Investigating the role of arcuate nucleus and paraventricular nucleus glucokinase in glucose homeostasis

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Abstract

The brain is as an important regulator of appetite and metabolism. Glucokinase (GK) is a key component of glucose sensing, and is expressed in many glucose sensitive neurones (Rosario et al., 2016, Pocai et al., 2005b). Two regions where glucokinase is expressed are the arcuate nucleus and the paraventricular nucleus of the hypothalamus (Jetton et al., 1994b). Using viral techniques to specifically alter glucokinase expression, I show that upregulation of glucokinase in these nuclei individually, significantly improves glucose tolerance and increases insulin secretion. Conversely down regulation significantly worsens glucose tolerance and reduces insulin secretion. Interestingly the incretin effect is negligible within the arcuate nucleus but within the paraventricular nucleus, glucagon like peptide-1 (GLP-1) secretion is blunted when glucokinase expression is reduced. GLP-1 secretion has been believed to be entirely regulated by the enteroendocrine L-cells of the small intestine however these results suggest that the brain may play a role. The pathway of action of both the arcuate and paraventricular GK appears to be via ATP-sensitive potassium channels (K_{ATP}) channels (Maekawa et al., 2000, Hussain et al., 2015b, Ma et al., 2018a). These results suggest an important role for arcuate and paraventricular nuclei glucokinase in glucose homeostasis via K_{ATP} channels.

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Declaration of Contributors

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

Chapter 2:

In vivo studies were performed in collaboration with Dr Ma Yu.

Some of the in vivo studies were further assisted by Dr Chioma Izzi-Engbeya and previous background studies performed by Errol Richardson, Sufyan Hussain, Ivan De Backer and Christopher Holton

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Abbreviations

AgRP agouti related peptide

Alpha MSH alpha melanocyte stimulating hormone

ARC arcuate nucleus

ATP adenosine triphosphate

CART cocaine- and amphetamine-regulated transcript

CCK Cholecystokinin

CNS central nervous system

CpdA compound A

DPP Dipeptidyl Peptidase

DPP-IV Dipeptidyl Peptidase-4

ENS enteric nervous system

FGF fibroblast growth factor

fMRI function magnetic resonance imaging

GABA γ-aminobutyric acid

GFAF Anti-Glial Fibrillary Acidic Protein

GFP green fluorescent protein

GIP gastric inhibitory polypeptide

GK glucokinase

GLP-1 glucagon like peptide

GLUT-2 glucose transporter 2

GTT glucose tolerance test

ICV Intracerebroventricular

IR insulin receptor

ITT insulin tolerance test

KATP potassium adenosine triphosphate

Lep ob/ob mutation in the leptin gene which renders leptin unable to bind to its receptors

LH lateral hypothalamus

MODY 2 maturity onset diabetes of the young type 2

NPY neuropeptide Y

NTS nucleus solitary tract

OGTT oral glucose tolerance test

P13K phosphoinositide 3-kinase

PCR polymerase chain reaction

PGP9.5 Protein gene product 9.5

POMC pro-opiomelanocortin

PVN paraventricular nucleus

PYY peptide YY

rAAV recombinant adeno associated virus

T2DM Type 2 diabetes

VMH ventromedial hypothalamus

VMN ventromedial nucleus

ZDF Fa/Fa Zucker fat

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Chapter 1

General Introduction

1.1 Obesity and Diabetes: A Clinical Problem

Obesity is a growing problem. National surveys suggest that 58% of women and 68% of men in the United Kingdom are overweight or obese. Obesity results from an imbalance between energy intake and energy expenditure and Body Mass Index (BMI) is typically used to define obesity. BMI gives an indication of an individual's weight relative to their height. The equation used to calculate BMI is weight in kilograms divided by height in cm^2. According to the World Health Organisation, being overweight is defined as a BMI ≥ 25 kg/m^2 and obesity is defined as a BMI ≥ 30 kg/m^2. The rising incidence of type 2 diabetes (T2DM) correlates closely with a rising BMI (Diabetes Care 1994). Current figures from the charity Diabetes UK suggest that 1 in 16 people have diabetes and that this figure is on the rise. Alarmingly, 1 in 8 deaths in those aged between 20 to 79 years are attributable to diabetes (Yorkshire and Humber Public Health Authority 2008: Diabetes attributable deaths). Death from diabetes reflects the multi-systemic effects of the disease and results from a range of causes including infections, renal failure and cancer. Whilst obesity is certainly a huge contributing factor, other pre-disposing non-modifiable risk factors such as age, family history and ethnicity also play a causative role. Type 2 diabetes, with its microvascular and macrovascular consequences, is a major health burden for patients and to the health service with disabling outcomes such as blindness, renal failure and amputation. There are an array of treatments currently available to treat Type 2 diabetes with the National Health Service spending 24 million pounds per day managing this disease process (NHS Confederation (2007a) Key statistics on the NHS London).

1.1.2 Treatment Premise behind Type 2 Diabetes

The treatment of diabetes has traditionally centered around the pancreas and a belief that the fundamental issue lies with the hormone insulin, either through its deficiency, resistance to its end tissue action or a combination of the two. Therefore, therapies revolve around this premise and often ultimately require replacement of insulin itself delivered through subcutaneous injections. Superficially, this system seems sufficient to explain most of the physiology behind blood glucose control. However, there is emerging evidence that the control of glucose homeostasis is far more complex than a simple peripheral network headed by the pancreas. From an evolutionary perspective, an alternative more complex system seems plausible as regulating the body's main fuel for life, glucose, is critical to sustain life. All organs need glucose however glucose in both excess or deficit causes harm and can result in death. It therefore follows to have a network which is not purely dependent on the function of a single peripheral organ and is instead reliant on a web of systems working in unison to achieve the final goal of optimal glucose homeostasis. Following from this, the brain is now emerging as a key player in this proposed new system. Its precise role is yet to be defined and debates as to the extent and importance of its contribution are ongoing amongst researchers in the field. For instance, it is likely that different areas of the brain play different roles. It is also possible that the brain's role is not significant all the time and perhaps only becomes relevant once blood sugars fall outside physiological norms. There is much uncertainty in this field, but the two things that are certain are that the brain does have a role in glucose homeostasis and that there are still many unanswered questions as to the nature of this role. In order to investigate the contribution of the brain further we need to first

understand the core historical pancreas-centred model which has dominated the glucose homeostatic scene.

1.2 The Traditional Glucose Homeostatic Model

Mainstream research and therefore treatment approaches have historically been based on the traditional glucose homeostatic model. In this model raised blood glucose levels are detected by the beta cells within the pancreatic islets. They secrete insulin in response to rising blood glucose concentrations following a meal. The insulin acts on insulin-sensitive tissues including muscle, liver, and adipose tissue stimulating glucose uptake thereby decreasing blood glucose levels to within the euglycaemic range (Figure 1).

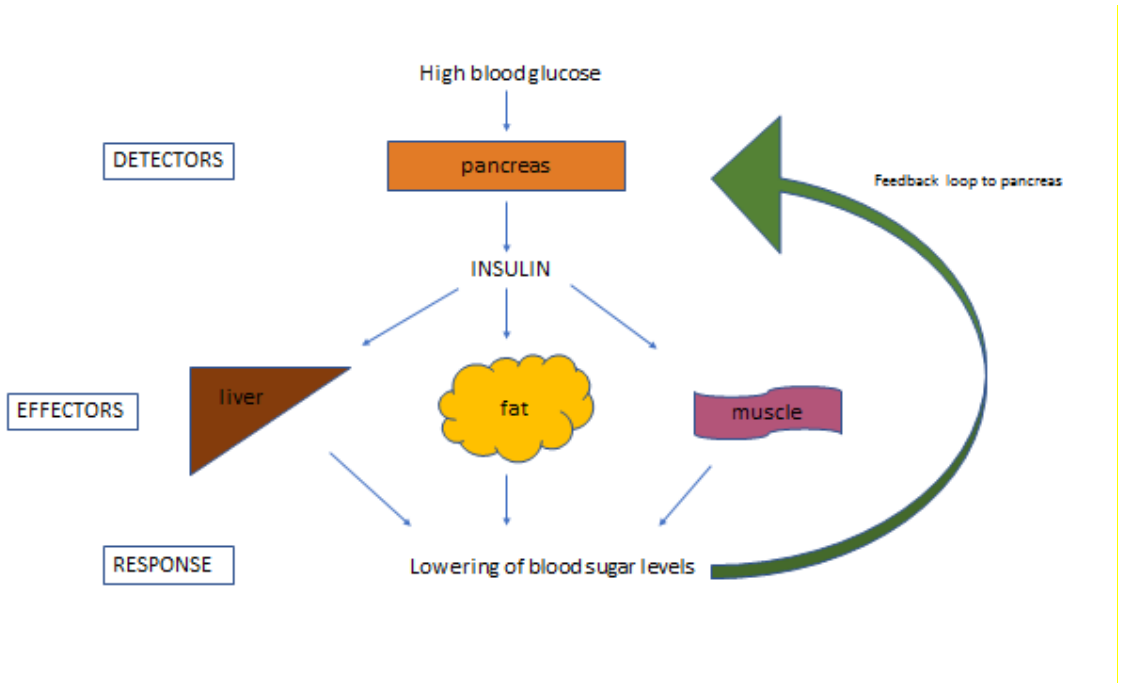


Figure 1: Diagrammatic representation of the traditional glucose homeostatic model. This model is headed by the pancreas which detects raised glucose levels and in response, releases insulin. Insulin has downstream effects on other organs to ultimately decrease blood sugar levels.

1.2.1 Insulin release from the beta pancreatic islets

At a beta pancreatic cellular level and prior to insulin release, glucose enters the cells via the glucose transporter 2 (GLUT 2). The enzyme glucokinase (GK) phosphorylates glucose to glucose-6-phosphate and this generates adenosine triphosphate (ATP). ATP is the major energy currency of cells. The generation of ATP closes the K_{ATP} channel which in turns leads to membrane depolarisation through calcium channel influx. This depolarisation leads to the movement of insulin secretory granules to the cell surface and the eventual release of insulin into the circulation (Fu et al., 2013) (Figure 2).

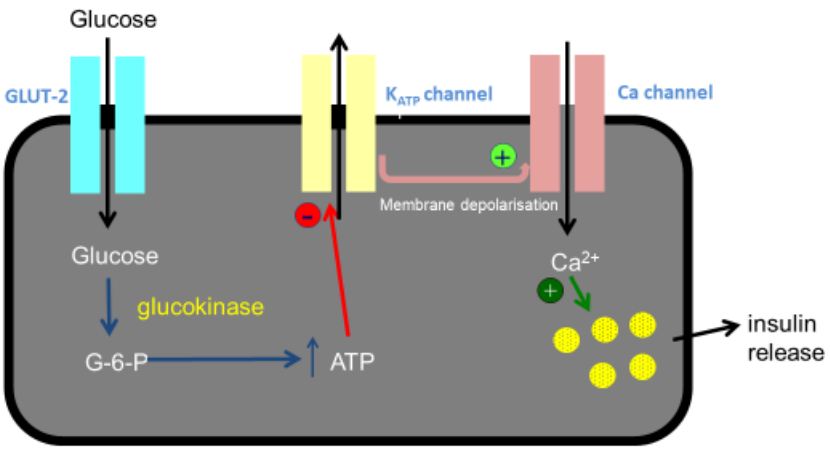


Diagram courtesy of PhD supervisor, Dr James Gardiner.

Figure 2: Diagrammatic representation of glucose triggering insulin release from the beta pancreatic islet cell.

1.2.2 Cellular action of insulin

Once released and at the level of the target tissues, insulin binds to its receptor (IR) which is a member of the tyrosine kinase family of receptors. This binding then increases the number of glucose transporter proteins by activation of the phosphoinositide 3-kinase (PI3K) intracellular signaling pathway which results in increased glucose uptake. Figure 3 summarises the effects of insulin at the major target sites. At the liver, insulin inhibits hepatic gluconeogenesis and glycogenolysis, both of which decrease circulating glucose levels (Petersen et al., 1998, Edgerton et al., 2006). Insulin also stimulates the uptake of glucose into fat and muscle which also decreases glucose levels (Dimitriadis et al., 2011). Insulin suppresses the hormone glucagon which is released from the alpha cells of the islets of Langerhans within the pancreas in response to low levels blood sugar levels. Glucagon at the level of the liver stimulates glycogenolysis and gluconeogenesis thereby releasing glucose into the blood stream. Similarly to other stress hormones such as catecholamines, cortisol, growth hormone, glucagon is important in the counter-regulatory response to hypoglycaemia (Schwartz et al., 1987) .

Key Sites	Action of insulin	Effect on plasma glucose
liver	suppresses glycogenolysis stimulates glycogen synthesis suppresses gluconeogenesis	decrease
muscle	suppresses lipolysis suppresses gluconeogenesis suppresses protein catabolism stimulates glycogen synthesis	decrease
fat	suppresses lipolysis stimulates lipogenesis stimulates glucose uptake	decrease

Figure 3: Table showing the key sites where insulin acts and the effects it has at these organs

According to this traditional glucose homeostatic model, the pathophysiology of diabetes is thought to be simple; either there is failure of the pancreas such that it is unable to produce enough insulin to control glucose levels and/or there is resistance to the action of insulin at the level of peripheral tissues.

1.2.3 Innervation of the islet cells

Islet cells express a large number of cell surface receptors to include receptors for Glucagon-Like Peptide 1 (GLP-1) and Gastric Inhibitory Peptide (GIP) which are incretin hormones. These hormones are secreted by the gut following a meal and the islets respond to these hormones in the circulation as well as to a number of other circulating hormones. Islets also have receptors for glucagon which respond to paracrine signaling. These receptors are G-protein coupled receptors which potentiate glucose stimulated insulin secretion. Some of these G Protein Coupled receptors also respond to signals from the parasympathetic nervous system e.g. receptors for acetylcholine (Gilon and Henquin, 2001). These acetylcholine receptors

are important and mice with beta cell specific inactivation of the acetylcholine receptor have glucose intolerance with reduced insulin secretion despite normal insulin content and normal insulin sensitivity (Gautam et al., 2010, Gautam et al., 2006).

1.2.4 Neural Input to Glucose Homeostasis

Beyond the islets themselves and on a larger scale, the nervous system makes a significant contribution to maintenance of euglycemia through vagal innervation of the gut, portal vein and liver. Whilst it is well established that the liver has the ability to convert glucose to glycogen and vice versa, there does appear to be an indirect pathway where both blood glucose levels and liver glucose output is maintained despite the hepatocytes being unable to respond to insulin directly (Lu et al., 2012). For instance, where hepatic insulin receptors have been knocked out there is very little effect on glucose homeostasis (Mittelman et al., 1997). The vessel which carries blood containing nutrients from the gastrointestinal tract to the liver is the hepatic portal vein. This vein is innervated with visceral sensory axons and glucose orientated machinery such as glucose transporters and GLP-1 receptors thereby making it a key area conveying the glucose and nutrient status of a meal (Thorens, 2001). Once digested, nutrients are absorbed into the circulation through the hepatic portal vein. Both hepatic glucose uptake and peripheral glucose uptake increase as a consequence of increased glucose detected within the portal vein. This process occurs through neural mechanisms (Moore et al., 1991). Interventional studies, such as those where the hepatic branch of the vagus nerve is ligated or chemically inactivated demonstrate loss of hepatic contribution to glucose homeostasis (Xie and Lautt, 1996) and serve as important pieces of evidence highlighting the significance of this signaling mechanism.

The parasympathetic nervous system in particular plays a critical role in glucose stimulated insulin release. Electrical activation of the vagus nerve leads to increased insulin secretion and this effect is heightened by elevated glucose concentrations (Kaneto et al., 1975, Berthoud et al., 1980, Lundquist, 1982). This observation in animal studies led to speculation as to whether these findings could be translated into humans in a similar way to the electrical stimulation provided by cardiac pacemaker devices. Additionally, food in the oral cavity is known to trigger metabolic responses even before glucose is detected in the circulation. The insulin response is no exception to this. This is known as the cephalic phase of insulin secretion. Consistently with the studies showing that increased vagal activity improves glucose levels, insulin secretion can be suppressed by vagotomy (Berthoud and Jeanrenaud, 1982, Berthoud et al., 1980, Berthoud et al., 1981, Berthoud and Powley, 1990). Furthermore, when very small doses of glucose are injected via the carotid artery small increases in insulin secretion are seen despite the injected glucose levels being too small to directly change peripheral glucose levels (Guillod-Maximin et al., 2004). This observation supports the idea of additional neural mechanisms in glucose homeostasis. Tracking neuronal activation through C Fos labelling of neurons shows activity at the arcuate nucleus (ARC) and paraventricular nucleus (PVN) within the hypothalamus during this period (Guillod-Maximin et al., 2004). The use of alternative labelling methods, such as retrograde transsynaptic viral labelling studies, illustrate more clearly the synaptic contact exists between the pancreatic parasympathetic nerves and the PVN and arcuate nucleus (Jansen et al., 1997a) hence reinforcing this idea of a nervous connection. In addition to this neural link, the parasympathetic system may also have additional roles in glucose homeostasis. Interestingly, lesioning the ventromedial hypothalamus (VMH) and hence

deactivating the parasympathetic nervous supply to the pancreas, leads to a reduction in beta cell proliferation and mass (Kiba et al., 1996, Kiba, 2004) which suggests that the central contribution to pancreatic activity does not just relate to physiological responses but also to pancreatic structure. There is therefore a distinct possibility that the brain is playing a more important role in glucose homeostasis than was first thought and research into energy balance and the gut lend support to this.

1.3 The brain and glucose homeostasis

1.3.2 Energy Balance

The brain has an important role in the regulation of energy balance particularly in areas relating to the gut and to appetite. Evidence suggests that energy balance and glucose homeostasis may be partly regulated by hormones which control both. Evidence in support of this dates back decades, with studies showing that rodent lesioning of the ventromedial hypothalamus lead to increased appetite and obesity whilst lesions of the lateral hypothalamus (LH) result in starvation (Hervey, 1959). Additionally, tumours within the pituitary gland lead to increased appetite and obesity (Elmquist et al., 1999). Important discoveries in signaling molecules such as leptin, insulin and gut hormones have further paved the way for re-aligning the research mentality towards the significance of peripheral versus central control in the glucose homeostatic debate.

1.3.3 Leptin and the Brain

The discovery of leptin in 1994 was also a pivotal step in embracing the interplay between central and peripheral signals in energy balance. Leptin is an important

signaler of body adiposity. It is able to cross the blood brain barrier and is therefore able to act within the hypothalamus. The hypothalamus contains neurons which express the leptin receptor, LepRb. Through direct actions on this receptor, leptin is able to convey information on energy homeostasis. These receptors are mainly found within the arcuate nucleus, dorsomedial hypothalamus, ventromedial hypothalamus and lateral hypothalamus (Patterson et al., 2011, Scott et al., 2009). Mice with homozygous mutations in the leptin gene (Lep ob/ob) and lean mice, both have reduced food intake and glucose concentrations in response to intracerebroventricular (ICV) leptin injections as well as to peripheral leptin administration (Pelleymounter et al., 1995, Mistry et al., 1997). However, leptin is not only important in fat storage signaling. Leptin also has an effect on glucose homeostasis. Where the leptin receptor is mutated in Lepr db/db mice as well as in Lep ob/ob mice where there is no leptin, severe hyperglycemia is seen (Pelleymounter et al., 1995). Administering leptin to Lep ob/ob mice corrects the glucose intolerance independently of nutritional status (Koch et al., 2014, Kievit et al., 2006, Hill et al., 2008, Hedbacker et al., 2010, German et al., 2010) suggesting that deficient leptin signaling contributes to their diabetic phenotype (Schwartz et al., 1996). Furthermore, severely diabetic mice whose beta cells are destroyed using streptozocin are also leptin deficient. In these mice, administering low doses of leptin corrects their raised blood sugars (Hidaka et al., 2002, German et al., 2011, Lin et al., 2002, Fujikawa et al., 2010). Leptin is therefore an interesting centrally acting signaling molecule. Looking more closely at leptin's relationship with specific nuclei within the brain, in rodent models with no leptin receptor expression whatsoever who then have leptin receptors re-expressed only within the arcuate POMC neurons show normalization of their blood sugar levels whilst having only moderate effects on

energy homeostasis (Berglund et al., 2012). Of particular interest is that the action of leptin in the brain lowers blood glucose but does not cause hypoglycaemia. This interesting and potentially therapeutically significant phenomena has been described by key academic players such as Schwartz as a central ‘resetting’ of glucose to within the normal range through a process that does not require functional beta cells (Deem et al., 2017). The centrally mediated role of leptin is now well accepted and this has been a pivotal academic step in recognising and exploring the potential contribution of the brain to other metabolic functions of the body.

1.3.4 Insulin and the brain

Insulin is another important regulator of blood sugar levels and is not only essential for glucose homeostasis through its direct action in the periphery for example in liver and skeletal muscle, but it also signals to the brain to convey information on energy stores. Insulin acts both centrally and peripherally to regulate glucose homeostasis. In transgenic insulin receptor knock out models, insulin resistance is seen (Bruning et al., 2000b). The arcuate nucleus contains insulin receptors. Specific activation of insulin receptors here results in a reduction of hepatic gluconeogenesis and glycogenolysis independent of food intake (Obici et al., 2002a). It also appears that insulin receptor signaling on anabolic but not catabolic neurons improves glucose homeostasis. For instance, alteration of insulin receptor activity or presence (Konner et al., 2007, Lin et al., 2010) on pro-opiomelanocortin C (POMC) neurons has no effect on hepatic insulin sensitivity whereas insulin receptor alterations on Agouti-related peptide (AgRP) neurons does have an effect on insulin sensitivity (Konner et al., 2007).

Insulin and leptin penetrate the blood brain barrier and bind to their receptors within the brain in notable areas such as the arcuate nucleus (Tartaglia et al., 1995). It is most likely that insulin and leptin work together in conveying signals pertaining to the body's energy stores. Indeed, some POMC neurons express leptin receptors and some express insulin receptors (Williams et al., 2010). Deletion of leptin receptors on POMC neurons results in obesity. However, deletion of both insulin and leptin receptors on the POMC neurons partially protects from this obesity thus suggesting a collaborative approach. Interestingly however, compared to control animals, these leptin and insulin knockout models have worse insulin tolerance and hepatic insulin resistance (Hill et al., 2010).

1.3.5 Fibroblast Growth Factor and the brain

Other key molecules are fibroblast growth factors (FGF) which are a family of peptides believed to have broad mitogenic and cell survival activities. In addition to this FGF is thought to have some antidiabetic effects and administering FGF-19 and 21 peripherally lowers blood glucose levels in rodents with Type 2 diabetes (Fu et al., 2004). Intracerebral injection of FGF-19 and 21 at small doses which would not have significant peripheral effects results in decreased blood glucose levels in leptin deficient ob/ob mice (Morton et al., 2013). Not only this but where FGF-1 is administered centrally, it induces diabetes remission in rodent models of T2DM without resulting in hypoglycaemia in either these models nor in healthy models (Scarlett et al., 2016). This is intriguing as again, similarly to leptin, there is an inference of *'re-setting to a defended level of glycaemia'* as described by Schwartz et al.

1.3.6 Glucagon and the brain

Another hormone and important regulator of glucose homeostasis is glucagon. Glucagon is secreted from the alpha cells of the pancreas when blood glucose levels fall below euglycaemic levels. In hypoglycaemia there is rapid activation of the autonomic nervous system which triggers a counter-regulatory response to bring blood glucose levels back within the normal range. This response is mediated by an increase in glucagon secretion, the release of catecholamines and inhibition of insulin secretion. Both the sympathetic and parasympathetic nervous system activate glucagon secretion by direct alpha cell stimulation (Taborsky et al., 1998, Cryer, 2004). In T2DM there is an elevation of blood glucagon levels (D'Alessio, 2011) which leads to excessive glucose production from the liver. Potential mechanisms for the increase in glucagon production are based on evidence that insulin directly inhibits alpha cells and hence this inhibition is lost in states of reduced insulin production (Gosmanov et al., 2005). Further studies involving the VMN suggest that optogenic activation of this nucleus stimulates glucagon secretion whereas silencing it blunts glucagon's response to hypoglycaemia (Meek et al., 2016). At a receptor level, central GLUT-2 knockout mice have raised glucagon levels (Marty et al., 2005) which suggests central involvement of this receptor. These findings again support a role for the brain acting through the pancreas in coordinating glucose homeostasis.

Whilst the precise mechanism is as yet unclear, what is striking is the evidence in support of a coordinated response by the peripheral organs and tissues with the brain in maintaining glucose homeostasis. Understanding the interaction between

the digestive system and the brain provides further insight into the potential nature of the coordinated mechanism by which glucose may also be controlled.

1.4 Neural connections between the gut and the brain

Appetite and energy balance control are complex. Hypothalamic neurons are influenced by numerous short and long term signals.

1.4.1 The gut and the Central Nervous System (CNS)

Gastrointestinal vagal afferents are activated following a meal by both mechanoreceptors and chemoreceptors. Indeed before food even enters the mouth, there are a myriad of sensory signals which trigger exocrine secretions, endocrine signals and gut motility. Once food is consumed, the gastrointestinal tract transmits nervous signals to the brain. The vagus nerve transmits afferent nervous signals to the dorsal vagal complex in the medulla and to the nucleus of solitary tract (Altschuler et al., 1989, Altschuler et al., 1993, Altschuler et al., 1991). Some afferents end directly on distal dendrites of gastromotor vagal neurons. Further afferents communicate with the dorsal motor vagal nucleus which innervates the entire gastrointestinal tract (Berthoud et al., 1980). The conscious perception of fullness is a consequence of the NTS projection to the visceral sensory thalamus and subsequent communication with the visceral sensory cortex (Schwartz et al., 2000, Grill and Hayes, 2009).

1.4.2 The enteric nervous system (ENS)

In additional to projections to the brain, the gastrointestinal tract has an enteric nervous system which is able to regulate gastrointestinal function independently of the CNS. This is thought to be almost a brain localised only to the gut itself. The enteric nervous system is comprised of two major ganglionic plexi. Firstly, the

myenteric plexus which is located between the circular and longitudinal muscle layers and runs the length of the gut, oesophagus and stomach. Secondly, the submucosal plexus which is located in the submucosa which is between the mucosa and circular muscles. The submucosal plexus predominantly serves the small and large intestine (Furness et al., 2012). This additional independent nervous system regulates epithelial fluid movement, intestinal motility, exocrine secretions and blood supply to the gut (Furness et al., 2012).

1.4.3 The gut and hypothalamic nuclei

The hypothalamus and the brainstem are the main parts of the central nervous system involved in regulating energy homeostasis. They receive information from the periphery which signal both short term and long term nutrient availability. Neural afferents and hormonal signals from the periphery are integrated with other brain signals such as those relating to reward and addiction to regulate appetite and energy expenditure (Schwartz et al., 2000).

The hypothalamus is recognised to be key in coordinating central energy balance with a particular emphasis placed on the arcuate nucleus. The hypothalamus is located at the base of the brain. It lies below the thalamus and above the brain stem and is comprised of multiple nuclei each with varying roles in temperature control, energy and fluid balance as well as glucose homeostasis. The hypothalamus houses the arcuate nucleus which contains two well studied populations of neurons with regards to energy homeostasis. Firstly, the orexigenic neurons which stimulate appetite and express neuropeptide Y (NPY) and AGRP (Broberger et al., 1998, Bewick et al., 2005). Secondly there are anorexigenic neurons which express POMC which gives rise to alpha melanocyte stimulating hormone (alpha MSH) and also

cocaine and amphetamine regulated transcript (CART). The POMC neurons reduce food intake and the AGRP/NPY neurons increase food intake and weight gain. These neurons send signals to neighbouring nuclei as well as signaling to each other. One example of this cross-talk is that NPY-AGRP neurons can suppress the activity of the POMC neurons through gamma-aminobutyric acid (GABA) input (Rau and Hentges, 2017). These nuclei also respond to peripheral signals such as those from insulin and leptin. In fact, insulin and leptin receptors are expressed throughout the hypothalamus (Bruning et al., 2000a, Halaas et al., 1995) as well as being expressed on POMC, NPY-AGRP neurons in the arcuate nucleus and are necessary for the normal regulation of body weight. Injecting insulin or leptin into the third ventricle, which lies next to the arcuate nucleus, in a dose dependent manner suppresses food intake and reduces body weight (Pelleymounter et al., 1995, Seeley et al., 1996, Chavez et al., 1995, Woods et al., 1979). Additionally, the actions of insulin and leptin are partly mediated by increased expression of POMC and reduced expression of AGRP-NPY (Woods et al., 1998). Furthermore, genetic mutations which reduce function of the POMC system lead to obesity (Vaisse et al., 1998).

1.5 Enteroendocrine hormones

Within the gastrointestinal system, food stimulates the intestine to release hormones. Satiety factors such as cholecystokinin (CCK), GLP-1 and Peptide YY (PYY) are released into the circulation and decrease appetite. In contrast, ghrelin, released predominantly from the stomach but also released from the small intestine, stimulates feeding. Many of these peptides predominantly enter the brain through the area postrema which is located above the NTS thereby enabling neurons to respond directly to circulating gut hormones (De Silva and Bloom, 2012, Leibowitz et al., 1981).

There are over 15 different types of enteroendocrine cells lining the gut epithelium (Sjolund et al., 1983). Gut hormones produced by some of these cells contribute to the short term sensations of hunger and satiety (Badman and Flier, 2005). They have various actions such as decreasing food intake by downregulating hypothalamic orexigenic signaling and increasing anorectic signaling (Batterham et al., 2002). Additionally, these peptides also slow gastric emptying thereby prolonging gastric distension and increase satiety through inhibitory feedback mechanisms (Lin et al., 1996). The NTS and area postrema are thought to be the interface through which gut hormones exert their central effects. In studies where the area postrema has been ablated, there is a resultant delay in the action of PYY (Baraboi et al., 2010). Furthermore, the enteroendocrine cells appear to form synapses which directly innervate the brain (Bohorquez et al., 2015) and the gut hormones themselves alter the activity of the ascending pathways between the gut and the brainstem (Jobst et al., 2004). Hence, it is believed that there are two ways in which the enteroendocrine cells convey information to the brain. One is through hormones such as GLP-1, which is a relatively slow process and the other is through neuronal

firing which is much faster (Bohorquez et al., 2015, Spreckley and Murphy, 2015, Kaelberer et al., 2018).

1.5.1 Glucagon Like Peptide-1

GLP-1 is a 30 amino acid peptide produced by cleavage of preproglucagon (Dhanvantari et al., 1996). Preproglucagon is expressed in α cells of the pancreas, L cells of the gut and in the NTS of the brainstem (Larsen et al., 1997, Sandoval and D'Alessio, 2015) and produces a variety of peptides including glucagon, GLP-1 and oxyntomodulin. GLP-1 receptors are found in key nutrient rich areas such as on afferent neurons innervating the hepatic portal vein (Vahl et al., 2007) and enteric neurons innervating the gastrointestinal tract (Amato et al., 2010). Within the portal tract, these afferent neurons are key in the regulation of glucose tolerance (Vahl et al., 2007). GLP-1 is an incretin hormone which stimulates glucose stimulated insulin release (MacDonald et al., 2002). GLP-1 inhibits endogenous glucose production through inhibition of glucagon secretion (Willms et al., 1996). These are the more peripheral effects of GLP-1, yet GLP-1 is known to have many central effects too. Where GLP-1 has been administered into the brains of rodent models, both direct central and peripheral administration resulted in reduced food intake whilst chronic GLP-1 delivery minimised weight gain (Meeran et al., 1999). GLP-1 delays gastric emptying and increases satiety (Meeran et al., 1999, Punjabi et al., 2011). Central areas identified in the control of appetite through the actions of GLP-1 include the arcuate nucleus, paraventricular nucleus and supraoptic nucleus (Shughrue et al., 1996). In humans, the results are similar. Intravenous GLP-1 injection into both lean and obese humans results in decreased food intake and slowing of gastric emptying (Verdich et al., 2001, Naslund et al., 1999, Nauck et al., 1997).

GLP-1 within the CNS also regulates glucose homeostasis. In rodent models, GLP-1 administered directly into the arc (Sandoval et al., 2008) decreases hepatic glucose output and similarly, intracerebral GLP-1 injection also stimulates insulin secretion (Knauf et al., 2005, Sandoval et al., 2008).

The combined appetite and glucose regulatory effects of GLP-1 make it a powerful force in glucose homeostasis. The pathway of its action is believed to be through vagal pathways to the brainstem as in rodents, GLP-1 activates neurons within the brainstem with no activation following vagotomy (Abbott et al., 2005, Imeryuz et al., 1997). In humans, functional magnetic resonance imaging (fMRI) conducted during peripheral administration of GLP-1 show activation within the VMH and PVN thereby lending support to the animal data (Parkinson et al., 2009). These key and beneficial effects have made GLP-1 the focus of much therapeutic research.

1.5.1.1 Glucagon Like Peptide-1 therapies for obesity and type 2 diabetes mellitus

With such powerful appetite and glucoregulatory actions, it is unsurprising that GLP-1 agonists have been at the forefront of therapeutic advancement in treating type 2 diabetes where obesity is also a feature.

1.5.1.1.1 Gila lizard

Originally isolated from the venom of the gila lizard, Exendin-4 is a naturally occurring GLP-1 mimetic (Eng et al., 1992). Interestingly, long before gaining scientific prominence, the gila lizard was believed to be a powerful force by the Native American Navajo tribe. Originating in New Mexico and Arizona, the gila monster (Heloderma suspectum) and the closely related bearded lizard *(*Heloderma horridum*)* produce a poisonous venom which has been shown to contain powerful

constituents (Raufman, 1996). The venom is made up of toxic elements, similar to snake venom, as well as biologically active peptides. Of the biologically active peptides particular interest was devoted to helospectins and helodermin which are members of the glucagon, secretin, vasoactive intestinal peptide family (Raufman et al., 1982). Further analyses of these led to the identification of exendin 3 (Eng et al., 1990) and exendin 4 (Eng et al., 1992) which have paved the way for translation into human therapeutics. Different forms of Exendin-4 (Exendin 9-39) act as a competitive antagonist at the GLP-1 receptor where it acutely increases food intake and chronically increases body weight in rats (Goke et al., 1993, Kolligs et al., 1995)

1.5.1.1.2 Synthetic GLP-1

The synthetic version of Exendin-4 is called Exenatide and was the first GLP-1 analogue to be created and prescribed as diabetes treatment for Type 2 diabetics (Drucker and Nauck, 2006). It has numerous benefits; stimulation of insulin release, suppression of glucagon release, lowering of blood sugar levels, reduction in body weight. Since Exenatide's release many other agents are now on the market which have longer half-lives and therefore reduced dosing which is important as these agents are injectable subcutaneously (Tzefos et al., 2012) and therefore patient tolerability is a factor in treatment. The most recently approved drug for the treatment of diabetes is Semaglutide which shares 94% homology with human GLP-1 and an orally administered GLP-1 agonist is in the pipeline. Native GLP-1 is rapidly inactivated by dipeptidyl peptidase (DPP) which is found in the bloodstream and hence dipeptidyl peptidase IV (DPP-IV) inhibitors have been developed as treatments for T2DM to prolong the activity of GLP-1. These agents reduce blood glucose levels without having a substantial effect on food intake or weight. The

reason for the difference in effect between DPP-IV inhibitors and GLP-1 agonists is not clear but may be due to them blocking the action of DPP-IV on other target hormones.

However, GLP-1 is not the only signal relaying information between the gut and the brain. This area is the focus of much research and consists or endocrine and neural elements.

1.5.2 Cholecystokinin (CCK) and the Gut-Brain-Liver axis

The gut serves as an important interface between input into the body and subsequent responses by the body. In addition to GLP-1, CCK has been extensively studied. GLP-1 and CCK share some similarities. CCK is secreted from the proximal intestine in response to lipid ingestion. Infusion of small amounts of lipids which are too small to trigger any peripheral plasma rise (Wang et al., 2008) or direct infusion of CCK (Cheung et al., 2009) into the upper intestine lead to a reduction in glucose output by the liver thereby implying the presence of a neural link between the gut and the liver, most likely involving channels in the dorsal vagal complex of the hindbrain (Ross et al., 2010), but not simply involving substances travelling through the circulation. Similarly to GLP-1, CCK also promotes satiety in both rats and humans (Lieverse et al., 1994) and this is believed to be through the CCK receptors on vagal afferent axons (Strader and Woods, 2005).

Hence, the gut provides an important model reminding us of the interplay between the central systems and peripheral organs and gives a framework on which to investigate other areas within energy balance and metabolism where this concept may also be relevant.

1.6 The Modified Glucose Homeostatic Model

The research surrounding the cooperative relationship between the gut and the brain has provided a strong case for exploring whether the brain has a role in glucose homeostasis. However, this concept is not new. As far back as the 1800s, this was eluded to when Claude Bernard punctured the floor of the fourth ventricle in the brain rendering a rabbit diabetic. However, the discovery of insulin in 1921 and glucagon shortly after that resulted in diabetes being considered to be predominantly a failure of the pancreas. More recent evidence has resurrected the idea of the brain playing a role in glucose homeostasis and challenges the age-old assumption that there is only one controller of glucose homeostasis - the pancreas (Figure 4).

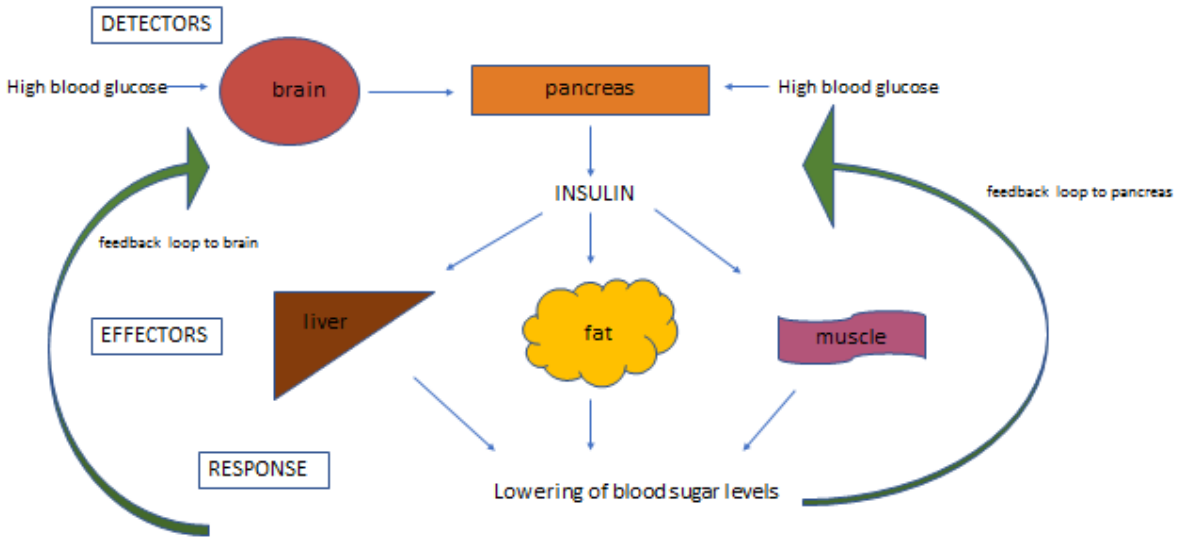


Figure 4: Diagrammatic representation of the brain and the pancreas working in unison to control glucose homeostasis.

1.6.1 The brain and glucose

The pure signaling role of glucose centrally is difficult to demonstrate as glucose also serves as a fuel. It is probable however that glucose may well signal short term fuel availability to the brain. Additionally, glucose is thought to interact with various nuclei within the hypothalamus to alter neuronal activity and therefore signaling (Schwartz et al., 2000). Administration of glucose directly into the arcuate nucleus inhibits total hepatic glucose production by reducing both gluconeogenesis and glycogenolysis (Lam et al., 2005, Obici et al., 2002b). Exposing nuclei such as the NTS, arcuate and VMH to glucose show that they can be can be excited or inhibited ex vivo through increasing levels of glucose (Levin, 2006).

1.6.2 The brain and hypoglycaemia

The most established role of the brain with regards to glucose homeostasis comes from studies involving hypoglycaemia. The difficulty with studies of hypoglycaemia as a measure of glucose deprivation however are that it is a state of stress and hence distinguishing the effect of stress from glucose fuel deprivation is tricky. A subset of neurons expressing leptin and cholecystokinin have been discovered within the lateral parabrachial nucleus which on activation raise blood sugar levels in response to hypoglycaemia (Garfield et al., 2014). These effects are also thought to involve the VMN which has been repeatedly cited as a key player in the counter-regulatory response to hypoglycaemia both through viral interventional studies (Levin et al., 2008) as well as optogentic activation studies (Meek et al., 2016). In order to elicit a response to the stress of hypoglycaemia, neurons are thought to extend from the VMN to the anterior bed of the stria terminalis which integrates the signals for a response. Inhibiting these neurons dampens down the counter-regulatory response to hypoglycaemia (Meek et al., 2016).

1.6.3 Insulin, leptin and the brain

Insulin and leptin are also useful signals in glucose homeostasis. Injection of insulin acutely into the arcuate nucleus suppresses hepatic glucose production by reducing both gluconeogenesis and glycogenolysis (Obici et al., 2002c). Interestingly, injection of leptin into the brain leads to hepatic gluconeogenesis and decreases glycogenolysis with the combination leading to overall hepatic glucose production being the same as in saline controls (Liu et al., 1998). In rodent models central injection of leptin at doses too low to have systemic effects, bring blood glucose levels back to normal despite uncontrolled insulin deficient diabetes in rats (Fujikawa et al., 2010, German et al., 2011). The use of transgenic mice has helped to identify the specific role of glucose related receptors in various nuclei. Knockout of insulin receptors only from AGRP neurons renders insulin unable to suppress hepatic glucose production during hyperinsulinaemic-euglycaemic clamp studies (Konner et al., 2007). Re-activation of some of these insulin receptors then restores normal hepatic glucose production (Konner et al., 2007) which suggests that AGRP neuronal insulin receptors are required to control hepatic glucose production. Insulin receptors on POMC neurons however seem to have a different role. Their activation has no effect on hepatic insulin sensitivity (Konner et al., 2007) whereas insulin receptor re-activation increases energy expenditure and hepatic glucose production. One method of knocking down insulin receptors throughout the arcuate nucleus is via the intraventricular injection of an antisense oligodeoxynucleotide. In these models, there is an increase in output of glucose from the liver (Obici et al., 2002a) through a reduction in the liver's sensitivity to insulin. As the rodents were pair fed, this effect was independent of food intake (Obici et al., 2002a). However, in this model the effect on glucose homeostasis is offset by the rise in insulin secretion

meaning that the ultimate effect on glucose homeostasis was negligible (Jordan et al., 2010, Hill et al., 2010). Similarly, in human conditions of leptin deficiency such as lipodystrophy, blood glucose levels are raised and accompanied by increased hepatic glucose output despite raised insulin levels. The phenotype of this condition suggests that lack of insulin is not the sole pathology and contributions are made from other systems.

1.6.4 The brain and K_{ATP} channel

ATP is a key energy currency within the body and its generation through glucokinase related phosphorylation is important both centrally and peripherally. Potassium ATP (K_{ATP}) channels are found within neurons in various hypothalamic nuclei including the ARC, VNM, NTS and closure of the K_{ATP} channels leads to neuronal depolarisation and activation (Levin et al., 2004). Conversely, opening these channels within the hypothalamus for instance attenuates changes in hepatic glucose production when glucose is administered centrally in rodents (Lam et al., 2005, Obici et al., 2002b). Closure of K_{ATP} channels on POMC neurons disable the neuron from responding to glucose (Parton et al., 2007) whereas K_{ATP} closure within the arcuate nucleus leads to increased hepatic glucose production (Pocai et al., 2005b, Pocai et al., 2005a).

1.6.5 The Autonomic Nervous system

Further signaling occurs via the autonomic nervous system which consists of the sympathetic nervous system and the parasympathetic nervous system. In relation to metabolism and energy balance, the parasympathetic branch is activated post prandially and its actions are mediated predominantly by the vagus nerve. Many studies have demonstrated the importance of the hepatic branch of the vagus for the

ability of insulin and nutrients infused directly into the CNS to suppress hepatic glucose production (Lam et al., 2005, Pocai et al., 2005a, Cheung et al., 2009). The sympathetic branch is activated at times of physiological stress such as during exercise and hypoglycaemia. The major neurotransmitters released are adrenaline and noradrenaline which act on the liver to increase glucose production and on skeletal muscle to decrease glucose uptake in order to maintain glucose levels.

1.6.6 Tanycytes and glucose homeostasis

Aside from neurons, the hypothalamus also houses specialised glial cells called tanycytes. These cells line the third ventricle of the hypothalamus with some projecting to the arcuate nucleus and VMH. Tanycytes bear some similarity to the beta pancreatic cell. Firstly, they express glucose transporters (Peruzzo et al., 2000, Garcia et al., 2003). Secondly, glucose is metabolised to generate ATP which ultimately results in cellular depolarization (Dale, 2011, Frayling et al., 2011). Finally the tanycytes are also believed to have a glucose sensory role, albeit primarily through glucose detection within the cerebrospinal fluid (Rodriguez et al., 2005).

1.7 Theories in the field of central mediated glucose homeostasis

There is convincing evidence in support of the brain playing a role in glucose homeostasis but the nature of this role is yet to be identified. There are some within the field who would argue that the brain's role is critical to basal daily maintenance of euglycemia and others who believe that the pancreas takes responsibility for basal control, whilst the brain defends and protects euglycaemia only when it is significantly threatened by extreme stress. Following on from that argument, one alternative but plausible theory is that Type 2 diabetes may be a gradual and progressive failure of the brain to sense and respond to glycaemia. Hence in an attempt to compensate for this lack of detection, the body gradually shuts down the pancreas in order to deliver more glucose to the brain (Deem et al., 2017). Hence over time, the acceptable glucose set point gets higher and higher. This 'chicken and egg' theory offers an alternative perspective on the traditional glucose dysregulation that is believed to occur in diabetes and may indeed have a part to play even if it does not explain the entire phenomenon. Furthermore, it is also possible that loss of neural input to the beta pancreatic cell causes its subsequent decline in function. Pancreatic islet cells receive a rich nervous supply from sympathetic, parasympathetic, and sensory nerves (Salvioli et al., 2002, Woods and Porte, 1974). Glucose stimulated insulin release is inhibited by increased sympathetic activity whereas increased parasympathetic activity stimulates insulin secretion through muscarinic receptors (Karlsson and Ahren, 1993). Not only does the sympathetic nervous system influence insulin secretion, it is also involved in islet cell development which may itself impact on metabolic impairment (Borden et al., 2013). Further interesting evidence comes from studies into the GLUT-2 transporter. The GLUT-2 transporter is an important component of the glucose sensing machinery

and is expressed in the pancreatic beta cells and the hepatocytes as well as various hypothalamic nuclei. In rodent studies, deletion of central GLUT-2 impairs insulin secretion, reduces beta cell mass and alters the response to hypoglycaemia (Tarussio et al., 2014, Thorens, 2014). However, these deletions were performed in the prenatal period and hence results must be interpreted with caution when extrapolating for type 2 diabetes which typically occurs in mature animals. Even with this in mind, it does not remove the possibility that the signalling from the brain is a contributing factor, if not the major determinant of beta cell dysfunction. Indeed, diabetic Chinese hamsters show islet cell dysfunction with reduced islet cell innervation (Kohnert et al., 1999). Additionally, when cholinergic stimulation is applied to high fat diet mouse models an improvement is seen in both glucose tolerance and insulin secretion (Ahren et al., 1999).

1.8 The brain and bariatric surgery

Further evidence in support of the brain's role in glucose homeostasis is from studies from bariatric surgery. In diabetic rat models undergoing duodenal exclusion, there appears to be correction of blood sugars into the euglycaemic range through activation of a neural circuit that inhibits hepatic glucose production (Breen et al., 2012). This is mechanism is insulin independent. It could be argued that many of the positive effects of bariatric surgery on glucose homeostasis are secondary to weight loss. However, studies into obese rat models undergoing duodenal bypass surgery with or without subdiaphragmatic vagotomy, show that the improvements in glycaemia are independent of weight and do in fact require intact vagal input into the central nervous system (Jiao et al., 2013). This particularly relates to the beneficial effects on hepatic glucose output and that this involves insulin signaling in the VMH (Paranjape et al., 2013).

In clinical practice bariatric surgery can be recommended for individuals who have a BMI >40 kg/m2 or a BMI >35kg/m2 with comorbidities which would benefit from weight loss according to guidelines from the National Institute of Clinical Excellence. However bariatric surgery involves an operation which automatically carries risk from both the surgery and the anaesthetic. Whilst recent figures suggest that current laparoscopic techniques have reduced serious morbidity and mortality to 4% and 0.1-0.3% (Nguyen and Varela, 2017) it is nonetheless an invasive procedure in a population with multiple comorbidities.

Hence, whilst we are still trying to understand how bariatric surgery influences metabolic parameters so impressively, one very plausible explanation is that there is

some involvement of the brain. Hence the brain once again becomes an interesting therapeutic target.

1.10 Targeting the brain in treatments for diabetes

Current therapy for diabetes is largely pancreas centred. In light of recent progression in gut hormone based therapies, bariatric surgery and emerging evidence in glucose homeostasis, re-aligning our minds to consider brain centred diabetes treatments is not unreasonable. Firstly, it gives a greater repertoire of treatments which may help to bring blood sugar levels down which can be a clinical minefield due to co-existing morbidities, weight and lifestyle issues. Although some individuals get good control many do not (Ali et al., 2013). Treatments cause hypoglycaemia which is not only unpleasant for patients, but also dangerous and leads to compliance issues. If indeed the brain's role is to restore to a defended level of glucose then treatments targeting the brain should in theory eliminate hypoglycaemic risk making brain centred therapy infinitely attractive.

From an evolutionary perspective, considering two or more systems to be controlling blood sugar levels gives a sense of ease, as disruption of one system may be compensated for by another. It affords greater protection from harm and a greater chance of survival. From a modern day perspective, it gives increased scope for therapy development. Yet, the brain is a complex structure and unpicking the contribution of each nuclei or indeed each neuron to glucose homeostasis is a complicated task. However, identification of a key glucose sensing enzyme, glucokinase, in both peripheral glucose regulatory organs as well as key metabolic areas within the brain offers some insight into central systems which may be contributing to glucose homeostasis.

1.11 Glucokinase (GK)

Glucokinase is a member of the hexokinase family and has important roles in glucose sensing and glucose disposal. Glucokinase is an enzyme which converts glucose to glucose-6-phospate (Richter et al., 2016). It is unique within the hexokinase family as it is not inhibited by its end product (Cardenas et al., 1979). There are two isoforms of glucokinase, a hepatic form and a neuroendocrine form (Iynedjian, 1993). Hepatic and pancreatic glucokinase are enzymatically identical but utilise two different promoters which each drive the synthesis of GK messenger ribonucleic acid (mRNA) (Jetton et al., 1994b). Hepatic glucokinase has been well studied in rodent models where it is known to be important in glucose disposal and is sensitive to systemic insulin levels (Bosco et al., 2000, Fisher and Kahn, 2003). In insulin deficient rats GK mRNA levels drop markedly and rise again after treatment with insulin (Iynedjian et al., 1988). Pancreatic glucokinase has a different role to that in the liver. Here glucokinase behaves as a glucose sensor, is present within beta islet cells and its activity is glucose dependent (Iynedjian et al., 1989). GK mRNA levels within the islets of Langerhans are not significantly affected by feeding or by fasting (Iynedjian et al., 1989). Knockout models of hepatic glucokinase in only suffer mild hyperglycaemia thus suggesting that hepatic GK is helpful but not critical in glucose homeostasis (Postic et al., 1999b). Interestingly however GK has been identified within the brain and, in particular, within the hypothalamus. The structure and role of glucokinase is summarised in Figure 5.

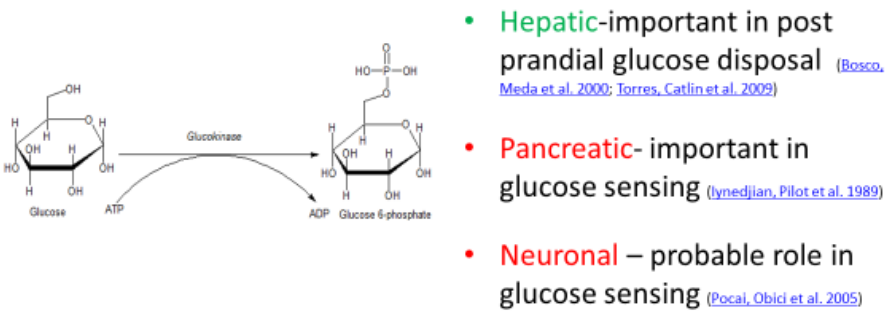


Figure 5: Chemical structure of the enzyme glucokinase and a brief summary of the key sites where it is expressed and its function at these sites

1.11.1 Glucokinase within the hypothalamus

Glucokinase has been identified in various nuclei within the hypothalamus. The most extensively studied is the VMN. Here glucokinase has an important role in the counter-regulatory response to hypoglycaemia (Levin et al., 2008). Within the arcuate nucleus it has a role in feeding which again supports a possibility of a role in glucose homeostasis (Hussain et al., 2015a). The greatest concentration of glucokinase however is found within the paraventricular nucleus but its role here is unclear.

Understanding the role of glucokinase within various nuclei is therefore of great importance in unpicking the role of the brain in glucose control as well as to potentially identify future central therapeutic targets. This forms the basis of my thesis. Two key nuclei which contain GK are the arcuate nucleus and the paraventricular nucleus. These nuclei are known to be important in metabolism and energy balance. This thesis will discuss in detail the role of GK in glucose homeostasis within each of these nuclei in rodent models of health and disease.

Chapter 2

The Role of the Arcuate Nucleus in Glucose Homeostasis

2.1 Introduction

The demand for better understanding of diabetes is growing as the incidence of Type 2 diabetes increases dramatically. According to the charity Diabetes UK, 1 in 11 people are believed to have diabetes and it is attributable to 1 in 6 deaths. These deaths are predominantly from cardiovascular disease but also from liver disease, kidney disease, cancer and infection. Figures from the NHS Health Survey 2016 suggest that well over half the UK population is overweight or obese and in light of this, the burden of Type 2 diabetes is on the rise.

The rise in diabetes has dramatic effects on quality of life as well as an impact on health service spending. From a patient perspective, the multisystem nature of diabetes threatens organs supplied by both large blood vessels such as the heart, brain and legs as well as smaller vessels supplying the eyes, nerves and kidneys. Resulting dysfunction of these systems and the associated disability from amputation, blindness and renal failure impacts on an individual's quality of life and also their economic contribution to society. Life expectancy in a Type 2 diabetic can be reduced by up to 10 years. Unsurprisingly diabetes is therefore a very topical issue amongst politicians, scientists and the public alike with pursuit of treatments and cures gaining momentum. However, a rate limiting step in fully understanding and treating diabetes has been our focus as researchers on diabetes being a pancreas-centred disease process. Whilst this theory holds some truth, new evidence suggests that it is too simplistic. Emerging studies show that the central nervous system is a critical player in the regulation of glucose homeostasis and there is a complex signalling network uniting the brain and other glucose regulatory organs such as the pancreas and liver. This work is in its infancy, entering unchartered scientific territory with many areas still to be explored.

Although a small structure within the brain, the hypothalamus is critical and contains numerous nuclei which communicate with the systemic circulation and with other neighbouring nuclei. Some of these nuclei, such as the ventromedial nucleus, have been studied in detail. However, the role of many of the other hypothalamic nuclei such as the arcuate nucleus have not been studied in as much detail particularly in the context of glucose homeostasis.

2.1.2 The brain and glucose homeostasis

There is accumulating evidence supporting a role for the brain in glucose homeostasis. Acute administration of insulin into the arcuate nucleus of rodents reduces hepatic gluconeogenesis and glycogenolysis (Obici, Feng et al. 2002). Knock-down models lend further support to a central contribution to glucose homeostasis. Insulin receptor knockdown and reactivation models in mice show an effect on hepatic glucose processing. Whilst only a fraction of peripheral insulin crosses the blood brain barrier, knockdown of insulin receptors in the brain leads to loss of suppression of hepatic glucose production (Obici, Feng et al. 2002; Fisher and Kahn 2003), an effect which is mediated through AgRP neurones. The ultimate effect is to reduce hepatic gluconeogenesis (Konner, Janoschek et al. 2007). This supports the idea that the brain is involved, alongside other glucose modulatory organs such as the pancreas and liver in adjusting blood glucose levels. The liver and brain are thought to be linked through nervous connections. However, there are also known to be extensive neuronal connections between the hypothalamus and the pancreas (Rosario, Singh et al. 2016) which supports the idea that similar physiological links may occur here too. The arcuate nucleus lies within the hypothalamus and is the closest nucleus to the blood brain barrier. It has known

connections between itself and the pancreas suggesting that the two structures may be functionally linked.

2.1.3 The arcuate nucleus and glucose homeostasis

Previous work within the laboratory has investigated the role of the arcuate nucleus in the context of food intake (Hussain et al., 2015a). It was shown here that glucokinase activity effects glucose intake. One further unifying observation is that the glucose regulatory enzyme glucokinase has been localised to the arcuate nucleus as well as to other nuclei within the hypothalamus. Interestingly glucokinase is also found in other key glucose modulatory organs namely the liver and pancreas where it has a role in glucose disposal and glucose sensing respectively. The identification of glucokinase within the brain raises questions surrounding its purpose within the arcuate and how it links to peripheral glucose modulatory organs such as the pancreas.

2.1.4 Glucokinase

Glucokinase is a member of the hexokinase family and converts glucose to glucose-6-phospate (Richter et al., 2016). It is unique within the hexokinase family as it is not inhibited by its end product (Cardenas et al., 1979) which means it can continue its role even in the context of escalating glucose levels. There are two isoforms of glucokinase, a hepatic form and a neuroendocrine form (Iynedjian, 1993). Hepatic and pancreatic glucokinase have two different promoters which each drive the synthesis of GK mRNA (Jetton et al., 1994b). Hepatic glucokinase has been well studied in rodent models where it is known to be important in glucose disposal and is

sensitive to systemic insulin levels (Bosco et al., 2000, Fisher and Kahn, 2003). Hence where rats are insulin deficient, glucokinase mRNA levels drop markedly. Insulin treatment causes the mRNA levels to rise again (Iynedjian et al., 1988). Pancreatic glucokinase has a different role to that in the liver. Within the pancreas glucokinase is present within the beta islet cells where it behaves as a glucose sensor. Unlike the hepatic glucokinase which is insulin sensitive, glucokinase activity within the pancreas is glucose dependent (Iynedjian et al., 1989). However, glucokinase mRNA levels within the islets of Langerhans are not significantly affected by feeding or by fasting (Iynedjian et al., 1989). Knockout models suggest that hepatic glucokinase is helpful but not critical in glucose homeostasis as knock out of hepatic glucokinase in mice only results in mild hyperglycaemia (Postic et al., 1999b). In rodent models of Type 2 diabetes, pancreatic and hepatic glucokinase levels decrease with reduced beta cell mass and function and hepatic glucokinase decreases with the reduced insulin levels seen in severe diabetes (Matschinsky, 1990). Interestingly, the brain contains an identical isoform of glucokinase to the pancreas and they are both believed to act in a similar way.

The importance of glucokinase in other glucoregulatory systems and its identification within discrete hypothalamic nuclei, begs the question; what is the function of glucokinase within these discrete hypothalamic nuclei? Indeed, if it is shown to have an important function here, then can it serve as a drug target for future therapeutic intervention? The specific role of glucokinase within the arcuate nucleus and the paraventricular nucleus have not been studied in the context of glucose homeostasis which therefore exposes a gap in our scientific understanding of exactly how glucose is sensed and processed by the body as well as leaving a potential drug target

unexplored. In this chapter I will investigate the role of arcuate nucleus glucokinase in the regulation of glucose homeostasis.

2.2 The role of arcuate nucleus glucokinase in glucose homeostasis

2.2.1 Hypothesis

Glucokinase in the arcuate nucleus has a role in glucose homeostasis.

2.2.2 Aims

1. To use sense recombinant adeno-associated virus (rAAV-sGK) to increase expression of glucokinase or anti-sense recombinant adeno-associated virus (rAAV-asGK) to reduce the expression of glucokinase within the arcuate nucleus of rats.

2. To determine the effect increasing and decreasing expression of glucokinase on glucose homeostasis in response to an insulin tolerance test and glucose tolerance test.

3. To use pharmacological agents to establish whether GK exerts its effects through the K_{ATP} channel.

4. To determine if the effects of arcuate GK are maintained in obese models.

2.3 Methods

2.3.1 Animals

Rats provide a good model for investigation into human disease processes. In particular, the appetite and glucose regulatory systems are thought to be similar although not identical in rats and humans. The rat pancreas does however differ from the human pancreas physiologically and anatomically, but when considering whole organ rather than very specific functions, rats provide a good model of pancreatic response and function (Case 2016). Male Wistar rats (specific pathogen free, Charles River UK Ltd) were individually housed and maintained under a controlled environment (temperature 21-23°C, 12 hour light-dark cycle, lights on at 07:00). They had ad libitum access to standard chow (RM1 diet, Special Diet Services UK Ltd) and water. All animal procedures were approved under the British Home Office Animals (Scientific Procedures) Act 1986 (Project Licence no. 70/7229). Animals were acclimatised to the Imperial College animal holding rooms for 7 days prior to undergoing any procedures. In order to limit bias, animals were block-randomised into either the control group or the study group after being weighed immediately prior surgery. This method of randomisation reduces bias due to uneven distribution of body weight within groups, a factor which is known to affect glucose tolerance. Animals and their food were weighed thrice weekly post operatively for the duration of the study to ensure that differences in food intake and body weight were not variable which would ultimately affect glucose tolerance and insulin sensitivity. Animals were subject to glucose tolerance tests (GTT) at 4 weeks post operatively and insulin tolerance tests (ITT) at 5 weeks post operatively. The recombinant adeno associated vector (rAAV) virus takes 4 weeks to take full expression and after 5 weeks post operation the weights of the animals begin to diverge thereby introducing

weight as a variable which is well known to affect glucose homeostasis. Hence studies were performed at 4 and 5 weeks post operatively to remove this bias. Animals were killed after completion of the study.

2.3.2 Recombinant adeno associated vector production

The rAAV has been used in previous studies within my laboratory and was generated by my supervisor Dr James V Gardiner (Division for Diabetes, Endocrinology and Metabolism). Full length glucokinase complementary deoxyribonucleic acid (cDNA) was isolated and amplified by polymerase chain reaction (PCR) from the plasmid pCMV4.GKB1 encoding full length GK cDNA (gift from Prof. Magnuson, Nashville). In order to construct the GK sense (sGK) and antisense (asGK), GK DNA underwent cloning in the forward (GKS) and reverse (GKAS) orientation into the plasmid pTR-CGW (gift from Dr. Verhaggen, Amsterdam).

2.3.4 GK expression modulation via bilateral intra arcuate rAAV injection using a stereotaxic technique

0.5 microlitres of rAAV-asGK (titre: $3.42x10^{12}$ genome particles/ml) or rAAV-sGK (titre: $2.96x10^{12}$ genome particles/ml) was bilaterally injected into ARC of male Wistar rats using a stereotactic frame, coordinates determined by the Paxinos Watson criteria. This volume of virus is known to cause no additional swelling in the brain and does not produce any additional viral particles once injected (Hussain et al., 2015b). The control used was rAAV encoding green fluorescent protein (GFP). GFP (titre: $5.04x10^{12}$ genome particles/ml) is often used as an indicator of successful gene transfer and has been shown to be effective in mammalian cells (Zhang, Gurtu et al. 1996).

2.3.5 Intra-arcuate administration of pharmacological agents

A permanent stainless steel cannula was inserted unilaterally into the rat arcuate nucleus using the same stereotactic technique described above. The cannula was secured into place using screws and dental cement. Cannulas were capped. Animals were then fasted overnight and one of the following pharmacological agents was injected the following morning in a volume of 0.5 microliter;

- saline, 0.5nmol
- Compound A (2-Amino-5-(4-methyl-4H-(1,2,4)-triazole-3-yl-sulfanyl)-N-(4-methyl-thiazole-2-yl)benzamide) CAS 603108-44-7 (Merck-Millipore, Beeston, UK) which is a pharmacological glucokinase activator
- 1nmol diazoxide (a K_{ATP} activator)
- 2nmol of glibenclamide (a K_{ATP} blocker).

The doses used were identical to those used in a previous study within the laboratory. Animals underwent an oral glucose tolerance test (OGTT) thirty minutes after the injection as described below. The experiment was a cross-over design with each animal receiving each injection performed in a random order at least three days apart thereby ensuring that the minimal number of animals were used to power the study.

At the end of study, 1µl of India ink was injected through the cannula and the brains were dissected out. Cresyl violet staining was performed on brain slices to determine the accuracy of the ARC cannulation.

2.3.6 Oral glucose tolerance tests

All rats underwent an OGTT 4 weeks following arcuate surgery to determine glucose tolerance. Animals were fasted overnight prior to the study. A 24 gauge/19mm cannula was inserted into the tail vein. The baseline blood sample (approximately 250µl) was collected at 0 minutes. A standard accepted dose of 2.5g/kg of glucose (20% dextrose diluted in GDW) for rat glucose tolerance tests was then administered orally to each animal. Following glucose consumption, blood was taken at 15, 30, 60 and 120 minutes as per usual rodent and human glucose tolerance tests. These time points cover the first and second phase insulin responses and by 120 minutes glucose levels in healthy rodents should return to baseline. Plasma was separated from blood cells by centrifugation at 13,000xg for 5 minutes at 4˚C and was stored at -80°C.

2.3.7 Insulin tolerance tests

All rats underwent an insulin tolerance test 5 weeks following surgery injecting rAAV into the arcuate nucleus to determine insulin sensitivity. A 24 gauge/19mm cannula was inserted into the tail vein. The baseline blood sample (approximately 250µl) was collected at 0 minutes. 2 units/kg of insulin was injected intraperitoneally. Blood was taken at 15, 30, 60 and 120 minutes. Plasma was separated from blood cells by centrifugation at 13,000xg for 5 minutes at 4˚C and was stored at -80°C.

2.3.8 Collection of tissue samples

Animals were killed and brains dissected.

2.3.9 Glucokinase activity assay in isolated ARC, VMN and PVN samples

200 µl ice-cold buffer:

- 0.0107 M MgCl2
- 5 mM sodium EDTA
- 0.15 M KCl
- 0.07% w/v 2-mercaptoethanol

1 ml reaction reagent:

- 100 mM GlyGly
- 45 µM 5-thio-d-glucose-6-phosphate
- 1 M MgCl2,
- 0.5 mM 3-O-methyl-N-acetylglucosamine
- 200 mM ATP
- 12.5 mM NADP
- 2 M glucose
- 0.4 units glucose-6-phosphate dehydrogenase (type IX, from Baker's yeast).

In order to assess the change in GK activity produced by the stereotactic injection of rAAV-sGK or rAAV-asGK, a GK activity assay was performed. Sections 880-µm thick were cut from frozen brains using a freezing Shandon sled microtome and mounted on slides. Slides were kept on dry ice. Arcuate nucleus, VMN, and PVN were removed using a 22-gauge neuro punch (Fine Science Tools). Nuclei were homogenized in 200 µl ice-cold buffer containing 0.0107 M MgCl2, 5 mM sodium EDTA, 0.15 M KCl, and 0.07% w/v 2-mercaptoethanol. The homogenate was centrifuged at 16,000 g for 40 minutes at 4°C. Glucokinase activity was determined spectrophotometrically using an NADP+-coupled assay with glucose-6-phosphate

dehydrogenase. Twenty microliters of homogenate was added to 1 ml reaction reagent containing 100 mM GlyGly, 45 μM 5-thio-d-glucose-6-phosphate, 1 M MgCl2, 0.5 mM 3-O-methyl-N-acetylglucosamine, 200 mM ATP, 12.5 mM NADP, 2 M glucose, and 0.4 units glucose-6-phosphate dehydrogenase (type IX, from Baker's yeast). Each reaction was undertaken in triplicate. The reaction was incubated at 37°C for 1 hour. Absorbance at 340 nm was measured and compared with a standard curve for glucokinase. Sample protein concentration was determined by BCA protein assay (Pierce).

2.3.10 Measurement of glucose in plasma samples using a glucose oxidase assay

A glucose oxidase assay (Randox, Crumlin, UK) was used to measure the plasma glucose levels using the manufacturer's protocol. The change in colour intensity of the reaction product at 570 nm or fluorescence intensity at λex/em = 530/585 nm is directly proportional to glucose oxidase activity in the sample. Plasma rather than capillary glucose was measured as previous studies within my laboratory have shown results to be significantly different between glucometer readings and laboratory samples.

2.3.11 Measurement of insulin in plasma samples using enzyme linked immunosorbent assay (ELISA)

Plasma insulin levels were analysed using Ultra-sensitive rat Insulin ELISA Kit from Crystal Chem.

2.3.12 Measurement of active glucagon-like peptide 1 (GLP-1) in plasma samples using ELISA

Plasma active GLP-1 level was measured using Glucagon-like Peptide-1 (Active) ELISA from Millipore following the manufacturer's instructions.

2.3.13 Immunohistochemistry

Four weeks after intra-arcuate injection of rAAV, the rats were killed with pentobarbitone. Brains were fixed by transcardial perfusion with phosphate-buffered saline (PBS) then 4% phosphate-buffered formaldehyde. Brains were removed, equilibrated in 20% sucrose prior to snap freezing in liquid nitrogen and stored at –80°C until use. Twenty micrometer coronal sections were cut on a freezing sled microtome (Shandon, Runcorn, UK) and mounted on poly-lysine slides. A standard dual immunohistochemistry protocol was performed using the following antibodies mouse anti-GFP antibody (1:500, ab38689) with rabbit anti-PGP9.5 antibody (1:200, ab27053) or mouse anti-GFP antibody with rabbit anti-GFAP antibody (1:500, ab33922). The secondary antibodies donkey antirabbit IgG Alexa Fluor® 647 (1:200, ab150075) and goat anti-mouse IgG Alexa Fluor® 488 (1:500,ab150113) diluted in TBS. These antibodies were chosen as they had no cross reaction with each other and no cross reaction with the sample serum. Dilutions were established using a previous protocol within the laboratory for immunohistochemistry for GK. Cover slips were a fixed using flouroshield mounting medium with DAPI (ab104139). Slides were examined with a Zeiss Axiovert 100 deconvoluting microscope, using an EC Plan-Neofluar 40x/0.75 objective.

2.3.14 Western Blot analysis

The arcuate nucleus of male Wistar rats injected with rAAV were collected by punch biopsy and were lysed in RIPA buffer containing protease inhibitor cocktail tablets (Roche) and centrifuged at 15000g for 15 min to pellet cell debris. Protein concentration was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific) according to manufacturer's instructions. Twenty-eight micrograms of protein per sample were separated by SDS-PAGE under reducing conditions and transferred onto a polyvinylidene fluoride membrane (0.45 µm, Amersham). The membrane was blocked with 5% milk in PBS-T0.1 (Phosphate Buffer Saline, Tween 0.1%) for 1h and incubated overnight at 4˚C with the primary antibodies anti-GLUT2 (Rabbit, dilution 1/2000) generous gifts from Bernard Thorens, Lausanne or anti-Kir6.2 (Mouse, dilution 1/500)2. The expression of α-tubulin (dilution 1/20000; T5168, Sigma) was used as a loading control. After washing, the membrane was incubated for 1h in horseradish peroxidase coupled secondary antibodies (Goat Anti-Rabbit-HRP, Abcam, AB6721, dilution 1/8000) and the signal was detected using Amersham Hyperfilm ECL, after exposure to Amersham ECL WB Detection Reagent. Band intensity was measured using Image J.

2.3.15 Statistical Analysis

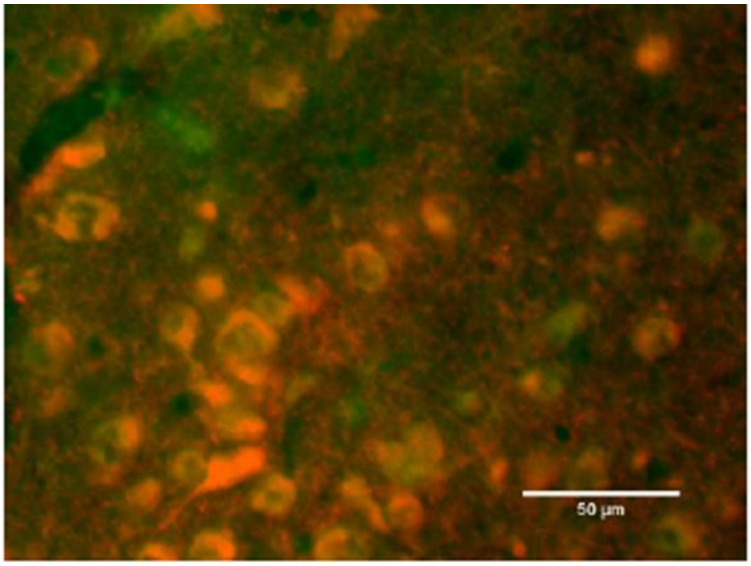
All data are shown as mean ± SEM. Analysis was by either one-way or two-way analysis of variance (ANOVA; as appropriate) with a post hoc Holm–Sidak test or *t* test (GraphPad Prism 8.0). Significance was set at $P < 0.01$ for all analyses.

2.4 Results

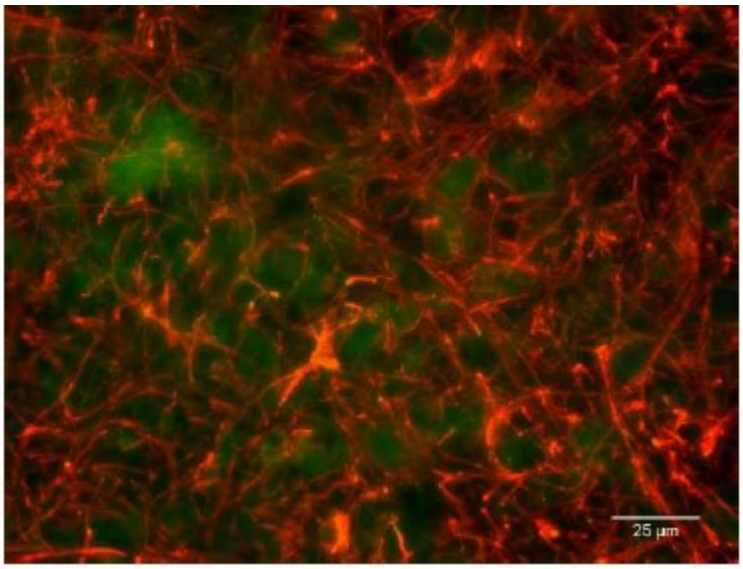
2.4.1 Neuronal specificity of technique

To determine the validity of any subsequent findings, it was crucial to establish the specificity of rAAV injection to neurones and not to other nerve bodies which themselves make different contributions to glucose sensing (Dale 2011, Lanfray et al 2013). Immunohistochemistry was used to see if the rAAV localises purely to neurones. Brains were sectioned and stained brains via dual immunohistochemistry for green fluorescent protein (GFP) and protein gene product 9.5 (PGP9.5) which is a neuron specific protein and confirmed localisation of GFP to neurons (Figure 6a). Dual immunohistochemistry for GFP and glial fibrillary acidic protein (GFAP), an astrocyte specific protein did not detect any co-localisation (Figure 6b) hence establishing that the technique of intra-arcuate rAAV injection to alter glucokinase expression is specific to neurones.

Figure 6: Localisation of GFP using immunohistochemistry following bilateral rAAV injection into the arcuate nucleus



a: Detection of both GFP (green) and neurone specific PGP9.5 (red) following injection of rAAV encoding GFP into the arcuate nucleus using immunohistochemistry which shows clear co-expression of GFP and PGP9.5.



b. Detection of both GFP (green) and GFAP (red) following injection of rAAV encoding GFP into the arcuate nucleus using immunohistochemistry. There is clear separation of the GFP and GFAP with no overlap of expression.

2.4.2 Arcuate Study 1: Establishing the effects on glucose homeostasis of chronically increasing glucokinase activity using rAAV injection into the arcuate nucleus

2.4.2.1 Summary method

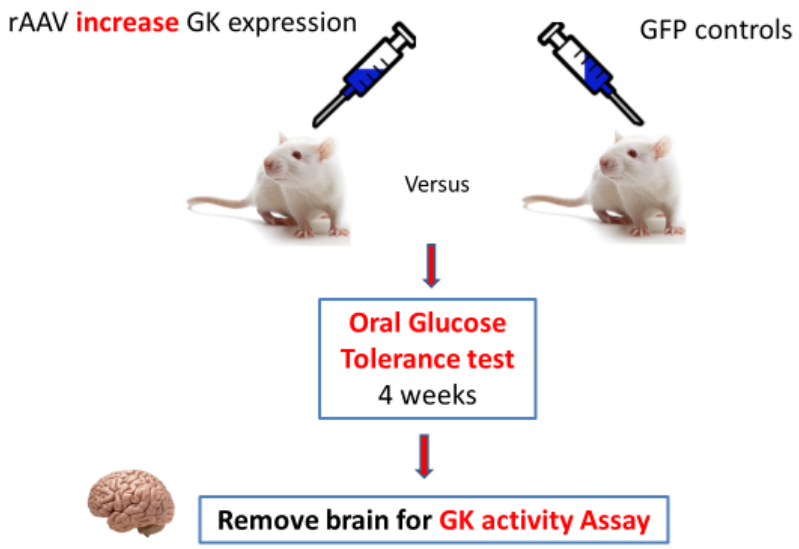


Figure 7: 0.5 microlitres of rAAV-sGK (titre: $2.96x10^{12}$ genome particles/ml) was bilaterally injected into ARC of male Wistar rats. GFP was used as a control. All rats underwent an OGTT at 4 weeks following arcuate surgery to determine glucose tolerance and an ITT at 5 weeks to determine insulin sensitivity.

The effects of increased glucokinase expression was investigated by virally increasing glucokinase activity in the arcuate nucleus through stereotactic intra-arcuate injection of recombinant AAV expressing full length glucokinase (iARC-sGK). A control group were injected with rAAV expressing GFP (iARC-GFP) to investigate the role of arcuate nucleus glucokinase activity on glucose homeostasis. This method is summarised in Figure 7.

2.4.2.2 Determining accuracy of arcuate injections

To ensure accuracy, brains were extracted after the study was complete. All brains underwent punch biopsy and GK activity was measured by enzymatic activity. Glucokinase activity was increased by 2 fold specifically in the arcuate nucleus of iARC-sGK compared to controls. Glucokinase activity in neighbouring nuclei (VMN and PVN) was unaffected (Figure 8) thereby confirming that GK activity was increased specifically in the arcuate nucleus.

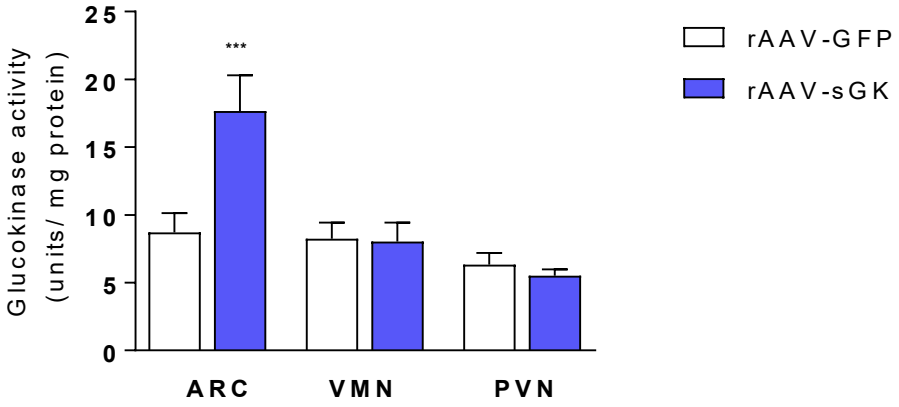


Figure 8: Effect of injection of rAAV encoding either sense glucokinase or GFP into the arcuate nucleus on glucokinase activity in hypothalamic nuclei. Groups of adult male Wistar rats (n=10) were injected with 1x 10^{12} genome particles of either rAAV-GFP or rAAV-sGK bilaterally into the arcuate nucleus. They were culled and the specified hypothalamic nuclei isolated by punch biopsy. Glucokinase activity was measured by enzymatic assay and expressed as units per mg protein. Results are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.

2.4.2.3 Weights of controls versus iARC-sGK

There was no significant difference between the weights of the iARC-sGK and controls during the study period (Figure 9).

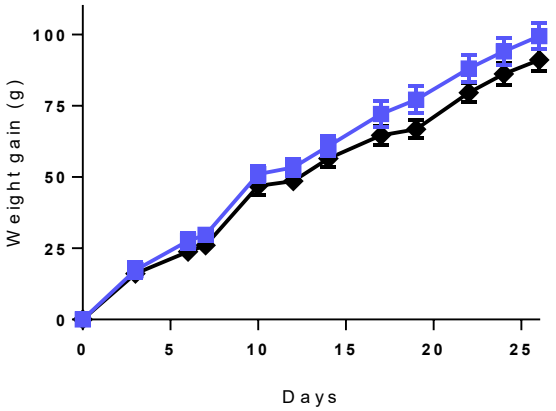
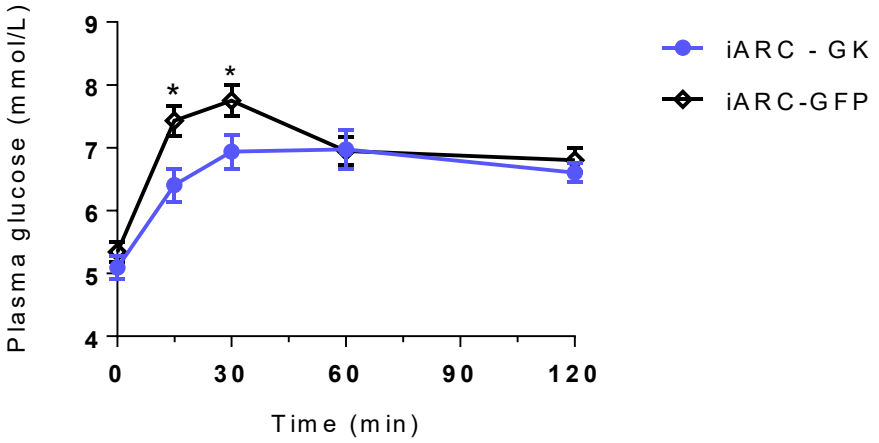


Figure 9: Body weight in male Wistar rats following intra-arcuate injection of either rAAV-GFP (black) or rAAV-sGK (purple). Results analysed by GEE.

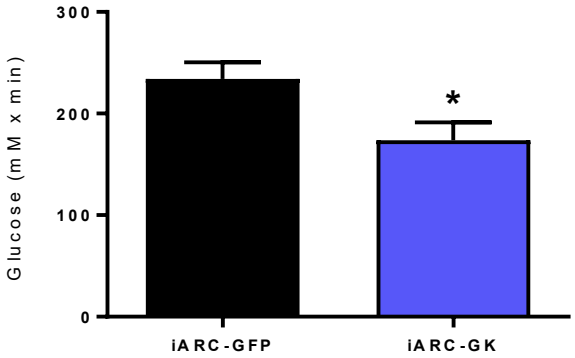
2.4.2.4 Dynamic glucose testing in iARC-sGK

During an OGTT iARC-sGK rats demonstrated 14% lower glucose excursion than control iARC-GFP rats 15 minutes after ingestion of glucose (7.43±0.23 mmol/L iARC-GFP vs 6.4±0.27 mmol/L iARC-GK) and 11% lower at 30 minutes (7.75±0.24 mmol/L iARC-GFP vs 6.93±0.27mmol/L iARC-GK) (Figure 10a and 10b). This suggests that increasing arcuate glucokinase expression improves glucose levels. Theoretically, glucose levels can only improve through increased insulin secretion or increased insulin sensitivity and hence both possibilities were investigated.

Figure 10: Effect of injection of rAAV encoding either glucokinase or GFP into the arcuate on glucose tolerance. Groups of adult male Wistar rats (n=10) were injected with 1x 10^{12} genome particles of either rAAV-GFP or rAAV-GK bilaterally into the arcuate nucleus. Four weeks later rats were subject to a GTT.



a. Results are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.

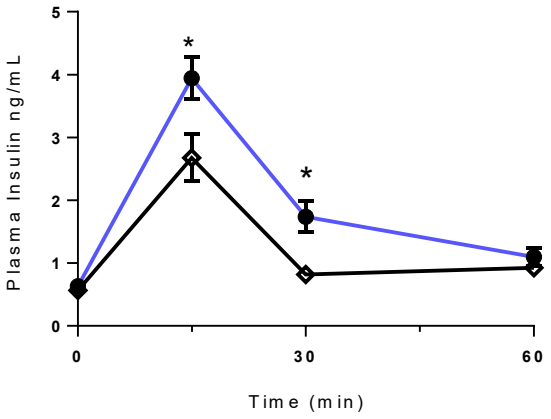


b. Glucose levels shown as area under the curve by t-Test * =p<0.01

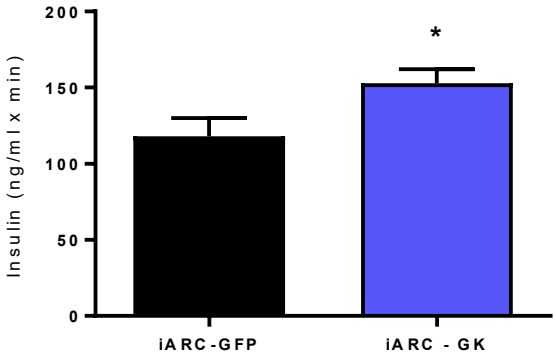
2.4.2.5 Dynamic insulin testing in iARC-sGK

Measurement of insulin levels showed that insulin levels were higher in the iARC-sGK group compared to the iARC-GFP group during the OGTT. Insulin levels were approximately 1.5 times higher at 15 minutes (2.67±0.44ng/ml iARC-GFP vs 3.94±0.31 ng/ml iARC-sGK) and 2 fold higher at 30minutes (0.81±0.19ng/ml iARC-GFP vs 1.74±0.34 ng/ml) iARC-sGK (Figure 11a and 11b).

Figure 11: Effect of injection of rAAV encoding either sense glucokinase or GFP into the arcuate on insulin release. Groups of adult male Wistar rats (n=10) were injected with 1x 10^{12} genome particles of either rAAV-GFP or rAAV-sGK bilaterally into the arcuate nucleus. Four weeks later they were subject to a GTT.



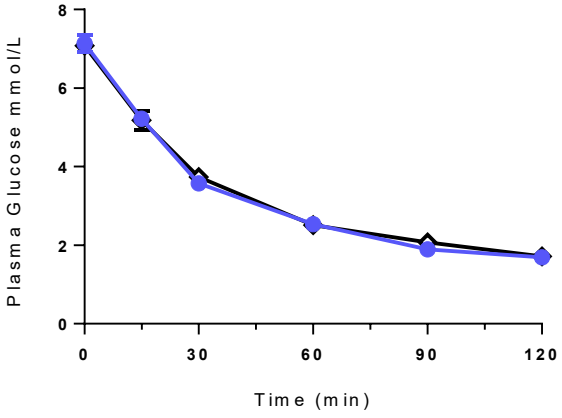
a. Insulin levels are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.



b. Insulin levels shown as area under the curve by t-Test * =p<0.01

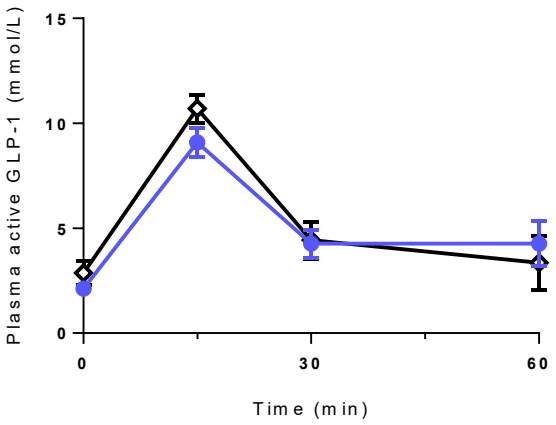
Insulin sensitivity on the other hand, measured during an ITT (Figure 12) was unchanged, as were fasting glucose and insulin levels (Figures 10a and 11a). This suggests that that increased glucokinase activity within the arcuate nucleus improves blood glucose levels by increasing insulin secretion but not insulin sensitivity.

Figure 12: Effect of injection of rAAV encoding either sense glucokinase or GFP into the arcuate on insulin tolerance. Groups of adult male Wistar rats (n=10) were injected with 1x 10^{12} genome particles of either rAAV-GFP or rAAV-sGK bilaterally into the arcuate nucleus. Five weeks later they were subject to an ITT. Results are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.



It is known that the incretin effect contributes to glucose stimulated insulin secretion and hence active GLP-1 levels were measured. These were the same in iARC-GK animals as iARC-GFP animals during the OGTT (Figure 13) hence suggesting that there is no contribution from the incretin effect.

Figure 13: Effect of injection of rAAV encoding either sense glucokinase or GFP into the arcuate on acute GLP-1 levels during a glucose tolerance. Groups of adult male Wistar rats (n=10) were injected with 1x 10^{12} genome particles of either rAAV-GFP or rAAV-sGK bilaterally into the arcuate nucleus. Four weeks later they were subject to a GTT. Results are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.



2.4.3 Arcuate Study 2: Establishing the effects of acute pharmacological manipulation of K_{ATP} channels within the arcuate nucleus

2.4.3.1 Summary method

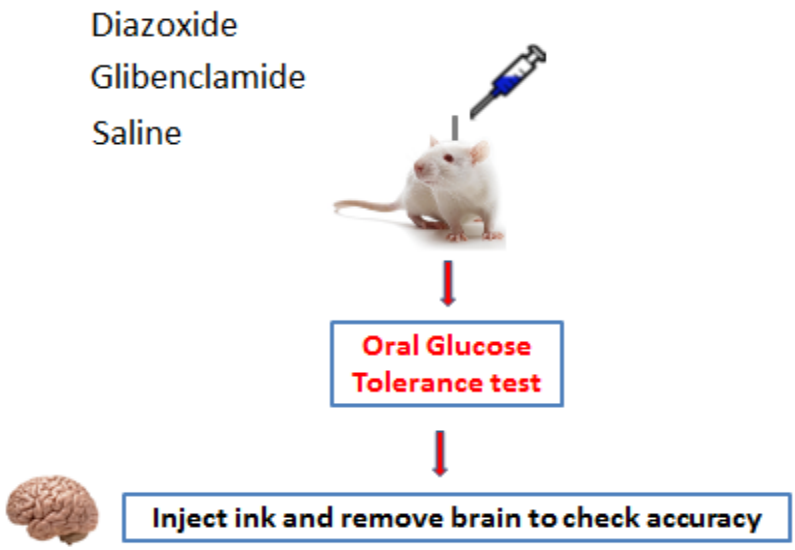


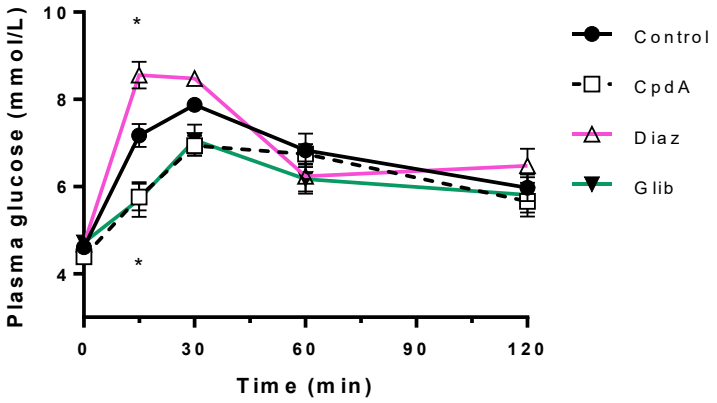
Figure 14: Adult male Wistar rats were injected into the arcuate nucleus through a stainless steel cannula with either saline vehicle (control) or 0.5nmol of the glucokinase activator compound A (CpdA), 2nmol Glibenclamide or 1nmol Diazoxide. The rats then underwent an oral glucose tolerance test to measure plasma glucose and insulin levels.

Evidence suggests that the mechanism through which glucokinase exerts its effects is via the K_{ATP} channel to trigger insulin release (Henquin 2000). In order to establish whether this is true of neuronal glucokinase, glucose homeostasis was assessed during OGTT following intra-arcuate administration of Compound A (a glucokinase activator) glibenclamide (K_{ATP} blocker) or diazoxide (K_{ATP} activator) in healthy Wistar rats (who had not undergone rAAV surgery). This method is summarised in Figure 14.

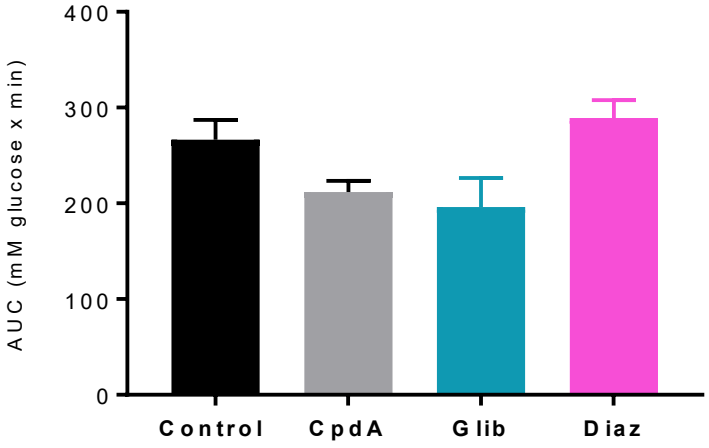
2.4.3.2 Dynamic glucose and insulin testing in iARC cannulated animals

As expected Cpd A reflected the effect of virally increasing GK activity within the arcuate nucleus and led to significantly lower glucose levels at 15 minutes (7.17±0.27 mmol/L iARC-vehicle vs 5.76±0.31mmol/L iARC-CpdA) $p<0.01$ (Figure 15) and increased insulin levels at 15 minutes (2.63±0.17ng/mL iARC-vehicle vs 3.40±0.10ng/mL iARC-CpdA) $p<0.001$ (Figure 16). Glibenclamide is a sulphonylurea which is frequently prescribed as a treatment of Type 2 diabetes. It acts by closure of the K_{ATP} channel leading to increased insulin release and mimicking increased GK activity. Glibenclamide significantly reduced glucose levels at 15 minutes (7.17 ±0.27 mmol/L iARC-vehicle vs 5.70±0.40mmol/L iARC-Glib) $p<0.01$ (Figure 15) and significantly increased insulin secretion at 15 minutes (2.63±0.17 ng/mL iARC-vehicle vs 3.60±0.11 ng/mL iARC-Glib) $p<0.001$ (Figure 16). Diazoxide acts by opening the KATP channel. Diazoxide administration into the arcuate nucleus significantly increased glucose levels at 15 minutes (7.17±0.27 mmol/L iARC-vehicle vs 8.55±0.31mmol/L iARC-Diaz) $p<0.01$(Figure 15) and significantly reduced insulin secretion at 15 minutes (2.63±0.17 ng/mL iARC-vehicle vs 1.91±0.15 ng/mL iARC-Diaz) $p<0.001$ (Figure 16). As with virally increased GK activity, insulin sensitivity during an ITT was unaltered by CpdA injection into the arcuate nucleus.

Figure 15: Effect of pharmacologically increased arcuate glucokinase activity and manipulating KATP activity on glucose homeostasis in Wistar rats. Adult male Wistar rats were injected into the arcuate nucleus with either vehicle (control) or 0.5nmol of the glucokinase activator compound A (CpdA), 2nmol Glibenclamide or 1nmol Diazoxide

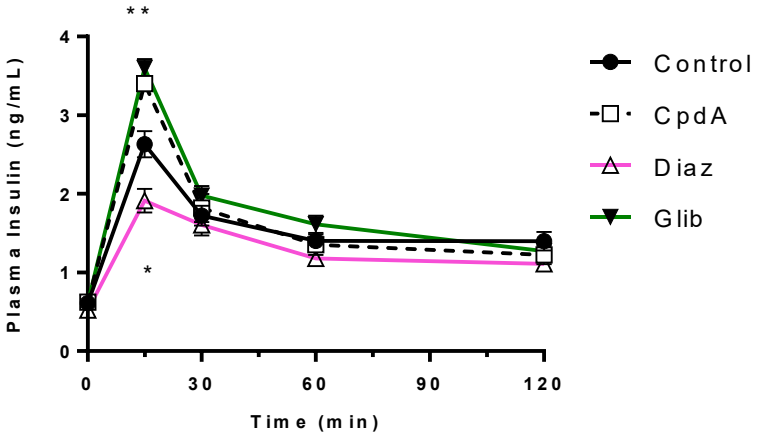


a. Plasma glucose during an oral glucose tolerance test in control (black circle black line); CpdA injected (white square dotted line); Glibenclamide (black triangle green line) and Diazoxide injected (white triangle magenta line) rats. Results are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.

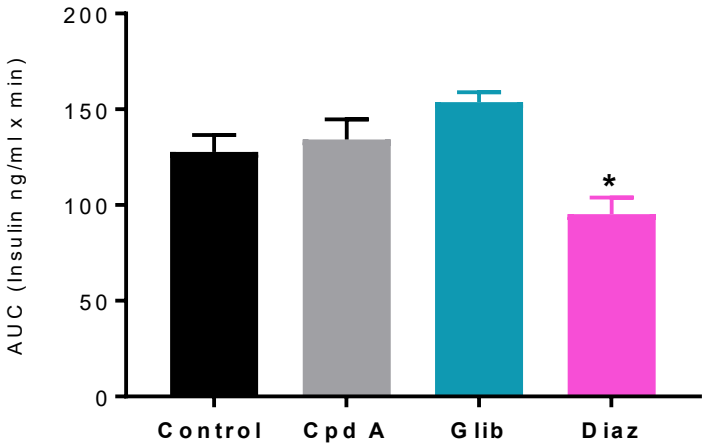


b. Plasma glucose during an oral glucose tolerance test in control, CpdA, Glibenclamide and Diazoxide injected in rats. Results shown as area under the curve versus control * p<0.01 by ANOVA and post-hoc Holm-Sidak test

Figure 16: Effect of pharmacologically increased arcuate glucokinase activity and manipulating KATP activity on insulin levels in Wistar rats. Adult male Wistar rats were injected into the arcuate nucleus with either vehicle (control) or 0.5nmol of the glucokinase activator compound A (CpdA), 2nmol Glibenclamide or 1nmol Diazoxide



a. Plasma insulin during an oral glucose tolerance test in control (black circle black line); CpdA injected (white square dotted line); Glibenclamide (black triangle green line) and Diazoxide injected (white triangle magenta line) rats. Results are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.



b. Plasma insulin during an oral glucose tolerance test in control, CpdA, Glibenclamide and Diazoxide injected in rats. Results shown as area under the curve versus control * p<0.01 by ANOVA and post-hoc Holm-Sidak test

2.4.4 Arcuate Study 3: Chronically decreased glucokinase activity within the arcuate nucleus worsens glucose tolerance

2.4.4.1 Summary method

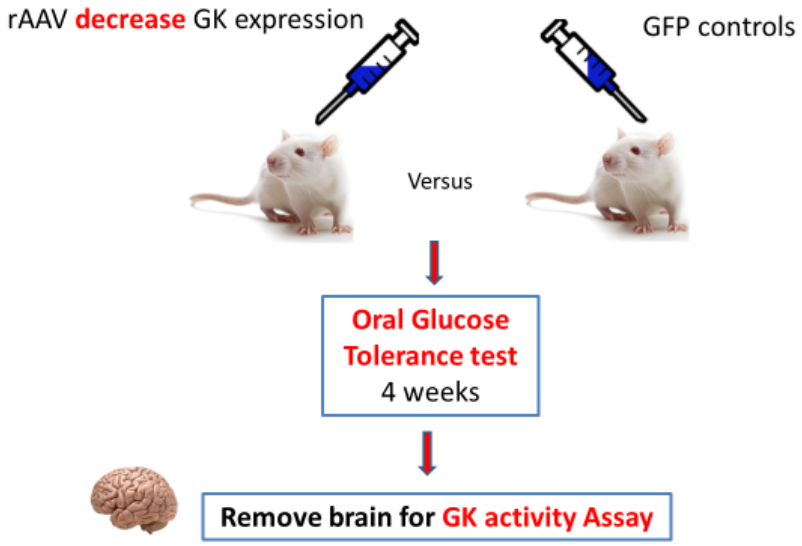


Figure 17: 0.5 microlitres of rAAV-asGK (titre: 2.96 x10^{12} genome particles/ml) was bilaterally injected into ARC of male Wistar rats. GFP was used as a control. All rats underwent an OGTT at 4 weeks following arcuate surgery to determine glucose tolerance and an ITT at 5 weeks to determine insulin sensitivity.

It could be argued that overexpression of GK in the arcuate was studying a pharmacological effect. Therefore studies were repeated using the anti-sense strand of glucokinase to downregulate expression of glucokinase within neurones to establish if this was a physiological effect. A summary of this method is shown in Figure 17.

2.4.4.2 Determining accuracy of arcuate injections

The effect of decreasing arcuate glucokinase activity on glucose homeostasis was studied by stereotactic injection of rAAV encoding anti-sense glucokinase into the arcuate nucleus (iARC-asGK) again rAAV-GFP was injected as a control (iARC-GFP). The anti-sense glucokinase construct has previously been shown to reduce glucokinase activity in vivo (Hussain et al., 2015a).

Glucokinase activity was decreased by 2 fold specifically in the arcuate nucleus of iARC-asGK versus controls (Figure 18). Glucokinase activity in other nuclei was unaffected (Figure 18).

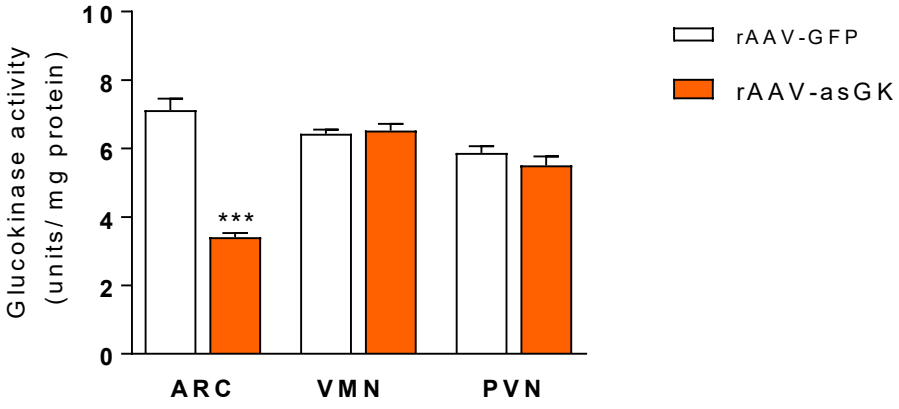


Figure 18: Effect of injection of rAAV encoding either anti-sense glucokinase or GFP into the arcuate nucleus on glucokinase activity in hypothalmic nuclei. Groups of adult male Wistar rats (n=12) were injected with 1x 10^{12} genome particles of either rAAV-GFP or rAAV-asGK bilaterally into the arcuate nucleus. They were culled and the specified hypothalamic nuclei isolated by punch biopsy. Glucokinase activity was measured by enzymatic assay and expressed as units per mg protein. Results are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test

2.4.4.3 Weights of control rats versus iARC-asGK

There was no significant difference between the weights of the iARC-asGK and controls during the study period (Figure 19).

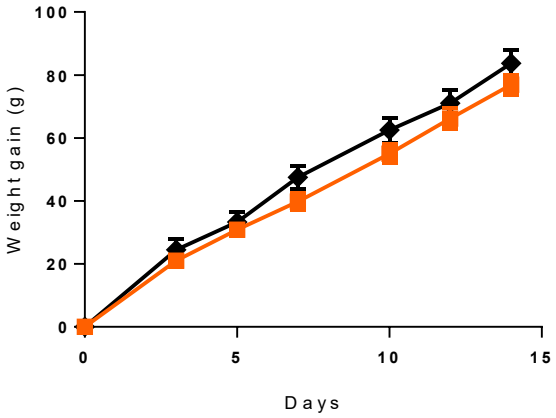
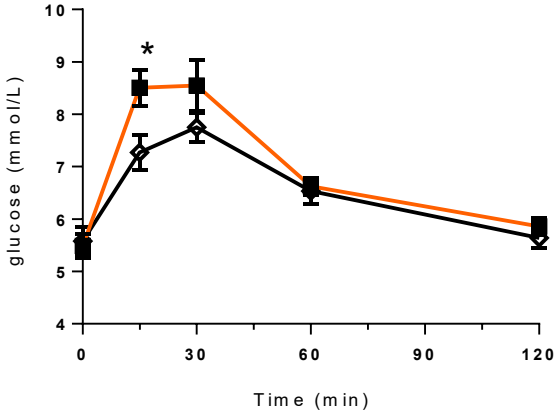


Figure 19: Body weight in male Wistar rats following intra-arcuate injection of either rAAV-GFP (black) or rAAV-asGK (orange). Results analysed by GEE.

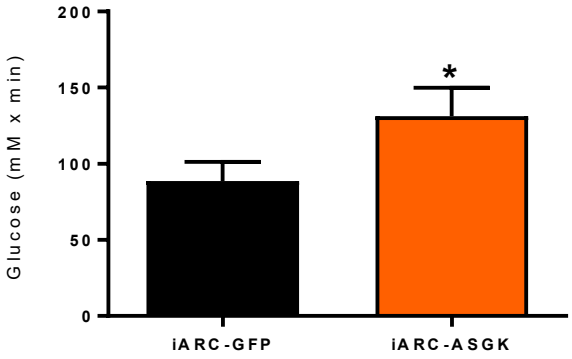
2.4.4.4 Dynamic glucose testing in iARC-asGK

Post operatively and as expected, iARC-asGK rats demonstrated higher glucose excursions compared with control iARC-GFP rats 15 minutes after glucose ingestion with glucose levels being 17% higher than controls (7.27±0.34mmol/L iARC-GFP vs 8.5±0.34mmol/L iARC-asGK) (Figure 20). Given the results of the overexpression study, it seemed likely that the increase in glucose levels was a consequence of reduced insulin secretion and indeed this was shown to be true. Insulin levels were 20% lower in the asGK group than controls at 15 minutes (3.63±0.12ng/ml iARC-GFP vs 2.89±0.20 ng/ml iARC-asGK) and area under the curve was decreased (Figure 21) thereby suggesting that total insulin secretion was reduced.

Figure 20: Effect of injection of rAAV encoding either anti-sense glucokinase or GFP into the arcuate on glucose. Groups of adult male Wistar rats (n=10) were injected with 1x 10^{12} genome particles of either rAAV-GFP or rAAV-asGK bilaterally into the arcuate nucleus. Four weeks later they were subject to a GTT.

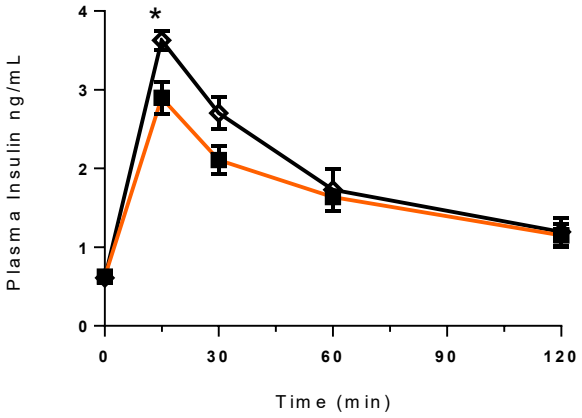


a. Plasma glucose levels are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.

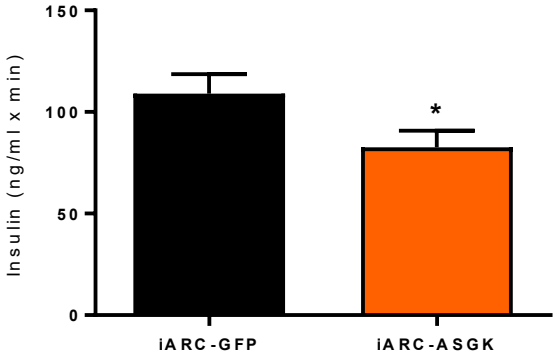


b. Plasma glucose levels are shown as area under the curve by t-Test * =p<0.01

Figure 21: Effect of injection of rAAV encoding either anti-sense glucokinase or GFP into the arcuate on insulin. Groups of adult male Wistar rats (n=10) were injected with 1x 10^{12} genome particles of either rAAV-GFP or rAAV-asGK bilaterally into the arcuate nucleus. Four weeks later they were subject to a GTT.



a. Plasma insulin levels are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.



b. Plasma insulin levels are shown as area under the curve by t-Test * =p<0.01

2.4.4.5 Dynamic insulin testing in iARC-asGK

Insulin sensitivity on ITT was unchanged (Figure 22) as were fasting insulin and glucose levels. Active GLP-1 levels remain unchanged and showed the same profile of release as control animals (Figure 23). These results show that glucose stimulated insulin release is decreased in iARC-asGK rats compared with controls.

Figure 22: Effect of injection of rAAV encoding either anti sense glucokinase or GFP into the arcuate on insulin tolerance. Groups of adult male Wistar rats (n=11) were injected with 1x 10^{12} genome particles of either rAAV-GFP or rAAV-asGK bilaterally into the arcuate nucleus. Five weeks later they were subject to an ITT. Results are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.

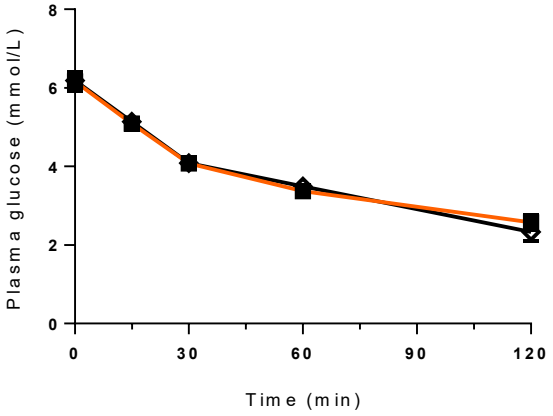
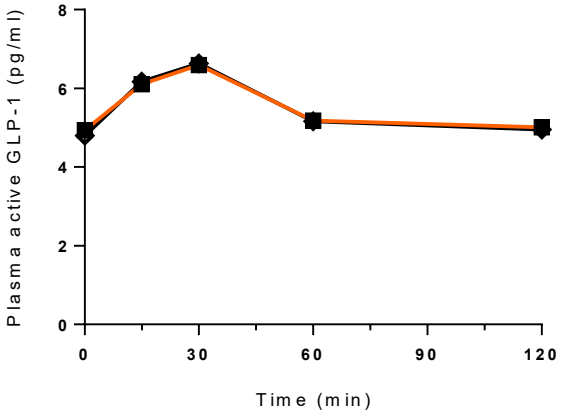


Figure 23: Effect of injection of rAAV encoding either anti sense glucokinase or GFP into the arcuate on acute GLP-1 levels during a glucose tolerance. Groups of adult male Wistar rats (n=11) were injected with 1x 10^{12} genome particles of either rAAV-GFP or rAAV-asGK bilaterally into the arcuate nucleus. Four weeks later they were subject to a GTT. Results are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.



The effects described so far have been in healthy lean rats. The effect of glucokinase modification in models of disease would be of significant clinical interest for investigation into therapeutic potential.

2.4.5 <u>Arcuate Study 4:</u> Establishing the effects of pharmacological activation of arcuate GK activity on glucose homeostasis In Zucker Diabetic Fat rats (ZDF Fa/Fa) and the effects of K_{ATP} channel modification

The effects of glucokinase over-activation was investigated in a model of type-2 diabetes, the Zucker diabetic fatty rat (ZDF Fa/Fa). These rats are developed from the Zucker Fat rat which have a missense mutation in the leptin receptor gene which thereby predisposes them to obesity and diabetes.

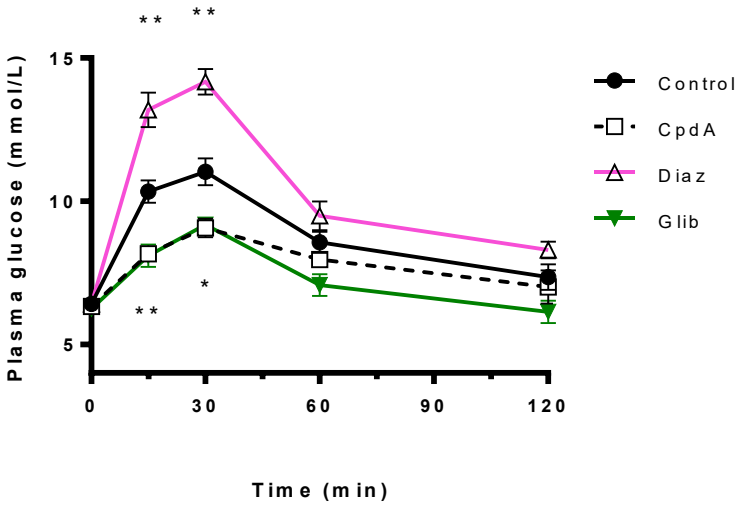
Firstly, the glucokinase activator Compound A was injected into the arcuate nucleus of these Fa/Fa rats to ensure that activation of GK had the same effects of improving glucose homeostasis in these models of disease. In Fa/Fa rats, Cpd A significantly lowered glucose levels at 15 minutes (10.34±0.39 mmol/L iARC-vehicle vs 8.16±0.25mmol/L iARC-CpdA) $p<0.001$ and 30 minutes (11.03±0.47mmol/L iARC-vehicle vs 9.06±0.33mmol/L iARC-CpdA) $p<0.01$ (Figure 24). CpdA increased insulin levels at 15 minutes (11.05±0.28mmol/L iARC-vehicle vs 13.65±0.48mmol/L iARC-CpdA) $p<0.001$, 30 minutes (9.48±0.53mmol/L iARC-vehicle vs 12.91±1.06mmol/L iARC-CpdA) $p<0.001$ and at 60 minutes (7.64±0.48mmol/L iARC-vehicle vs 10.85±1.10mmol/L iARC-CpdA) $p<0.001$ (Figure 25).

Following proof of activation, manipulation of KATP channel activity was examined. Similarly to leaner models, in obese Zucker rats K_{ATP} channel blocker glibenclamide significantly decreased glucose levels at 15 minutes (10.34±0.39 mmol/L iARC-vehicle vs 8.09±0.40mmol/L iARC-Glib) $p<0.001$ and at 30 minutes (11.03±0.47 mmol/L iARC-vehicle vs 9.18±0.21mmol/L iARC-Glib) $p<0.01$ (Figure 24). Glibenclamide also significantly increased insulin secretion at 15 minutes (11.05±0.28 mmol/L iARC-vehicle vs 13.29±0.75mmol/L iARC-Glib) $p<0.01$ and at

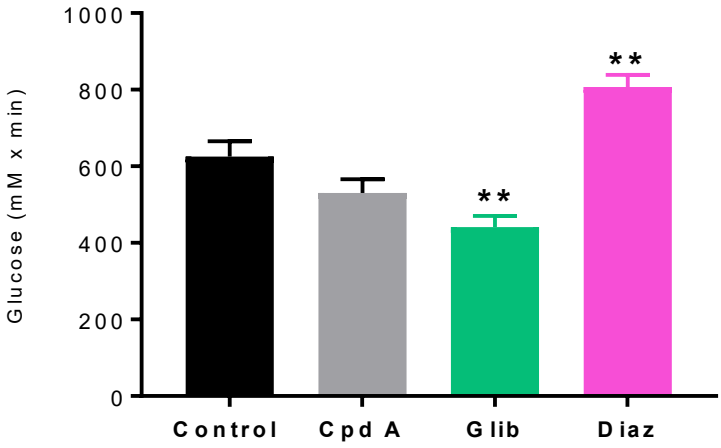
30 minutes (9.48±0.53mmol/L iARC-vehicle vs 11.73±0.76mmol/L iARC-Glib) $p<0.01$ (Figure 25).

Conversely, K_{ATP} channel activator diazoxide significantly raised glucose levels at 15 minutes (10.34±0.39 mmol/L iARC-vehicle vs 13.19±0.61mmol/L iARC-Diaz) $p<0.001$ and at 30 minutes (11.03±0.47 mmol/L iARC-vehicle vs 14.17±0.45mmol/L iARC-Diaz) $p<0.001$ (Figure 24). Diazoxide significantly reduced insulin secretion at 15 minutes (11.05±0.28 mmol/L iARC-vehicle vs 8.81±0.28mmol/L iARC-Diaz) $p<0.01$ and at 30 minutes (9.48±0.53mmol/L iARC-vehicle vs 7.38±0.31mmol/L iARC-Diaz) $p<0.05$) (Figure 25). These results show that, similarly to healthy rats, glucose homeostasis is improved following arcuate glucokinase activation and arcuate sulphonylurea administration in obese diabetic rodent models. The extension of these findings into obese diabetic models is key in show casing the clinical potential of central glucokinase as a possible therapeutic target in human Type 2 diabetes.

Figure 24: Effect of pharmacologically increased arcuate glucokinase activity and manipulating K_{ATP} activity on glucose in Fa/Fa rats. Fa/Fa rats were injected into the arcuate nucleus with either vehicle (control) or 0.5nmol of the glucokinase activator compound A (CpdA), 2nmol Glibenclamide or 1nmol Diazoxide

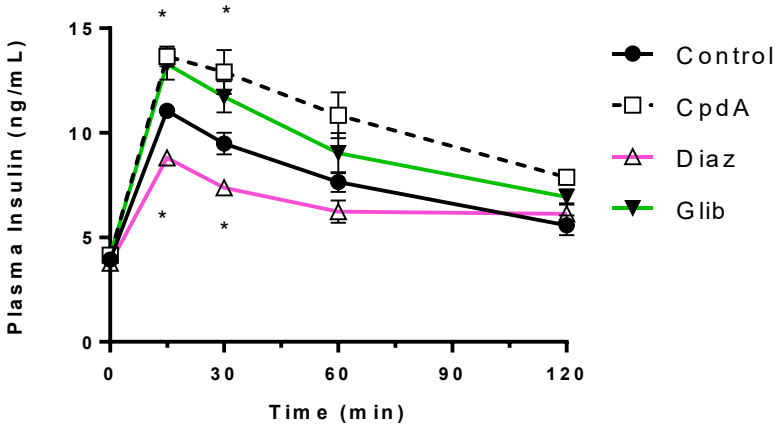


a. Plasma glucose was measured during an oral glucose tolerance test in control (black circle black line); CpdA injected (white square dashed line); Glibenclamide (black triangle green line) and Diazoxide injected (white triangle magenta line) rats. Results are shown as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.

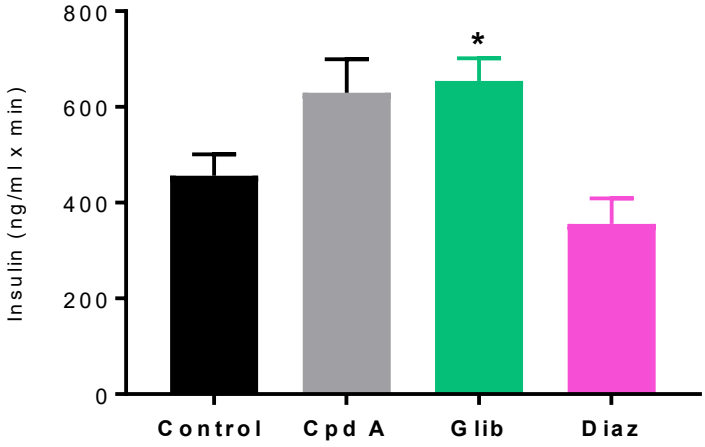


b. Plasma glucose was measured during an oral glucose tolerance test in control; CpdA; Glibenclamide and Diazoxide injected rats. Results shown as area under the curve versus control *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.

Figure 25: Effect of pharmacologically increased arcuate glucokinase activity and manipulating K_{ATP} activity on plasma insulin in Fa/Fa rats. Fa/Fa rats were injected into the arcuate nucleus with either vehicle (control) or 0.5nmol of the glucokinase activator compound A (CpdA), 2nmol Glibenclamide or 1nmol Diazoxide



a. Plasma insulin was measured during an oral glucose tolerance test in control (black circle black line); CpdA injected (white square dashed line); Glibenclamide (black triangle green line) and Diazoxide injected (white triangle magenta line) rats. as mean ± SEM, *=p<0.01 by ANOVA and post-hoc Holm-Sidak test.



b. Plasma insulin was measured during an oral glucose tolerance test in control; CpdA; Glibenclamide and Diazoxide injected rats. Results shown as area under the curve versus control *=p<0.01 by ANOVA and post-hoc Holm-Sidak test

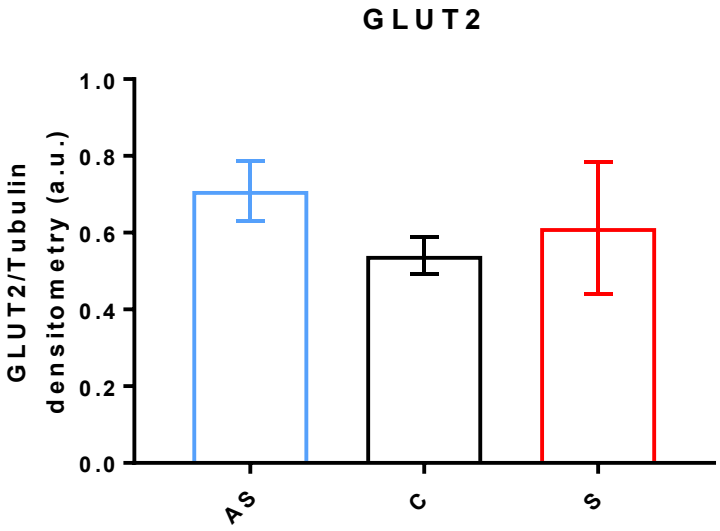
Exactly how altering GK activity leads to changes in glucose homeostasis is a huge area of speculation ranging from potential alterations within the arcuate itself, changes in signalling pathways to the pancreas or even modifications at the pancreatic cellular level.

2.4.6 Arcuate Study 5: Establishing the expression of proteins known to be important in glucose homeostasis in animals where glucokinase is over-expressed

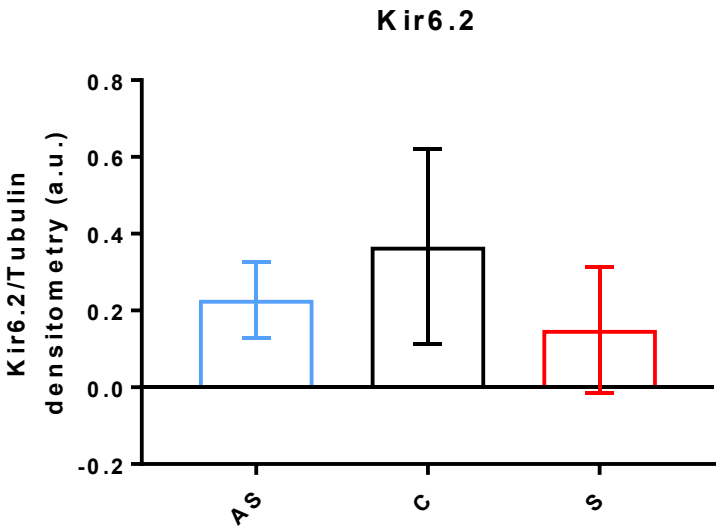
One possibility of the downstream effect of increased glucokinase expression is a change in the expression of proteins known to be important in glucose homeostasis. The GLUT-2 receptor is an important glucose transporter (Henquin 2000). Changing levels of GLUT-2 could contribute to how much or how little glucose is detected by the brain and the amount of insulin that is subsequently released. Kir 6.2 is a major sub unit of the K_{ATP} channel and is also important in glucose homeostasis (Tinker 2001). Alterations in this subunit could affect the opening and closing of the K_{ATP} channel which would ultimately affect insulin release. Western blot analysis was used to assess the expression of these proteins in arcuate samples from rats injected with rAAV expressing either GFP (iARC-GFP), glucokinase (iARC-GK) or antisense glucokinase (iARC-asGK). No significant difference in expression of GLUT-2 and Kir 6.2 between groups (Figure 26) was observed and hence the changes in glucose homeostasis elicited by glucokinase are not governed by changes in GLUT-2 or Kir 6.2 levels in the arcuate nucleus.

Experiments performed courtesy of collaborator Professor Guy Rutter's laboratory.

Figure 26: Effect of genetically increased glucokinase activity in the arcuate nucleus on expression of other proteins: Quantification using western blot of arcuate nucleus samples following intra-arcuate injection of either rAAV-GFP (C), rAAV-sGK (S) or rAAV-asGK (AS). Data are represented as mean ± SEM analysed with ANOVA and post Hoc Holm-Sidak



a. GLUT2 expression corrected for loading using tubulin



b. Kir6.2 expression corrected for loading using tubulin

Findings from these studies taken in the context of current evidence suggest that another mechanism by which GK exerts its effects might be indirectly via NPY neurones which are typically inhibited by glucose (Ziotopoulou et al., 2000). GK appears to lift this inhibition (Hussain et al., 2015b). However, on a genetic level, pancreatic and ileum proglucagon mRNA expression levels also remained unchanged in both iARC-sGK animals and controls. The findings suggest that the physical neural connection allowing the GK within arcuate nucleus to exert its effect on insulin secretion is via acute vagal stimulation and not an incretin effect involving glucagon or active GLP-1 action. Further detailed work, with vagotomy studies and specialised pancreatic cellular studies would be needed to explore this idea further.

2.5 Discussion

GK has been studied in other areas of the brain where it has been shown to have role in glucose homeostasis. In the VMN GK has an important role in the counter regulatory response to hypoglycaemia (Levin et al., 2008). Activation of glucokinase expressing neurones within the VMH using radio waves results in increased blood glucose levels with decreased insulin levels and increased glucagon levels (Stanley et al., 2016b). Using rAAV expressing full length glucokinase my laboratory have previously shown that glucokinase within the arcuate nucleus has a role in food intake (Hussain et al., 2015b) and in this chapter I have investigated the role of arcuate glucokinase in glucose homeostasis.

Using neurone specific viral alteration of glucokinase activity within the arcuate nucleus of rats, I have shown that increased arcuate GK improves glucose tolerance and increases insulin secretion. These effects are independent of changes in food intake and body weight between groups. Fasting glucose and insulin levels were also unaltered compared to controls in these rats. As insulin tolerance tests were identical in both study and control groups, these improvements in glucose tolerance are unlikely to be due to changes in insulin sensitivity. Conversely, decreasing GK activity within the arcuate nucleus results in higher glucose levels and lower insulin levels. Plasma GLP-1 levels were unaltered in both upregulated and downregulated models which suggests that there is little input from the incretin effect. These results suggest that arcuate GK has a role in glucose stimulated insulin release. Stanley et al report neural connections between the arcuate nucleus and the pancreas in an elegant set of studies and it is likely that changes in glucose tolerance demonstrated in Chapter 2 are due to the same neural connections between the pancreas and glucokinase expressing neurones in the arcuate nucleus (Stanley et al., 2016b). My

functional findings are however in contrast to the effects reported following over expression of hexokinase in the arcuate nucleus (Rosario et al., 2016) where increased expression of hexokinase resulted in decreased glucose sensitivity and a decrease in insulin release. In this context it is important to note the difference in the kinetic properties between glucokinase and hexokinase. Hexokinase is fully active at all physiological glucose concentrations and is not responsive to changes in blood glucose levels. My findings are however consistent with other studies where, although no specific nuclei was identified, an infusion of glucose into the third ventricle increased glucose stimulated insulin secretion whereas infusion of a non-selective glucokinase inhibitor decreased it (Osundiji et al., 2012).

The suggestion that glucokinase in the arcuate nucleus has a physiological role in the regulation of glucose homeostasis is further strengthened by the effect of decreasing glucokinase activity in the arcuate nucleus. Using my laboratory's previously validated anti-sense construct I reduced glucokinase activity specifically in the arcuate nucleus. There was no effect on fasting glucose and insulin levels or insulin sensitivity during an insulin tolerance test. Under expression of GK showed an opposite effect to that of over expression and glucose excursions were higher than those of controls. During the glucose tolerance test glucose levels were increased beyond that of the controls and the area under the curve was greater. This was accompanied by decreased insulin release. These results further support the suggestion that glucokinase within the arcuate nucleus increases glucose stimulated insulin release. It is possible that the effects observed in the upregulation of glucokinase are due to ectopic expression of glucokinase in neurones in which it is not normally expressed, however both the anti-sense data as well as the pharmacological studies with compound A go against this. Hence these findings,

together with the previous laboratory data suggesting arcuate glucokinase regulates glucose intake, suggest the possibility that glucokinase within the arcuate nucleus coordinates a physiological response to glucose in the diet.

The precise pathway through which glucokinase exerts its effects remains unknown. Findings from my studies taken in the context of current evidence suggest that another mechanism by which GK exerts its effects might be indirectly via NPY neurones which are typically inhibited by glucose (Ziotopoulou et al., 2000). GK appears to lift this inhibition (Hussain et al., 2015b). However, on a genetic level, pancreatic and ileum proglucagon mRNA expression levels also remained unchanged in both iARC-sGK animals and controls. These findings suggest that the physical neural connection allowing the GK within arcuate nucleus to exert its effect on insulin secretion is via acute vagal stimulation and not an incretin effect involving glucagon or active GLP-1 action. Further detailed work, with vagotomy studies and specialised pancreatic cellular studies would be needed to explore this idea further.

Therapeutically, my findings show that upregulation of glucokinase centrally can improve glucose tolerance and increase insulin release significantly in normal rodent models. This intervention mirrors what might be expected from a therapeutic intervention such as administration of a central glucokinase activator. My pharmacological data suggests also that GK exerts these effects though acting via the KATP channel and that administration of a sulphonylurea (glibenclamide) mirrors the effects of GK over expression. The fact that these effects are maintained in obese models is clinically significant.

Down regulation of glucokinase causes deterioration in glucose tolerance. This down regulated model is reminiscent of the human genetic disease Maturity Onset

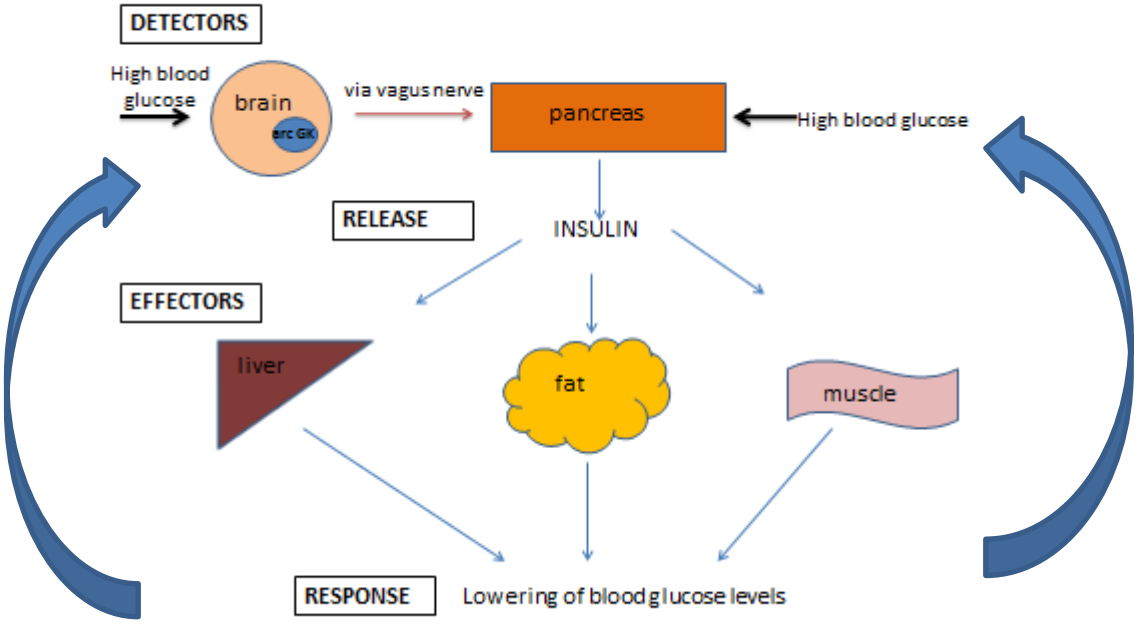
Diabetes of the Young Type 2 (MODY-2). MODY-2 is one of the more common forms of genetic diabetes seen in clinical practice and is caused by a mutation in the GK gene. MODY-2 leads to diabetes to varying degrees of severity (Velho and Robert, 2002). Humans born without any GK have severe neonatal and often fatal hyperglycaemia. GK knockout mice die at birth (Matschinsky et al., 2006). There were no significant differences between the rats in either the sense or anti-sense groups versus control rats. Incidentally, GK anti-sense animals were 3g lighter than control animals and yet had worse glucose tolerance tests than controls.

Glucokinase has a key role in glucose homeostasis in other organs within the body. The recent identification of GK within the brain has been an area of much interest. Here I show that increased glucokinase activity improves glucose homeostasis through increased glucose stimulated insulin release. Additionally, my data suggests that activating the pathway through which GK acts, closure of the KATP channel, also improves glucose tolerance. The clinical potential of this is intriguing. Over a decade ago GK activators went to Phase 2 trials. The studies were aborted do to hypoglycaemia, lack of sustained effect and hyperlipidaemia (Nakamura and Terauchi, 2015). At this point glucokinase had only been identified within the liver and pancreas. More recently a group in China carried out a multi-centre randomised controlled trial using dorzagliatin, a GK activator again targeting the liver and pancreas. The results were more promising with sustained improvements in blood glucose levels at 12 weeks with no difference in the number of side effects in placebo versus treatment group (Zhu et al., 2018). Glucokinase activation as a therapy is therefore showing promise and the new knowledge afforded by the presence of GK within central nuclei offers not only new insight into the complexity of the networks controlling glucose homeostasis, but also offers hope for central GK or

its downstream pathways as a potential therapy. There is a shift towards recognising central therapies as important players in disease treatment. GLP-1 agonists are an important example of this. However, it is not easy to develop drugs to cross the blood brain barrier and indeed if there were reports of previous peripheral adverse events, it would be difficult to develop a drug which is centrally active without adverse effects peripherally. With this in mind, ligand directed therapy using centrally acting glucokinase activators is an exciting although untrodden possibility in the field of diabetes treatments. This form of therapy has shown promise in the treatment of cancer (Zhong 2014) and extending this into other chronic disease such as diabetes in the future should not be dismissed. Additionally, centrally permeable sulphonylureas which act upon a pathway by which GK is known to act, is another interesting therapeutic possibility.

Regardless of whether glucokinase itself holds a therapeutic potential or not, understanding its role is contributing to our appreciation of the importance of the brain's role in glucose homeostasis and moves us away from the historical pancreas-centred model (Figure 27).

Figure 27: Proposed role of arcuate glucokinase in glucose homeostasis. Both the pancreas and brain are involved in sensing of raised blood glucose levels either in part or predominantly through the actions of glucokinase. Evidence suggests that the arcuate signals to the pancreas through the vagus nerve which then releases insulin. Insulin has its effects via various organs such as fat, liver and muscle which results in the lowering of the blood glucose levels.



Chapter 3

The role of glucokinase within the hypothalamic paraventricular nucleus

3.1 Introduction

The hypothalamic paraventricular nucleus is known to have roles in the regulation of various endocrine functions as well as energy homeostasis. The PVN neurones produce and release neuropeptides from magnocellular neurones which can ultimately affect appetite and metabolism (Swanson and Sawchenko, 1980). Neurones from the PVN project to several other regions of the brain including the NTS (Geerling et al., 2010b, Zhang et al., 1999). Second order neurones project from the NTS to peripheral metabolically active organs such as the gastrointestinal tract (Rogers and Hermann, 1985) and pancreas (Jansen et al., 1997b). Interestingly, both lesioning of the PVN and deletion of a critical transcription factor required for PVN development lead to hyperphagia and obesity in rats (Leibowitz et al., 1981) which suggests that the PVN has a role in appetite. The role of the PVN in glucose homeostasis is not well understood. Intriguingly however both the enzyme glucokinase (Jetton et al., 1994a, Matschinsky et al., 2006, Teichgraber et al., 1973) and binding sites for the hormone glucagon-like peptide-1 are expressed in abundance within the PVN (Katsurada et al., 2014) and that decreased expression of these receptors is found in Type 2 diabetes (Ten Kulve et al., 2016). Therefore this suggests that this small nucleus within the hypothalamus makes some contribution to the regulation of blood sugar levels.

3.1.2 Glucokinase

Glucokinase, an enzyme that has a critical role in glucose sensing (Rutter et al., 2015, Dunn-Meynell et al., 2002) and disposal (Iynedjian, 2009, Pocai et al., 2005b) is highly expressed within the PVN. Glucokinase is also expressed in numerous other hypothalamic nuclei, including the VMN and arcuate nucleus (Jetton et al.,

1994a, Matschinsky et al., 2006, Teichgraber et al., 1973). GK within the VMN is important in the counter-regulatory response to hypoglycaemia (Levin et al., 2008, Kang et al., 2006, McCrimmon et al., 2005) and within the arcuate nucleus, it has a role in food intake (Hussain et al., 2015a, Ma et al., 2018b, Stanley et al., 2016a, Rosario et al., 2016). In neurons, GK forms part of the glucose sensing mechanism and is co-expressed with GLUT-2 and ATP sensitive potassium channels, KATP (Maekawa et al., 2000, Li et al., 2003b).

3.1.3 Glucagon Like Peptide-1

GLP-1, a potent anorexogenic agent as well as mediator of glucose homeostasis, has binding sites which are expressed in great abundance within the PVN (Larsen et al., 1997, Katsurada et al., 2014). GLP-1 is one of the hormones responsible for the incretin effect (Kreymann et al., 1987). The incretin effect is where an oral glucose load elicits a more substantial insulin response than a peripheral glucose load. GLP-1 is released from enteroendocrine L-cells in the gut in response to oral glucose intake (Baggio and Drucker, 2007). It increases both insulin synthesis and secretion (Holst, 2007). Release of GLP-1 thus far is thought to be solely dependent upon gastrointestinal tract mechanisms. GLP-1 is produced by both the intestine and the brain, specifically the nucleus of solitary tract which has neuronal projections to the PVN (Larsen et al., 1997, Merchenthaler et al., 1999, Katsurada et al., 2014). Interestingly, GLP-1 binding sites have been demonstrated in high densities within the PVN (Ten Kulve et al., 2016). Additionally, GLP-1-IR nerve fibre density is highest in the PVN compared to all other hypothalamic nuclei (Goke et al., 1995).

The role of the PVN in glucose homeostasis is as yet unknown, but the presence of both glucokinase and GLP-1 fibres and receptors within the PVN raise questions as to whether the PVN has a role in coordinating the gut and brain in the regulation of glucose homeostasis.

3.2 The role of paraventricular nucleus glucokinase in glucose homeostasis

3.2.1 Hypothesis

Glucokinase in the paraventricular nucleus has a role in glucose homeostasis.

3.3.2 Aims

1. To use sense recombinant adeno-associated virus (rAAV-GK) to increase expression of glucokinase or anti-sense recombinant adeno-associated virus (rAAV-asGK) to reduce the expression of glucokinase within the paraventricular nucleus of rats.

2. To determine the effect of increasing and decreasing expression of glucokinase on glucose homeostasis in response to an insulin tolerance test and glucose tolerance test.

3. To use pharmacological agents to establish whether glucokinase within the PVN exerts its effects through the K_{ATP} channel.

4. To determine if GLP-1 plays a role in glucose homeostasis in the context of altered glucokinase expression.

3.3 Methods

3.3.1 Animals

Adult male Wistar rats (230–280 g, Charles River UK Ltd) were individually housed and maintained in a controlled environment (temperature 21 °C–23 °C, 12-hours light–dark cycle, lights on at 07:00 hours). They had ad libitum access to standard chow (RM1 diet; Special Diet Services UK Ltd, Witham, UK) and water except where stated. All animal procedures were approved under the British Home Office Animals (Scientific Procedures) Act 1986 (Project Licence no. 70/7229).

3.3.2 Recombinant adeno associated vector production

Full length glucokinase (GK) complementary deoxyribonucleic acid (cDNA) was isolated and amplified by polymerase chain reaction (PCR) from the plasmid pCMV4.GKB1 encoding full length glucokinase cDNA (gift from Prof. Magnuson, Nashville). In order to construct the glucokinase sense (sGK) and antisense (asGK), glucokinase DNA underwent cloning in the forward (GKS) and reverse (GKAS) orientation into the plasmid pTR-CGW (gift from Dr. Verhaggen, Amsterdam). We used helper plasmid pDG to produce rAAV particles in a two-plasmid system (Grimm et al., 1998). The rAAV particles were then recovered and purified using an iodixanol gradient to make rAAV sense glucokinase (rAAV-GK) and rAAV anti-sense glucokinase (rAAV-asGK) (Zolotukhin et al., 1999).

3.3.4 Glucokinase expression modulation via bilateral intra paraventricular rAAV injection using stereotaxic technique

Half a microliter of either rAAV-sGK (titre: $2.96x10^{12}$ genome particles/ml) or rAAV-asGK (titre: $3.42x10^{12}$ genome particles/ml) or GFP (titre: $5.04x10^{12}$ genome particles/ml) as a control was bilaterally injected into PVN of male Wistar rats using a stereotactic surgery. Coordinates were determined from determined by the Paxinos Watson criteria (Paxinos et al., 1980) and were 1.8 mm posterior to bregma ± 0.5mm mm lateral to bregma and 8mm below the skull surface.

The stereotactic surgery was carried out as previously described(Hussain et al., 2015a). Male Wistar rats were anesthetised with oxygen (2L/min) and 4% isoflurane and held in a stereotactic frame. The surgical area was shaved then cleaned with betadine. A rostro-caudal incision (approximately 1cm) was made in the skin over the vertex of the skull and the periosteum from the underlying bone was removed to expose bregma. Bilateral burr holes were drilled using an electric drill according to coordinates calculated previously. Each animal received an iPVN injection of 0.5µl rAAV into both sides at a rate of 12µl/hour over five minutes using a stainless steel injector and an infusion pump. The cannula and injector were left in position for five minutes to limit back diffusion and then slowly withdrawn. The scalp incision was sutured with a 4.0 polypropylene suture (Ethicon).

3.3.5 Intra-paraventricular administration of pharmacological agents

Animals were prepared and mounted onto a stereotactic frame as described previously. Anaesthetic, analgesia and antibiotics were administered as previously described. A unilateral burr hole was drilled using an electric drill according to coordinates listed previously (1.8 mm posterior to bregma ± 0.5mm mm lateral to bregma and 8mm below the skull surface). A stainless steel guide cannula held by an arm mounted onto the stereotactic frame was inserted 9.5mm below the skull surface. Three stainless steel screws were then gently inserted into the skull using a hand drill to act as anchors. Dental acrylic was used to form a pedestal fixing the cannula into a stationary position with support from the mounting screws. This was left to dry and a dummy cap was screwed onto the cannula to prevent infection.

Subsequently rats were fasted overnight and the following morning injected with one of the following in a volume of 0.5 microliter; saline, 0.5nmol Compound A (a glucokinase activator), Cpd A – (2-Amino-5-(4-methyl-4H-(1,2,4)-triazole-3-yl-sulfanyl)-N-(4-methyl-thiazole-2-yl)benzamide) CAS 603108-44-7 (Merck-Millipore, Beeston, UK), 1nmol diazoxide (a K_{ATP} activator) or 2nmol of glibenclamide (a K_{ATP} blocker). These doses are identical to those used previously (Hussain et al., 2015a). Thirty minutes after injection the animals underwent an oral glucose tolerance test (OGTT) as described below. The experiment was a cross-over design with each animal receiving each injection which were performed in a random order at least three days apart.

In another study, animals were injected with one of the following in a volume of 0.5 microliter; saline, 1.5 ug D-glucose (Chaleek, Kermani et al. 2012) or 0.25 mg 2-

deoxy-d-glucose (Egawa, Yoshimatsu et al. 1989), then underwent an oral glucose tolerance test (OGTT) as described below.

At the end of study, 1µl of India ink was injected through the cannula and the brains were dissected out. Cresyl violet staining was performed on brain slices to determine the accuracy of the cannulation.

3.3.6 Oral glucose tolerance tests

All rats underwent an oral glucose tolerance test (OGTT) four weeks post-surgery. Animals were fasted overnight prior to the study. A 24 gauge/19mm cannula was inserted into the tail vein. The baseline blood sample was collected at 0 minutes. A 2.5g/kg dose of glucose (20% dextrose diluted in GDW) was then administered orally to each animal. Following glucose consumption, blood was taken at 15, 30, 60 and 120 minutes. Heparin was administered after each sample to prevent clotting. Plasma was separated from blood cells by centrifugation at 13,000xg for 5 minutes at 4˚C and was stored at -80°C.

3.3.7 Insulin tolerance tests

All rats underwent an insulin tolerance test (ITT) 5 weeks post PVN surgery. A 24 gauge/19mm cannula was inserted into the tail vein. The baseline blood sample was collected at 0 minutes. 2 units/kg of insulin was injected intraperitoneally. Blood was taken at 15, 30, 60 and 120 minutes. Plasma was separated by centrifugation at 13,000xg for 5 minutes at 4˚C and was stored at -80°C

3.3.8 Collection of tissue samples

Unless otherwise stated in the methods, animals from all studies were euthanized in early light phase. Pancreas, ileum and brain were collected from all animals following the completion of a study.

3.3.9 Glucokinase activity assay in isolated PVN, VMN and ARC samples

200 µl ice-cold buffer:

- 0.0107 M MgCl2
- 5 mM sodium EDTA
- 0.15 M KCl
- 0.07% w/v 2-mercaptoethanol

1 ml reaction reagent:

- 100 mM GlyGly
- 45 µM 5-thio-d-glucose-6-phosphate
- 1 M MgCl2,
- 0.5 mM 3-O-methyl-N-acetylglucosamine
- 200 mM ATP
- 12.5 mM NADP
- 2 M glucose
- 0.4 units glucose-6-phosphate dehydrogenase (type IX, from Baker's yeast).

The glucokinase activity in the hypothalamic nuclei was measured as previously described (Hussain et al., 2015a). In brief, 300µm thick coronal section of brain tissue was cut using a cryostat with a chamber temperature of -7°C and transferred

to glass slides. Slides were kept on dry ice to keep frozen and the PVN, ARC and VMN were collected using a 22-gauge neuro punch (Fine Science Tools) using a rat brain atlas as a guide(Paxinos et al., 1980). Nuclei were homogenised in 200µl extraction buffer containing 0.0107M $MgCl_2$, 5 mM sodium EDTA, 0.15 M KCl, and 0.07% w/v 2-mercaptoethanol. Fifty microliters of each supernatant were added to 500µl reaction mix containing 100 mM Gly-Gly, 45 µM 5-thio-d-glucose-6-phosphate,1 M MgCl2, 0.5 mM 3-O-methyl-N-acetylglucosamine, 200 mM ATP, 12.5 mM NADP, 2 M glucose, and 0.4 IU (type IX, from Baker's yeast) and incubated at 37°C for thirty minutes. Each sample was set up in triplicate. Absorbance of each sample was read at 340nm and GK concentration was calculated by extrapolating values from the standard curve for glucokinase. Samples were normalised to protein content using a Pierce bicinchoninic acid (BCA) assay (Pierce).

3.3.10 Measurement of glucose in plasma samples using a glucose oxidase assay

Plasma glucose levels were measured using a glucose oxidase assay (Randox) according to the manufacturer's instructions and the absorbance read using by ELx808 Microplate Reader (Biotek Instruments Ltd).

3.3.11 Measurement of insulin in plasma samples using enzyme linked immunosorbant assay (ELISA)

Plasma insulin levels were analysed using Ultra-sensitive rat insulin ELISA Kit from Crystal Chem (intra and inter assay precision co-efficient of variant $\leq$ 10.0%) as per

manufacturer's instructions. Microplates were read by ELx808 Microplate Reader (Biotek Instruments Ltd).

3.3.12 Measurement of active glucagon-like peptide 1 (GLP-1) in plasma samples using ELISA

Plasma active GLP-1 levels were measured using Glucagon like peptide-1 (Active) ELISA from Millipore (Inter-assay precision co-efficient of variant 8±4.8 and inter assay precision co-efficient of variant 7.4±1.1) or GLP-1 (Active) ELISA Kit from Shibayagi as per manufacturer's instructions. For accurate measurement of active-GLP-1 we used protease inhibitors to prevent rapid degradation by DPP4. The microplates were read by SpectraMax® i3x Platform (Molecular Devices).

3.3.13 Quantitative RT-PCR

Frozen tissues were homogenized by TissueLyser II (QIAGEN) in TRIzol (Invitrogen), and total RNAs were isolated by the RNeasy Mini kit with on-column DNase treatment (QIAGEN). cDNA were synthesized by High-Capacity cDNA Reverse Transcription Kit (Thermofisher). qPCR was conducted using TaqMan Gene Expression Assays (primer assay ID: ins1 Rn02121433_g1, Gcg Rn00562293_m1 and 18S 4310893E) in 7900 HT Fast Real-Time PCR System (Thermo Fisher Scientific). mRNA expression relative to housekeeping gene (18S) was determined by using the ΔΔCT method.

3.3.14 Statistics

All data are shown as mean ± SEM. Analysis was by either one-way or two-way ANOVA (as appropriate) with post-hoc Holm-Sidak test or t-test (GraphPad Prism 8.0). Significance was set at $p < 0.05$ for all analyses.

3.4 Paraventricular Study 1: To determine the effects of chronically increased glucokinase activity in the paraventricular nucleus on glucose tolerance

3.4.1 Summary method

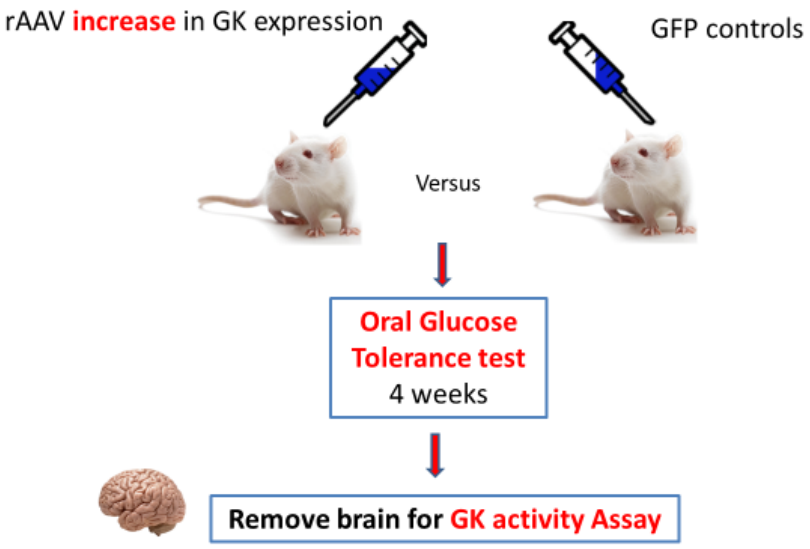


Figure 28: 0.5 microlitres of rAAV-sGK (titre: $2.96x10^{12}$ genome particles/ml) was bilaterally injected into PVN of male Wistar rats. GFP was used as a control. All rats underwent an OGTT at 4 weeks following arcuate surgery to determine glucose tolerance and an ITT at 5 weeks to determine insulin sensitivity.

Given the sparsity of data regarding the PVN's effects on glucose regulation, I first sought to establish whether GK within the PVN is important in glucose homeostasis. Using a stereotactic technique, rAAV encoding glucokinase gene (iPVN-sGK) was injected into the PVN of male Wistar rats (Figure 28). This full-length glucokinase construct has been demonstrated to increase glucokinase activity *in vivo*.

3.4.2 Determining accuracy of the paraventricular nucleus injections

Glucokinase activity was increased 40% in iPVN-sGK animals compared to controls, and there was no difference in glucokinase activity between groups in neighbouring nuclei such as the VMN and ARC (Figure 29).

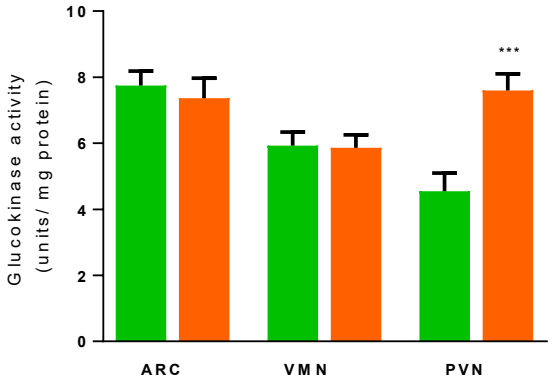


Figure 29: Effect of injection of rAAV encoding either sense glucokinase or GFP into the paraventricular nucleus on glucokinase activity in neighbouring hypothalamic nuclei. Groups of adult male Wistar rats (n=10) were injected with 1x 10^{12} genome particles of either rAAV-GFP or rAAV-sGK bilaterally into the PVN. They were culled and the specified hypothalamic nuclei isolated by punch biopsy. Glucokinase activity was measured by enzymatic assay and expressed as units per mg protein. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.

3.4.3 Changes in weight and food intake

There was no significant difference between the weights or food intake of the iPVN-sGK and controls during the study period (Figure 30 and Figure 31)

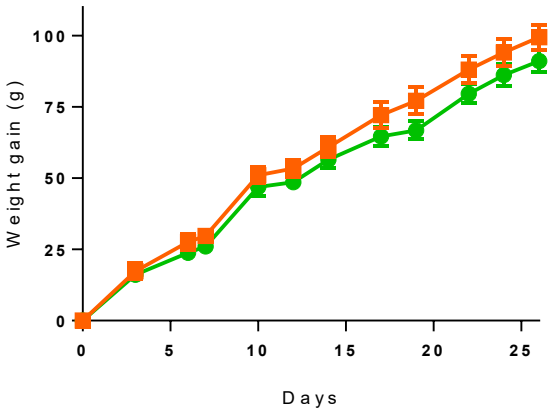


Figure 30: Body weight in male Wistar rats following intra-PVN injection of either rAAV-GFP (green) or rAAV-sGK (orange). Data analysed by GEE.

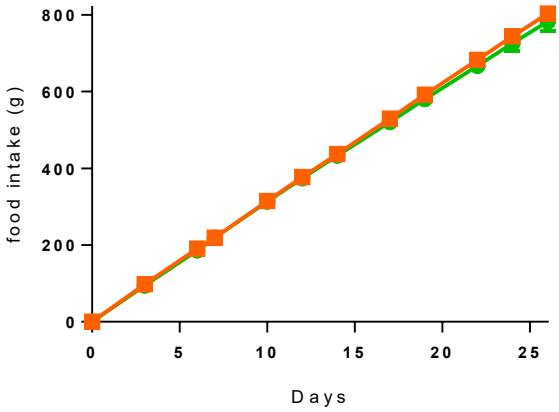
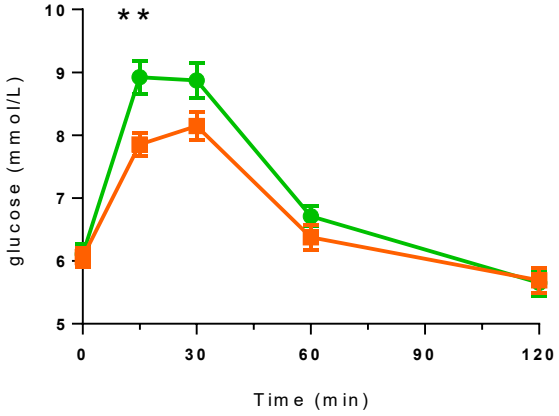


Figure 31: Body weight in male Wistar rats following intra-PVN injection of either rAAV-GFP (green) or rAAV-sGK (orange). Data analysed by GEE.

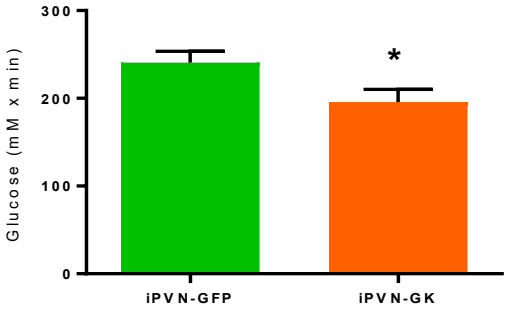
3.4.4 Effects of glucokinase over-expression on dynamic glucose testing

iPVN-GK rats had significantly lower plasma glucose levels than controls at 15 minutes (GFP: 8.93±0.27mmol/L, n=11; GK: 7.72±0.22mmol/L, n=12; $p<0.01$), 30 minutes (GFP: 8.88±0.27mmol/L, n=11; GK: 8.02±0.25mmol/L, n=12; $p<0.05$) and reduced total glucose levels shown by the iAUC during OGTT (Figure 32a and b). Initial insulin release also increased after 15 minutes (GFP: 2.84±0.14mmol/L, n=11; GK: 3.73±0.27mmol/L, n=12; $p<0.01$) and 30 minutes (GFP: 1.93±0.21mmol/L, n=11; GK: 2.69±0.26mmol/L, n=12; $p<0.05$) compared to iPVN-GFP animals and iAUC was increased (Figure 32c and d). Insulin sensitivity was unchanged as demonstrated by insulin tolerance test (ITT) (Figure 32e).

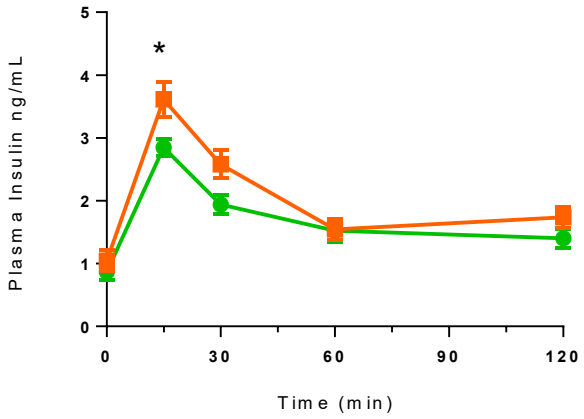
Figure 32: Effect of genetically increased paraventricular nucleus glucokinase activity on oral glucose testing: Groups of adult male Wistar rats were injected with either rAAV-GFP (iPVN-GFP) or rAAV-GK (iPVN-GK) bilaterally into the paraventricular nucleus. They then underwent an OGTT and an ITT.



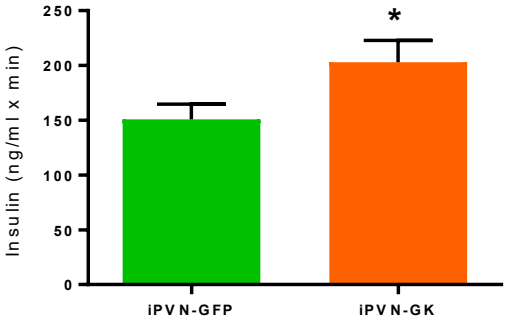
(a) Plasma glucose during an oral glucose tolerance test in iPVN-GFP (green) and iPVN-GK (orange) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



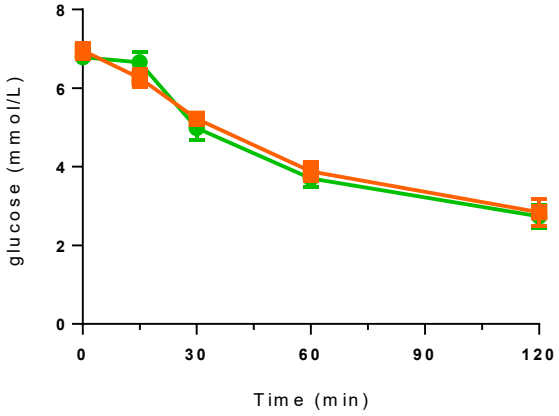
(b) Area under the curve analysis of plasma glucose during an oral glucose tolerance test in iPVN-GFP (green) and iPVN-GK (orange) rats. Data analysed by t-test * p<0.05.



(c) Plasma insulin levels during an oral glucose tolerance test in iPVN-GFP (green) and iPVN-GK (orange) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



(d) Area under the curve analysis of plasma insulin during an oral glucose tolerance test in iPVN-GFP (green) and iPVN-GK (orange) rats. Data analysed by t-test * p<0.05.

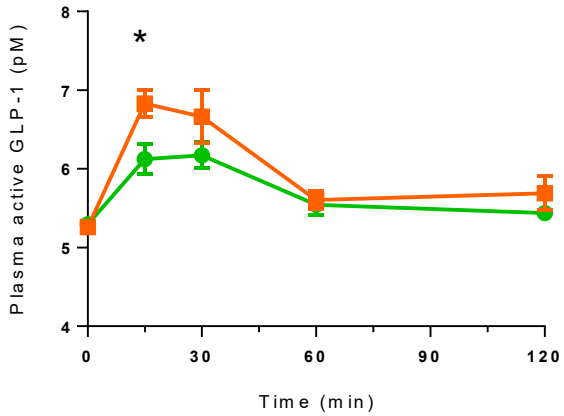


(e) Plasma glucose levels during an insulin tolerance test in iPVN-GFP (green) and iPVN-GK (orange) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.

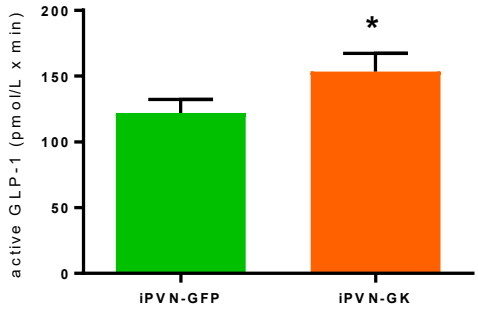
Data are represented as mean ± SEM, n=9-10. *p< 0.05, ** p< 0.01, *** p<0.001. Data for a,c and e were analysed by ANOVA and post-hoc Holm-Sidak. Data for b and d were analysed by t-test.

The ability of the body to process a glucose load is influenced by various factors. In recent years the contribution of the incretin hormones has increasingly been recognised. In addition to this, GLP-1 receptor binding sites are expressed in high density within the PVN thereby suggesting a role for GLP-1 here. I measured both blood glucagon and GLP-1 levels in response to an OGTT in iPVN-GK animals. Whilst glucagon levels remained unchanged, GLP-1 secretion was significantly higher in iPVN-GK than in control rats at 15 minutes (GFP: 6.16±0.18mmol/L, n=11; GKS: 6.90±0.26mmol/L, n=12; $p<0.01$) (Figure 33a) and iAUC was increased (Figure 33b). This intriguing result suggests GK within the PVN plays a role in co-ordinating peripheral GLP-1 release.

Figure 33: Effect of genetically increased paraventricular nucleus glucokinase activity on oral glucose testing: Groups of adult male Wistar rats were injected with either rAAV-GFP (iPVN-GFP) or rAAV-GK (iPVN-GK) bilaterally into the paraventricular nucleus. They then underwent an oGTT and an ITT.



(a) Plasma GLP-1 levels during an oral glucose tolerance test in iPVN-GFP (green) and iPVN-GK (orange) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



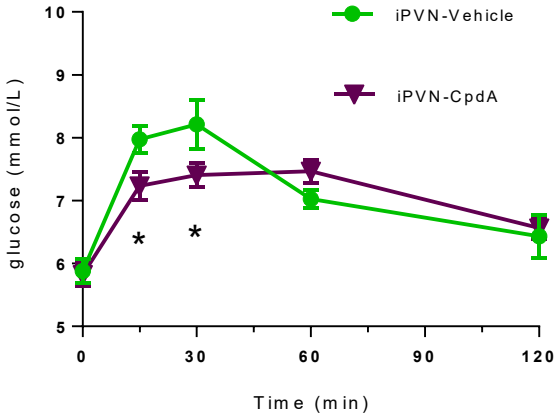
(b) Area under the curve analysis of plasma GLP-1 during an oral glucose tolerance test in iPVN-GFP (green) and iPVN-GK (orange) rats. Data analysed by t-test * p<0.05

Data are represented as mean ± SEM, n=9-10. *p< 0.05, ** p< 0.01, *** p<0.001. Data for a was analysed by ANOVA and post-hoc Holm-Sidak and data for b was analysed by t-test.

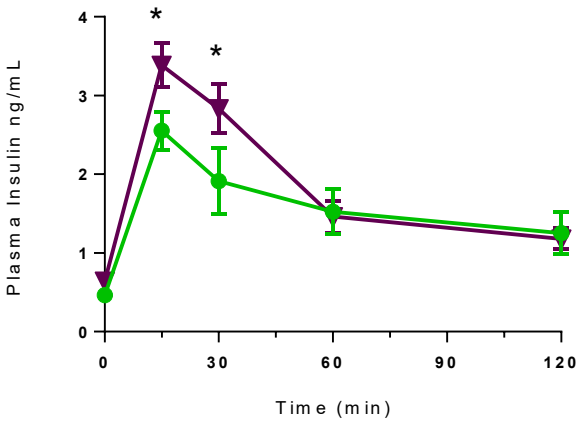
3.4.5 Acute increases in GK activity within the PVN also stimulate GLP-1 release

In order to establish if the effects on glucose homeostasis were acute or occurred only as a consequence of chronic changes within the PVN, the pharmacological agent Compound A was used. Compound A, a specific glucokinase activator, was injected into the PVN of male Wistar rats. Injection of Cpd A reduced glucose levels, increased insulin levels during an OGTT (Figure 34a and 34b). Importantly this acute GK activation also increased GLP-1 levels during an OGTT (Figure 34c) which reflects the increases seen in long term viral overexpression of PVN GK.

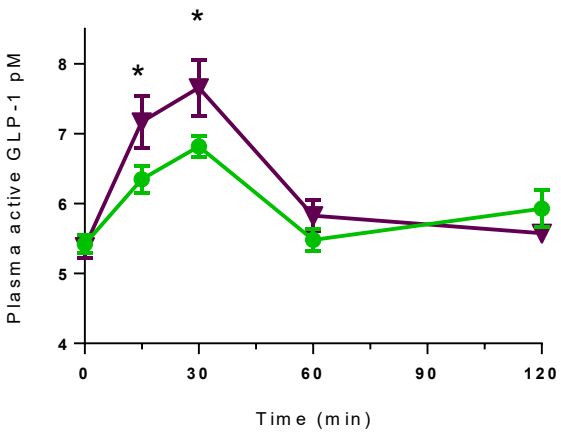
Figure 34: Effect of acutely increased paraventricular nucleus glucokinase activity on oral glucose testing: Groups of adult male Wistar rats were injected with either vehicle or Compound A (glucokinase activator) through a stainless steel cannula. They then underwent an oGTT and an ITT.



(a) Plasma glucose levels during an oral glucose tolerance test after injection of iPVN vehicle (green) and iPVN Compound A (dark purple). Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



(b) Plasma insulin levels during an oral glucose tolerance test after injection of iPVN vehicle (green) and iPVN Compound A (dark purple) . Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



(c) Plasma GLP-1 levels during an oral glucose tolerance test after injection of iPVN vehicle (green) and iPVN Compound A (dark purple). Results are shown as mean ± SEM, *=$p<0.05$ by ANOVA and post-hoc Holm-Sidak test.

3.5 Paraventricular Study 2: Establishing the effects of chronically decreased glucokinase activity in the paraventricular nucleus on glucose tolerance

3.5.1 Summary method

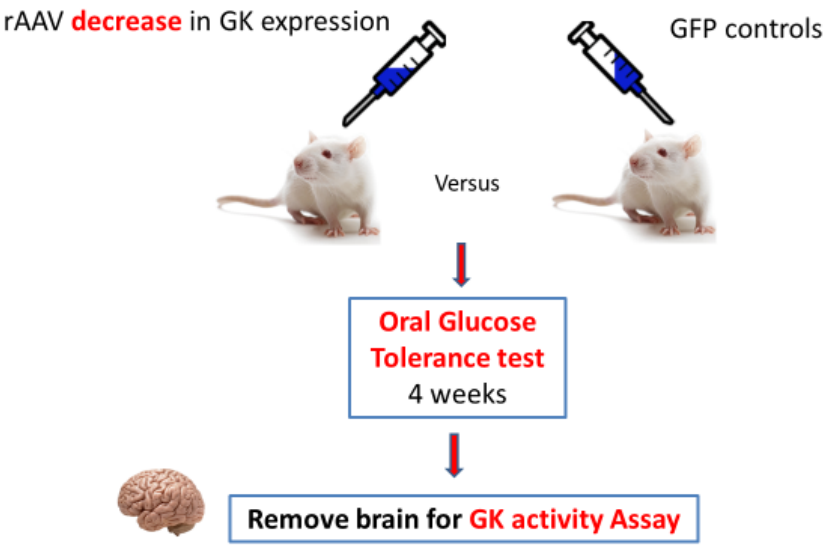


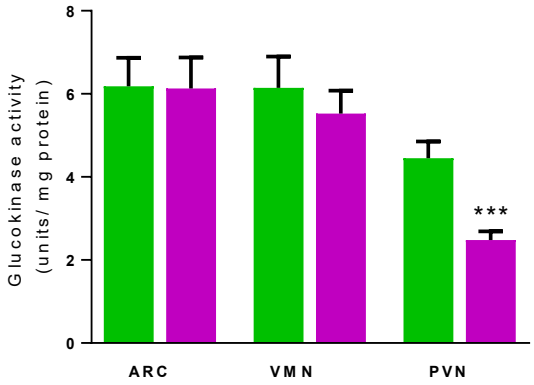
Figure 35: 0.5 microlitres of rAAV-asGK (titre: $2.96x10^{12}$ genome particles/ml) was bilaterally injected into PVN of male Wistar rats. GFP was used as a control. All rats underwent an OGTT at 4 weeks following arcuate surgery to determine glucose tolerance and an ITT at 5 weeks to determine insulin sensitivity.

In order to establish the physiological significance of GK within the PVN, I reduced expression of GK levels to examine whether the effect observed in iPVN-GK on glucose homeostasis is maintained. A summary of the method used is shown in Figure 35.

3.5.2 Determining accuracy of paraventricular injections (under-expression)

GK activity was reduced by approximately 50% in the PVN compared to those inject with GFP. There was no difference in glucokinase activity in the neighbouring arcuate nucleus or ventromedial nucleus (Figure 36).

Figure 36: Effect of injection of rAAV encoding either anti-sense glucokinase (purple) or GFP (green) into the paraventricular nucleus on glucokinase activity in neighbouring hypothalmic nuclei. Groups of adult male Wistar rats (n=10) were injected with 1x 10^{12} genome particles of either rAAV-GFP or rAAV-asGK bilaterally into the PVN. They were culled and the specified hypothalamic nuclei isolated by punch biopsy. Glucokinase activity was measured by enzymatic assay and expressed as units per mg protein. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



3.5.3 Changes in weight and food intake

There was no significant difference between the weights or food intake of the iPVN-asGK and controls during the study period (Figure 37 and Figure 38).

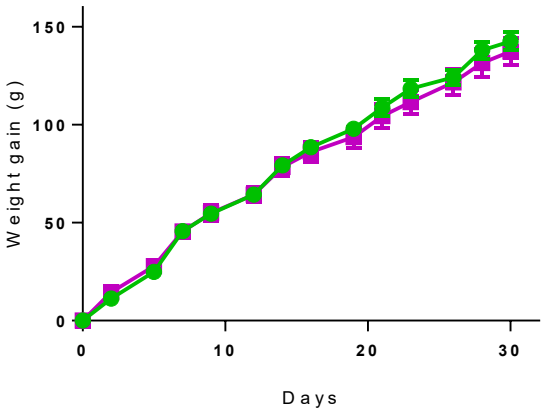


Figure 37: Body weight in male Wistar rats following intra-PVN injection of either rAAV-GFP (green) or rAAV-asGK (purple). Data analysed by GEE.

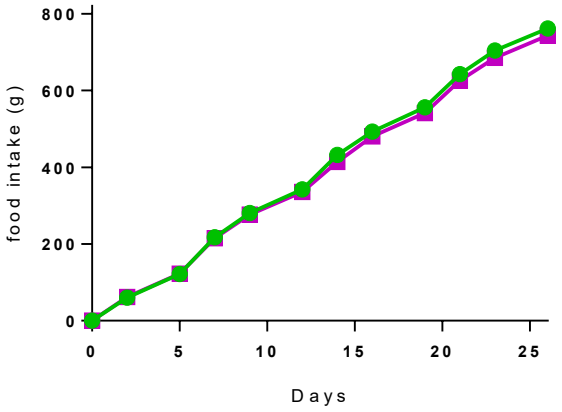
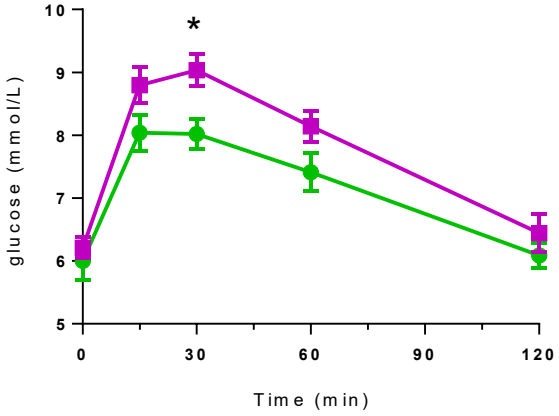


Figure 38: Body weight in male Wistar rats following intra-PVN injection of either rAAV-GFP (green) or rAAV-asGK (purple). Data analysed by GEE.

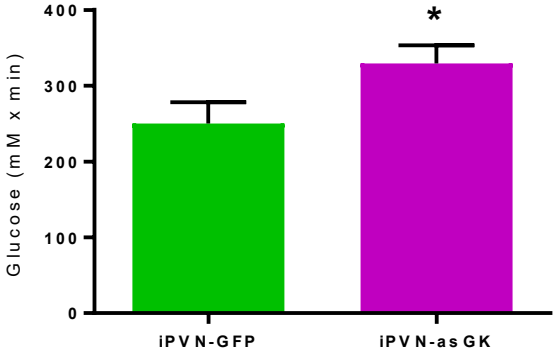
3.5.4 Effects of reduced glucokinase activity in the PVN on glucose homeostasis

iPVN-asGK rats had significantly higher plasma glucose levels at 30 minutes (GFP: 8.22±0.28mmol/L; asGK: 9.46±0.24mmol/L, n=8; $p<0.01$) (Figure 39a) compared to controls and total glucose levels were increased as shown by the iAUC (Figure 39b). Plasma insulin levels were also significantly reduced at 15 minutes compared (GFP: 4.07±0.37mmol/L; ASGK: 2.25±0.17mmol/L, n=8; $p<0.001$) (Figure 39c) and iAUC also reduced (Figure 39d). In conjunction with this, secretion of active GLP-1 (7-36) was markedly impaired in iPVN-ASGK rats. The initial GLP-1 response to the oral glucose challenge was blunted at both 15 minutes (GFP: 6.12±0.33pMol/L, n=8; $p<0.01$ASGK: 5.36±0.08pMol/L, n=8; $p<0.01$) and 30 minutes (GFP: 6.93±0.25pMol/L, n=8; $p<0.01$ASGK: 5.47±0.13pMol/L, n=8; $p<0.001$) (Figure 39e) and the overall amount of GLP-1 secreted was also reduced (Figure 39f). Insulin tolerance was unaffected (Figure 39g).

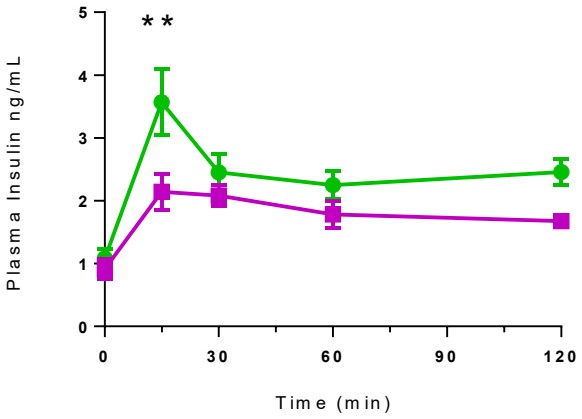
Figure 39: Effect of genetically decreased paraventricular nucleus glucokinase activity on oral glucose testing: Groups of adult male Wistar rats were injected with either rAAV-GFP (iPVN-GFP) or rAAV-GKAS (iPVN-asGK) bilaterally into the paraventricular nucleus. They then underwent an OGTT and an ITT.



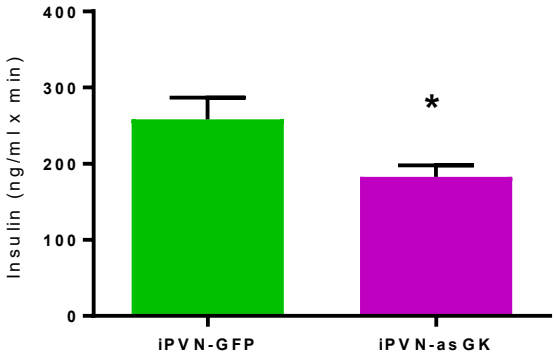
(a) Plasma glucose during an oral glucose tolerance test in iPVN-GFP (green) and iPVN-asGK (purple) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



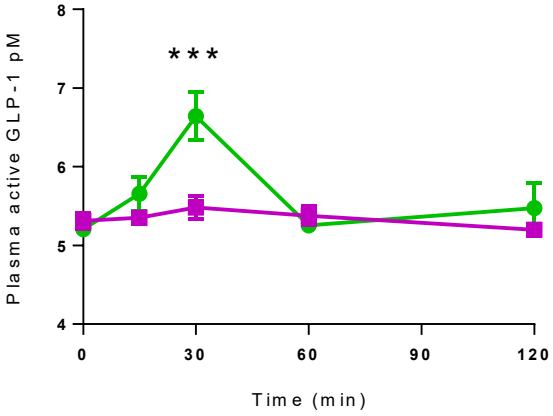
(b) Area under the curve analysis of plasma glucose during an oral glucose tolerance test in iPVN-GFP (green) and iPVN-asGK (purple) rats. Data analysed by t-test * p<0.05.



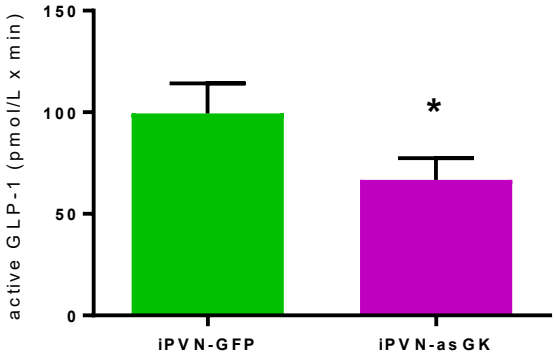
(c) Plasma insulin levels during an oral glucose tolerance test in iPVN-GFP (green) and iPVN-asGK (purple) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



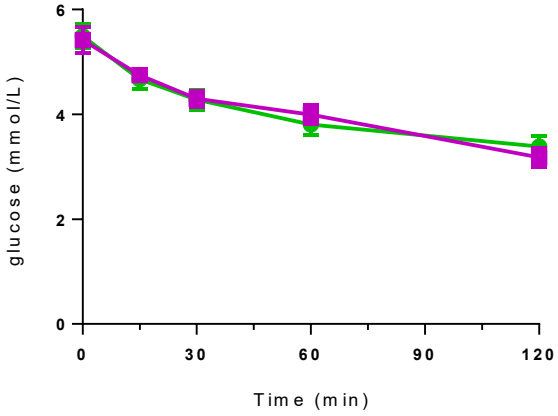
(d) Area under the curve analysis of plasma insulin during an oral glucose tolerance test in iPVN-GFP (green) and iPVN-asGK (purple) rats. Data analysed by t-test * p<0.05.



(e) Plasma GLP-1 levels during an oral glucose tolerance test in iPVN-GFP (green) and iPVN-asGK (purple) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



(f) Area under the curve analysis of plasma GLP-1 during an oral glucose tolerance test in iPVN-GFP (green) and iPVN-asGK (purple) rats. Data analysed by t-test * p<0.05



(g) Plasma glucose levels during an insulin tolerance test in iPVN-GFP (green) and iPVN-asGK (purple) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.

Data are represented as mean ± SEM, n=9-10. *p< 0.05, ** p< 0.01, *** p<0.001. Data for a, c and e were analysed by ANOVA and post-hoc Holm-Sidak and data for b, d and f were analysed by t-test.

There are two plausible broad explanations for the observed results. Firstly, that GK within the PVN is solely responsible for plasma GLP-1 release or, and more probably, that GK within the PVN is part of a wider system which involves, for instance the gut.

To establish the contribution of the incretin effect, intraperitoneal glucose administration was compared to orally consumed glucose during OGTTs in the context of both increased (iPVN-GK) and decreased (iPVN-asGK) PVN GK activity.

3.6 Paraventricular Study 3: Establishing the effect of an intra-peritoneal glucose tolerance test in rats with chronic under-expression of glucokinase (iPVN-asGK)

3.6.1 Summary Method

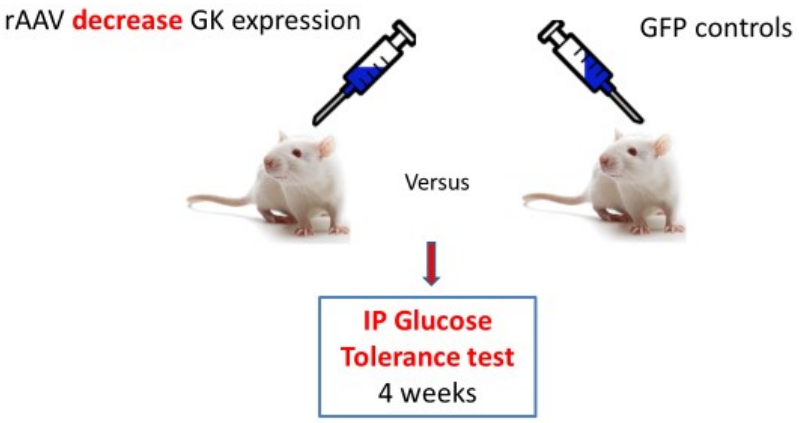


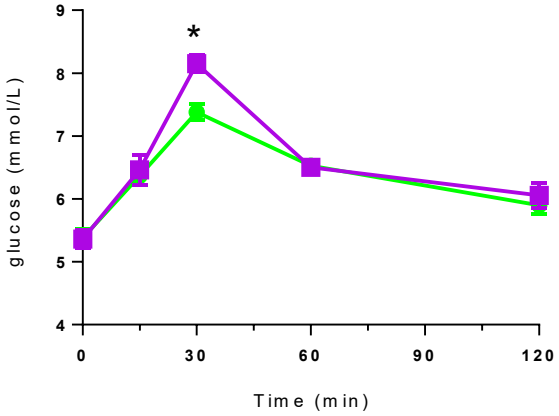
Figure 40: 0.5 microlitres of rAAV-asGK (titre: $2.96x10^{12}$ genome particles/ml) was bilaterally injected into PVN of male Wistar rats. GFP was used as a control. All rats underwent an GTT at 4 weeks following PVN surgery to determine glucose tolerance in response to an intraperitoneal glucose load.

The previous results suggest that reduced GK expression worsens glucose tolerance through an effect potentially mediated via GLP-1 release. In order to establish whether this effect is purely through glucose within the gastrointestinal tract or whether glucose detected within the circulation rAAV-asGK was bilaterally injected into PVN of male Wistar rats. GFP was used as a control. All rats underwent an intra-peritoneal GTT at 4 weeks following paraventricular nucleus surgery. This method is summarised in Figure 40.

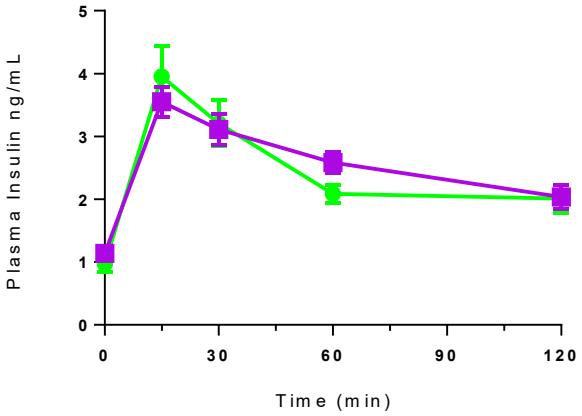
3.6.2 Establishing whether the effects on glucose homeostasis in under-expression of GK are maintained when the oral route of glucose ingestion is bypassed

iPVN-asGK rats had a higher plasma glucose level at 30 minutes, compared to the controls (GFP: 7.38±0.13mmol/L, asGK: 8.15±0.14mmol/L, n=9; $p<0.05$) (Figure 41a). However, no differences in insulin release were found between two groups during IPGTT (Figure 41b). Plasma GLP-1 levels did not differ between the two groups either (Figure 41c). No difference in plasma glucagon concentration between the two groups was observed.

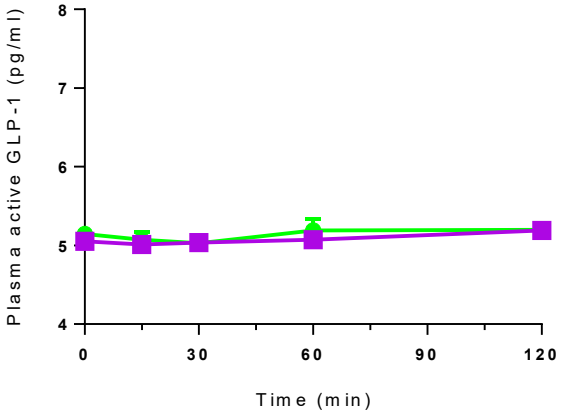
Figure 41: Effect of genetically decreased paraventricular nucleus glucokinase activity on intraperitoneal glucose testing: Groups of adult male Wistar rats were injected with either rAAV-GFP (iPVN-GFP) or rAAV-ASGK (iPVN-asGK) bilaterally into the paraventricular nucleus. They then underwent an intraperitoneal (IP) glucose tolerance test (GTT)



(a) Plasma glucose during IP glucose tolerance test in iPVN-GFP (green) and iPVN-asGK (purple) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



(b) Plasma insulin levels during IP glucose tolerance test in iPVN-GFP (green) and iPVN-asGK (purple) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



(c) Plasma GLP-1 levels during IP glucose tolerance test in iPVN-GFP (green) and iPVN-asGK (purple) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.

Data are represented as mean ± SEM, n=9-10. *p< 0.05, ** p< 0.01, *** p<0.001. Data analysed by ANOVA and post-hoc Holm-Sidak.

3.7 Paraventricular Study 4: Establishing the effect of an intra-peritoneal glucose tolerance test in rats with chronic over-expression of GK (iPVN-GK)

3.7.1 Summary Method

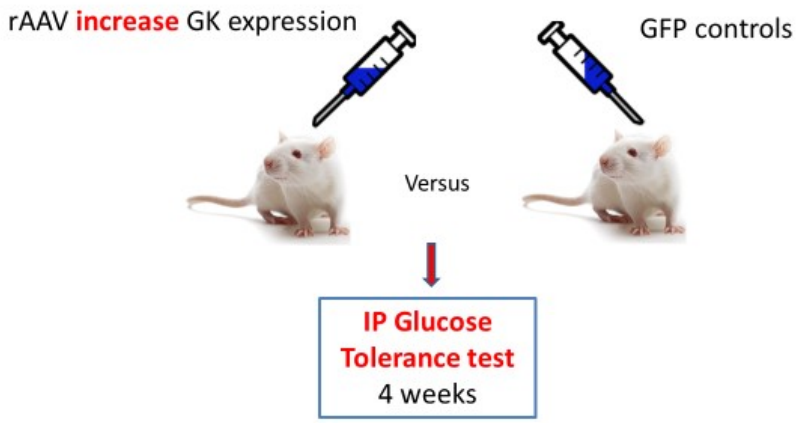


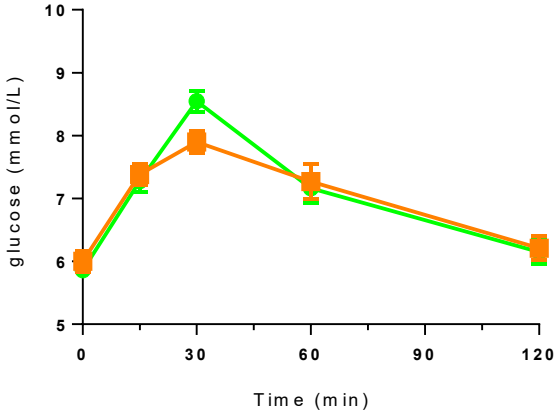
Figure 42: 0.5 microlitres of rAAV-GK (titre: $2.96x10^{12}$ genome particles/ml) was bilaterally injected into PVN of male Wistar rats. GFP was used as a control. All rats underwent an GTT at 4 weeks following PVN surgery to determine glucose tolerance in response to an intraperitoneal glucose load.

In order to establish whether the lack of effect of an intra-peritoneal load was maintained in rats with over-expression of GK, rAAV-GK was bilaterally injected into PVN of male Wistar rats. GFP was used as a control. All rats underwent an intra-peritoneal GTT at 4 weeks following paraventricular nucleus surgery. This method is summarised in Figure 42.

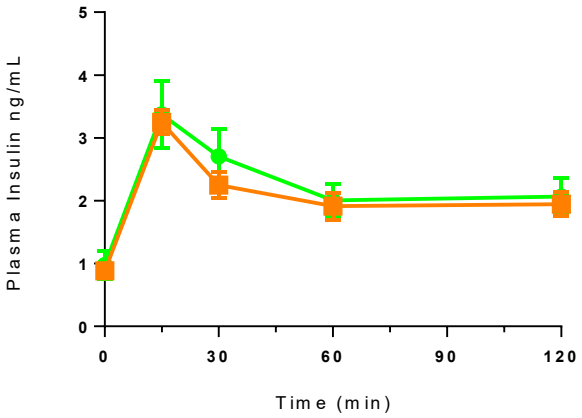
3.7.2 Establishing whether the effects on glucose homeostasis in over-expression of GK are maintained when the oral route of glucose ingestion is bypassed

Similarly to the under-expressed group, iPVN-GK rats had no significant difference in plasma glucose levels compared to control animals (Figure 43a) There was no significant difference in total plasma insulin and GLP-1 release between the two groups (Figure 43b and 43c).

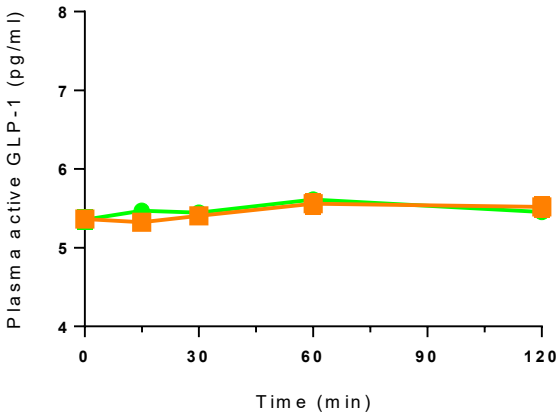
Figure 43: Effect of genetically increased paraventricular nucleus glucokinase activity on intraperitoneal glucose testing: Groups of adult male Wistar rats were injected with either rAAV-GFP (iPVN-GFP) or rAAV-SGK (iPVN-GK) bilaterally into the paraventricular nucleus. They then underwent an intraperitoneal (IP) glucose tolerance test (GTT)



(a) Plasma glucose during IP glucose tolerance test in iPVN-GFP (green) and iPVN-GK (orange) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



(b) Plasma insulin levels during IP glucose tolerance test in iPVN-GFP (green) and iPVN-GK (orange) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



(c) Plasma GLP-1 levels during IP glucose tolerance test in iPVN-GFP (green) and iPVN-GK (orange) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.

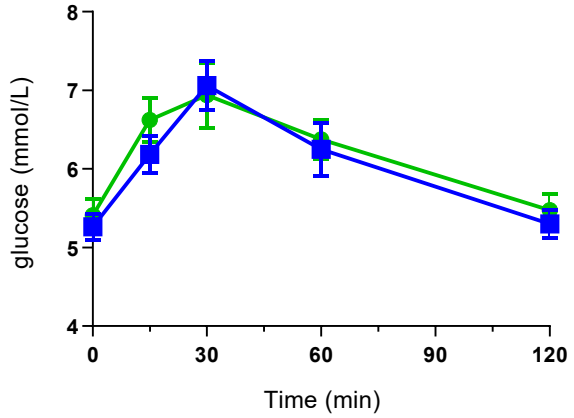
These results suggest that the observed effects of PVN glucokinase alteration on initial GLP-1 secretion is specific to the oral route of administration of glucose as GLP-1 and glucagon release were comparable to controls in both groups during IP GTT. Hence there appears to be a necessary contribution by the incretin effect of GLP-1 to maximise on paraventricular GK activity.

3.8 Paraventricular Study 5: Determining the glucose homeostatic response to alternative nutrient loads

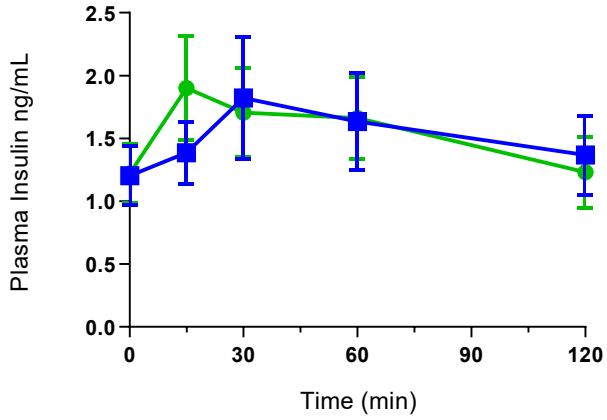
To determine whether oral ingestion of other nutrients has a similar effect as oral glucose on initial GLP-1 release, L-arginine was administered via oral gavage to iPVN-asGK and iPVN-GFP rats. There were no significant differences in plasma glucose, insulin, GLP-1 and glucagon levels between the groups at any time point (Figure 44). Therefore, the impairment of the initial GLP-1 response produced by reduced PVN glucokinase activity observed during the OGTT is specific to glucose, and it does not appear to apply to the other nutrients studied.

Oral gavage courtesy of Phil Rawson, CBS Unit at Hammersmith Campus, Imperial College

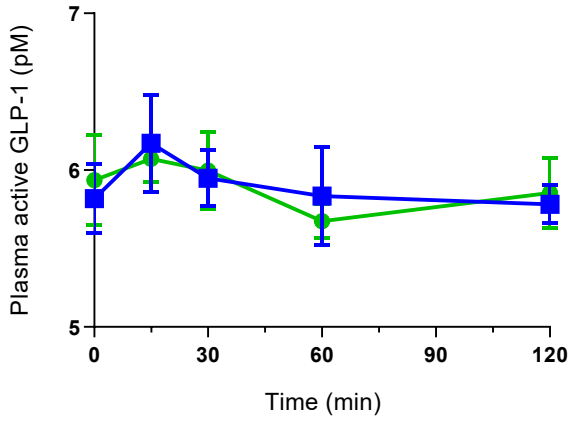
Figure 44: Effect of oral gavage with L-arginine on genetically decreased paraventricular nucleus glucokinase activity. Groups of adult male Wistar rats were injected with either rAAV-GFP (iPVN-GFP) or rAAV-ASGK (iPVN-asGK) bilaterally into the paraventricular nucleus. They then underwent oral gavage with L-arginine followed by a glucose tolerance test (GTT)



(a) Plasma glucose during oral gavage in iPVN-GFP (green) and iPVN-asGK (blue). Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



(b) Plasma insulin during oral gavage in iPVN-GFP (green) and iPVN-asGK (blue). Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



(c) Plasma active GLP-1 during oral gavage in iPVN-GFP (green) and iPVN-asGK (blue). Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.

Hence this effect appears to be specific to orally consumed glucose which is present within the gastrointestinal tract. However, this raises questions as to whether the lack of response to intra-peritoneal glucose is secondary to delayed transit time of IP glucose into the brain or whether glucose must be detected specifically within the gut.

3.9 Paraventricular Study 6: Determining which combination of glucose delivery gives maximal GLP-1 release and benefit to glucose tolerance

3.9.1 Summary Method

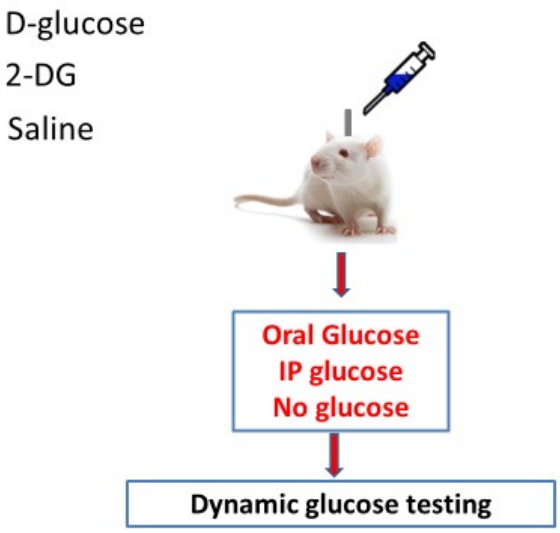


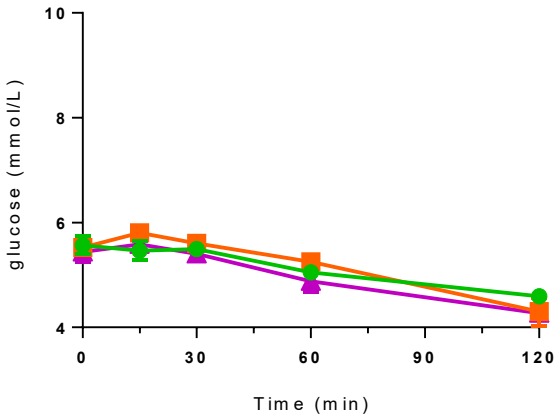
Figure 45: Adult male Wistar rats were injected into the paraventricular nucleus through a stainless steel cannula with either saline vehicle (control), D-glucose or 2-DG. They then underwent either an oral glucose tolerance test, an IP glucose tolerance test or glucose tolerance test with no glucose given. Plasma glucose and insulin levels were measured.

Thus far, glucose within the gut appears to be important in GLP-1 release and subsequent improvement in glucose tolerance. To clarify this, I investigated whether the PVN's glucose-sensing ability is affected by the mode in which the glucose originally enters the system i.e. no glucose load, OGTT or IPGTT. Hence, D-Glucose, 2-Deoxy-D-glucose (2-DG), a glucose derivative which binds to the glucose receptor but does not induce any of the downstream actions of glucose receptor binding, or vehicle were injected into the PVN followed by either no glucose load, OGTT or IPGTT. This method is summarised in Figure 45.

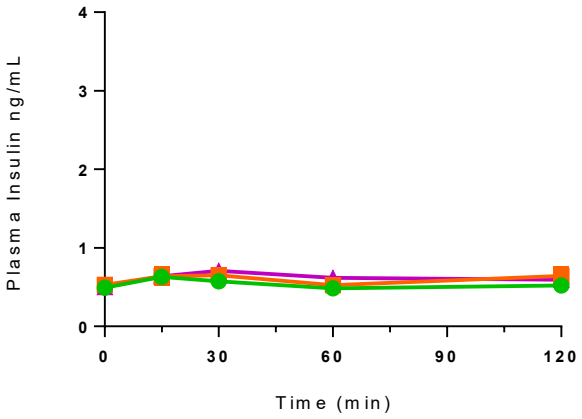
3.9.2 Effect of intra-paraventricular injection of D-Glucose, 2-DG or vehicle when no glucose load is delivered peripherally

In the absence of a glucose load, there was no difference in plasma glucose, insulin or GLP-1 levels, irrespective of iPVN administration of either D-Glucose, 2-DG or vehicle (Figure 46).

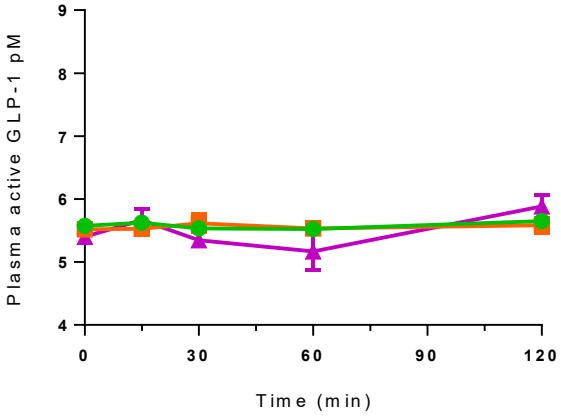
Figure 46: Effect of pharmacological administration of D-glucose and 2-Deoxyglucose versus vehicle administration followed by no peripheral glucose load on glucose homeostasis in Wistar rats: Adult male Wistar rats were injected into the paraventricular nucleus with either vehicle (control) or the competitive antagonist to glucose-6-phosphate (2-DG) or D-glucose.



a. Plasma glucose when no peripheral glucose is given in control (green circle); 2-deoxyglucose injected (purple triangle); D-glucose (orange square) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



b. Plasma insulin levels when no peripheral glucose is given in control (green circle); 2-deoxyglucose injected (purple triangle); D-glucose (orange square) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



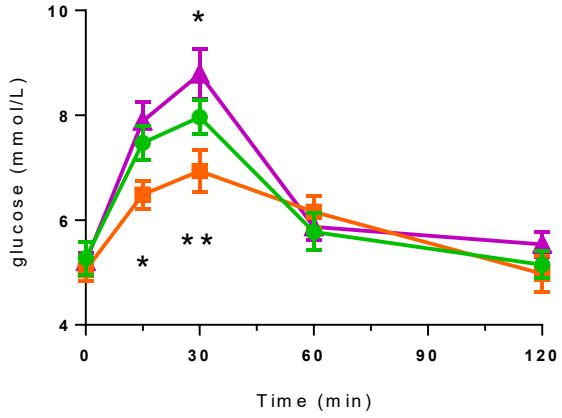
c. Plasma GLP-1when no peripheral glucose is given in control (green circle); 2-deoxyglucose injected (purple triangle); D-glucose (orange square) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.

3.9.3 Effect of intra-paraventricular injection of D-Glucose, 2-DG or vehicle when glucose is given orally

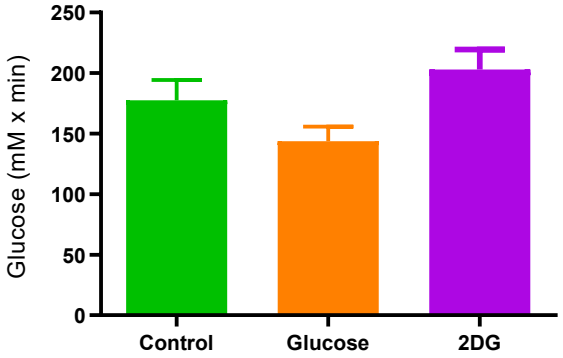
During OGTT, iPVN D-Glucose significantly suppressed plasma glucose levels, whereas 2-DG induced a slight increase in plasma glucose compared to vehicle (15 minutes: iPVN-vehicle: 7.60±0.27 mmol/L, n=10; iPVN-D-glucose: 5.66±0.26 mmol/L, p<0.001 vs vehicle; iPVN-2-DG: 8.39±0.27 mmol/L, p<0.05 vs iPVN-vehicle; 30 minutes: iPVN-vehicle: 8.02±0.32 mmol/L, n=10; iPVN-D-glucose: 6.27±0.30 mmol/L, p<0.001 vs iPVN-vehicle; iPVN-2-DG: 9.32±0.36 mmol/L, p<0.001 vs iPVN-vehicle; 60 minutes: iPVN-vehicle: 5.54±0.41 mmol/L, n=10; iPVN-2-DG: 6.45±0.31 mmol/L, p<0.05 vs iPVN-vehicle) and it resulted in an reduced iAUC in iPVN-D-glucose and an increased iAUC in iPVN-2-DG (Figure 47a and 47b). Correspondingly, insulin levels were significantly higher at 15 minutes and 30 minutes in D-Glucose-injected animals than vehicle-injected animals. Conversely, insulin levels were lower in animals injected with 2-DG (15 minutes: iPVN-vehicle: 2.77±0.19 ng/mL, n=10; iPVN-D-glucose: 3.93±0.15 ng/mL, p<0.001 vs iPVN-vehicle; iPVN-2-DG: 1.88±0.26 ng/mL, p<0.01 vs iPVN-vehicle; 30 minutes: iPVN-vehicle: 2.21±0.25 ng/mL, n=10; iPVN-D-glucose: 3.00±0.31 ng/mL, p<0.01 vs iPVN-vehicle; iPVN-2-DG: 1.48±0.19 ng/mL, p<0.05 vs iPVN-vehicle) and iAUC was increased in iPVN-D-glucose and reduced in iPVN-2-DG (Figure 47c and 47d). Compared with vehicle injection, iPVN D-glucose administration induced a significant rise in GLP-1 secretion at 15 minutes and 30 minutes, whereas, 2-DG administration inhibited GLP-1 secretion (15 minutes; iPVN-vehicle: 6.29±0.19 pMol/L, n=10; iPVN-D-glucose: 8.33±0.29 pMol/L, p<0.001 vs iPVN-vehicle; iPVN-2-DG: 6.01±0.15 pMol/L, p=ns vs iPVN-vehicle; 30 minutes: iPVN-vehicle: 7.07±0.19 pMol/L, n=10; iPVN-D-glucose: 8.44±0.29 pMol/L, p<0.001 vs iPVN-vehicle; iPVN-2-DG: 6.04±0.15

pMol/L, $p<0.001$ vs iPVN-vehicle) and iAUC was increased in iPVN-D-glucose and reduced in iPVN-2-DG (Figure 47e and 47f). Hence, for a significant GLP-1 response, glucose needs to be present in the gut as well as within the PVN.

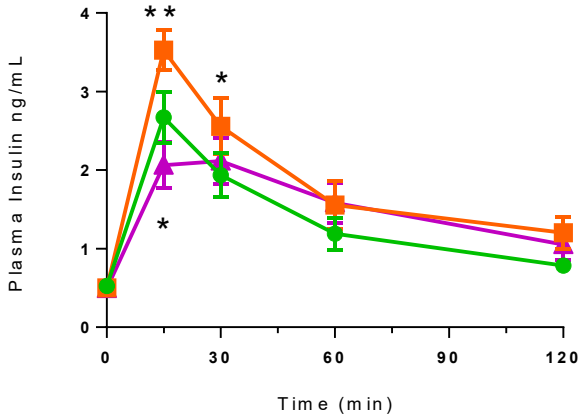
Figure 47: Effect of pharmacological administration of D-glucose and 2-Deoxyglucose versus vehicle administration followed by OGTT on glucose homeostasis in Wistar rats: Adult male Wistar rats were injected into the paraventricular nucleus with either vehicle (control) or competitive antagonist to glucose-6-phosphate (2-DG) or D-glucose.



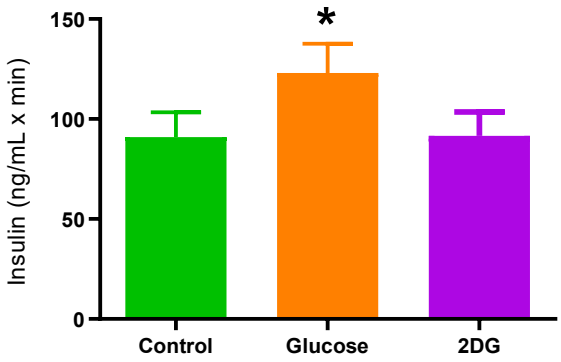
a. Plasma glucose during an oral glucose tolerance test in control (green circle); 2-deoxyglucose injected (purple triangle); D-glucose (orange square) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



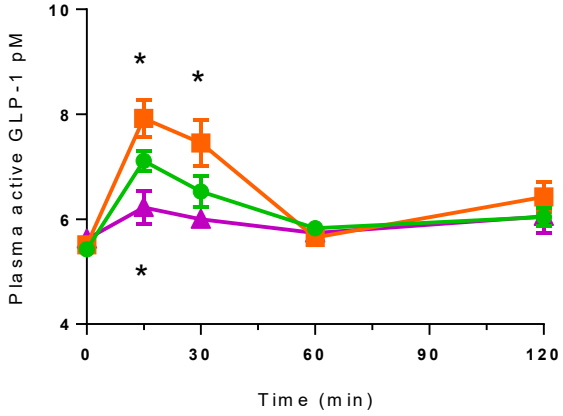
b. Plasma glucose during an oral glucose tolerance test in control (green); 2-deoxyglucose injected (purple); D-glucose (orange) rats. Results shown as area under the curve versus control by ANOVA and post-hoc Holm-Sidak test.



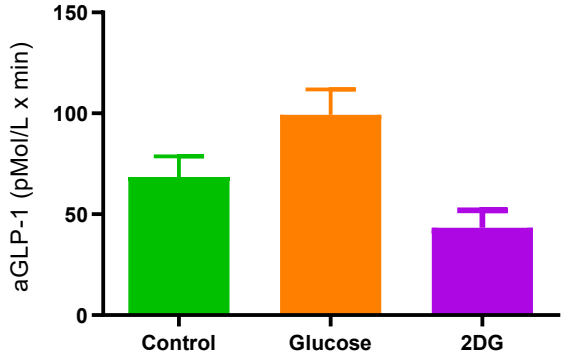
c. Plasma insulin during an oral glucose tolerance test in control (green circle); 2-deoxyglucose injected (purple triangle); D-glucose (orange square) rats. . Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



d. Plasma insulin during an oral glucose tolerance test in control (green); 2-deoxyglucose injected (purple); D-glucose (orange) rats. Results shown as area under the curve versus control by ANOVA and post-hoc Holm-Sidak test.



e. Plasma active GLP-1 during an oral glucose tolerance test in control (green circle); 2-deoxyglucose injected (purple triangle); D-glucose (orange square) rats. . Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.

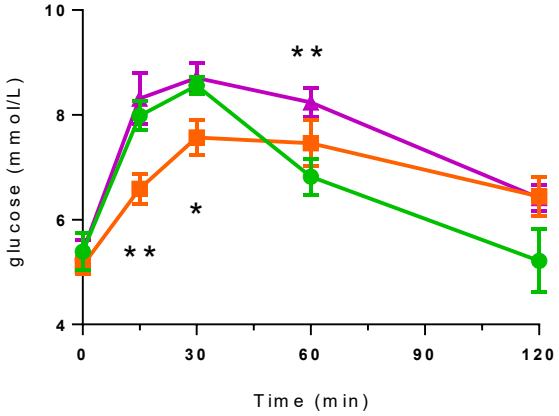


f. Plasma active GLP-1 during an oral glucose tolerance test in control (green); 2-deoxyglucose injected (purple); D-glucose (orange) rats. . Results are shown as area under the curve versus control by ANOVA and post-hoc Holm-Sidak test.

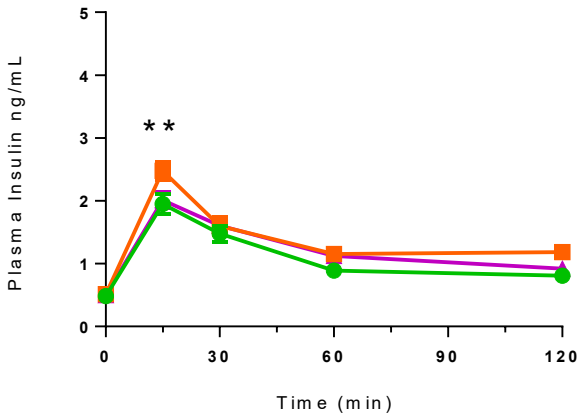
3.9.4 Effect of intra-paraventricular injection of D-Glucose, 2-DG or vehicle when no glucose load is delivered intra-peritoneally

During IPGTT, compared to vehicle administration, iPVN D-Glucose administration (but not 2-DG) produced lower plasma glucose levels at 15 minutes and 30 minutes (15 minutes: iPVN-vehicle: 7.98±0.28 mmol/L, n=10; iPVN-D-glucose: 6.59±0.28 mmol/L, $p<0.01$ vs iPVN-vehicle; iPVN-2-DG: 8.31±0.48 mmol/L, p=ns vs iPVN-vehicle; 30 minutes: iPVN-vehicle: 8.56±0.17 mmol/L, n=10; iPVN-D-glucose: 7.57±0.34 mmol/L, $p<0.05$; iPVN-2-DG: 8.70±0.28 mmol/L, p=ns vs iPVN-vehicle;). However, at 60 minutes, 2-DG injected animals had a higher glucose level (iPVN-vehicle: 6.84±0.33 mmol/L, n=10; iPVN-D-glucose: 7.46±0.44 mmol/L, p=ns vs iPVN-vehicle; iPVN-2-DG: 8.24±0.28 mmol/L, $p<0.01$ vs iPVN-vehicle) (Figure 48a) but neither treatment altered iAUC. Insulin levels were higher in D-glucose injected animals than vehicle-injected animals at 15 minutes (iPVN-vehicle: 1.95±0.16 ng/mL, n=10; iPVN-D-glucose: 2.48±0.15 ng/mL, $p<0.001$ vs vehicle; iPVN-2-DG: 2.01±0.13 ng/mL, p=ns vs iPVN-vehicle) (Figure 48b) but neither treatment altered iAUC. Active GLP-1 levels were higher in D-glucose injected animals than vehicle-injected animals at 15 minutes and 30 minutes (15 minutes: iPVN-vehicle: 5.55±0.04 pMol/L, n=10; iPVN-D-glucose: 6.08±0.30 pMol/L, $p<0.01$ vs vehicle; iPVN-2-DG: 5.60±0.11 pMol/L, p=ns vs iPVN-vehicle; 30 minutes: iPVN-vehicle: 5.56±0.08 pMol/L, n=10; iPVN-D-glucose: 5.96±0.30 pMol/L, $p<0.05$ vs vehicle; iPVN-2-DG: 5.46±0.05 pMol/L, p=ns vs iPVN-vehicle) (Figure 48c) but neither treatment altered iAUC.

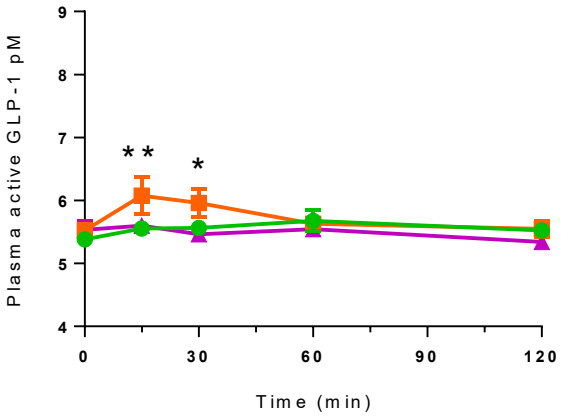
Figure 48: Effect of pharmacological administration of D-glucose and 2-Deoxyglucose versus vehicle administration followed by IPGTT on glucose homeostasis in Wistar rats: Adult male Wistar rats were injected into the paraventricular nucleus with either vehicle (control) or competitive antagonist to glucose-6-phosphate (2-DG) or D-glucose.



a. Plasma glucose during an intraperitoneal glucose tolerance test in control (green circle); 2-deoxyglucose (purple triangle); D-glucose (orange square) rats. . Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



b. Plasma insulin during an intraperitoneal glucose tolerance test in control (green circle); 2-deoxyglucose (purple triangle); D-glucose (orange square) rats. . Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



c. Plasma GLP-1 during an intraperitoneal glucose tolerance test in control (green circles); 2-deoxyglucose (purple triangle); D-glucose (orange square) rats. . Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.

Summary of key findings so far:

I have shown that there is no effect of altered PVN glucokinase activity during an intraperitoneal GTT. However, it is worth noting that following intraperitoneal administration of glucose in rats it is reported to take up to twenty minutes for glucose to reach the brain (Jones et al., 1983). Therefore, the lack of GLP-1 response to IP glucose may well be a consequence of a delay in reaching the brain. To mitigate this, D-glucose, 2-DG and vehicle were injected directly into the PVN of fasted rats. Injection of glucose directly into the PVN was without effect on GLP-1, glucose or insulin levels demonstrating that activation of glucokinase by local glucose levels within the PVN is not sufficient to stimulate GLP-1 release.

However, it is possible that other factors associated with increased glucose, for example secreted insulin, in conjunction with administration of glucose directly in to the PVN, leads to stimulation of GLP-1 release. To test this theory, glucose was injected into the PVN simultaneously with intra-peritoneal glucose. Injection of glucose into the PVN during an intraperitoneal glucose tolerance test (ipGTT) did result in a slight increase in plasma GLP-1 but this was not to the same significance as when glucose was present within the gastrointestinal tract. This slight increase in GLP-1 was associated with minor changes in both glucose and insulin levels. In addition to this, it is important to recognise that the levels of glucose in the PVN following direct injection of iPVN glucose are likely to be markedly higher than those achieved under any normal physiological conditions. These data suggest that the combined presence of elevated glucose levels in the PVN and within the circulation as a whole are not sufficient to significantly stimulate the release of GLP-1. Instead, the combination of glucose being detected both the PVN and by the enteroendocrine L-cells is required to stimulate the release of GLP-1.

3.10 Paraventricular Study 8: Determining the effect of acute pharmacological manipulation of KATP channels within the PVN on glucokinase activity

3.11.1 Summary method

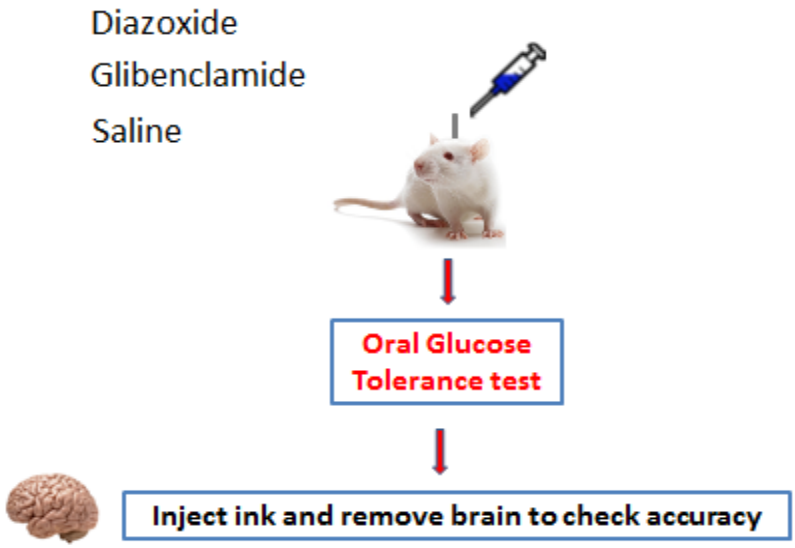


Figure 49: Adult male Wistar rats were injected into the paraventricular nucleus through a stainless steel cannula with either saline vehicle (control) or 0.5nmol of the glucokinase activator compound A (CpdA), 2nmol Glibenclamide or 1nmol Diazoxide. The rats then underwent an oral glucose tolerance test to measure plasma glucose and insulin levels.

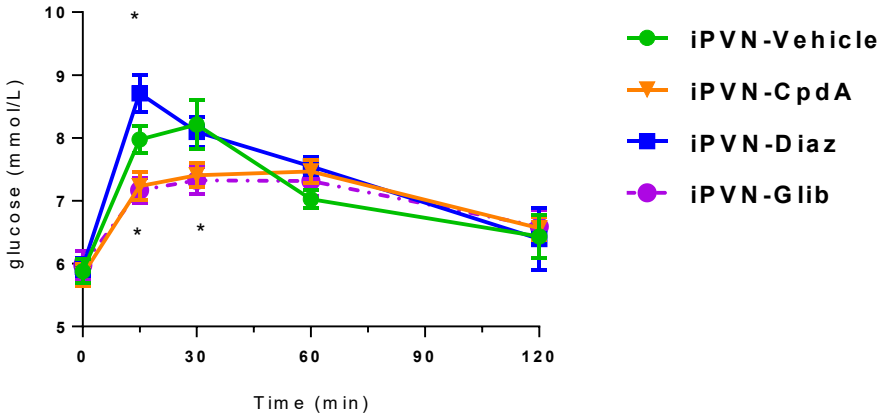
Evidence suggests that glucokinase acts via the K_{ATP} channel to trigger insulin release (Henquin 2000) and the studies in Chapter 2 support this. It is reasonable therefore to hypothesise that PVN glucokinase may be acting via a similar downstream mechanism. To investigate this, glucose homeostasis during OGTT following intra-PVN administration of Compound A (a glucokinase activator) glibenclamide (K_{ATP} blocker) or diazoxide (K_{ATP} activator) was measured in healthy Wistar male rats. A summary of the method is shown in Figure 49.

3.10.2 Dynamic glucose and insulin testing in iPVN cannulated animals

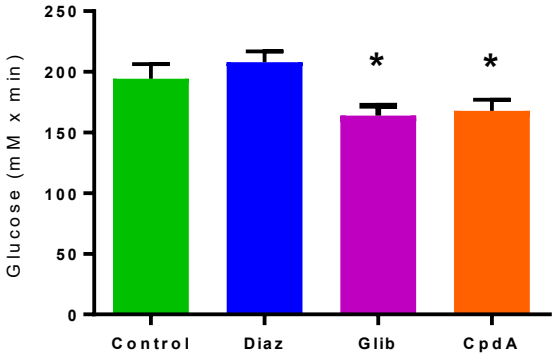
Diazoxide had effects comparable to those obtained in iPVN-ASGK, whereas both glibenclamide and Cpd A had the opposite effects and yielded responses similar to those seen in iPVN-GKS animals (Figure 50a). Diazoxide significantly increased glucose levels at 15 minutes (8.07±0.16 mmol/L iPVN-vehicle vs 9.05±0.22mmol/L iPVN-Diaz) $p<0.01$ (Figure 50a) but it did not affect iAUC (Figure 50b). Diazoxide significantly reduced insulin secretion at 15 minutes (2.62±0.22 ng/mL iPVN-vehicle vs 1.65±0.19 ng/mL iPVN-Diaz) $p<0.01$ (Figure 50c) but it did not affect iAUC (Figure 50d). Diazoxide significantly reduced active GLP-1 release at 15 minutes (6.35±0.19 pMol/L iPVN-vehicle vs 5. 25±0.15 pMol/L iPVN-Diaz) $p<0.05$ (Figure 50e) but it did not affect iAUC (Figure 50f). Glibenclamide significantly reduced glucose levels at 15 minutes (8.07±0.16 mmol/L iPVN-vehicle vs 7.31±0.21 mmol/L iPVN-Glib) $p<0.05$ and 30 minutes (8.38±0.36 mmol/L iPVN-vehicle vs 7.19±0.20 mmol/L iPVN-Glib) $p<0.01$ (Figure 50a) and it resulted in an affected iAUC (Figure 50b). Glibenclamide significantly increased insulin secretion at 15 minutes (2.62±0.22 ng/mL iPVN-vehicle vs 3.88±0.24 ng/mL iPVN-Glib) $p<0.001$ and at 30 minutes (1.88±0.27 ng/mL iPVN-vehicle vs 2.84±0.24 ng/mL iPVN-Glib) $p<0.01$ (Figure 50c) and it resulted in an affected iAUC (Figure 50d). Glibenclamide significantly increased active GLP-1 release at 15 minutes (6.35±0.19 pMol/L iPVN-vehicle vs 7.15±0.30 pMol/L iPVN-Glib) and at 30 minutes (6.82±0.15 pMol/L iPVN-vehicle vs 7.56±0.33 pMol/L iPVN-Glib) $p<0.05$ (Figure 50e) and it resulted in an affected iAUC (Figure 50f). Cpd A significantly reduced glucose levels at 15 minutes (8.07±0.16 mmol/L iPVN-vehicle vs 7.18±0.20 mmol/L iPVN-Cpd A) $p<0.05$ and 30 minutes (8.38±0.36 mmol/L iPVN-vehicle vs 7.43±0.18 mmol/L iPVN-Cpd A) $p<0.01$ (Figure 50a) and it resulted in an affected iAUC (Figure 50b). Cpd A significantly increased insulin secretion at 15

minutes (2.62±0.22 ng/mL iPVN-vehicle vs 4.03±0.27 ng/mL iPVN-Cpd A) $p<0.001$ and at 30 minutes (1.88±0.27 ng/mL iPVN-vehicle vs 2.72±0.32 ng/mL iPVN-Cpd A) $p<0.05$ (Figure 50c) and it resulted in an affected iAUC (Figure 50d). Cpd A significantly increased active GLP-1 release at 15 minutes (6.35±0.19 pMol/L iPVN-vehicle vs 7.13±0.11 pMol/L iPVN-Glib) $p<0.05$ and at 30 minutes (6.82±0.15 pMol/L iPVN-vehicle vs 7.69±0.31 pMol/L iPVN-Cpd A) $p<0.05$ (Figure 50e) and it resulted in an affected iAUC (Figure 50f).

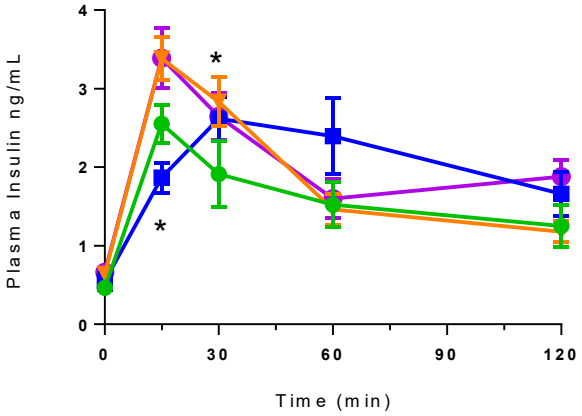
Figure 50: Effect of pharmacologically increased paraventricular glucokinase activity and manipulating activity on glucose homeostasis in Wistar rats: Adult male Wistar rats were injected into the paraventricular nucleus with either vehicle (control) or 0.5nmol of the glucokinase activator compound A (CpdA), 2nmol Glibenclamide or 1nmol Diazoxide



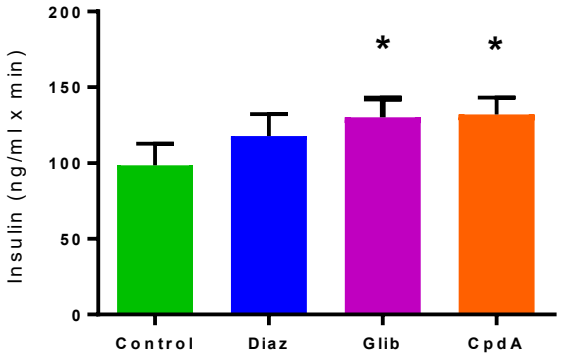
a. Plasma glucose during an oral glucose tolerance test in control (green circle); CpdA injected (orange triangle); Glibenclamide (purple circle) and Diazoxide injected (blue square) rats. . Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



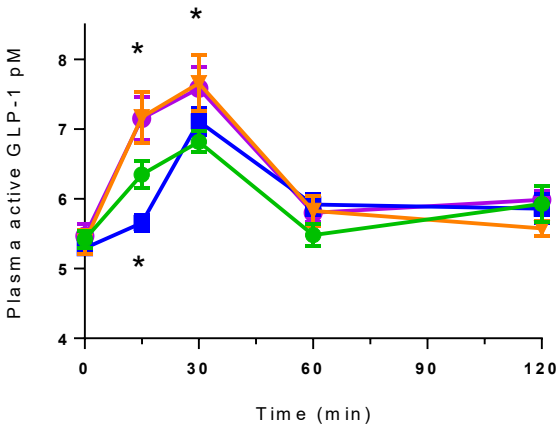
b. Incremental area under the curve analysis of plasma glucose during an oral glucose tolerance test in control; Cpd A injected (CpdA); glibenclamide injected (Glib) or diazoxide (Diaz) injected rats. . Results are shown as area under the curve versus control *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



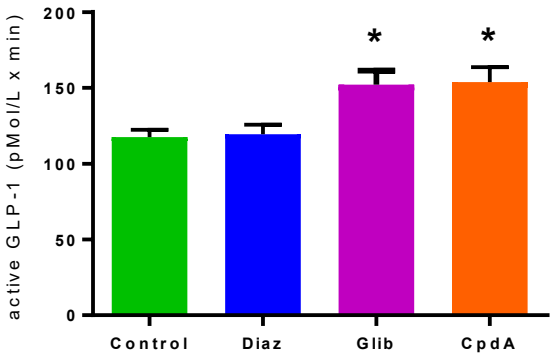
c. Plasma insulin during an oral glucose tolerance test in control (green circle); CpdA injected (orange triangle); Glibenclamide (purple circle) and Diazoxide injected (blue square) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test



d. Incremental area under the curve analysis of plasma insulin during an oral glucose tolerance test in control; Cpd A injected (CpdA); glibenclamide injected (Glib) or diazoxide (Diaz) injected rats. . Results are shown as area under the curve versus control *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.



e. Plasma GLP-1 during an oral glucose tolerance test in control (green circle); CpdA injected (orange triangle); Glibenclamide (purple circle) and Diazoxide injected (blue square) rats. Results are shown as mean ± SEM, *=p<0.05 by ANOVA and post-hoc Holm-Sidak test



f. Incremental area under the curve analysis of plasma GLP-1 during an oral glucose tolerance test in control; Cpd A injected (CpdA); glibenclamide injected (Glib) or diazoxide (Diaz) injected rats. Results are shown as area under the curve versus control *=p<0.05 by ANOVA and post-hoc Holm-Sidak test.

3.11 Discussion

Glucokinase serves as a glucose sensor both in the pancreas and in many glucose sensitive neurons (Li et al., 2003a) such as the VMN and arcuate nucleus (Levin et al., 2008, Hussain et al., 2015b). In this chapter, I identify a role for GK within the PVN in regulating glucose homeostasis specifically through the actions of GLP-1.

From a physiological perspective, down-regulating PVN glucokinase activity *via* stereotactic injection of rAAV containing anti-sense RNA had no effect on the rate of body weight gain, food intake, glucose intake or total energy intake. During an OGTT, iPVN-ASGK rats had a significantly higher plasma glucose level than control animals at 30 minutes hence suggesting that GK within the PVN has a role is restoring euglycaemia after an oral glucose load. Conversely, up-regulating GK within the PVN using rAAV expressing full length glucokinase has the opposite effects to iPVN-asGK and resulted in an improvement in glucose levels compared to controls. These observations support a role for GK within the PVN in glucose homeostasis.

During an OGTT, insulin levels were initially reduced in iPVN-asGK animals and enhanced in iPVN-GK animals, thus suggesting that the hypothalamic PVN has a role in glucose stimulated insulin release. In support of this, infusions of the glucokinase inhibitor, glucosamine, into the third ventricle decreases glucose stimulated insulin release. On the contrary glucose infusions into the third ventricle increases insulin release (Osundiji et al., 2012).

Interestingly, the initial GLP-1 response to oral glucose loading was abolished in rats where GK expression was reduced within the PVN. However, where GK activity was increased, GLP-1 release was increased. This suggests that GK within the PVN may

be exerting its beneficial effects on glucose tolerance by altering GLP-1 release. GLP-1 is predominantly released from the intestinal L-cells and is a potent incretin hormone which acts on the GLP-1 receptor on the pancreatic beta cell to increase insulin secretion (MacDonald et al., 2002). GLP-1 is also thought to promote insulin synthesis (Wang et al., 1995). The effect of reduced PVN glucokinase on GLP-1 release may be due to reduced signalling from the PVN to L- cells, as ileal GCG and PYY gene expression remain unchanged in iPVN-asGK animals (unpublished data from my collaboration with Professor Guy Rutter's laboratory). However, when the incretin effect of GLP-1 is bypassed by administering glucose intraperitoneally, iPVN-asGK animals still have a delayed initial insulin response, raising the possibility that PVN glucokinase may also act directly on pancreatic β-cells to induce insulin release. Together, this suggests that following an oral glucose load, PVN glucokinase can sense plasma glucose changes and act on both pancreatic β-cells and intestinal L- cells to maintain regulate glucose homeostasis. Additionally, this response appears to be specific to glucose as the nutrient rather than an alternative amino-acid load.

Unlike within the VMN where the role of GK is in the counter-regulatory response to hypoglycaemia (Levin et al., 2008), PVN glucokinase does not appear to share a similar role as evidenced by the absence of change of insulin sensitivity. This is in keeping with previous finding within the laboratory showing that glucokinase mRNA levels decrease during fasting (Hussain et al., 2015b).

The downstream pathways by which GK within the PVN exerts its effects is unknown. However, the arcuate work detailed in Chapter 2 suggests that GK acts via the KATP channel. Additionally, Compound A, a GK activator, has been successfully used previously to acutely activate GK. To examine the possible downstream

mechanisms by which PVN glucokinase produces its effects, various pharmacological agents were injected directly into the PVN through an implanted cannula. CpdA mirrored the effects of GK over expression and resulted in an improvement in glucose homeostasis with increased plasma GLP-1 levels. This conforms that the observed responses in the studies are due to a physiological function of PVN glucokinase rather than the result of ectopic expression of glucokinase in neurones in which it is not normally expressed. Additionally, the K_{ATP} channel activator diazoxide and inhibitor glibenclamide, mimicked the effects of virally up and down regulation of GK respectively. These findings suggest that K_{ATP} channels are a key component in the signalling pathway following PVN glucokinase activation. These results are in keeping with previously reported ARC glucokinase activation via K_{ATP} channels (Hussain et al., 2015b) as well as leptin modulation of neurons via hypothalamic K_{ATP} channels (Spanswick et al., 1997, Mirshamsi et al., 2004).

Administration of glucose directly into the PVN promoted insulin and GLP-1 release and decreased plasma glucose levels during OGTT, compared to animals injected with vehicle. Hence, PVN neurons appear to be glucose-responsive neurons, which are capable of sensing the local increase in glucose levels and initiating insulin secretion from β-cells and GLP-1 release from L-cells to restore euglycaemia. This is supported by evidence that administration of a glucose blocking agent such as 2-DG inhibits both insulin and GLP-1 release during OGTT. Interestingly however, the ability of PVN neurons to promote GLP-1 release from L-cells appears to be dependent on the presence of glucose within the gut i.e. oral glucose ingestion, as neither iPVN administration of D-glucose or 2-DG during an IPGTT affected insulin release.

One possible way in which glucokinase within the PVN glucokinase exerts its effects on glucose homeostasis is by the transmission of parasympathetic efferent signals from the PVN to β-cells and L-cells via the vagus nerve. PVN neurons project to the NTS, the DMV, and the intermediolateral cell column of the spinal cord, which contain sympathetic preganglionic neurons (Geerling et al., 2010a) which innervate organs such as the stomach, pancreas and intestines through the vagus nerve (Richards et al., 2014, Duca et al., 2015, Nishizawa et al., 2013). The NTS also produces GLP-1 (Larsen et al., 1997). Electrical vagal stimulation produces an increase in insulin secretion from isolated rat pancreata (Nishi et al., 1987) and this response is partially suppressed by atropine, a nonspecific antagonist for the muscarinic receptors expressed on pancreatic β-cells. Furthermore, vagal innervation of the rat duodenum increases GLP-1 secretion (Rocca and Brubaker, 1999) which is also controlled by muscarinic receptors expressed by L-cells (Anini et al., 2002, Brubaker and Anini, 2003). Additionally, GLP-1 stimulates insulin secretion both directly by acting on GLP-1 receptors (GLP-1Rs) on pancreatic β-cells (Holst, 2013) as well as indirectly through the vagus nerve. For instance, the hepatic and gastrointestinal branches of the vagus nerve can sense endogenous GLP-1 to ameliorate hyperglycemia (Nishizawa et al., 2013, Duca et al., 2015). GLP-1 may also interact with GLP-1Rs in the nodose ganglion. The afferent signal from the nodose ganglion sends impulses back to the NTS and then to the ARC and PVN (Nakagawa et al., 2004, Richards et al., 2014). The efferent signal from the hypothalamus travels to the dorsal vagal complex (DVC) and onwards to the pancreas leading to insulin release. This pathway is supported by the fact that microinjection of the GLP-1 analogue, Exendin-4, into the DVC increases insulin secretion (Babic et al., 2012) and vagotomy blocks the effects of GLP-1 (Abbott et

al., 2005, Iwasaki et al., 2018) hence suggesting that GLP-1 production in the NTS is likely to mediate this signal transmission.

Hence this data does suggest that glucokinase within the PVN plays a role in glucose homeostasis and this is predominantly through stimulation of insulin secretion from β-cells and by promoting GLP-1 release from intestinal L-cells. As with the arcuate nucleus, K_{ATP} channels appear to be involved in the signalling mechanism of PVN neurons. The action of the GLP-1 is likely to be both direct and indirect through stimulation of insulin release through β-cell GLP-1Rs and activation of vagal afferent-mediated neuronal pathways linking the pancreas and intestines. This work provides an important basis for further investigation into the exact way in which GLP-1 release is mediated centrally.

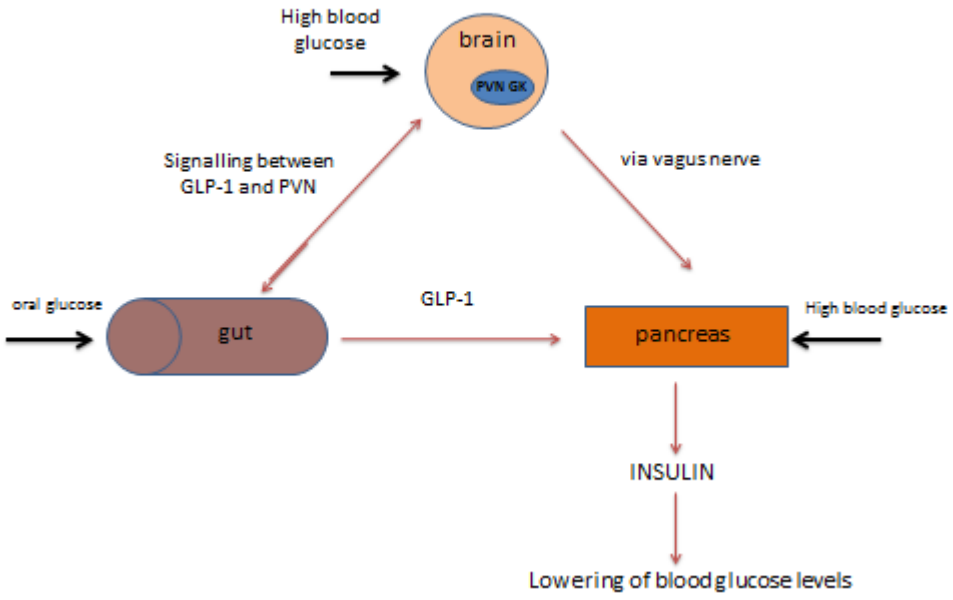


Figure 51: Diagrammatic representation of the hypothesised role of the PVN GK in glucose homeostasis. High blood glucose levels are detected by glucokinase within the PVN. This acts via the vagus nerve to stimulate insulin release. Additionally, when oral glucose reaches the gut, GLP-1 signals to the PVN. This also leads to the release of insulin via the pancreas.

Chapter 4

General Discussion

4.1 Overview of findings

Glucokinase has long been understood to have a role in glucose homeostasis. In humans the glucokinase gene is located on chromosome 7p15.3-p15.1 and is expressed in the brain, liver, pancreas and intestine (Iynedjian, 1993). The hepatic isoform differs from the pancreatic and central isoforms due to tissue specific promotors. This means that regulation and transcription vary in the different tissues but very little is known about specific promoters in the brain. Current knowledge suggests that the different promoters give rise to different transcripts and different sizes of exon 1 (Exon 1a in the pancreas and exons 1b and 1C in the liver) (Osbak et al., 2009). The upstream promoter is functional in the pancreas and the downstream promoter is functional in the liver. Within the liver phosphorylation of glucose by GK promotes glycogen synthesis (Barzilai et al., 1996) and in the pancreatic beta cells GK acts as a glucose sensor (Matschinsky, 1990) ultimately resulting in insulin release.

The pancreas and the brain share the same isoform of GK which is involved in glucose sensing. GK has been studied in other areas of the brain where it has been shown to have role in glucose homeostasis. In the VMN GK has an important role in the counter regulatory response to hypoglycaemia (Levin et al., 2008). Activation of glucokinase expressing neurones within the VMH, using radio waves results in increased blood glucose levels with decreased insulin levels and increased glucagon levels (Stanley et al., 2016b). My laboratory has previously shown that glucokinase within the arcuate nucleus has a role in regulating food intake (Hussain et al., 2015b). In my study I investigated the role of arcuate and paraventricular

glucokinase in glucose homeostasis using neurone specific viral alteration of glucokinase activity in rats. I have shown that increased arcuate GK improves glucose tolerance and increases insulin secretion, at a time point where food intake and body weight are not confounding factors and that the opposite is true when GK activity is virally decreased. The role of glucokinase within the PVN also has an additional role in GLP-1 secretion.

4.1.2 The scientific significance of these findings

Historically, the control of glucose homeostasis has been deemed a pancreas centred process. Findings from my studies combined with the expanding body of evidence supporting the role for the brain in appetite, metabolism and glucose homeostasis suggest that this model is too simplistic.

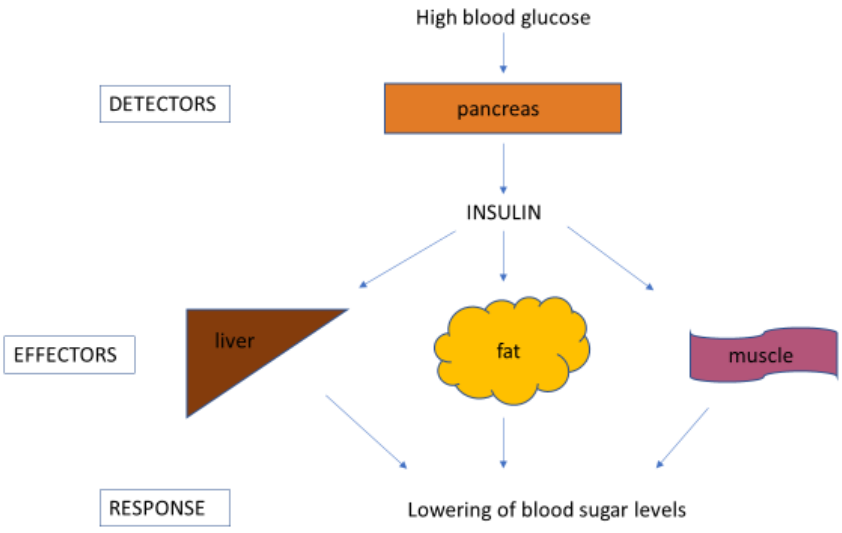


Figure 52: Diagrammatic representation of the traditional glucose homeostatic model

Controlling blood glucose levels is undoubtedly important. Uncontrolled hyperglycaemia acutely can be fatal and in the long term hyperglycaemia has significant microvascular and macrovascular complications which can worsen quality of life and reduce life expectancy. From an evolutionary perspective it seems sensible for such an important process to be shared across organ systems, where failure of one system can be compensated for, to a degree, by another system. The brain is well placed for this role as the hypothalamus is a central hub with connections to the periphery as well as projections within the brain thereby equipping logistically to co-ordinate signals relating to appetite and energy balance.

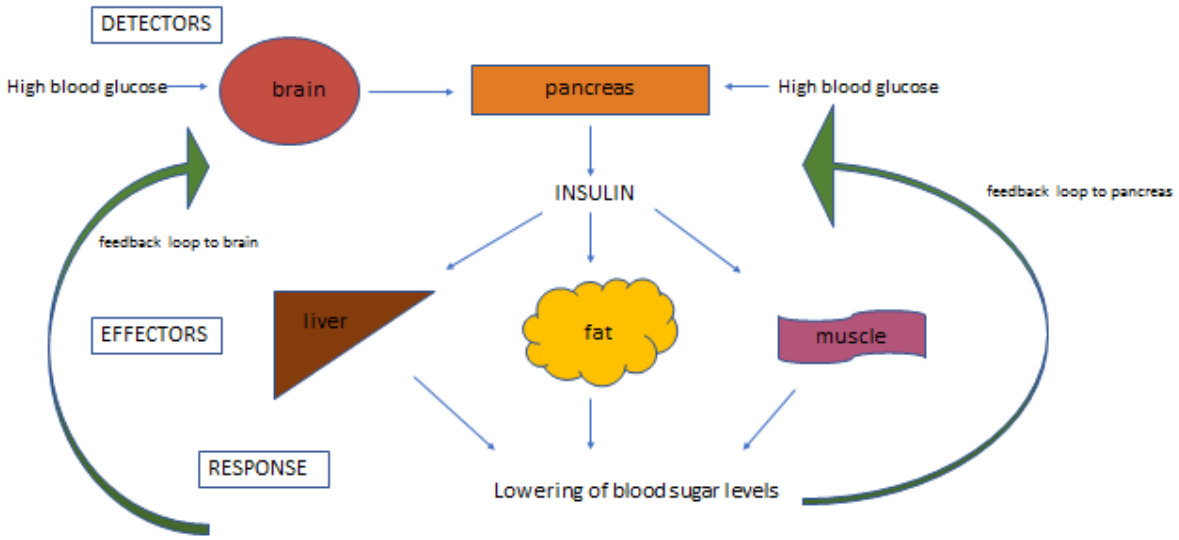


Figure 53: Diagrammatic representation of the brain and the pancreas working in unison to control glucose homeostasis

The arcuate nucleus has long been implicated in energy and metabolism. It's location as the closest nuclei to the blood brain barrier and its extensive connections to other downstream hypothalamic areas implicated in feeding and energy balance fit

well with a possible role in glucose homeostasis. The paraventricular nucleus on the other hand receives signals from the nucleus of solitary tract which itself produces GLP-1. Furthermore, GLP-1 binding sites have been demonstrated in high densities within the PVN (Larsen et al., 1997). These nuclei are likely to be important mediators of glucose homeostasis and this knowledge is important as obesity and diabetes grow in our population. Whilst they might not be the sole controllers or indeed feasible therapeutic targets, understanding their contributions and the pathways that they use as well as their downstream effectors is important academically if we are to understand and better treat type 2 diabetes.

4.2 Therapeutic Potential of Glucokinase Activation

4.2.1 Current therapies for diabetes

There are many classes of oral hypoglycaemic agents which are used to treat Type 2 diabetes. After diet and exercise, the next agent of choice is metformin in the absence of any contraindications. Metformin is a biguanide which decreases intestinal glucose absorption and improves peripheral glucose uptake. There is lowering of fasting plasma glucose and increase of insulin sensitivity (Grzybowska et al., 2011). Metformin also inhibits gluconeogenesis with the activation of AMP-activated protein kinase (AMPK) (Matthaei and Greten, 1991). AMP-activated protein kinase (AMPK) is an important player in the regulation of energy metabolism, which has a key role in diabetes and related metabolic diseases. Where metformin fails to reach therapeutic target or side effects are experienced, available second line agents include insulin secretagogues such as sulphonylureas, insulin sensitisers such as peroxisome proliferator activated receptor γ agonists, incretin mimetics such as GLP-1 agonists and dipeptidyl peptidase inhibitors which prevent the breakdown of endogenous GLP-1. Newly developed inhibitors of the sodium-dependent glucose co-transporter (SGLT) are increasingly being used and these agents reduce renal absorption of glucose. Hence, agents to treat Type 2 diabetes are not in short supply. However, in most cases ongoing β-cell failure (Kahn, 2007) means that sustained improvements in blood sugar levels are not maintained. Furthermore, all these agents have side effects such as weight gain, diarrhoea and genital ulceration which mean that despite there being numerically multiple treatment options, in reality the available therapies are more restricted. Hence, in many cases insulin is introduced. Insulin therapy directly replaces the declining intrinsic hormonal deficit. However, insulin replacement therapy is not without its own issues. These difficulties

relate to problems maintaining healthy, constant blood sugars, problems with patient compliance as insulin is delivered through a subcutaneous injection and unpleasant and dangerous hypoglycaemia. The addition of insulin therapy also has significant lifestyle implications and more so in certain professions such as those operating large transportation vehicles e.g. lorries and planes. Therefore, for many patients, delaying the commencement of insulin is favoured.

4.2.2 Treatment targets within the glucokinase activation pathway

Glucokinase within the brain is hypothesised to act through the same pathways as within the beta pancreatic islet.

Glucose enters the cell through GLUT-2 transporters and is sensed by glucokinase which is the rate limiting step in insulin secretion. Glucokinase controls glycolytic and oxidative ATP production. This determines the ratio of ADP to ATP. ATP generation regulates KATP channel closure. Closure of the KATP channel gradually depolarises the cell leading to opening of the L-Type calcium channel once the membrane potential is reached. This then triggers insulin release.

A major player in understanding the role of central GK is Schwartz et al whose extensive work hypothesises that the role of central glucokinase is to ‘defend a glycaemic set point’. According to this theory, the true significance of GK is only revealed during glycaemic extremities rather than during basal day-to-day glucose control which stays within the healthy range. Hence central activation of glucokinase such as within the arcuate nucleus does not lead to hypoglycaemia and instead restores glucose levels back to a defended glycaemic range. This suggestion is supported by my findings in both the arcuate nucleus and the PVN where baseline

glucose levels are unaltered by GK activation. The activation only shows significance during a glucose tolerance test where a large glucose load is delivered.

4.2.3 K_{ATP} Channels

My studies also confirm an important role for the K_{ATP} channels in glucose homeostasis. Sulphonylureas lead to insulin secretion through action on the K_{ATP} channels and are already commonly prescribed agents in clinical practice. Administration of the sulphonylurea glibenclamide centrally into both the arcuate nucleus and PVN mirrors the effects of glucokinase activation in my rodent models. This raises the possibility of development of centrally active sulphonylureas i.e. those that can cross the blood brain barrier. If indeed Schwartz's theory of defending a glycaemic setpoint is true then, unlike peripheral sulphonylureas, centrally acting sulphonylureas should not cause hypoglycaemia.

4.2.4 Glucose transporters

Yet the hypothesised GK activation pathway also identifies two other potential therapeutic targets; the GLUT-2 transporter and the L-type calcium channel. The GLUT-2 transporter moves glucose from the circulation into the beta islet cell. It therefore lies upstream of GK and is not a downstream consequence of GK activation. There are three different families of glucose transporter which have thus far been described: facilitative diffusion of glucose transporter family (GLUT), sodium-glucose transporter family (SGLT), and sugars will eventually be exported transporter family (SWEET) which is only found in plants (Wright and Turk, 2004, Zehendner et al., 2013, Zhang et al., 2009, Chandran, 2015). The SGLT receptor has been studied in detail and SGLT inhibitors have successfully been brought to market. These agents are featured in the NICE guidelines as potential second line

agents for the treatment of Type 2 diabetes. GLUT transporters are expressed in all tissues (Mueckler and Thorens, 2013) with 14 isoforms identified in humans (Mueckler et al., 1985). Although GLUT-2 transporters have been investigated for therapeutic potential nothing has shown success so far.

4.2.5 Calcium channels

Calcium channel opening occurs downstream of glucokinase activation. Opening of the calcium channels leads to insulin release in pancreatic beta cells. It is hypothesised that opening of calcium channels centrally leads to release of a neurotransmitter such as NPY. An association between calcium channel modifying drugs and diabetes has long been debated. Based on our understanding of GK activation, Ca channel activation should in theory increase insulin release. However, a study looking at human islet cells and murine models for type I and type II diabetes suggests the opposite and showed that a calcium channel blocker such as verapamil inhibit the expression of the proapoptotic proteins in cell lines to include human islets which enhances β-cell survival and function and prevents diabetes in obese mice (Xu et al., 2012). Perhaps, more in line with our hypothesised expectation, a metanalysis investigating the effect of calcium channel blockers on incidence of diabetes was conducted based on concerns that calcium channel blockers can cause or worsen existing diabetes, concluded that incidence of diabetes was not correlated with their use (Noto et al., 2013). Hence central modification of calcium channels would be another avenue of investigation if GK activation is felt to be a viable therapeutic target. However one major issue with this is that the effects would be so widespread that side effects may be an issue.

4.2.6 Glucokinase activity modification in vitro and in vivo

The interest in glucokinase activators stems from the historical understanding of its role in the liver and pancreas. Mice which have a heterozygote GK knockout and subsequent 50% reduction in GK activity specifically in the pancreas and not the liver, show mild diabetes and impaired glucose stimulated insulin release at 10 weeks (Terauchi et al., 1995). In contrast homozygous GK knock out models develop a significantly more severe metabolic phenotype which is incompatible with life beyond 7 days. These studies are supported by other studies using a different system of under expression of GK through the Cre/loxP systems where (Postic et al., 1999a) heterozygotes for GK defects in the pancreas showed a greater impact on glucose tolerance than those with a heterozygous knockout for the liver only. This finding suggests that the pancreatic isoform has a more pivotal role than hepatic isoform in maintaining glucose homeostasis. It is important to note however that although liver GK knockouts showed only mild hyperglycaemia there were pronounced defects in glycogen synthesis and glucose turnover rates. In vivo work also supports these findings, with cell studies showing that glucose stimulated insulin release in these GK knockout models was lower (Grupe et al., 1995). On the other hand, GK over-expression in pancreatic islets resulted in lower blood sugars despite insulin levels being relatively similar to controls in hyperglycaemic clamp models. This finding suggests that glucose modification is not occurring at the level of the pancreas and is instead a consequence of hepatic glucose metabolism leading to a reduction in blood glucose levels. At a cellular level, this group observed that lowering of blood sugar levels through GK over activation interestingly resulted in down regulation of the islet glucokinase expression which, in turn, may have then contributed to the lack of insulin rise (Niswender et al., 1997). These mice also

appear to be protected against hyperglycaemia and hyperinsulinemia associated with consuming a high-fat diet (Shiota et al., 2001). Extending these findings into models of disease, in rodent models of Type 2 diabetes, chronic administration of GK activator (Ro-28-1675) prevented hyperglycaemia in obese mice and administration of four other GKA also did the same in Zucker Fa/Fa rats (GKA20, GKA30, GKA31 and GKA50) (Grimsby et al., 2003).

4.2.7 Glucokinase activators

Glucokinase activators were first described by Grimsby *et al*. in 2003. Since then there have been a series of activators which have gone to trial.

Extending rodent knock out and upregulation findings into models of disease, obese rodent models of Type 2 diabetes exposed to chronic administration of a GK activator (GKA) Ro-28-1675 did not develop hyperglycaemia (Grimsby et al., 2003) and this finding was mirrored in Zucker Fa/Fa rats administration of four other GKAs.

4.2.8 Glucokinase activators in humans

Naturally, the optimistic findings supporting glucokinase activation in animal studies stirred much interest and as a consequence, there have been multiple single and multiple-dose placebo-controlled trials since 2008 in both healthy adults as well as those with type 2 diabetes. One example is Piragliatin, a glucokinase activator, which was trialled in those with mild Type 2 diabetes as part of a randomised double blind three way cross over trial. Piragliatin administration resulted in a dose dependent reduction in blood glucose levels and increase in beta cell function. Another glucokinase activator, AZD1656, was trialled in Type 2 diabetics on metformin in a double blind placebo controlled trail. Here a significant reduction in HBA1c was

observed for 6 months but this was not sustained beyond this. Similarly, the glucokinase activator MK-0941 was trialled in those with Type 2 diabetes receiving insulin. In these individuals who have more severe diabetes marked improvements in HbA1c were seen at 14 weeks but again this improvement was not sustained at 30 weeks. In the latter two, an increased incidence of hypoglycaemia was also reported. In attempt to further salvage the potential therapeutic benefit of GKA, steps were taken to develop partial agonists to GK as well as liver specific glucokinase activators in order to harness the benefits of increased GK activity whilst limiting the risk of hypoglycaemia. However, these efforts were not fruitful. MK-0941 and AZD1656 (Meininger et al., 2011) which also have activity at the level of the liver resulted in an increase in plasma triglyceride levels. However, this data is inconclusive as the findings remain unproven in other studies (Kiyosue et al., 2013). Furthermore, there is some suggestion that despite these issues, co-prescription with a GLP1 agonist may prevent many of the harmful side effects.

4.2.9 Glucokinase activation and effects on beta islet cells

Aside from the systemic effects on circulating blood glucose levels, some studies looking at the beta islet cells in the context of glucokinase expression modification have shown that mice with a genetic deficiency of GK show insufficient B cell hyperplasia, decreased beta cell replication and impaired upregulation of the insulin receptor substrate-2 (IRS2) (Terauchi et al., 2007). This has been further shown in mice fed a high fat diet and administered a GKA for 20 weeks where beta cell proliferation was increased in both GK deficient mice and wild type mice who received a GKA versus those which were not exposed to a GKA (Porat et al., 2011, Oh et al., 2014). Whilst unfortunate, the lack of a sustained effect of GK activation seen in the human trials is not entirely unsurprising. In vitro studies suggest that in

histological samples genetic activation of glucokinase in the beta cells does initially trigger proliferation but then double stranded breaks ensue which trigger activation of the p53 tumour suppressor and subsequent cell death which may account for the transient nature of the improvements seen.

4.3 Adjusting our mindset to the treatment of Type 2 diabetes

My data shows a clear role for arcuate and paraventricular glucokinase in glucose homeostasis. This role does not affect fasting blood sugars but it does improve glucose tolerance in response to a glucose load. Furthermore, unlike the peripheral administration of glucokinase activators, no rats had hypoglycaemic episodes with central glucokinase activation either through viral upregulation or pharmacological activation with Compound A. Studies have shown that the GK activity using virally induced upregulation is maintained for years but this does not mean that its effects on glucose homeostasis are also long term. My studies spanned 8 weeks which is deemed chronic but not of sufficient duration to show benefit for longer than that seen in the clinical trials. Targeting central glucokinase is attractive but difficult. The arcuate nucleus lies closest to the blood brain barrier and thus serves as an accessible target for agents which are able to pass from the circulation and into the brain. Trials into humans with glucokinase activators pre-dated the detailed modern knowledge of central glucokinase and hence it is unclear whether these agents crossed the blood brain barrier when they went to trial. Certainly brain penetrance was not detailed in the published literature from the time. Even if we had a better understanding of the precise mechanism by which central GK exerts its peripheral effects, obvious issues lie with developing a GKA which can cross the blood brain barrier without having the undesirable effects of hypoglycaemia and hyperlipidaemia through direct action on the pancreas and liver. Furthermore, GK has different effects in different nuclei within the brain. I have shown an improvement in glucose tolerance within the arcuate nucleus, and effect on GLP-1 release in the PVN. Others have shown a role in the counter-regulatory response to hypoglycaemia in the VMN. Restricting the effect of a peripherally administered GKA to just one or two

nuclei within the brain without allowing permeation into other nuclei seems challenging.

4.3.1 Ligand Directed Therapy

Looking to the future, perhaps it is our perception of treatments for T2DM that need to change. T2DM is after all a chronic condition which can cause death or be present at death. In that sense, it does not differ massively from other chronic conditions in for example oncology and rheumatology where huge leaps of innovation have been taken.

One example is the fascinating advancement made in ligand directed targeting of genes which enable delivery of a gene vector to a specific site which can ultimately drive gene expression. Major considerations when developing such therapies are: barriers presented by the cell such as in uptake, physical barriers presented by the target tissue and broader barriers relating to the organism as a whole such as immunity and clearance (Wickham, 2003). Applying this to my findings with central glucokinase activation, administering a gene delivery vector systemically in order to target a hypothalamic nucleus would require identification specific genetic features such as receptors or promoters present in central glucokinase which allow it to be targeted specifically without affecting unintended cells as well as for the vector to be able to cross the blood brain barrier with ease. Additionally, the host's immune system must not develop antibodies or serum proteins which inactivate the vector or rapidly clear it in the liver or another organ. It is not an easy task, not least because it is unchartered academic territory. However, similar techniques are being explored in other fields such as the treatment of aggressive variants of prostate cancer. These tumours express a cell surface glucose-regulated protein (GRP78) which can bind to

ligands such as the SNTRVAP motif. Human aggressive prostate cancer cells displaying this protein can be targeted both in vivo and vitro by SNTRVAP. Using this knowledge and ability, human *Herpes simplex* virus thymidine kinase type-1 *(HSVtk)* gene was delivered to tumour cells through a variant of the SNTRVAP motif. HSVtk within the target tissue serves as both a molecular-genetic sensor/reporter and a cell suicide-inducing transgene (Ferrara et al., 2016).

4.4 Future Work

4.4.1 Neuronal signalling

Understanding the mechanism of how the brain is involved in glucose homeostasis is critical to furthering our understanding of diabetes but may also be pivotal in bringing brain-centred diabetes therapy from bench to bedside. Future steps would be to map out the bigger picture extending outside neuronal GK with the arcuate or paraventricular nucleus. It is likely that unlike the pancreatic islet cell which releases insulin in response to GK activation, the neuronal GK results in the release of a neurotransmitter such as NPY. Establishing that neurotransmitter and where it signals to would be the first important step.

4.4.2 Neuronal connection between brain and pancreas

Tracking the path by which the brain signals to the pancreas is also key and is most likely to be through the vagus nerve. An example of experiments to establish the role of the vagus nerve in the regulation of glucose homeostasis by arcuate/PVN glucokinase would be to divide adult Wistar rats into two groups and inject bilaterally into the arcuate/paraventricular nucleus with 1 X 10^{12} genome particles of rAAV encoding either GFP (rAAV-GFP) or sense glucokinase (rAAV-GK). At the same time half of each group will be subject to a sub-diaphragmatic vagotomy while the other half undergo a sham operation. After recovery animals undergo glucose tolerance tests and insulin tolerance tests every three weeks for twelve weeks. This would pave the way to a better understanding of the connection between the brain and the pancreas in rodent models.

4.4.3 Cellular changes at the pancreatic beta islet cell

Beyond this, understanding the changes that central manipulation of GK has on the pancreatic islet cells is critical. Certainly from a peripheral organ perspective, pancreatic homeobox-1 is the major regulator of glucose stimulated insulin gene transcription. Interestingly, its mRNA level was significantly increased by administration of peripheral glucokinase activators which suggests that pharmacological glucokinase activation improves the function of the b-cells at the transcriptional level (Nakamura et al., 2012). Additionally, piragliatin administered to isolated islets from Type 2 diabetic organ donors shows that it repairs defective insulin secretion, improves oxygen consumption and the calcium response to glucose (Doliba et al., 2012b, Doliba et al., 2012a). Furthermore, glucokinase knock out at the level of the beta cells leads to decreased b cell replication and impaired upregulation of insulin receptor substrate-2 (IRS-2) despite similar levels of insulin resistance (Terauchi et al., 2007). Additionally, gain of function mutations of GK in humans are associated with increased beta cell proliferation (Kassem et al., 2010). My data suggests that increased arcuate and paraventricular glucokinase results in increased glucose stimulated insulin release to mediate its beneficial effects on glucose homeostasis. However, the downstream effects of central activation of glucokinase is currently unknown. To determine the mechanisms by which increased arcuate glucokinase results enhancement of insulin secretion at the level of the beta cell various experiments will be needed such as (1) beta cell mass estimation as a percentage of pancreatic volume using optical projection tomography of whole pancreata (Sun et al., 2010) (2) beta cell : alpha cell ratio establishment by quantitative immunohistochemical analysis of pancreatic slices stained for insulin or glucagon (antibodies from Dako) (Sun et al., 2010); (3) understanding the dynamics

of insulin secretion from isolated islets during perifusion with varying concentrations of glucose (3,11, 16 mM) or other secretagogues (glutamine plus leucine, KCl), as well as potentiators of secretion including GLP-1 (0.1 – 10 nM), acetyl choline (1-10 μM) and sulphonylureas (tolbutamide, 1-100 μM).

The cellular mechanisms underlying the changes could also be examined. Glucose metabolism producing ATP can be measured using confocal imaging of the adenovirally-expressed ATP/ADP sensor Perceval) (Tarasov et al., 2012) and biochemical measurements of glucose oxidation and utilisation in isolated islets with radiotracers (Sekine et al., 1994). The effect on membrane excitability can be measured using patch clamp electrophysiology (Tarasov et al., 2012) and intracellular cAMP generation in response to elevated glucose with the FRET-based probe Epac2-camps (Everett and Cooper, 2013). Calcium imaging can be performed in whole islets using the trappable calcium probe fluo2, assessing both maximal responses and the degree of intercellular connectivity (Hodson et al., 2013). Secretory granule distribution and dynamics can be determined using electron microscopy and total internal reflection of fluoroscence (TIRF) imaging, respectively (da Silva Xavier et al., 2009). Finally, changes in the expression of key genes involved in beta cell development (Pdx1, MafA, neuroD1), glucose sensing (*Glut2, Gck, Kcnj11, Abcc8*) or responses to secretory potentiators (*Glp1r, Gipr sstr etc*) can be quantified by qRT-PCR. These studies will identify the effects of increased arcuate or paraventricular glucokinase on pancreatic beta cell function.

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Appendix

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WILEY

ORIGINAL ARTICLE

Hypothalamic arcuate nucleus glucokinase regulates insulin secretion and glucose homeostasis

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Aims: To investigate the role of arcuate glucokinase (GK) in the regulation of glucose homeostasis.

Materials and methods: A recombinant adeno-associated virus expressing either GK or an antisense GK construct was used to alter GK activity specifically in the hypothalamic arcuate nucleus (arc). GK activity in this nucleus was also increased by stereotactic injection of the GK activator, compound A. The effect of altered arc GK activity on glucose homeostasis was subsequently investigated using glucose and insulin tolerance tests.

Results: Increased GK activity specifically within the arc increased insulin secretion and improved glucose tolerance in rats during oral glucose tolerance tests. Decreased GK activity in this nucleus reduced insulin secretion and increased glucose levels during the same tests. Insulin sensitivity was not affected in either case. The effect of arc GK was maintained in a model of type 2 diabetes.

Conclusions: These results demonstrate a role for arc GK in systemic glucose homeostasis.

KEYWORDS
glucokinase activator, glycaemic control, neuropharmacology, pharmacogenetics

1 | INTRODUCTION

Glucokinase (GK) is a member of the hexokinase family[1] with important roles in glucose sensing and disposal. There are two isoforms of GK, a hepatic form, expressed exclusively in the liver, and a neuroendocrine form expressed in pancreatic β cells and the central nervous system (CNS).[2] These two isoforms have identical kinetic properties and differ only in the promoter utilized.[3]

In the CNS, GK is expressed in neurons, astrocytes and tanycytes. In neurons it is co-expressed with GLUT-2 and ATP-sensitive potassium channels (K_{ATP})[4,5] and is part of the glucose-sensing mechanism,[4,7] acting in a manner analogous to its role in pancreatic

Yue Ma and Risheka Ratnasabapathy contributed equally to this work.

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