0.

Mice were group housed and moved into single housing test cages for each experiment. Mice were trained in the test cages until consistent consumption of wet mash was observed with no injection, followed by training with saline injection until consistent intake of wet mash was conserved. Following consistent wet mash intake, CCK (20µg/kg) or saline (volume matched) was injected (i.p) in a crossover counter balanced designed study.

After successful confirmation of CCK induced hypophagia, mice were randomly assigned to CCK or saline groups. Mice received an injection of either CCK or saline and wet mash intake was measured 60 minutes and 90 minutes after dark onset. Mice were immediately transcardially perfused after the 90 minutes time point and processed for C-Fos immunohistochemistry.

2.9 Brain GLP-1 Concentration

Glu-CRE-RFP mice were group housed and deeply anaesthetised using isoflurane followed by decapitation. Animals were sacrificed in groups at 4-hour intervals during the light and dark phase throughout a 24-hour circadian cycle.

2.9.1 Active GLP-1 assay

Brains were rapidly extracted from the skull, the lower brainstem (including the NTS), hypothalamus, cerebellum, olfactory bulbs and spinal cord tissue were isolated and immediately snap frozen on tin foil embedded into dry ice. Tissue was stored at -80°c for 24 hours prior to mechanical disruption with a pestle and mortar and by trituration in 500μl aCSF supplemented with dipeptidyl peptidase – IV (DPP-4) inhibitor (Millipore) through a 29G insulin syringe until completely homogenous. Samples were refrozen on dry ice and stored for another 24 hours at -80°C. To generate a crude protein lysate samples were thawed on wet ice and clarified twice by centrifugation at 500g for 10 min and the supernatant aspirated into a new sterile reaction tube. The concentration of active GLP-1 was determined using an MSD kit (K150JWC-1; Meso Scale Diagnostics, Rockville, Maryland, USA). A Bradford protein assay was performed to allow expression of the results per mg of protein for comparisons. A Bradford assay is method of measuring the concentration of protein in a solution, based on the absorbance shift of Coomassie blue dye.

2.9.2 Bradford assay

The protein concentration of crude tissue lysates was determined using Bio-Rad's protein assay using BSA standards. Absorbance was determined using a Lab Systems Multiscan MS.

2.10 Statistical analysis

Statistical analysis was performed using Graphpad Prism version 7.00. An F-test was used to confirm whether data was normally distributed. If normative distribution was confirmed a unpaired t-test was used to test statistical significance on data that provided two means with equal variance. If data did not have equal variance the non-

parametric Mann-Whitney U test was performed. Repeated measure ANOVA was performed on data with normative variance with several dependent data points over time. Following a repeated measure ANOVA, the bonferroni multiple comparison test was used to test the difference in means at specific time points, as this removes the chance of type 1 error due from performing multiple test. Experiments with a number of treatments were tested by a repeated measure ANOVA, followed by a Dunnett's test, comparing the values back to one treatment (usually the control, unless stated otherwise).

3. Role of the preproglucagon neurons in control of food intake and glucose homeostasis

3.1 Background

3.1.1 Preproglucagon neurons and glucose homeostasis

Central (i.c.v) injection of GLP-1 has been demonstrated to transiently reduce blood glucose and increase insulin secretion (Göke *et al.*, 1993; Ahrén *et al.*, 1999; Zander *et al.*, 2001; Sandoval *et al.*, 2008; Jessop, 2015; Jessen *et al.*, 2017). Moreover, i.c.v injection of Ex-9 increased blood glucose, suggesting a role for endogenous brain GLP-1 to regulate glucose homeostasis (Knauf *et al.*, 2005). In further studies the ARC was identified to be crucial in regulating glucose homeostasis in response to central GLP-1 (Sandoval *et al.*, 2008). However, contradicting studies following similar methodology have demonstrated that central GLP-1 does not have role in glucose homeostasis.

Based on the literature (discussed in more detail in section 1.2) it is unclear whether central GLP-1 has a role in regulating blood glucose. If there is a role for central GLP-1 in blood glucose it is likely that this response is mediated through the ARC. The ARC is innervated by the PPG neurons and expresses the GLP-1R (Merchenthaler, Lane and Shughrue, 1999; P. Richards *et al.*, 2014). As the ARC has been implicated in the blood glucose response to central GLP-1 and is innervated by the PPG neurons, it seems that the PPG neurons would be the physiological pathway to regulate glucose homeostasis, through release of GLP-1.

3.1.2 Preproglucagon neurons and food intake

Central injection of GLP-1R agonists induces hypophagia in mice and rats (Tang-christensen *et al.*, 1996; Turton *et al.*, 1996; Gutzwiller *et al.*, 1999; Chelikani, 2005; Scott and Moran, 2007; Burmeister *et al.*, 2017). Studies have identified brainstem, hypothalamic and mesolimbic nuclei to be involved in the hypophagic response, demonstrating that central GLP-1 has can induce hypophagia through homeostatic, stress-related and hedonic feeding pathways (Hayes and Schmidt, 2016).

The PPG neurons project from the NTS and IRT to hypothalamic and mesolimbic sites involved in homeostatic control of food intake (food intake concerned with the regulation of energy balance) and hedonic control of food intake (food intake concerned with the reward associated with food intake)(Jin *et al.*, 1988; Merchenthaler, Lane and Shughrue, 1999; Llewellyn-Smith *et al.*, 2011, 2013). The PPG neurons innervate the PVN, ARC, DMH, VTA and NAc most strongly. All of these have been demonstrated to mediate food intake through action on the GLP-1R expressed in each nuclei (discussed in more detail in chapter 1.3)(Craddock et al., 1998; McMahon and Wellman, 1998; Merchenthaler, Lane and Shughrue, 1999; Ll Schick *et al.*, 2003; Dossat et al ., 2011, 2013; Llewellyn-Smith *et al.*, 2011 Dickson et al., 2012; Mietlicki-Baase et al., 2013, 2014; Secher *et al.*, 2014; Cork *et al.*, 2015; Katsurada and Yada, 2016; Burmeister *et al.*, 2017; Williams *et al.*, 2018). Additionally, Barrera et al (2011) knocked down PPG mRNA in the NTS of rats, reducing central GLP-1 levels by 50% using short hairpin RNA (shRNA). They reported an increase in food intake and body weight, suggesting that the PPG neurons are essential for maintaining food intake and body weight (Barrera *et al.*, 2011).

3.1.2 Aims & Objective

Whilst a principal role for GLP-1 within the brain is undisputed, the role of the preproglucagon (PPG) neurons, which are the main potential endogenous source of GLP-1 within the brain, is less clear. This chapter will focus on investigations to clarify the exact role of the PPG neurons in food intake control and glucose homeostasis.

I hypothesise that activation of the PPG neurons would lead to the release of GLP-1 in the brain. The release of GLP-1 in the brain would activate the GLP-1R expressed in the CNS, mimicking the anorexigenic effect of the exogenous central application of GLP-1R agonist on food intake, therefore the PPG neurons will act to reduce food intake. Additionally, I hypothesise that the release of GLP-1 from the PPG neurons in the CNS would also lead to a reduction in blood glucose, mimicking the effect of exogenous GLP-1 injection on glucose tolerance. In this chapter I will investigate:

- If the PPG neurons are necessary to regulate glucose homeostasis through ablating the NTS-PPG neurons and measuring glucose tolerance.
- If the PPG neurons have the capacity to regulate glucose homeostasis through measuring whether activation of the NTS-PPG increases glucose tolerance.
- The effect of ablating the PPG neurons on GLP-1 concentration in the CNS.
- The effect of pharmacogenetic activation of the PPG neurons on food intake.

Additionally, all manipulations are only introduced in the adult mouse in order to avoid any potential developmental compensation in these experiments.

3.2 Results

3.2.1 Validation of the Glu-CRE-RFP mouse

Before proceeding to study the PPG neurons in the Glu-CRE-RFP mouse it is important to validate that these mice indeed express CRE and RFP selectively in the

PPG neurons. In order to ascertain that this is the case, the Glu-CRE-RFP mouse strain was crossed with the Glu-YFP strain to produce the Glu-YFP-CRE-RFP mouse strain. The Glu-YFP mouse strain has previously been verified to selectively express YFP in PPG neurons. Hisadome et al (2010) extracted the cytoplasm from the YFP expressing NTS neurons and performed single-cell RT-PCR for preproglucagon mRNA. They demonstrated that 100% of the YFP expressing neurons tested positive for preproglucagon mRNA, compared to 0% in the control (negative for YFP) cells (Hisadome *et al.*, 2010)**.** Adult Glu-YFP-CRE-RFP mice were transcardially perfused and the extracted brains were processed for YFP and RFP immunohistochemistry. As demonstrated in Fig 3.1 there was clear YFP and RFP expression in the NTS of the Glu-YFP-CRE-RFP mice, and RFP and YFP expression co-localised in the vast majority of positive neurons (white colour). 85% of YFP expressing neurons co-localised with RFP, and less than 5% of RFP neurons did not express YFP (Fig 3.1). This high level of co-localisation between the YFP and RFP positive neurons demonstrates that the Glu-CRE-RFP mouse is a suitable animal model to study the PPG neurons.

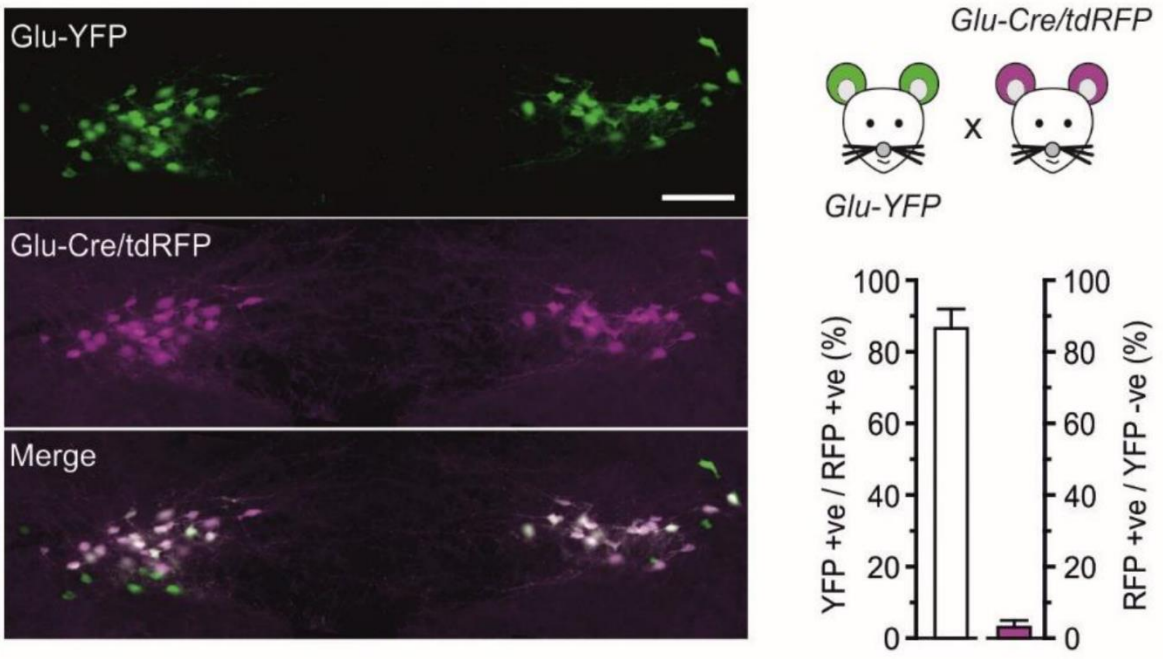


Figure 3.1 Validation of the Glu-CRE-RFP transgenic mouse for the study of the PPG neurons: To validate the Glu-CRE-RFP mouse for study of the PPG neurons, the Glu-CRE-RFP mouse was cross bred with Glu-YFP mouse. Glu-CRE-RFP X Glu-YFP animals (n=3) were transcardially perfused and processed for YFP and RFP expression. There is clear co-localisation between the NTS YFP expressing neurons and the NTS RFP expressing neurons. 85% of YFP positive neurons expressed RFP, whereas only 5% of RFP neurons did not express YFP. As the majority of YFP and RFP expressing neurons co-localise, it is clear that the Glu-CRE-RFP mouse is suitable to study the PPG neurons (n=3). **SB: 100µm**

3.2.2 Preproglucagon neurons are the source of central GLP-1

To study the function of the PPG neurons in mice, I aimed to selectively ablate the NTS-PPG neurons, using the Glu-CRE mouse strain. To selectively ablate the PPG neurons AAV-mCherry-Flex-DTA was injected into the NTS of Glu-CRE-RFP mice, expressing diphtheria toxin sub-unit A (DTA) selectively in the NTS-PPG neurons, in turn ablating these neurons. I refer to these animals as DTA mice.

For proof of concept, AAV-mCherry-Flex-DTA was injected unilaterally into the NTS of the Glu-CRE-GCaMP3 mice (Fig 3.2), to selectively ablate the PPG neurons in one hemisphere. The GCaMP3 mouse was used as this expresses a GFP, allowing easier identification of the PPG neurons, with the mCherry encoded and expressed through

the virus. As the mCherry encoded in the viral construct is not cre-dependent, it is expressed in all infected cells and represents the spread of the virus when injected into the brain. Fig 3.2A,B demonstrates that where the virus is expressed (mCherry), there is no longer GCaMP3 expression, therefore demonstrating that the PPG neurons are ablated when infected by the virus.

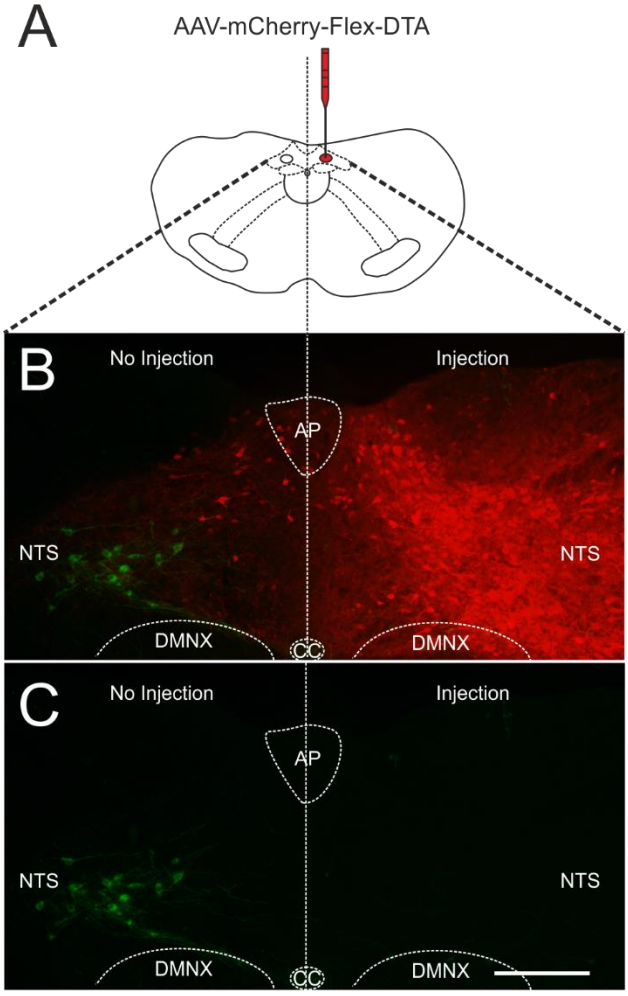


Figure 3.2 Selective ablation of the PPG neurons: To demonstrated selective ablation of the PPG neuron, a Glu-CRE mouse was unilaterally (right side) stereotaxically injected with AAV-mCherry-Flex-DTA, into the brainstem (A). 3 weeks after stereotaxic injection, the mouse was transcardially perfused with PFA and stained for GFP and mCherry. The spread of the virus is represented by mCherry (Red) expression, as the mCherry is not Cre-dependent. GFP expression represents the PPG neurons. (B) mCherry expression is spread widely across the right half of the brainstem (the midline is indicated by the dashed line), from the site of injection. Where mCherry is expressed there is no expression of GFP, therefore the virus selectively ablates the PPG neurons. To more clearly demonstrates the ablation of the PPG neurons (C) is the same image showing GFP immunoreactivity only. Here it is clear that the PPG neurons on the side of the injection are ablated, therefore we can selectively ablate the PPG neurons using AAV-mCherry-Flex-DTA. **AP: area postrema, DMNX: dosal motor nucleus of the vagus, NTS; nucleus tractus solitarius SB: 200µm**

To study whether the PPG neurons are the source of GLP-1 in the brain, we measured GLP-1 concentration in the brainstem, hypothalamus, spinal cord, olfactory bulbs, cerebellum and blood in DTA mice and compared them to GLP-1 concentration after stereotaxic injection of AAV-Flex-eGFP (Control)(Fig 3.3A). DTA mice received a

bilateral injection of AAV-mCherry-Flex-DTA into the NTS, ablating the NTS-PPG neurons in both hemispheres. In control mice, GLP-1 levels in the brainstem, hypothalamus, and spinal cord were measurable high whereas GLP-1 concentration in the blood, cerebellum and olfactory bulb were negligible.

Selective ablation of the NTS-PPG neurons significantly reduced GLP-1 concentration in the brainstem, hypothalamus, and spinal cord (Fig 3.3). GLP-1 concentration in the cerebellum, olfactory bulb, and blood was negligible. This was expected as the PPG neurons cell bodies reside in the NTS, and highly innervate hypothalamic and spinal cord nuclei but do not innervate the cerebellum. As GLP-1 concentration is significantly reduced after ablation of the NTS-PPG neurons, this data demonstrates that the PPG neurons are the main source of central GLP-1. The remaining GLP-1 concentration in each area of the CNS is most likely the result of the intact IRT-PPG neurons, however, this cannot ultimately be concluded at this stage. This experiment was performed in collaboration with Dr. James Richards.

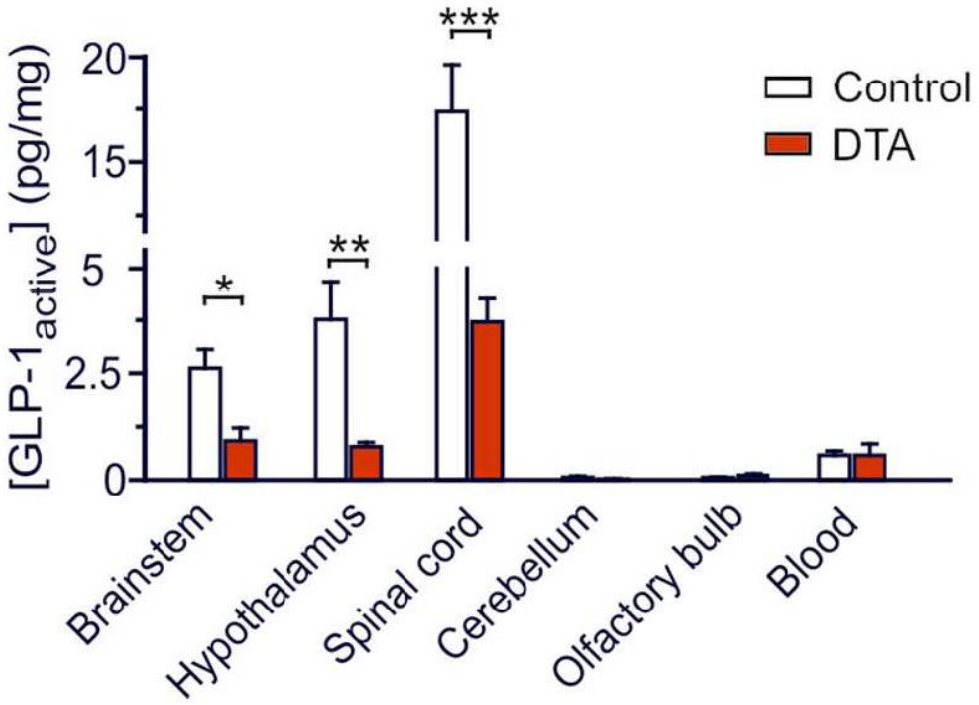


Figure 3.3 Selective ablation of the NTS-PPG neurons reduces GLP-1 concentration in the brain: Glu-CRE-RFP mice were stereotaxically injected into the NTS with either AAV-mCherry-Flex-DTA or AAV-Flex-eGFP, to selective ablate the NTS-PPG neurons or as a control respectively. Ablation of the NTS-PPG neurons significantly reduced GLP-1 concentration in the brainstem, hypothalamus and spinal cord. GLP-1 concentration was not changed in the cerebellum, olfactory bulb and blood. This data demonstrated that the PPG neurons are the central source of GLP-1. Brainstem P= 0.0317; Hypothalamus P=0.0079 (Mann – Whitney U-Test); Spinal cord P=0.0004 (unpaired-test), n=5 in each group, *p<0.05, **p<0.01, ***p < 0.001

As the PPG neurons appear to be the source of central GLP-1, it is most probable that the PPG neurons have a role in glucose homeostasis and energy homeostasis, as demonstrated by exogenous injection of central GLP-1.

3.2.3 The role of the preproglucagon neuron in glucose homeostasis

3.2.3.1 Ablation of the NTS-PPG neurons does not affect glucose tolerance

After successfully demonstrating that we can ablate the PPG neurons using the AAV-mCherry-Flex-DTA and that the PPG neurons are indeed the main source of central GLP-1, I decided to study the effect of ablating the PPG neurons on glucose tolerance. Glucose tolerance was tested in DTA mice (n=7) and GFP mice (referred to as control) (n=7) with an intraperitoneal glucose tolerance test (IPGTT). Both groups of mice were

injected i.p with glucose (1g/kg) and blood glucose was measured. Overall there was no significant difference in glucose tolerance between the DTA mice and the control mice (Fig 3.4A,B) nor when the area under the curve was calculated and statistically tested with an unpaired t-test (Fig 3.4G). There was no obvious trend between the two groups (Fig 3.4B). As the ability to regulate blood glucose highly differs between sexes the cohort was split into male and female (Ingvorsen, Karp and Lelliott, 2017). When the cohort was split into sex groups, the male DTA cohort (n=4) appeared to have a slight trend for slower glucose clearance, driven by the timepoint 60 minutes after glucose injection compared to control mice (n=4), however, this was not significant (Fig 3.4C, D, H). There was no significant difference in glucose tolerance or any obvious trend in the female cohort (n=3 in both cohorts) (Fig 3.4E, F, I). Ablation of the PPG neurons was confirmed in all mice by visual inspection following immunohistochemistry for mCherry and GFP. Overall this shows that ablation of the NTS - PPG neurons does not affect glucose tolerance in mice. Statistical significance was tested with a repeated measure ANOVA (Fig 3.4 A-F) or an unpaired t-test (Fig G-I).

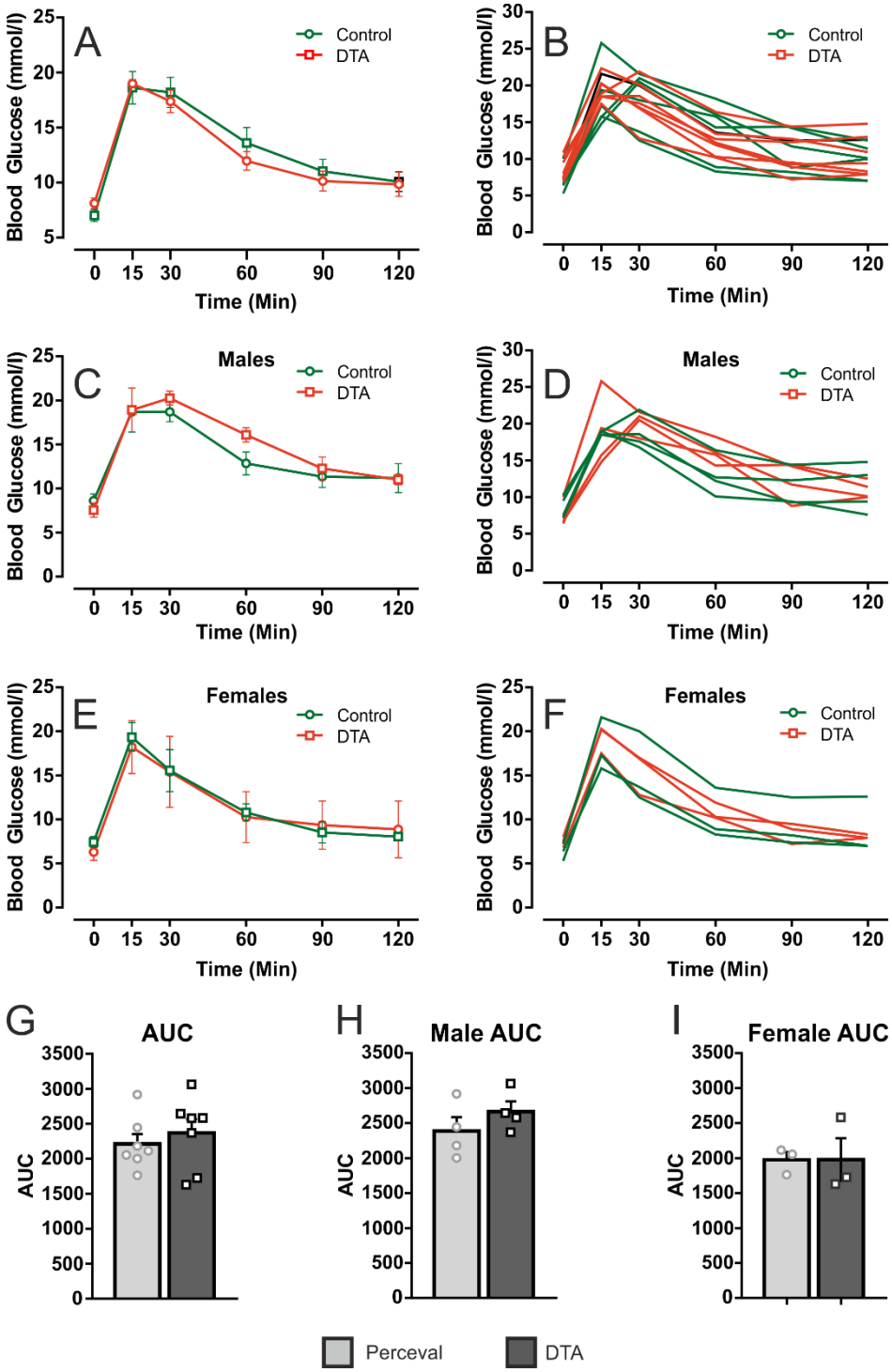


Figure 3.4 Ablation of the PPG neurons has no effect on glucose tolerance: Glu-CRE mice were injected with either AAV-Flex-eGFP (n=7) or AAV-Flex-hM3Dq:mCherry (n=7) into the NTS and their glucose tolerance was tested using an IPGTT. Mice received a 1% glucose i.p injection (1g/kg) at timepoint 0 and blood glucose was measured at 0, 15, 30, 60, 90 and 120 minutes, respective to glucose injection. Overall there was no difference be control and PPG-NTS ablated mice (A,B,G) ($F_{(5,60)}$ = 1.124, P= 0.3574). As glucose tolerance varies between sexes, the sexes were separated. There was no difference in glucose tolerance in males (C, D) ($F_{(5, 30)}$ = 1.361, P=0.267) and female PPG-NTS ablated mice compared to their respective controls (E, F) ($F_{(5,20)}$ = 1.046, P=0.419). Overall this experiment demonstrates that ablation of the PPG neurons does not affect glucose tolerance. Statistical significance was tested using a repeated measure ANOVA (A-F) or an unpaired t-test (G-I).

However, given the earlier results from rat suggesting a role for brain GLP-1R activation in glucose homeostasis, I wondered whether the current result might reflect a compensatory mechanism masking the loss of PPG neurons.

As measuring an effect from ablation of the PPG neurons requires the PPG neurons to be recruited in the situation presented in the experiment to produce a measurable effect, it is also possible in this study that the PPG neurons were not recruited under the paradigm. Furthermore, it is possible that the remaining IRT-PPG neurons are sufficient to modulate glucose homeostasis in the DTA mice. Consequently, I repeated these experiments under a paradigm of acute short-term activation of PPG neurons.

3.2.3.2 Expression of functional hM3Dq in the PPG neurons

To study the effects of activating the PPG neurons on glucose tolerance the stimulatory hM3Dq was expressed in the PPG neurons.

To confirm successful selective expression of hM3Dq, AAV-Flex-hM3Dq:mCherry was stereotaxically injected into the GCaMP3 mouse. The GCaMP3 mouse was used to confirm that the virus is CRE specific as it expresses a green fluorescent protein, which allows easier identification of hM3Dq expression (red). As both the RFP mouse and GCaMP3 mouse produce their reporter protein through the same CRE dependent mechanism, CRE is produced in the equivalent cells in each transgenic mouse line. In fig 3.5, expression of hM3Dq in the NTS (Fig 3.5B, magenta) was limited to cells that also express GCaMP3 (Fig 3.5A). All hM3Dq expressing cells co-localised with cells that expressed GCaMP3 (Fig 3.5C), demonstrating selective targeting of the NTS-PPG neurons with hM3Dq:mcherry.

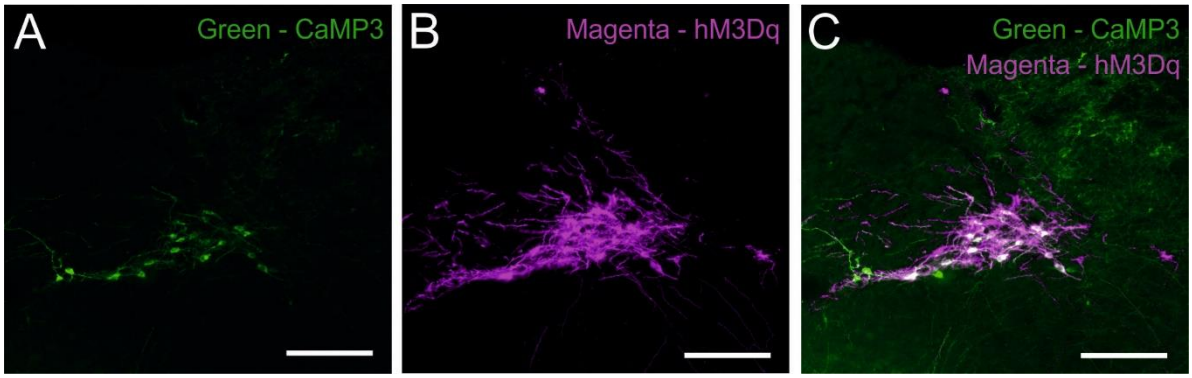


Figure 3.5 Selective expression of hM3Dq:mCHerry in the NTS PPG neurons: Immunofluorescent images demonstrating selective expression of hM3Dq in the NTS-PPG neurons. Glu-CRE-GCaMP3 mice were stereotaxically injected into the NTS with hM3Dq:mCherry. Following 3 weeks incubation the mouse was sacrificed and processed for GFP (A) and RFP (B) immunofluorescence. Co-localisation was visualised by overlaying both images with neurons expressing both hM3Dq and GCaMP3 visualised in white (C). All hM3Dq expressing neurons also expressed GCaMP3, demonstrating selective targeting of the NTS-PPG neurons with hM3Dq:mCherry **SB:100µm**

Before proceeding to behavioural experiments, it was necessary to confirm that the hM3Dq expressed in the NTS-PPG neurons is functional. This was also accomplished with the GCaMP3 mice (described in section 2.3.1). The GCaMP3 protein is a circular permutated GFP protein fused to calmodulin binding domain from the M13 myosin light chain kinase. In the absence of $Ca^{2+,}$ the permutated GFP protein exists in the poorly fluorescent state. As Ca^{2+} ions are introduced, Ca^{2+} binds to the 4 E-F motifs, induces a conformational change, which in turn increases fluorescence of the permutated GFP protein. Therefore, an increase in fluorescence is indicative of an increase in intracellular Ca^{2+}, which in turn indicates neuronal activation (Tian *et al.*, 2009; Zariwala *et al.*, 2012). hM3Dq was expressed in the NTS of the GCaMP3 mouse and the mouse brain was harvested, sliced and the NTS was imaged in *in vitro* brainstem slices. hM3Dq and GCaMP3 expression were visually confirmed by red (mCherry) and green (GCaMP3) fluorescence, respectively, before beginning of the experiment. Recorded cells exhibited both red and green fluorescence. Baseline green fluorescence was measured, followed by application of 1µM CNO in aCSF for 3 min.

Fig 3.6A depicts a pseudocoloured example cell to indicate GCaMP3 fluorescence intensity levels. The fluorescence of this cell increased in the presence of 1μM CNO, compared to baseline and washout of CNO. Fig 3.6B demonstrates the increase in intracellular Ca^{2+} of 11 cells in response to CNO, displayed in two different ways. Fluorescence increased in response to CNO application and began to decay following the wash. CNO increasing intracellular Ca^{2+} demonstrates that the hM3Dq expressed in the NTS-PPG neurons is functional, therefore the PPG neurons can be activated by CNO (this experiment was performed by Marie Holt).

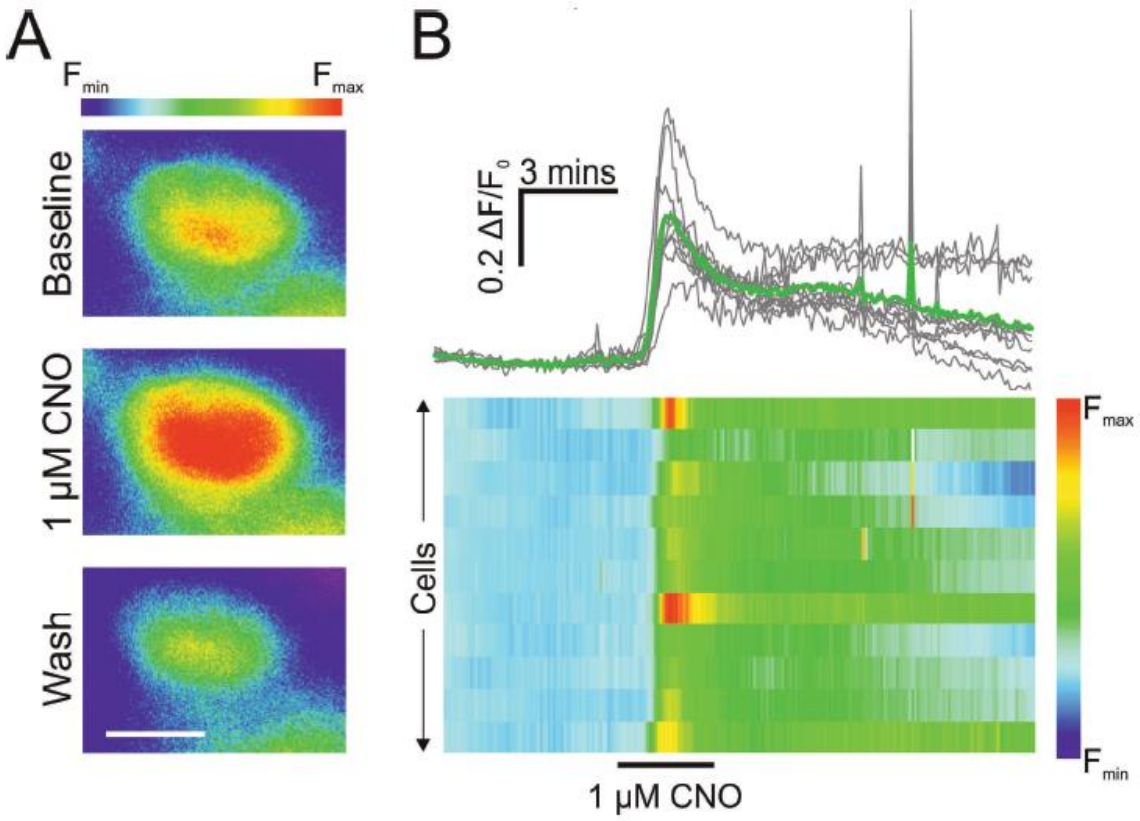


Figure 3.6 Functional expression of hM3Dq in the PPG neurons: Glu-CRE-GCaMP3 mice were stereotaxically injected with AAV-Flex-Hm3Dq:mCherry into the NTS. After 3 weeks incubation, mice were anaesthetised, the brain was harvested, and brainstem was section into 200µM slices. Sections were imaged using a microscope and fluorescence was measured. hM3Dq expression was confirmed by mCherry expression and mCherry expression colocalised with GCaMP3 expression. Baseline fluorescence was recorded in aCSF, followed by 3 minutes of 1µM CNO, followed by aCSF (wash). (A) A pseudocoloured example cell increasing its fluorescence in response to CNO, followed by a reduction in fluorescence during the wash. (B) present the fluorescence response to CNO across 11 hM3Dq expressing PPG cells (grey trace). The green trace represents the mean fluorescence across all 11 cells. As CNO is applied fluorescence increases in each cell, demonstrating functional expression of hM3Dq. **SB:10µm**

3.2.3.3 Acute pharmacogenetic activation of the PPG neurons does not affect glucose tolerance

After confirmation of functional hM3Dq expression in the PPG neurons, I studied the effects of activation of the PPG neurons on glucose tolerance as the experiment represents the ability of the PPG neurons to affect glucose tolerance independent of the conditions tested. To study the effects of activation of the PPG neurons on glucose

tolerance, Perceval (control) mice (n=4), and hM3Dq mice (n=4) received an IPGTT. Both cohorts of mice were injected (i.p.) with CNO (2mg/kg), 30 minutes before injection (i.p.) of glucose (1g/kg) and blood glucose was measured. To avoid any effect on blood glucose due to sex difference, this study was performed only in male mice. Overall there was no difference between the hM3Dq NTS injected animals and the controls (Fig 3.7A, B, C). There was a minor reduction in blood glucose concentration in the hM3Dq mice, 60 minutes after injection, although this was not significant. There was no obvious trend between the control and hM3Dq mice (Fig 3.7B). Following the experiment, all mice were transcardially perfused and expression of hM3Dq and Perceval in the PPG neurons was confirmed through immunohistochemistry and visual identification. This experiment demonstrated that activation of the PPG neurons does not affect glucose tolerance in mice, therefore the PPG neurons do not seem to have a role in glucose homeostasis. Lack of statistical significance was confirmed by repeated measure ANOVA $F_{(6, 36)} = 0.4908$, $P=0.811$.

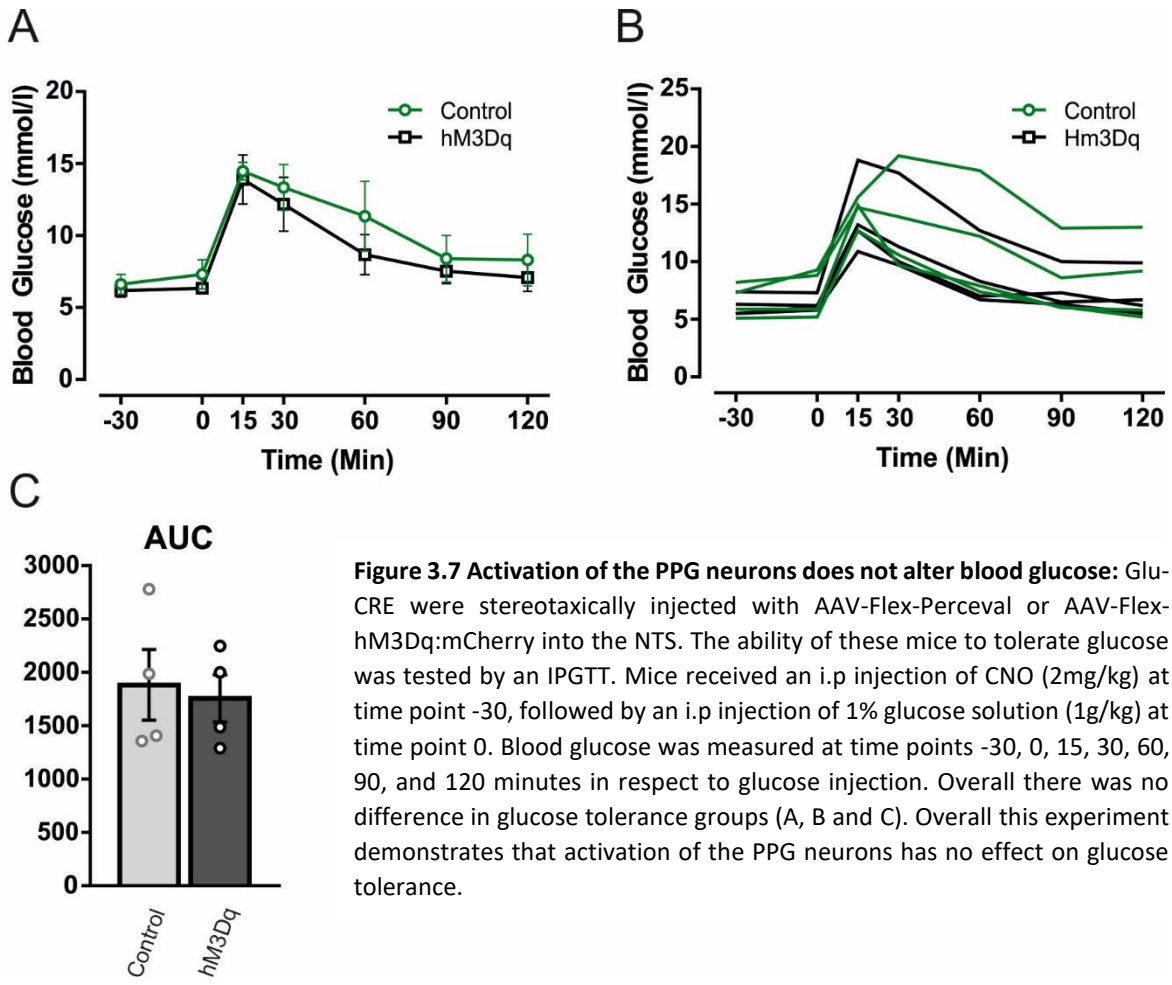


Figure 3.7 Activation of the PPG neurons does not alter blood glucose: Glu-CRE were stereotaxically injected with AAV-Flex-Perceval or AAV-Flex-hM3Dq:mCherry into the NTS. The ability of these mice to tolerate glucose was tested by an IPGTT. Mice received an i.p injection of CNO (2mg/kg) at time point -30, followed by an i.p injection of 1% glucose solution (1g/kg) at time point 0. Blood glucose was measured at time points -30, 0, 15, 30, 60, 90, and 120 minutes in respect to glucose injection. Overall there was no difference in glucose tolerance groups (A, B and C). Overall this experiment demonstrates that activation of the PPG neurons has no effect on glucose tolerance.

3.2.4 Activation of NTS-PPG neurons reduces food intake

Following the successful demonstration of functional hM3Dq expression in the NTS-PPG neurons, I wanted to use the paradigm to assess whether the PPG neurons had a role in food intake. As they are the main source of central GLP-1, I hypothesised that upon activation of the PPG neurons, the PPG neurons will release GLP-1 at the synapse, inducing hypophagia comparable to that seen in various studies upon i.c.v injection of GLP-1 and GLP-1R agonists.

Previous studies have claimed that activation of the PPG neurons reduce highly palatable diet intake and the leptin receptor expressed on the GLP-1 neurons is required in energy homeostasis (Scott *et al.*, 2011; Wang *et al.*, 2016). However, these

claims were derived from experiments using transgenic mice expressing CRE under Phox2B, as a method of targeting the PPG neurons. However, there is no co-expression between Phox2B and the PPG neurons, as demonstrated in Fig.3.8, where bilateral injection of the lentivirus PRSx8-AlstR-eGFP-LV (expressing eGFP in Phox2B expressing neurons) demonstrates two distinct populations between the Phox2B and PPG neurons (red). Therefore, Viral targeting of the NTS of Phox2B-CRE transgenic mice is not a valid approach to study the PPG neurons.

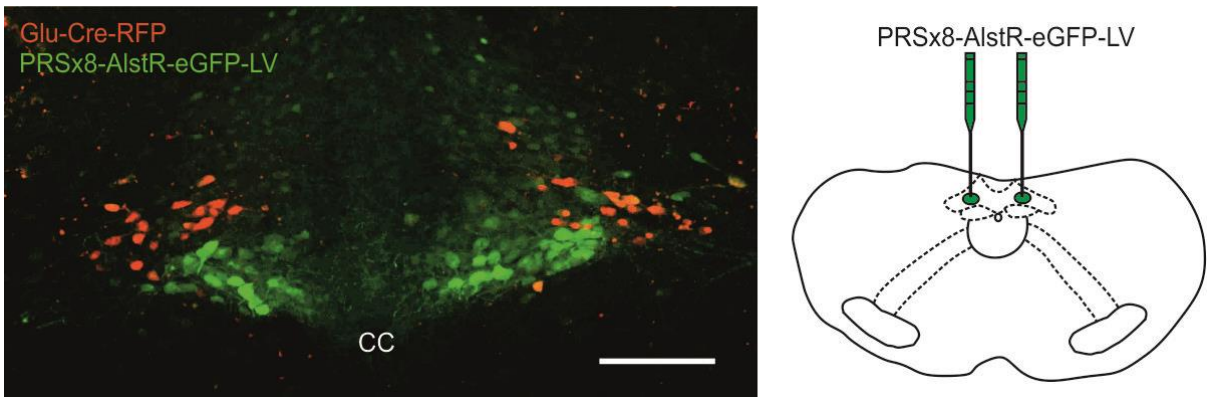


Figure 3.8: PPG neurons do not co-express Phox2B: Glu-CRE-RFP (Red) mouse stereoraxiaclly injected into the NTS bilaterally with PRSx8-AlstR-eGFP-LV (Green). PRSx8-AlstR-eGFP-LV expresses eGFP in neurons that express Phox2B. There is clearly no co-localisation between the Phox2B population and the PPG neuron population, therefore the PPG neurons do not express Phox2B.

To study whether the PPG neurons can induce hypophagia hM3Dq or Perceval (control) was selectively expressed in the NTS-PPG neurons of the PPG-RFP mice. All animals were fasted for 18 hours to drive feeding. Mice were initially tested with no injection to ensure that there was no intrinsic effect of the viral injection on food intake (data not shown). Following confirmation that both cohorts eat similar amounts of standard chow under this paradigm, mice received saline, or CNO (2mg/kg) injection. Standard chow was returned 30 minutes after injection and food intake was measured 1 hour and 2 hours after return of standard chow. There was no significant difference between control and hM3Dq after saline injection, revealed by repeated measure

ANOVA (Fig 3.9A). Injection of CNO significantly reduced food intake, with the repeated measure ANOVA revealing a significant effect of viral injection ($F_{(1,8)}$=17.44, P=0.0031), with a non-significant interaction. This suggests that the effect of CNO in the hM3Dq is not dependent on time within this time period, however I would expect this to change over a longer time period. Bonferroni multiple comparison test revealed significant effect at both time points (p= P=0.0173 and P=0.0011 in 1 hour and 2 hours respectively) (Fig 3.9B). This experiment demonstrates that activation of the PPG neurons acutely reduces food intake in fasted mice. It also confirmed that CNO had no effect on food intake in the absence of hM3Dq and that hM3Dq had no intrinsic activity to reduce food intake in the absence of CNO.

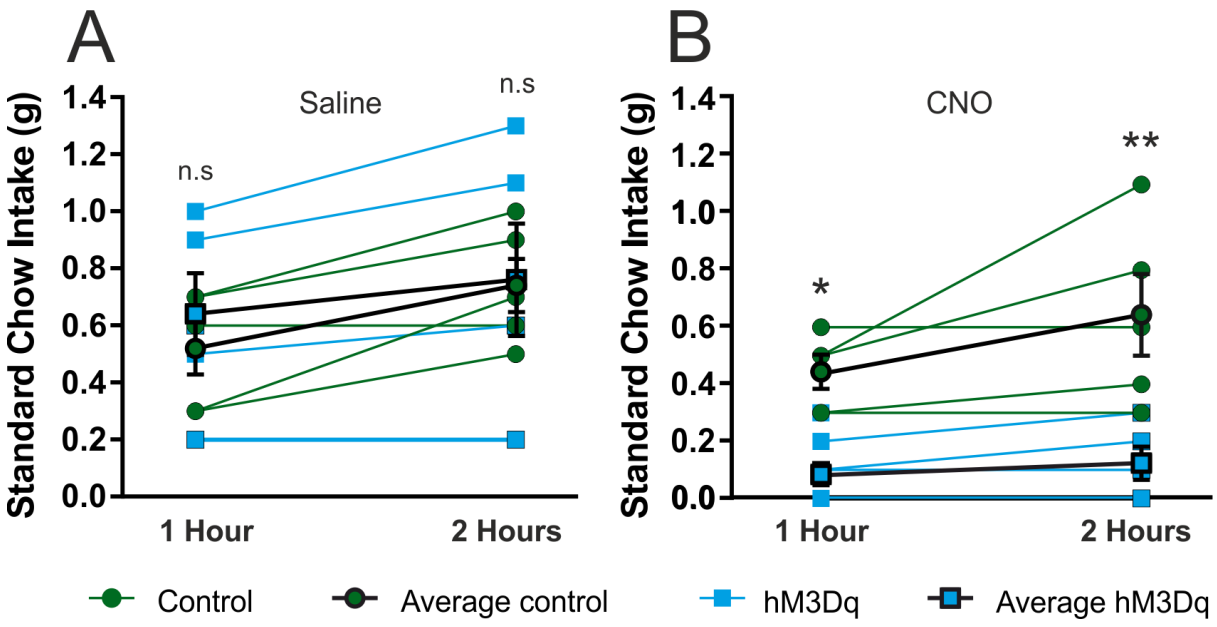


Figure 3.9 Activation of the PPG neurons acutely decreases food intake: Glu-CRE-RFP mice were stereotaxically injected with either AAV-Flex-Perceval or AAV-Flex-hM3Dq:mCherry. At least 3 weeks after the injection, mice were food restricted for 16 hours overnight. Mice were then injected with saline, or CNO (2mg/kg). Standard chow was returned 30 minutes later, and food intake was measured at 1 and 2 hours after the return of standard chow. Data is displayed as individual data points with mean ± SEM depicted as a black outline. CNO injection significantly reduced food intake compared to control when injected with CNO, whereas saline injection did not produce a significant difference at each time point. (Control mice n=5, hM3Dq mice n=5). * P<0.05, ** P<0.01, n.s not significant according to Bonferroni multiple comparison test.

As activation of the PPG neurons reduced food intake and that the PPG neurons are the central source of central GLP-1, it is likely that this was due to release of GLP-1. However, the PPG neurons produce other preproglucagon products such as GLP-2 and oxyntomodulin and are also glutamatergic (Zheng *et al.*, 2014; Cork *et al.*, 2015). Therefore, the hypophagia induced by the PPG neurons could be due to the release of any of these potential transmitters. To assess whether the food intake response to activation of the GLP-1R, Ex-9 was dual injected with CNO peripherally (i.p.) into the hM3Dq (n=5) and control mice (n=5). Ex-9 was dual injected with Ex-4 simultaneously to avoid the stress induced by two consecutive injection affecting overall food intake, which occurred during previous attempts at this experiment when Ex-9 was injected 15min before injection of Ex-4. In both the control and hM3Dq a repeated measure ANOVA revealed a significant effect of injection ($F_{(2, 12)}$= 4.743, P=0.0303, and $F_{(2, 12)}$= 5.924, P=0.0162 respectively). According to the Dunnett's multiple comparison test CNO + Ex-9 significantly increased food intake compared to CNO only injection in the 2nd hour in the control cohort (Fig 3.10A). No other results compared to the Ex-9 group was revealed to be significant. There was no significant difference between Saline and CNO + Ex-9 standard chow intake in the hM3Dq suggesting that Ex-9 attenuates the effect of activation of the PPG neurons on food intake (Fig 3.10B). However, there was also no significant difference between CNO and CNO + Ex-9 suggesting that Ex-9 does not attenuate the food intake effects of activation of the PPG neurons. Therefore, it is unclear whether the increase in food intake seen in the hM3Dq mice is due to blocking GLP-1 action released from the PPG neurons. As there was a significant increase in food intake in the controls when injected with CNO + Ex-9 compared to CNO only, it seems that systemic Ex-9 at a concentration of (100µg/kg) increases food intake, therefore any possible attenuation effect of food intake maybe due to the

intrinsic action of Ex-9 on food intake. In summary, this experiment demonstrates that activation of the PPG neurons can reduce food intake in fasted mice, and that the hypophagia induced by the PPG neurons is possibly attenuated by systemic Ex-9, however, this is unclear.

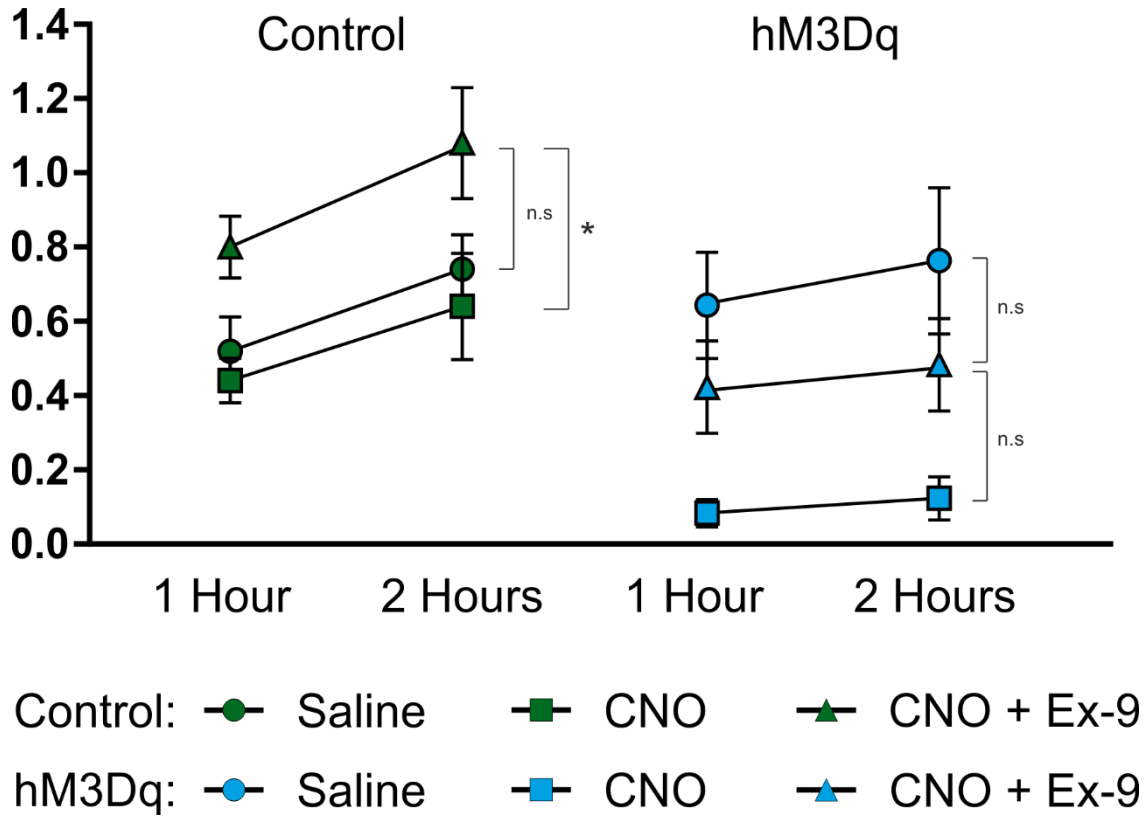


Figure 3.10 Systemic injection of exendin-9 possibly attenuates PPG neuron induced hypophagia: hM3Dq and Perceval mice were fasted overnight, followed by i.p injection of Saline, CNO (2mg/kg) or CNO (2mg/kg) + Exendin-9 (100µg/kg). Standard chow was returned 30 minutes later and food intake was measured at 1 and 2 hours, after the return of standard chow. Data displayed as mean ± SEM. There was a significant main effect in both the controls and hM3Dq revealed by repeated measure ANOVA. CNO + Ex-9 significantly increased food intake compared to CNO injection in 2 hours in the controls. There was no other significant effect when compared to CNO + Ex-9. Dunnett's test: * P<0.05

3.2.5 The reduction in food intake induced by the PPG neurons is transient

To further explore the regulation of food intake by the PPG neurons, I studied the effects of repeated activation of the PPG neurons. Here hM3Dq and perceval (control) mice were individually housed, and body weight and food intake were recorded every

24 hours. From day 1 to 5 the mice received injections of saline twice daily. hM3Dq (n=7) and control (n=6) mice show no significant difference in food intake and percentage change in body weight relative to day 0 (Δbodyweight), at baseline (day 1 to day 5). CNO (2mg/kg) was injected twice a day (i.p) on days 5, 6, 7, and 8. Two-way repeated ANOVA demonstrated a significant interaction between time and virus in both food intake ($F_{(12,132)}$ =1.62, P=0.0372) and bodyweight ($F_{(12, 132)}$ = 2.105, P=0.0206) After CNO injection began (Day 5), food intake of the hM3Dq animals significantly decreased to 2.14 ± 0.48g on the following day compared to the Perceval animal's food intake of 3.55 ± 0.23g (P=0.0119) (Fig 3.10A). Although Bodyweight of the animals decreased at day 7 and demonstrated a significant interaction there was no significant time point (Fig 3.10B). Interestingly, food intake in the hM3Dq animals returned to baseline despite injection of CNO from day 7 onwards (Fig 3.10A). Overall, the experiment demonstrates that activation of the PPG neurons reduces food intake over 24 hours with *ab libitum* access to standard chow. However, the reduction in food intake induced by activation of the PPG neurons is transient only. This experiment was performed in collaboration with Dr. James Richards.

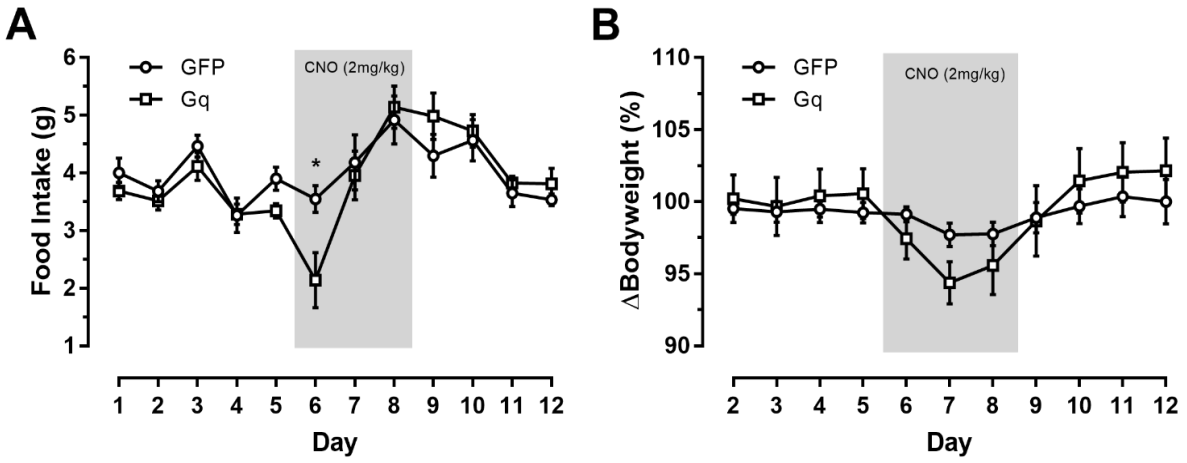


Figure 3.10. Activation of the PPG neurons transiently reduces food intake: Glu-CRE mice were stereotaxically injected with AAV-Flex-eGFP (n=6) or AAV-Flex-hM3Dq (n=7). Food intake (A) and bodyweight (B) was measured for 13 consecutive days. CNO (2mg/kg) was injected, twice a day (i.p) from day 5 to day 8. CNO significantly reduced daily food intake at day 6 in the hM3Dq injected mice compared to the controls. However, food intake in the hM3Dq injected mice returned to baseline levels (day 7, 8) despite CNO injection. There was no significant effect on bodyweight. This study demonstrates that activation of the PPG neurons reduces food intake in *ad libitum* fed mice, but the effect on food intake is only transient. Statistical significance was tested using a repeated measure ANOVA (Interaction ($F_{(12,132)}$ =1.62, P=0.0372) followed by Bonferroni multiple comparison test (Day 6 P=0.119).

3.3 Discussion

This chapter demonstrated that neither ablation of the PPG neurons nor their activation affects glucose tolerance. Furthermore, this study demonstrated that selective chemogenetic activation of the PPG neurons reduced food intake in food-restricted mice (fasted). The hypophagia induced by PPG neuronal activation was attenuated by systemic injection of Ex-9, however, whether this is due to blocking GLP-1R activation by GLP-1 released from the PPG neurons is unclear. Continuous activation of the PPG neurons reduced food intake in *ad libitum* fed mice, however, this hypophagia was transient only. Overall these experiments demonstrated that the PPG neurons have no role in the control of blood glucose but does have a role in the regulation of food intake.

3.3.1 Validation of mouse models

As the study of the PPG neurons in this thesis relies on the validity of the Glu-CRE-RFP mouse, the transgenic mouse strain needed to be verified before proceeding onto further experiments. As the Glu-YFP mouse has already been validated by Hisadome et al (2011), and the mechanism of reporter gene expression is entirely different between the two strains, cross breeding allowed verification of the Glu-CRE-RFP mouse (Hisadome *et al.*, 2011). 85% of YFP expressing cells co-localised with RFP, whereas only 5% of RFP expressing neurons did not express YFP. 5% of RFP expressing neurons not also expressing YFP is unsurprising, as once CRE is expressed the gene of interest is activated. Therefore, if there was historical expression of glucagon products in neurons during development the cre-dependent RFP is inverted and permanently activated, and would continue to be expressed, whereas the non-cre specific YFP will only be produced when glucagon products are being produced. Although unfortunate during visualisation of these neurons, this would not affect targeting these neurons with a cre-specific virus as CRE is no longer being produced therefore the cre-specific gene encoded by the virus will not be translated.

However, it is surprising that 15% of YFP neurons did not co-localise with RFP, as this cannot be explained by historical expression. It is possible that this anomaly is introduced because of the method in which the transgenic mice were generated. The PPG-YFP mouse was generated by Professor Frank Reimann and Professor Fiona Gribble using bacterial artificial chromosomes (BACs), compared to the traditional gene targeting approach (Reimann et al, 2008). In transgenic mice generated using a BAC, the BAC vector encodes a gene of interest (GOI) and randomly inserts the GOI into the mouse genome. The new founder mouse can germline transmit the inserted

gene to its offspring. Although BACs have been used frequently to generate transgenic mice, it does have its limitation generated by to the random gene insertion. These limitations have been reported to induce ectopic, mosaic or diminishing expression of the transgene (Yung and Gong, 2005). This method of transgenic mice development may be responsible for the 15% YFP expressing but not RFP expressing neurons, however I dispute this as it would suggest that GLP-1 is not produced in these ectopic transgene expressing neurons. As stated earlier, Hisadome et al demonstrated that 100% of YFP neurons expressed mRNA transcripts for PPG (Hisadome, et al., 2010). Additionally, Reimann et al verified the mouse line in the L-Cells of the gut demonstrating co-localisation of YFP with GLP-1 cells identified by immunohistochemistry, and proglucagon expression was 11,000 fold higher in YFP expressing cells compared to non-YFP expressing cells sorted by FACS (Reimann et al, 2008). Therefore, this exact transgenic mouse line has been verified to be specific to GLP-1 producing cells in different cell types, from two different labs, therefore it is more likely that the PPG-YFP mouse is PPG/GLP-1 cell specific.

As 95% of RFP cells co-localised with YFP expression, this data demonstrates that RFP, and therefore CRE is expressed in the majority of PPG neurons, and therefore the Glu-CRE-RFP mouse strain is appropriate to study the PPG neurons. Although the total PPG neurons in the brainstem may not be targeted, a large proportion of the PPG neurons were using the cre-specific viral approach. Although, it is important to note that it is possible that the remaining cells that were not targeted may be necessary to induce an affect particularly in the ablation studies. This conclusion was further supported by the effects of the ablation experiments on brain GLP-1 levels as discussed in the following section.

3.3.2 The NTS - preproglucagon neurons are the majority source of central GLP-1

Ablation of the NTS-PPG neurons was achieved by selectively expressing DTA in the NTS-PPG neurons, using a cre-lox recombinase system. Ablation of the NTS-PPG neurons significantly reduced the concentration of GLP-1 in the brainstem, hypothalamus, and spinal cord compared to controls. As the PPG neuronal cell bodies reside in the NTS, a nucleus in the brainstem, a reduction in GLP-1 concentration here is expected. Additionally, the hypothalamus and spinal cord are both strong projection targets for the PPG neurons. 50% of the PPG neurons in the brainstem projecting to the spinal cord, therefore a reduction in GLP-1 concentration here is unsurprising (Llewellyn-Smith *et al.*, 2015). My experiment confirmed this strong innervation, as the spinal cord concentration of GLP-1 was the largest in my study, and ablation of the NTS PPG neurons reduced this concentration significantly. There are no NTS PPG neuronal projection to the cerebellum or olfactory bulb, and the PPG neurons in the olfactory bulb were not ablated so high GLP-1 concentration was not expected. However, given the confirmed expression of PPG neurons in the olfactory bulbs (Merchenthaler, Lane and Shughrue, 1999; Thiebaud *et al.*, 2016), the measured concentration was quite low and could raise questions about whether these bulb cells indeed produce significant amounts of GLP-1. GLP-1 concentration in the blood was measured to assess if ablation of the PPG neurons affects peripheral GLP-1 levels. As ablation of the NTS-PPG neurons did not affect blood GLP-1 concentration it is probable that the PPG neurons do not modulate peripheral GLP-1 release, although this was already considered improbable.

Although ablation of the NTS-PPG neurons reduced the GLP-1 concentration in the brainstem, hypothalamus, and spinal cord, GLP-1 was not completely abolished from the brain. The remaining GLP-1 in each area could possibly be due to peripheral GLP-1 entering the brain. However, as the concentration of GLP-1 in the blood was significantly lower, this is unlikely. The IRT-PPG neurons were not ablated but have a similar projection pattern to the NTS PPG neurons and make up about 1/3 of all PPG neurons in the brainstem. Therefore, it is most likely that these neurons are the source of the remaining GLP-1. The OB-PPG neurons are granule cells and short axon cells (inter-neurons), therefore do not send projections to other parts of the brain and the PPG neurons do not have ascending projections to the brain, meaning they do not contribute to the brainstem and hypothalamic GLP-1 concentrations (Llewellyn-Smith *et al.*, 2015; Thiebaud *et al.*, 2016). Overall, this data suggests that the brainstem PPG neurons are the main source of central GLP-1, and possibly the only source. Moreover, the PPG neurons being the main source of brain GLP-1 implicates the PPG neurons to induce similar responses demonstrated by central injection of GLP-1 analogues.

3.3.3 Do the preproglucagon neurons have a role in glucose tolerance?

Firstly, I demonstrated that ablation of the NTS PPG neurons did not affect blood glucose, suggesting that the PPG neurons are not necessary to regulate glucose homeostasis. However, it is possible that the PPG neurons in the IRT are sufficient to regulate blood glucose in this case, as the NTS-PPG neurons were ablated, and the IRT-PPG neurons were left intact. Additionally, it is possible that the experimental paradigm was not sufficient to study the role of PPG neurons in glucose homeostasis, as the experiment requires to PPG neurons to be active in the situation presented in

the experimental paradigm in order to induce an effect. For example, it has been demonstrated that the PPG neurons are activated due to gastric distension in rat (Vrang *et al.*, 2003). If gastric distension is the physiological route of PPG neuronal activation the experiment, i.p injection of glucose would not initiate this pathway, therefore the PPG neurons would not be recruited, and therefore ablation of the PPG neurons would not induce a differential effect.

To overcome this limitation, I investigated whether activation of the PPG neurons affects glucose tolerance. This experiment explored the capacity of the PPG neurons to alter glucose homeostasis. However, activation of the PPG neurons did not alter glucose homeostasis demonstrating that the PPG neurons do not have the capacity to alter glucose homeostasis, and therefore have no role in glucose homeostasis.

Interestingly this result contradicts a previous paper published by Shi et al (2017). Here they demonstrated that chemogenetic activation of the PPG neurons using a hM3Dq in the GCG-CRE (PPG-CRE) mice increased glucose tolerance and reduced blood glucose in a glucose tolerance test 30 minutes after CNO (0.3mg/kg, i.p) injection (Shi et al., 2017). As this data follows the same experimental paradigm, the different result is unexpected. The discrepancy in results may be due to the under-powered nature of the experiment presented in this thesis, however if this was the case, I would expect a trend in the experiment in Fig 3.7, which cannot be found in the data and is not suggested by the P value at any timepoint. Interestingly the IPGTT experiments in the DTA mice did exhibit a trend towards decreased glucose tolerance which would be expected if activation of the PPG neurons increases glucose tolerance, however this only occurred in the males. The Shi et al study does not state whether the experiment occurred in males or females, which suggests that the experiment was performed in

both sexes. As the trend was only seen in DTA males it seems more likely that the trend is an anomaly in the data. If I assume that the Shi et al paper is correct, the data presented in this chapter demonstrates that ablation of the NTS-PPG neurons does not affect glucose tolerance, overall suggesting that the NTS-PPG neurons are sufficient to reduce blood glucose but are not necessary in the regulation of glucose tolerance.

3.3.4 Is the arcuate nucleus responsible for the regulation of glucose tolerance by the preproglucagon neurons

As described earlier, the PPG neurons are the central source of GLP-1, and have been demonstrated to effect glucose homoeostasis upon activation (Shi et al., 2017). However, ablation of the PPG neurons did not affect glucose tolerance suggesting that central endogenous GLP-1 has a non-necessary role in glucose homeostasis. This conclusion is supported by the studies that demonstrated a blood glucose response to exogenous application of central GLP-1 (Knauf *et al.*, 2005). Additional reports have identified the ARC as a brain region in mediating the central GLP-1 blood glucose response (described in chapter 1.2.2). The POMC neurons in the ARC have previously been shown to be 'glucose excited', and genetic disruption of this POMC glucose excitation impaired glucose tolerance in response of the glucose load (Parton et al., 2007)**.** The GLP-1R has been shown to be expressed in the POMC/CART neurons and GLP-1 has been demonstrated to directly depolarise the POMC/CART neurons in vitro (Secher et al., 2014). Additionally, the POMC/AgRP neurons have been shown to bind and uptake fluorescence labelled liraglutide, all suggesting that GLP-1 influences glucose homeostasis by modulating the POMC/CART neurons in the ARC (Secher et al., 2014). Although direct apposition of the PPG neurons to the POMC

neurons in the ARC have not been observed, it is possible that the PPG neurons activate the POMC neurons, through release of GLP-1, to induce a reduction of glucose homeostasis.

3.3.5 The preproglucagon neurons reduce food intake

Chemogenetic activation of the NTS-PPG neurons induced hypophagia in fasted mice during the light phase, demonstrating a role for the PPG neurons in food intake. However, food-restriction generated an impetus for the mice to consume a large meal in the light phase. As a light phase large meal is an unusual feeding condition for mice, it does not demonstrate that the PPG neurons reduce food intake in normal satiated mice. However continuous activation of the PPG neurons initially reduced food intake in *ab libitum* fed mice, therefore suggesting that the PPG neurons can reduced food intake under physiological conditions.

As the PPG neurons are the source of central GLP-1 and potentially use GLP-1 as a neurotransmitter, it is most likely that the hypophagia induced by NTS-PPG neuronal activation is due to release of GLP-1 at the synapse of the PPG neurons. This is supported by the studies demonstrating the effects of exogenous i.c.v GLP-1 and during specific injection of GLP-1 and Ex-9 into brain nuclei expressing associated with regulation of appetite (as stated in chapter 1.3) (Schick *et al.*, 2003; Hayes, Bradley and Grill, 2009; Alhadeff, Rupprecht and Hayes, 2012; Dossat *et al.*, 2012; Alhadeff *et al.*, 2014; Katsurada *et al.*, 2014; Secher *et al.*, 2014). As the PPG neurons project to the PVN, ARC, DMH, NAc, and VTA (among other nuclei), it is most likely that the PPG neurons reduce food intake through releasing GLP-1 from its projections to the PVN, ARC, DMH, NAc, and VTA, activating the GLP-1Rs expressed in the nuclei. Further evidence has been generated, that begins to delineate the pathways

in each nucleus that may be responsible for the food intake response to central GLP-1.

The necessity of the hypothalamus in the regulation of food intake in response to GLP-1 was demonstrated using a GLP-1RKD$^{\Delta Nkx2.1CRE}$ mouse by Burmeister et al. This mouse strain exhibited increased food intake, with no net effect on body weight, with only ~50% KD of hypothalamic GLP-1R (Burmeister et al., 2016).

In the PVN GLP-1 has been shown to evoke Ca^{2+} signals in the anorexigenic CRH, nesfatin-1 and oxytocin neurons, suggesting that GLP-1 induces hypophagia through activation of these neurons in the PVN (Katsurada et al., 2014)**.** Additionally, 80% of CRH and 38% of the Oxytocin positive neurons demonstrated positive C-Fos-IR 90 minutes after i.c.v infusion of GLP-1 in rats (Larsen et al., 1997) and central GLP-1 agonist increase circulating corticosterone a stress outcome induced by activation of the CRH neurons (Jessen et al., 2017). As GLP-1 activates these neurons it would seem most probable that the PPG neurons activate the oxytocin, CRH and nesfatin-1 neurons to induce hypophagia. To support this further the PVN projections of the PPG neurons have shown to establish synapses on both the perikaya and dendrites of the CRH neurons and the oxytocinergic neurons receive close appositions with GLP-1 positive boutons projecting from the NTS (Sarker et al., 2003; Tauchi et al., 2008). To my knowledge close apposition of the PPG neurons to the nesfatin-1 neurons has yet to be studied. To further test this hypothesise dual immunohistochemistry for C-Fos-IR and either CRH, nesfatin-1 or oxytocin following PPG activation would reveal activation of these neuronal pathways. Although the data for the PVN regulating the homeostatic appetite regulation of central GLP-1 is convincing it is not the only hypothalamic region that has been demonstrated to induce hypophagia in response

to central GLP-1. Interestingly, it has been demonstrated that GLP-1R KD solely in the PVN using the transgenic mouse strain (GLP-1RKDΔ^{SIM1CRE}) does not exhibit a food intake or bodyweight phenotype in these mice (Burmeister et al., 2016). As widespread GLP-1RKD in the hypothalamus induce a food intake phenotype the data suggest that other hypothalamic areas are involved in the regulation of appetite by central GLP-1.

The POMC/CART and NPY/AgrP neurons are two appetite regulatory counter-acting neuronal population that reside in ARC. The POMC/CART are anorexigenic population of neurons that produce α-melanocyte stimulating hormone (α-MSH) that act in the PVN reduce food intake, whereas the NPY/AgRP produce neuropeptide Y and AgRP which stimulate food intake and decreases melanocortin signalling respectively (Cowley et al., 2001, Roseberry et al., 2004). They provide inhibitory inputs to each other further regulating appetite. These two neuronal populations have been demonstrated to be crucial in the regulation of appetite by selective ablation of the POMC neurons in the ARC inducing an obese phenotype (Zhan et al., 2013) and selective ablation of the NPY neurons in adults induced rapid starvation (Luquet et al., 2005). As stated previously GLP-1R is expressed on the POMC/AgRP neurons and GLP-1 depolarises these neurons suggesting that GLP-1 influences appetite by modulating the POMC/CART neurons in the ARC (Secher et al., 2014). Furthermore, GLP-1 has been shown to act on the GABAergic neurons inhibiting the NPY/AgRP neurons, suggesting the GLP-1 indirectly inhibits the strong orexigenic signal in the ARC. As the PPG neurons have strong innervation of the ARC it seems probable that the PPG neurons would influence appetite through these neuronal population, however direct synapses to these neurons from the PPG neurons have yet to be observed. As well as specific KD of GLP-1R expressed in the PVN, specific KD of the

GLP-1R in the POMC neurons using a transgenic mouse strain (GLP-1RKD^{POMCCRE}) did not exhibit a food intake phenotype (Burmeister et al., 2016).

NPY neurons also reside in the DMH and have been implicated in controlling bodyweight and food intake. Interestingly, it seems a similar mechanism to NPY neurons in the ARC is employed here for GLP-1 to reduce food intake. Specific GLP-1R KD using AAV-GLP-1R shRNA in the DMH induced a small increase in bodyweight (Lee,S et al,. 2018). This DMH GLP-1RKD was associated with an increase in NPY mRNA expression in the DMH. As the GLP-1R is localised to the GABAergic neurons inhibiting the NPY neurons in the DMH, it appears that GLP-1 increases GABAergic (inhibitory) inputs to the NPY neurons to reduce food intake.

As widespread hypothalamic GLP-1R KD was required to induce a phenotype on food intake the data suggest that the hypothalamus as a whole is necessary in homeostatic regulation of food intake by GLP-1, but the individual nuclei are not necessary.

This food reward role of central GLP-1 would most likely be implemented through its projections to the VTA, and NAc as these nuclei have all been demonstrated to reduce food reward related behaviour upon injection of GLP-1R agonist, as stated in chapter 1.3.2. Food reward behaviour is regulated by dopaminergic pathways in the VTA and NA (Volkow et al,. 2012)**.** Dopaminergic pathways in the VTA project to the NA, amygdala and hippocampus, and reinforce food reward related behaviour (Wise., 2006)**.** Although the direct innervation of the PPG neurons to the dopaminergic neurons in the VTA and NA have not been observed, it is most plausible that they would act through activation of these dopaminergic pathways.

The NTS has also been implicated to have role in hedonic food intake in response to GLP-1, where microinjection of GLP-1 into the NTS (affected some sort of hedonic measure). The mechanism for this hedonic role of GLP-1 in the NTS has been suggested to be through the NA neurons. Injection of GLP-1 into the NTS increase DBH mRNA production in the NTS and increased dopamine-2 receptor nd TH mRNA production in the VTA (Richards et al,. 2015)**.** As the NA neurons directly project to the VTA and NA and have previously identified to have a role in hedonic food intake it is plausible that GLP-1 in the NTS regulates hedonic food intake through the NA neurons. The PPG neurons could also induce this effect as it has recently been demonstrated that the PPG neurons have close appositions with the NTS NA neurons (Patrick Card et al., 2018).

The literature discussed here suggests that central GLP-1 reduces both homeostatic and hedonic feeding and therefore suggest that the PPG neurons would exhibit the same role. It is important to note that the regulation of homeostatic and hedonic food intake is not considered entirely separate and the two systems interact to overall regulate food intake, therefore it appears that central GLP-1 and the perhaps the PPG neurons are regulating food intake at several levels of a complex regulatory system.

3.3.6 Do the PPG neurons reduce food intake through the release of GLP-1?

Systemic Ex-9 attenuates the reduction in food intake induced by chemogenetic activation of the NTS-PPG neurons, suggesting that blocking the GLP-1R stops the hypophagia induced by activation of the PPG neurons, therefore the PPG neurons release GLP-1 to reduce food intake. However, Ex-9 also significantly increased food intake in the control mice where CNO did not cause hypophagia. This does not exclude the possibility that the restoration of food intake by Ex-9 in the hM3Dq mice under

CNO is due to systemic effects of Ex-9 increasing intake, rather than preventing the GLP-1 released at the synapse of the PPG neurons from acting on the central GLP-1R's. Although it is most likely that the PPG neurons induce hypophagia through the release of GLP-1 these experiments are not conclusive.

It is possible that other neurotransmitters mediate the response. Firstly, the PPG neurons co-express glutamate (Zheng *et al.*, 2014; Cork *et al.*, 2015). As glutamate is widely used in the brain as an excitatory neurotransmitter, it is possible that the PPG neurons induce hypophagia by the release of glutamate. Secondly, the PPG neurons also produce other preproglucagon products (described in section 1.1), including GLP-2 and oxyntomodulin, both of which have been demonstrated to reduce food intake when injected into the CNS (Lovshin *et al.*, 2001; Cline *et al.*, 2008). It is unlikely that hypophagia induced is due to the release of GLP-2 as the PPG neurons projection pattern and GLP-1R expression is highly complementary, suggesting the PPG neurons act through GLP-1R activation, and GLP-2 does not act through the GLP-1R (Amato, Baldassano, and Mulè, 2016).

Furthermore, i.c.v injection of Ex-9 increased food intake in satiated rats, and microinjection of Ex-9 into the PVN and the NAc increased food intake, further suggesting that the food intake response is mediated through the GLP-1R (Turton *et al.*, 1996; Dossat *et al.*, 2011; Katsurada *et al.*, 2014). However, this does not rule out that GLP-2 acts to induce hypophagia through a different pathway. Additionally, this does not exclude oxyntomodulin, as oxyntomodulin acts on the GLP-1R to induce hypophagia when injected centrally (Kerr et al., 2010).

Based on the evidence, hypophagia induced by pharmacogenetic activation of the PPG neurons is most likely due to the release of GLP-1. To confirm that the

hypophagia induced by the PPG neurons is through the release of GLP-1, a dose of Ex-9 that does not induce hyperphagia should be injected i.c.v, before activation of the PPG neurons. If i.c.v Ex-9 attenuates the hypophagia induced by activation of the PPG neurons, then the hypophagia induced is dependent on the GLP-1R. A more complex experiment to demonstrate that the hypophagia is/is not induced by glutamate would be to cross the PPG-CRE mouse line with a CRE specific knockout VGLUT2. As VGLUT2 mostly expressed in the brainstem and is required for glutamate release, this would produce PPG neurons that lacked the ability to release glutamate. Activation of the PPG neurons in this mouse, and examining whether it would impact food intake would demonstrate whether glutamate release is required for the PPG neurons to induce hypophagia.

3.3.7 Why is PPG neuron induced hypophagia transient?

Although activation of the NTS-PPG neurons clearly reduced food intake, this effect was transient. Continuous activation of the PPG neurons initially reduced food intake compared to controls, however food intake then increased to levels comparable to the control mice. This transient effect of central GLP-1 is supported by central GLP-1R knockout having no overall effect on body weight and food intake, suggesting that central GLP-1 is not necessary for regulation of food intake in mice (Scrocchi *et al.*, 1996; Sisley *et al.*, 2014). In support of this, Baggio et al generated a transgenic mouse that expresses preproexendin under the metallothionein promoter (MT-Ex), which produces Ex-4 in response to drinking water supplemented with 25mM $ZnSO_4$. After activation of Ex-4 release, mice were food-restricted for 18 hours and food intake was measured. The study reported an initial decrease in food intake within the first 2 hours, but this effect was transient with food intake returning to the same level as the WT

controls for the remainder of the 24 hour experiment (Baggio *et al.*, 2000). In further support, central injection of GLP-1 initially reduced food intake in rats, but this effect was transient also (Donahey *et al.*, 1998)**.** In a similar experiment, Roman et al (2016) demonstrated that activation of the brain cholecystokinin (CCK) neurons and dopmain β-hydroxylase (DBH) neurons reduces intake. They found that chronic activation of the CCK and DBH neurons reduced food intake, but this reduction in food intake was also transient (Roman, Derkach and Palmiter, 2016). As the PPG, CCK, and DBH neurons are a few among other NTS populations that have the capacity to impact food intake, and many of the populations are activated by similar external signals including gastric distention, it is possible that when targeting one population, the other populations including the CCK and DBH can compensate for the increase in activation of the other, either through inhibition between populations or inhibition by the external signal.

Control of food intake is regulated by a multitude of hormonal and neuronal factors. It is possible that the drive for nutrition overcomes the hypophagia induced by the PPG neurons, leading to the decreased release of other anorexigenic hormones such as leptin, CCK, insulin, GIP, etc, or an increased release of orexigenic hormones such as ghrelin. A shift in the release of anorexigenic or orexigenic hormones could overcome the effects of the central GLP-1 release. Another reason could be downregulation/desensitisation of the GLP-1R at the synapse or downregulation/desensitisation of the hM3Dq. In support of this, the GLP-1R has been demonstrated to be internalised in response to overactivation (Roed *et al.*, 2014). Additionally, pre-treatment of Ex-4 in INS-1 cells reduced GLP-1R dependent cAMP accumulation, therefore reducing the recruitment of the secondary messenger under

continuous activation, reducing the release of GLP-1 at the synapse (Baggio, Kim, and Drucker, 2004). The loss of the hypophagic response could also be due to the reduction of GLP-1 stored at the synapse. As the NTS- PPG neurons are continuously activated in this experiment, it is possible that the consistent release of GLP-1 has depleted the stores at the synapse, and that axonal transport is not quick enough to replenish GLP-1 stores. Finally, the transient nature of the hypophagia induced could be due to the nature of the experimental paradigm. As the hM3Dq expressing in the NTS-PPG neurons is continuously activated it is possible that the hM3Dq is downregulated in the CNS, removing the ability of CNO to activate the PPG neurons. This effect would explain why the same transient nature of the hypophagia induced is exhibited in the CCK and DBH neurons in the Roman et al (2016) study. However, a similar lack of long term effect was demonstrated with i.c.v injection of GLP-1, therefore leading more credence to compensatory mechanism, or downregulation/desensitisation of the GLP-1R attenuating hypophagia induced by PPG neuronal activation (Donahey *et al.*, 1998).

6.6 Circadian Rhythm

A limitation found throughout the experiment in this chapter was the choice to perform many of the experiments within the light cycle period of the mouse day/night cycle. The circadian rhythm regulates a multitude of aspects within the body including hormone secretions, hepatic metabolism, sleep-wake cycle and body temperature (Jensen et al., 2013). As mice are nocturnal mammals' experiments performed during the light cycle will be affected by the natural activity of mouse. More specifically, both blood glucose and food intake has been shown to be affected by the circadian rhythm where bolus injection of glucose at differing time points demonstrated a different blood

glucose peak (Fleur et al., 2001) and mice have been shown to consume more food and water during the dark phase compared to the light phase (Szentirmai et al,. 2010). Furthermore, secretion of GLP-1 has ben shown to be released in a circadian rhythm from mGLUtag cells in response to a known secretagogue suggesting that GLP-1 release itself is regulated by the day night cycle (Gil-Lozano et al., 2014, 2015).

This could have affected both the ablation and activation of the PPG neuron experiments as all the experiments in this chapter were performed during the light cycle. The ablation experiment may be affected by the PPG neurons not being recruited naturally during the light cycle, therefore ablation of the PPG neurons would have no affect at this time. The activation experiments could also be affected as the concentration of GLP-1 released at the synapse of the PPG neurons would be lower during the light cycle and the GLP-1R expression on the post-synaptic cell would be lower as well inducing a lower response on food intake and glucose tolerance. To improve the experiments in this chapter it is recommended to simply move the timing of the experiment to be performed at the start of the dark cycle where the PPG neurons and GLP-1 release naturally occur.

3.3.8 Are DREADDS experimentally viable?

A recent study on the use of DREADDS has suggested that DREADDS are preferentially activated by the metabolite clozapine, rather than CNO. CNO is quickly metabolised to clozapine *in vivo* (Manvich *et al.*, 2018). CNO was demonstrated to have low affinity for the DREADD receptor, whereas clozapine was shown to have a higher specific affinity in HEK293 cells expressing hM3Dq and mouse brain tissue. Clozapine has non-specific effects within the brain, as demonstrated by its original use as anti-psychotic medication. Clozapine is a known serotonin receptor antagonist,

strongly binding to the 5-$HT_{2A/2C}$ receptor. Additionally, high concentration CNO was demonstrated to inhibit endogenous receptors (Histamine, H1; 5-HT_{2A}; Muscarinic, M1, M3, M4; and dopamine D1 and D2). This finding highlights the crucial nature of proper and thorough controls when using DREADDS. To prevent the aforementioned issue, in the experiments involving DREADDS I used controls that consist of non-DREADD viral injected mouse (mice injected with AAV-Flex-Perceval) and an injection of CNO in those animals, to ensure the effects seen are not due to the non-specific effects of the CNO and the CNO metabolite clozapine (Gomez *et al.*, 2017).

3.4 Conclusion

This chapter demonstrates that the PPG neurons are the source of central GLP-1. This insight demonstrates the potential for the PPG neurons to have a role in energy homeostasis, cardiovascular control, and glucose homeostasis, as injection of GLP-1 into the brain affects in all these regulatory systems. Building on this, potential chemogenetic activation of the PPG neurons reduces food intake in mice, suggesting that the PPG neurons have the capacity to regulate food intake. It is most likely the hypophagia induced is driven by the release of GLP-1 onto hypothalamic and mesolimbic sites that are known to regulate energy homeostasis and food reward behaviour in response to GLP-1R activation. Although the hypophagia induced was transient, it is possible that selective targeting of the PPG neurons could be used as a treatment for obesity. This chapter demonstrated that activation and ablation of the PPG neurons did not alter blood glucose, although previous studies have identified that activation of the PPG neurons did reduce blood glucose. Together with the data presented in this thesis it seems that the PPG neurons can alter blood glucose but are not necessary in resting conditions. Overall, this data expands and clarifies the role of

the PPG neurons. It demonstrates that the PPG neurons have the capacity to induce hypophagia but cannot regulate blood glucose. Future experiments should identify whether the PPG neurons are necessary to regulate food intake through ablation or inhibition of the PPG neurons during or preceding feeding sessions.

4. The role of GLP-1 and the preproglucagon neurons in cardiovascular control

4.1 Background

As mentioned in the introduction (Section 1.4.1) peripheral injection (i.p and i.v) of GLP-1 and GLP-1R agonist induces tachycardia in mice, rats, and humans (Barragan, Rodriguez and Blazquez, 1994; Yamamoto *et al.*, 2002; Hayes, Skibicka and Grill, 2008; Griffioen *et al.*, 2011a; Kang and Jung, 2016) and central (i.c.v) injection of GLP-1R agonist induces tachycardia in mice and rats (Yamamoto *et al.*, 2002; Griffioen *et al.*, 2011). As i.c.v injection of Ex-9 attenuated the tachycardia induced by peripheral GLP-1, and peripheral injection (i.p) of Ex-9 did not block i.c.v GLP-1 induced tachycardia, it is most probable that tachycardia induced by GLP-1 is caused by GLP-1 acting in the brain of rats (Barragán *et al.*, 1999).

4.1.1 The preproglucagon neurons

As demonstrated in chapter 3, the PPG neurons are the main source of central GLP-1, therefore the PPG neurons might be the physiological source of central GLP-1 induced tachycardia. The PPG neurons project to nuclei involved in cardiovascular control (Merchenthaler, Lane and Shughrue, 1999; Llewellyn-Smith *et al.*, 2011, 2013, 2015). They project to the preparasympathetic nuclei, the NA, and the DMNX. Additionally, the PPG neurons project to the PVN, ARC and the RVLM areas that modulate the preganglionic sympathetic nuclei in the spine IML and central CAA of the spinal cord (Coote, 2005; Llewellyn-Smith *et al.*, 2011, 2013; Rahmouni, 2014). Moreover, the PPG neurons themselves project to IML and CAA, with close

appositions to the sympathetic preganglionic neurons (SPN) (Llewellyn-Smith *et al.*, 2015). The NA, DMNX, RVLM, ARC and PVN nuclei express the GLP-1R, suggesting a direct mechanism for the PPG neurons to release GLP-1 onto the NA, DMNX, RVLM, ARC and PVN, possibly decreasing parasympathetic tone and increasing sympathetic outflow, increasing heart rate (Merchenthaler, Lane and Shughrue, 1999; P. Richards *et al.*, 2014; Cork *et al.*, 2015).

As peripheral and central injection of GLP-1 and GLP-1R agonists induced tachycardia, I hypothesised that the PPG neurons have the capacity to induce tachycardia. The PPG neurons could induce tachycardia through a decrease in parasympathetic tone or an increase in sympathetic drive. Parasympathetic tone to the heart is regulated by the cardiac vagal neurons which reside in the NA and DMNX. From here the cardiac vagal neurons project to the parasympathetic ganglia. The parasympathetic ganglia then release acetylcholine (ACh) in the sinoatrial (SA) and atrioventricular node (AV), where it binds to the muscarinic acetylcholine receptor, acting to decrease heart rate. As the PPG neurons project to the NA and DMNX, and the DMNX expresses the GLP-1R it is possible that the PPG neurons can inhibit the cardiac vagal neurons through release of GLP-1, therefore increasing heart rate through a reduction of parasympathetic tone.

It also possible that the PPG neurons increase heart rate through an increase in sympathetic outflow. The possible mechanism by which the PPG neurons could increase heart rate through an increase in sympathetic drive to the heart is through the projections to the presympathetic nuclei, the PVN, ARC, and RVLM and projections directly to the spinal sympathetic preganglionic neurons in the IML and CAA (Llewellyn-Smith *et al.*, 2011, 2015) . These pathways are outlined in Fig 4.1.

Additional to studying the effects of peripheral GLP-1 on cardiovascular parameters in mice, in this chapter I aimed to clarify whether PPG neurons can induce tachycardia and whether the PPG neurons are involved in the regulation of heart rate, using the Glu-CRE-RFP mice and the pharmacogenetic techniques used previously in this thesis.

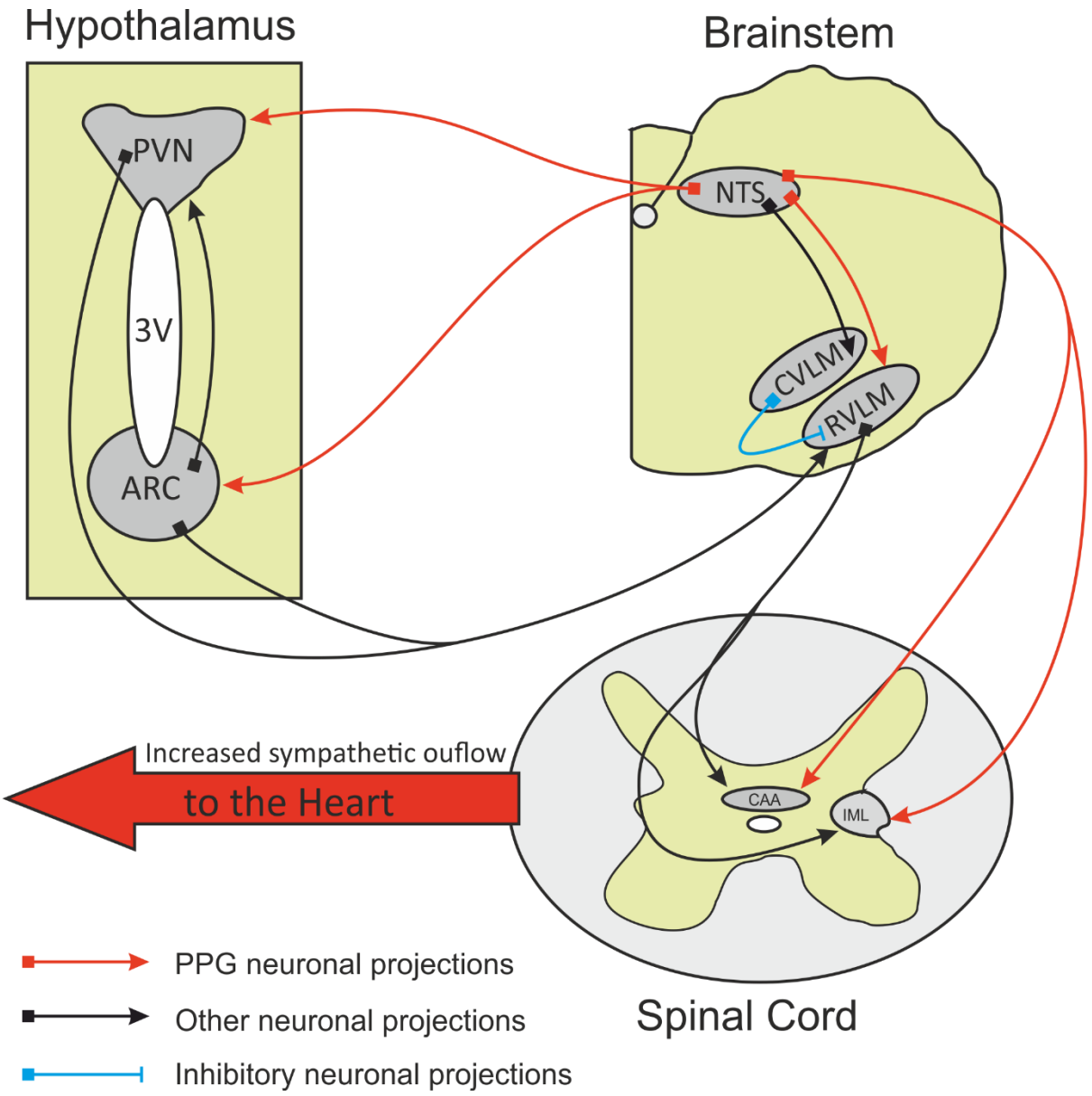


Figure 4.1: Possible mechanism for the preproglucagon neurons to induce tachycardia: Injection of GLP-1 into the brain induces tachycardia, through an increase in sympathetic drive. As the preproglucagon (PPG) neurons are believed to use GLP-1 as a neurotransmitter in the brain, it is plausible that the PPG neurons could also induce tachycardia. The possible mechanism to induce tachycardia is outlined in this figure. The PPG neurons project to the paraventricular nucleus (PVN), the arcuate nucleus (ARC) and the rostral ventral lateral medulla (RVLM). The PVN, ARC and RVLM have all been demonstrated to increase heart rate. Additionally, the ARC has been shown to have ascending circuitry to the PVN and descending circuitry to the RVLM, and the PVN has been demonstrated to have descending circuitry to the RVLM. From the RVLM, there are descending projections to the sympathetic preganglionic neurons (SPN's) in the IML and CAA, where activation leads to increased sympathetic drive to the heart, increasing heart rate. Additionally, the PPG neurons have descending projection to the SPN's in the IML and CAA. Upon activation of the PPG neurons, GLP-1 is released onto the aforementioned nuclei, activating the SPN''s, increasing sympathetic drive to the heart, increasing heart rate. **PVN: Paraventricular Nucleus, ARC: Arcuate nucleus, CVLM: Caudal ventral lateral medulla NTS: Nucleus tractus solitauis, DMNX: Dosal motor nucleus of the vagus, RVLM: Rostral ventral lateral medulla, CAA: Central autonomic area, IML: Intermediolateral column, 3V: 3rd ventricle.**

In this thesis the effect of GLP-1 and the PPG neurons on heart rate is studied in mice under general anaesthesia. Anaesthetised models have both advantages and disadvantages. Anaesthetics have been shown to influence cardiovascular parameters in mice and rats; for instance, isoflurane dose-dependently decreases mean arterial blood pressure and increase heart rate in mice (Constantinides, Mean and Janssen, 2011), and urethane has been shown to increase heart rate and sympathetic outflow in rats (Shimokawa *et al.*, 1998). Urethane also induces secretion of mucous in the trachea of the mouse, this excess production of mucous can lead to asphyxiation of the mouse without intervention from the experimenter (Moldestad *et al.*, 2009). Additionally, anaesthesia removes the ability of rodents to control their temperature, therefore they are at risk of hypothermia, without intervention from the experimenter. However, there are also clear advantages to using an anaesthetised model. Firstly, the stress response is removed. When studying the cardiovascular system in conscious mice, there is an initial peak in heart rate and blood pressure, due to the presence of the experimenter and any handling of the animal during the experiment (i.e injection) (the stress response in the CVS is demonstrated in Fig 4.12). Any effect on heart rate and blood pressure induced must persist longer than the stress response for it to be detectable in conscious mice. Secondly, to consistently record cardiovascular parameter in conscious animals, biotelemetry probes have to be implanted. Biotelemetry probes are costly and involve recovery surgery, putting additional stress and pain onto the animal. After considering all the advantages and disadvantages, an anaesthetic model was chosen for the following experiments.

4.1.2 Aims and Objective

In this study, I first investigated the effects of Ex-4 and GLP-1 on heart rate in mice. I aimed to clarify if Ex-4 increases heart rate through an increase in sympathetic outflow or a decrease in parasympathetic tone in mice. Then, using the Glu-CRE-RFP mouse strain, I investigated whether the PPG neurons are involved in cardiovascular control. I hypothesised that the tachycardia induced by Ex-4 is due to Ex-4 bypassing the PPG neurons and directly activating the nuclei expressing the GLP-1R, that are innervated by the PPG neurons. Therefore, activation of the PPG neurons should increase heart rate through the prospective release of GLP-1 in the cardiovascular control regions in the brain. I aimed to determine if:

- GLP-1 induces tachycardia in anaesthetised mice
- Ex-4 induces tachycardia in anaesthetised mice
- The tachycardia induced by Ex-4/GLP-1 is mediated through an increase in sympathetic outflow or a decrease in parasympathetic tone
- Assess whether the PPG neurons have the capacity to alter heart rate through their chemogenetic activation
- Assess whether PPG neurons-controlled tachycardia is necessary through chemogenetic inhibition

4.2 Results

4.2.1 ECG recordings in anaesthetised mice

In my experiments, heart rate was determined from ECG recordings in anaesthetised mice as depicted in Fig. 4.2. Using the ECG probes, an ECG was recorded that clearly depicted the R wave of an ECG allowing direct extrapolation of heart rate by measuring

the frequency of the R wave and converting the frequency into beats per minutes (Fig 4.2A). Displaying the ECG trace at higher time resolution clearly resolves the typical PQRST waves of an ECG (Fig 4.2B).

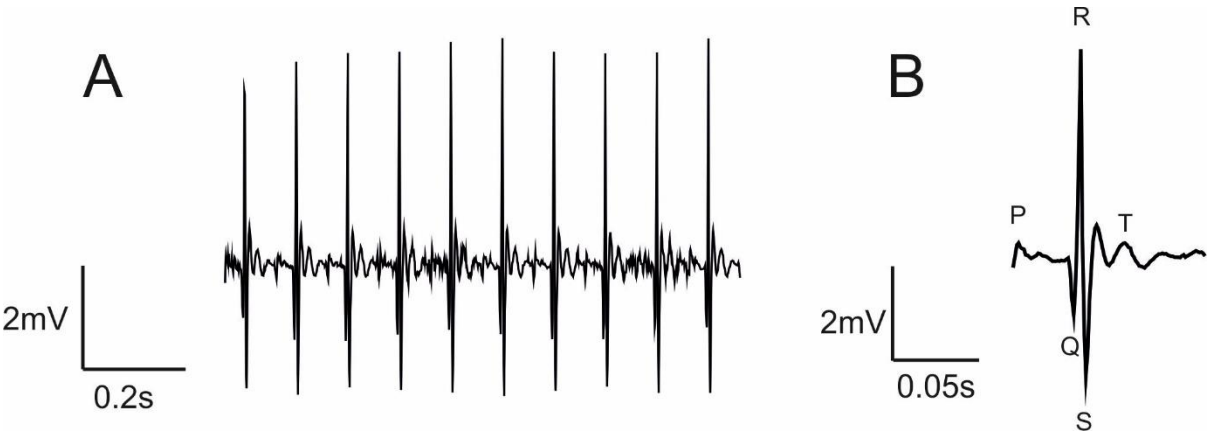


Figure 4.2 Electrocardiogram recorded from an anaesthetised mouse: ECG was recorded using a 2 core cable, with each wire soldered to syringe needles (22G). ECG was digitised and displayed in Spike7. (A) example image of ECG recorded from an anaesthetised mouse over 1s (B) example image of individual ECG wave, demonstrating the PQRST traces of the ECG trace.

4.2.2 Exendin-4 increases heart rate in urethane anesthetised mice

Knowing that GLP-1R agonists increase heart rate in conscious mice and rats (Barragan et al, 1999;Yamamoto *et al.*, 2002; Griffioen *et al.*, 2011;), I first confirmed that GLP-1R agonists can induce tachycardia in the urethane anaesthetised mouse. Fig 4.3 demonstrates the change in heart rate compared to baseline (Δheart rate), after i.p injection of saline (n=3), Ex-4 (10µg/kg, n=3) or Ex-4 (100µg/kg, n=3). Fig 4.3 demonstrates that i.p injection of Ex-4 (10µg/kg) significantly increased Δheart rate, whilst saline did not increase heart rate compared to baseline, demonstrating that injection of a volume of fluid has no effect *per se*. This also confirmed the absence of a stress response (comp. Fig 4.12) in the anaesthetised mouse. Two-way repeated measure ANOVA revealed a significant interaction ($F_{(8, 28)}$ = 2.53, P=0.0329) and the post-hoc Dunnett test revealed significant increase in heart rate from the 10 min timepoint in both Ex-4 groups.10µg/kg Ex-4 induced a maximum recorded heart rate

increase of 24.13± 6.64 BPM, 20 minutes after injection. Injection of 100µg/kg Ex-4 also significantly increased heart rate, 10 minutes after injection, compared to saline injection. This dose of Ex-4 increased the maximum recorded rise in heart rate to 32.74 ± 6.90 BPM, 20 minutes after injection. Although Ex-4 (10µg/kg) induced a significant Δheart rate, the amplitude of this response is very small compared to the amplitude of the tachycardia induced in conscious mice injected with Ex-4 (10µg/kg)**.** Marie Holt demonstrated an increase of median resting heart rate from 517.4 ± 23 BPM to 647.6 ± 36 BPM in response to i.p Ex-4 (10µg/kg) in conscious mice (Fig 4.12, unpublished). This increase in heart rate of 130 BPM is drastically larger than the increase in heart rate of 24.13 BPM produced in this experiment. Moreover, even the higher dose of Ex-4 (100µg/kg) induced a much smaller increase in heart rate in anaesthetised mice compared to conscious mice injected with Ex-4 (10µg/kg), suggesting that the anaesthetic might interfere with the mechanism of Ex-4 induced tachycardia. The dose of Ex-4 (10µg/kg) was chosen based on current literature, reporting effects on food intake, heart rate and blood pressure in rats and mice.

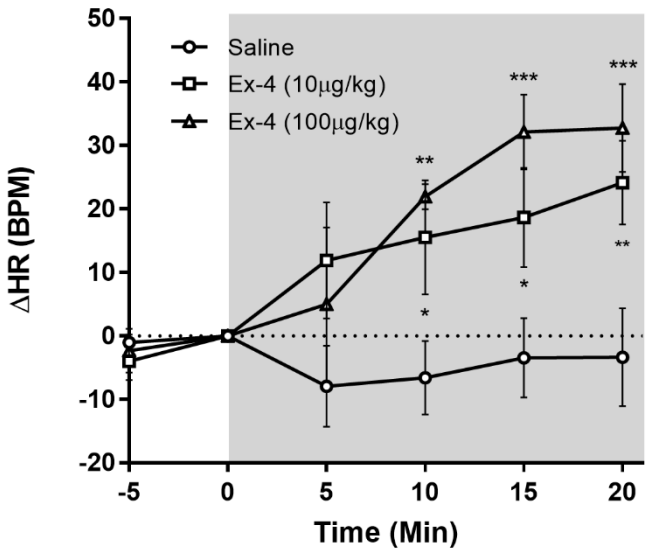


Figure 4.3: Exendin-4 increases heart rate in anaesthetised mice: Urethane anaesthetised C57/Bl6 mice were injected i.p with either saline (n=4), Ex-4 (10µg/kg, n=3) or Ex-4 (100µg/kg n=3) at time point 0. Ex-4 (10µg/kg) significantly increased ΔHeart Rate, 20 minutes after injection, compared to saline. Ex-4 (100µg/kg) significantly increased Δheart rate, 10 minutes after injection, compared to saline. There was no significant difference between Ex-4 (10µg/kg) and Ex-4 (100µg/kg) at any time point. Overall this demonstrates that Ex-4 induces tachycardia in anaesthetised mice. Statistical significance was confirmed by repeated measure two-way ANOVA ($F_{(8, 28)}$ = 2.53, P=0.0329), followed by post-hoc Dunnett's test (* P<0.05, **P<0.01, ***P<0.001)

4.2.3 Glucagon-like peptide-1 induces tachycardia without affecting vagal tone

After demonstrating that Ex-4 increases heart rate in the previous experiment, I wanted to determine whether GLP-1 also induces tachycardia. As described in section 1.1.3, Ex-4 is likely to cross the BBB, whilst this is less clear for GLP-1. Therefore, Ex-4 can act directly on central GLP-1R, whereas GLP-1 may not be able to reach central GLP-1R in sufficient amounts to activate the central GLP-1R. Furthermore, most studies use Ex-4 to examine the effects of GLP-1R activation on heart rate in mice. Currently, there is no study that has demonstrated the effects of peripheral GLP-1 injection on heart rate in mice.

When examining the effect of GLP-1 on heart rate, I recorded both the ECG and vagal nerve activity, in order to establish whether GLP-1 might increase heart rate through a decrease in parasympathetic tone. I injected 100µg/kg GLP-1 (i.v), and measured heart rate and vagal tone. An example of raw vagal recording is demonstrated in Fig 4.4C, where the expected oscillation of vagal activity occurs due to regulation of respiratory rhythm (Silverman et al, 2018). Heart rate increased following GLP-1 (100µg/kg) injection ($F_{(5,40)}$=4.824, P=0.0015) and reached significance after 10 minutes. GLP-1 increased heart rate by 28.63 ± 8.54 BPM, 20 minutes after injection (Fig 4.4A) but had no effect on vagal tone (Fig 4.4B). Vagal tone was measured by direct recording of vagal electrical activity. Vagal electrical activity was integrated over 10s periods, to obtain average electrical activity. The experiment demonstrated that GLP-1 can increase heart rate, without affecting vagal tone, suggesting that GLP-1 increases heart rate through an increase in sympathetic outflow, not a decrease in parasympathetic tone. Statistical significance was tested by repeated measure ANOVA, followed by post-hoc Bonfferoni's multi comparison test.

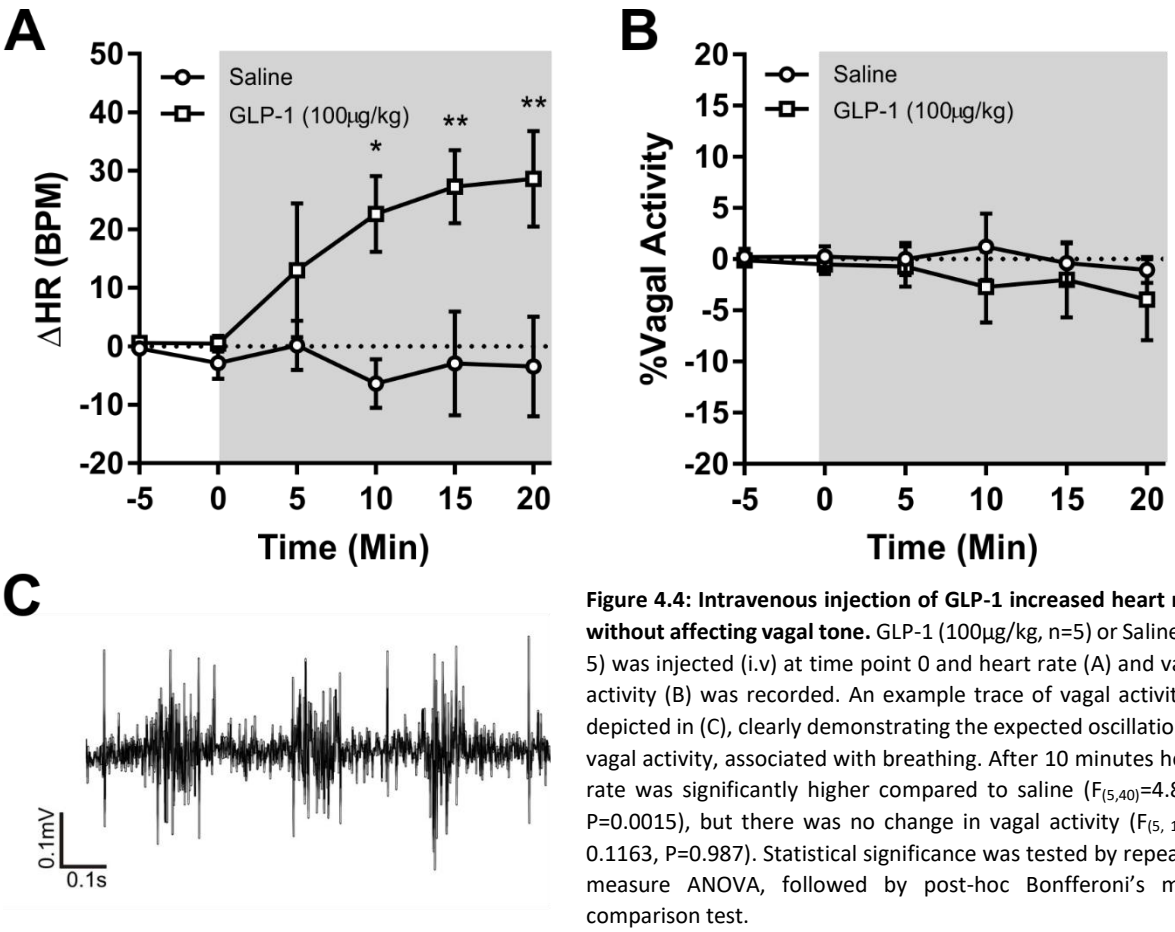


Figure 4.4: Intravenous injection of GLP-1 increased heart rate without affecting vagal tone. GLP-1 (100µg/kg, n=5) or Saline (n-5) was injected (i.v) at time point 0 and heart rate (A) and vagal activity (B) was recorded. An example trace of vagal activity is depicted in (C), clearly demonstrating the expected oscillation of vagal activity, associated with breathing. After 10 minutes heart rate was significantly higher compared to saline ($F_{(5,40)}$=4.824, P=0.0015), but there was no change in vagal activity ($F_{(5, 18)}$ = 0.1163, P=0.987). Statistical significance was tested by repeated measure ANOVA, followed by post-hoc Bonfferoni's multi comparison test.

Most anaesthetics affect the activity of the autonomic nervous system in some manner. Rats anaesthetised with urethane (i.p) display an increase in sympathetic outflow, through tachycardia and increase in renal sympathetic nerve activity but leave vagal tone intact (Shimokawa *et al.*, 1998). It has been demonstrated that concentrations of urethane exerts an effect on neurotransmitter gated ion channels, dose dependently potentiating the current response of $GABA_A$ and Glycine receptors, while inhibiting NMDA and AMPA receptors among others (Hara and Adron Harriss, 2014)**.** This fundamental change in neurotransmitter receptors could attenuate the response to GLP-1 and Ex-4, explaining the reduction in amplitude of the tachycardia induced by Ex-4 and GLP-1 in urethane anaesthetised mice. To address this potential limitation, the anaesthetic regime was changed in the following experiments.

4.2.4 Exendin-4 substantially increases heart rate in urethane/α-chloralose anaesthetised mice

To explore the possibility that urethane reduces the amplitude of Ex-4 induced tachycardia, heart rate recordings were performed in C57/Bl6 mice, under urethane/α-chloralose anaesthesia. At a 50% effective anaesthetic concentration of urethane, the effects on ligand gated ion channels is modest, therefore reducing the dose of urethane to 50% and supplementing with the sedative α-chlorlose should achieve anaesthesia, while reducing the effect of urethane in the CNS and on ligand gated ion channels (Hara and Adron Harriss, 2014). The following experiments in Fig 4.5 were statistically tested together using a repeated measure ANOVA ($F_{(40,144)}$= 6.512, P<0.0001), followed by post-hoc turkey test. The critical results were separated into sub-figures to clarify the significance of the pertinent results.

Mice were injected i.p with either saline (n=4) or Ex-4 (n=4)(10µg/kg), and heart rate was recorded. There was no significant difference between absolute heart rate between groups at baseline (Fig 4.5F). Ex-4 (10µg/kg) significantly increased heart rate, compared to saline injection. This increase was significant 10 minutes after injection and heart rate increased to a maximum of 71.10 ± 17.17 BPM (mean ± SEM), 30 minutes after injection (Fig 4.5B). The increase in heart rate of on average 71.10 BPM in this experiment is substantially larger than that of Ex-4 induced tachycardia in the urethane anaesthetised mice (24.13BPM). This experiment demonstrated that Ex-4 increases heart rate in anaesthetised mice and that the choice of anaesthetic is critical.

4.2.5 Atropine does not attenuate the positive chronotropic effect of exendin-4 on heart rate

To clarify the whether Ex-4 induces tachycardia through changes in sympathetic or parasympathetic tone in mouse, the following experiments were performed.

Firstly, to assess whether a reduction in parasympathetic tone contributes to Ex-4 induced tachycardia, the muscarinic acetylcholine receptor antagonist, atropine, was used to block parasympathetic tone to the heart (Just, Faulhaber and Ehmke, 2000; Baudrie, Laude and Elghozi, 2007; Janssen, Leenders and Smits, 2013; Nunn *et al.*, 2013). Atropine (2mg/kg) was injected i.p, 30 minutes before Ex-4. There was no significant difference in heart rate between the groups before injection of atropine (Fig 4.5F). Upon i.p. injection of atropine heart rate increased by 24.86 ± 11.97 BPM, measured 25 minutes after injection when the increase in heart rate reached a plateau (Fig 4.5E). In Atropine-Ex-4 mice (n=4), heart rate had significantly increased 10 minutes after injection of Ex-4 compared to Atropine-saline mice (n=4) (Fig 4.5A). There was no significant difference between the tachycardia induced by Ex-4 after atropine injection compared to after saline injection (Fig 4.5D). Ex-4 increased heart rate in the presence of atropine, therefore Ex-4 does not induce tachycardia through a decrease in parasympathetic tone.

4.2.6 Sympathetic blockade abolishes the positive chronotropic effect of exendin-4 on heart rate

To determine if the increase in heart rate by Ex-4 is due to increased sympathetic outflow, atenolol, a β1 receptor antagonist, was used to block sympathetic outflow to the heart (Burns *et al.*, 2004). Atenolol (2mg/kg) was injected i.p, 30 minutes before injection of Ex-4 (10µg/kg) or saline (Just, Faulhaber and Ehmke, 2000; Khor *et al.*,

2016). There was no significant difference in baseline absolute heart rate between Aten-Sal (n=4) and Aten-Ex-4 (n=4) mice, before injection (Fig 4.5F). Atenolol decreased resting heart rate by 48.77 ± 18.42 BPM, 25 minutes after the injection (Fig 4.5E).

There was a significant difference in Δheart rate between the Atenolol-Saline injected mice and Atenolol-Ex-4 injected mice (Fig 4.5B). The Δheart rate increase in the Sal-Ex-4 group first reached significance at timepoint 20min and maintained significance until the end of the experiment compared to the Atenolol-Ex-4 group (Fig 4.5C). Injection of Atenolol 30 minutes before injection of Ex-4 abolished the tachycardia induced by Ex-4 (Fig 4.5C).

These results demonstrate that Ex-4 induces tachycardia through an increase in sympathetic outflow and not through a decrease in parasympathetic tone.

There was no significant difference in absolute baseline between all cohorts, tested by one way ANOVA ($F_{(2.5,\ 7.7)}$=1.631, P=0.260). The effect of parasympathetic and sympathetic blockade was revealed as significant using a one-way ANOVA $F_{(2,19)}$=9.122, P=0.0017). The post-hoc Dunnett's test revealed significant effect of atenolol compared to saline, however the effect of atropine was not significant. At the end of all experiment's mice received an i.v bolus of phenylephrine (1mg/kg) and a bolus of sodium nitroprusside (1mg/kg) to ensure the baroreflex was intact (Fig.2.5G)

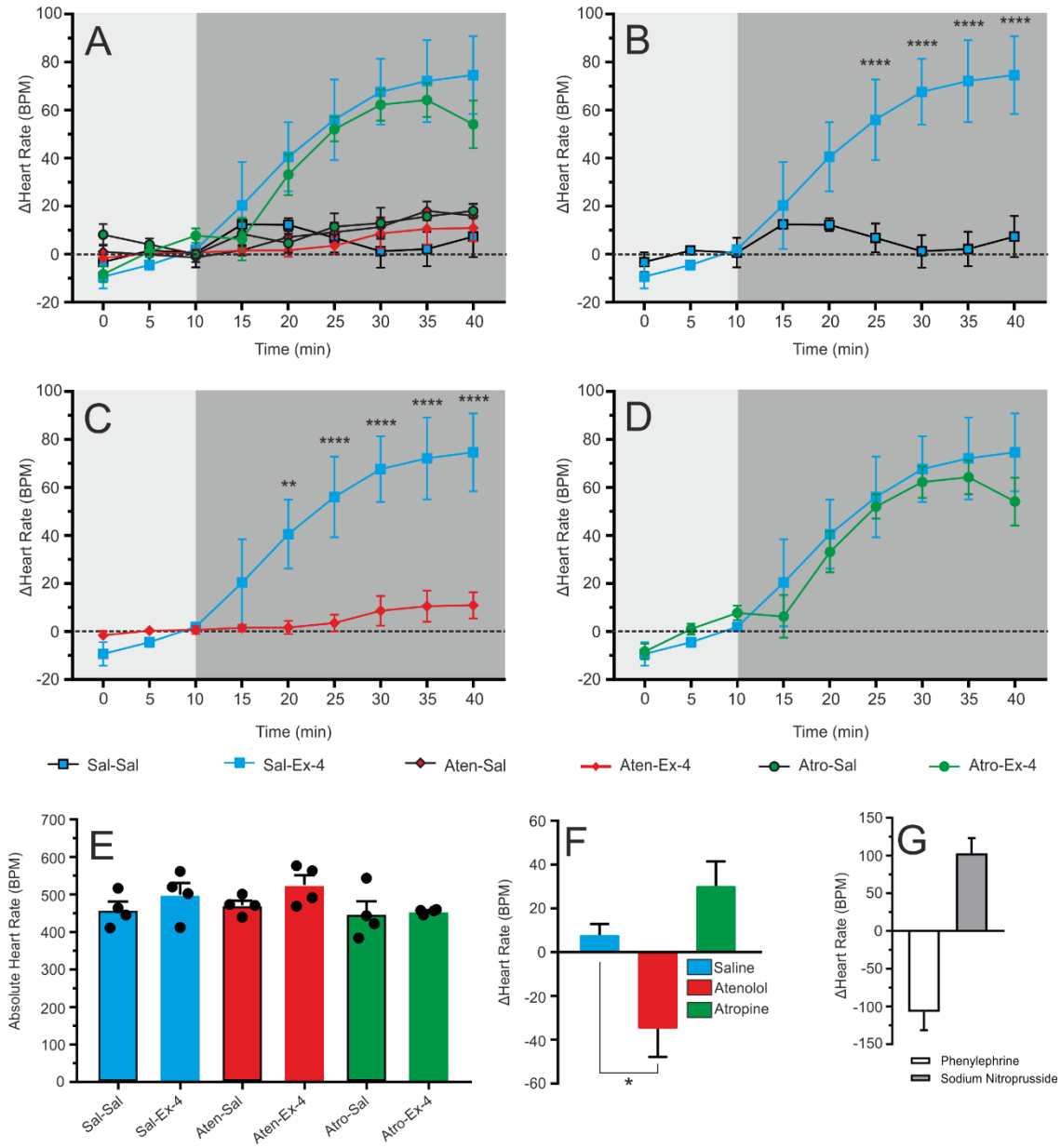


Figure 4.5 Autonomic blockade of exendin-4 induced tachycardia: C57/Bl6 mice were anaesthetised and heart rate was recorded. After a 10-minute baseline, mice received an i.p injection of either saline, atropine or atenolol. Following the first i.p injection, mice received a second injection of either saline or Ex-4, 30 minutes later, demonstrated in A,B,C,D by change in background colour (at t=10). Ex-4 (i.p) in anaesthetised mice substantially increased heart rate, which reached significance at time point 25 min(B). This increase in heart rate can be due to either an increase in sympathetic outflow or a decrease in parasympathetic tone. Atropine (2mg/kg) failed to block the heart rate response when injected 30 minutes before Ex-4 (D), while Atenolol (2mg/kg) attenuated the Ex-4 response (C). As expected Atenolol decreased heart rate and Atropine increased heart rate compared to baseline, 25 minutes after injection. One-way ANOVA revealed a significant interaction ($F_{(2,19)}$=9.122, P=0.0017), however post-hoc Dunnett's test only revealed significance in the atenolol cohort (E)(* p<0.05). There was no significant difference between baseline absolute heart rates between groups according to one-way ANOVA ($F_{(2.5, 7.7)}$=1.631, P=0.260) (F), (n=4 in each group). Phenylephrine (1mg/kg) and Sodium nitroprusside (1mg/kg) were injected i.v to test the baroreflex at the end of every experiment, ensuring the experimental preparation was still viable at the end of each experiment (G). Atenolol attenuates the heart rate increase induced by Ex-4, therefore Ex-4 increases heart rate through an increase in sympathetic drive. Statistical significance was tested using a repeated measure ANOVA, followed by Bonferroni multi comparison test, unless stated otherwise (* P<0.05, ** P<0.01, ***P<0.001, **** P<0.0001)

4.2.7 Expression of perceval, hM3Dq, and hM4Di in the NTS-preproglucagon neurons

To study the effects of PPG neuron activation or inhibition on heart rate, the Glu-CRE-RFP mice were injected either with AAV-Flex-Perceval, AAV-Flex-hM3Dq:mCherry, or AAV-Flex-hM4Di:mCherry, allowing me to inhibit or activate the neurons with CNO. At the end of the experiment each animal was transcardially perfused, sliced and stained for GFP and RFP to verify that Perceval, hM3Dq, and hM4Di, were selectively expressed within the PPG neurons. To confirm that the AAV-Flex-hM4Di is CRE specific the GCaMP mouse used, as used previously in Fig 3.5. hM4Di (Fig 4.6B), falsely coloured in magenta, co-localised with GCaMP3 (Fig 4.6A) expression when the images are overlaid (Fig 4.6C), confirming hM4Di is selectively expressed in the NTS PPG neurons in Glu-CRE mice.

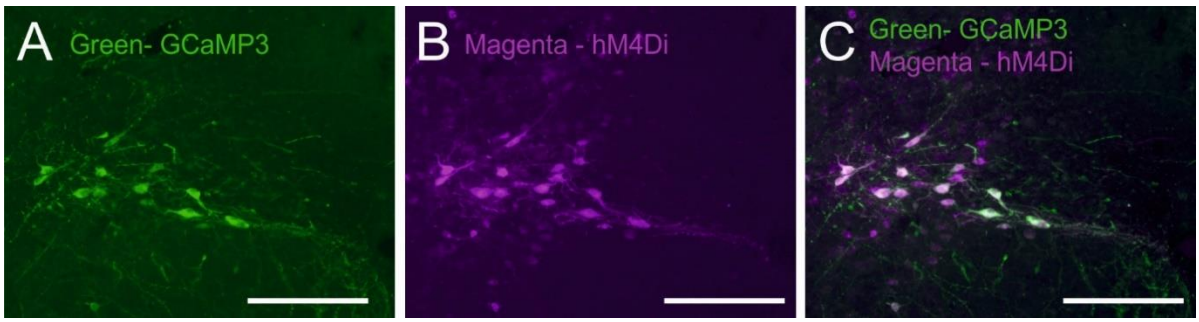


Figure 4.6: Selective expression of hM4Di in the NTS-PPG neurons: Glu-CRE-GCaMP3 mice were stereotaxically injected with AAV-Flex-hM4Di:mCherry, into the NTS. After 3 weeks mice were transcardially perfused and expression of hM4Di:mCherry was confirmed. PPG neurons expressing GCaMP3 are shown in green (A) and hM4Di:mCherry expression is fasley coloured in magenta (B). Co-localisation was visualised by merging both channels with neurons expressing hM4Di and GCaMP3 visualised in white (C). All hM4Di expressing neurons also expressed GCaMP3, demonstrating selective targeting of the NTS-PPG neurons with hM4Di:mCherry **SB: 200µm**

4.2.8 Pharmacogenetic activation of the PPG neurons induces tachycardia

It has been demonstrated, that Ex-4 and GLP-1 induce tachycardia when injected into the brain (Barragan, Rodriguez and Blazquez, 1994; Yamamoto *et al.*, 2002; Griffioen *et al.*, 2011; Goodwill *et al.*, 2014)**.** However, the physiological effects of endogenous

central GLP-1 on heart rate is yet to be determined. Having demonstrated in the previous chapter that the GLP-1 found in the brain originates from the PPG neurons, I next examined whether activation of the PPG neurons induces tachycardia, through the release of GLP-1 onto the projection sites of the PPG neurons involved in cardiovascular control, including the PVN, DMH, RVLM, IML, and CAA. To investigate this I initially used Glu-CRE mice stereotaxically injected with either AAV-Flex-eGFP or AAV-Flex-hM3Dq:mCherry. Both urethane and urethane/α-chloralose preparations were used to study the effects of PPG neuronal activation on heart rate as urethane keeps vagal tone intact, which allows more optimised study of effects induced by changes in vagal tone. Urethane/α-chloralose studies were also used as this mitigates some of the effects of urethane on the CNS neurotransmitters ion gated channels and sympathetic outflow, which allows more optimised study of effects induced by changes in sympathetic outflow (O'Leary and Joes, 2003).

In mice anaesthetised with urethane (1300mg/kg, i.p), heart rate was recorded for 15-minutes, before i.p injection of CNO (2mg/kg). There was no significant difference in heart rate between the control or hM3Dq group before injection of CNO (Fig 4.7B). Following injection of CNO (2mg/kg), there was no significant difference in the Δheart rate at any time point (Fig 4.7A) and there was no visible trend between two groups (Fig 4.7C). However, variation in ΔHeart rate increased over time (Fig 4.7C) suggesting that small changes in ΔHeart rate would be hard to distinguish. Statistical significance was determined with a repeated measure ANOVA $F_{(12, 72)}$= 0.205, P = 0.0079. This lack of effect of PPG neuron stimulation could be due to the fact that urethane itself maximises sympathetic drive to the heart, as demonstrated by the

resting heart rate under urethane (Fig 4.7B) compared to that under urethane/α-chloralose (Fig. 4.8C) might suggest.

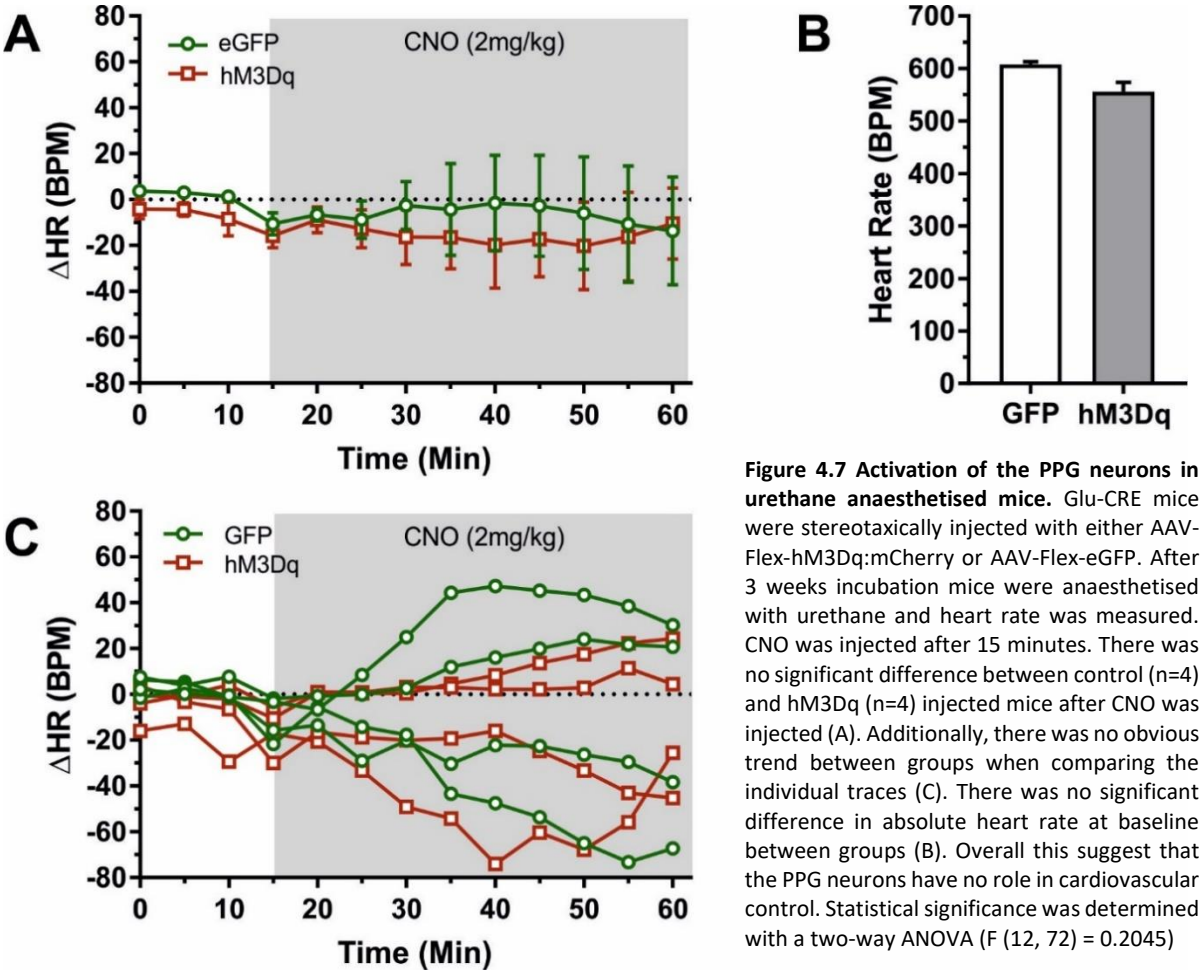


Figure 4.7 Activation of the PPG neurons in urethane anaesthetised mice. Glu-CRE mice were stereotaxically injected with either AAV-Flex-hM3Dq:mCherry or AAV-Flex-eGFP. After 3 weeks incubation mice were anaesthetised with urethane and heart rate was measured. CNO was injected after 15 minutes. There was no significant difference between control (n=4) and hM3Dq (n=4) injected mice after CNO was injected (A). Additionally, there was no obvious trend between groups when comparing the individual traces (C). There was no significant difference in absolute heart rate at baseline between groups (B). Overall this suggest that the PPG neurons have no role in cardiovascular control. Statistical significance was determined with a two-way ANOVA ($F(12, 72) = 0.2045$)

In mice anaesthetised with urethane/α-chloralose mix a 10-minute baseline was recorded and CNO (2mg/kg) was injected i.p. There was no significant difference in heart rate between groups prior to CNO (Fig 4.8C). A significant increase in heart rate was observed 20 minutes after CNO injection and reached a peak at 74.24 ± 8.91 BPM, 40 minutes after injection in hM3Dq expressing, but not in control mice (Fig 4.8A,B). This clearly indicates that PPG neurons can induce tachycardia and suggests a role for the PPG neurons in cardiovascular control. Sodium Nitroprusside (1mg/kg) and Phenylephrine (1mg/kg) were injected i.v to test the pressor and depressor

response (respectively) testing that the baroreflex was intact at the end of each experiment, ensuring that changes in heart rate can be measured and that the CVS is functioning as expected (Fig 4.8D). Statistical significance was explored using a repeated measure ANOVA (interaction F (12, 84) = 7.19, P < 0.0001), followed by bonferonni multicomparison test

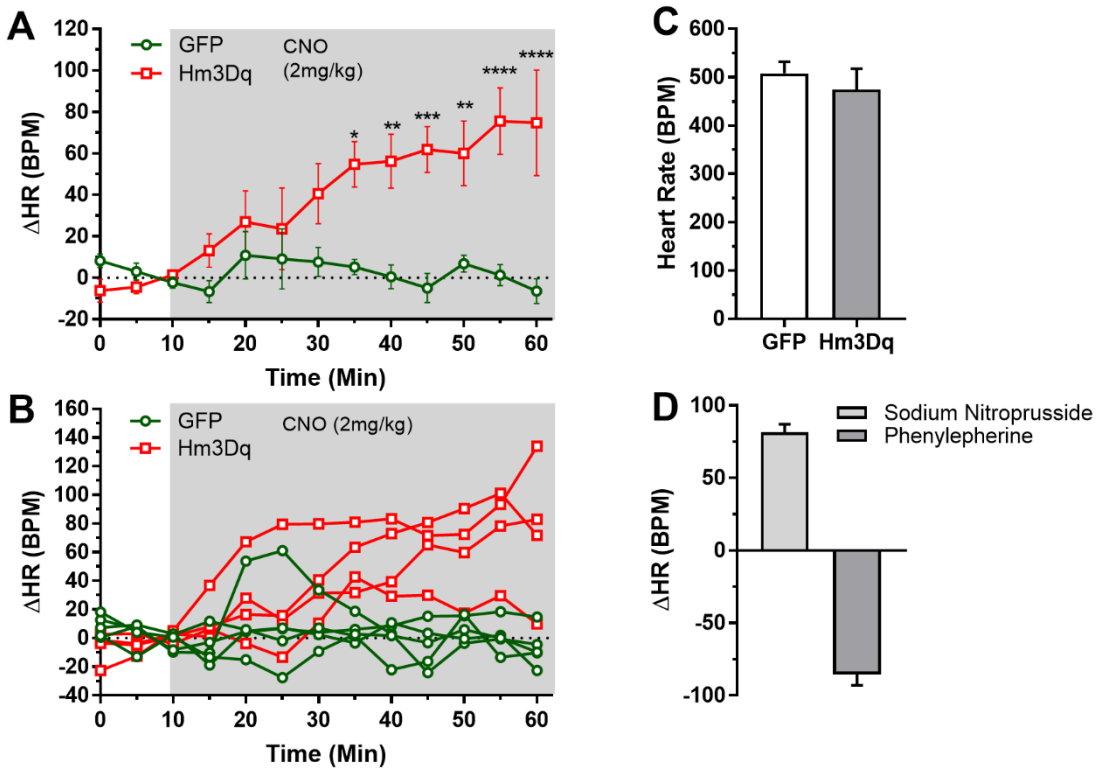


Figure 4.8 Activation of the PPG neurons increases heart rate in urethane/α-chloralose anaesthetised mice. Glu-CRE-RFP mice were stereotaxically injected with either AAV-Flex-eGFP (n=5) or AAV-Flex-hM3Dq:mCherry (n=5). Following a 3-week incubation, mice were anaesthetised with a urethane/α-chloralose mix through the femoral vein and heart rate was recorded. CNO (2mg/kg) was injected after 10 minutes. Activation of the PPG neurons increased heart rate significantly by 20 minutes post CNO injection (A). Individual heart rate traces are depicted in B. There was no significant difference between the baseline heart rate of both groups (unpaired t-test, P<0.05) (C). Sodium Nitroprusside (1mg/ml, 50µl) and Phenylephrine (1mg/ml, 50µl) were used to test the baroreflex of every mouse, ensuring each mouse still maintained cardiovascular control under anaesthesia. Statistical significance was achieved by repeated measure two-way ANOVA interaction F (12,84) = 7.19, P < 0.0001), followed by Bonferroni multiple

4.2.9 Inhibition of the NTS-PPG neurons does not reduce heart rate

Although pharmacogenetic activation of the PPG neurons induced tachycardia in mice, this does not necessarily mean that the PPG neurons have a major physiological role in cardiovascular control, as this experiment forces activation of the PPG neurons under resting conditions where they may not be intrinsically recruited. To assess whether the PPG neurons are required in control of the CVS, the next experiment assessed whether acute inhibition of PPG neurons affects heart rate, Glu-CRE-RFP mice were stereotaxically injected with either AAV-Flex-eGFP (same controls as animals in fig 4.2.8) or AAV-Flex-hM4Di:mCherry (n=3). Mice were anaesthetised initially with isoflurane, followed by i.v urethane/α-chloralose mix and heart rate was recorded. A 10-minute baseline was recorded, CNO was injected i.p (2mg/kg) and heart rate was recorded for the following 40 minutes (Fig 4.9A). There was no difference in heart rate between groups prior to CNO (Fig 4.9B). There was no difference in heart rate between groups at any timepoint following injection of CNO. Lack of statistical significance was confirmed by repeated measure ANOVA ($F_{(10, 60)}$ = 0.461, P=0.905). Therefore, inhibition of the PPG neurons does not decrease heart rate in anaesthetised mice. This experiment demonstrates that the PPG neurons are not significantly activated during resting, anaesthetised conditions, and thus do not significantly contribute to resting heart rate. It is important to note that the same eGFP control group as the previous experiment was used here.

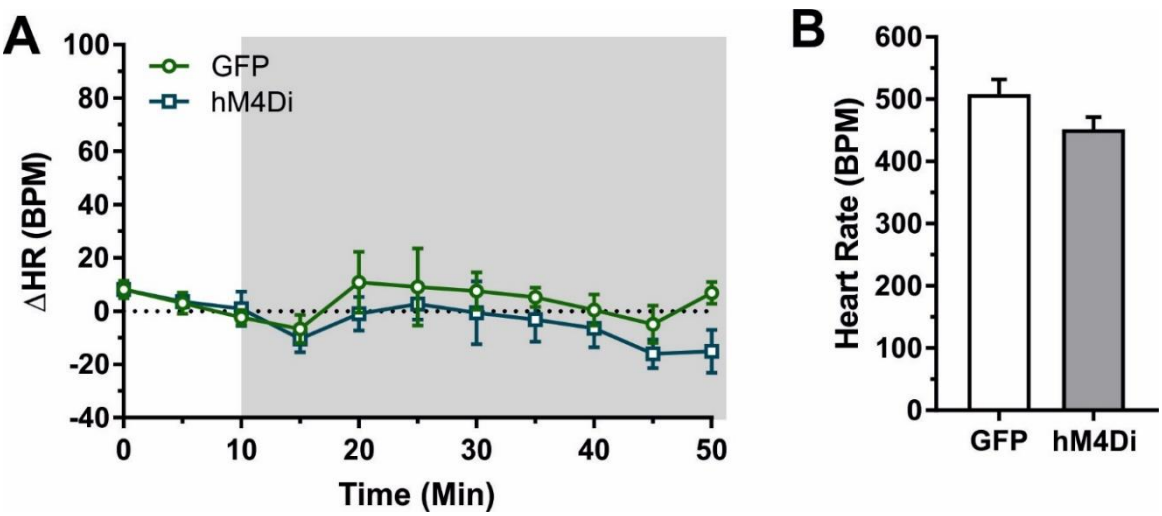


Figure 4.9 Inhibition of the preproglucagon neurons did not decrease heart rate: Glu-CRE mice were stereotaxically injected with AAV-Flex-eGFP (n=5) or AAV-Flex-hM4Di:mCherry (n=3). (A) Mice were anaesthetised, and heart rate was recorded. CNO (2mg/kg) was injected i.p 10 minutes later (transition to grey background, t=10). Inhibition of the PPG neurons did not reduce heart rate compared to the control. There was no difference in absolute heart rate at baseline between both groups (B) (unpaired t-test, $p>0.05$).

4.3 Discussion

In this study I demonstrated, that Ex-4 and GLP-1 induce tachycardia in anaesthetised mice through an increase in sympathetic outflow. This result confirms similar findings seen in rats (Yamamoto *et al.*, 2002; Gardiner *et al.*, 2010; Saraiva and Sposito, 2014). and refutes the suggestions that these drugs work via a decrease of vagal tone to the heart (Griffioen *et al.*, 2011). Additionally, I showed that chemogenetic activation of the PPG neurons also induced acute tachycardia in anaesthetised mice, at a similar magnitude as seen with the direct injection of GLP-1 or Ex-4, demonstrating that the PPG neurons have the capacity to regulate heart rate, and suggesting that they activate the same pathways as employed by the peripherally injected drugs. However, inhibition of the PPG neurons did not reduce heart rate in the anaesthetised model, demonstrating that the PPG neurons do not have a tonic effect on heart rate under

resting (anaesthetised) conditions. These novel findings replicate the tachycardia induced by central injection GLP-1 and Ex-4, providing further evidence that the PPG neurons are the central source of GLP-1.

4.3.1 Anaesthetic in mice

The use of anaesthetics in mice is particularly challenging as mice hold several differences to other animals that effect the properties that the anaesthetic induces in mice. The small size of a mouse leads to rapid drug metabolism and excretion, reducing the half-life of the anaesthetic causing a reduction in length of appropriate anaesthetic depth. Maintaining anaesthetic depth is crucial in ensuring that no excess pain and stress is brought onto the animal and that experimental outputs, in particular heart rate, are not affect by stressful stimuli. The increased surface area of mice also promotes severe heat loss and anaesthesia reduces the ability of mice to regulate body temperature inducing hypothermia in anaesthetised mice and the increased oxygen demand in rodents reduces the survival rate in response to hypoxia. Hypothermia induces an initial tachycardia in mild hypothermia and bradycardia in more moderate hypothermia, demonstrating that body temperature can fundamentally effect the cardiovascular system (Deussen,. 2007)**.** Hypoxia increases the oxygen demand of the biological system, and will act to increase heart rate and blood pressure to try to meet this demand for oxygen. A few seconds of hypoxia has been demonstrated to produce irreversible CNS damage in mice, fundamentally effecting the health of the mouse (Gargiulo et al., 2012)**.** These limitations require anaesthetised mice to be closely monitored to avoid these effects tainting the experimental outcome. To counter these effects a set of procedures and criteria were used to assess the experimental viability of each mouse used in the experiment.

At the start of the procedure successful depth of anaesthetic was assessed using a toe pinch to review the withdrawal of the pedal reflex. At the point of anaesthetic induction mice were kept on a heat mat and body temperature was kept consistently at 37°C to avoid induction of hyperthermia. During the procedure mice were intubated to ensure mice could breath as freely as possible to avoid the induction of hypoxia. Before the start of the experiment anaesthetic depth was assessed again using a toe pinch to review withdrawal of the pedal reflex. Additionally, as the ECG was implanted at this time anaesthetic depth was measured by lack of heart rate response to toe pinch. The length of experiment was kept to a maximum of 90 minutes to avoid significant anaesthetic washout. At the end of each experiment withdrawal of pedal reflex and ECG response was tested once again to ensure anaesthetic depth was kept throughout.

The inclusion of data of each mouse in the experimental outcome was limited by particular criteria:

- Mice that were visibly seen to be gasping for air were removed form experimental data, to avoid the effect of hypoxia on the experimental result.
- Mice that did not exhibit a baroreflex in response to sodium nitroprusside and phenylephrine were not included in the experimental data
- If a response to toe pinch was measured at the end of the experiment (either heart rate or pedal reflex response) data was not included in the experiment

4.3.2 The effect of urethane on heart rate

The differential effect of Ex-4 on heart rate between two different methods of inducing anaesthesia highlights the importance of selecting the most appropriate anaesthetic for a study. Here urethane (i.p) as sole anaesthetic proved unsuitable to study the

effects of GLP-1, Ex-4 and the effects of stimulating the PPG neurons on heart rate. The precise reason is unclear. Urethane inhibits the responses of the glutamate receptors AMPA and NMDA and potentiates the responses of the inhibitory Glycine and $GABA_A$ receptors (Hara and Adron Harriss, 2014). As these receptors are found widespread throughout the CNS and are involved in a multitude of signalling pathways this effect of urethane will affect the physiology of the anaesthetised mice, including the cardiovascular system. This effect of urethane was observed by Shimokawa et al (1998) who demonstrated that urethane increases renal sympathetic nerve activity, and induced tachycardia in rats (Shimokawa *et al.*, 1998). Furthermore, when comparing baseline heart rate of the mice anaesthetised with urethane, compared to urethane/α-chloralose in these experiments mice anesthetised with urethane exhibited on average a baseline heart rate of 581.9 ± 12.21 BPM (n=8) compared to mice anaesthetised with urethane/α-chloralose exhibited an average baseline heart rate of 480.8 ± 21.32 BPM (n=10). Mice anaesthetised with urethane have a baseline heart rate roughly 100BPM greater than those anaesthetised with the urethane/α-chloralose, suggesting a change in the balance of the autonomic nervous system to the heart, and in combination with Shimokawa observation, most likely an increase in sympathetic outflow. Whether the reason for the lack of effect of GLP-1 receptor stimulation under these circumstances was an already maximal sympathetic drive to the heart, or a requirement for glutamate receptor activation, that is suppressed by urethane, remains unclear.

4.3.3 Cardiovascular effects of exendin-4

Ex-4 increased heart rate in mouse, as has been demonstrated before in rats. However, it is well documented that Ex-4 and GLP-1 also increase blood pressure in rats (Barragan, Rodriguez and Blazquez, 1994; Yamamoto *et al.*, 2002; Goodwill *et*

al., 2014), this, however, does not occur in mice (Fig 4.12). Therefore, it was important to understand the cardiovascular response to Ex-4 in mice before I proceeded to study the PPG neurons, if as hypothesised they both induce tachycardia through central GLP-1R activation.

In this study, Ex-4 induced tachycardia was inhibited by atenolol. Atenolol (a β1 adrenoreceptor antagonist) blocks the β1 adrenoreceptor, blocking binding of NA on the heart that is released in response to an increase in sympathetic activity. NA acts on the heart to increase heart rate, by binding to the β1 adrenergic receptor on the heart. Therefore, Ex-4 induces tachycardia through an increase in sympathetic tone. In support of this, GLP-1 did not alter vagal nerve activity (Fig 4.2B) and atropine (a muscarinic acetylcholine receptor antagonist) did not attenuate the effects of Ex-4 on heart rate (Fig 4.5D). This data contradicts that published by Griffioen et al (2011). Here they inferred a decrease in parasympathetic activity due to an increase in heart rate variability (HRV). As HRV is a secondary measure, it can be influenced by other factors and the results demonstrated in this thesis are in line with those demonstrated in rat, demonstrating that Ex-4/GLP-1 induced tachycardia is due to an increase in sympathetic tone. However, it is possible that this discrepancy reflects a difference between central and systemic GLP-1R activation, as Griffioen et al (2011) measured the change in heart rate variability in response to central injection of GLP-1, although I would expect Ex-4 to activate the central GLP-1R.

Although it is now clear that Ex-4 induces tachycardia through an increase in sympathetic tone in mice, the point in which GLP-1/Ex-4 acts to induce tachycardia is unknown. Ex-4 possibly increases heart rate through activation of GLP-1R expressing neurons in brain nuclei associated with cardiovascular control through an increase in sympathetic outflow. In support, Yamamoto et al demonstrated that Ex-4 injected i.p

and i.c.v induced C-Fos-IR in the PVN, ARC, VMH, and RVLM, demonstrating that Ex-4 activates nuclei that modulate the sympathetic preganglionic nuclei in rat (Yamamoto *et al.*, 2002). It is also possible that Ex-4 activates GLP-1R within the spine, in areas such as the IML and CAA, however GLP-1R expression in these areas is yet to be confirmed.

Although GLP-1R is expressed in the cardiomyocytes in atria of the heart, it is unlikely that GLP-1 induces tachycardia through this pathway as perfusion of GLP-1 or lixisenatide in a Langendorff isolated heart preparation did not change heart rate (Baggio *et al.*, 2017). It is likely that the GLP-1R expressed in the heart has a role in GLP-1 induced cardioprotection (Giblett *et al.*, 2016). Additionally, a direct effect of GLP-1 on the cardiomyocytes on the heart to induce tachycardia would not be abolished by sympathetic blockade.

Although mice do not develop hypertension in response to GLP-1 or GLP-1R agonist, it seems that the tachycardia induced by GLP-1 in mice and in rats is both induced through an increase in sympathetic outflow, and is most likely induced by the same pathways and mechanisms, as the GLP-1R expression is mostly conserved across both species, particularly in the cardiovascular control regions i.e PVN, ARC, and RVLM (Merchenthaler, Lane and Shughrue, 1999; Cork *et al.*, 2015).

4.3.4 Preproglucagon neurons induce tachycardia

In this study, the PPG neurons were shown to have the capacity to regulate the cardiovascular system but are not necessary in the regulation of heart rate in anaesthetised conditions.

Activation of the PPG neurons is believed to induce release of PPG products including GLP-1, GLP-2, Oxyntomodulin, or GRPP. GLP-2 has been demonstrated to not affect

heart rate or blood pressure when injected peripherally, and the effects of GRPP and IP-2 on cardiovascular parameters has yet to be documented (Barragan, Rodriguez and Blazquez, 1994)**.** Both Oxyntomodulin and GLP-1 increase heart rate when injected peripherally, but only GLP-1 has been demonstrated to induce tachycardia when injected centrally (Mukharji *et al.*, 2013)**.** Therefore, it is likely that the tachycardia induced by activation of the PPG neurons is due to release of GLP-1 or Oxyntomodulin release. As both act through the GLP-1R, they could induce tachycardia through activation of GLP-1R expressed in the projection targets of the PPG neurons PVN, ARC and RVLM and possible CAA and IML (although expression of GLP-1R in these areas is unknown) (Alvarez *et al.*, 1996; Cork *et al.*, 2015).

Additional to releasing PPG products, the PPG neurons are glutamatergic, (Zheng *et al.*, 2014; Cork *et al.*, 2015). Therefore, the tachycardia induced by PPG activation could be due to the release of glutamate, PPG products or both. However, as central injection of GLP-1/Ex-4 induces tachycardia, this would suggest that GLP-1R activation alone is sufficient to induce tachycardia, suggesting that the tachycardia induced by activation of the PPG neurons is due to release of PPG products. To demonstrate if the PPG neurons increase heart rate through the release activation of the GLP-1R, the experiment described in section 3.2.8 should be repeated with prior i.c.v injection of the GLP-1R antagonist, Ex-9. If the tachycardia is attenuated, then the PPG neurons induce tachycardia through the release of GLP-1 or oxyntomodulin.

It is plausible that the tachycardia induced by Ex-4 is due to Ex-4 bypassing the PPG neurons and directly activating GLP-1Rs expressed in the PVN, ARC, and RVLM. As the PPG neurons project to the PVN, ARC, and RVLM and release GLP-1 or oxyntomodulin, it might be assumed that activation of the PPG neurons and injection of Ex-4 both induce tachycardia through the same autonomic pathways. In support of

this hypothesis, central Ex-9 attenuated the tachycardia induced by systemic Ex-4 injection (Barragán *et al.*, 1999)**.** To study this further the central GLP-1R KD mouse strain used in Sisley et al (2014) should be used while measuring heart rate during systemic Ex-4 injection and PPG activation. If tachycardia is attenuated in both cases, the result would suggest that both effects are induced by activation of the central GLP-1R's.

Inhibition of NTS PPG neurons did not reduce heart rate in anaesthetised mice, indicating that there is no tonic effect of PPG neurons on sympathetic outflow to the heart under these conditions. This conclusion is supported by the finding that sodium nitroprusside and phenylephrine injection could induce an effect on heart rate under the same conditions, excluding an anaesthetic interference. Additionally, peripheral and central injection of Ex-9 did not induce any changes in blood pressure and heart rate in rat, suggesting that central GLP-1 does not have a tonic influence on cardiovascular parameters under resting conditions (Barragan *et al.*, 1996)**.**

It is possible that the PPG neurons are only activated in specific situations. Central GLP-1 has been suggested to be involved in the stress response (Holt and Trapp, 2016). When an animal meets a stressor, tachycardia and hypertension are induced in order to provide enough oxygen to the muscles, in preparation for the fight and flight response. Therefore, the increase in tachycardia induced by activation of the PPG neurons could represent the role of the PPG neurons during a stress response and would explain why inhibition of the neurons does not reduce heart rate under resting conditions.

4.3.5 Is GLP-1 tachycardia linked with PPG neuron induced tachycardia?

The mechanism by which GLP-1 acts to induce tachycardia is unknown. As discussed in section 1, one possible mechanism in which peripheral GLP-1 induces tachycardia is through detection and activation of the GLP-1R expressed on the vagal afferents. This induces an increase in firing rate of the vagal afferent, relaying the signal into the brain, where it acts to increase heart rate through an unknown neuronal pathway.

Another mechanism is detection of GLP-1 in the bloodstream by the GLP-1R expressed in the area postrema (AP). As the AP is a circumventricular organ and exhibits a leaky blood-brain barrier, GLP-1 could be detected directly in the bloodstream without crossing the BBB. The AP can then relay the signal into the CNS.

The final mechanism is direct detection of GLP-1, by the GLP-1R expressed in the autonomic nuclei, described earlier.

If the GLP-1 acts through the GLP-1R expressed on the vagal afferents, it is possible that the vagal afferents activate the PPG neurons in the NTS, inducing tachycardia. In support of this the PPG neurons receive synaptic input from the vagus nerve (Hisadome *et al.*, 2010). However, I found that peripheral GLP-1 induce tachycardia without modulating vagal activity, suggesting that GLP-1 does not induce an effect through the vagal nerve. The same is true, for detection of GLP-1 by the AP neurons expressing GLP-1R receptors. Ex-4 activates the catecholaminergic neurons (identified by tyrosine hydroxylase immunoreactivity) in the AP, measured by induction of C-Fos-IR (Yamamoto *et al.*, 2003)**.** The catecholaminergic neurons have been demonstrated to project to the cNTS, the area where the PPG neuron cell bodies reside. Therefore, GLP-1 can activate the AP neurons, without crossing the BBB, leading to downstream activation of the PPG neurons, inducing tachycardia. In support

of both theories, Orskov et al demonstrated that radiolabelled GLP-1 distinctively binds to the circumventricular organs, the subfornical organs (SFO) and the AP (Orskov *et al.*, 1996). Additionally, GLP-1 binding only to the circumventricular organs supports the hypothesis that GLP-1 cannot cross the BBB, and can induce an effect in the CNS, through upstream signalling. In further support, i.c.v injection of Ex-9 attenuated the tachycardia and hypertension induced by peripheral injection of Ex-4 (Barragán *et al.*, 1999). As Ex-9 blocks the GLP-1R the tachycardia induced by peripheral GLP-1 must act through the central GLP-1R (Barragán *et al.*, 1999). Therefore, GLP-1 must act directly in the brain to induce tachycardia, either through activation of the PPG neurons to release GLP-1 or direct activation by peripheral GLP-1. However, if GLP-1 only binds to the circumventricular organs, and GLP-1 has to directly activate the GLP-1R in CNS, the most likely explanation is that the tachycardia induced is due to activation of the PPG neurons, releasing GLP-1 in the CNS.

However, it is important to note that GLP-1 doses used in experiments demonstrating systemic GLP-1 induced tachycardia are very large. The GLP-1 dose used in these experiments was based on current literature demonstrating the effects of GLP-1 on heart rate in rat (Yamamoto *et al.*, 2002, 2003). The dose of GLP-1 used is 10x greater than the dose commonly used in Ex-4 studies. As Ex-4 is not inactivated by DPP-IV, Ex-4 can maintain a higher concentration in the bloodstream than GLP-1. The higher dose of GLP-1 is used to counteract the inactivation by DPP-IV. It is likely this large dose of GLP-1 exceeds physiological concentrations in the blood. It might produce effects that are not seen with endogenous GLP-1, as GLP-1 will be able to cross the BBB in a sufficient enough concentration to induce tachycardia. Therefore, it is plausible that peripheral GLP-1 does not have a role in cardiovascular control, and the effects on tachycardia that are measured are due to the high concentration of GLP-1

entering the brain and activating central GLP-1R directly, which wouldn't happen under physiological conditions. Nevertheless, the results from both the experiments injecting GLP-1 or Ex-4 as well as the activation of the PPG neurons show the capacity of the GLP-1 system to modulate cardiovascular function.

4.3.6 Possible circuitry that the PPG neurons act through to increase HR

As the PPG neurons induce tachycardia and are the main source of central GLP-1, it would seem likely that the PPG neurons would induce tachycardia through the same mechanism of exogenous GLP-1, through an increase in sympathetic outflow. The PPG neurons most likely increase heart rate through its projections to the PVN, RVLM, IML, and CAA. The RVLM is major contributor to sympathetic activity and modulates the SPNs in the spinal cord. As the PPG neurons project to the RVLM and the RVLM express the GLP-1R, is would seem obvious that this would be the major contributor of the tachycardia induced through the PPG neurons. However, a study demonstrated that 20 out of 22 neurons in the RVLM were hyperpolarised by GLP-1, suggesting that GLP-1 inhibits the presympathetic RVLM (Oshima et al,. 2017). This data contradicts that the hypothesises that the PPG neurons would increase heart rate through an increase in sympathetic outflow. Interestingly, this suggests that the PPG neurons would in fact decrease heart rate, through release of GLP-1 in the RVLM, therefore this leads me to believe to that GLP-1 is not released in the RVLM to induce tachycardia, leading to two possible outcome: 1) that another neurotransmitter is released i.e glutamate, GLP-2, etc or 2) that the RVLM is not involved in the regulation of heart rate by direct activation by the PPG neurons.

One region that could be responsible for the tachycardia induced in the PVN. The PVN has been demonstrated to be one of the hypothalamic centres that modulates

sympathetic activity to the heart. It is believed that this modulation of sympathetic activity emanates from the presympathetic neurons that project to the RVLM and the IML. The oxytocin population in the PVN that project to the IML in the spine have been demonstrated to induce tachycardia through an increase in sympathetic activity (Yang et al, 2004). Additionally, oxytocin injected directly into the thoracic spinal cord increase heart rate without effecting blood pressure (Yashpal et al., 1987). As the GLP-1 was shown to depolarise and induce C-Fos-IR the PVN oxytocin neuron it is possible that central GLP-1 induces tachycardia through this neuronal population. Additionally, the PPG neurons have been demonstrated to have boutons in close apposition with the PVN oxytocin neurons, suggested that the PPG neurons may also act through the oxytocin neurons to induce tachycardia. Furthermore, this fits with the results seen in mice, and humans where exogenous GLP-1 increases heart rate without affecting blood pressure (Bharucha *et al.*, 2008).

Additional to the projections to the presympathetic nuclei in the brain, the PPG neurons also have been demonstrated to have close appositions to the SPNs in the IML, which mediate sympathetic outflow to the sympathetic ganglion chain. This close apposition suggest that the PPG neurons can directly modulate sympathetic outflow through SPNs. However, GLP-1R expression on the SPNs has not been confirmed and overall GLP-1R expression in the spinal cord is controversial, with contradicting studies detecting GLP-1R mRNA expression in the spinal cord (Li et al., 2012, Vahl et al., 2007). GLP-1 has been shown to have a role in the spinal cord where intrathecal injection of GLP-1R agonist reduced pain hypersensitivity in mice suggesting that GLP-1 does act in the spine, which was attenuated by GLP-1R KD, however the study here suggests that this is due to GLP-1R expressed on microglia (Nian Gong et al., 2014). As 61.5 ± 6.3% of NTS- PPG neurons project to the spinal cord, I would expect

this to be a major source of the tachycardia induced by activation of the PPG neurons (llewelyn- smith 2011).

4.3.7 GLP-1 analogues do not increase blood pressure in mice

In this chapter, the effect of GLP-1 and GLP-1 analogues on heart rate was studied. Yamamoto et al demonstrated that i.c.v and i.v GLP-1 increases blood pressure in rats, therefore it would be desirable to study the effect of GLP-1 on hypertension in mice as well. However, my aneasthetised model did not allow the study of blood pressure. Initial blood pressure recordings were performed by cannulating the carotid artery with PE tubing, flushed with heparinised saline. The PE tube was then connected to a pressure transducer, and blood pressure was recorded. As demonstrated in Fig 4.10, the blood pressure recordings were not correctly measuring the blood pressure of the mouse. The transducer recorded a very low systolic and diastolic blood pressure, and the pulse pressure (the difference between systolic and diastolic) was extremely low, in many cases 10mm.Hg compared to the physiological 40mm.Hg.

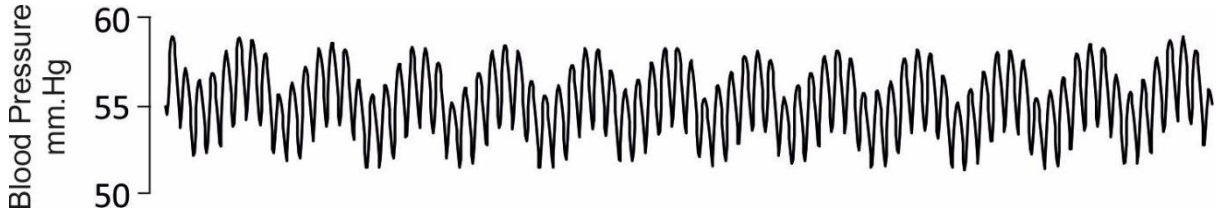


Figure 4.10 Example Blood pressure of an anaesthetised mouse measured using a pressure transducer. Blood pressure of an anaesthetised mouse was measured by cannulating the carotid artery, with a PE tubing, flushed with saline. The PE tubing was connected to a pressure transducer, plugged into a blood pressure neurolog, and digitised through Spike7. The whole recording shown here was taken over 10 seconds. The blood pressure measured ranges from a diastolic 53mm.Hg, to a systolic pressure of 59mm.Hg. This blood pressure trace representative of other traces achieved. This figure clearly demonstrates that recording blood pressure through this method is insufficient.

Further methods would have been sought out to measure blood pressure, however, Marie Holt (a fellow Ph.D. student in our laboratory) demonstrated that GLP-1 does not induce hypertension in wake mice (Fig 4.11). Marie recorded heart rate and blood pressure using biotelemetry in conscious mice and injected saline (control) or Ex-4 (10µg/kg, i.p) at zeitgeber time 4. A significant increase in heart rate was recorded after injection of Ex-4, compared to saline injection (Fig.4.11A). After a spike in mean arterial blood pressure (MAP) due to the stress caused by handling and injection, there was no significant difference in the MAP between Ex-4 and saline injection, demonstrating that Ex-4 does not increase MAP, and therefore did not require further study (Fig.4.11B).

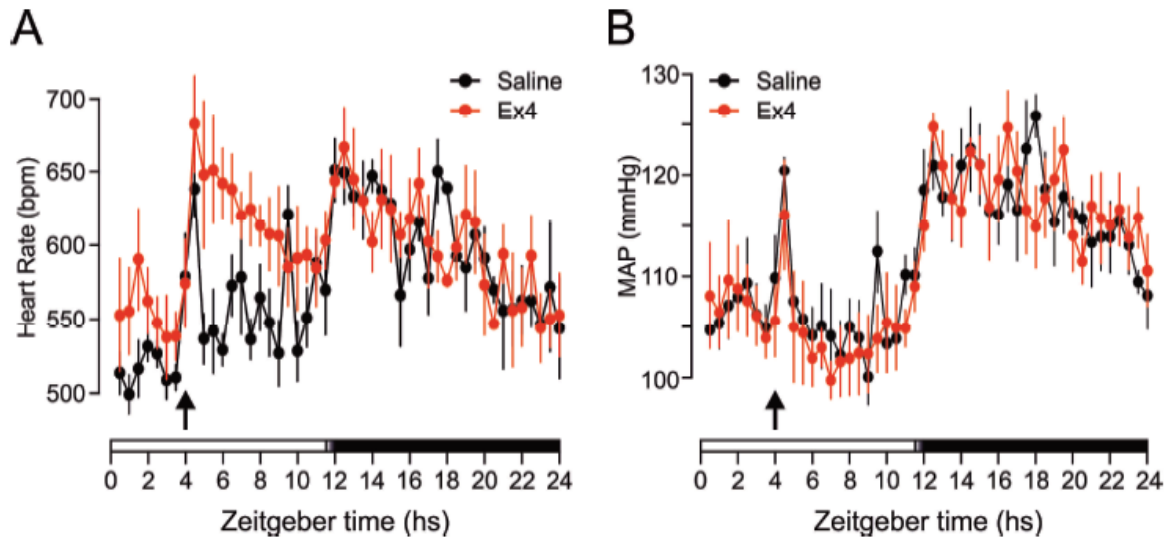


Figure 4.11 Exendin-4 induces tachycardia but does not increase blood pressure in conscious mice: C57/Bl6 mice were implanted with biotelemetry probes in carotid artery to measure heart rate, blood pressure and activity. Telemetry mice were then injected i.p with either saline (Black, n=3) or exendin-4 (10µg/kg, Red, n=3) at zeitgeber time 4 (arrow), while measuring heart rate (A) and mean arterial blood pressure (MAP) (B). Injection of Ex-4 significantly increase heart rate, however there was no significant difference in MAP between the saline injected and Ex-4 injected cohorts, demonstrating that Ex-4 does no increase blood pressure.

4.4 Conclusion

GLP-1 and Ex-4 induce tachycardia in anaesthetised mice through an increase in sympathetic outflow. Activation of the PPG neurons also increased heart rate, but

inhibition of the PPG neurons did not decrease heart rate. This is supported by data observed in conscious mice using telemetry probes, where ablation of the NTS-PPG neurons did not affect heart rate compared to controls (M.Holt, unpublished). Taken together this data suggests that the PPG neurons have the capacity to regulate heart rate but are not necessary for cardiovascular control under resting conditions. However, it is noteworthy that the IRT-PPG neurons were not ablated in this study, and it is possible that the IRT-PPG neurons are sufficient to maintain cardiovascular control. Peripheral and central injection of Ex-9 under different paradigm (i.e stress, etc), while measuring heart rate changes for bradycardia will be sufficient to answer whether GLP-1 is necessary in cardiovascular control.

It is plausible that Ex-4 and activation of the PPG neurons induce tachycardia through the same mechanism. Although, the GLP-1R is expressed on atrial cardiomyocytes, current evidence suggests that Ex-4 most likely acts through the GLP-1R expressed in the CNS, however further study is required to assess whether it is through direct activation of the GLP-1R in the CNS, or through activation of the PPG neurons. Further insight could be achieved through genetic knockdown of the GLP-1R selectively in the brain, using sh-RNA and assessing whether Ex-4 injection can still induce tachycardia. Specific nuclei can be assessed by ablation of GLP-1R using the Glu-CRE-GLP-1R-RFP transgenic mouse and the AAV-mCherry-Flex-DTA virus to ablate GLP-1R expressing neurons in each nucleus

5. Recruitment of the preproglucagon neurons

5.1 Background

In chapters 3 and 4 I have demonstrated that activation of the PPG neurons induces hypophagia and tachycardia in mice. As these effects were seen during selective pharmacogenetic activation of the PPG neurons using genetic tools, it is likely the endogenous activation of the PPG neurons would induce similar effects. In this chapter, I aim to identify which endogenous signals activate the PPG neurons to possibly induce hypophagia and tachycardia.

5.1.1 The immediate early gene - C-Fos

In this chapter, I use C-Fos-immunoreactivity (C-Fos-IR) to study what signals activate the PPG neurons. Expression of C-Fos is induced in response to external stimulation and acts as a transcription factor in neurons (Kovács, 1998). Induction of C-Fos was first observed after seizure activity and in response to noxious stimuli in the spinal cord. Since then many stimuli have been demonstrated to rapidly induce C-Fos within neuronal populations, leading to C-Fos-IR being widely used as a marker for neuronal activation (Kovács, 1998).

C-Fos is an immediate early gene and a proto-oncogene. It is part of the Fos family of transcription factors including FosB, Fra-1, and Fra-2. Upon activation C-Fos binds to the secondary messenger c-jun, forming activator protein-1 (AP-1). AP-1 then binds to DNA, at the AP-1 promoter sites, initiating transcription. Overall, C-Fos acts in the cell to convert extracellular signals into an intracellular response.

It is thought that neuronal activity directly correlates with C-Fos expression within the cell. However, areas with high native neuronal activity do not continually express C-Fos,, for example, sensory stimulation only induces C-Fos in the visual cortex after periods of sensory deprivation (Kaczmarek and Chaudhuri, 1997). In further support of this, it was shown in cultured dorsal root ganglion cells that C-Fos induction is inversely correlated to the burst interval of the action potential within the cell, suggesting that C-Fos is induced after a threshold increase in neuronal activity over baseline (Fields *et al.*, 1997).

Expression of C-Fos is low under resting conditions and strongly induced by a stimulus. It has been demonstrated that peak mRNA expression occurs between 30-60 minutes, and peak protein expression between 1-3 hours after the stimulus (Kovács, 1998). Therefore, when measuring C-Fos induction from a stimulus, the animals should be transcardially perfused 45 minutes after the stimuli if detecting C-Fos using *in situ* hybridisation, or 1-2 hours after the stimulus if measuring C-Fos-IR using immunohistochemistry.

Although C-Fos-IR has often been used as a marker for neuronal activation, it does have limitations. As a neuronal mapping tool, it works for stimulatory inputs, however neuronal inhibition does not induce C-Fos, therefore lack of a C-Fos response does not necessarily suggest that there is no response in the target neurons. Additionally, C-Fos-IR has been demonstrated to be induced widely in the brain in response to stress (Senba *et al.*, 1993; Cullinan *et al.*, 1995) . As stress induces C-Fos-IR widely across the brain, it can be a limitation in studies involving conscious animals. As handling and injecting animals induces stress, it can be difficult to differentiate whether the neuronal activation is induced by stress or the experimental variable. However,

thorough experimental controls such as handling control, or injecting saline as a control for the experimental injection, can overcome this limitation.

It is also currently unclear whether all neuronal cells can induce C-Fos. It has already been demonstrated that different nuclei have a different threshold for C-Fos induction, therefore it is feasible that some neuronal populations do not induce C-Fos in response to neuronal activation by the stimulus (Cullinan *et al.*, 1995).

Although the ability of C-Fos to be induced by a wide variety of signals is initially a benefit, it does have its disadvantages. C-Fos-IR does not convey any information about the pathway in which the neuronal population is active. For example, further study is required to discover whether C-Fos expression in the individual cell is direct or indirect activation, as C-Fos is induced whether the cell is activity by the experimental variable directly, is activated by the neuronal population initially activated by the experimental variable, or the action of inhibitory inputs are reduced, which also increases C-Fos expression within the neuronal cell.

Despite these limitations C-Fos-IR is a valuable tool to gauge neuronal activity, consequently I use C-Fos-IR to measure activation of the PPG neurons, as it allows the study of this neuronal cell population in conscious mice.

The previous chapters demonstrated that the PPG neurons reduce food intake (Fig 3.8 and Fig 3.9) and induce tachycardia (Fig 4.9) when activated using pharmacogenetics. It has been demonstrated that the PPG neurons are activated by CCK, LiCl, large intake of highly palatable diet, and gastric distension in rats (Rinaman, 1999; Vrang *et al.*, 2003; Kreisler and Rinaman, 2016). However, it is unknown what physiological conditions activate the PPG neurons to induce these responses in mice.

As the differential effects of GLP-1 between species has become more clear, it is important to further delve into what activates the PPG neurons in different species. To further determine when the PPG neurons are activated in mice, C-Fos-IR was used, as it has been previously used in rats. The following experiments measure C-Fos-IR in the Glu-YFP mouse (described in methods section 2.3) after experimental stimuli.

In this chapter I investigate if the:

- PPG neurons are activated by peripheral anorexigenic hormones GLP-1 and CCK
- The PPG neurons are activated due to the intake of a large meal

5.2 Results

5.2.1 Exendin-4 does not activate the PPG neurons

Initially, I aimed to determine if the PPG neurons are activated by GLP-1. The pathway by which peripheral GLP-1 induces hypophagia and tachycardia is unknown.

The three potential pathways described in the introduction include direct detection of peripherally released GLP-1 by brain GLP-1R, detection of peripheral GLP-1 by GLP-1Rs expressed on vagal afferent neurons, which then relay the signal into the NTS, and detection of peripheral GLP-1 by the GLP-1Rs expressed in the AP, which in turn relays the signals to the NTS. As GLP-1 is thought not to reach the CNS in a sufficiently high concentration to initiate a response, the latter two hypotheses seem more likely.

However, wide expression of GLP-1R across the brain and the hypophagia induced by central injection of GLP-1 strongly suggest that GLP-1 acts directly in the CNS to reduce food intake and tachycardia (Merchenthaler, Lane and Shughrue, 1999; Cork

et al., 2015). Based on this, it is likely that the PPG neurons are the link in this chain, as they are the source of brain GLP-1. I hypothesised that peripheral GLP-1 is detected by GLP-1R expressed in either the AP or vagal afferents, where both relay the signal into the NTS. This, in turn, activates the PPG neurons, leading to release of GLP-1 in the CNS, inducing hypophagia and tachycardia, therefore, peripheral GLP-1 should activate the PPG neurons. To study if the PPG neurons are involved in Ex-4 induced tachycardia and hypophagia, Glu-YFP mice were injected i.p. with either saline or Ex-4 (10µg/kg). 1.5 hours later mice were transcardially perfused and brains were processed for C-Fos and YFP immunohistochemistry. C-Fos-IR (red) positive YFP expressing neurons were counted and expressed as a percentage of total YFP-PPG neurons. C-Fos-IR in the Area Postrema (AP) after Ex-4 was taken as positive control, as Ex-4 has been demonstrated to induce C-Fos-IR in the AP in rats and GLP-1 receptors or binding sites have been found in many studies in both rat and mouse (Merchenthaler, Lane and Shughrue, 1999a; Yamamoto *et al.*, 2002; Cork *et al.*, 2015). The AP showed substantial amounts of C-Fos-IR in the Ex-4 (Fig 5.1B) injected group compared to the saline-injected cohort (Fig 5.1A). DAPI nuclear staining was used to confirm nuclear localisation of the C-Fos-IR as expected of an immediate early gene. DAPI is a known marker of the nucleus of the cell, as it binds strongly to A-T rich regions of DNA, and DNA is localised to the nucleus of a cell (Kubota, Kubota and Tani, 2000). A representative image of C-Fos-IR co-localisation with DAPI is presented in Fig 5.1C,D,E.

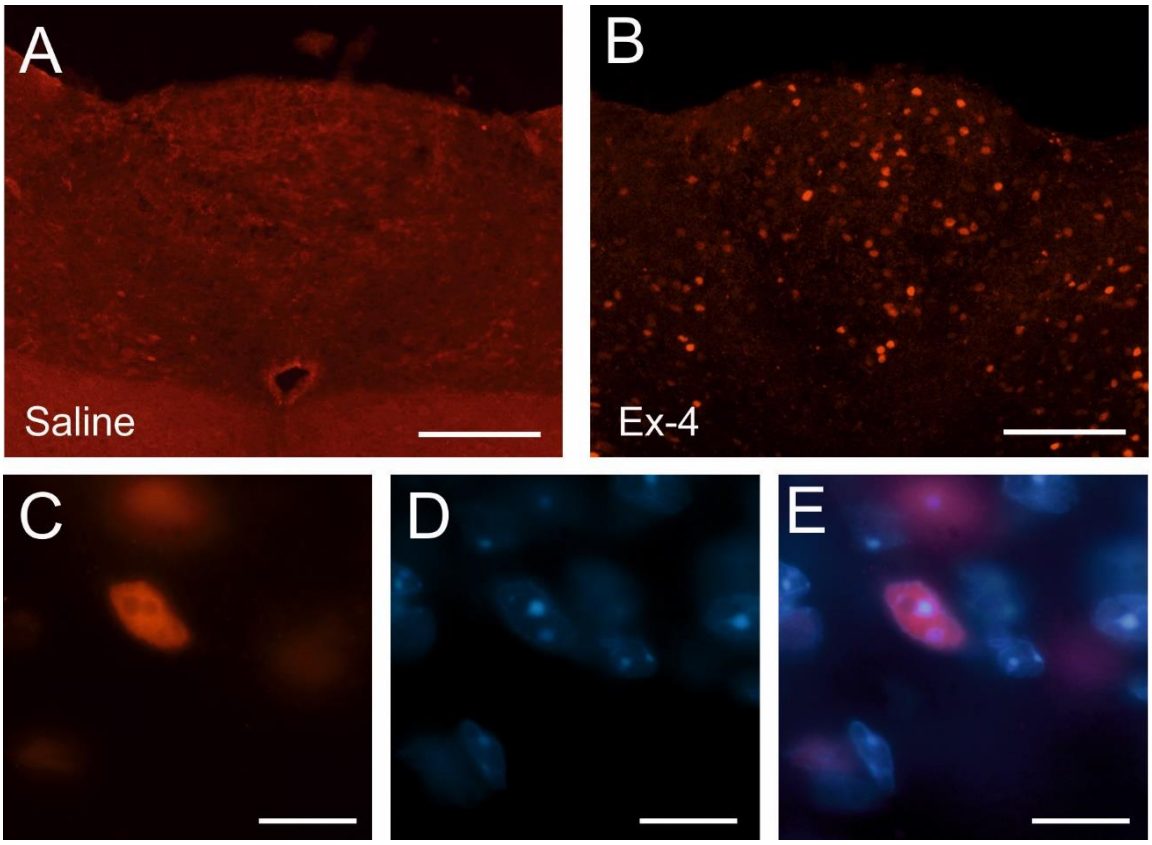
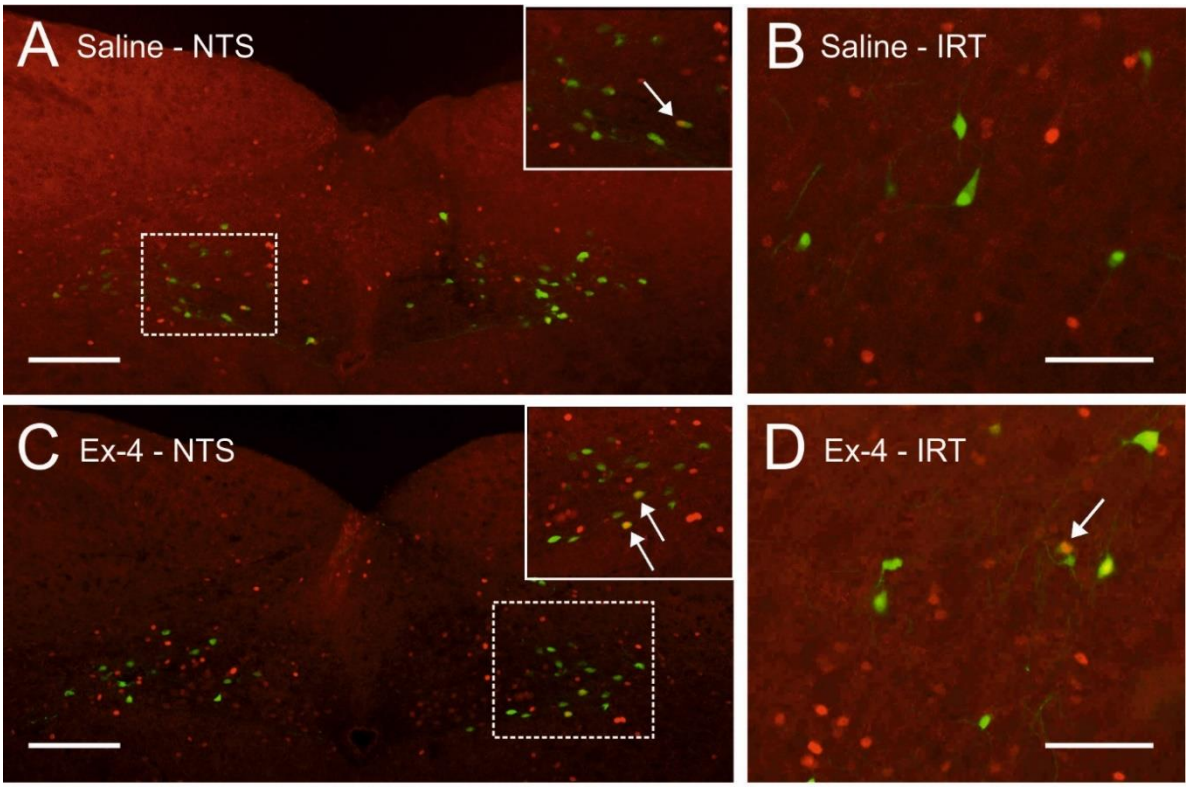


Figure 5.1 Example images of C-Fos-IR in the area postrema following intraperitoneal injection of exendin-4: To confirm that the exendin-4 (Ex-4) can induce C-Fos-IR in the brain, the area postrema (AP) was used as an example nuclei, as it has been demonstrated to induced C-Fos-IR in response to Ex-4 previously in rats (Yamamoto et al., 2002, 2003). Ex-4 clearly induced C-Fos-IR in the AP (B), compared to saline injection (A). Therefore, Ex-4 induces C-Fos-IR in the brain and can be used to assess C-Fos-IR in the PPG neurons. As C-Fos is nuclear bound, examining C-Fos-IR against the nucleus is a method of confirming functional C-Fos-IR. DAPI staining was used to identify the nucleus of the neurons (D), C-Fos-IR was stained and imaged (C) and colocalised with DAPI Staining at x80 magnification (E). E demonstrates that C-Fos-IR co-localised with the nucleus of the cell, demonstrating the C-Fos-IR is correct and functional. **$SB_{(A,B)}$: 200µm, $SB_{(C,D,E)}$: 20µm.**

Following successful C-Fos-IR detection, C -Fos-IR positive (Fos +ve) YFP cells were counted in Ex-4 injected Glu-YFP mice (n=4) and in saline injected Glu-YFP mice (n=4). The number of Fos +ve YFP cells was determined in the NTS, Midline, and IRT and expressed as a percentage of total YFP cells within the NTS, IRT and in total (the midline was excluded due to few YFP expressing cells). There was no significant difference in the percentage of Fos +ve YFP cells in the NTS (Fig 5.2B, D, E), IRT (Fig

5.2C, E) and in total between the saline and Ex-4 cohorts. There were a low number of Fos +ve YFP cells in both the saline and Ex-4 injected animals, indicated by the arrows in the example image (Fig 5.2A, B, C, D) demonstrating that C-Fos-IR can be detected in the PPG neurons. This experiment demonstrates that systemic Ex-4 does not activate the PPG neurons. This leads me to assume that gut-released GLP-1 would not activate the PPG neurons either, as Ex-4 is a long-lasting analogue of GLP-1. Therefore, this data suggest that the PPG neurons are not the missing link in peripheral GLP-1 induced hypophagia and tachycardia. Furthermore, this data suggests that the peripheral GLP-1 and central GLP-1 are separate systems, that induce similar effects and are possibly activated by similar stimuli.



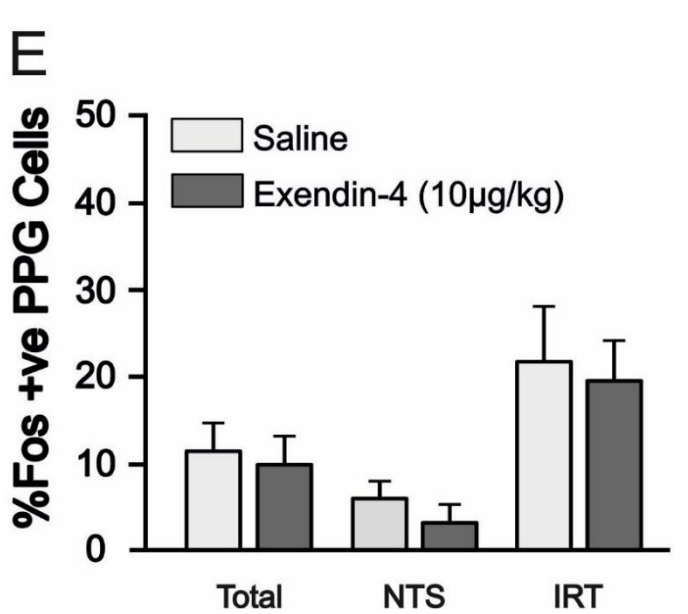


Figure 5.2 Exendin-4 does not induce C-Fos-IR in the PPG neurons: Following intraperitoneal injection of saline (n=4) or exendin-4 (10µg/Kg) (n=4), mice were perfuse- fixed and immunostained for C-Fos-IR. C-Fos-IR positive PPG cells were counted and expressed as a percentage of total PPG neurons in the NTS, IRT and in Total (including the midline neurons). There was no difference in % Fos positive YFP cells in NTS, IRT and in Total (E). C-Fos-IR was induced in both the Saline injected NTS (B) and IRT (C) and the Ex-4 NTS (D) and IRT (E). C-Fos-IR positive PPG neurons are indicated by arrows. Statistical significance was tested using an unpaired t-test ($P<0.05$) This experiment demonstrates that Ex-4 does not activate the PPG neurons. **$SB_{(A,C)}$: 200µm, $SB_{(B,D)}$: 100µm**

5.2.2 CCK does not activate the PPG neurons

Cholecystokinin (CCK) was the first discovered anorexigenic hormone (Gibbs, Young and Smith, 1973). CCK is released from the I cells in the GI tract in response to a meal (Mitchell and King, 2014). Exogenous administration of CCK slows gastric emptying,

reducing meal size and duration of a meal, without affecting the inter-meal interval in rodents and increasing the perception of fullness and decreases hunger in humans (Mitchell and King, 2014). Surgical and chemical vagotomy ablates the anorexigenic effect of CCK, demonstrating the CCK activates vagal afferents to induce satiety. As CCK is an anorexigenic hormone, that induces hypophagia through vagal afferents, it is possible that CCK induces hypophagia through the PPG neurons. As the vagal afferent nerve terminates in the NTS, it seems feasible that CCK activates vagal afferents, relaying the signal into the NTS, where the vagal afferents activate the PPG neurons to induce satiety. In support of this, exogenous administration of (i.p) CCK induced C-Fos-immunoreactivity (C-Fos-IR) in 60% of PPG neurons in rats. Additionally, CCK indirectly depolarises the PPG neurons *in vitro* in mice, through α1-adrenoreceptor signaling and glutamatergic inputs, although this effect relies on CCK receptors within the brainstem, as this experiment was performed in coronal brain stem slice (Hisadome *et al.*, 2011). As the PPG neurons have been demonstrated to reduce food intake, it is possible that the PPG neurons form part of the pathway by which CCK induces hypophagia. To explore this possibility Glu-YFP mice were trained with i.p. injection of saline until mean food intake (wet mash) was consistent over 3 experimental days (day 7-9, intraclass correlation coefficient (1,1)= 0.667, $P<0.01$) (Fig 5.3)

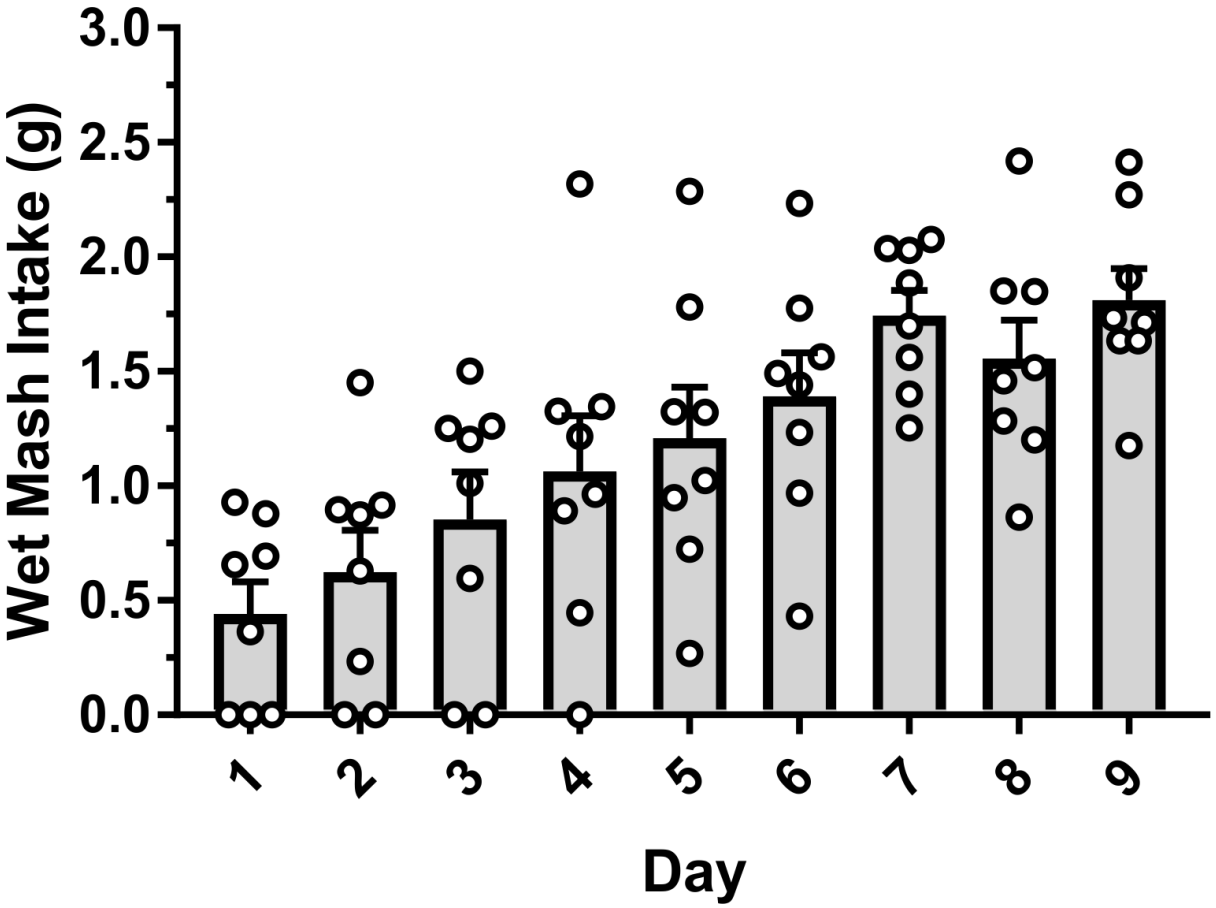


Figure 5.3 Consistent intake of wet mash. Glu-YFP mice were trained to consume wet mash over a 2-hour period after dark onset, after a 3 hour food restriction. PPG mice were trained until all mice consumed wet mash, and there was no significant difference between 3 consecutive days of training (day 7-9, intraclass correlation coefficient (1,1)= 0.667, $P<0.01$). Mice consumed on average 1.81 ± 0.14g over the 2 hour final feeding session (day 9). There was no significant difference between feeding session day 7, 8 and 9. Once consistent intake was reached, C-Fos-IR study was commenced. Data from individual mice is shown as circles. Note that until day 4 individual mice did not consume any food over the 2-hour test period, presumable due to the stress of the i.p. injection.

After consistent wet mash intake mice received both CCK injection (i.p, 20µg/kg, n=8) and saline injection (n=8) in a cross over design to confirm that CCK injections are functional and act to reduce food intake as expected (Fig 5.4). CCK injections did not reduce significantly reduce food intake at any timepoint, however there was a trend towards a decrease in food intake at the 60min timepoint (p=0.0077). As a significant interaction was observed ($F_{(2, 28)}$=5.823, P=0.0077) between hourly intake and drug

administration, due to a trend decrease in wet mash intake following CCK injection I deemed it sufficient data to proceed with the C-Fos-IR experiment in these mice.

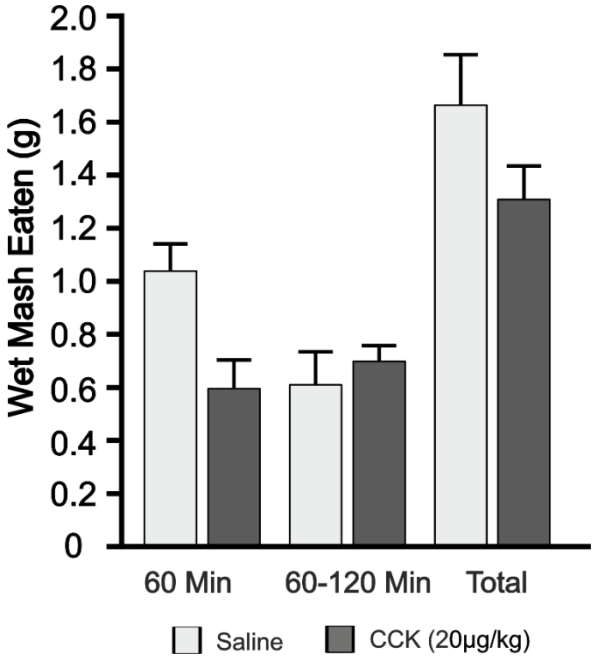


Figure 5.4. Confirmation of the hypophagic effect induced by i.p CCK. Following wet mash training C57/Bl6 mice received i.p injection of either saline (n=8) or CCK (20µg/kg, n=8) in a cross over design. Repeated measure ANOVA produced a significant interaction (p=0.0077). CCK injection demonstrated a trend towards a decrease wet mash intake compared to saline at the 60 min timepoint, however it was not significant (60min P=0.0638). There was no significant difference in wet mash intake between saline and CCK injection in the 60-120min and in total time point. Statistical significance was tested by repeated measure ANOVA, followed by Bonferroni multiple comparison test $F_{(2,\ 28)}$=5.823, P=0.0077. Bonferroni multiple comparison test was not significant at each time point.

After the effect of CCK on wet mash intake was confirmed, mice were split into two cohorts, saline or CCK. Saline or CCK-8 (20µg/kg) was injected i.p at dark onset and wet mash was returned Wet mash intake was measured at 60min and 90min after the return of wet mash. Both cohorts were transcardially perfused 90 minutes after the return of wet mash. A reduction in wet mash intake from 1.09 ± 0.07g to 0.67 ± 0.13g (Fig 5.4A) was seen within the first-hour, however this was not significant (p=0.0987). There was no significant difference in wet mash intake in the second hour or in cumulative 90-min intake (Total) (Fig 5.5A). PPG neuron activation by CCK was assessed by counting C-Fos-IR within the Glu-YFP neurons and expressed as a percentage of the total YFP positive neurons in the NTS, IRT, and Midline. Representative images of the Saline treated NTS and IRT depicting C-Fos-IR and YFP neurons are shown in Fig 5.5C and E respectively. Representative images of the CCK

treated NTS and IRT depicting C-Fos-IR and YFP neurons are shown in Fig 5.5D and E respectively. CCK treated mice demonstrated an increase in C-Fos-IR in the NTS and IRT. There was an significant overall interaction $F_{(2,10)}$ = 11.69, p=0.0024, however, there was no difference in C-Fos-IR within the PPG neurons in the Total, NTS, and IRT between the CCK and Saline-injected animals, although the IRT did display a trend towards an increase C-Fos-IR in response to CCK (p=0.087) (Fig 5.4B). Therefore, i.p. CCK does not induce C-Fos in the PPG neurons, suggesting systemic CCK hypophagia does not involve the PPG neurons in mice.

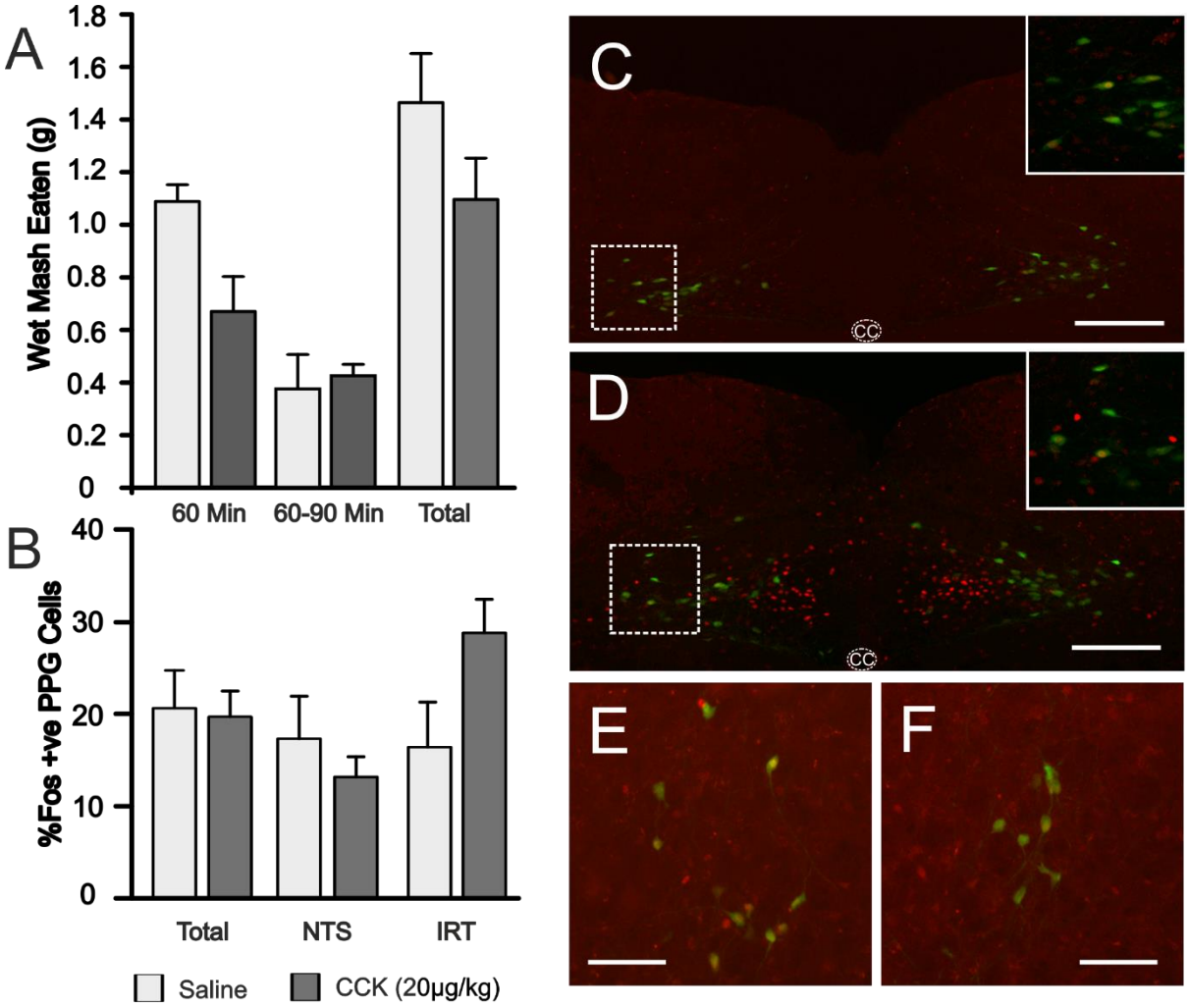


Figure 5.5 CCK-8 does not induce C-Fos-IR in the preproglucagon neurons YFP-PPG mice were fasted for 3h and injected, i.p, with either saline (n=3) or CCK-8 (20µg/kg) (n=4). After injection mice were allowed to consume wet mash and intake was measured after 60min and 90min. CCK significantly reduced food intake after 60 minutes (A). 90 Minutes after injection mice were transcardially perfused fixed, and the brains were removed. The brains were sliced and stained for C-Fos-IR and YFP. C-Fos-IR was counted in the NTS, IRT and in Total YFP neurons (including the midline), in both groups. There was no significant difference between %Fos +ve PPG neurons in the CCK injected animals compared to the control, saline injected, mice (B). CCK does not activate the PPG neurons. Statistical significance was tested using a repeated measure ANOVA followed by Bonferroni multiple comparison test Food intake: $F_{(2, 12)}$= 1.47, p=0.269. C-Fos: $F_{(2,10)=}$ 11.69, p=0.0024, Bonferroni N.S in each region. **$SB_{(C,D)}$: 500µm, $SB_{(E,F)}$: 200µm**

5.2.3 Intake of Ensure activates the PPG neurons

As the PPG neurons were not been activated by injection of the anorexigenic gut hormones GLP-1 and CCK, I wanted to determine if the PPG neurons could be

activated by the ingestion of a large meal. To ensure that a large meal was ingested voluntarily, Glu-YFP mice were trained to consume Ensure (a highly palatable vanilla milkshake). Glu-YFP mice were fasted for 3 hours before dark onset and given 30 minutes access to Ensure. This regime was repeated daily until all mice consumed Ensure and mean Ensure intake was consistent for 3 consecutive feeding sessions. Across all mice Ensure intake was consistent on acclimatisation days 4-6 (intraclass correlation coefficient (1,1)= 0.739, P<0.01) (Fig 5.6) (Kreisler., 2016).

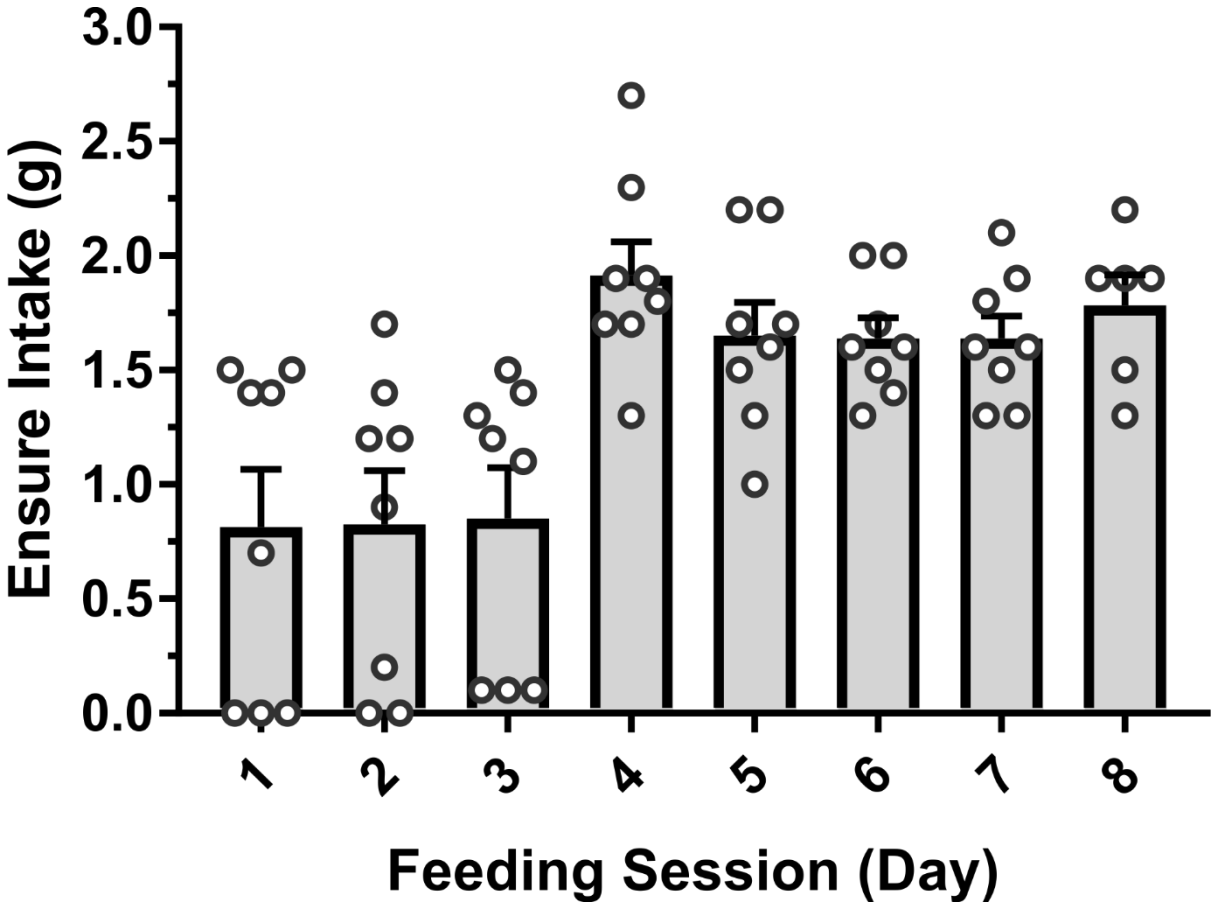


Figure 5.6: Consistent intake of liquid diet in Glu-YFP mice. Glu-YFP mice (n=8) were trained to consume Ensure in a 30-minute period, after 3 hour food restriction. Mice were trained until all mice consumed Ensure, and there was no significant difference in average intake over 3 feeding sessions (intraclass correlation coefficient (1,1)= 0.739, P<0.01). Mice consumed 1.78 ± 0.13g over the 30 minutes in the final feeding session (Day 8). There was no significant difference between Feeding session 6, 7 & 8. Once Ensure intake was consistent, the C-Fos-IR study was commenced.

Following training, the mice were split into two groups: Ensure Fed and fasted. The Ensure Fed cohort was given *ad libitum* access to Ensure at dark-onset whilst the fasted cohort had access to water only. Ensure intake was measured at 30 min and 90 min, followed by immediate transcardial perfusion. Average Ensure intake was 1.92 ± 0.18g or 2.88 ± 0.27 kcal in 30 minutes, and 3.42 ± 0.24 or 5.13 ± 0.37 in 90 minutes (Fig 5.7A,B); when intake was expressed as % bodyweight mice consumed 7.74 ± 0.83% in 30 minutes and 14.09 ± 1.79% in 90 min (Fig 5.7C). In comparison to figure 3.8 where mice consumed on average 0.8g standard chow in a two-hour period, following an overnight fast, intake of 1.92g in 30 minutes and 3.42g in 90 minutes can be considered a large meal. Following immunohistochemistry, Fos +ve YFP expressing PPG neurons in the NTS, IRT, and Midline were counted and expressed as a percentage of total YFP neurons in these areas.

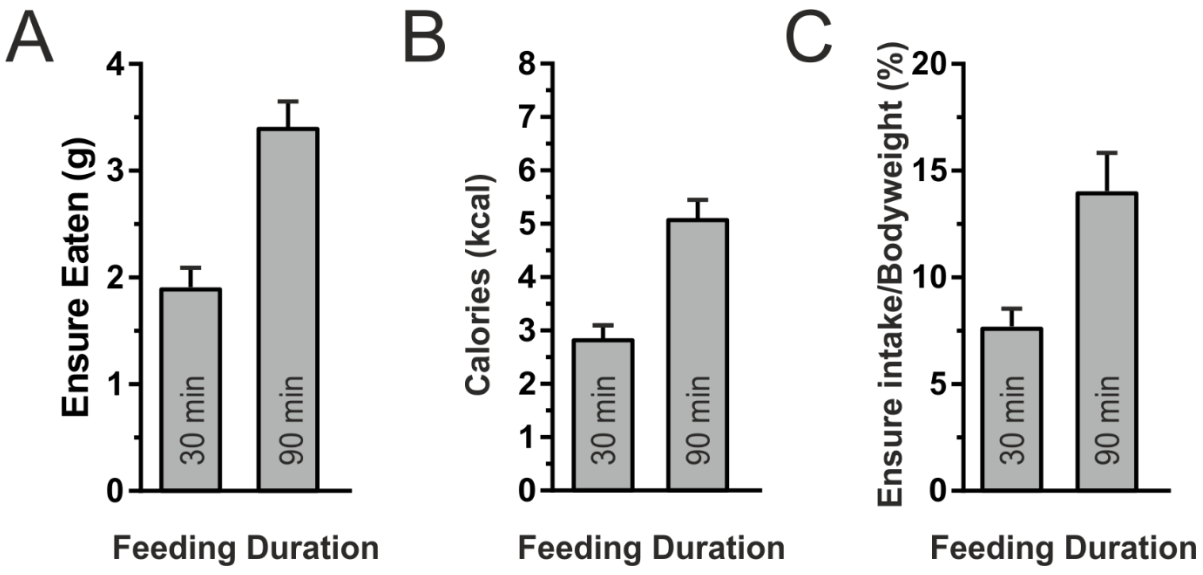


Figure 5.7 Ensure intake of Glu-YFP mice before transcardial perfusion: Glu-YFP mice were provided Ensure after a 3 hour fast at dark-onset. (A) Ensure intake of Glu-YFP 30 minutes and 90-minutes after provision of Ensure. (B) Esnure intake expressed as calories ingested 30-minutes and 90-minutes after provision of Ensure. (C) Ensure intake as a percentage of body 30-minutes and 90-minutes after provision of Ensure.

There was an overall noticeable increase in C-Fos-IR in the NTS of Ensure Fed mice (Fig 5.8C) compared to the food-restricted cohort (Fig 5.8A). The Ensure fed cohort had significantly more Fos +ve YFP cells in all nuclei in with the repeated measure ANOVA providing a significant column factor ($F_{(1,10)}$ =32.69, P=0004), without producing a significant interaction ($F_{(1,20)}$ = 1.607, P=0.23) demonstrating the Ensure intake induces C-Fos-IR in the PPG neurons independent of the nuclei. More specifically, in the NTS control mice expressed 8.74 ± 1.25% C-Fos-IR positive YFP neurons (Fig 5.8A), which increased to 46.98 ± 5.79% C-Fos-IR positive YFP neurons in the Ensure Fed cohort (Fig 5.8C) Additionally, the Ensure Fed cohort increased C-Fos-IR YFP neurons in the IRT from 12.12 ± 7.17% in the fasted control (Fig 5.8B), to 37.74 ± 5.18%. Overall, this represents a total increase of C-Fos-IR positve YFP neurons from 11.70 ± 1.55% to 45.44 ± 5.04% from intake of liquid diet (Ensure). Therefore, this experiment demonstrates that intake of a large meal activates the PPG neurons in mice and that C-Fos-IR is sufficient to initially study neuronal activation of the PPG neurons, before moving onto more complex experiments. Additionally, the amount of Ensure consumed is positively correlated with the number of total Fos +ve PPG neurons, suggesting the larger the meal or larger the calorific intake, the more PPG neurons are recruited (Fig 5.8F). Statistical significance was tested with a repeated measure ANOVA, followed by a Bonferroni multiple comparison test. Correlation significance was testing using the Pearson correlation test.

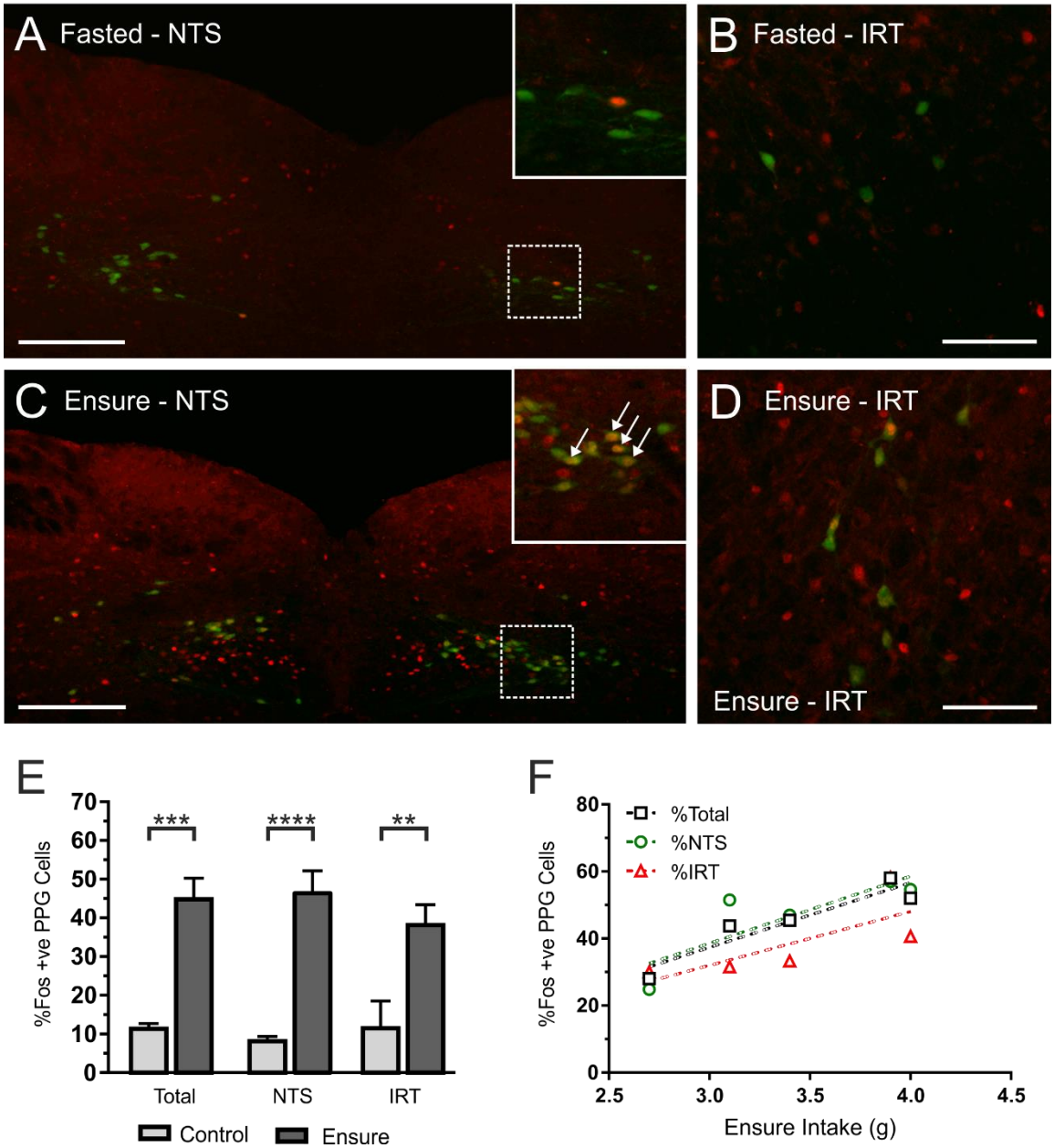


Figure 5.8 Intake of a large liquid meal induces C-Fos-IR in the PPG neurons: YFP-PPG mice were trained to consume Ensure (Vanilla milkshake) in a 30-minute period. Follwing 3 consistent days of Ensure intake, mice were split into fasted (n=5) and Ensure Fed Groups (n=5). The ensure fed group were given Ensure and the ensure intake was measured over 30 minutes and 120 minutes (F). Both groups were transcardially perfused fixed 2 hours after Ensure was provided. The brains were sliced and stained for C-Fos-IR and YFP. C-Fos-IR was counted in the NTS, IRT and in Total YFP neurons (including the midline), in both groups. Ensure heavily expressed C-Fos-IR in the NTS and IRT (C,D), compared to the food restricted mice (A,B). Ensure fed animals expressed C-Fos-IR in 45% of the YFP expressing neurons, compared to 10% in the food restricted group in total (E) . Ensure significantly increased C-Fos-IR in the PPG neurons, therefore high intake of large meal activates the PPG neurons (Bonferroni ** P<0.01, ***P<0.001, ****P< 0.0001). There is strong positive correlation between Ensure intake and total %Fos +Ve cells, suggesting that amount of Ensure consumed directly relates in amount of PPG neurons activated (Pearson correlation: Total=0.93, P=0.020; NTS= 0.825, P=0.073; IRT=0.7562, P=0.014) **$SB_{(A,C)}$: 500µm, $SB_{(B,D)}$:100µm**

5.3 Discussion

This chapter explores what factors activate the PPG neurons in free-moving conscious mice. I explored agents known to cause hypophagia and/or tachycardia including the postprandially released peptides GLP-1 and CCK. Additionally, I explored activation of the PPG neurons by refeeding. C-Fos-IR was not induced in the PPG neurons under systemic injection of CCK and Ex-4. Both have been previously demonstrated to induce a hyperphagic response when injected into the peritoneum, suggesting that the PPG neurons do not have a role in the hypophagia induced by CCK. Additionally, these data suggest that peripheral GLP-1 does not induce tachycardia through activation of the PPG neurons. However, voluntary intake of a large volume of liquid diet did induce C-Fos-IR in the NTS and IRT PPG neurons, demonstrating that the PPG neurons are activated during intake of a large meal or intake of highly palatable diet.

5.3.1 Exendin-4

Systemic injection of the GLP-1R analogue Ex-4 did not induce C-Fos-IR in PPG neurons. This finding was surprising, because whilst it is known that PPG neurons in mouse do not express functional GLP-1Rs (Hisadome *et al.*, 2010), it is generally assumed that activation of GLP-1Rs on vagal afferents or on the AP neurons would indirectly activate the PPG neurons (Yamamoto *et al.*, 2002, 2003). The results presented here indicate a distinct lack of GLP-1Rs upstream from the PPG neurons and therefore also suggest that the post-prandial release of GLP-1 from L-cells in the gut does not activate the PPG neurons. Thus, the hypophagic action due to activation of PPG neurons must be initiated by different signals. This strongly suggests that post-

prandial activation of both the peripheral and the central GLP-1 system is not obligatory linked, although it might occur in parallel.

In regard to peripheral GLP-1 induced hypophagia this is not surprising as central Ex-9 does not attenuate the hypophagia induced by peripheral GLP-1, suggesting it does not require the use of central GLP-1 (Williams et al., 2009). However, in regard to tachycardia this is more surprising as central injection of Ex-9 did attenuate peripheral GLP-1 induced tachycardia, demonstrating that tachycardia induced by peripheral GLP-1 is induced through central GLP-1R activation (Barragan et al., 1999).

Ex-4 did however induce C-Fos-IR in neuronal populations other than PPG neurons in the NTS and IRT, demonstrating GLP-1R activation by systemic Ex-4 at a dose of 10µg/kg as seen in similar studies in rat (Yamamoto et al, 2002). Since C-Fos-IR only indicates that the cell in question is activated by the stimulus, but not whether this activation is direct or indirect, these findings could be explained either by activation of peripheral GLP-1Rs on vagal afferents, which then in turn innervate the C-Fos +ve NTS neurons, or by action of Ex-4 on GLP-1Rs within the lower brainstem, which then directly or indirectly cause the observed C-Fos activation.

Ex-4 induces hypophagia and tachycardia when injected systemically. If Ex-4 does not activate the central GLP-1 system, how does it induce hypophagia and tachycardia? As Ex-4 is not inactivated by DPP-IV it is likely to cross the BBB to induce an effect in the CNS. Therefore, it seems likely that Ex-4 crosses the BBB and activates GLP-1Rs expressed in the brain, reducing food intake and increasing heart rate. Ex-4 would thus activate GLP-1Rs accessible to endogenous gut-derived GLP-1 from L-cells, as well as GLP-1Rs accessible to endogenous brain-derived GLP-1 from PPG neurons.

This may reflect a pharmacological situation where peripheral and central GLP-1R activation is obligatory linked but does not reflect what happens physiologically.

However, peripheral GLP-1, whilst unlikely to cross the BBB in a sufficient concentration to activate central GLP-1R's, reduces food intake. This is demonstrated as peripheral exogenous GLP-1 induced hypophagia is not attenuated by central injection of Ex-9 (Williams, Baskin and Schwartz, 2009). If the peripheral release of GLP-1 does not activate the PPG neurons, how does peripheral GLP-1 induce hypophagia? Studies using systemic injection of GLP-1 to induce hypophagia could be using supraphysiological doses of GLP-1, therefore GLP-1 in the bloodstream could cross the BBB to exert an effect on the CNS, however central blockade with Ex-9 should attenuate the hypophagia induced in this case, assuming that the hypophagia induced is through activation of the GLP-1R.

My data suggests that peripheral and central GLP-1 are distinctly different systems. In support of this idea, peripheral injection of Ex-9 blocks the hypophagia induced by peripheral injection of GLP-1 but did not block hypophagia induced by i.c.v GLP-1 and central injection of Ex-9 did not block the hypophagia induced by peripheral GLP-1 but did block the hypophagia induced by central GLP-1 (Barragán *et al.*, 1999; Williams, Baskin and Schwartz, 2009). Furthermore, an experiment performed in our laboratory by my fellow PhD student Marie Holt demonstrated that the tachycardia induced by i.p Ex-4 is not attenuated by ablation of the NTS PPG neurons, using the PPG-CRE mice and AAV-mCherry-Flex-DTA virus used in chapter 4 (data not shown, unpublished). Overall these data suggest that the PPG neurons are not involved in the tachycardia and hypophagia induced by Ex-4. As hypophagia and tachycardia are also induced by activation of the PPG neurons (Chapter 3 and 4) it is possible that Ex-4 enters the

brain and activates the same GLP-1R in the CNS as the PPG neurons to induce tachycardia or hypophagia. To study this further an experiment measuring heart rate or food intake after injection of a GLP-1 analogue that does not cross the BBB should be performed. If tachycardia or hypophagia is not induced, it would demonstrate the tachycardia and hypophagia induced by GLP-1 is through mechanism within the CNS.

5.3.2 Cholecystokinin

In this chapter exogenous systemic CCK did not induce C-Fos-IR in the PPG neurons, suggesting that CCK does not activate the PPG neurons to induce hypophagia. As systemic injection of CCK has been demonstrated to induce C-Fos-IR in the PPG neurons in rats (Rinaman., 1999), and CCK indirectly increases the electrical activity of the PPG neurons in mouse brain slice (Hisadome et al., 2010) this result is surprising. As CCK did not significantly reduce wet mash intake or display an interaction it is possible that the CCK injected was not functional, however it is more likely that the lack of significance was driven by the low n numbers as the wet mash intake was similar to that of the experiment demonstrating the effect of CCK on food intake which did exhibit a significant interaction and a trend towards a reduction in wet mash in the same mice.

Interestingly, the CCK injected mice exhibited a trend towards an increase in C-Fos-IR in the IRT-PPG neurons. If this trend is true it represents the ability of CCK to activate the IRT-PPG neurons, and more substantially it represents an anatomical differential role between the IRT and NTS PPG neurons. However, as no obvious difference between the IRT and NTS PPG neurons (besides anatomical location) has been demonstrated previously to my knowledge, I consider this result unlikely.

CCK not activating the PPG neurons could possibly be due to the difference in action of peripheral CCK and central CCK. Both peripheral and central CCK have been demonstrated to reduce food intake.

The effects of central CCK have been demonstrated by microinjection of CCK into hypothalamic sites (DMH, PVN, lateral hypothalamus, supraoptic nucleus), and the hindbrain NTS inducing food intake suppression (Blevins, Stanley and Reidelberger, 2000). Furthermore, a role for endogenous central CCK has been demonstrated through injection of the CCK_B antagonists PD-135158 into the lateral ventricle, and microinjection of the CCK_A antagonist into the PVN increased food intake (Dorré and Smith, 1998). i.c.v injection of proglumide, a mixed CCK_A and CCK_B antagonist also increased food intake in rats (Corp *et al.*, 1997). It has also been demonstrated the peripheral CCK acts solely through CCK_A to induce hypophagia, whereas central CCK acts through both the CCK_A and CCK_B receptor, suggesting that peripheral and central CCK act through different mechanisms to induce hypophagia. Overall the data here suggests that peripheral CCK acts in the periphery on the vagus to induce hypophagia, but there is also a role for direct action of CCK in the brain.

It is unknown whether there is a direct link between peripheral and central CCK induced hypophagia. Firstly, it is possible that peripheral CCK enters the brain and activates the central CCKR, to induce what is currently described as central CCK induced hypophagia. However, peripheral CCK has been demonstrated to not cross the BBB in significant concentration to be able to induce an effect. This was demonstrated by lack of detection of ^{125}I – labelled CCK in the CNS after intraarterial injection (Oldendorf, 1981). Therefore, peripheral CCK does not activate the central CCK receptors directly and only acts in the peripheral system. If peripheral CCK is not

responsible for the effects of central CCK administration, it is likely that the CCK neurons in the brainstem are responsible for the effects of central CCK (Roman, Derkach and Palmiter, 2016).

It is entirely possible that peripheral CCK can induce activation of central CCK system, however lateral ventricular injection of the CCK_A antagonists (lorglumide and devazepide) did not attenuate peripheral CCK induced hypophagia, and neither did the CCKB antagonist, L 365, 260, suggesting that peripheral CCK does not induce hypophagia through central CCK release (Corp *et al.*, 1997). However, It is important to note that CCK_A and CCK_B receptors were antagonised independently, not simultaneously. As both receptor types have been demonstrated to induce hypophagia separately in the brain, it is possible that the lack of attenuation of peripheral CCK induced hypohagia by either CCK_A antagonism or CCK_B antagonism is due to action of the other receptor. Further experiments in this study found that central injection of the mixed CCK_A/CCK_B receptor antagonist, proglumide (260nmoles), did in fact attenuate the hypophagia induced by peripheral injection of CCK, but did not abolish it (Corp *et al.*, 1997). However, in the Corp et al (1997) study higher dose concentration of i.c.v proglumide (1400nmoles) did not attenuate or abolish the hypophagic effect of peripheral exogenous CCK, suggesting some discrepancy within their results. Based on the evidence presented it is hard to conclude whether peripheral CCK activates the central CCK.

I hypothesise that peripheral CCK and central CCK are in fact different systems. If this hypothesises is correct, the study presented in this thesis is focussing on the action of peripheral CCK, not central CCK, on the PPG neurons. Therefore, this study demonstrates that peripheral CCK does not activate the PPG neurons to induce

hypophagia. However, this does not explain how systemic injection of CCK activated the PPG neurons in rat, which suggests that peripheral CCK can activate the PPG neurons (Rinaman,. 1999). However, the dose of CCK used in the study (100µg/kg) was significantly higher than what is considered physiological (1-3µg/kg), therefore it is entirely plausible that the CCK used in this study could cross the BBB in a sufficient concentration to induce an effect. However, as the dose used in the study presented in thesis used a dose that was also significantly higher (26µg/kg) than the physiological dose, I would have also expected C-Fos-IR in the PPG neurons.

Based on this evidence I conclude that peripheral CCK does not activate the PPG neurons, however it seems entirely plausible that central CCK can activate the PPG neurons. Further study would focus on whether central injection of CCK into the NTS activates the PPG neurons as it has been demonstrated *ex vivo* where CCK increased electrical activity in the PPG neurons.

5.3.3 Ensure

Intake of a large liquid meal (Ensure) induced C-Fos-IR in the PPG neurons. Mice consumed on average 1.92 ± 0.18g in 30 minutes, and 3.42 ± 0.24 in 90 minutes. In comparison to bodyweight mice consumed 7.74 ± 0.83% of their body weight in 30 minutes and 14.09 ± 1.79% in 90 min. Intake of a large meal induces gastric distention and constitutes a high-calorie intake. Interestingly, Ensure is calorically less dense than standard chow (Ensure:1.5kcal/ml, Standard Chow: 2.9kcal/g), so if intake were driven by the calories ingested, the mice would be expected to eat a larger volume of Ensure than chow to reach the same calorific load. However, mice allowed to consume standard chow after 3 hour fast before dark onset consumed 1.04 ± 0.07 kcal (0.36 ± 0.02g) of standard chow 1 hour after dark onset (n=8) (data not shown). This intake of

calories is drastically lower than the 2.88 ± 0.27 kcal consumed by mice allowed to consume Ensure after a 3 hour fast before dark onset. Although not directly comparable, the fact that a lot more Ensure is consumed in a short period of time compared to standard chow suggests that the intake is driven by its hedonic or reward value rather than a need for calories. When considering which parameter is the main driver for the activation of PPG neurons, this experiment does not allow clear conclusions, because it relies on the voluntary intake of the meal, and this could not be achieved e.g. with a meal that contains minimal calories, as it wouldn't provide an incentive for ingestion. Gastric distension has been demonstrated to induce C-Fos-IR in the PPG neurons in rats. As intake of Ensure was on average 14% of bodyweight, this can be classed as a large meal. However, this is high in both volume and calories, so in this case, it is unknown whether the C-Fos-IR measured in the PPG neurons is induced by gastric distention or calorific intake. Based on the previous observations in rat, where high consumption of a low-calorie meal induced C-Fos-IR in the PPG neurons, it seems more likely that the C-Fos-IR induced by Ensure intake is due to gastric distention.

5.3.4 Fos as a study of neuronal activation

C-Fos as a tool to study neuronal activity is limited to whether the neurons are activated, it cannot measure inhibition. Therefore, a lack of C-Fos does not necessarily mean that the stimulus has no effect on the target neurons. Additionally, different neuronal populations exhibit different thresholds to induce C-Fos. Therefore, one has to be cautious when concluding that a stimulus does not activate the neuronal population, so whilst C-Fos activation clearly indicates neuronal activation, the absence of C-Fos does not rule out a subtle effect on the neuron under investigation.

In this study, Ensure intake induced C-Fos-IR in the PPG neurons, demonstrating that C-Fos can be readily induced in the mouse PPG neurons. Therefore, this activation demonstrated that the PPG neurons can induce C-Fos-IR, and therefore C-Fos-IR can be used to study activation of the PPG neurons mice.

5.4 Conclusion

The anorexigenic hormones GLP-1 and CCK, when applied peripherally, did not activate the PPG neurons, therefore they do not induce their hypophagic effects through activation of the PPG neurons. This adds further evidence to peripheral and central GLP-1 to be two distinct systems. As distinct systems, peripheral and central GLP-1 could have different actions within the system, creating a need to study individually the effects of peripheral and central GLP-1. It seems likely that central GLP-1 is involved in appetite suppression of GLP-1, whereas peripheral GLP-1 influences glucose homeostasis and has insulinotropic effects. Intake of a large liquid meal did induce C-Fos-IR in the PPG neurons suggesting a physiological role for the PPG neurons in food intake; whether the PPG neurons act as a meal termination signal or are involved in the food-reward response or overeating as a stressor is unknown. Overall these data suggest that the PPG neurons form a separate, distinct, GLP-1 system within the brain, and that the central GLP-1 system is activated by intake of a meal and possibly acts to generate a meal termination signal within the CNS.

6. Summary & Perspective

Overall, in this thesis, I have expanded the current insight into the role of the central GLP-1 system. Although many studies have indicated a physiological role for GLP-1 in the brain through overactivation of the central GLP-1R, using exogenous central GLP-1 and GLP-1R agonist, here I have expanded the role of the putative physiological and endogenous source of central GLP-1. Firstly, I demonstrated that the PPG neurons are indeed the main central source of GLP-1. Once confirmed I proceeded to study the role of the PPG neurons on energy homeostasis, glucose homeostasis, and cardiovascular regulation by selectively targeting the NTS-PPG neurons and manipulating the activity the PPG neurons, using pharmacogenetic techniques.

Through activation, inhibition or ablation of the NTS-PPG neurons I demonstrated that the PPG neurons are not capable, or intrinsic in the regulation of blood glucose, that the PPG neurons are capable of reducing food intake, and that the PPG neurons can impact heart rate by inducing tachycardia but are not required for the regulation of resting heart rate. All the data presented here and from other studies suggest that the PPG neurons and central GLP-1 are not involved in normal regulation of heart rate and need to be recruited in specific situations. To further discover when the PPG neurons are recruited to induce hypophagia and tachycardia, I examined PPG neuronal activation in response to systemic injection of Ex-4, CCK and in response to large intake of a highly palatable meal. Here systemic CCK and Ex-4 did not activate the PPG neurons, but the PPG neurons were activated in response to intake of a large highly palatable meal, to possible induce satiety or tachycardia.

The data presented largely supports a role for the PPG neurons in the regulation of food intake and heart rate. In this chapter, I will expand on the evidence presented in this thesis, suggesting the potential mechanism and involvement of the PPG neurons in the biological system, and detailing experimental methods that could be used to further study the role of the PPG neurons.

6.1 Glucose tolerance

The experiments in chapter 3 of this thesis suggested that the PPG neurons have no role in the control of blood glucose, however it seems more likely based in other studies that the PPG neuron can impact glucose tolerance, but are not necessarily required in resting control of blood glucose.

A putative mechanism for central GLP-1 to regulated glucose homeostasis was hypothesised by Sandoval (2009). Here it was suggested that peripheral GLP-1 release acts directly on the vagal nerve, where the afferent feedback stimulates the central GLP-1 system in the hind brain (i.e PPG neurons). The PPG projections to the ARC activate the GLP-1R expressed in the ARC. Activation in the ARC leads to output to peripheral systems to regulate blood glucose. However, the data presented in this thesis and from other studies disagrees with hypothesis. Firstly, Sisley et al (2014) demonstrated that KD of the GLP-1R expressed on the vagal nerve did not attenuate blood glucose lowering effect of liraglutide**.** Additionally, in this study i.v GLP-1 did not alter vagal activity in the mouse. Both sets of data rule out a role for the vagal nerve. Secondly, systemic Ex-4 did not induce C-Fos-IR in the PPG neurons, suggesting the peripheral and central GLP-1 does not activate the PPG neurons. It seems that the PPG neurons are recruited by intake of a large meal, suggesting that the regulation of blood glucose by the PPG neurons is induced when a large meal is ingested.

6.2 PPG neuron induced hypophagia

Chapter 3 demonstrated that activation of the PPG neurons induces hypophagia when activated pharmacogenetically. As central injection of GLP-1R agonists and antagonist have demonstrated a role for central GLP-1R's in both homeostatic and food-reward behaviour (hedonic) related food intake, I would expect the PPG neurons to have a role in both homeostatic and hedonic food intake.

My study demonstrated that the PPG neurons have the capacity to impact food intake, but not necessarily a role in regulation of normal energy homeostasis. Experiments demonstrating an increase in food intake in response to central injection of Ex-9 suggests a role for central GLP-1 normal regulation of food intake. As the GLP-1 released from the PPG neurons is most likely blocked by Ex-9 to induce hyperphagia, it would be expected that the PPG neurons have role in resting food intake. However, my fellow PhD student Marie Holt demonstrated that ablation of the NTS-PPG neurons does not affect food intake or bodyweight over 70 days post ablation (unpublished) and inhibition of the NTS-PPG neurons didn't increase food intake either (unpublished). Furthermore, Sisley et al demonstrated that central KD of the GLP-1R did not alter bodyweight or 24hr food intake (Sisley et al,. 2014). Together this data suggests that there is not role for PPG neurons in normal regulation of food intake. However, it is important to note that the IRT-PPG neurons were left intact in Marie Holts study, therefore it is possible that the IRT-PPG neurons are sufficient to maintain regular feeding, and that complete loss or inhibition of the PPG neurons may result in a role for the PPG neurons in normal regulation of food intake. The ability for a small amount of neurons to maintain regulation of food intake was demonstrated in orexin neurons where greater than 90% population loss was required to exhibit the catyplexy

phenotype (Tabuchi *et al.*, 2014). Further experimentation targeting the complete brainstem population of PPG neurons with ablation or inhibition would negate this issue and reveal whether the PPG neurons have a role in regulation of normal food intake.

6.2.1 Central Vs Peripheral GLP-1: Hypophagia

This study clarified a role for the PPG neurons in the regulation of food intake, demonstrating that the central GLP-1 system can induce hypophagia. This discovery adds credence to the previous hypothesis that peripheral GLP-1, released from the L-cells, acts on the GLP-1R in the vagus or the AP, which relays the signal into the NTS, as it demonstrates the PPG neurons can in fact induce hypophagia. It was hypothesised that activation of the GLP-1R in the vagus would lead to activation of the PPG neurons, inducing hypophagia through release of central GLP-1. However, in chapter 5 systemic injection of Ex-4 did not activate the PPG neurons, suggesting that peripheral GLP-1R activation or in-fact central GLP-1R activation does not activate the PPG neurons to induce hypophagia. In support of this, central injection of Ex-9 abolished hypophagia induced by central injection of GLP-1 but did not abolish hypophagia induced by peripheral injection of Ex-4 (Williams, Baskin and Schwartz, 2009). Additionally, 4th ventricular infusion of Ex-9 attenuated the reduction in food intake induced by gastric distention but did not attenuate the food intake reduction induced by duodenal infusion of nutrients in the gut (peripheral GLP-1 activation) (Hayes, Bradley and Grill, 2009). As gastric distention induces C-Fos-IR in the PPG neurons, this data suggests that the PPG neurons are activated by gastric distention and act to induce hypophagia through GLP-1 release in the central nervous system, whereas peripheral GLP-1 is released in response to nutrients in the duodenum

induces hypophagia by acting on peripheral GLP-1R's. From combining this literature with my results, I conclude that peripheral and central GLP-1 system are independent, and both may act to induce hypophagia through different pathways.

It was made apparent in this thesis that central GLP-1 induced hypophagia is most probably due to activation of the PPG neurons. However, if peripheral GLP-1 does not act through the central GLP-1 system, how does GLP-1 induce hypophagia? As peripheral injection of Ex-9 abolished hypophagia induced by peripheral injection of GLP-1, peripheral GLP-1 must act on receptors in the periphery to induce hypophagia (Williams, Baskin and Schwartz, 2009)**.** As this is the case, peripheral GLP-1 is mostly likely to act through the GLP-1R expressed on the vagal afferents, where the vagal afferents terminate in the NTS and induce hypophagia through a pathway that does not require central GLP-1. In support, sub-diaphragmatic vagotomy required higher doses of Ex-4 and liraglutide to induce a significant reduction in food intake (Kanoski *et al.*, 2011)**.** This data suggests that the GLP-1R expressed on vagal afferents has a role in food intake, as Ex-4 and liraglutide are believed to enter the CNS, the difference in food intake between the vagotomised and control rats could possibly be attributed to the loss of peripheral GLP-1R activation induced hypophagia on the vagal GLP-1R's. Furthermore, peripheral GLP-1R agonist have been demonstrated to increase firing rate and of the vagal nerve, demonstrating the GLP-1 agonists alter vagal activity (Vahl *et al.*, 2007; Krieger *et al.*, 2016)**.** However, Sisley et al 2014 found that selective KD of the GLP-1R expressed in the vagus did not attenuate Ex-4 induced hypophagia (Sisley *et al.*, 2014)**.** This approach specifically targets the GLP-1R's expressed on vagus nerve without affecting vagal signalling from other constructs, thus providing evidence that the vagus is not required for peripheral GLP-1 to induce hypophagia.

Additionally, in this thesis it was demonstrated that peripheral GLP-1 injection does not alter integrated vagal activity, further suggesting the GLP-1 does not recruit the vagus. Unfortunately, many of these studies suffer the same limitation where peripheral GLP-1R's and Central GLP-1R's are activated simultaneously, therefore it difficult to determine the what effect can only be attributed to loss of vagal GLP-1R's.

Another mechanism in which peripheral GLP-1 can act to induce hypophagia is yet to be widely explored. The AP widely expresses the GLP-1R. As the AP is a CVO, peripheral GLP-1 can directly activate GLP-1R in this nucleus from the blood stream. In support, peripheral injection of GLP-1 has been demonstrated to induce C-Fos-IR in the AP, and this was observed in the experiments in this thesis with injection of Ex-4 (Yamamoto *et al.*, 2002, 2003). However, liraglutide induced weight loss was not attenuated in AP lesioned rats, although rats with a lesioned AP presented with significant weight loss compared to sham surgery rats before liraglutide injection, therefore it is very possible that the effect of liraglutide in the AP is precluded by this effect. Additionally, liraglutide enters the CNS and acts on the central GLP-1R system to induce hypophagia, therefore masking any measurement of peripheral GLP-1 on hypophagia (Secher *et al.*, 2014).

These unanswered questions suggest a role for AP in regard to peripheral GLP-1 induced hypophagia. To explore the possible role of the AP in peripheral GLP-1 induced hypophagia, lesioning, ablating (DTA) or inhibiting (hM4Di) the GLP-1R expressing neurons in the AP before peripheral injection of GLP-1 and measuring the effect on food intake would reveal a role or lack of a role for the GLP-1R expressing neurons in the AP. Firstly, to ensure that the GLP-1R expressing neurons in the AP

do have the capacity to induce hypophagia I would express hM3Dq in the AP GLP-R neurons and measure food intake upon activation.

It is also possible that peripheral GLP-1 induces hypophagia indirectly through increasing release of insulin from the β-cells in the pancreas. Peripheral GLP-1 is a strong insulinotropic and insulin has been demonstrated to induce hypophagia through interaction in the ARC, therefore peripheral GLP-1 could be reducing food intake through an increased release of insulin (Ikeda *et al.*, 1986)**.**

As stated previously, many of these studies suffer a similar limitation: central GLP-1R is being activated as well as peripheral GLP-1R. A study using the BBB impermeable albumin conjugated GLP-1 analogue, CJC-1131, found that peripheral GLP-1 did not impact food intake but did decrease blood glucose (demonstrating that modified GLP-1 is still functional), therefore peripheral GLP-1 does not have a role in the control of food intake, at least in this study.

Based on the evidence presented it is difficult to conclude whether peripheral GLP-1 has a role in food intake, however I suspect that peripheral GLP-1 does have a role in food intake. Overall, I hypothesise that both peripheral and central GLP-1 are activated by intake of a meal (peripheral by the presence of nutrients in the gut, central GLP-1 by gastric distension (Vrang *et al.*, 2003). Upon release, peripheral GLP-1 predominantly acts to reduce blood glucose as its primary action, but also acts reduce food intake. Peripheral GLP-1 could either reduce food intake through action on the GLP-1R expressed on the vagus or GLP-1R expressed in the AP, relaying the signal into the brain and reducing food intake through a non-GLP-1 related pathway, or through the indirect method of insulin release acting on the ARC to reduce food intake. Based on the evidence presented, it seems that action through the AP and the vagus

is most likely responsible for this effect, however current data supporting both pathways needs further study. Central GLP-1 system appears to at least be activated by gastric distension, suggesting that the PPG neurons are activated only by intake of a large meal. This leads to release of GLP-1 acting on the GLP-1R expressed in the CNS to reduce food intake.

6.3 GLP-1 and PPG neuron induced tachycardia

The experiments in this thesis demonstrated systemic activation of the GLP-1R, using Ex-4, induces tachycardia through an increase in sympathetic tone. The results in this chapter significantly expanded our insight into the mechanism by which GLP-1R activation induces tachycardia, specifically in mice. Previously it was identified that Ex-4 induces tachycardia and hypertension in rats, whereas only tachycardia is induced in response to systemic Ex-4 in mice. Notably, this highlights the species-specific effects of Ex-4 on the cardiovascular system and demonstrates the necessity to understand the effects of Ex-4 across multiple species. Furthermore, the autonomic mechanism by which Ex-4 induced tachycardia is probably an increase in sympathetic tone in rats. However, whether the tachycardia induced was due to an increase in sympathetic tone was not so clear in mice with data from Griffioen et al (2011) suggesting that the tachycardia induced by Ex-4 is due to a decrease in parasympathetic tone, with limited other studies suggesting it is due to an increase in sympathetic tone (Griffioen *et al.*, 2011). Whilst atenolol abolished Ex-4 induced tachycardia, atropine did not, and GLP-1 did not alter vagal activity, the data in this thesis undeniable demonstrated that systemic GLP-1R activation induces Ex-4 through an increase in sympathetic tone in mice. This further clarifies the mechanism by which Ex-4 induces tachycardia and indicates that both mice and rats increase

heart rate response to tachycardia through the same autonomic mechanism, even if mice do not exhibit hypertension.

Further experimentation in chapter 4 demonstrated that the PPG neurons have the capacity to induce tachycardia but are not necessary to regulate heart rate in anaesthetised, and perhaps resting mice. Although it was not studied in this thesis, it is most probable that the PPG neurons induce tachycardia through the release of PPG products, acting through GLP-1Rs in the cardiovascular regulatory sites in the brain. Further experiments to clarify the mechanism of PPG neuron-induced tachycardia could include targeting specific GLP-1R nuclei such as the PVN, ARC, IML, and CAA with Ex-9, before activation of the PPG neurons, specifically identifying the nuclei involved in the induced PPG neurons induced tachycardia.

6.3.1 Central Vs Peripheral GLP-1: Tachycardia

Regarding peripheral and central GLP-1 induced tachycardia the evidence points towards a different story than to that of peripheral and central GLP-1 induced hypophagia. I hypothesis that peripheral GLP-1 induced tachycardia and central GLP-1 induced tachycardia act through the same mechanism. Here I suspect that both peripheral and central GLP-1 act on the central GLP-1R expressed in the PVN, ARC, RVLM, and possible the IML and CAA to induce tachycardia. Evidence towards this is that central Ex-9 attenuates the tachycardia produced by peripheral Ex-4, suggesting that Ex-4 acts centrally on the GLP-1R to induce tachycardia (Barragán *et al.*, 1999). This implies that either Ex-4 enters the brain and activates the GLP-1R or the PPG neurons are recruited to release GLP-1 and induce tachycardia. However, the PPG neurons do not express the GLP-1R and it was demonstrated in this thesis that Ex-4 injection does not activate the PPG neuron (Hisadome *et al.*, 2010. Therefore, the only

source of GLP-1 available to act in the brain is the systemically injected Ex-4. As PPG neurons activation is hypothesised to act through activation of the central GLP-1R's as well, this data suggests that both systemic Ex-4 and PPG neurons induce tachycardia through the same mechanism. Evidence against this hypothesis is that peripheral injection of GLP-1 also induces tachycardia, and peripheral GLP-1 is believed not to cross the BBB in a high enough concentration to exert an effect above threshold on the central GLP-1R's. However, in all cases where peripheral GLP-1 injection has induced tachycardia, the concentration of GLP-1 injection is significantly higher than what would be expected in to be released physiologically, therefore the exogenous GLP-1 will enter the brain above threshold concentration to induce an effect on heart rate. In this case I would expect that endogenous release of GLP-1 from the L-Cells does not induce tachycardia. Overall, I hypothesis that central GLP-1 system can induce tachycardia, whereas native peripheral GLP-1 does not. To study this I would peripherally inject the albumin conjugate GLP-1 that does not cross the BBB (CJC-1131) and measure heart rate. If tachycardia is induced the data would suggest the peripheral GLP-1 does induce tachycardia. Further, study to demonstrate whether endogenous release of peripheral GLP-1 acts to regulate heart rate could be achieved by creation of a GLP-1R antagonist that does not cross the BBB.

To study whether the PPG neurons also induce tachycardia through the same mechanism, a similar experiment should be employed as conducted by Barragan et al (1999) (i.e injection Ex-9 centrally, followed by activation of the PPG neurons). If central injection of Ex-9 abolishes the tachycardia induced by activation of the PPG neurons, it would suggest that the PPG neurons induce tachycardia through activation of the central GLP-1R expressing neurons. Further experiments could include

targeting specific GLP-1R nuclei such as the PVN, RVLM, IML, and CAA with Ex-9, before activation of the PPG neurons or before systemic injection of Ex-4, specifically identifying the nuclei involved in the tachycardia induced by peripheral and central GLP-1.

6.4 The difference between the IRT and NTS PPG neurons

The experiments within the thesis focuses on the NTS-PPG neurons. It is important to note that there is a population of PPG neurons in the IRT, however the difference between the IRT and the NTS PPG neurons, besides anatomical, has yet to be confirmed. There are limited studies that have looked into the characteristics of each population, and current data looks as if the populations are extremely similar. Both the NTS and IRT PPG neurons project to the spinal cord (through T9 fluorogold injections) in a similar proportion (Llewelyn-Smith, 2014). Additionally, both populations project to the PVN and DMH and receive similar innervation of 5-HT neurons (Holt, 2017). Based on the current literature it seems that the IRT population would have the same role as the NTS-PPG neurons. To further clarify the role of the IRT-PPG solely studying the effect of activation and inhibition of the IRT-PPG neurons and whether they also induce hypophagia and tachycardia would begin to determine the role for the IRT-PPG neurons.

6.5 Viral Vectors

A flaw within many of the experiments in this thesis appears through the use of viral vectors with differing capsid proteins i.e serotypes. The serotype of the AAV used affects the ability of the viral vector to infect specific cell types. In this study AAV serotype 1/2 was used for most vectors, however the GFP viral vector was in fact

AAV8. Although both AAV1/2 and AAV8 have been designated an optimal serotype for infection in the CNS there is most likely a differing rate of infection and expression of the GOI between the serotypes (Naso et al,. 2017). This difference in gene expression weakens the use of the AAV8-Flex-eGFP vector as a control against the AAV1/2 serotype viral vectors, however in the experiments where the AAV8 serotype is used there is no difference between the hM3Dq and hM3Di to the GFP on baseline heart rate (Fig 4.7, 4.8, 4.9) and no difference between the DTA to GFP in glucose tolerance (Fig 3.4).

Another issue present with viral vectors is the use of Perceval as a viral control. Perceval is an ATP sensor. There is a thought that expression of Perceval will cause ATP to be chelated within the neuron changing the physiology of the cell when compared normal healthy neurons (Berg et al,. 2008). Although possible once again there was no difference between glucose tolerance when the Perceval mice were tested against the hM3Dq (Fig 3.7)

Although it appears that the affect of serotype and Perceval ATP chelation did not affect the outcome of the physiological parameters measured it still remains unknown whether it altered different parameters or affected the neurons on a more cellular level.

6.5 The physiological role of the PPG neurons: Increased oxygen delivery to the digestive organs

Feeding-induced cardiac changes have been known since 1929, where Grollman et al documented an increase in heart rate and systolic blood pressure in response to an ingestion of a meal in humans (Grollman, 1929)**.** This effect was confirmed in dogs and primates where ingestion of a meal induced an increase in heart rate in

both (Kelbæk *et al.*, 1989)**.** This amplitude of the effect on heart rate has been demonstrated to be impacted by the size of the meal, where the larger meal induced a larger increase in heart rate (Waaler, Eriksen and Toska, 1991)**.** The biological reason for this response is believed to allow increased oxygen delivery to digestive organs, as they perform the task of digestion.

It is possible that the PPG neurons are at least partially the source of this response. Firstly, the PPG neurons are activated in a response to a large meal, and the number of neuronal cells activated is proportional to the size of the meal. Secondly, the PPG have the capacity to induce tachycardia, but do not regulate heart rate under resting conditions and finally, they act to inhibit further intake of food. However, there is an extremely limited number of studies examining the effects of large meals on cardiac output in rodents, therefore making this hypothesis highly speculative but nevertheless possible.

6.6 The physiological role of the PPG neurons: Stress

The central GLP-1 system has also been implicated in stress responses (Holt and Trapp, 2016). i.c.v GLP-1 has been demonstrated to increase peripheral release of the stress hormones corticosterone and adrenocorticotropic hormone (ACTH) in rats (Gil-Lozano *et al.*, 2010). Additionally, i.c.v GLP-1 increase anxiety like behaviour in rats on the elevated plus maze (Kinzig *et al.*, 2003). Furthermore, GLP-1 microinjection of GLP-1 into the bed nucleus of the stria terminalis, a brain nucleus heavily associated with stress, reduced food intake. Additionally, microinjection of Ex-9 into the BNST increased food intake in mouse (Williams et al., 2018). Together these data indicate that central GLP-1 induces a stress response. As demonstrated in this thesis the PPG neurons are the main physiological source of central GLP-1, therefore

it is plausible that the PPG neurons would induce a similar stress response to i.c.v, assuming the stress response demonstrated by i.c.v GLP-1 is physiologically relevant.

In this case, I hypothesise that one of the physiological roles of the PPG neurons is in the stress response. In support of this, hypophagia has been demonstrated to be induce by stress, where the forced swim test and restraint test both reduced meal size and duration (Calvez *et al.*, 2011)**.** This is a mechanism within the system to divert the body's resources from the less pressing need of searching for food, when presented with a stressor. As this thesis demonstrated that the PPG neurons can induce hypophagia, it is possible that the PPG neurons are at least partially responsible for this response in stress. Substantial evidence was produced by M.Holt (a fellow PhD student in the laboratory). M.Holt demonstrated that selective inhibition of the PPG neurons reduced the hypophagia induced by 30-minute restraint stress, demonstrating the PPG neurons are recruited during a stress response (M.Holt, Diabetes manuscript).

Alongside hypophagia stress induces tachycardia through the release of stress hormones and an increase in sympathetic drive (Fight or Flight). As this thesis demonstrated that the PPG neurons can induce tachycardia, this presents another role for the PPG neurons in stress. Furthermore, this would explain why no effect on heart rate was seen when inhibiting the PPG neurons in the anaesthetised mouse. As the anaesthetised mouse is not under stressful stimulus, the PPG neurons would not be recruited, and therefore inhibition would have no effect. Therefore, I hypothesise that a role of the PPG neurons is in the stress response, here they act to increase heart rate to deliver oxygen to the muscle and inhibit food intake to avoid the increased risk involved when searching for a meal.

However, if the PPG neurons are in fact involved in stress, it is surprising that they would be activated by intake of a large meal. It has been suggested that intake of food, particularly a large meal, is a disruptive event in the sense of homeostasis, and that intake of meal initiate release of stress hormones, similar to that of a stressor (Al-Damluji et al,. 1987; Woods., 1991). Therefore, a large meal can be seen a stressor and further suggests that the PPG neurons have a role in stress.

It is surprising that the PPG neurons do not exhibit a role in glucose homeostasis, if stress is the role of the PPG neurons. During a stress response blood glucose is elevated to ensure the body has the energy source available to flee is necessary (Marik and Bellomo, 2013). However, as the PPG neurons do not inhibit a rise in blood glucose, the lack of role in glucose homeostasis does not exclude the PPG neurons from having a role in stress.

6.7 Peripheral Vs Central GLP-1: Hypothesis

Overall, I hypothesise that the peripheral and central GLP-1 system are in fact distinct system, activated in response to intake of a meal outlined. Upon ingestion of meal peripheral GLP-1 is released from L-Cells after detecting intraluminal nutrients. GLP-1 enters the bloodstream, and acts on the pancreatic β-cells to induces insulin release. In the bloodstream GLP-1 acts on the GLP-1R expressed on the vagal afferents or the AP. The vagal afferents or the AP relay the signal into the NTS, where it relayed into higher brain centres to induce hypophagia, through unknown neuronal pathway. Peripheral GLP-1 did not activate the PPG neurons, therefore GLP-1 action on the AP and the vagal afferents does not induce hypophagia through activation of the PPG neurons. Additionally, I do not believe the endogenous peripheral GLP-1 contributes to the tachycardia induced by GLP-1 injection, I believe that tachycardia is solely

induced by central GLP-1R activation. Upon ingestion of a large meal, the PPG neurons are activated by gastric distention or large intake of calories. Upon activation the PPG neurons release GLP-1 on higher brain centres to reduce food intake. These higher brain centres most likely include not exclusive to the PVN, ARV, DMH, NAc, VTA and BNST. Additionally, the release of GLP-1 by the PPG neuron induces tachycardia through activation of the GLP-1R expressed in the CNS. These nuclei most likely include but is not exclusive to the PVN, ARC, DMH, RVLM, IML and CAA.

Interestingly, if this hypothesis is true this represents the ability to produce a GLP-1 analogue that does not induce tachycardia but can regulate appetite and blood glucose. A GLP-1 analogue that does not cross the BBB will not be able to enter the CNS, and therefore will not alter the cardiovascular system, reducing any possible cardiovascular complications when treating obesity and type 2 diabetes.

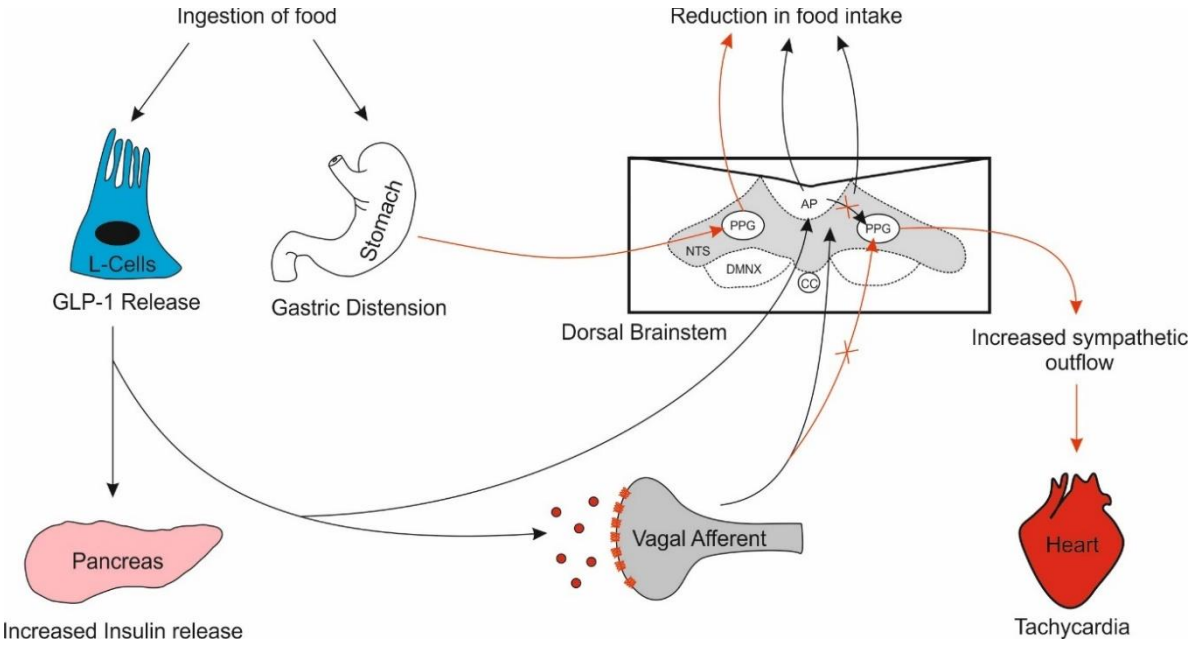


Figure 6.1: Hypothesised pathway of peripheral and central GLP-1 to induce hypophagia and tachycardia in mice: Following ingestion of a meal, nutrients in GI tract lead to release of GLP-1 from the L-cells in the gut (peripheral GLP-1). Peripheral GLP-1 then acts on the pancreas to increase insulin release and maintain glucose homeostasis. Peripheral GLP-1 also acts on the GLP-1R expressed on the vagal afferents and the area postrema (AP). From here both act in the CNS to reduce food intake, however as demonstrated in this study is not induced through activation of the PPG neurons. Ingestion of a large meal also leads to gastric distension in the stomach. Gastric distension activates the PPG neurons which in turn release GLP-1 in PPG neurons project sites which acts to induce hypophagia and induce tachycardia through an increase in sympathetic outflow (pathways demonstrated or clarified in this thesis are represented by red arrows). **AP: Area postrema, CC: Central Canal, DMNX: Dorsal motor nucleus of the vagus, NTS: Nucleus tractus solitarius, PPG: Preproglucagon neurons.**

6.8 Conclusion

This thesis present crucial insight into the role of central GLP-1 and the PPG neurons. I demonstrated that the PPG neurons are indeed the source of central GLP-1. I identified that the PPG neurons can impact food intake, possibly through release of GLP-1, however it was not determined whether the PPG neurons are required in normal regulation of satiety. Furthermore, this thesis provides the first evidence that the PPG neurons do in fact induce tachycardia, but this regulation of heart rate is not necessary in the regulation of the cardiovascular system under resting anaesthetised condition. Surprisingly, the results suggested that the PPG neurons have no role in the control of blood glucose, suggesting that central GLP-1 does not regulated glucose

homeostasis, however this result directly opposes recent data demonstrating a role for central GLP-1 in glucose homeostasis, therefore whether the PPG neurons affect blood glucose is unclear. Upon discovering the role of the PPG neurons in the mouse, I wanted to understand what conditions these neurons would be activated to induce tachycardia, and hypophagia. This thesis demonstrated that the PPG neurons are activated upon ingestion of a large calorific meal. Collectively, this thesis expands and clarifies the role of the PPG neurons, while raising the question when and where these neurons are recruited to induce their effect on the heart and food intake. Future studies should focus on this question, discovering the situation in which the PPG neurons will be recruited, whether it is in normal regulation of the food intake or heart rate or whether specific situation is required for the PPG neurons to implement their effect.

Conference abstracts

Cook DR, Richards JE, Holt MK, Trapp S (2017). Acute Activation of the PPG neurons in the mouse brainstem induces meal termination in mice. EASD Study group. Pisa

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