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Investigation of KATP channel function in response to metabolic and pharmacological manipulation, in the hypothalamic GT1-7 cell line

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Chapter 1

Introduction
1.1 Diabetes

Compared to previous human existence, modern day society has a much more sedentary lifestyle which, coupled with an increasingly high fat and high sugar diet, has led to a rise in the worldwide prevalence of type 2 diabetes and obesity (Flier, 2004). For reasons not entirely understood, the occurrence of type 1 diabetes is also on the rise. Type 1 diabetes results from autoimmune reactive destruction of the insulin secreting pancreatic \( \beta \)-cells, rendering the patient glucose intolerant. However, it is now thought that environmental factors, such as early life diet, and genetic predisposition may contribute to the development of this condition (Vehik and Dabelea, 2011). Intensive insulin therapy is the main form of treatment for individuals with type 1 diabetes, due to the lack of endogenous insulin secretion, in order to avoid postprandial hyperglycaemia. Hyperglycaemia, when left untreated for a long period of time, can lead to serious health complications such as diabetic retinopathy, neuropathy and nephropathy (1993). However, intensive insulin therapy can also cause dangerous levels of hypoglycaemia.

1.2.1 Hypoglycaemia

Hypoglycaemia is defined as a fall in systemic plasma glucose levels to \( \leq 4.4 \) mM, at which point the release of the glucose-lowering hormone insulin, along with zinc and \( \gamma \)-amminobutyric acid (GABA), from the pancreatic \( \beta \)-cell are decreased (Slucca et al., 2010, Zhou et al., 2007, Xu et al., 2006). The reduction in these \( \beta \)-cell secretory factors alleviates inhibition of the pancreatic \( \alpha \)-cell and allows the release of glucagon which acts on the liver to stimulate glycogenolysis and gluconeogenesis. If circulating glucose levels fall further (3.3 – 3.8 mM) efferent neurons of the sympathetic nervous system are activated and stimulate the release of adrenaline from the adrenal medulla which acts to mobilise intracellular fuel stores from skeletal muscle and adipose tissue, such as fatty acids, glutamine and glucose from
muscle glycogen breakdown. This can then be utilised for gluconeogenesis in the liver to increase blood glucose. Hypoglycaemia below the aforementioned levels will activate the release of growth hormone and cortisol which stimulate lipolysis and further upregulate gluconeogenesis (Cryer, 2008). These neuronal and hormonal counter-regulatory responses (CRR) to hypoglycaemia, along with behavioural responses such as carbohydrate ingestion, are crucial for ensuring that the brain has a constant supply of glucose, the preferred form of fuel for this organ (Sokoloff et al., 1977).

1.2.2 Recurrent hypoglycaemia and hypoglycaemia unawareness.

For individuals with type 1 diabetes, the main obstacle in the way of good glycaemic control is recurrent hypoglycaemia (RH), resulting from supraphysiological exogenous insulin supplementation. Patients with type 1 diabetes can experience on average two hypoglycaemic episodes per week (Ovalle et al., 1998). Studies in both humans and rodents have demonstrated that recurrent episodes, or even just a single exposure, of antecedent hypoglycaemia will blunt the neuronal and hormonal CRRs to further hypoglycaemia on the following day, in both healthy and diabetic subjects (Adamson et al., 1984, Davis and Shamoon, 1991, Sanders et al., 2006, Inouye et al., 2002, Heller and Cryer, 1991, Jacobson et al., 2006). However, the behavioural responses to CRR, such as food seeking, are not always attenuated indicating that these processes may be regulated through separate mechanisms to the behavioural and neuronal (Sanders et al., 2006). This results in the dangerous phenomena of hypoglycaemia unawareness, whereby the glycaemic threshold for the activation of CRRs to hypoglycaemia has been reset so that blood glucose levels have to fall further than before in order to initiate a response. Although the evolutionary and molecular mechanisms underlying hypoglycaemia unawareness are still unclear, a vast amount of evidence now exists in the literature that implicates a role for central glucose-sensing in hypoglycaemia
detection and that adaptations may be occurring in the brain following RH which are contributing to hypoglycaemia unawareness.

1.3.1 Brain glucose-sensing

In order to monitor and respond appropriately to fluctuations in circulating glucose levels, peripheral and central glucose sensors have evolved in order to maintain glucose levels within a healthy euglycaemic range (3.9 – 5.5 mM). Peripheral glucose sensors exist within the pancreas, gut, the carotid body and the portal/mesenteric vein (PMV) of the liver, the latter of which has been found to relay information to the lateral hypothalamus (LH) and the nucleus of the solitary tract (NTS) through vagal afferent nerves. This was shown to reduce the activity of neurons in these brain areas, that were also found to be sensitive to inhibition by glucose (Lopez-Barneo, 2003, Adachi et al., 1984, Shimizu et al., 1983, Parker et al., 2009, Reimann et al., 2008). Peripheral glucose sensors seem to play a role in the immediate sensing of hypoglycaemia and relaying that information to the CNS, where the appropriate response to the level of hypoglycaemia is then regulated. Evidence for this comes from the observation that more than 70% of hypoglycaemia in humans occurs at a slow onset (≤0.05 mM/min), a rate at which the PMV senses hypoglycaemia and relays this information to the brain (Kovatchev et al., 2005). In vivo hyperinsulinaemic-hypoglycaemic clamp studies examining the effects of denervated PMV found that the CRR to slow onset hypoglycaemia was attenuated, in the form of reduced adrenaline and noradrenaline release, and coincided with a decrease in hindbrain neuronal activity (Saberi et al., 2008, Routh et al., 2012). In contrast, there was no difference in the CRR with fast onset hypoglycaemia (≥1.11 mM/min) in denervated PMV rodents, implying that central glucose sensors are important for the detection of fast onset hypoglycaemia (Routh et al., 2012, Saberi et al., 2008). However, as less than 10% of hypoglycaemia experienced by patients is fast onset, PMV hypoglycaemia
detection in likely to be important in the initial detection of low circulating glucose in most cases (Kovatchev et al., 2005).

The earliest study that demonstrated the importance of the hypothalamus in sensing and modulating the CRR to hypoglycaemia was by Borg et al. in 1994. This study showed that lesioning of the VMH disrupted the sympatho-adrenal and glucagon responses during glucose deprivation in rats (Borg et al., 1994). The same group also demonstrated that neuroglucopenia specifically in the VMH, during systemic euglycaemia, stimulated the neuronal and hormonal CRRs (Borg et al., 1995). Conversely, this group also found that glucose perfusion into the VMH blunted the CRR during systemic hypoglycaemia (Borg et al., 1997). Taken together, these studies provided strong evidence for a role of VMH glucose sensors in modulating the CRR to hypoglycaemia, especially the sympatho-adrenal and glucagon response. However, the exact neurocircuitary linking the VMH to glucose control in the CRR remains largely unknown due to lack of information regarding the neurotransmitter/peptide phenotype of glucose-sensing neurons in this brain region.

Hypothalamic “glucoreceptors” were first proposed by Jean Mayer in the early 1950s as part of his “glucostatic hypothesis” whereby the hypothalamus was considered capable of translating changes in cerebral ambient glucose levels into electrical activity in order to regulate food intake (Mayer, 1955). 10 years later, specialised glucose-sensing neurons were first reported by the labs of Oomura et al. and Anand et al. who showed that the electrical activity of hypothalamic neurons could be directly regulated by extracellular glucose availability, in both in vivo and ex vivo brain slices (Oomura et al., 1964, Anand et al., 1964, Ono et al., 1982). Unlike most neurons, glucose-sensing neurons use glucose not only as a fuel source but also as a signalling molecule in order to regulate electrical activity. The activity of these specialised neurons is regulated over the physiological glucose concentration
range found the brain 0.5-3.5 mmol/l, which is about 30% of glucose levels found in the periphery (Kang et al., 2004). The evolutionary purpose of central glucose sensors is most likely due to the fact that the brain has an absolute need for glucose due to high energy demanding processes such as action potential firing and neurotransmitter/hormone release (Sokoloff et al., 1977). Two distinct phenotypes of neurons were first observed in the aforementioned early studies. Glucose excited (GE), whereby the appearance of glucose increased neuronal firing, and glucose inhibited (GI) where neuronal excitability reduced in response to glucose. GE and GI neurons have been found to co-exist in similar brain regions such as the LH, ventromedial hypothalamus (VMH) encompassing the arcuate nucleus (ARC) and ventromedial nucleus (VMN), amygdala, NTS and hindbrain (Adachi et al., 1984, Mizuno and Oomura, 1984, Shimizu et al., 1983, Spanswick et al., 2000, Rowe et al., 1996a, Nakano et al., 1986, Ritter et al., 1998, Burdakov et al., 2005, Tovar et al., 2013). The mechanisms by which GE and GI neurons sense glucose and the physiological processes that they regulate have been the focus of many studies. In the case of GI neurons, the reduction in activity observed during a rise in ambient glucose levels has been attributed to activation of an outward K⁺-selective leak current, opening of an ATP-sensitive K⁺ (K_{ATP}) channel and stimulation of an inward Cl⁻ current (Rowe et al., 1996b, Murphy et al., 2009, Burdakov et al., 2006). The most well characterised mechanism for the increased firing of GE neurons, in response to an increase in ambient glucose concentration, is the inhibition of K_{ATP} channels through a rise in intracellular ATP production from glucose metabolism (Ashford et al., 1990a, Ashford et al., 1990b, Lee et al., 1999). This K_{ATP}-dependent mechanism is similar to that which regulates the excitation of the pancreatic β-cell (Fig. 1) (Ashcroft et al., 1984). The mechanisms outlined for the function of GE and GI neurons tend to be regulated either metabolism-dependent pathways or metabolism-independent pathways. The focus of this
introduction will be mainly on metabolically regulated glucose-sensing mechanisms, although both will be discussed in more detail further on.
Figure 1 Schematic diagram of a glucose-excited neuron

(A) When extracellular glucose levels are high, glucose is metabolised which increases the intracellular ATP:ADP ratio. This results in closure of the $K_{ATP}$ channel, membrane potential depolarisation and increased action potential firing. (B) When extracellular glucose levels are low, the intracellular ATP:ADP ratio falls. This causes activation of $K_{ATP}$ channels and hyperpolarisation of the cell membrane, which silences action potential firing.
1.4 The role of glucose-sensing neurons in nutrient homeostasis.

Glucose-sensing neurons are mainly located in hypothalamic nuclei involved in the regulation of feeding, energy homeostasis and whole body nutrient metabolism. Hypothalamic glucose-sensing neurons also respond to nutrient metabolites such as lactate, ketones and free fatty acids (Himmi et al., 2001, Minami et al., 1990, Oomura et al., 1975). Glucose-sensing neurons express receptors for hormones such as insulin and leptin, which in a healthy individual correlates with nutritional status and fat stores (Spanswick et al., 1997, Spanswick et al., 2000, Kang et al., 2004). Both leptin and insulin are elevated during high blood glucose levels and under these conditions reduce the firing rate of some hypothalamic GE neurons [32, 41].

Anabolic neuropeptide Y (NPY) and catabolic proopiomelanocortin (POMC) neurons of the ARC are important modulators of energy homeostasis, producing orexigenic and anorexigenic outcomes upon activation, respectively (Schwartz et al., 2000). These opposing outcomes are brought about through the differing effects of hormones, such as leptin and insulin, which signal whole-body energy status to POMC and NPY neurons. For example, while anorexigentic leptin increases POMC firing rates and mRNA expression, the firing and expression of NPY is reduced (Elias et al., 1999, Cowley et al., 2001, Baver et al., 2014). To coincide with the role of these neurons in energy homeostasis, there is also some evidence in the literature to suggest that a proportion of POMC and NPY neurons exhibit GE and GI properties, respectively (Ibrahim et al., 2003, Muroya et al., 1999, Sergeyev et al., 2000, Marston et al., 2011). The mechanisms by which POMC and NPY neurons detect glucose differs in that POMC neurons are thought to do so through glucose metabolism-dependent inhibition of $K_{ATP}$ channel whereas NPY neurons react to a rise in glucose through an as of yet unidentified Cl$^{-}$ channel (Parton et al., 2007, Claret et al., 2007, Fioramonti et al., 2007).
However, there are discrepancies in terms of glucose-sensing in the regulation of energy homeostasis in relation to the actions of leptin on GE neurons. Early electrophysiological studies of unidentified VMH GE neurons demonstrated that leptin reduces the activity of GE neurons through activation of the K\textsubscript{ATP} channel but more recently leptin has been shown to excite POMC neurons through PI3-kinase-dependent activation of a transient receptor potential cation channel (TRPC), resulting in a depolarising inward current (Qiu et al., 2010, Spanswick et al., 1997, Cowley et al., 2001). Other electrophysiological studies have shown that leptin has no effect on the electrical activity of non-POMC expressing GE neurons in the ARC (Wang et al., 2004). Therefore, while VMH glucose-sensing neurons may have a role in modulating systemic metabolic processes through nutrient metabolite sensing, it is still unclear how much of a role glucose-sensing plays in meal initiation. However, the role of glucose-sensing neurons in hypoglycaemia detection, and maintaining whole-body glucose homeostasis, is more well defined and will be the focus of the rest this discussion.

1.5.1 Metabolism-dependent glucose-sensing

The most well characterised of the two glucose-sensing neuronal phenotypes are GE. These were first identified in the VMH (which will be defined here as encompassing the VMN and ARC) and show functional similarities with glucose-sensing mechanisms in the pancreatic β-cell (Ashford et al., 1990b, Ashcroft et al., 1984). Both of these studies demonstrated similar glucose sensing mechanisms by which membrane depolarisation was dependant on a rise in extracellular glucose and inhibition of the K\textsubscript{ATP} channel. In the β-cell model, glucose enters the cell via the GLUT2 transporter, is phosphorylated by glucokinase (GK) to glucose-6 phosphate which is then metabolised by glycolytic enzymes to pyruvate, which enters the mitochondria through pyruvate dehydrogenase (PDH). Pyruvate can also be converted to lactate by lactate dehydrogenase (LDH) in the cytoplasm, which is then extruded from the
cell by monocarboxylic acid transporters (MCT). Once in the mitochondria, pyruvate is further processed by the tricarboxylic acid (TCA) cycle and subsequently oxidative phosphorylation by the electron transport chain. This results in an increase in the global adenosine triphosphate (ATP):adenosine diphosphate (ADP) ratio, which consequentially inhibits the $K_{ATP}$ channel in the plasma membrane, depolarises the cell membrane and increases electrical activity (Ashcroft and Rorsman, 2004). However, it is still unclear whether a rise in wide-spread cellular ATP, from oxidative metabolism, or localised ATP concentration at the plasma membrane, through glycolysis, is more important in the regulation of $K_{ATP}$ channel activity (Ainscow et al., 2002). It has also been demonstrated that $K_{ATP}$ channel (isoform Kir6.2/SUR1) containing GE neurons are at least partially reliant on intact function of cellular glucose metabolising machinery such as glucose transporters (GLUT2, 3 and 4), GK, mitochondrial respiration and the energy deficit sensing enzyme, AMP-activated protein kinase (AMPK) for normal function (Beall et al., 2010, Parton et al., 2007, Arluison et al., 2004, Dunn-Meynell et al., 2002). Evidence for this type of metabolic regulation of electrical activity has been demonstrated in a number of GE populations such as LH melanocortin–concentrating hormone (MCH) neurons and POMC neurons of the ARC (Parton et al., 2007, Kong et al., 2010).

As well as metabolic dependent GE neurons, the electrical activity of some GI neurons has also been found to be reflective of the metabolic state of the cell. Electrophysiological studies have demonstrated that the low glucose-induced excitation of GI neurons in the VMN may be due to the closure of an ATP-activated chloride ion channel. When active, this channel facilitates an inhibitory inward $Cl^-$ current which silences the neuron (Song et al., 2001, Murphy et al., 2009). In a separate study, recordings from an unidentified population of VMH GI neurons showed that these cells reduce action potential firing in response to a rise in
extracellular glucose and displayed an increase in single-channel activity of an ATP-activated 
K⁺ channel (Rowe et al., 1996b).

In vivo studies have been conducted where the normal function of the cellular glucose 
metabolising machinery was genetically or pharmacologically altered or removed from 
specific brain areas and populations of neurons. These studies have demonstrated that 
metabolic-dependent glucose-sensing has an important role in maintaining whole body 
glucose homeostasis and will now be discussed in more detail.

1.5.2 The role of central K\textsubscript{ATP} channels in glucose-sensing

K⁺ selective ion channels that are regulated by adenosine nucleotides (ATP and MgADP) are 
present in many tissues including pancreatic β-cells, cardiac, skeletal muscle and the brain 
(Ashford et al., 1990a, Ashcroft et al., 1984, Budas et al., 2004, Tricarico et al., 2010). The 
physiological function of K\textsubscript{ATP} channels is to couple cellular metabolism to the electrical 
activity of the plasma membrane.

K\textsubscript{ATP} channels are octomeric structures with a 4:4 stoichiometric ratio of inward rectifying, 
K⁺-selective, pore forming subunits (K\textsubscript{ir}x) and regulatory sulphonylurea receptor subunits 
(SUR). K\textsubscript{ir}x and SUR must be expressed together with this stoichiometry in order to form a 
fully functioning K\textsubscript{ATP} channel, in terms of ATP sensitivity and inward rectifying K⁺ 
conductance (Clement et al., 1997). The inward rectifying, pore forming subunits are 
expressed as two isoforms K\textsubscript{ir}6.1 and K\textsubscript{ir}6.2, the former is present in smooth muscle whereas 
the latter is found in the pancreatic islets, cardiac muscle, skeletal muscle and GE neurons 
(Proks and Ashcroft, 2009). There are three isoforms of SUR subunits expressed in different 
tissues throughout the body: SUR1 in pancreatic β-cells and neurons, SUR2A in cardiac 
muscle and SUR2B in vascular smooth muscle. The SUR isoforms all display differing
pharmacological properties, allowing for tissue specific drug therapies to be developed (Gribble and Reimann, 2002). Sulphonylureas mimic the effect of a rise in circulating glucose levels by closing the $K_{ATP}$ channels. However, unlike glucose, sulphonylureas do not rely on metabolism to create a rise in intracellular ATP in order to close the channels. These compounds bind to and directly inhibit the channels, as demonstrated by electrophysiological studies on isolated inside-out patches of membrane (Ashford et al., 1990b). In contrast, the activation of the channel, specifically Kir6.2/SUR1, by potassium channel openers such as Diazoxide require the presence of intracellular MgATP or MgADP in order to cause activation (Kozlowski and Ashford, 1992, Dabrowski et al., 2003, Kozlowski et al., 1989). Mg$^{2+}$ also facilitates the inactivation of this channel over time in isolated excised patches, known as run-down (Kozlowski and Ashford, 1990).

As mentioned previously, in the case of pancreatic β-cells, during a rise in extracellular glucose, glucose is taken up by cells and metabolised, resulting in the generation of ATP. ATP then binds to an inhibitory site on Kir6.2 subunit and inhibits activation of the channel. This causes the cell membrane to depolarise, due to the reduced efflux of $K^+$, and drives activation of voltage-dependent calcium $Ca^{2+}$ channels. This results in an influx of calcium and stimulation of insulin secretion. Conversely, a fall in extracellular glucose results in a rise in intracellular ADP which, when it associates with Mg$^{2+}$ binds to the nucleotide binding domain of the SUR1 subunit and activates the $K_{ATP}$ channel. This facilitates an efflux of $K^+$ which hyperpolarises and electrically silences the cell (Ashcroft et al., 1984, Proks et al., 1999, Shyng et al., 1997, Ashcroft and Gribble, 1998). This is also the mechanism by which some GE neurons in the hypothalamus respond to fluctuations in cerebral glucose levels, facilitating neurotransmitter release and firing frequency to be appropriately modulated (Ashford et al., 1990a, Ashford et al., 1990b).
The pore forming subunit of K\textsubscript{ATP} channels in GE neurons, K\textsb{ir}6.2, has been shown to have a role in maintaining whole-body glucose homeostasis. *In vivo* studies examining the response of K\textsb{ir}6.2\textsuperscript{−/−} mice to systemic hypoglycaemia demonstrated that these animals displayed a compromised CRR in the form of reduced glucagon secretion, compared to wild type (WT) mice. This phenotype was even more pronounced by neuroglucopenia, brought about through intracerebroventricular (ICV) injection of 2-deoxyglucose (2DG, a non-metabolisable glucose analogue), with complete loss of glucagon secretion in K\textsb{ir}6.2\textsuperscript{−/−} mice. This was probably due to the fact that neuroglucopenia does not allow activation of peripheral glucose sensors which will contribute to the detection and response of hypoglycaemia, as mentioned previously. Neuroglucopenia also failed to stimulate food intake in K\textsb{ir}6.2\textsuperscript{−/−} mice, indicating a role for central K\textsubscript{ATP} channels in food intake. However, glucagon secretion from isolated pancreatic α-cells of K\textsb{ir}6.2\textsuperscript{−/−} mice was comparable to WT mice, implying that the sympathetic innervation of the islets, to stimulate glucagon release during hypoglycaemia, is likely to be regulated by VMH glucose-sensing neurons, which express K\textsb{ir}6.2 containing K\textsubscript{ATP} channels (Miki et al., 2001). While this study provides evidence that functional K\textsubscript{ATP} channels in hypothalamic GE neurons are important for hypoglycaemia detection, it could not attribute the phenotype of K\textsb{ir}6.2\textsuperscript{−/−} mice to a specific neuronal population. Further evidence for the role of K\textsubscript{ATP} channels in ARC GE neurons comes from studies examining the effects of a specific mutation in K\textsubscript{ATP} channels in the GE POMC neurons (Kir6.2[ΔN2−30,K185Q]−GFP). This mutation rendered the channels insensitive to inhibition by intracellular ATP, as demonstrated by electrophysiological recordings showing that POMC neurons no longer increased action potential frequency in response to an increase in extracellular glucose. This is likely to be through the loss of inhibition by ATP, generated through metabolism during a rise in extracellular glucose. Interestingly, POMC neurons were depolarised by the application of leptin in this study, a response which was not altered in neurons containing...
mutated $K_{\text{ATP}}$ channels (Parton et al., 2007). This indicates that either $K_{\text{ATP}}$ channels in POMC neurons are not dependent on ATP inhibition in order to be inhibited by leptin or that this population of neurons is depolarised by leptin through a $K_{\text{ATP}}$-independent mechanisms. The latter was shown to be true in later studies demonstrating that the increased firing of POMC neurons in response to leptin is dependent on activation of TRP channels (Qiu et al., 2010).

Insulin receptors have been identified in regions of the hypothalamus that are involved in regulating appetite and body weight. In normal, lean animals, binding of insulin to these receptors reduces food intake and body weight (Air et al., 2002). Electrophysiological studies have demonstrated that stimulation of intracellular insulin signalling pathways results in silencing of hypothalamic GE neurons via activation of $K_{\text{ATP}}$ channels. The insulin-stimulated opening of $K_{\text{ATP}}$ channels is phosphatidylinositol 3-kinase (PI3K) dependent, as pharmacological inhibition of this downstream kinase in the insulin-signalling pathway, prevents the hyperpolarising effects of insulin. However, this response is lost in obese animals, indicating that hypothalamic $K_{\text{ATP}}$ channels are involved in linking peripheral signals of energy and nutritional status to food intake and modulation of energy expenditure (Spanswick et al., 2000). In vivo studies have shown that the opening of hypothalamic $K_{\text{ATP}}$ channels, during insulin-clamp studies, is required to produce a decrease in blood glucose levels through reduced hepatic glucose production due to inhibition of gluconeogenesis. Pharmacological inhibition of hypothalamic $K_{\text{ATP}}$ channels through ICV infusion of Glibenclamide blocked the effect of peripheral insulin to reduce hepatic glucose production. In an attempt to investigate if activation of hypothalamic $K_{\text{ATP}}$ channels by insulin was responsible for this effect, Diazoxide was applied to the hypothalamus in order to activate these channels. This resulted in reduced hepatic glucose production, indicating that activation of hypothalamic $K_{\text{ATP}}$ channels during the insulin-clamp is required reduce hepatic glucose
output. Indeed, SUR1⁺/− mice display an impaired ability to reduce hepatic glucose production during insulin clamp studies (Pocai et al., 2005). This result demonstrates that SUR1 is required for insulin-dependent activation of hypothalamic K\textsubscript{ATP} channels and has a role in reducing endogenous glucose production in response to a rise in circulating insulin.

Electrophysiological studies have also demonstrated that K\textsubscript{ATP} channels in glucose-sensing neurones are defective in animal models of glucose intolerance, such as obesity. K\textsubscript{ATP} channels of GE neurons in the VMH of obese rats are less responsive to a fall in circulating glucose levels, in terms of slower onset of membrane hyperpolarisation and activation of K\textsubscript{ATP} channels, in comparison to lean animals. Reversal of this effect, upon the readmission of glucose, was also abnormal in obese animals, in relation to recovery of resting membrane potential and K\textsubscript{ATP} channel inactivation. This result implies that disruption to K\textsubscript{ATP} channel function contributes to glucose intolerance during obesity (Rowe et al., 1996a).

Further evidence that VMH K\textsubscript{ATP} channels are involved in glucose-sensing comes from their role in regulating the CRR to systemic hypoglycaemia. Hyperinsulinaemic-hypoglycaemic clamp studies in rodents have shown that pharmacologically opening VMH K\textsubscript{ATP} channels, as they would normally be during glucose deprivation, amplifies the CRR, in terms of adrenaline and glucagon release, in animals with both normal and defective CRR. The amplified response observed in the animals was greatest when the SUR1-selective K\textsubscript{ATP} channel opener, NN414, was administered as opposed to the SUR1/SUR2B-selective K\textsubscript{ATP} channel opener, diazoxide. This gives strength to the argument that VMH glucose-sensing neurons are comprised of SUR1 subunits, and that this subunit is essential for initiating an appropriate CRR to hypoglycaemia (McCrimmon et al., 2005). Alternatively, it has also been shown that the infusion of sulphonylureas, such as glibenclamide, into the VMH of rats results in defective CRR to systemic hypoglycaemia (Evans et al., 2004).
1.5.3 The role of glucose transport in central glucose-sensing.

Glucose metabolism is essential for maintaining the function of mammalian cells and therefore glucose requires a specific transport system in order to enter cells from the circulation. GLUT2, GLUT3 and GLUT4 are all expressed in the brain, however it is their differences in tissue expression and transport kinetics that determine their role in glucose-sensing neurons. GLUT2 has a low affinity, high $K_m$ for glucose ($\sim$11.2 mM), which prevents saturation of the transporter at glucose concentrations found within the physiological range in the periphery, making it a suitable transporter for the $\beta$-cell and liver (Guillam et al., 1997, Gould and Holman, 1993). Expression of GLUT2 in the brain is much less compared to other glucose transporters. Interestingly, GLUT2 has been shown to be expressed in $\frac{1}{3}$ of glucose-sensing neurons within the VMN (Kang et al., 2004, Arluison et al., 2004). However, GLUT2 is also expressed in an equally low number of non-glucose-sensing neurons in the VMN, making it unlikely to be the sole determinant of glucose-sensing behaviour in glucose-sensing neurons (Kang et al., 2004). GLUT2 may also have a role in regulating the leptin sensitivity of POMC and NPY neurons. Mice lacking functional GLUT2 centrally were found to be compromised in terms of their thermoregulation as they did not upregulate adaptive thermogenesis to either cold exposure or ICV leptin injection. These mice also entered a state of food deprivation-induced torpor, a state whereby energy is conserved through inactivity and lowered body temperature along with reduced metabolic rate, more readily than wild type mice (Mounien et al., 2010). In another study from the same group, loss of central GLUT2 function prevented reduced food intake when glucose was injected centrally and increased food intake when neuroglucopenia was induced with ICV 2DG injection. There was also a loss in the appropriate regulation of POMC and NPY expression in response to these conditions (Bady et al., 2006). However, there is no evidence that GLUT2 is expressed in POMC and NPY neurons and so it may be more likely that GLUT2 containing neurons
outside of the VMH and which innervate POMC and NPY neurons are regulating the response of these neurons to changes in circulating glucose and leptin. GLUT2 has also been shown, through immunohistochemical staining of rat brain, to be co-expressed in some areas with GK. Therefore, in some neurons these two structures may work together, possibly in conjunction with the K<sub>ATP</sub> channel, to sense glucose through a metabolic-dependent mechanism (Arluison et al., 2004). These data indicate that although expression is low, neuronal GLUT2 may have a role in the glucose-dependent regulation of anabolic and catabolic neuropeptide function/expression.

GLUT3 is expressed in almost all VMN glucose-sensing neurons but is also ubiquitously expressed throughout the brain as the predominant neuronal glucose transporter (Kang et al., 2004). GLUT3 has a high affinity, low K<sub>m</sub> for glucose at ~1.4 mM (Gould and Holman, 1993). This means that GLUT3 is fully saturated at physiological brain glucose levels, making it unlikely to be a rate-limiting step in glucose sensing (Kang et al., 2004).

GLUT4 is co-expressed with the insulin receptor in a high percentage of GE neurons (75%) and GI neurons (65%) in the VMN, and in less than half of non-glucose-sensing neurons (40%) (Kang et al., 2004). However, it is unlikely that GLUT4 determines glucose-sensing, as both GI and GE neurons are able to sense changes in extracellular glucose in the absence of insulin. GLUT4 may be more likely to enhance the capacity of glucose-sensing in the presence of insulin.

1.5.4 The role of Glucokinase in central glucose-sensing.

GK (hexokinase IV) is the rate-limiting step in glucose metabolism and is expressed in a variety of tissues involved in glucose-sensing such as the liver, pancreas and hypothalamus. Following uptake into the cell, GK acts to phosphorylate glucose to glucose-6-phosphate which goes on to be processed through glycolysis and oxidative phosphorylation, resulting in
an increase the intracellular ATP:ADP ratio. In the pancreatic β-cell, the rise in ATP inhibits K_{ATP} channels, causing a depolarisation of the cell membrane and resulting in insulin secretion (Iynedjian, 2009). Mutations in pancreatic GK, resulting in decreased activity of this enzyme and a failure to secrete insulin because K_{ATP} is active and the membrane potential is hyperpolarised, have been shown to be the underlying cause of some forms of monogenic diabetes in humans. Individuals with GK-mutation related diabetes were once considered to have type 1 or 2 diabetes and were treated with insulin. However, the β-cells of patients with GK related diabetes were found to be functional and so sulphonylureas, which inhibit K_{ATP} channels and depolarise the cell membrane to facilitate insulin secretion, are now used to treat this form of diabetes (Gloyn et al., 2008). GK has a low affinity for glucose and does not experience end-product inhibition. However, it is still not clear how GK, which has a K_m of 8-10mmol/l glucose, can regulate glucose-sensing in neurons when brain glucose levels only range from 0.5-3.5mmol/l (Kang et al., 2004).

The pancreatic form of GK is expressed in the VMN and has been shown to be present in some glucose-sensing neurons, 63% GE and 43% of GI compared to only 8% of glucose unresponsive cells (Kang et al., 2004). GK has been located to specific population of GE and GI neurons in the ARC including POMC and NPY which, as mentioned previously, are also involved in feeding (Dunn-Meynell et al., 2002). Dissociated VMH neurons, taken from animals where GK inhibitors had been perfused into the hypothalamus, showed that GK activation produced different effects in GE and GI neurones. Normally, a rise in extracellular glucose produces depolarisation of the cell membrane and an influx of Ca^{2+} through voltage-dependent Ca^{2+} channel in GE neurones, and induces hyperpolarisation and a fall in [Ca^{2+}]_i in GI neurons. However, fura-2 Ca^{2+} imaging showed that when GK was inhibited in GE neurons, [Ca^{2+}]_i oscillations were suppressed, with the opposite occurring in GI neurons, in response to a rise in extracellular glucose levels. Activation of the GE neurons was restored
upon application of the SUR1 specific sulfonylurea, Tolbutamide, providing evidence that GK is expressed with $K_{ATP}$ channels in GE neurons (Dunn-Meynell et al., 2002).

Inhibition of GK through injection of alloxan into the third ventricle of the brain blunts the CRR resulting in an impaired ability to increase blood glucose levels and increase feeding in response to systemic hypoglycaemia and neuroglucopenia in rats. Post-mortem mRNA analysis found that this effect was associated with an increase in GK expression in the VMN and ARC (Sanders et al., 2004). A separate study from the same group also found that GK expression is upregulated in the VMH following a antecedent period of insulin-induced hypoglycemia known to cause defective CRR (Dunn-Meynell et al., 2002). This demonstrates that central GK expression is increased in states of defecion CRR and therefore may have a role in regulation neuronal and hormonal responses to hypoglycaemia.

In relation to the role of GK in hyperglycaemia, *in vivo* studies examining the role of GK activity in glucose stimulated insulin secretion (GSIS) found that while glucose infusion into the third ventricle of rats increased GSIS during an intravenous glucose tolerance test, inhibition of GK with glucosamine or mannoheptulose compromised glucose tolerance and reduced GSIS (Ousndiji et al., 2012). While this provides evidence of a role for GK in responding to hyperglycaemia through regulation of GSIS, it is unclear if the GK mediated effects are neuronal dependent, as GK is also expressed in tanycytes and astrocytes or whether GK expressed in GE or GI neurons is more important for hyper- or hypoglycaemia detection (Guillod-Maximin et al., 2004).

### 1.5.5 The role of AMPK in central glucose-sensing.

Adenosine monophosphate activated kinase (AMPK) is an evolutionarily conserved serine/threonine kinase which acts as a fuel sensor in multiple cell types. It has a trimeric structure composed of a catalytic $\alpha$ and regulatory $\beta$ and $\gamma$ subunits. AMPK is activated
during situations that cause an increase in the intracellular AMP:ATP ratio, such as hypoglycaemia. Switching on AMPK results in a suppression of ATP consuming cellular processes and an increase in events that lead to a rise in intracellular ATP (Kahn et al., 2005).

AMPK is expressed in many neuronal populations, the majority of which are found in the VMH with a small amount residing in the ARC (Kahn et al., 2005). Strong evidence suggests that hypothalamic AMPK is a fundamental component of the glucose-sensing machinery in GE neurons and plays a role in regulating hormonal CRR. In vivo hyperinsulinaemic-hypoglycaemic clamp studies have demonstrated that activation of AMPK during hypoglycaemia, through microinjection of 5-aminoimidazole-4-carboxamide (AICAR), amplified the CRR in the rats with defective CRR following 3 days of insulin-induced hypoglycaemia (McCrimmon et al., 2006). Conversely, another study from the same group found that downregulation of the AMPK catalytic α-subunit, using short hairpin RNA adenovirus microinjection into the VMH, suppressed both glucagon and adrenaline responses to hypoglycaemia. Both α1- and α2-subunits are expressed in the hypothalamus but it is interesting to note that it was the expression of AMPKα2 but not AMPKα1 that was reduced in the aforementioned study (McCrimmon et al., 2008). Other studies examining the phenotype of AMPKα2−/− mice, where AMPKα2 is specifically knocked out of cells expressing the rat insulin promoter sequence such as the pancreatic β-cell and some hypothalamic neurons, animals displayed glucose intolerance and reduced GSIS. Isolated islets from AMPKα2−/− mice failed to completely cease insulin secretion in response to low extracellular glucose. The underlying mechanism for this appeared to be attenuated ability of the β-cells to hyperpolarise in response to low extracellular glucose, meaning that the membrane potential remained more depolarised and insulin secretion was occurring at lower glucose concentrations than in wild type β-cells. This phenotype was not observed in AMPKα1−/− mice (Beall et al., 2010). AMPKα2 has also been shown to be important for the
hyperpolarisation of the GT1-7 mouse hypothalamic GnRH cell line to low glucose exposure (Beall et al., 2012). This implies that AMPKα2 activity in particular may be important for GE mechanisms of glucose-sensing.

AMPK also appears to be important for the regulation of GI neurons in the VMN through nitric oxide production (NO). During low glucose, when AMPK is activate, AMPK phosphorylates neuronal NO synthase (nNOS) which stimulates the release of NO. When NO binds to a receptor on guanylate cyclase which increases levels of cyclic guanosine monophosphate and further stimulates AMPK. This activation of AMPK has been shown to lead to closure of an inhibitory Cl⁻ channel, leading to depolarisation of the neuron during low glucose. Inhibition of AMPK with Compound C, inhibition of nNOS and inhibition of the Cl⁻ conducting CFTR all prevented depolarisation of GI neurons in response to low glucose (Murphy et al., 2009). Complementary in vivo studies by the same group demonstrated that VMH injection of a NOS inhibitor during a hyperinsulinaemic-hypoglycaemic resulted in blunted CRR to hypoglycaemia in the form of reduced adrenaline and increased glucose infusion rates in rats (Fioramonti et al., 2010).

In addition to the involvement of AMPK in hormonal counter regulation, in vivo studies have also shown that pharmacological inhibition of AMPK activity in the hypothalamus suppressed hepatic glucose output, during euglycaemic-pancreatic clamp studies, and is independent of effects on food intake and body weight. However, activation of hypothalamic AMPK does not increase endogenous glucose production but rather inhibits the reduction of hepatic glucose output in response to a rise in brain glucose levels, further implying that AMPK activity in glucose-sensing neurons is important for regulation whole-body glucose homeostasis (Yang et al., 2010). It was not established from this study if this effect was due to AMPK activity in GI or GE neurons.
1.5.6 The role of mitochondrial respiration in central glucose-sensing.

The general hypothesis of the mechanism for GE neurons is that changes in glycolytic ATP, possible compartmentalised at the plasma membrane, are responsible for regulating the $K_{ATP}$ channel response to changes in extracellular glucose rather than a requirement for oxidative metabolism by the mitochondria and a global change in intracellular ATP (Ainscow et al., 2002). However, evidence has emerged recently to suggest a role for mitochondrial processes in glucose sensing. UCP2 is a mitochondrial uncoupling protein which facilitates the non-ATP producing leak of protons across the mitochondrial membrane, dissipates the proton gradient and reduces ATP production. UCP2 is upregulated during times of increased substrate flux through the mitochondria in order to facilitate movement of the electron transport chain and reduce oxidative stress (Divakaruni and Brand, Produit-Zengaffinen et al., 2007). Transgenic UCP2$^{-/-}$ mice display better pancreatic $\beta$-cell function compared to wild type littermates following conditions that cause hyperglycaemia or obesity induced $\beta$-cell dysfunction (Zhang et al., 2006). In isolated islets, inhibition of UCP2 within genipin was found to raise intracellular ATP, inhibit $K_{ATP}$ channel currents and stimulate insulin secretion (Zhang et al., 2006). These data indicate that UCP2 may be partially responsible for $\beta$-cell failure during type 2 diabetes. UCP2 is expressed in 95% LH MCH neurons, along with $K_{ATP}$ channels in some of these neurons (Kong et al., 2010). Deletion of UCP2 from MCH neurons resulted in increased glucose clearance during the glucose tolerance test on these mice and reduced fasting blood glucose levels. This was not due to increased insulin secretion from the pancreas and indicates a possible role for MCH neurons in the uptake of glucose by peripheral tissues (Kong et al., 2010). UCP2 mRNA was also found to be expressed in the ARC and was increased in mice following a high fat diet. This was in conjunction with a loss of glucose stimulated increase in activity of POMC neurons, which are excited by glucose through $K_{ATP}$ dependent mechanisms. Application of genipin to these neurons during
electrophysiological recordings resulted in an increase in action potential firing through inhibition of the $K_{ATP}$ channel and this effect was absent in cells with mutated ATP-insensitive channels (Parton et al., 2007). Loss of POMC glucose-sensing following high fat diet was assessed by the release of $\alpha$-MSH, the neuropeptide secreted from these neurons, during increased extracellular glucose. $\alpha$-MSH release was stimulated by increasing extracellular glucose in mice fed normal chow diet but was not stimulated in the hypothalami of mice on a high fat diet who also displayed glucose intolerance. $\alpha$-MSH release could however be stimulated in both groups with the application of genipin. This indicates that obesity upregulates UCP2 in GE neurons resulting in a loss of glucose-sensing centrally and glucose tolerance systemically (Parton et al., 2007).

The way in which genipin increases the firing of GE neurons is thought to be the same as in the $\beta$-cell which is a rise in intracellular ATP resulting in inhibition of the $K_{ATP}$ channel. However, inhibition of UCP2 also increases reactive oxygen species (ROS) which have also been demonstrated to have a role in glucose sensing (Pi et al., 2009). Increasing extracellular glucose concentration from 5 to 20 mM was found to increase the ROS production in hypothalamic slices by 70% but could be blocked by adding catalase as an antioxidant (Leloup et al., 2006). Application of Rotenone and Antimycin A, mitochondrial complex I and III inhibitors respectively, also stimulated ROS that could be quenched by catalase. 20 mM glucose, Rotenone and Antimycin A increased neuronal activity in the ARC and stimulated insulin secretion, in vivo, actions which were prevented by co-infusion with catalase (Leloup et al., 2006). This may be a separate GE mechanism to $K_{ATP}$ channel mediated glucose-sensing as $K_{ATP}$ has been shown to be activated by $H_2O_2$ which, according to this study, would be produced by a rise in glucose (Chai and Lin, 2010). Although 20 mM glucose is a supraphysiological concentration of glucose for the brain, these data may suggest a role for ROS in hypothalamic glucose sensing during hyperglycaemia.
1.6 Non-metabolic-dependent glucose-sensing neurons.

While the β-cell model of GE neurons applies to some populations of neurons, increased electrical activity can still be achieved through non-glucose metabolism-dependent mechanisms. In relation to the $K_{ATP}$ channel, many neurons, glucose-sensing or otherwise, express Kir6.2 or Kir6.1 (Dunn-Meynell et al., 1998). Interestingly, Kir6.2 was found to be expressed in NPY positive neurons, although no functional $K_{ATP}$ channel has been observed in this neuronal population and electrophysiological studies have demonstrated that GI neurons are unaffected by sulphonylureas (Spanswick et al., 2000). Therefore, the expression of this metabolically regulated ion channel cannot be used alone to identify a glucose-sensing neuron. While GK appears to be important for glucose-sensing in neurons, as it is in the β-cell, only 70% of GE neurons and 40% of GI neurons express GK (Kang et al., 2004, Matschinsky et al., 1993). It is also important to note that intracellular ATP in VMH GE neurons has not yet been shown to increase in response to glucose and that these cells were still found to be capable of hyperpolarising in response to low glucose in the presence of relatively high (3 mM) intracellular ATP (Ainscow et al., 2002).

LH Orexin neurons are important for regulating wakefulness and also have a role in food intake, as disruption of normal orexin signalling leads to narcolepsy and obesity in animal models (Hara et al., 2001). Electrophysiological recordings have demonstrated that orexin neurons display GI behaviour and that this is regulated through an unidentified extracellular glucose sensor which results in the activation of a tandem-pore-domain $K^+$ channel ($K_{2P}$), which is a family of $K^+$-selective leak channels, during a rise in extracellular glucose, resulting in an outward $K^+$ current (Burdakov et al., 2006). The activation of this channel was found to be metabolism independent as it was unaffected by changes in intracellular glucose, ATP and $Ca^{2+}$ (Burdakov et al., 2006) $K_{2P}$ can be categorised into subfamilies which include
two-pore domain acid-sensitive potassium (TASK) channels which are inhibited by extracellular acidification (Bayliss et al., 2003). Lowering the pH of the extracellular environment (from pH 7.3 to 5.9) reversed the glucose induced hyperpolarisation of orexin neurons and inhibited the outward K⁺ current produced by glucose, indicating that a TASK K₂P channel mediates the GI behaviour in this neuronal population (Burdakov et al., 2006). The observations from this study suggest a mechanism for glucose-sensing that is independent of glucose metabolism and reliant on an extracellular glucose sensor. However, genetic deletion of TASK1 and 3 from orexin neurons did not disrupt the response of these GI neurons to glucose or pH, although there was a reduction in high frequency firing (Gonzalez et al., 2009). A similar mechanism of glucose induced inhibition of VMH neurons was observed involving activation of a K⁺ channel during a rise in extracellular glucose which, like the TASK channels found in orexin neurons, was sensitive to depolarisation by a change in extracellular acidification (from pH 7.3 to 6) following the application of glucose. Extracellular acidification did not have any effect in low glucose, when the K⁺ was predominantly closed (Williams and Burdakov, 2009). However, from this study it was not concluded if the GI behaviour of VMH neurons was dependent of glucose metabolism or activated though an external glucose sensor. LH NPY neurons are also thought to respond to glucose through a GI mechanism involving a glucose activated K⁺ channel, independently of glucose metabolism, although the molecular evidence for this is yet to be determined (Marston et al., 2011).

In addition to metabolic independent mechanisms of GI neurons, GE neurons have also been demonstrated to function in the absence of glucose metabolism. The evidence that GE neurons can function without metabolising glucose came from the observation that not all GE neurons in the VMH express GK (Kang et al., 2004). Some GK absent GE neurons were found to express a sodium-glucose transporter (SGLT1) which couples glucose transport with
Na\(^+\) transport, resulting in a depolarising inward current in response to a rise in extracellular glucose (Kang et al., 2004). ICV injection of the SGLT1 inhibitor, phlorizin, into the brains of rats upregulated food intake in these animals (Tsujii and Bray, 1990). In a separate study, phlorizin was also found to inhibit activation of GE neurons in response to glucose in the VMH (Yang et al., 1999). Conversely, application of the SGLT1 specific substrate, alpha-Methylglucopyranoside (α-MDG), activated dissociated rat hypothalamic GE neurons. Interestingly, neurons which are activated by α-MDG were not activated by the K\(_{ATP}\) channel inhibitor, tolbutamide, as determined by calcium imaging (O'Malley et al., 2006). This indicates a separate GE mechanism in neurons other than typical K\(_{ATP}\) channel mediated signalling. It is worth noting that the aforementioned study examined the response of neurons when raising the glucose concentration from 3 to 15 mM which is unlikely to be physiological for the brain in a healthy individual but perhaps infers a role for SGLT1 in responding to extreme hyperglycaemia.

1.7 Non-neuronal central glucose-sensing

In addition to glucose sensing neurons, astrocytes and tanycytes may also be involved in hypoglycaemia detection. Tanycytes line the third ventricle of the brain and are in contact with cerebrospinal fluid (CSF), which will contain about \(\frac{2}{3}\) the concentration of glucose in the blood (Ono et al., 1983). Tanycytes have projections into both the Arc and the VMN which may be able to convey information about CSF and hence peripheral glucose status (Sanders et al., 2004). This population of ependymal cells also express typical glucose metabolising machinery, such as GLUT2 and GK. Functional studies have shown that application of alloxan, a glucose-analogue toxin which is taken up by GLUT2 and inhibits GK, destroys tanycytes (Sanders et al., 2004). Tanycytes appear to communicate through calcium signalling as both glucose and the non-metabolisable glucose analogue, 2-deoxy-D-
glucose, increased intracellular Ca\(^{2+}\) content (Dale, 2011). Therefore, these cells may sense glucose through metabolic-independent mechanisms. One of the most interesting findings into the role of tanycytes in hypoglycaemia detection came from RH rodent studies which demonstrated that the projections from tanycytes into the VMH retracted following RH. The same study also found that destroying tanycytes with alloxan impaired the CRR to hypoglycaemia, suggesting a role for these cells in hypoglycaemia detection or modulation of the CRR (Sanders et al., 2004).

Another non-neuronal cell type which may be important for maintaining whole body glucose homeostasis is the astrocyte. These cells are often described as being metabolically coupled to neurons in that they supply fuel substrate from glycogen breakdown and glycolysis, especially during times of increased neuronal energy demand or stress (Chih and Roberts Jr, 2003). Astrocytes in the ARC express GLUT2 and have been shown to be activated by a rise in intracellular glucose, through c-fos staining rat brain. Inhibition of astrocytic metabolism, by application of methionine sulfoximine, inhibits astrocyte activation in response to ICV glucose injection and the associated stimulation of insulin secretion (Guillod-Maximin et al., 2004). GLUT1 expression in hypothalamic astrocytes has also shown to be important for hyperglycaemia detection and initiating insulin secretion. This is based on the observation that astrocytic GLUT1 expression, in the hypothalamus is reduced, in hyperglycaemic streptozotocin (STZ) induced diabetic rats. Injection of GLUT1 adenovirus into the mediobasal hypothalamus of STZ diabetic rats reduced endogenous glucose production from the liver and restored plasma glucose levels to within a normal range (Chari et al., 2011).

Evidence that hypothalamic astrocytes are involved in hypoglycaemia detection, as well as hyperglycaemia detection, comes from studies examining whole-body GLUT2\(^{-}\) mice. These animals display impaired CRR in the form of glucagon secretion to both systemic and central
glucopenia. However, genetic restoration of GLUT2, specifically in astrocytes, restored the glucagon response to hypoglycaemia in these animals but not the feeding response to hypoglycaemia (Marty et al., 2005). This suggests that increased food intake in response to hypoglycaemia, in healthy individuals, may be regulated by functional GLUT2 in neurons. Interestingly, a small population of astrocytes in the NTS may also be involved in hypoglycaemia detection as they were found to be activated during low glucose, indicated through increased intracellular calcium (McDougal et al., 2013). Low glucose-activated astrocytes in the NTS may be of particular importance to hypoglycaemia detection by the PMV as this is a site where efferent neurons from PMV converge (Adachi et al., 1984).

The communication between hypothalamic astrocytes has also been shown to be important for glucose sensing as both systemic hypo- and hyperglycaemia increase the expression of connexin proteins in the mediobasal hypothalamus of rats. Inhibition of these proteins blunted insulin secretion in the animals during ICV glucose injection (Allard et al., 2014). These data suggest that astrocytic glucose-sensing contributes towards modulating the appropriate response to both hypo- and hyperglycaemia.

1.8 Aim of investigation

The mechanisms underlying glucose-sensing neurons are extremely diverse and appear to play individual roles in relation to whole body glucose homeostasis. In respect to metabolism-dependent glucose-sensing neurons, multiple studies have shown that pharmacological and genetic alterations to the function of key components in glucose metabolism disrupt different aspects of glucose tolerance and homeostasis. However, due to the heterogeneity of many GE and GI populations, investigation of glucose metabolism in a homogenous population of glucose-sensing neurons during a state where glucose-sensing is known to be disrupted, such as following RH, has not been possible. Therefore, the aim of
this investigation was to model RH in vitro in the mouse hypothalamic GT1-7 neuronal cell line and examine adaptations that may occur in glucose metabolism and/or electrical activity following recurrent low glucose exposure. This immortalised cell line displays hypoglycaemia-sensing behaviour, typical of GE neurons, which has been demonstrated to be directly regulated by the modulation of glucose-sensing cellular components, such as glucose transporters (Glut1, Glut3 and Glut4), $K_{\text{ATP}}$ channel subunits (Kir6.2, SUR1 and SUR2B), glucokinase and AMPK (Mellon et al., 1990, Beall et al., 2012). GT1-7 cells also express gonadotrophin releasing hormone (GnRH), which is synthesised and released within the hypothalamus and has a fundamental role in successful reproductive function. The release of this neurohormone has also been shown to be regulated by fluctuations in ambient glucose levels and $K_{\text{ATP}}$ channel activity (Zhang et al., 2007). Therefore, the GT1-7 model seemed appropriate for this project. This type of investigation will determine if adaptations occur intrinsically to glucose-sensing neurons following recurrent low glucose exposure, in the absence of astrocytic and synaptic influences, and possibly uncover new therapeutic targets for the treatment of defective CRR.
Table 2.1 Chemicals, antibodies and reagents

<table>
<thead>
<tr>
<th>Supplier</th>
<th>Chemical / antibody / reagent</th>
</tr>
</thead>
<tbody>
<tr>
<td>SIGMA</td>
<td>Calcium chloride (CaCl$_2$), dimethyl sulphoxide (DMSO), EGTA, HEPES, ADP, poly-l-lysine,</td>
</tr>
<tr>
<td></td>
<td>Bradford reagent, bovine serum albumen (BSA), amphotericin B, tolbutamide, diazoxide,</td>
</tr>
<tr>
<td></td>
<td>sodium chloride (NaCl), PMSF, bovine trypsin, H$_2$O$_2$</td>
</tr>
<tr>
<td>Roche Diagnostics, Germany</td>
<td>ATP</td>
</tr>
<tr>
<td>VWR International</td>
<td>Ethanol, hydrochloric acid (HCl), magnesium chloride hexa-hydrate (MgCl$_2$·6H$_2$O),</td>
</tr>
<tr>
<td></td>
<td>methanol, potassium chloride (KCl), sodium hydroxide (NaOH), sodium fluoride (NaF),</td>
</tr>
<tr>
<td></td>
<td>sodium pyrophosphate (NaPPi)</td>
</tr>
<tr>
<td>Fisher Scientific</td>
<td>D-glucose, TRIS base</td>
</tr>
<tr>
<td>Novo Nordisk</td>
<td>NN414</td>
</tr>
<tr>
<td>University of Dundee</td>
<td>Polyclonal antibodies against AMPKα1 and AMPKα2 were kind gifts from Dr Simon</td>
</tr>
<tr>
<td></td>
<td>Hawley, University of Dundee, U.K.</td>
</tr>
</tbody>
</table>

2.1 Cell culture

Cells were cultured under sterile conditions to avoid infection and were maintained at 37°C in a humidified atmosphere consisting of 95% air and 5% CO$_2$. Wild-type cells were maintained in a category-1 biological safety-rated cell culture suite, whereas virally infected cells were generated and maintained in a category-2 facility. Media components for the GT1-7 cell line are listed as follows in table 2.2:
Table 2.2 GT1-7 media components

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Stock media</th>
<th>Plating media</th>
</tr>
</thead>
<tbody>
<tr>
<td>GT1-7</td>
<td>DMEM (Sigma D5671), 10% (v/v) Fetal Bovine Serum (Hyclone), 2% (v/v) L-glutamine, 2% (v/v) penicillin-streptomycin</td>
<td>DMEM (Gibco 11966), 2.5 mM glucose, 10% (v/v) Fetal Bovine Serum (Hyclone), 2% (v/v) penicillin-streptomycin</td>
</tr>
</tbody>
</table>

Cell cultures were passaged every 3-5 days and kept between 50-85% confluency in T-75 NUNC flasks. GT1-7 cells are non-adherent and so plasticware had to first be coated with poly L-lysine (PLL) in order to aid attachment (used at 0.2 mg/ml for 20 minutes). Cell cultures were passaged by removing the media, washing the cells gently with warmed PBS, incubating with 2 ml 0.05% trypsin-EDTA for 3-5 minutes at 37 °C after which trypsination was stopped by adding 5 ml of media to the flask. Cells were then centrifuged at 800 rpm for 4 minutes to remove the trypsin. This produced a pellet which was resuspended in 3-10 ml of media and 1 ml of cell suspension was then added to a T-75 NUNC flask, along with 10 ml of stock media, in order to create a stock flask. Stock flasks were given fresh media every 48 hours in order to replenish glucose and other nutrients. For experiments, cells were plated in plating media which contained glucose at a concentration of 2.5 mM. Preliminary experiments with GT1-7 cells found that the response of these cells to low glucose, in terms of AMPK activation, diminishes with increasing confluency (data not shown). Therefore, in order to ensure the confluency ≤70%, at the time of lysing/assay, cells were plated in the following way for experiments that would take place on the following day:

- 60 mm NUNC dish, 50% confluency = 450,000 cells plus 5 ml plating media.
- 100 mm NUNC dish, 50% confluency = 2,650,000 cells plus 10 ml plating media.
- 96-well NUNC plate, 50% confluency = 1000 cells/well plus 200 µl plating media.
• 35 mm NUNC dish, 15% confluency = 30,000 cells plus 2 ml plating media.

2.2.1 Cell treatments

For all treatment conditions, cells were first seeded into plating media for 24 hours and incubated in serum free (SF) media (plating media containing no serum) for 1 hour prior to the treatment. This is because GT1-7 cells do not respond to low glucose, in the form of membrane hyperpolarisation, when they are assayed straight from serum containing media for reasons which remain unclear. Cells were incubated at 37°C in a humidified atmosphere consisting of 95% air and 5% CO₂ during all treatments.

2.2.2 Recurrent hypoglycaemia, in vitro

In order to replicate recurrent hypoglycaemia in vitro, a protocol was adapted from human and rodent studies which robustly resulted in a defective counterregulatory response to further hypoglycaemia (McCrimmon et al., 2006). GT1-7 cells were the only cells used for these experiments in order to investigate if functional and/or metabolic adaptations occur in glucose-sensing neurons following recurrent hypoglycaemia. 24 hours after seeding, the plating media was removed, cells were gently washed with warmed PBS and SF media was applied for 1 hour. After 1 hour, SF media was removed, cells were washed with PBS and 0.1 mM glucose Normal Saline (table 2.2) was applied to replicate hypoglycaemia (or 2.5 mM glucose Normal saline for the corresponding controls) for 3 hours. Both 2.5 and 0.1 mM glucose were selected as the euglycaemic and hypoglycaemic stimuli, respectively, as these concentrations have been shown to be in the physiological extracellular glucose concentration range in the rat brain during these conditions (Silver and Erecinska, 1994). Although this model for replication recurrent hypoglycaemia in vitro is reductionist in the fact that it is also absent of free fatty acids (FFAs) and other alternative fuel substrates (such as lactate and ketones) there is evidence within the literature to suggest that levels of these circulating...
factors are lower in individuals with defective glucose counterregulation, compared to control (Ovalle et al., 1998). Therefore, in order to control for this, alternate fuel substrates were withheld during the euglycaemic or hypoglycaemic incubations so that the effect of altering ambient glucose levels could be observed. After 3 hours, the Normal Saline was replaced with plating media and cells were left to incubate for 24 hours. This process was repeated twice more and is illustrated in Fig. 2.1. Due to the timescale that cells had to be cultured for in order to replicate recurrent hypoglycaemia, cells were seeded at 30% confluency so that they would reach 60-70% confluency on the last day of the protocol:

- 100 mm NUNC dish and T-75 NUNC flask, 30% confluence = 785,400 cells plus 10 ml plating media.
- 35 mm NUNC dish, 10% confluency = 20,000 cells plus 2 ml plating media.

100 mm NUNC dishes were used to generate samples for RNA analysis and AMPK activity assays. 35 mm NUNC dishes were used to treat cells for electrophysiology. T-75 NUNC flasks were used for assays that would be carried out in 96-well, 12-well or 24-well plates on the final day of the recurrent hypoglycaemia protocol, in order to avoid the adverse effects high confluency. Cells were split and seeded at the required density for these assays 2-3 hours after the last hypoglycaemic incubation.
2.3.1 Electrophysiological recordings

Patch-clamp electrophysiology, in conjunction with voltage-clamp, was used to examine micro- and macroscopic currents as well as monitoring real-time changes in membrane potential across the cell membrane. The formation of a high resistance giga-ohm (GΩ) seal between the cell membrane and the glass patch pipette allows high resolution of single ion channel events to be monitored as well as mechanical manipulation of the patch in order to obtain different configurations (Fig 2.2) (Neher and Sakmann, 1976). This system also utilises a negative feedback amplifier in order to hold the membrane at a constant potential, when required. The amplifier will inject more or less current into the cell in response to a change in the membrane resistance, brought about through the opening and closing of ion channels. Borosilicate glass capillaries containing a filament with an outer diameter of 1.2
mm and inner diameter of 0.69 mm were used to create the patch pipettes and were pulled using a Flaming/Brown micropipette puller (Model P97, Sutter Instrument Co. USA). The tips of the pipettes were fire polished with a micro forge (MF-830, Narishige, Japan) and filled with the required solution to a depth of 100-150 mm from the tip of the pipette. The opposite ends of the electrodes were also briefly fire polished using a Bunsen burner in order to avoid damaging the rubber grommet and the silver-chloride wire in the electrode holder.

Table 2.3 Electrophysiological Recording Solutions

<table>
<thead>
<tr>
<th></th>
<th>NaCl (mM)</th>
<th>KCl (mM)</th>
<th>MgCl$_2$·6H$_2$O (mM)</th>
<th>CaCl$_2$ (mM)</th>
<th>HEPES (mM)</th>
<th>EGTA (mM)</th>
<th>pH</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pipette Solution A</td>
<td>140</td>
<td>5</td>
<td>3.8</td>
<td>10</td>
<td>10</td>
<td>7.2 (KOH)</td>
<td></td>
</tr>
<tr>
<td>Pipette Solution B</td>
<td>140</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td></td>
<td>7.2 (KOH)</td>
<td></td>
</tr>
<tr>
<td>External Solution A (Normal Saline)</td>
<td>135</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>7.4 (NaOH)</td>
<td></td>
</tr>
<tr>
<td>External Solution B</td>
<td>140</td>
<td>1 (0.65 nM free Mg$^{2+}$)</td>
<td>2.7 (100 nM free Ca$^{2+}$)</td>
<td>10</td>
<td>10</td>
<td>7.2 (KOH)</td>
<td></td>
</tr>
</tbody>
</table>

2.3.2 Whole-cell Patch Clamp Configuration

Glass electrodes (5-10 MΩ) were lowered onto the cell membrane and, once touching the cell, gentle negative pressure was applied through mouth pipetting in order to form a GΩ seal. This is deemed as cell-attached configuration. Once in this configuration, the negative pressure was increased in order to rupture the cell membrane and gain access to whole-cell macroscopic currents. Over time (10-15 minutes), the intracellular contents of the cell was dialysed and exchanged with the contents of the glass electrode. The advantage of this configuration is that the intracellular contents of the cell can be manipulated whilst still
allowing whole-cell macroscopic currents to be visualised and usually manipulated using voltage-clamp protocols via the amplifier (Axopatch 200B, Axon Instruments). The voltage-clamp protocol used in the outlined experiments in the Results section was performed by applying a stepped voltage pulses across the cell membrane. The test pulses ranged from -90 to +30 mV, from a holding potential of -70 mV (net membrane potential steps were -160 to -40 mV), in 20 mV steps which were 400 ms in duration with a 20 ms interval between pulses. The steady-state currents elicited by each test pulse allow the reversal potential, slope conductance, total whole-cell current and voltage-dependent kinetics to be analysed which will allow the type of ion channels present to be identified based on their electrical characteristics. In order to calculate the conductance density of the cell, the current amplitude elicited by each voltage step was plotted against the magnitude of the test pulse, producing a linear current-voltage relationship. Linear regression analysis of the best fit line of the current-voltage relationship allows the slope conductance (nS) to be determined from the gradient of this line. The reversal potential (mV) can also be determined from this relationship as the point where the line intercepts the x-axis. The slope conductance can then be normalised to the cell size (pF) in order to determine the conductance density of the cell (ps/pF). In terms of examining maximum $K_{ATP}$ channel conductance, this was done in response to dialysing intracellular ATP out of the cell (Pipette solution A) and then applying the voltage-clamp protocol, outlined above. In line with Ohm’s Law (voltage = current x resistance), as the $K_{ATP}$ channels open in response to the removal of ATP the input resistance will reduce and therefore the amplitude of the current achieved with each voltage step will increase. All recordings in whole-cell voltage clamp configuration were sampled at 2 kHz and filtered at 10 kHz using Axopatch 200B amplifier and PCLAMP7 software.
2.3.3 Perforated Patch Clamp Configuration

Once in cell-attached configuration, the addition of antibiotics, such as amphotericin B, into the glass electrode (5-8 MΩ) will form small pores in the cell membrane, under the electrode. This allows access to the membrane potential without the loss of soluble intracellular content/processes, which occurs in the whole-cell configuration, as these pores are only permeable to monovalent cations. However, the input resistance is often too high in order to gain good resolution of whole-cell macroscopic currents. All recordings in perforated patch clamp configuration were sampled at 2 kHz and filtered at 10 kHz using Axopatch 200B amplifier and PCLAMP7 software.

2.3.4 Inside-out Patch Clamp Configuration

Once in cell attached configuration, rapid withdrawal of the glass electrode (8-12 MΩ) from the cell obtained in an inside-out configuration of the excised patch of membrane. Recordings were made in equimolar 140 mM KCl and the patch was held at a potential -50 mV so that inward K⁺ currents were driven by the pipette holding potential (+50 mV) rather than by an electrochemical gradient. -50 mV is also the resting membrane potential of GT1-7 cells and so seemed like a logical potential to study K_ATP channel activity at in these cells (Beall et al., 2012). Once the patch was excised, channel activity was observed for 2-3 minutes before drugs or nucleotides were applied to the bath solution. For concentrations of nucleotides ≥ 100 µM, the bath concentration of MgCl₂ was increased from 1 to 1.1 mM in order to avoid a fall in the free Mg²⁺ concentration. Recordings were not made for longer than 15 minutes in order to avoid channel run-down, as previously reported (Larsson et al., 1993). The average channel activity in a patch was defined as N.P(o), where N = the number of functional channels in the patch and P(o) = the open state probability which was determined by measuring the total time spent at each unitary current level and expressed as a proportion of
the total time of the segment of recording (90-120 seconds) that was analysed. N.P(o) was calculated using the PCLAMP7 software which incorporates a 50% threshold parameter in order to detect single channel events which are $\geq 50\%$ of a predetermined unitary current amplitude. All recordings in inside-out patch clamp configuration were sampled at 2 kHz and filtered at 10 kHz using Axopatch 200B amplifier and PCLAMP7 software.
Figure 2.2 Patch-clamp configurations

Initially a glass micropipette is lowered onto the cell surface, followed by gentle suction which results in the formation of a gigohm seal. This forms the cell-attached configuration (A). From this point, the patch of membrane can be excised from the cell to form inside-out patch configuration (B). Alternatively, whole-cell patch clamp configuration can be obtained by applying strong suction to rupture the plasma membrane (C). Excision of the patch at this point results in outside-out patch configuration (D).

(Diagram adapted from http://neuronresearch.net/neuron/patchclamp.htm)
2.4 The Nernst equation

All ions have an equilibrium potential (i.e. the potential at which there is no net flux of a particular ion) which can be used in order to predict the membrane potential in response to the concentration of a certain ion on either side of a permeable membrane. The equilibrium potential of $K^+$ can be calculated using the Nernst equation as follows:

\[
E_K = \frac{RT}{zF} \ln \left( \frac{[K^+ \text{ extracellular}]}{[K^+ \text{ intracellular}]} \right)
\]

$E_K$ = the equilibrium potential of $K^+$ (mV)

$R$ = the universal gas constant (8.314472 J/K/mol)

$T$ = temperature (K)

$z$ = the valency of the ion

$F$ = Faraday’s constant (C/mol)

The $E_K$ at room temperature (25°C) with an intracellular $K^+$ concentration of 140 mM and 5 mM $K^+$ in the pipette is -85.38 mV. However, this equation is based on the assumption that the membrane is only permeable to $K^+$ and in reality other ion transport processes will be ongoing, so the equilibrium potential in practice will differ slightly from that calculated. For example, in experiments where the aim was to chemically clamp the membrane potential at -60 mV, 140 mM KCl was inside the glass electrode and 13.5 mM KCl was present in the extracellular bath solution. In whole-cell configuration, in response to removal of intracellular ATP, the membrane potential of GT1-7 cells did not hyperpolarise more negatively than -55 mV on average.

2.5 $[^3\text{H}]-2$-Deoxyglucose uptake assay

$[^3\text{H}]-2$-Deoxyglucose ($[^3\text{H}]-2\text{DG}$) uptake assay were performed on GT1-7 cells at room temperature, as previously described (Tsakiridis et al., 1994). 100,000 cells/well were seeded
into a 12-well cell culture plate, 16 hours prior to the assay being performed. Cells were washed twice with pre-warmed (37°C) Hepes Buffered Saline (HBS: 140 mM NaCl, 20 mM Hepes, 5 mM KCl, 2.5 mM MgSO4, 1 mM CaCl2, pH 7.4) before they were incubated for 10-12 minutes in HBS containing 10 µM 2-DG, 10 µM [3H]-2DG (1 μCi/ml, Perkin Elmer) and 0-2.5 mM glucose. 2 wells on each plate were also treated with 10 µm cytochalasin B in order to disrupt the actin cytoskeleton and inhibit [3H]-2DG in order to determine non-specific binding of radioactivity to the cell membrane, which would be subtracted from samples in the final analysis. After the incubation time, the HBS containing the [3H]-2DG was removed and cells were washed twice with ice-cold 0.9% NaCl to halt substrate uptake. 1 ml of 50 mM NaOH was added to the wells, for at least 1 hour, in order to lyse the cells. 850 µl of cell lysate was used to quantify the amount of [3H]-2DG taken up by the cell, using a Beckman LS6000IC scintillation counter. Protein content of the lysate was determined on the remaining 150 µl of lysate using the Bradford method. The rate of [3H]-2DG uptake was determined by subtracting the counts of cytochalasin B treated wells from the counts in the samples. These values were then divided by the specific activity of the assay buffer (quantified from 10 µl of assay buffer) to determine the amount of [3H]-2DG (pmol) taken up by the whole well. This value was then divided by 850 µl to determine [3H]-2DG uptake in pmol/µl. This value was then multiplied by 1000 to obtain the amount of [3H]-2DG taken up by the whole well. This value was then divided by the total protein content in the well and the time of the assay incubation time to give rate of [3H]-2DG in pmol/min/mg of protein in the lysate.

2.6 14C-Glucose oxidation assay

14C-glucose oxidation assays were carried out at 37°C, as described previously (Hamilton et al., 2014). 200,000 cells/well were seeded into a 6-well cell culture plate at least 16 hours prior to performing the assay. At the start of the assay, GT1-7 cells were washed twice with
pre-warmed (37 °C) HBS and then incubated for 4 hours at 37 °C in HBS containing 2 µCi/ml ¹⁴C-glucose (U-¹⁴C-D-glucose, Perkin Elmer) and 0.1, 0.5 or 2.5 mM glucose. Following the incubation period, 1 ml of the assay buffer was transferred to a 15 ml tube (Falcon) which contained a Whatman (GF/B) filter paper which had been pre-soaked in 1 M KOH. 200 µl of 60% perchloric acid was then added to the assay buffer in the tube in order to facilitate the release of ¹⁴C-CO₂, which had been released from the cells through the metabolism of ¹⁴C-Glucose. This reaction was then left overnight to allow the filter paper in the tube lids to capture a quantifiable amount of ¹⁴C-CO₂. The next day, filter papers were soaked in scintillation fluid and the radioactivity was quantified using a Beckman LS600IC scintillation counter. After the assay buffer was removed from the wells, the cells were washed twice with ice-cold 0.9% NaCl and then lysed in 1 ml of 50 mM NaOH. 850 µl of lysate was used to quantify the ¹⁴C incorporation into the cell, through scintillation counting. 150 µl of the remaining lysate was used to determine the protein content of the well, using the Bradford method. Glucose oxidation in each ¹⁴C-CO₂ sample was calculated by dividing the counts in each sample by the specific activity of the assay buffer. This value was then divided by the incubation time and then by the protein content of the well in order to achieve a ¹⁴C-Glucose oxidation rate in pmol/min/mg protein in the lysate. ¹⁴C incorporation was calculated by dividing the counts in each sample by the specific activity of the assay buffer. This value was then divided by the assay incubation time, then divided by 850 µl to give pmol/min/µl. This value was then multiplied by 1000 in order to determine the ¹⁴C incorporated in the whole well. This was then divided by the protein content of the well in order to obtain the ¹⁴C incorporation rate in pmol/min/mg of protein in the lysate.

2.7 AMPK activity assay

The AMPK activity assay was performed as previously described (Beall et al., 2012). GT1-7 cells were seeded in 100 mm NUNC dishes and were maintained in plating media while
being subjected to the RH protocol. On the last day of the RH protocol, cells were washed with warm PBS and serum starved for 1 hour in order to the AMPK activity in the cells down to a more physiological level as the serum in the plating media contains unknown levels of AMPK activators such as leptin and insulin. Following the serum free period, cells were again washed with warm PBS before being challenge with 2.5, 0.5 or 0.1 mmol/l glucose for 15 minutes or 3 hours. The purpose of treating for 15 minutes was to try and gauge the level of AMPK activity that would be present in the same time frame as the electrical response was taken during the electrophysiology. The 3 hour incubation was performed to examine whether maximal AMPK activation was altered between groups. Cells were lysed in low salt Immunoprecipitation Buffer (IP) (table 2.4) and lysates were immediately frozen in liquid nitrogen. Lysates were then thawed and centrifuged at 13000 rpm for 15 minutes at 4°C in order to remove insoluble material. The protein content of the supernatant was determined using a BCA assay (Fisher Scientific, Loughborough, UK) as the DTT in the IP buffer makes the Bradford protein assay unsuitable. Lysates were then immunoprecipitated with the desired α-AMPK subunit antibody and Protein-G Sepharose (PGS) beads. PGS beads are provided in a slurry of 20% ethanol so in order to prepare the beads and remove the ethanol the following calculation was used, allowing 5 µl of beads per sample: (no. of samples x 5 µl) x 120%. The slurry was then briefly centrifuged at 5000 rpm at room temperature in order to pellet the beads. The ethanol layer could be seen clearly on top of the PGS beads and 20% of the total volume was removed to leave behind 100% PGS beads. The beads were then washed with 1 ml of low salt IP buffer and briefly spun down, as mentioned previously, after which the low salt IP buffer was removed. This was repeated once more and then the beads were suspended in a volume of low salt IP buffer that would allow a volume of no less than 20 µl of bead slurry to be added to each lysate sample. The prepared PGS beads were therefore divided into eppendorfs, for each sample that would be run, and incubated with α1-, α-2 or both α-subunit
AMPK specific antibodies at 4°C for 1 hour, whilst being gently mixed. After the beads and antibodies were bound, lysate was added (100 µg for mixed α1- and α2-AMPK or 200 µg for looking at individual isoforms) along with 500 µl of low salt IP buffer plus fresh inhibitors (table 2.4). Samples were then incubated at 4°C on an IP shaker overnight. The following day, samples were spun down and washed twice with high salt IP buffer and once with HEPES assay buffer. Once the HEPES assay buffer wash was removed, 320 µl of fresh HEPES assay buffer was added to each sample and then 100 µl was aliquoted in 3 eppendorfs in order to obtain 33.3 µg of protein for examining mixed α1- and α2-AMPK or 66.6 µg for individual isoforms. The 3 aliquots were then designated as two positive samples that would receive the AMPK substrate AMARA (AMARAASAAALARRR) and one negative sample which would be used as a blank to subtract non-specific radioactivity from each positive sample. Samples were then centrifuged briefly and 80 µl of supernatant was removed, leaving behind 20 µl of beads, antibody and lysate. The ATP stock solution and sample mixes were then prepared as follows:

- ATP stock solution (1 ml total volume) – 20 µl 32P-ATP (Perkin Elmer), 10 µl 100 mM ATP, 10 µl 2.5 M MgCl2 and 960 µl HEPES assay buffer
- Mix 1 (AMARA positive samples) - 1mM ATP stock solution, 1 mM AMP and AMARA (equal volumes of each component should be added to the mix)
- Mix 2 (AMARA negative samples) – 1mM ATP stock solution, 1 mM AMP and HEPES assay buffer (equal volumes of each component should be added to the mix)

The reaction was started when 30 µl of either mix 1 or 2 was added to the samples (the eppendorfs already contained 20 µl of beads, antibodies and lysate so total volume for the assay is 50 µl). Reactions were staggered 20 seconds apart and incubated at 30°C in a bench top air incubator on an IP shaker for 20 minutes. The reactions were stopped in the
order they were started, 20 seconds apart, by removing 30 µl of sample and pipetting it onto a square of P81 paper. After a few seconds the sample had soaked into the paper which was then dropped into 1% (v/v) phosphoric acid. This terminated the reaction and washed away any unbound \(^{32}\)P-ATP. Once all the reactions were stopped, the P81 papers were left stirring in phosphoric acid for 5 minutes at room temperature. The phosphoric acid was poured off and the P81 papers were stirred in fresh phosphoric acid for another 5 minutes. This process was then repeated once more. The P81 papers were then left to dry on a radioactive spill and then loaded into scintillation vials containing scintillation fluid. The radioactivity was quantified using a Beckman LS600IC scintillation counter. In order to calculate the activity in each sample, the counts of the two AMARA positive samples were averaged and the AMARA negative values were subtracted. This value was then divided by the specific activity of the ATP stock solution and then divided by the 20 minute incubation time. This value was divided by the 30 µl of sample that was pipetted on the P81 paper and multiplied by 50 µl to give the total activity present in the sample. The activity was then normalised to the amount of protein in the sample (either 33.3 or 66.6 µg). Final activity was calculated as nmol/min/mg but data were normalised to “control 3 hours 2.5 mmol/l” and expressed as fold change.
Table 2.4 AMPK Activity Assay Buffers

<table>
<thead>
<tr>
<th>Buffer Type</th>
<th>Composition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Immunoprecipitation Buffer (low salt)</td>
<td>50 mM Tris-HCl (pH 7.25), 150 mM NaCl, 50 mM NaF, 5 mM NaPPi, 1 mM EDTA, (Inhibitors to be added just before use - 1 mM dithiothreitol (DTT), 0.1 mM benzamidine, 0.1 mM phenylmethanesulfonyl fluoride (PMSF), 5 µg/ml soya bean trypsin inhibitor, 1% (v/v)) Triton-X 100</td>
</tr>
<tr>
<td>Immunoprecipitation Buffer (high salt)</td>
<td>Low salt Immunoprecipitation Buffer + 0.5 M NaCl</td>
</tr>
<tr>
<td>HEPES Assay Buffer</td>
<td>50 mM HEPES (pH 7.4), 1 mM DTT, 0.02% Brij-35</td>
</tr>
</tbody>
</table>

2.8 Seahorse XF24 Extracellular Flux Analyser

The Seahorse XF24 Extracellular Flux Analyser (Seahorse Bioscience) was used to measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) produced from oxidative phosphorylation and the release of lactate from glycolysis, respectively. 30,000 cells/well were seeded into 24 well Seahorse XF24 culture plates, at least 18-24 hours before the assay was performed. Optimal cell number for GT1-7 cells had been determined by previous experiments using titrations of increasing cell numbers. The OCR and ECAR can become unstable and inconsistent between wells when cells are too confluent and so 30,000 cells/well was determined as the optimal number. To start the assay, cells were changed from 2.5 mM glucose plating media into unbuffered, serum free, phenol-red free DMEM containing 2.5 mM glucose and placed in a non-CO₂ incubator at 37°C, for 1 hour prior to being placed in the Seahorse XF24. The purpose of this was to de-gas the cells and the cell culture plate of any CO₂ that may disrupt the reading from the Seahorse XF24. During the cell de-gassing incubation, any inhibitors/compounds were loaded into the XF24 cartridge, in
order to allow these compounds to be injected during the assay. When mitochondrial function was being examined, these compounds were Oligomycin, Antimycin A, Rotenone and FCCP. The cartridge was then loaded into the Seahorse XF24 in order to calibrate. After successful cartridge calibration and de-gassing of the cells, the cells were placed into the Seahorse XF24 and the cell culture plate was aligned with the cartridge so that injections of compounds into wells could take place when required. The respiration of the GT1-7 cells determined that 3 minute mix, 2 minute wait and 3 minute measure cycles were used during the assay. 4-5 baseline measurements were taken at the start of the assay before compounds were injected in order to make sure that the basal respiration was stable. 3 mix, wait, measure cycles were performed after the addition of a compound.

The above protocol was altered slightly when performing the glycolysis stress test, designed to examine glycolytic function and capacity. On the day of the assay, cells are incubated for 1-2 hours in unbuffered, serum free, phenol-red free DMEM with pyruvate and L-glutamine. As no glucose is present in this culture media but pyruvate and L-glutamine are still able to maintain mitochondrial respiration through by-passing glycolysis. This resulted in very minimal glycolytic activity being observed at the start of the assay. After baseline measurements were made, glucose was injected into the wells in order to stimulate basal glycolysis, as represented by an increase in ECAR. Oligomycin was then applied in order to inhibit mitochondrial ATP synthesis, making the cell reliant of glycolytic ATP production for maintaining bioenergetics and so this will stimulate the maximal glycolytic rate. 2DG (5x the concentration of the injected glucose) was added at the end of the assay in order to inhibit glucose uptake and glycolysis.
2.9.1 RNA extraction

RNA was extracted from GT1-7 cells for use with the Qiagen RT² Profiler PCR Array Kit (mouse glucose metabolism - PAMM-006Z, Qiagen). Cells were grown to <70% confluency in 100 mm² NUNC cell culture plates and were washed twice with ice-cold PBS before 1 ml of Tri reagent (Sigma, T9424) was applied to each plate. Tri reagent detached the cells from the dish and they were collected by pipetting into an eppendorf. 200 µl of chloroform was then added to each sample, after which the eppendorf was shaken vigorously for 30 seconds and left to incubate at room temperature for 15 minutes. Samples were centrifuged at 12,000 x g for 15 minutes at 4 °C in order to achieve phase separation. The RNA was contained in a colourless upper phase which was carefully removed, ensuring not to disturb the pink interphase which contained DNA. The RNA was placed into a separate eppendorf with 500 µl of isopropanol (Fisher Scientific, P/7490/17) and the sample was mixed by gently inverting the tube. The samples were then left to react for 10 minutes at room temperature. Samples were centrifuged again at 12,000 x g for 10 minutes at 4°C in order to pellet the RNA. The supernatant was removed and the RNA pellet was washed with 1 ml of 75% (v/v) ethanol, mixed by briefly vortexing and then centrifuged at 7,500 x g for 5 minutes at 4°C. The ethanol was poured off and the RNA pellet was left to air dry for at least 10 minutes. The dried RNA pellet was re-suspended in RNase-free H₂O by gentle pipetting. RNA concentration (absorbance at 240 nm) and purity were determined using a Nano-Drop spectrophotometer (ND-8000). Purity was determined by the 260:280 ratio and samples were accepted if > 1.9.

2.9.2 cDNA synthesis

1 µg of DNA was prepared from the purified RNA samples, outlined above, for use with the Qiagen RT² Profiler PCR Array Kit (mouse glucose metabolism - PAMM-006Z, Qiagen)
using the RT² First Strand reagent kit (330401, Qiagen). A genomic DNA elimination mix was prepared from components within the kit as follows: 1 µg RNA, 2 µl Buffer GE, enough RNase-free H₂O to bring the total volume of the mix to 10 µl. The genomic DNA elimination mix was then incubated at 42°C for 5 minutes before being placed on ice. The reverse-transcription mix was prepared as follows: 12 µl 5x Buffer BC3, 3 µl Control P2, 6 µl RE3 Reverse Transcriptase Mix and 9 µl RNase-free H₂O. 10 µl of reverse-transcription mix was then added to each genomic DNA elimination mix and mixed by gentle pipetting. The samples were then incubated at 42°C for 15 minutes, after which the reaction was halted by incubating the sample at 95°C for 5 minutes. Samples were then either stored at -20°C or loaded straight away on the RT² Profiler PCR Array plate.

2.9.3 Real-time PCR

Real-time PCR was carried out on the cDNA synthesised previously and was carried out using a Qiagen RT² Profiler PCR Array Kit (mouse glucose metabolism - PAMM-006Z, Qiagen). The PCR array assay mix was prepared for each sample as follows: 1350 µl 2x RT² SYBR Green Mastermix, 102 µl cDNA and 1248 µl RNase-free H₂O. 25 µl of the PCR array assay mix was then loaded into each well of the RT² Profiler PCR Array plate, which was ran on the 7900HT Fast Real-time PCR system. Results were analysed using the Qiagen RT² Profiler PCR Array online software and finally expressed as fold change from control.

2.10 NADP⁺/NADPH assay

The levels of nicotinamide nucleotides, NADP⁺ and NADPH, were quantified in GT1-7 cells using a colourimetric assay kit, according to the manufacturer’s instructions (ab65349, Abcam). On the day on the assay 200, 000 cells were trypsinised and centrifuged at 800 rpm for 4 minutes in order to pellet the cells. The pellet was washed with 5 ml ice-cold PBS and briefly vortexed. Cells were then centrifuged again at 2000 rpm for 5 mins, after which time
the PBS was removed and 400 µ of extraction buffer was added. The cells were then lysed with 2x freeze-thaw cycles on dry-ice. After this time, the samples were briefly vortexed and centrifuged at 13,000 rpm at 4°C for 15 minutes. The supernatants were then transferred to new eppendorfs. Each sample was divided into two, with one sample being left on ice and used for the detection of total NADP (NADP + NADPH) while the other half of the sample was incubated at 60°C for 30 minutes in order to decompose all NADP in the sample, leaving behind only NADPH. 50 µl of each sample, standard and decomposed, were added to the plate in duplicate along with an NADP standard curve (0, 20, 40, 60, 80 and 100 pmol/well). NADP Cycling Mix was then prepared, as per manufacturer’s instructions by adding NADP Cycling buffer and NADP Cycling Enzyme Mix together in a 50:1 ratio. 100 µl of NADP Cycling Mix was added to all wells and incubated at room temperature for 5 minutes. 10 µl of NADPH developer was then added to all wells and the plate was left to incubate for 4 hours at room temperature, whilst being gently mixed on a rocker. The reaction was stopped by adding 10 µl of Stop Solution to each well and the plate was then read at 450 nm. NADPH was read by comparing the absorbance against the NADP standard curve, whereas NADP\(^+\) was calculated as the difference in absorbance between total NADP and NADPH.

2.11 ROS detection assay

Levels of reactive oxygen species (ROS) were semi-quantified using a fluorometric detection assay kit, according to the manufacturer’s instructions (ab113851, Abcam). This assay utilises the cell permeable reagent 2’,7’-dichlorofluorescein diacetate (DCFDA) which is a fluorogenic dye. DCFDA is oxidised by ROS and converted into the fluorescent compound 2’,7’-dichlorofluorescein which can be detected and used to indirectly semi-quantify levels of ROS within the cell. GT1-7 cells seeded at a density of 1000 cells/well in a black, opaque 96-well microplates 16 hours prior to the start of the assay. At the start of the assay, cells were washed with 100 µl sterile PBS and then stained with 100 µl 25 µM DCFDA for 45 minutes
at 37°C. After this time, cells were washed with 100 µl of the provided 1x buffer before any cell treatments were applied. After the duration of the desired treatment, fluorescence was analysed using a fluorescent plate reader (FluoSTAR OPTIMA) with the excitation wavelength at 485 nm and the emission wavelength at 535 nm. Data were analysed as fluorescence intensity per 1000 cells.

2.12 ATP assay

The intracellular concentration of ATP was measured using a bioluminescence assay, according to the manufacturer’s instructions (ATPlite, PerkinElmer). This assay is based on the production of light from the reaction of ATP with luciferase and D-luciferin. The emitted light is directly proportional to the intracellular ATP concentration. The reaction is as follows:

\[
\text{ATP} + \text{D-Luciferin} + \text{O}_2 \xrightarrow{\text{Mg}^{2+}, \text{luciferase}} \text{Oxyluciferin} + \text{AMP} + \text{PPi} + \text{CO}_2 + \text{Light}
\]

GT1-7 cells were seeded at 1000 cells/well in plating media, 16 hours before the assay was performed. On the day of the assay, the media was replaced with fresh plating media in order to replenish glucose availability. Cell treatments were applied in NS for the desired time, after which 50 µl of mammalian lysis buffer was added to the wells to lyse the cells and stabilise the released ATP. The plate was mixed gently on an orbital shaker for 5 minutes, after which time 50 µl of substrate solution was added to the wells and the plate was placed back on the orbital shaker for 5 minutes. After this time, the plate was dark adapted for 10 minutes and then read on a LB 962 CentroPro Luminescence Microplate reader. A standard curve for ATP (0, 0.001, 0.01, 0.1, 1 and 10 pmols) was also included on the plate. Data were analysed pmol ATP/1000 cells by correlating the luminescence intensity for each well against the ATP standard curve.
2.13 ATP/ADP assay

The intracellular ATP:ADP ratio was examined using a bioluminescence assay (Ab65313, Abcam). GT1-7 cells were seeded at 1000 cells/well 16-24 hours before the assay in plating media. The media was replaced with fresh on the day of the assay. Cell treatments were applied in NS for a desired time, after which the treatment was removed and cells were washed with 200 µl pre-warmed PBS. 50 µl of reaction mix (1:19 mix of ATP Monitoring Enzyme and Nucleotide Releasing Buffer) were added to all wells. Cells were left to lyse in the reaction mix for 2 minutes and then the plate was read on a LB 962 CentroPro luminescence microplate reader \((data\ A)\). The reaction was left for 10 minutes and read again \((data\ B)\). 10 µl of ADP Converting Enzyme was then added to each well, left for 1 minute and then read again \((data\ C)\). Results were analysed as \(data\ C\ (=ATP) / (data\ A - data\ B)\)(=ADP).

2.14 Pyruvate dehydrogenase activity assay

Pyruvate dehydrogenase activity was examined using a colourimetric assay, according to the manufacturer’s instructions (Ab109902, Abcam). This assay utilises immunocapture of PDH from samples, the activity of which is determined from the reduction of \(NAD^+\) to \(NADH\) which will produced a yellow colour. This output can then be measured by monitoring the absorbance at 450 nm. 200,000 cells per sample were collected in 200 µl ice-cold PBS and then lysed with 2x freeze thaw cycles on dry-ice. Protein content was determined using the Bradford method. Cells were solubilised by the addition of detergent (9:1 sample volume:detergent volume) and incubated on ice for 10 minutes. Lysates were centrifuged at 1000 x g for 10 minutes at 4°C. Samples were then diluted to 500 µg in 200 µl, in duplicate, which was then loaded onto a 96-well microculture plate and incubated at room temperature
for 3 hours. The wells were washed twice with 1x stabiliser before 200 µl of assay buffer was added. The plate was then read at OD450 nm, at room temperature.

2.15 Isocitrate dehydrogenase assay

Isocitrate dehydrogenase (IDH) activity was examined using a colourimetric assay, according to the manufacturer’s instructions (Ab102528, Abcam). 1, 000, 000 cells were collected and lysed in 200 µl of ice-cold IDH activity assay buffer. Samples were then centrifuged at 13, 000 x g for 10 minutes and the supernatant was transferred to a separated eppendorf. 50 µl of the supernatant was loaded onto a 96-well microculture plate with 50 µl of Master Mix which contained IDH substrate and either NADP⁺ or NAD⁺ as a co-factor, depending on the IDH isoform. A standard curve of NADH was also prepared (0, 2, 4, 6, 8, 10 nmol/well). The reaction was incubated for 3 minutes at 37°C before the first reading was made at OD450nm (A₀). The plate was incubated at 37°C for another 5 minutes before the second reading was taken (A₁). The change in absorbance of each sample (A₀-A₁) was compared to the NADH standard curve in order to calculated how much NADH or NADPH was produced during the assay (B). IDH activity was analysed as (B x sample dilution factor)/(reaction time x sample volume) in nmol/min/ml.

2.16 Bradford protein assay

Protein concentration was determined for each lysate using the Bradford method (Bradford, 1976). Protein samples were diluted 1 µl of lysate in 9 µl distilled H₂O with the addition of 250 µl of Bradford reagent (Sigma), in a 96-well microculture plate. The reaction was left to incubate at room temperature for 10-20 minutes followed by analysis using at 96-well plate spectrometer (2104 Envision, PerkinElmer). Absorbance was read at OD595 nm and compared to a standard curve of known BSA protein concentrations.
2.17 Data analysis

One-way ANOVA, with a post-hoc Bonferroni test, was used to determine statistical differences between 3 or more groups, such as in the electrophysiological recordings. All other data sets were analysed using an unpaired Student’s t-test (Graphpad, Prism 5 software). Data are presented as mean ± SEM. Statistical significance was accepted at the 95% confidence value with a $P$ value of less than 0.05. Significance was allocated to the following $P$ values: * < 0.05; ** < 0.01 and *** < 0.001.
Chapter 3

*In vitro* modelling of recurrent hypoglycaemia in GT1-7 cells
3.1 Introduction

There is now an accumulating body of evidence in the literature demonstrating that the CRR to hypoglycaemia is regulated by the central nervous system (Borg et al., 1994, Borg et al., 1997, Borg et al., 1995). However, little is known in regards to the underlying mechanisms by which the brain appears to become desensitised to hypoglycaemia following antecedent/recurrent hypoglycaemia. Some molecular adaptations have been identified centrally following RH, in relation to altered neuronal metabolism. *In vivo* studies examining GK expression in rats with defective CRR following antecedent hypoglycaemia found that GK mRNA expression in the VMH was increased (Kang et al., 2008, Dunn-Meynell et al., 2002, Sanders et al., 2004). Due to the fact that GK generates ATP through the phosphorylation of glucose, this may result in an increase in cytosolic ATP. If this occurred in $K_{ATP}$-dependent GE neurons following RH, an increase in ATP concentration localised at the plasma membrane could act to inhibit the channel and keep the cell membrane depolarised during hypoglycaemia. Following 4 days of ICV 2DG-induced neuroglucopenia, in rats, the activity of AMPK$\alpha_1$ and AMPK$\alpha_2$ in some hypothalamic nuclei was found to be attenuated in response to further 2DG stimulation (Alquier et al., 2007). In addition to this finding, a separate study also showed that AMPK$\alpha_2$, but not AMPK$\alpha_1$, mRNA expression in the VMH was reduced following antecedent hypoglycaemia (Kang et al., 2008). The discrepancies between the two studies may be due to the fact that AMPK would have been determined from slightly different proportions of mixed neuronal populations in each study (i.e. GE, GI, non-glucose-sensing). Nevertheless, activation of AMPK during hypoglycaemia has been shown to amplify the CRR and so a reduction in this response in the glucose-sensing neurons of the aforementioned studies may contribute to a blunted ability of the brain to detect hypoglycaemia (McCrimmon et al., 2006, McCrimmon et al., 2008).
In addition to adaptations occurring intrinsically to glucose-sensing neurons, there have also been reports of glycogen supercompensation following RH and recurrent neuroglucopenia in rats (Alquier et al., 2007). The theory of glycogen supercompensation following antecedent hypoglycaemia is based on the fact that astrocytes, like the liver, are capable of storing glycogen. As neurons are able to utilise lactate for energy, which has been shown to increase activity of GE neurons and reduce the activity of GI neurons, increased glycogen storage and then breakdown to lactate by astrocytes during hypoglycaemia may prevent GE neurons from hyperpolarising and initiating the CRRs (Song and Routh, 2006, Beall et al., 2012).

Conversely, studies of glycogen compensation in the brains of rats following RH found an overall reduction in brain glycogen content at the end of a hypoglycaemic episode and no overshoot of glycogen restoration in RH animals when euglycaemia had been restored, compared to controls (Herzog et al., 2008). Interestingly, the same group did find a slight increase in lactate uptake from the periphery and utilisation by the brain, following RH compared to controls (Herzog et al., 2013). Although lactate cannot sustain the same level of metabolic activity as glucose, these authors suggest that it may be able to support neuronal function during hypoglycaemia and therefore minimise damage to the brain during recurrent hypoglycaemia.

While the studies mentioned above have shown that adaptations can occur in physiological processes that are involved in central glucose-sensing, the focus of these studies was on whole hypothalamic nuclei or unidentified populations of neurons. The aim of the current study was to investigate if adaptations occur following recurrent low glucose exposure in a homogenous population of GE neurons. To do this, the mouse hypothalamic GT1-7 cell line was utilised as a model of a homogenous population of GE neurons and the adaptations to recurrent low glucose exposure were examined in terms of both metabolism and electrical activity. This type of investigation will aim to decipher if adaptations occur intrinsically
within glucose-sensing neurons following recurrent low glucose exposure, in the absence of astrocytic and synaptic influences, and possibly uncover molecular therapeutic targets for the treatment of defective CRR.
3.2 Results

3.2.1 Antecedent low glucose exposure attenuates glucose sensing, in GT1-7 cells

One episode of antecedent hypoglycaemia has been shown to attenuate the counter regulatory response to further episodes of hypoglycaemia in both humans and rodents (Heller and Cryer, 1991, Adamson et al., 1984, Fioramonti et al., 2013, Inouye et al., 2002, Jacobson et al., 2006, McCrimmon et al., 2005). In an attempt to determine the suitability of the mouse hypothalamic GT1-7 cell line for investigating the neuronal adaptations to hypoglycaemia, cells were exposed to 3 hours of antecedent 0.1 (antecedent hypoglycaemia = AH), or 2.5 mM glucose (control) normal saline (NS) for the corresponding controls, before being placed back into 2.5 mM glucose plating media and examined electrically 18-24 hours later.

Perforated patch-clamp recordings were performed in order to monitor the response of the membrane potential to low glucose without disrupting the intracellular metabolism of the cell. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). 25-40 μg/ml amphotericin B was also present in the pipette in order to perforate the cell membrane and no current was injected during the recordings. A stable recording was obtained for ~10 minutes at 2.5 mM glucose NS before 0.5 mM glucose was superfused. This concentration of glucose was chosen to test the response of GT1-7 cells to low glucose as these cells have been shown to respond to glucose in a dose dependent manner (0-2.5 mM) with respect to membrane potential (Beall et al., 2012). 0.5 mM is in the middle of the glucose-sensing range and so was used in order to decipher if there was a rightward shift in the glucose dose response of cells exposed to AH which might not be apparent at lower concentrations which cause near maximal $K_{ATP}$ activity. The response of GT1-7 cells to 0.5 mM glucose under control conditions was a hyperpolarisation with an average response of $-13.7 \pm 1.7$ mV (Fig 3.1A),
which was significantly attenuated following AH, with the mean membrane potential only hyperpolarising by $-8.7 \pm 1.6$ mV (Fig 3.1B) ($n = 19-23$; $P < 0.05$, Fig 3.1C).

Because the defect in the counterregulatory response (CRR) to hypoglycaemia appears to be enhanced by increased duration, frequency and depth of AH in humans and animal models, a recurrent hypoglycaemia (RH) protocol was applied to GT1-7 cells in order to verify whether a further loss of hypoglycaemia sensitivity could be recapitulated in vitro.

### 3.2.2 Recurrent low glucose exposure attenuates glucose sensing, in GT1-7 cells.

GT1-7 cells were exposed to 0.1 (RH) or 2.5 mM glucose NS (control) for 3 hours on 3 consecutive days. Cells were maintained in 2.5 mM glucose plating media in between hypoglycaemia or euglycaemic incubations and examined electrically 18-24 hours later. Perforated patch-clamp recordings were used to examine glucose-sensing. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). 25-40 µg/ml amphotericin B was also present in the pipette and no current was injected during the recordings. As before, a stable recording was obtained in 2.5 mM glucose before 0.5 mM glucose NS was superfused. GT1-7 cells under control conditions hyperpolarised from 2.5 to 0.5 mM glucose by an average of $-14.7 \pm 0.9$ mV (Fig 3.2A) whereas this response was significantly attenuated following RH with an average response of $-4.5 \pm 1.2$ mV (Fig 3.2B) ($n = 7-10$ respectively; $P < 0.001$, Fig 3.2C).

In an attempt to investigate if the attenuated glucose-sensing observed at 0.5 mM glucose following RH was also true for lower glucose concentrations (i.e. if there was a change in the maximal response), a subset of cells were tested at 0.1 mM glucose NS, as the hyperpolarisation obtained with this glucose concentration is near maximal. Perforated patch-clamp recordings were used to examine glucose-sensing. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP.
ATP (pipette solution A). 25-40 μg/ml amphotericin B was also present in the pipette and no current was injected during the recordings. As before, a stable recording was obtained in 2.5 mM glucose before 0.1 mM glucose NS was superfused. GT1-7 cells under control conditions hyperpolarised from 2.5 to 0.1 mM glucose by an average of -23.0 ± 2.8 mV (Fig 3.3A) whereas this response was significantly attenuated following RH with an average response of -8.6 ± 2.5 mV (Fig 3.3B) (n = 7 respectively; P < 0.005, Fig 3.3C). Although the membrane hyperpolarisation was greater when tested at 0.1 compared to 0.5 mM glucose, the response was still attenuated at both glucose concentrations following RH compared to control. This suggests a blunting of hypoglycaemia detection in GT1-7 cells at all low glucose concentrations following RH, rather than just a rightward shift at intermediate concentrations between 2.5 and 0.1 mM.

Due to the fact that GT1-7 cells respond to glucose electrically through $K_{ATP}$ channel dependent mechanisms, the availability of the channel to open maximally was then investigated.

**3.2.3 Recurrent low glucose exposure does not alter the availability of the $K_{ATP}$ channel to conduct maximally.**

In order to measure the maximum availability of the $K_{ATP}$ channel to open, whole cell patch-clamp configuration was used to dialyse out the intracellular ATP from the cell, alleviating channel inhibition. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). Currents were evoked by a voltage-clamp protocol from -160 to -40 mV steps which were 400 milliseconds in duration and 20 milliseconds apart. This was applied when the cell was first ruptured and when intracellular ATP had been dialysed out of the cell (run-up) in order to obtain the maximum conductance density. The maximum conductance density, at run-up, did
not differ between cells under control conditions (Fig 4.4A) compared to RH (Fig 4.4B) (n = 10; 1.4 ± 0.3 vs 1.2 ± 0.3 pS/pF, respectively, Fig 4.4C). As the $K_{\text{ATP}}$ channel is regulated by intracellular metabolism and RH attenuates the ability of the membrane potential to hyperpolarise to low glucose yet there is no change in the conductance of the channel, alterations in glucose metabolism were next then investigated.
Figure 3.1 Antecedent low glucose exposure results in attenuated glucose-sensing at 0.5 mM glucose.

(A) Representative perforated-patch current clamp recording from a GT1-7 cell exposed to 2.5 mM glucose NS for 3 hours on day 1 before electrical activity was examined on day 2. Supernusion of 2.5 mM glucose depolarised the cell membrane where as substitution with 0.5 mM glucose induced hyperpolarisation. Pooled membrane potential data, including response to acute NN414, for antecedent 2.5 mM glucose (n = 19). (B) Representative perforated-patch current clamp recording from a GT1-7 cell exposed to 0.1 mM glucose NS for 3 hours on day 1 before electrical activity was examined on day 2. Under these conditions, the cells displayed a significantly attenuated hyperpolarisation to superfusion of 0.5 mM glucose. Pooled membrane potential data, including response to acute NN414, for antecedent 0.1 mM glucose (n = 23). For illustrative purposes, the change in membrane potential from 2.5 to 0.5 mM glucose is shown in panel C (AH = antecedent hypoglycaemia). Statistical significance was analysed by one-way ANOVA in panels A and B and student’s unpaired t-test in panel C (** P < 0.01, *** P < 0.001).
Figure 3.2 Recurrent low glucose exposure results in attenuated glucose-sensing at 0.5 mM glucose.

(A) Representative perforated-patch current clamp recording from a GT1-7 cell exposed to 2.5 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. Supertusion of 2.5 mM glucose depolarised the cell membrane where as substitution with 0.5 mM glucose induced hyperpolarisation. Pooled membrane potential data for antecedent 2.5 mM glucose (n = 7). (B) Representative perforated-patch current clamp recording from a GT1-7 cell exposed to 0.1 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. Under these conditions, the cells displayed a significantly attenuated hyperpolarisation to supertusion of 0.5 mM glucose. Pooled membrane potential data for antecedent 0.1 mM glucose (n = 10). For illustrative purposes, the change in membrane potential from 2.5 to 0.5 mM glucose is shown in panel C (RH = recurrent hypoglycaemia). Statistical significance was analysed by one-way ANOVA in panels A and B and student’s unpaired t-test in panel C (*P < 0.05, ***P < 0.001).
Figure 3.3 Recurrent low glucose exposure results in attenuated glucose-sensing at 0.1 mM glucose.

(A) Representative perforated-patch current clamp recording from a GT1-7 cell exposed to 2.5 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. Superfusion of 2.5 mM glucose depolarised the cell membrane whereas substitution with 0.1 mM glucose induced hyperpolarisation. Pooled membrane potential data for antecedent 2.5 mM glucose (n = 7). (B) Representative perforated-patch current clamp recording from a GT1-7 cell exposed to 0.1 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. Under these conditions, the cells displayed a significantly attenuated hyperpolarisation to superfusion of 0.5 mM glucose. Pooled membrane potential data for antecedent 0.1 mM glucose (n = 10). For illustrative purposes, the change in membrane potential from 2.5 to 0.5 mM glucose is shown in panel C (RH = recurrent hypoglycaemia). Statistical significance was analysed by one-way ANOVA in panels A and B and student’s unpaired t-test in panel C (∗ P < 0.05, ∗∗ P < 0.001).
Figure 3.4 Recurrent low glucose exposure does not alter $K_{ATP}$ channel conductance in response to dialysis of cell with 0 ATP

(Ai) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 2.5 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4, as it is dialysed with 0 ATP. (Aii) Corresponding families of currents evoked by the voltage-clamp protocol stated in “Methods”. Whole-cell macroscopic currents were examined immediately after the cell membrane was ruptured (control) and after the cell had been dialysed with 0 ATP (Run-up) and reached a steady state ($n = 10$). (Bi) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 0.1 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4, as it is dialysed with 0 ATP. (Bii) Corresponding families of currents ($n = 10$) (RH = recurrent hypoglycaemia). (C) Pooled conductance densities at run-up. Statiscal significance was analysed in panel C by student’s unpaired t-test.
3.2.4 Glucose uptake is not altered by recurrent low glucose exposure, in GT1-7 cells.

Glucose uptake is the rate-limiting first step in glucose metabolism and therefore this process was assessed in GT1-7 cells subjected to RH or control conditions (Fink et al., 1992). This was performed using the 2-deoxy-d-[³H]glucose (non-metabolisable analogue of glucose) uptake assay as described earlier. The calculations associated with this assay, which determine glucose uptake, assume that the rate of substrate transport is linear during the experiment. Therefore an initial set of experiments were run in order to determine the time over which glucose uptake was linear in GT1-7 cells. The assay was performed over 12, 15, 20 and 25 minutes, the results of which were all shown to be within a linear range (Fig 3.5). All subsequent assays were then performed at 15 or 20 minutes as these time points fell within the linear glucose uptake range. Unlabeled concentrations of glucose, physiologically relevant for the brain, were added to the assay buffer containing 2-deoxy-d-[³H]glucose in order to set the rate of substrate uptake. Increasing the substrate pool with unlabelled glucose increased the competition for uptake with [³H]2DG at the glucose transporter, which decreased the radioactivity that is taken up into the cell. The hypothesis behind performing this assay was to investigate if GT1-7 cells displayed increased glucose uptake, following RH, at lower glucose concentrations compared to controls. This could lead to increased intracellular ATP which would inhibit the K̂ATP channel and prevent hyperpolarisation. However, no change in glucose uptake was observed following RH compared to controls at 0, 0.1, 0.5 or 2.5 mM glucose (n = 4; 3.66 ± 0.78 vs 3.62 ± 0.86; 2.79 ± 0.79 vs 2.63 ± 0.63; 2.06 ± 0.62 vs 2.20 ± 0.61; 0.79 ± 0.21 vs 0.83 ± 0.17 pmol/min/mg, respectively, Fig 3.6).

Although glucose uptake was not altered following RH in GT1-7 cells, compared to controls, this does not inform us regarding possible adaptations that may occur in glucose oxidation and glycolysis. Therefore, glucose oxidation was next investigated following RH.
3.2.5 Glucose oxidation and incorporation are unaltered following RH, in GT1-7 cells

Glucose oxidation was examined using a radiolabeled glucose (\(^{14}\text{C}-\text{glucose}\)) oxidation assay. This assay involves the metabolism of \(^{14}\text{C}-\text{glucose}\) resulting in the production of \(^{14}\text{CO}_2\) which can be captured and taken as a measure of glucose oxidation. Glucose which has been incorporated into the cell through anabolic processes can also be investigated through measurement of \(^{14}\text{C}\) in the cell lysate. The assay was performed over 4 hours at 37°C in assay buffer that contained \(^{14}\text{C}-\text{glucose}\) and a range of unlabelled glucose, physiologically relevant for the brain, which will aim to set the rate of oxidation. The hypothesis behind this experiment was that RH might make GT1-7 cells more efficient at using lower levels of glucose, which would produce more ATP to keep the \(\text{K}_{\text{ATP}}\) closed channel during low glucose exposure. However, no change in glucose oxidation was observed following RH compared to controls at any of the unlabelled glucose concentrations 0.1, 0.5 and 2.5 mM (\(n = 3\); 4.41 ± 0.63 vs 4.60 ± 0.02; 14.46 ± 1.61 vs 14. 19 ± 1.8; 27.79 ± 2.60 vs 28.98 ± 2.17 pmol/min/mg, respectively, Fig 3.7A). There was also no change in the glucose incorporation observed at 0.1, 0.5 and 2.5 mM glucose (\(n = 3\); 46.47 ± 5.08 vs 46.78 ± 8.33; 177.03 ± 16.34 vs 163.53.53 ± 24.51; 228.43 ± 34.07 vs 214.33 ± 31.30 pmol/min/mg, respectively, Fig 3.7B).

These results indicate that there are no alterations in the levels of glucose oxidation and incorporation in GT1-7 cells following RH compared to controls. However, the oxidation quantification component of the assay works through radiolabeled \(\text{CO}_2\) release. As other metabolic pathways within the cell, such as the pentose phosphate pathway, also process glucose-6-phosphate and release \(\text{CO}_2\) as a by-product, there could still be a reduction of one pathway and an increase in another which, would produce no overall change in glucose oxidation (Takahashi et al., 2012). As mitochondria are the main site of ATP generation within the cell, adaptations in mitochondrial function were next investigated as an increase in
the efficiency of ATP production may contribute to keeping the $K_{\text{ATP}}$ channel closed during low glucose exposure.

**3.2.6 Recurrent low glucose exposure alters glucose utilisation, in GT1-7 cells.**

Due to the fact that actively respiring mitochondria is an oxygen consuming process, oxygen consumption rate (OCR) can be taken as a measure of substrate flux through the mitochondria. Mitochondrial function and real-time analysis of cellular respiration were investigated using the Seahorse XF-24 Extracellular Flux Analyser (Seahorse Bioscience, Copenhagen, Denmark). GT1-7 cells were subjected to the RH or control protocols and then seeded at 30,000 cells/well in a 24-well culture microplate on the 3rd day of the protocol, at least 3 hours following the last hypoglycaemic incubation. Cells were then left for 24 hours in 2.5 mM glucose plating media. Cells were placed in serum-free seahorse media 1 hour before the assay, which contained 2.5 mM glucose as the only fuel source, after which time they were then placed in the XF-24 and oxygen OCR was measured in real-time. GT1-7 cells subjected to RH displayed a significantly lower OCR compared to controls ($n = 8$; $4.60 \pm 0.51$ vs $7.97 \pm 0.63$ pmoles O$_2$/min/μg, respectively; $P < 0.001$, Fig 3.8A).

Extracellular acidification rate (ECAR), which is a measure of protons extruded from the cell in the form of lactic acid from glycolysis, was obtained from the same wells that the OCR results were taken from. GT1-7 cells subjected to RH displayed a significantly lower ECAR compared to controls, indicative of reduced glycolysis ($n = 5$; $0.59 \pm 0.07$ vs $1.03 \pm 0.14$ pmoles O$_2$/min/μg, respectively; $P < 0.05$, Fig 3.8B).

In order to confirm a reduction in glycolysis, a glycolysis stress test was applied to GT1-7 cells following RH and control protocols. Cells were started off in 0 mM glucose containing media supplemented with 4 mM L-glutamine and 2.5 mM Na-pyruvate in order to shut off glycolysis yet maintain cellular energetics through oxidative metabolism. 2.5 mM glucose
was injected into the wells to stimulate glycolysis. Oligomycin was applied in an attempt to upregulate and obtain maximal glycolysis, as the cells will need to maintain bioenergetics through glycolysis rather than oxidative metabolism. Finally 2-deoxyglucose (2DG) was added to inhibit glucose uptake and metabolism through hexokinase inhibition. Data were quantified as the change in ECAR from baseline (i.e. 0 mM glucose). As can be seen in Fig 3.9 ECAR starts to fall after the 1st or 2nd measurement following injection of 2.5 mM glucose. This was only observed when 2.5 mM glucose was applied during the glycolysis stress test, whereas glycolysis seemed to be more stable at higher glucose concentrations (Appendix Fig. 1). However, because the metabolic measurements following RH were required to correlate with the conditions at which the cells are subjected to during electrophysiology, 2.5 mM glucose seemed the most physiologically relevant. In order to try and quantify any differences in glycolysis between RH and control treated cells, the first measurement following addition of glucose or Oligomycin was compared. The ΔECAR from baseline at the first measurement (40 minutes) after 2.5 mM glucose was added was significantly reduced following RH compared to control (n = 10; 3.39 ± 0.59 vs 4.94 ± 0.41, respectively, P < 0.05, Fig 3.9). Oligomycin failed to upregulate glycolysis in GT1-7 cells, following both RH and control protocols. This effect was not limited to performing the assay with 2.5 mM glucose, as Oligomycin failed to stably increase glycolysis at higher glucose concentrations (Appendix Fig. 1). The ΔECAR from baseline at the first measurement (64 minutes) after 1 μM Oligomycin was added was also reduced following RH compared to control. But this was not significant (n = 10; 3.76 ± 0.22 vs 4.56 ± 0.36 respectively, Fig 3.9). 25 mM 2DG was then applied to the wells in order to inhibit ECAR and show that it is dependent on glucose uptake and hexokinase activity.

Taken together these results indicate that glucose utilisation, in the form of oxidative metabolism and glycolysis, are reduced following RH in GT1-7 cells compared to controls.
Due to the fact that hexokinase is the first rate-limiting step in glycolysis, hexokinase activity was then measured in order to assess if RH had led to adaptations in the kinetics.

**3.2.7 Recurrent low glucose exposure does not alter hexokinase activity, in GT1-7 cells.**

Glucokinase (GK) is the rate-limiting enzyme in glucose metabolism and studies in both the pancreatic β-cell and glucose-sensing neurons have shown that it has a pivotal role in glucose sensing (Gloyn et al., 2008), (Beall et al., 2012), (Kang et al., 2006). GT1-7 cells express GK and it has been shown to regulate the electrical glucose-sensing of these cells, as direct activation of GK by GKA50 prevents hyperpolarisation of the cell membrane in response to low glucose exposure (Beall et al., 2012). Due to the observation that both OCR and ECAR are reduced following RH compared to control, in GT1-7 cells, it could be possible that GK activity is altered following RH. GK consumes ATP in order to phosphorylate glucose, therefore it could be possible that a reduction in GK activity would lead to an increase levels of ATP in the cytosol, localised at the plasma membrane, which in turn may inhibit the $K_{ATP}$ channel (Iynedjian, 2009). Therefore, GK activity was examined using a fluorometric assay which indirectly measures GK activity via the appearance of β-NADPH from the conversion of glucose-6-phosphate (produced from the phosphorylation of glucose by GK) to 6-phospho-D-gluconate by glucose-6-phosphate dehydrogenase (Meakin et al., 2007). This assay was kindly performed by Dr Craig Beall. Fig 3.10 demonstrates that increasing glucose increases GK activity in a concentration dependant manner, displaying Michaelis-Menton kinetics, reaching $V_{max}$ between 0.25 and 0.5 mM glucose for both RH and control cells (10.07 ± 0.70 vs 10.25 ± 0.50 mU/mg, respectively). There was a significant reduction in hexokinase activity following RH when tested at 0.1 mM glucose, compared to control ($n = 5$; 5.65 ± 0.88 vs 8.02 ± 0.31 mU/mg, respectively, Fig 3.10). However, there was no significant difference in hexokinase activity at 0, 0.25, 0.5, 1, 2.5 or 5.0 mM glucose, following RH compared to control ($n = 5$; 0.26 ± 0.07 vs 0.20 ± 0.10; 7.50 ± 0.83 vs 9.50 ± 0.60; 9.36 ±
0.96 vs 11.39 ± 0.61; 9.35 ± 1.12 vs 10.12 ± 0.79; 9.10 ± 1.31 vs 9.75 ± 1.17; 10.26 ± 1.97 vs 9.33 ± 1.63 mU/mg, respectively, Fig 3.10). It is worth noting that the Km of glucokinase activity in GT1-7 cells is not the low affinity, 8-10 mM glucose reported in the pancreatic β-cell but in fact ~0.05 (as calculated from vehicle control treated cells), much more akin to HKI or II (Matschinsky et al., 1998).

The trend for reduced hexokinase activity following RH in GT1-7 cells correlates with the reduction in glycolysis observed with the Seahorse XF-24 measurements. However, this result is puzzling as inhibition of GK has been shown to hyperpolarise glucose-sensing neurons and pancreatic β-cells, presumably through a consequential reduction in cellular ATP levels, leading to K\textsubscript{ATP} channel activation (Beall et al., 2012, Beall et al., 2010). Although reduced hexokinase activity may explain reduced glycolysis, it does not explain reduced OCR following RH. Reduced mitochondrial respiration would lead to an appearance in reduced cellular OCR, therefore mitochondrial efficiency was examined to see if adaptations were occurring here following RH.

3.2.8 Mitochondrial efficiency is altered following recurrent low glucose exposure, in GT1-7 cells.

The Seahorse XF-24 was used to examine mitochondrial efficiency. Inhibitors of electron transport chain complexes were injected into wells so that the percentage of OCR contributing to the activity of specific mitochondrial processes could be assessed. GT1-7 cells were seeded as stated previously, following the RH or control protocol, and placed into serum-free Seahorse media, containing 2.5 mM glucose as the only fuel source, 1 hour before the assay. It is important to note that the OCR shown in Fig 3.8 has been normalised to make baseline 100% in order to show the percentage of OCR contributing towards mitochondrial processes and also because wells injected with drugs were not normalised to protein in case
cell death had occurred (Fig 3.11A). Mitochondrial ATP generation was examined by applying 1 μM Oligomycin to inhibit ATP-synthase. The reduction in OCR after Oligomycin was applied represents the percentage oxygen contributing towards mitochondrial ATP generation. There was a significant increase in the OCR% associated with ATP-synthase following RH compared to control (n = 9; 52.57 ± 1.61 vs 48.37 ± 0.73 % change in OCR, respectively; \( P < 0.05 \), Fig 3.11B). Once the Oligomycin response had stabilised, Rotenone and Antimycin A were co-applied to the wells in order to inhibit mitochondrial complexes I and III, respectively, which inhibited the function of the electron transport chain. Complexes I and III are responsible for facilitating the extrusion of protons from the mitochondria and generating a protonmotive force across the inner membrane of the organelle, which is then dissipated by re-entry of protons back into the matrix via ATP-producing (ATP-synthase) and non ATP producing processes (i.e. mitochondrial leak uncoupling proteins) (Divakaruni and Brand). Because ATP-synthase is already inhibited at the point of rotenone and antimycin A addition, the change in OCR observed is indicative of non-ATP producing mitochondrial leak. There was a significant increase in mitochondrial leak following RH, compared to control (n = 9; 36.79 ± 1.15 vs 32.10 ± 0.83 % change in OCR, respectively; \( P < 0.005 \), Fig 3.11C). The percentage of OCR left once Rotenone and Antimycin A were applied represents the OCR generated from non-mitochondrial oxidation, such as fatty acid oxidation by peroxisomes (Kaikaus et al., 1993). There was a significant reduction in non-mitochondrial OCR following RH compared to control (n = 9; 10.63 ± 1.53 vs 19.53 ± 1.015 % change in OCR, respectively, \( P < 0.001 \), Fig 3.11D).

The spare respiratory capacity of the GT1-7 cells was examined using the mitochondrial uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP), which exerts its action through inserting pores in the mitochondrial membrane which acts to dissipate the proton gradient resulting in an increased demand and flux of substrate in order to maintain
the proton motive force. This is an oxygen consuming process and so the increase in OCR% when FCCP is applied relates to the ability of the mitochondria in the cell to upregulate their respiration in response to increased energy demand (i.e. spare respiratory capacity) (Fig 3.12A). FCCP was titrated into the wells in increments of 0.2 μM (in order to establish a dose that would uncouple the mitochondria without causing cell death) with a maximum final concentration being 0.8 μM. There was no significant difference observed in the spare respiratory capacity of GT1-7 cells following RH compared to controls, when 0.8 μM FCCP was applied (n = 4; 58.81 ± 6.771 vs 66.09 ± 4.91 % change in OCR, respectively, Fig 3.12B).

Taken together, these data indicate that while there is no change in the spare respiratory capacity of the mitochondria following RH there are small yet significant increases in the oxygen consumed by ATP-synthase and complexes which facilitate the leak of protons back into the mitochondria through non-ATP producing pathways, such as uncoupling proteins (as ATP-synthase is already inhibited by Oligomycin at the point that Rotenone and Antimycin A are added). The increase in ATP-synthase activity indicates that there is an increased demand for ATP within the cell, although the increased leak activity suggests that the mitochondria are in a more uncoupled state and functioning inefficiently following RH. These results are puzzling because if the mitochondria were more uncoupled following RH then, when FCCP was applied to examine spare respiratory capacity, it would be expected that the OCR would not increase as much as control as the mitochondria are already in an uncoupled state. This was not the case and there was no difference between groups in respect to spare respiratory capacity. However, these experiments were performed on intact cells where the spare respiratory capacity will also be determined by glucose uptake, substrate availability, glycolysis and substrate delivery to the mitochondria by pyruvate dehydrogenase (PDH). Therefore, isolated mitochondria may have yielded a different result.

Also,
mitochondrial ATP synthesis is an oxygen consuming process and therefore if more ATP was being generated following RH it would be expected to result in an increase in OCR when in fact a reduction in OCR is observed following RH. In an effort to examine if alterations were occurring following RH in terms of substrate delivery to the mitochondria, PDH activity was next measured.

3.2.9 Pyruvate dehydrogenase activity is reduced following recurrent low glucose exposure, in GT1-7 cells.

PDH activity was examined using a colourimetric assay, utilising the reduction of NAD+ to NADH when pyruvate is converted to acetyl-CoA (Abcam). GT1-7 cells were subjected to the RH and control protocols and then lysates were generated for the assay, 24 hours following the last hypoglycaemic incubation. Cells were incubated for 1 hour in 2.5 mM glucose NS before lysing. There was a significant reduction in the PDH activity, as determined by absorbance at 450 nm, following RH compared to control cells (n = 5; 0.008 ± 0.001 vs 0.120 ± 0.001 respectively; P < 0.05, Fig 3.13). A reduction in PDH activity would go towards explaining the reduction in OCR, through reduced substrate delivery into the mitochondria, following RH. In an attempt to examine the consequences of both a reduction in PDH and a possible increase in the activity of ATP-synthase, levels of ATP and the ATP/ADP ratio were next examined.

3.2.10 Recurrent low glucose exposure prevents the ATP/ADP ratio from falling during acute low glucose exposure and reduces ATP, in GT1-7 cells.

ATP/ADP ratios were examined using a bioluminescent assay, which utilises luciferase in order to catalyse the formation of light from ATP and luciferin (Abcam). GT1-7 cells were subjected to the RH or control protocol before being seeded at 1000 cells/well in a 96-well plate on the third day of the protocol, at least 3 hours following the last hypoglycaemic
incubation. Cells were plated into 2.5 mM glucose plating media for 24 hours before the assay. Fresh media was applied to the wells first thing on the day of the assay as preliminary experiments had shown that the ATP/ADP ratio was very low in cells that had been in 2.5 mM glucose for 24 hours, likely due to the glucose being metabolised in such a small volume of media (200 μl/well). Cells were washed with PBS and placed into 2.5 mM glucose NS for 3 hours (to establish a euglycaemic basal ATP/ADP ratio) or 0.1 mM glucose NS for 3 hours or 15 minutes. The ATP/ADP fell significantly from 2.5 mM glucose when 0.1 mM glucose was applied for 15 minutes and 3 hours (n = 7; 2.34 ± 0.14; 1.80 ± 0.17, P < 0.05 compared to 2.5 mM glucose; 1.71 ± 0.10 P < 0.005 respectively, compared to 2.5 mM glucose, Fig 3.14A). However, following RH, GT1-7 cells displayed a lower ATP/ADP ratio at 2.5 mM glucose (although not significantly different from the control 2.5 mM glucose response) which did not fall in response to 0.1 mM glucose at 15 minutes and 3 hours (n = 7; 1.91 ± 0.18; 1.78 ± 0.27; 1.854 ± 0.32 respectively, Fig 3.14B). An absence of a reduction in the ATP/ADP ratio during low glucose may explain the attenuated hyperpolarisation following RH in response to low glucose. However, the reduced basal ATP/ADP ratio could be due to either increased ADP or reduced ATP following RH. As there is currently no method to directly measure ADP, ATP levels were examined using another bioluminescence assay (Abcam). The RH and control protocols were applied to GT1-7 cells and on the third day, at least 3 hours after the last hypoglycaemic incubation, were seeded at a density of 1000 cells/well and left in 2.5 mM glucose plating media for 18-24 hours before the assay. As with the ATP/ADP ratio assay, fresh media was applied to the cells first thing on the day of the assay. Cells were later washed with PBS and incubated in 2.5 or 0.1 mM glucose NS for 15 minutes and 3 hours. GT1-7 cells under control conditions displayed a fall in ATP from 2.5 to 0.1 mM glucose at 15 minutes but this was absent in cells following RH. The level of ATP in GT1-7 cells following RH was significantly lower in wells containing 2.5 mM glucose for 15
minutes compared to controls (n = 4; 5.49 ± 0.21 vs 8.54 ± 0.52 ** P < 0.005 pmoles/1000 cells, respectively, Fig 3.15) but levels did not differ significantly after 15 mins of 0.1 mM glucose (n=4; 5.17 ± 0.08 vs 6.07 ± 0.98 pmoles/1000 cells, respectively, Fig 3.15). Curiously, when tested at 2.5 mM glucose for 3 hours both RH and control treated cells displayed reduced ATP compared to 2.5 mM glucose for 15 minutes, with RH cells displaying significantly lower ATP compared to control (n = 4; 3.67 ± 0.46 vs 5.87 ± 0.31 pmoles/1000 cells, respectively, Fig 3.15). This may be due to the limited amount of glucose available in the wells of the assay (200 μl) which is metabolised to a greater degree over 3 hours than it is at 15 minutes. This may be an artefact of the assay protocol, as the conditions under which cells are cultured for other biochemical and electrophysiological assay have a far higher media to cells ratio. Unlike the 15 minute treatments, the ATP did not fall from 2.5 to 0.1 mM glucose after 3 hours in control treated cells. There was also no ATP reduction from 2.5 to 0.1 mM glucose after 3 hours in the RH treated cells, although ATP was significantly lower after 3 hours in 0.1 mM glucose compared to controls (n = 4; 3.87 ± 0.53 vs 6.32 ± 0.79 pmoles/1000 cells, respectively; P < 0.05, Fig 3.15).

Taken together these data indicate that, following RH, GT1-7 cells reduce even basal (euglycaemic) ATP reduction and lose the metabolic flexibility to reduce ATP/ADP ratio in response to acute low glucose exposure. The reduced basal ATP correlates with the conditions under which reduced OCR and PDH activity were observed, therefore it is feasible that reduced substrate delivery to the mitochondria results in reduced ATP generation. However, reduced ATP should activate the KATP channel, which is not the case following RH. A potential reason for this could be that the KATP channel has become habituated to reduced levels of ATP, and possibly increased levels of ADP, and so it is not open at 2.5 mM glucose following RH, even though the levels of ATP after 15 minutes of 2.5 mM glucose in RH cells
are similar to the levels after 15 minutes of 0.1 mM glucose in control cells (conditions under which the channel would be open).

Adaptations in both levels of ATP and the ATP/ADP ratio, following RH, may lead to alterations in the activity of AMPK, as the activity of this kinase is regulated by levels of adenosine nucleotides (Kahn et al., 2005). In vivo studies have shown that recurrent neuroglucopenia, through 2DG ICV into brain, blunts the activity of AMPK in some regions of the hypothalamus in response to further ICV 2DG (Alquier et al., 2007). Therefore, the activity of AMPK following RH was assessed in GT1-7 cells in order to ascertain if there was a correlation with the in vivo RH studies.

3.2.11 Recurrent low glucose exposure blunts the ability of AMPKα1, but not AMPKα2, to activate in response to acute low glucose, in GT1-7 cells.

AMPK activity assay were performed by immunoprecipitation from GT1-7 lysates with specific antibodies against α1- and α2-AMPK catalytic subunits and kinase activity was measured using synthetic AMARA peptide and [γ-32P]ATP. GT1-7 cells were treated with the RH or control protocol and 18-24 hours following the last hypoglycaemic incubation, were treated with 2.5 mM glucose NS for 3 hours or 0.1 or 0.5 mM glucose NS for 15 minutes or 3 hours. These time points were chosen in order to determine the AMPK activities which correlate with the conditions at which electrical activity was assessed (15 minutes) and a longer low glucose incubation to see if a greater stimulus resulted in different levels of near maximal activity (3 hours). Data were quantified as fold change from control cells in 2.5 mM glucose for 3 hours because even though AMPK activity displayed the same trends between experiments the overall levels of activity differed slightly probably due, to slightly variations in the time it took to lyse and snap-freeze the samples and the decay of [γ-32P]ATP. Where possible, assays were performed on consecutive days. Low glucose exposure increased
AMPKα2 activity and there was no significant difference between RH and control groups at 2.5 mM glucose for 3 hours, 0.1 and 0.5 mM glucose for 15 minutes or 0.1 and 0.5 mM glucose for 3 hours (n = 4-6; 0.818 ± 0.18 vs 1.00 ± 0.00; 1.34 ± 0.25 vs 1.58 ± 0.41; 1.017 ± 0.29 vs 1.59 ± 0.34; 4.09 ± 0.99 vs 6.87 ± 1.12; 2.03 ± 0.72 vs 2.76 fold change from to 2.5 mM glucose 3 hours in control cells; Fig 3.16). Interestingly, the activity of AMPKα1 appeared to be reduced following RH, compared to control, at 2.5 mM glucose for 3 hours, 0.1 and 0.5 mM glucose for 15 minutes or 0.1 and 0.5 mM glucose for 3 hours (n = 5-7; 0.61 ± 0.05 vs 1.00 ± 0.00, P < 0.001; 0.91 ± 0.12 vs 1.48 ± 0.09, P < 0.005; 1.25 ± 0.31 vs 1.67 ± 0.41; 1.07 ± 0.20 vs 3.98 ± 0.67, P < 0.001; 0.79 ± 0.11 vs 2.02 ± 0.44, P < 0.05 fold change from to 2.5 mM glucose 3 hours in control cells; Fig 3.17). This result was unexpected as it has been demonstrated in the literature that removal or reduction of AMPKα2 activity in both pancreatic β-cells, hypothalamic neurons and GT1-7 cells results in glucose intolerance in vivo and attenuated glucose-sensing in vitro (Beall et al., 2010, Beall et al., 2012, Sun et al., 2010). However, a hypothesis was generated that perhaps AMPKα1 activation during the initial hypoglycaemic incubations may be driving the metabolic and electrical adaptations observed at the end of the RH protocol in GT1-7 cells. As there are currently no specific inhibitors or activators of AMPKα1, expression was reduced using shRNA lentiviral vector targeting AMPKα1.
Figure 3.5 Glucose uptake in GT1-7 cells is linear over 12-25 minutes.

Glucose uptake kinetics in GT1-7 cells were examined over 12, 15, 20 and 25 minutes using a $^3$H-2Deoxyglucose assay ($n = 2$ in triplicate). 15 and 20 minutes was chosen to perform all future uptake assays as this was in the middle of the linear range.
Figure 3.6 Glucose uptake is not altered by recurrent low glucose exposure, in GT1-7 cells

2-deoxy-d-[³H]glucose ([³H]2DG) uptake was measured in GT1-7 cells following a 20 minute incubation in a range of unlabelled glucose concentrations, physiological for the brain. There was no change in [³H]2DG uptake, at any unlabelled glucose concentration, between cells exposed to 2.5 (Control) or 0.1 mM glucose (RH) NS for 3 hours on 3 days before glucose uptake was examined on day 4 (n = 4).
Figure 3.7 Glucose oxidation and incorporation is not altered by recurrent low glucose exposure, in GT1-7 cells

Glucose utilisation was measured in GT1-7 cells following a 4 hour incubation in $^{14}$C-Glucose and a range of unlabelled glucose concentrations, physiological for the brain. There was no change in glucose oxidation (A) or incorporation (B) between cells exposed to 2.5 (Control) or 0.1 mM glucose (RH) NS for 3 hours on 3 days before glucose uptake was examined on day 4 (n = 3).
Figure 3.8 Glucose utilisation is reduced following recurrent low glucose exposure exposure, in GT1-7 cells.

(A) Real-time analysis of the oxygen consumption rate (OCR) was performed, using the XF-24 Seahorse Extracellular Flux Analyser, on intact GT1-7 cells. 2.5 mM glucose was the only fuel substrate present during this test. OCR was significantly reduced following 3 hours of 0.1 mM glucose NS for 3 days (RH) compared to 3 hours of 2.5 mM glucose for 3 days (Control) (n = 13). (B) Real-time analysis of the extracellular acidification rate (ECAR) was also performed on intact GT1-7 cells. 2.5 mM glucose was the only fuel substrate present during this test. ECAR was significantly reduced in GT1-7 cells following RH compared to controls (n = 5). Statistical significance was analysed using unpaired student's t-test (** P < 0.001). Statistical significance was analysed using unpaired student's t-test (* P < 0.05, ** P < 0.001).
Figure 3.9 Glycolysis, in response to 2.5 mM glucose, is attenuated following recurrent low glucose exposure, in GT1-7 cells.

Real-time analysis of the glycolysis (extracellular acidification rate = ECAR) was performed, using the XF-24 Seahorse Extracellular Flux Analyser, on intact GT1-7 cells. Cells were started off in 0 mM glucose containing media supplemented with 4 mM L-glutamine and 2.5 mM Na-pyruvate in order to shut off glycolysis and maintain cell energetics through oxidative metabolism. 2.5 mM glucose was injected into the wells to stimulate glycolysis. Oligomycin was applied in an attempt to obtain maximal glycolysis. Finally 2-deoxyglucose (2DG) was added to inhibit glucose uptake and metabolism. ΔECAR from baseline was significantly reduced when 2.5 mM glucose was applied following 3 hours of 0.1 mM glucose NS for 3 days (RH) compared to 3 hours of 2.5 mM glucose for 3 days (Control) (n = 13). Maximal glycolysis appeared to be obtained at 2.5 mM glucose with no upregulation in response to Oligomycin. Statistical significance was analysed using unpaired student’s t-test (* P < 0.05, ** P < 0.005 compared to control).
Figure 3.10 Hexokinase activity in GT1-7 cells was unaltered following recurrent low glucose exposure, in GT1-7 cells.

Hexokinase activity was measured in GT1-7 cells at 0, 0.1, 0.25, 0.50, 2.50 and 5 mM glucose concentrations. There was a trend for reduced hexokinase activity following 3 hours of 0.1 mM glucose NS for 3 days (RH) compared to 3 hours of 2.5 mM glucose for 3 days (Control) but this was only significant at was only significantly reduced at 0.1 mM glucose (n = 5).

Data courtesy of Dr Craig Beall.
Figure 3.11 Efficiency of ATP synthesis was altered following recurrent low glucose exposure, in GT1-7 cells

Real-time analysis of the oxygen consumption rate (OCR) was performed, using the XF-24 Seahorse Extracellular Flux Analyser, on intact GT1-7 cells exposed to 3 hours of 0.1 mM glucose (RH) or 2.5 mM glucose for 3 days (Control). 2.5 mM glucose was the only fuel substrate present during this test. The OCR has been normalised to 100% before mitochondrial inhibitors were applied in order to examine the amount of oxygen contributing to specific mitochondrial processes. (A) Display of entire assay protocol and results. ATP-synthase was inhibited by addition of Oligomycin, followed by Rotenone and Antimycin A to inhibit processes which facilitate proton leak across the mitochondrial membrane (n = 9). For illustrative purposes, the % change in OCR from before and after Oligomycin and Rotenone plus Antimycin A were applied are displayed in panels B and C, respectively. The % OCR left after Rotenone plus Antimycin was applied is displayed in panel D and represents non-mitochondrial oxygen consumption. Statistical significance was analysed using unpaired student's t-test (* P < 0.05, ** P < 0.005, *** P < 0.001 compared to control).
Figure 3.12 Spare mitochondrial respiratory capacity was unaltered following recurrent low glucose exposure, in GT1-7 cells

Real-time analysis of the oxygen consumption rate (OCR) was performed, using the XF-24 Seahorse Extracellular Flux Analyser, on intact GT1-7 cells exposed to 3 hours of 0.1 mM glucose (RH) or 2.5 mM glucose for 3 days (Control). 2.5 mM glucose was the only fuel substrate present during this test. The OCR has been normalised to 100% before mitochondrial inhibitors were applied in order to examine the amount of oxygen contributing to specific mitochondrial processes. In panel A, cells were exposed to increasing concentrations of FCCP in order to uncouple the mitochondria (n = 4). For illustrative purposes, the % change in OCR from before and after 0.6 µM FCCP was applied is displayed in panel B. Statistical significance was analysed using unpaired student’s t-test.
Figure 3.13 Pyruvate dehydrogenase activity is attenuated following recurrent low glucose exposure, in GT1-7 cells.

Pyruvate dehydrogenase (PDH) was assessed using a colourimetric assay. Samples were prepared from GT1-7 cells exposed to 3 hours of 0.1 mM (RH) or 2.5 mM glucose NS (Control) for 3 days before being incubated in 2.5 mM glucose NS for 1 hour on day 4 before lysis. PDH activity was significantly reduced following RH compared to controls (n = 5) assessed by absorbance at 450 nm. Statistical significance was analysed using unpaired student’s t-test (* P < 0.05).
Figure 3.14 Acute low glucose reduces ATP/ADP ratios in control GT1-7 cells but not following recurrent low glucose exposure.

ATP/ADP ratios were measured using a luminescence assay and carried out at 2.5 and 0.1 mM glucose NS. (A) There was a reduction in the ATP/ADP ratio when glucose was lowered from 2.5 to 0.1 mM glucose in cells exposed to 2.5 mM glucose NS for 3 hours on 3 days (control; n = 7). (B) Cells that were treated with 0.1 mM glucose NS for 3 hours on 3 days (RH; n = 7) displayed a lower nucleotide ratio at 2.5 mM glucose which did not fall in response to 0.1 mM glucose NS. Statistical significance was analysed using unpaired student’s t-test (* P < 0.05, ** P < 0.005, compared to 2.5 mM glucose).
Figure 3.15 Acute low glucose reduces ATP/ADP ratios in control GT1-7 cells but not following recurrent low glucose exposure.

ATP was measured using a luminescence assay and carried out at 2.5 and 0.1 mM glucose NS for either 15 minutes or 3 hours. ATP levels were reduced in cells incubated in 0.1 mM glucose NS for 15 minutes compared to 15 minute incubation in 2.5 mM glucose NS. In cells that had been exposed to 2.5 mM glucose NS for 3 hours on 3 days (control, n = 4). Cells that were treated with 0.1 mM glucose NS for 3 hours on 3 days (RH, n = 4) displayed a lower levels of ATP at 2.5 mM glucose which did not fall in response to 0.1 mM glucose NS, at both 15 minute and 3 hour timepoints. For both control and RH cells, ATP was lower in 2.5 mM glucose NS after 3 hours and levels were not found to be any lower in the Data is represented as ATP (pmoles) in wells containing 1000 cells. Statistical significance was analysed using unpaired student’s t-test (* P < 0.05, ** P < 0.005, compared to control).
Figure 3.16 Acute low glucose increases AMPKα2 activity in GT1-7 cells and is unaffected by recurrent low glucose exposure.

AMPKα2 activity was measured using a kinase assay with radiolabelled substrate (AMARA) and immunoprecipitation in order to examine the activity of catalytic α2-subunit containing AMPK complexes. Kinase activity was measured after cells were exposed to 2.5, 0.5 and 0.1 mM glucose NS for either 15 minutes or 3 hours before lysis. AMPKα2 activity was increased in cells incubated in 0.1 or 0.5 mM glucose NS for 15 minutes or 3 hours compared to 3 hours in 2.5 mM glucose NS. There was no difference in AMPKα2 activity between cells that had been exposed to 2.5 (control; n = 5-7) or 0.1 mM glucose NS for 3 hours on 3 days (RH; n = 5-7). Statistical significance was analysed using unpaired student's t-test.
Figure 3.17 Acute low glucose increases AMPKα1 activity in GT1-7 cells but is attenuated following recurrent low glucose exposure.

AMPKα1 activity was measured using a kinase assay with radiolabelled substrate (AMARA) and immunoprecipitation in order to examine the activity of catalytic α1-subunit containing AMPK complexes. Kinase activity was measured after cells were exposed to 2.5, 0.5 and 0.1 mM glucose NS for either 15 minutes or 3 hours before lysis. AMPKα1 activity was increased in cells incubated in 0.1 or 0.5 mM glucose NS for 15 minutes or 3 hours compared to 3 hours in 2.5 mM glucose NS. In cells that had been exposed to 2.5 mM glucose NS for 3 hours on 3 days (control; n = 4-6), GT1-7 cells that had been exposed to 0.1 mM glucose NS for 3 hours on 3 days (RH; n = 4-6) displayed reduced AMPKα1 activity at all [glucose] timepoints except 15 mins 0.5 mM glucose). Statistical significance was analysed using unpaired student’s t-test (* P < 0.05, ** P < 0.005, compared to control 3 hours 2.5 mM glucose).
3.2.12 Lentiviral shRNA knock-down of AMPKα1 prevents reduced OCR associated with recurrent glucose exposure, in GT1-7 cells.

GT1-7 cells transfected shRNA lentiviral vector targeted towards AMPKα1 (shAMPKα1) [a gift from Dr Craig Beall] demonstrated a 30-40% reduction in expression of AMPKα1 compared to cells transfected with a scrambled control lentiviral vector (shC) (Fig 3.18A and B). However, the expression of AMPKα2 was unaltered in shAMPKα1 cells, compared to shC cells (Fig 3.18A and C). This protocol has also previously been shown to yield a significant reduction in activity of AMPKα1 in response to both 2.5 and 0.5 mM glucose stimulations (Beall et al., 2012). In an attempt to examine the effects of reducing AMPKα1 expression in GT1-7 cells, the XF-24 Seahorse Extracellular Flux Analyser was again used to examine oxidative metabolism (OCR) and glycolysis (ECAR). shC and shAMPKα1 GT1-7 cells were subjected to RH and control protocols and then seeded as stated previously on the third day of the protocol, at least 3 hours following the last hypoglycaemic incubation. Cells were then left for 24 hours in 2.5 mM glucose plating media. Cells were placed into serum-free seahorse media, 1 hour before the assay, which contained 2.5 mM glucose as the only fuel source, after which time they were then placed in the XF-24 and OCR was measured in real-time. shC GT1-7 cells subjected to RH displayed a significantly lower OCR compared to shC controls (n = 10; 9.79 ± 0.65 vs 16.51 ± 1.06 pmoles O\textsubscript{2}/min/μg, respectively; P < 0.001; Fig 3.19A). However, the OCR of shAMPKα1 GT1-7 cells subjected to RH was not reduced compared to the shAMPKα1 controls (n = 10; 13.98 ± 1.11 vs 14.94 ± 1.13 pmoles O\textsubscript{2}/min/μg, respectively, Fig 3.19A).

ECAR was obtained from the same wells that the OCR results were taken from. shC GT1-7 cells subjected to RH displayed a trend towards lower ECAR compared to controls, although this was not significant, as was found in wild type cells (n = 10; 0.48 ± 0.08 vs 0.71 ± 0.10 mPH/min/μg, respectively; Fig 3.19B). The ECAR of shAMPKα1 GT1-7 cells subjected to
RH was also not reduced compared to the shAMPKα1 controls (n = 10; 0.61 ± 0.14 vs 0.74 ± 0.13 mpH/min/μg, respectively; Fig 3.19B).

These results indicate that AMPKα1 may be driving adaptations that lead to reduced OCR following RH in GT1-7 cells. Therefore the implications of removing this RH-associated metabolic adaptation on electrical activity of GT1-7 cells was examined.

### 3.2.13 Lentiviral shRNA knock-down of AMPKα1 does not prevent attenuated hyperpolarisation to low glucose, following recurrent glucose exposure, in GT1-7 cells.

Glucose-sensing was examined in shC and shAMPKα1 GT1-7 cells using perforated patch-clamp recordings. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). 25-40 μg/ml amphotericin B was also present in the pipette and no current was injected during the recordings. As before, a stable recording was obtained in 2.5 mM glucose before 0.5 mM glucose NS was superfused. shC GT1-7 cells under control conditions hyperpolarised from 2.5 to 0.5 mM glucose on average by -17.0 ± 2.8 mV (Fig 3.20A) but this response was significantly attenuated following RH with an average response of -4.2 ± 1.4 mV (Fig 3.20B) (n = 5-9 respectively; P < 0.001; Fig 3.20C). shAMPKα1 GT1-7 cells under control conditions hyperpolarised from 2.5 to 0.5 mM glucose on average by -13.5 ± 1.5 mV (Fig 3.21A) whereas this response was significantly attenuated following RH with an average response of -4.2 ± 2.6 mV (Fig 3.21B) (n = 11-13 respectively; P < 0.005; Fig 3.21C).

Taken together, these results suggest that the reduced OCR observed in the wild type and shC GT1-7 cells following RH is unlikely to be responsible for the attenuated electrical glucose sensing in response to acute low glucose. Next, ATP/ADP ratios were examined in order to see if the adaptations observed here were retained in shAMPKα1 cells following RH.
3.2.14 Lentiviral shRNA knock-down of AMPKα1 does not prevent failure of ATP/ADP ratio to fall in response to acute low glucose following recurrent glucose exposure, in GT1-7 cells.

ATP/ADP ratios were examined using a bioluminescent assay, as stated previously (Abcam). shC and shAMPKα1 GT1-7 cells were subjected to the RH or control protocol before being seeded at 1000 cells/well in a 96-well plate on the third day of the protocol, at least 3 hours following the last hypoglycaemic incubation. Cells were plated into 2.5 mM glucose plating media for 24 hours before the assay. Fresh media was applied to the wells first thing on the day of the assay in order to replenish the fuel substrate availability. Cells were washed with PBS and placed into 2.5 mM glucose NS for 3 hours (to establish a euglycaemic basal ATP/ADP ratio) or 0.1 mM glucose NS for 3 hours or 15 minutes. The results for this set of experiments have been quantified as fold change from the ATP/ADP ratio obtained from shC or AMPKα1 cells incubated in 2.5 mM glucose for 3 hours. This is because although the results from each experiment displayed the same trends, the nucleotide ratios differed slightly between days on which the assays were performed. ATP/ADP ratios of shC GT1-7 cells, subjected to control conditions, fell from 2.5 mM glucose when 0.1 mM glucose was applied for 15 minutes and 3 hours (n = 4; 1.00 ± 0.04; 0.85 ± 0.05, P = 0.05 compared to 2.5 mM glucose; 0.80 ± 0.03, P < 0.005 compared to 2.5 mM glucose, respectively; Fig 3.22A). However, following RH, shC GT1-7 cells failed to display a fall in the ATP/ADP ratio from 2.5 mM glucose to 0.1 mM glucose at 15 minutes and 3 hours (n = 4; 1.00 ± 0.05; 1.10 ± 0.03; 1.01 ± 0.04 fold change from 2.5 mM glucose, respectively; Fig 3.22A). In respect to the effects of AMPKα1 knock-down, the ATP/ADP of shAMPKα1 GT1-7 cells, subjected to control conditions, fell from 2.5 mM glucose when 0.1 mM glucose was applied for 15 minutes and 3 hours (n = 4; 1.00 ± 0.06; 0.82 ± 0.03, P < 0.05 compared to 2.5 mM glucose; 0.80 ± 0.04, P < 0.05 compared to 2.5 mM glucose, respectively; Fig 3.23A). However,
Figure 3.18 Lentiviral shRNA knockdown of AMPKa1 specifically reduces expression of AMPK complexes containing catalytic α1-subunit containing AMPK complexes.

(A) Western blots showing that transfecting GT1-7 cells with shRNA lentiviral vector targeting AMPKa1 reduces the expression of AMPK complexes containing the α1 catalytic subunit but does not affect AMPKa2 expression, compared to cells transfected with a scrambled sequence (shControl) (n = 5–6). Actin was used as a loading control. Panels B and C display densitometric analysis of pooled data (data analysed as AMPKa1/2 + Actin expression and then fold change from shControl). Statistical significance was analysed using unpaired student’s t-test (* P < 0.05, ** P < 0.005, compared to shControl).

Data courtesy of Dr Craig Beall.
Figure 3.19 Glucose utilisation is reduced following recurrent low glucose exposure exposure, in shControl but not shAMPKα1 GT1-7 cells.

(A) Real-time analysis of the oxygen consumption rate (OCR) was performed, using the XF-24 Seahorse Extracellular Flux Analyser, on intact GT1-7 cells. 2.5 mM glucose was the only fuel substrate present during this test. OCR was significantly reduced in GT1-7 cells transfected with shControl lentivirus (shC) following 3 hours of 0.1 mM glucose NS for 3 days (RH) compared to 3 hours of 2.5 mM glucose for 3 days (Control) (n = 10). OCR was not reduced in GT1-7 cells transfected with AMPKα1 lentivirus following RH compared to control (n = 10). (B) Real-time analysis of the extracellular acidification rate (ECAR) was also performed on intact GT1-7 cells. 2.5 mM glucose was the only fuel substrate present during this test. ECAR was slightly reduced in GT1-7 cells transfected with shControl lentivirus (shC) following RH compared to controls (n = 10). ECAR was also slightly reduced in GT1-7 cells transfected with AMPKα1 lentivirus following RH compared to control (n = 10). Statistical significance was analysed using unpaired student’s t-test (*** P < 0.001).
Figure 3.20 Recurrent low glucose exposure results in attenuated glucose-sensing at 0.5 mM glucose, in GT1-7 cells transfected with control lentiviral vector.

(A) Representative perforated-patch current clamp recording from a GT1-7 cell treated with control lentiviral vector that was then exposed to 2.5 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. Superoxen 2.5 mM glucose depolarised the cell membrane where as substitution with 0.5 mM glucose induced hyperpolarisation. Pooled membrane potential data for antecedent 2.5 mM glucose (n = 5). (B) Representative perforated-patch current clamp recording from a GT1-7 cell exposed to 0.1 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. Under these conditions, the cells displayed a significantly attenuated hyperpolarisation to superfusion of 0.5 mM glucose. Pooled membrane potential data for antecedent 0.1 mM glucose (n = 9). For illustrative purposes, the change in membrane potential from 2.5 to 0.5 mM glucose is shown in panel C (shC = control lentiviral vector; RH = recurrent hypoglycaemia). Statistical significance was analysed by one-way ANOVA in panels A and B and student’s unpaired t-test in panel C (* P < 0.05, *** P < 0.001).
Figure 3.21 Recurrent low glucose exposure still results in attenuated glucose-sensing at 0.5 mM glucose, in GT1-7 cells when AMPKα1 is reduced

(A) Representative perforated-patch current clamp recording from a GT1-7 cell treated with shRNA lentiviral vector targeting AMPKα1 that was then exposed to 2.5 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. Superfusion of 2.5 mM glucose depolarised the cell membrane where as substitution with 0.5 mM glucose induced hyperpolarisation. Pooled membrane potential data for antecedent 2.5 mM glucose (n = 11).

(B) Representative perforated-patch current clamp recording from a GT1-7 cell exposed to 0.1 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. Under those conditions, the cells displayed a significantly attenuated hyperpolarisation to superfusion of 0.5 mM glucose. Pooled membrane potential data for antecedent 0.1 mM glucose (n = 13). For illustrative purposes, the change in membrane potential from 2.5 to 0.5 mM glucose is shown in panel C (shAMPKα1 = shRNA lentiviral vector targeting AMPKα1; RH = recurrent hypoglycaemia). Statistical significance was analysed by one-way ANOVA in panels A and B and student's unpaired t-test in panel C (** P < 0.005, *** P < 0.001).
Figure 3.22 Acute low glucose reduces ATP/ADP ratios, in GT1-7 cells transfected with control lentiviral vector, but not following recurrent low glucose exposure.

ATP/ADP ratios were measured using a luminescence assay and carried out at 2.5 and 0.1 mM glucose NS. Data has been normalised to 3 hours 2.5 mM glucose NS. (A) There was a reduction in the ATP/ADP ratio when glucose was lowered from 2.5 to 0.1 mM glucose in cells exposed to 2.5 mM glucose NS for 3 hours on 3 days (shC control; n = 4). (B) The nucleotide ratio of cells that were treated with 0.1 mM glucose NS for 3 hours on 3 days (shC RH; n = 4) did not fall in response to 0.1 mM glucose NS. Statistical significance was analysed using unpaired student’s t-test (* * P < 0.005, compared to 2.5 mM glucose).
Figure 3.23 Acute low glucose reduces ATP/ADP ratios, in GT1-7 cells transfected with shAMPKα1 lentivirus, but not following recurrent low glucose exposure.

ATP/ADP ratios were measured using a luminescence assay and carried out at 2.5 and 0.1 mM glucose NS. Data has been normalised to 3 hours 2.5 mM glucose NS. (A) There was a reduction in the ATP/ADP ratio when glucose was lowered from 2.5 to 0.1 mM glucose in cells exposed to 2.5 mM glucose NS for 3 hours on 3 days (shAMPKα1 control; n=4). (B) The nucleotide ratio of cells that were treated with 0.1 mM glucose NS for 3 hours on 3 days (shAMPKα1 RH; n=4) did not fall in response to 0.1 mm glucose NS. Statistical significance was analysed using unpaired student's t-test (** P < 0.005, compared to 2.5 mM glucose).
following RH, shAMPKα1 GT1-7 cells still failed to display a fall in the ATP/ADP ratio from 2.5 mM glucose to 0.1 mM glucose at 15 minutes and 3 hours (n = 4; 1.00 ± 0.03; 1.11 ± 0.11; 1.02 ± 0.07 fold change from 2.5 mM glucose, respectively; Fig 3.22B).

Coupled with the previous results demonstrating that, following RH, shAMPKα1 GT1-7 cells still display attenuated glucose-sensing, yet do not display the RH-associated reduction in OCR, a loss of ATP/ADP flexibility in response to acute low glucose application may be the underlying mechanism behind attenuated glucose-sensing following RH. These results also suggest a role for AMPKα1 in the regulation of substrate delivery to the mitochondria, based on the OCR results.

3.2.15 Inhibition of the pentose phosphate pathway reduces OCR and ECAR in GT1-7 cells following both control and recurrent low glucose.

Due to the observation that OCR was reduced, yet there were no differences in the results of the 14C-glucose oxidation assay following RH, an alternative glucose processing pathway was investigated. The pentose phosphate pathway (PPP) branches from glycolysis after glucose is phosphorylated to glucose-6-phosphate by hexokinase. Glucose-6-phosphate dehydrogenase is the first step and rate-limiting enzyme of the PPP and reduces nicotinamide adenine dinucleotide phosphate (NADP) to NADPH while oxidizing glucose-6-phosphate to 6-phosphogluconolactone (Takahashi et al., 2012). In turn, NADPH provides reducing equivalents for glutathione reductase which maintains levels of the reduced glutathione in order to protect against cellular oxidative stress. Because this pathway is reliant on glucose uptake and phosphorylation, it has been shown to be upregulated in neurons and astrocytes in response to high glucose conditions, in vitro. The result from the aforementioned study indicates that this metabolic pathway plays a key role in protecting the brain against the ROS that is generated during metabolic stress, such as hyperglycaemia (Takahashi et al., 2012).
The PPP has both oxidative and non-oxidative components therefore the activity of the oxidative part of this pathway, which produces 70% of intracellular NADPH along with CO₂, can be assessed using the OCR function of the Seahorse extracellular flux analyser XF-24. The PPP also feeds back into glycolysis, through synthesis of fructose 6-phosphate and glyceraldehyde 3-phosphate, and so any changes here can be monitored through ECAR. The hypothesis underlying this experiment was that perhaps there was an upregulation of this pathway following RH to combat repeated exposure to ROS caused by low glucose, which could be leading it to look like there is no change in a CO₂ in the ¹⁴C-glucose oxidation assay (Stincone et al., 2014). 6-aminonicatinamide (6-AN) and trans-dehydroepiandrosterone (DHEA) are competitive and non-competitive inhibitors of G6PDH respectively and have been shown to inhibit the PPP in pancreatic islets, cardiac tissue, astrocytes and neurons (Spegel et al., 2013, Takahashi et al., 2012, Gupte et al., 2002). GT1-7 cells were subjected to the RH and control protocols before OCR and ECAR were examined. Cells were seeded as before and either 500 μM 6-AN or 100 μM DHEA were applied to the wells for 16 hours or 1 hours, respectively, prior to the assay. 6-AN and DHEA were also present during the assay, as was 2.5 mM glucose as the only fuel source. Data have been analysed as fold change from vehicle in order to show the suppression of the PPP when inhibitors were applied and reflect the activity of the pathway in the cell. 6-AN application significantly reduced OCR, compared to vehicle treatment, in both control (n = 6-8; 0.60 ± 0.07 vs 1.00 ± 0.13 fold change from vehicle treatment, respectively; P < 0.05; Fig 3.24Ai) and RH GT1-7 cells (n = 6-8; 0.41 ± 0.04 vs 1.00 ± 0.09 fold change from vehicle treatment, respectively; P < 0.001; Fig 3.24Bi). 6-AN also significantly reduced ECAR, compared to vehicle treatment, in both control (n = 6-8; 0.45 ± 0.06 vs 1.00 ± 0.14 fold change from vehicle treatment, respectively; P < 0.005; Fig 3.24Aii) and RH GT1-7 cells (n = 6-8; 0.39 ± 0.07 vs 1.00 ± 0.20 fold change from vehicle treatment, respectively; P < 0.005; Fig 3.24Bii). When cells were treated with
DHEA, OCR significantly reduced, compared to vehicle treatment, in both control (n = 10; 0.29 ± 0.04 vs 1.00 ± 0.10 fold change from vehicle treatment, respectively; \( P < 0.001; \) Fig 3.25Ai) and RH GT1-7 cells (n = 10; 0.37 ± 0.06 vs 1.00 ± 0.11 fold change from vehicle treatment, respectively; \( P < 0.05; \) Fig 3.25Bi). DHEA also significantly reduced ECAR, compared to vehicle treatment, in both control (n = 10; 0.59 ± 0.12 vs 1.00 ± 0.10 fold change from vehicle treatment, respectively; \( P < 0.005; \) Fig 3.25Aii) and RH GT1-7 cells (n = 10; 0.47 ± 0.11 vs 1.00 ± 0.13 fold change from vehicle treatment, respectively; \( P < 0.005; \) Fig 3.25Bii).

These results imply that the activity of the PPP is high in GT1-7 cells. Studies of primary neuronal cultures have shown that PPP is relatively low compared to glycolysis and oxidative phopshorylation (Takahashi et al., 2012). However, the activity of this pathway have been found to be high in replicating cells, due to the synthesis of ribose 5-phosphate further down the pathway for the synthesis of nucleotides, and so this might explain the differences between primary neurons and the GT1-7 neuronal cell line (Stincone et al., 2014). ECAR and OCR were attenuated with both inhibitors, although to a slightly higher degree with 6-AN following RH. The functional differences between 6-AN and DHEA are that while 6-AN has been shown to be a specific inhibitor of the PPP inhibiting both glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase, DHEA has been found to have off-target effects such as activation of the nuclear receptor, peroxisome proliferator-activated receptor-\( \alpha \) (PPAR\( \alpha \)) (Spegel et al., 2013). Stimulation of this nucleic receptor will drives metabolism towards fatty acid \( \beta \)-oxidation, which consumes less oxygen than carbohydrate metabolism and so should theoretically reduce OCR (Peters et al., 1996, Kaikaus et al., 1993). Although, the expression of PPAR\( \alpha \) has not been shown in GT1-7 cells. Therefore, perhaps there is greater activity of the PPP following RH if conclusions are drawn from use of 6-AN. In an attempt to address this further, levels of NADP\(^+\) and NAPDH were measured.
3.2.16 Recurrent low glucose exposure results in increased levels of NADP⁺ and reduced NADPH, in GT1-7 cells.

Nicotinamide nucleotides, NADP⁺ and NADPH, were measured using a colourimetric assay (Abcam), which specifically detects NADPH. GT1-7 cells were treated with either the RH or control protocols and assayed 24 hours following the last hypoglycaemic exposure. Cells were incubated in 2.5 mM glucose NS for 1 hour before lysates were collected for analysis in an attempt to replicate the conditions that the cells are subjected to during the Seahorse XF-24 experiments and, to some extent, during the electrophysiological tests. There was a significant increase in NADP⁺ following RH, compared to control (n = 8; 0.083 ± 0.005 vs 0.050 ± 0.006 pmol/μg, respectively; P < 0.001; Fig 3.26). This result coincided with a significant reduction in NADPH following RH, compared to control (n = 8; 0.105 ± 0.007 vs 0.131 ± 0.006 pmol/μg, respectively; P < 0.05; Fig 3.26). Reasons for this result may be due to reduced activity of glucose-6-phosphate dehydrogenase and/or increased consumption of NADPH through ROS scavenging and anabolic pathway such as lipid and DNA synthesis (Stincone et al., 2014). ROS production was next examined in order to determine if increased oxidative stress, following RH, could be the underlying cause for the nicotinamide nucleotide changes.

3.2.17 Acute low glucose causes an increase in ROS but this is not exacerbated following recurrent low glucose exposure, in GT1-7 cells.

ROS was measured in GT1-7 cells using the cell permeable, fluorogenic dye, 2’,7’-dichlorofluorescin diacetate (DCFDA) (Abcam). DCFDA is oxidised by ROS and is converted into the fluorescent compound 2’,7’-dichlorofluorescein which can be detected and used to indirectly semi-quantify ROS. GT1-7 cells were treated with either the RH or control protocol before being seeded at a density of 1000 cells/well in a black, opaque 96-well
microplate at least 3 hours after the last hypoglycaemic exposure. Fresh 2.5 mM glucose plating media was applied to the wells first thing on the day of the assay, for the same reasons as stated in the ATP assays. Cells were stained with 25 μM DCFDA for 45 minutes before 2.5 or 0.1 mM glucose NS was applied for 15 minutes or 3 hours, after which fluorescence was analysed using a fluorescent plate reader. Data were analysed as fluorescence intensity per 1000 cells. There was no increase in ROS from 2.5 mM glucose when cells were treated with 0.1 mM glucose, for 15 minutes, following RH (n = 4; 11831.58 ± 2963.638 vs 11114.50 ± 2955.80 fluorescence intensity, respectively; Fig 3.27A) compared to controls (n = 4; 10692.17 ± 3140.96 vs 12877.00 ± 3482.06 fluorescence intensity, respectively; Fig 3.27A). However, ROS did increase from 2.5 to 0.1 mM glucose after 3 hour exposure but this was not significantly different between RH treated cells (n = 4; 6825.50 ± 645.18 vs 17625.58 ± 2139.64 fluorescence intensity, respectively; P < 0.005; Fig 3.27B) compared to controls (n = 4; 6003.08 ± 353.09 vs 12811.08 ± 1699.93 fluorescence intensity, respectively; P < 0.005; Fig 3.27B). These results show that low glucose exposure increases ROS in GT1-7 cells. This was most obvious at the longer low glucose incubation time although the results were too variable to draw conclusions after the 15 minute incubation. The lower levels of ROS following 3 hour incubations is most likely due to the fast acting antioxidant processes within the cell. These lower levels may also be due to the DCFDA leaking out of the cell. Taken together with the reduced NADPH levels obtained following RH, it could be speculated that the cell experiences recurrent oxidative stress during RH leading to increased PPP activity as a defence. The recurrent ROS may cause increased consumption of NADPH, which relies on glucose supply through the PPP and therefore fails to keep up with demand due to the cell recurrently having glucose withdrawn. Another way in which NADPH is generated is through isocitrate dehydrogenase isoforms 1 and 2, the activity of which were next examined.
3.2.18 Isocitrate dehydrogenase activity is unaltered by recurrent low glucose exposure, in GT1-7 cells.

Isocitrate dehydrogenase (IDH) activity was measured using a colourimetric assay (Abcam), utilising the conversion of isocitrate to α-ketoglutarate. There are 3 isoforms of IDH: IDH1 is cytosolic and uses NADP⁺ as a cofactor to produce NADPH and α-ketoglutarate; IDH2 is located in the mitochondria and also uses NADP⁺ as a cofactor to produce NADPH and α-ketoglutarate; IDH3 is cytosolic and uses NAD⁺ as a cofactor to produce NADH and α-ketoglutarate. The assay was used to examine the active of IDH in whole cell lysates so the activity of IDH1 and IDH2 could not be distinguished, although isolation of mitochondria would solve this. The activity of IDH3 was not altered following RH compared to controls (n = 4; 2.15 ± 0.18 vs 2.01 ± 0.38 nmol/min/ml, respectively; Fig 2.28). The activity of IDH1 and IDH2 were also unchanged following RH compared to controls (n = 4; 68.93 ± 1.15 vs 69.51 nmol/min/ml, respectively; Fig 2.28). These results indicate that there is no adaptation in IDH activity following RH, especially IDH1 and IDH2 which produce NADPH. Therefore the differences in NADPH levels shown previously must be due to altered PPP activity and/or consumption of NADPH following RH.

The results from the metabolic investigations do not explain the attenuated glucose-sensing observed in GT1-7 cells following RH. Therefore, the single channel activity of the $K_{ATP}$ channel was next examined.
Figure 3.24 6-AN reduces oxygen consumption rate in GT1-7 cells.

(A) Real-time analysis of the oxygen consumption rate (OCR) was performed, using the XF-24 Seahorse Extracellular Flux Analyser, on intact GT1-7 cells. 2.5 mM glucose was the only fuel substrate present during this test. 500 µM 6-Aminonicotinamide (6-AN), or 0.01 % DMSO vehicle, was applied to cells for 16 hours before and during the assay in order to inhibit glucose-6-phosphate dehydrogenase and hence the pentose phosphate pathway. OCR was significantly reduced in GT1-7 cells in the presence of 6-AN following 3 hours of 2.5 (control, n = 6-8) or (B) 0.1 mM glucose NS for 3 days (RH; n = 6-8).

Real-time analysis of the extracellular acidification rate (ECAR) was also performed on intact GT1-7 cells. 2.5 mM glucose was the only fuel substrate present during this test. ECAR was significantly reduced in both control (Aii) and RH (Bii) cells treated with 6-AN (n = 6-8) Data are displayed as fold change in OCR or ECAR from vehicle treated cells. Statistical significance was analysed using unpaired student’s t-test (* P < 0.05, ** P < 0.005 *** P < 0.001 compared to vehicle treated cells).
Figure 3.25 DHEA reduces oxygen consumption and extracellular acidification rates in GT1-7 cells.

Real-time analysis of the oxygen consumption rate (OCR) was performed, using the XF-24 Seahorse Extracellular Flux Analyser, on intact GT1-7 cells. 2.5 mM glucose was the only fuel substrate present during this test. 100 μM trans-dehydroepiandrosterone (DHEA) or 0.01 % DMSO vehicle, was applied to cells for 1 hour before and during the assay in order to inhibit glucose-6-phosphate dehydrogenase and hence the pentose phosphate pathway. OCR was significantly reduced in GT1-7 cells in the presence of DHEA following 3 hours of 2.5 (Ai, control, n = 10) or 0.1 mM glucose NS for 3 days (Bi, RH; n = 10). Real-time analysis of the extracellular acidification rate (ECAR) was also performed on intact GT1-7 cells. 2.5 mM glucose was the only fuel substrate present during this test. ECAR was significantly reduced in both control (Aii) and RH (Bii) cells treated with DHEA (n = 10). Data are displayed as fold change in OCR or ECAR from vehicle treated cells. Statistical significance was analysed using unpaired student’s t-test (* P < 0.05, ** P < 0.005, *** P < 0.001 compared to vehicle treated cells).
Figure 3.25 Recurrent low glucose exposure leads to increased NADP+ and reduced NADPH levels in GT1-7 cells.

Levels of NADPH and NADP+ were measured using a colourimetric assay. GT1-7 cells were incubated in 2.5 mM glucose NS for 1 hour before lysis. There was a significant increase in the oxidised state of nicotinamide adenine nucleotides (NADP+) in cells following 3 hours of 0.1 mM glucose NS (RH, n = 8) compared to 3 hours of 2.5 mM glucose NS (control, n = 8) for 3 days. There was also a significant reduction in the oxidised state of nicotinamide adenine nucleotides following RH (n = 8) compared to control (n = 8). Statistical significance was analysed using unpaired student's t-test (* P < 0.05, ** P < 0.001).
Figure 3.27 Acute low glucose increases levels of ROS, in GT1-7 cells, at 3 hours.

Quantification of the fluorescence intensity of the reactive oxygen species (ROS) sensitive probe H2DCFDA, in GT1-7 cells exposed to 3 hours of 0.1 mM (RH) or 2.5 mM glucose (control) for 3 days. (A) ROS induced fluorescence did not differ significantly between cells incubated in 2.5 and 0.1 mM glucose NS for 15 minutes in both control and RH treated cells (n = 4). (B) There was a significant increase in ROS induced fluorescence from cells incubated in 0.1 mM glucose NS for 3 hours compared to 2.5 mM glucose NS for 3 hours. There was no difference between ROS levels at 2.5 or 0.1 mM glucose NS following RH compared to control cells (n = 4). Statistical significance was analysed using unpaired student's t-test (**P < 0.005, compared to 2.5 mM glucose).
Figure 3.28 Recurrent low glucose does not alter IDH activity, in GT1-7 cells.

Isocitrate dehydrogenase (IDH) activity was assessed in GT1-7 cells using a colorimetric assay. Cells were incubated in 2.5 mM glucose NS for 1 hour before lysis. There was no difference in the activity of NAD+ or NADP+ dependent isoforms of IDH in cells following 3 hours of 0.1 mM glucose NS (RH, n = 8) compared to 3 hours of 2.5 mM glucose NS (control, n = 8) for 3 days. Statistical significance was assessed using unpaired student’s t-test.
3.2.19 Recurrent low glucose exposure reduces $K_{\text{ATP}}$ channel sensitivity to activation by MgADP, in the presence and absence of MgATP

In a final attempt to investigate the mechanism underlying the attenuated glucose sensing, following RH, single channel recordings were performed on inside-out membrane patches, isolated from GT1-7 cells exposed to RH or control protocols. Recordings were made in symmetrical 140 mM KCl, with the bath containing External Solution B and the electrode solution containing Pipette Solution B. +50 mV was injected during the recording in order to clamp the membrane potential at -50 mV (which is also the resting membrane potential of GT1-7 cells (Beall et al., 2012)). A stable recording was obtained for at least 2 minutes following patch excision in the presence of 0 adenine nucleotides (control). Channel activity was analysed by averaging the 90-120 seconds of the recording just before MgATP or MgADP was added and when the recording was stable following perfusion. Note that channel activity (inward current) is represented as downward deflections. N.P(o) was calculated, as previously described, where N = the number of functional channels in the patch and P(o) = the open state probability which was determined by measuring the total time spent at each unitary current level and expressed as a proportion of the total time of the segment of recording that was analysed. The N.P(o) control (0 nucleotides) value in cells exposed to RH (Fig 3.29B) was reduced compared to controls (Fig 3.29A) (n =6-9; 0.002 ± 0.001 vs 0.040 ±0.029 respectively; Fig 3.29C). This suggests that there is a reduction in the intrinsic $K_{\text{ATP}}$ channel activity following RH in GT1-7 cells. After 2 minutes of stable recording from patch isolation, 100 µM MgATP was perfused in to the bath in order to inhibit single channel events which reduced channel activity to a similar degree in both RH (Fig 3.29B) and control cells (Fig 3.29A) (n = 6; 0.002 ± 0.001 vs 0.005 ± 0.002 respectively; Fig 3.29C). Once MgATP perfusion had stopped and the trace had stabilised, a recording was made for at least 2 minutes before 100 µM MgADP was applied (in the absence of MgATP). There was a
significant reduction in the sensitivity of cells exposed to RH (Fig 3.29B) to activation by MgADP compared to vehicle treated cells (Fig 3.29A) (n = 4-7; 0.003 ± 0.001 vs 0.050 ± 0.012 respectively; P < 0.001; Fig 3.29A). 200 μM MgADP was applied to the patches in order to determine if there was the reduction in MgADP sensitivity was only present at lower [MgADP]. There was a trend for reduction in \( K_{ATP} \) activation in response to 200 μM ADP following RH (Fig 3.29B) but this was not significant compared to controls (Fig 3.29A) (n = 5-7; 0.047 ± 0.037 vs 0.184 ± 0.089 respectively; Fig 3.29C).

MgADP has been shown to activate \( K_{ATP} \) channels in the presence of MgATP, therefore the sensitivity of the channel to MgADP under these conditions was examined. In this set of experiments, the basal \( K_{ATP} \) channel activity was significantly lower following RH (Fig 3.30B) compared to control cells (Fig 3.30A) (n = 5; 0.001 ± 0.0005 vs 0.058 ± 0.014 respectively; P < 0.005; Fig 3.30C). 100 μM MgATP inhibited the channel to a similar degree in both RH (Fig 3.30B) and control treated cells (Fig 3.30A) (n = 5; 0.0002 ± 0.002 vs 0.008 ± 0.007 respectively; Fig 3.30C). When 200 μM MgADP was applied in the presence of 100 μM MgATP, there was a significant attenuation in the activation of the channel following RH (Fig 3.30B) compared to control (Fig 3.30B) (n = 3; 0.003 ± 0.001 vs 0.072 ± 0.010 respectively; P < 0.005; Fig 3.30C). 500 μM MgADP in the presence of 100 μM MgATP was also applied to the intracellular patch, yielding attenuated \( K_{ATP} \) activation following RH (Fig 3.31B) compared to control (Fig 3.31A), although this was not significant (n = 3-4; 0.001 ± 0.0007 vs 0.420 ± 0.203 respectively; P = 0.05; Fig 3.31C).
Figure 3.29 Recurrent low glucose exposure reduces $K_{ATP}$ channel sensitivity to activation by MgADP.

(A) Single channel recordings from an inside-out membrane patch isolated from a GT1-7 cell exposed to 2.5 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. Single channel currents were recorded in symmetrical 140 mM KCl at a membrane potential of -50 mV. The top trace displays channel activity directly after the patch is pulled, the trace below shows that channel activity is markedly reduced when 100 μM MgATP is applied to the intracellular side of the patch, the following trace demonstrates that channel activity is increased further by applying 200 μM MgADP (n = 4-6). (B) Single channel recordings from an inside-out membrane patch isolated from a GT1-7 cell exposed to 0.1 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. The top trace displays reduced channel activity directly after the patch is pulled and the bottom trace demonstrates that there is an attenuated ability to stimulate channel activity when 200 μM ADP to the intracellular side of the patch (n = 6-9). (C) Pooled NP(o) data (RH = recurrent hypoglycaemia). Statistical significance was analysed by student's unpaired t-test (*** P < 0.001).
Figure 3.30 Recurrent low glucose exposure reduces $K_{\text{ATP}}$ channel sensitivity to activation by 200 μM MgADP in the presence of MgATP.

(A) Single channel recordings from an inside-out membrane patch isolated from a GT1-7 cell exposed to 2.5 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. Single channel currents were recorded in symmetrical 140 mM KCl at a membrane potential of -50 mV. The top trace displays channel activity directly after the patch is pulled, the middle trace shows that channel activity is markedly reduced when 100 μM MgATP is applied to the intracellular side of the patch and the bottom trace displays channel activity is stimulated by addition of 200 μM MgADP, in the presence of 100 μM MgATP (n = 3-5). (B) Single channel recordings from an inside-out membrane patch isolated from a GT1-7 cell exposed to 0.1 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. The top trace displays channel activity directly after the patch is pulled, the middle trace shows that channel activity is markedly reduced when 100 μM MgATP is applied to the intracellular side of the patch and the bottom trace displays channel activity is stimulated by addition of 200 μM MgADP, in the presence of 100 μM MgATP (n = 3-5). (C) Pooled NPl(n) data (RH = recurrent hypoglycaemia). Statistical significance was analysed by student’s unpaired t-test (* * $P < 0.005$).
Figure 3.31 Recurrent low glucose exposure reduces $K_{ATP}$ channel sensitivity to activation by 500 μM MgADP in the presence of MgATP.

(A) Single channel recordings from an inside-out membrane patch isolated from a GT1-7 cell exposed to 2.5 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. Single channel currents were recorded in symmetrical 140 mM KCl at a membrane potential of -50 mV. The top trace displays channel activity directly after the patch is pulled, the middle trace shows that channel activity is markedly reduced when 100 μM MgATP is applied to the intracellular side of the patch and the bottom trace displays channel activity stimulated by addition of 500 μM MgADP, in the presence of 100 μM MgATP (n = 3-5). (B) Single channel recordings from an inside-out membrane patch isolated from a GT1-7 cell exposed to 0.1 mM glucose NS for 3 hours on days 1, 2 and 3 before electrical activity was examined on day 4. The top trace displays channel activity directly after the patch is pulled, the middle trace shows that channel activity is markedly reduced when 100 μM MgATP is applied to the intracellular side of the patch and the bottom trace displays channel activity stimulated by addition of 500 μM MgADP, in the presence of 100 μM MgATP (n = 4-5). (C) Pooled NP(o) data (RH = recurrent hypoglycaemia). Statistical significance was analysed by student’s unpaired t-test (** P<0.005).
3.3 Discussion

Animal and human studies have consistently demonstrated that recurrent, or even a single episode of, antecedent hypoglycaemia renders the individual less capable of responding to further hypoglycaemia (Heller and Cryer, 1991, Adamson et al., 1984, Fioramonti et al., 2013, Inouye et al., 2002, Jacobson et al., 2006, McCrimmon et al., 2005). While previous studies have shown that adaptations can occur in physiological processes that are involved in central glucose-sensing, the focus of those studies was on whole hypothalamic nuclei or unidentified populations of neurons. The aim of the current study was to investigate if adaptations occur following recurrent low glucose exposure in a homogenous population of GE neurons. To do this, the mouse hypothalamic GT1-7 cell line was utilised as a model of a homogenous population of GE neurons and the adaptations to recurrent low glucose exposure were examined in terms of both metabolism and electrical activity. This also allowed alterations in cellular functions following recurrent low glucose to be determined as cell autonomous because investigations were carried out in the absence of astrocyte/tanyocyte influences and synaptic inputs from neuronal networks.

Antecendent low glucose exposure on day 1 attenuated the ability of GT1-7 cells to hyperpolarise in response to 0.5 mM glucose (Fig 3.1). While this reduction in glucose-sensing was significant, a more robust attenuated response was observed in a model where cells were exposed to low glucose on 3 consecutive days (RH) (Fig 3.2). 0.5 mM glucose produces a submaximal activation of $K_{ATP}$ channels, as the hyperpolarisation of the membrane potential does not reach the level of hyperpolarisation that is produced in response to NN414 which, at -73 mV on average, is close to theoretical $E_K$. However, activation of the channel with NN414 is not driven by intracellular metabolism. The maximal activation of $K_{ATP}$ through metabolism-dependent processes can be achieved through application of 0.1 mM glucose, as this caused the membrane potential to hyperpolarise on average to -70 mV in
control cells, which is close to $E_K$ and is not significantly different from the membrane potential produced by NN414 (Fig 3.3). The maximum hyperpolarisation achieved with NN414 did not differ between control and RH treated cells, at -73 mV in both groups. However, the maximum metabolism-dependent hyperpolarisation in response to 0.1 mM glucose was attenuated following RH, with the membrane potential only reaching -56 mV on average. The attenuated hyperpolarisation of GT1-7 cells exposed to RH was greater compared to controls as the testing concentration of glucose was lowered. The membrane potential of RH cells only hyperpolarised by a further ~4 mV in response to 0.1 mM compared to 0.5 mM glucose, whereas control cells hyperpolarised by a further ~8 mV. These results indicate that near maximal $K_{ATP}$ channel activity through metabolic-dependent processes, in intact cells, is achieved at 0.5 mM glucose following RH as the membrane potential did not hyperpolarise by a great deal more from 0.5 to 0.1 mM glucose. However, activation of the channel through non-metabolic dependent processes was unaffected, as indicated by the intact response to NN414. Indeed, when the availability of the $K_{ATP}$ channel to open maximally in response to 0 ATP was examined using whole-cell patch clamp configuration, there was no difference in the conductance density between RH and control groups (Fig 3.4). This result demonstrates that there is no change in whole cell resistance following RH, as otherwise a reduction in whole cell macroscopic currents in response to 0 ATP would have been observed. However, it is still possible that input resistance is reduced during 0.5 mM glucose exposure (which produces sub-maximal membrane hyperpolarisation) which is missed when examining currents in response to the removal of intracellular ATP. Voltage clamp experiments would have to be performed during perforated patch clamp experiments, in the presence of 0.5 mM glucose in order to test this. Nevertheless, it appears that functional $K_{ATP}$ channels are still available in the plasma membrane of cells exposed to RH that maximal activity of these channels can still be achieved in response to the removal of
intracellular ATP or by pharmacological activation. These findings are consistent with observations already in the literature. Kang et al. found that animals with defective CRR following antecedent hypoglycaemia, displayed activation and maximal activation of VMH GE neurons at lower concentrations of glucose compared to control brains, as determined by intracellular Ca\textsuperscript{2+} imaging. GI neurons in the VMH were also inhibited at much lower concentrations of glucose compared to controls (Kang et al., 2008). Electrophysiological examination of VMN GI neurons following RH also demonstrated that extracellular glucose had to be lowered further, than for control brain slices, in order to initiate activation of GI neurons (Song and Routh, 2006). Taken together, the data from the current study and that in the literature demonstrates that both phenotypes of glucose-sensing neurons appear to lose sensitivity to glucose, whereby extracellular glucose must fall to lower levels before activation or inhibition of the neuron occurs. While this has occurred in GT1-7 cells in isolation, it also occurred in the brains of animals in the aforementioned studies in conjunction with defective CRR to hypoglycaemia. Therefore, recurrent low glucose exposure of GT1-7 cells in vitro appears to recapitulate the phenotype of GE neurons following antecedent/recurrent hypoglycaemia in vivo and seems to be an appropriate model to investigate the molecular adaptations in GE neurons in response to RH.

The attenuated response to hyperpolarise to low glucose indicates that metabolic adaptations were occurring in GT1-7 cells following RH. It was hypothesised that increased glucose uptake may have been occurring at lower glucose concentrations than controls, although there was no change between the groups at various glucose concentrations in terms of [\textsuperscript{3}H]2DG uptake (Fig 3.6). This result is in keeping with data from human studies that have shown that there is no increase in glucose uptake in the brains of individuals with poorly controlled type 1 diabetes or following antecedent hypoglycaemia, compared to controls (Fanelli et al., 1998, Segel et al., 2001). To coincide with the unchanged glucose uptake result, there was also no
change in the levels of glucose oxidation and incorporation following RH in this model, which otherwise may have indicated that increased glucose metabolism was occurring at lower glucose concentrations (Fig 3.7). However, the glucose oxidation assay examined substrate use over 4 hours. Real-time analysis of oxygen consumption rate using the Seahorse XF-24 revealed a decrease in the cellular OCR accompanied with a reduced ECAR, indicating a reduction in both aerobic and anaerobic glucose metabolism following RH (Fig 3.8). To examine this further, a glycolysis stress test was used with the Seahorse XF-24. Glycolysis was stimulated in GT1-7 cells when glucose was increased from 0 to 2.5 mM glucose, represented as an increase in ECAR, but this response was reduced after exposure to RH (Fig 3.9). Interestingly, when Oligomycin was applied in order to inhibit mitochondrial respiration, glycolysis was not upregulated in GT1-7 cells. This indicates that GT1-7 cells have no spare glycolytic capacity with which to try and maintain intracellular ATP when mitochondrial ATP production is inhibited. Whether or not this is a key feature of GE cells, by which no spare respiratory capacity allows glycolysis to be downregulated quickly in response to a fall in extracellular glucose and facilitate activation of the K_{ATP} channel, remains to be determined. When 2DG was applied to inhibit glucose uptake and hence metabolism, the fall in ECAR occurred more slowly in cells exposed to RH. It could be speculated that this could be due to the cell metabolising alternate fuel stores, such as glycogen although there is no evidence that neurons store glycogen. It is worth noting that GT1-7 cells do express gluconeogenic genes, although whether they express the functional proteins has not been determined (Appendix Fig 2).

In an attempt to explain the reduced glycolysis following RH, hexokinase activity was examined as this is the rate-limiting step in glycolysis (Fig 3.10). Although this assay is non-specific for hexokinase activity, GT1-7 cells do express the β-cell form of GK which has been shown to have a role in central glucose-sensing (Dunn-Meynell et al., 2002). HK
activity was significantly reduced at 0.1 mM glucose following RH and showed a trend for reduced activity at 0.25 and 0.5 mM glucose. This is in contrast to findings from in vivo studies examining GK expression in rats with defective CRR following antecedent hypoglycaemia where GK expression in the VMH was increased (Kang et al., 2008, Dunn-Meynell et al., 2002, Sanders et al., 2004). Although these investigations did not examine the activity of GK and the current study did not examine GK mRNA following RH it may be possible that expression increases in order to compensate for reduced activity, as inhibition of GK with alloxan into the third ventricle increases GK mRNA in the hypothalamus of rats (Sanders et al., 2004). While reduced activity of HK may explain the reduced ECAR following RH, previous studies with GT1-7 cells have shown that reducing GK activity with alloxan will hyperpolarise the membrane, likely due to reduced metabolic flux. Conversely, these cells are prevented from hyperpolarising to extracellular low glucose when GK activity is upregulated with GKA50 (Beall et al., 2012). Therefore, the GK results from the current study would be more inclined to activate the K\textsubscript{ATP} channel, especially during lower glucose concentrations, but this was not the case.

Because mitochondrial respiration is an oxygen consuming process, the OCR obtained from the Seahorse XF-24 can be taken as an indication of substrate flux through the mitochondria. This can be determined by mitochondrial efficiency and was examined in these cells using specific mitochondrial inhibitors of mitochondrial processes in order to determine the OCR\% contributing to these processes (Fig 3.11). There was an increase in O\textsubscript{2} consumption by ATP-synthase in conjunction with increased mitochondrial leak following RH, indicating that the mitochondria of these cells are possibly working to produce more ATP but in an inefficient manner, as mitochondrial leak transports protons across the mitochondrial membrane through non-ATP producing processes. This will act to dissipate the proton gradient across the mitochondrial membrane, which would normally result in reduced ATP synthesis as seen
when mitochondrial uncoupling proteins are active (Zhang et al., 2006, Divakaruni and Brand, 2011). There was also a reduction in the OCR% contributing towards non-mitochondrial oxygen consuming processes. Extramitochondrial oxidation includes the breakdown of long chain fatty acids through β-oxidation in peroxisomes. However, this process is usually upregulated in the liver following high fat feeding, which is not the case here (Kaikaus et al., 1993). The spare respiratory capacity of the mitochondria was determined by uncoupling the mitochondria and analysing the increased OCR% generated through the mitochondria trying to increase ATP production. There was no change in the spare respiratory capacity of the mitochondria following RH under these conditions (Fig 3.12). However, this test was performed with 2.5 mM glucose as the only fuel substrate and is reliant on other parameters such as glucose availability, glucose uptake and substrate delivery to the mitochondria. Differences may have been apparent in isolated mitochondria where this is not a determining factor of spare respiratory capacity.

While the changes in mitochondrial efficiency did not seem to explain the reduced cellular OCR, reduced substrate delivery to the mitochondria through decarboxylation of pyruvate to acetyl Co-A by pyruvate dehydrogenase (PDH) seemed like a logical hypothesis. PDH was significantly reduced following RH which may explain the reduced cellular OCR (Fig 3.13). This result coincided with an increase in the mRNA expression of pyruvate dehydrogenase kinase isoenzyme (pdk3), which phosphorylates and inhibits PDH, following RH (Appendix Fig. 2). PDK4 in particular has been shown to be upregulated in the heart, skeletal muscle, kidney and hypothalamus during fasting, as well as in response to hypoglycaemia in terms of the hypothalamus (Jeoung et al., 2006, Poplawski et al., 2011, Poplawski et al., 2010). Interestingly, mRNA expression of carnitine palmitoyltransferase 1a (cpt1a), the rate-limiting enzyme in free fatty acid metabolism through β-oxidation, is also upregulated in the hypothalamus of fasted mice and although it is unchanged by acute hypoglycaemia, cpt1a is
downregulated following RH (Poplawski et al., 2010, Poplawski et al., 2011). This indicates that during fasting and hypoglycaemia there is a reprogramming of neuronal metabolism towards lipid oxidation and away from glycolysis and glucose oxidation, but that metabolism reverts back to trying to utilise glucose following RH. While the current study did not find the same result as the aforementioned in vivo studies, this may be due to the lack of free fatty acids in the normal saline that the cells were incubated in during the low glucose incubations and directly before lysing for the gene array (cells were incubated in 2.5 mM glucose NS before being lysed for mRNA analysis). Nevertheless, increased mRNA expression of PDK3 may account for the reduced PDH activity and resulting OCR in this study.

The impact of reduced substrate delivery to the mitochondria in this study, along with possible inefficient ATP production, on the intracellular ATP/ADP ratio were examined. While the ATP/ADP ratio fell during low glucose exposure in control cells, the ratio was slightly lower at 2.5 mM glucose and did not fall further in response to low glucose in cells subjected to RH (Fig 3.14). Intracellular ATP levels were examined in order to determine if the reduced ratio was due to reduced ATP or otherwise, if ATP was conserved, may have been due to increased ADP. Intracellular ATP was reduced in control cells exposed to 0.1 mM glucose for 15 minutes compared to 2.5 mM glucose (Fig 3.15). ATP was lower in cells subjected to RH at 2.5 mM glucose and was not reduced in response to 0.1 mM glucose for 15 minutes (Fig 4.15). This is the time frame over which the electrophysiological recordings were made so a reduction in whole cell ATP may be driving the hyperpolarisation of the cell membrane during this time. It was previously thought that low glucose exposure resulted in a compartmentalised change in ATP that could not be detected by analysis of whole cell levels (Ainscow et al., 2002). A reduction in ATP should open the $K_{ATP}$ channel and, from the intracellular ATP results, it would be expected that channels would be more active even during euglycaemia following RH, but this is the opposite of the electrical phenotype
observed in these cells. The reduction in ATP and ATP:ADP ratio may also have consequences for PDH activity. At rest, PDK is regulated by multiple factors including activation by an increased ATP:ADP ratio coinciding with an increased Acetyl-CoA:CoA ratio and inhibition by increased pyruvate production through glycolysis (Fig. 3.32). The high ATP:ADP ratio and low pyruvate results in PDH being fairly inactive at rest. However, PDH is differentially regulated during exercise in skeletal muscle. During exercise, the Acetyl-CoA:CoA ratio will increase which should activate PDK and inhibit PDH, however PDH activity is increased during exercise. It is thought that the increase in pyruvate, intracellular \( \text{Ca}^{2+} \) and ADP at the onset of exercise override the activation of PDK and allow PDH activity (Spriet and Heigenhauser, 2002). Therefore, perhaps following RH, even though the ATP:ADP ratio and intracellular ATP levels were reduced in GT1-7 cells and should inhibit PDK and allow activation of PDH, the reduced glycolysis and theoretically reduced pyruvate production is overriding the inhibition of PDK by reduced ATP and stimulating it.

Figure 3.32 Schematic diagram of the regulation of PDH activity.

Pyruvate dehydrogenase (PDH) is phosphorylated by PDH kinase, resulting in PDH inhibition. PDK activity is promoted by high ratio of Acetyl-CoA (AcCoA):Coenzyme A (CoASH), NADH:NAD\(^+\) and ATP:ADP. PDH is dephosphorylated by PDH phosphatase, which is activated a rise in intracellular \( \text{Ca}^{2+} \). Activated PDH then converts pyruvate + CoASH +NAD\(^+\) to Acetyl-CoA + CO\(_2\) + NADH.

(Diagram adapted from [http://www.bmb.leeds.ac.uk/illingworth/metabol/2120lec3.htm](http://www.bmb.leeds.ac.uk/illingworth/metabol/2120lec3.htm))

As AMPK is activated during an increase in the AMP:ATP ratio, it would be expected that this kinase would be more active following RH. 0.1 and 0.5 mM glucose exposure for either
15 minutes or 3 hours increased AMPKα2 activity in control cells and, while there may have been a trend for reduced activity in RH cells at the longer times points, there was no significant difference between the groups (Fig 3.16). Reduced AMPKα2 activity at 3 hours may not be of functional importance, in terms of electrical glucose-sensing, as this is not the time frame over which the recordings were performed. Intriguingly, AMPKα1 activity was reduced following RH, even basally at 2.5 mM glucose exposure (Fig 3.17). While AMPKα1 activity was increased in control cells in response to 0.1 and 0.5 mM glucose for 15 minutes or 3 hours, the activation to low glucose was significantly blunted following RH. In line with the reduced AMPKα1 activity observed in the current study, AMPKα1 and AMPKα2 activity was reduced or occurred at a slower onset in some hypothalamic nuclei of rats following 4 days of ICV 2DG-induced neuroglucopenia, in response to further 2DG stimulation (Alquier et al., 2007). In a separate study, AMPKα2 but not AMPKα1 expression in the VMH was reduced following antecedent hypoglycaemia (Kang et al., 2008). The discrepancies between the current study and the in vivo studies may be due to the fact that AMPK would have been determined from a mixture of neuronal populations in the rodent studies whereas the data obtained here is from a homogenous GE neuronal population.

In an attempt to determine if AMPKα1 activation during low glucose exposure was driving metabolic adaptations to RH in GT1-7 cells, the expression of AMPKα1 was reduced using shRNA lentiral vector targeting AMPKα1 (Fig 3.18). Glucose utilisation was examined using the Seahorse XF-24 and demonstrated that OCR was no longer reduced in shAMPKα1 cells exposed to RH, whereas cells treated with a control scrambled sequence displayed reduced OCR following RH (Fig 3.19A). shAMPKα1 cells were also absent of reduced ECAR following RH (Fig 3.19B). However, the prevention of the reduced OCR did not translate into restoring glucose-sensing in these cells as they still displayed an attenuated ability to hyperpolarise to low glucose following RH, where as normal glucose-sensing was observed.
in shAMPKα1 cells under control conditions (Fig 3.21). The attenuated glucose-sensing observed in shAMPKα1 cells was accompanied with an absence of a reduction in the intracellular ATP:ADP ratio in response to low glucose (Fig 3.23). Unfortunately, in this set of experiments the ratios differed between assays and the data had to be normalised to baseline at 2.5 mM glucose so it is not possible in this case to conclude if the baseline ratio was lower in shAMPKα1 cells subjected to RH compared to controls. The observation that OCR is not reduced in shAMPKα1 RH cells but the glucose-sensing is still impaired implies that the changes in oxidative metabolism are not responsible for the attenuated ability of cells to hyperpolarise to low glucose following RH. The key feature in both wild type and shAMPKα1 GT1-7 cells is the loss of ATP:ADP reduction in response to low glucose in conjunction with attenuated glucose-sensing. Although the intracellular levels of ATP were not measured in shAMPKα1 cells, as they were in wild type cells, perhaps it is the acute change in the nucleotide ratio that results in activation of the K\textsubscript{ATP} channel during low glucose exposure. However, the preservation of OCR in shAMPKα1 cells suggests a possible role for AMPKα1 in the regulation of PDH, although PDH activity was not measured here. In skeletal muscle, activation of AMPK during hypoxia or pharmacologically with AICAR increases PDK4 which inhibits PDH and reduces glucose oxidation (Houten et al., 2009, Smith et al., 2005). Therefore, perhaps recurrent low glucose exposure, which activates AMPK during the initial hypoglycaemia incubations, is causing a repetitive inhibition of PDH which then adapts and remains in an inactive state, even when euglycaemic conditions are restored.

Due to the fact that there was no change was observed between control and RH groups in terms of glucose oxidation, as determined by the \textsuperscript{14}C-glucose oxidation assay which releases \textsuperscript{14}CO\textsubscript{2}, but RH cells displayed a reduced cellular OCR, the activity of an alternative CO\textsubscript{2} producing pathway was investigated. The activity of the pentose phosphate pathway (PPP)
was examined using the Seahorse XF-24 as this pathway feeds back into glycolysis and consequently mitochondrial respiration, hence OCR and ECAR could be obtained in response to PPP inhibitors (Spegel et al., 2013). The hypothesis behind this experiment was that if PPP activity was increased following RH resulting in an overall effect of no net change in CO$_2$ production, even when mitochondrial respiration was reduced, then there would be a greater suppression of OCR when PPP inhibitors were applied to the cells. Both 6-AN and DHEA inhibit the PPP through G6PDH and there was a bigger reduction in OCR obtained with 6-AN following RH but not DHEA (Figs 3.24 and 3.25). 6-AN is more specific for G6PDH than DHEA, which is also a PPAR-α agonist so may cause additional reduction of OCR by inhibiting oxygen consumption by peroxisomes (Peters et al., 1996). As an increase in PPP activity was not clear from these assays, the redox status of nicotinamide nucleotides was examined as PPP oxidises reduced NADP$^+$, producing NADPH. There was a significant increase in NADP$^+$ and a reduction in NADPH following RH (Fig 3.26). This implies that either the activity of the PPP is reduced following RH or that the consumption of NADPH for processes such as quenching ROS and fatty acid synthesis is increased (Fernandez-Fernandez et al., 2012). The latter seems unlikely as astrocytes are the main producers of fatty acids in the brain, although neurons are able to utilise fatty acids, therefore the ROS content of the cells were examined (Moore et al., 1991). ROS increased significantly in both control and RH GT1-7 cells when exposed to 0.1 mM glucose for 3 hours, although there was not a great deal of difference after 15 minutes incubation in 0.1 mM compared to 2.5 mM glucose (Fig 3.27). There was no significant difference between the levels of ROS produced at 3 hours in 0.1 mM glucose between groups but there may be a slight trend for increased ROS following RH. Although the nicotinamide nucleotide levels were measured at 2.5 mM glucose, the fact that ROS production was not hugely different between the groups but that there is more NADP$^+$ and less NADPH following RH suggests that RH cells are under a higher level of
oxidative stress and so are generating NADP$^+$ and consuming NADPH. To confirm this, NADP$^+$ and NADPH levels would have to be examined at 0.1 mM glucose exposure. This result also suggests that the activity of PPP is not reduced following RH as this would most likely have resulted in an increase in ROS. Another process within the cell which consumes NADP$^+$ and produces NADPH is IDH, while converting citrate to $\alpha$-ketoglutarate (Guay et al., 2013). However, there was no change in the activity of any of the isoforms of IDH following RH (Fig 3.28). This indicates that the change in NADP$^+$/NADPH is due to interactions between ROS production and the PPP.

Increased oxidative stress has been suggested to occur in the VMH following RH in rodent studies (Fioramonti et al., 2013). One episode of insulin-induced hypoglycaemia resulted in an increase in ROS, from euglycaemia, in VMH wedges whereas this response was lost in rats there were exposed to RH followed by an acute period of hypoglycaemia. This response was accompanied by an increase in the S-nitrosylation of sGC following RH which, as mentioned previously, is the receptor for NO and is thought to be involved in the AMPK-NO GI neuron activation pathway during hypoglycaemia. However, this study only examined the S-nitrosylation the sGC at euglycaemia in both control and RH animals so it is unclear if this process occurs during an acute episode of hypoglycaemia (Fioramonti et al., 2013). Preventing oxidative stress through giving animals an antioxidant, N-acetylcysteine (NAC), prevented defective CRR and S-nitrosylation of sGC following RH, indicating that oxidative stress from hypoglycaemia may be contributing to the development of hypoglycaemia unawareness (Fioramonti et al., 2013). S-nitrosylation is a post-translation modification which can be caused through NO reacting with ROS to form peroxynitrite which then covalently binds to thiol groups within the cysteine residues of proteins, resulting in a conformational change and/or altering the function of the protein (Jaffrey et al., 2001, Htet Hlaing and Clement, 2014). S-nitrosylation of SUR1 has been shown to occur in response to
NO production in dorsal root ganglion sensory neurons, resulting in activation of Kir6.2/SUR1 \(K_{ATP}\) channels. The modification was found to occur at a cysteine residue (Cys717) on the Walker A motif of nucleotide binding domain 1 (NBD1) and enhanced activation of the channel by intracellular MgATP, suggesting that S-nitrosylation amplifies \(K_{ATP}\) channel currents through enhancing MgATP binding at NBD1 of SUR1 (Kawano et al., 2009). This type of Kir6.2/SUR1 activation by NO may be another mechanism underlying inhibition of \(K_{ATP}\) channel containing GE neurons during hypoglycaemia, while GI neurons stay active. Interestingly, NO activation of recombinant Kir6.2/SUR1 in SH-SY5Y cells has been shown to be attenuated in the presence of catalase, which scavenges \(H_2O_2\) (Chai and Lin, 2010). This implies that NO activation of the channel is partially dependent on NO reacting with ROS. Although NO appears to activate \(K_{ATP}\) channels acutely, prolonged exposure to ROS and NO may produce S-nitrosylation of other cysteines including the Walker A motif of NBD2 which, although lysine residues of these motifs has been shown to be important for \(K_{ATP}\) channel opening in response to MgADP, may somehow alter MgADP binding or activation of the channel (Gribble et al., 1997). GT1-7 cells have been shown to express nitric oxide synthase (NOS) and histochemical investigation demonstrated that they are NADPH-diaphorase-positive, which is a marker of NOS activity (Lopez et al., 1997). Therefore perhaps S-nitrosylation is occurring in these cells in response to recurrent low glucose-associated ROS production and altering activity of the channel. Another post-translational modification which occurs during oxidative stress is S-glutathionylation and has been shown to inhibit \(K_{ATP}\) during increased oxidative stress through modification of cysteine residues at the N-terminus of transmembrane domains of Kir6.1, preventing the channel from entering an open state (Yang et al., 2011). However, this has not been shown in Kir6.2 containing \(K_{ATP}\) channels.
In an attempt to explain the electrical phenotype of GT1-7 cells exposed to RH, single channel analysis was performed in excised inside-out patches. Surprisingly, the intrinsic $K_{ATP}$ channel activity in RH treated cells was significantly lower than in control cells (Fig 3.29). This was unexpected as there was no change in the availability of the $K_{ATP}$ channel to activate maximally in the absence of ATP during the whole-cell voltage clamp experiments. This difference may be due to a process with removes an inhibiting modification or conformational change of the $K_{ATP}$ channel only being present/active in the intact cell and is lost when a patch of membrane is excised. 100 $\mu$M MgATP reduced channel activity in both groups, reaching a lower N.P(o) in the RH group, although these channels started from a lower NPo compared to controls. Channel activation was significantly blunted in response to 100 $\mu$M MgADP and there was a trend for reduced activation with 200 $\mu$M MgADP, applied in the absence of MgATP (Fig 3.29). Interestingly, there was a significant reduction in the activation of $K_{ATP}$ channels following RH in response to 200 $\mu$M MgADP, and a trend for reduction with 500 $\mu$M MgADP, in the presence of 100 $\mu$M MgATP. Taken together, these results suggest that while there may be a reduction in the sensitivity of the $K_{ATP}$ channel to be activated by MgADP following RH, there may also be reduced sensitivity to activation by MgATP. As mentioned previously, lysine residues in the Walker A motifs of NBD1 and NBD2 of SUR1 contribute to both channel activation by MgATP and MgADP (Gribble et al., 1997, Gribble et al., 1998). Therefore, perhaps post-translational modifications are occurring here which are either preventing nucleotide binding or causing a conformational change in the gating of the channel, preventing it from opening. Unfortunately, the single channel response to NN414 was not tested but considering the action of NN414 is dependent on intracellular MgATP and not MgADP, and no change was observed between groups in terms of the NN414 response in perforated-patch clamp configuration, it would be expected that single channel activation to NN414 would also be unchanged (Dabrowski et al., 2003).
However, as the action of Diazoxide is dependent on either intracellular MgATP or MgADP, a difference in the activation of the channel by Diazoxide may be observed if the sensitivity to MgADP is altered following RH (Dabrowski et al., 2003, Kozlowski et al., 1989, Larsson et al., 1993).

Following RH in GT1-7 cells, there is a reprogramming of glucose metabolism which results in reduced oxidative metabolism, glycolysis and ATP production (Fig 3.33). This ‘hypo-metabolic state’ should favor $K_{ATP}$ channel activation, whereas the opposite was observed (i.e. the channel appears to become disconnected from glucose metabolism). It appears as if the cell is becoming habituated to recurrent low glucose exposure and adapting a lowered metabolism, as if it were constantly in a low glucose environment, possibly in order to conserve energy which can be used during anticipated further hypoglycaemia in order to prevent intracellular ATP from dropping further. Unfortunately, the mechanism by which prolonged reduced metabolism relates to or alters the function of the $K_{ATP}$ channel has not been clarified in this study. Further investigations examining post-translational modification and/or protein associations with the channel need to be determined.
Figure 3.33 Hypothetical diagram of the adaptations which occur in glucose-excited neurons following recurrent hypoglycaemia

(Ai) In the absence of recurrent hypoglycaemia (RH), during euglycaemia (2.5 mM glucose), glucose is transported into the cell and metabolised to increase the intracellular ATP:ADP ratio. This results in closure of the $K_{ATP}$ channel, membrane potential depolarisation and increased action potential firing. (Aii) In the absence of RH, during hypoglycaemia (0.1 mM glucose), the intracellular ATP:ADP ratio falls. This causes activation of $K_{ATP}$ channels and hyperpolarisation of the cell membrane, which silences action potential firing. Hypoglycaemia also increases activation of AMPKα1/2 and levels of reactive oxygen species (ROS) within the cell. Increased ROS during acute hypoglycaemia may contribute to activation of the $K_{ATP}$ channel, as has been shown previously, however this has not been validated in GT1-7 cells. (Bii) Following RH, during euglycaemia, glucose transport was unaltered yet glycolysis, pyruvate dehydrogenase activity, oxidative metabolism and AMPKα1 activity were reduced. However, this did not result in a reduction of the ATP:ADP ratio, which remained high during acute hypoglycaemia and was associated with an attenuated hyperpolarisation of the membrane potential. Following RH, there was also an attenuated ability of the $K_{ATP}$ channel to be activated by MgADP which, in conjunction with an increased ATP:ADP ratio, may contribute to blunted hypoglycaemia detection. As acute low glucose exposure increases ROS production, perhaps repeated exposure to high levels of ROS during RH may be causing a post-translational modification (PTM) of the $K_{ATP}$ channel, resulting in an altered sensitivity to the rise in MgADP during hypoglycaemia and a loss of glucose-sensing?
Chapter 4

Chronic exposure of hypothalamic GT1-7 cells to the SUR1-selective agonist, NN414, induces a stable inactive state for $K_{\text{ATP}}$ channels
4.1 Introduction

$K_{ATP}$ channels have been shown to be present in GE neurons of the ventromedial hypothalamus (VMH). Electrophysiological studies have shown that VMH GE neurones increase their firing frequency in response to a rise in extracellular glucose and are hyperpolarised upon the removal of glucose. The glucose-associated depolarisation of these neurons was shown to be metabolism dependant as hexokinase inhibition prevented depolarisation in the presence of glucose, demonstrating that the activity of these neurons was modulated by a metabolically regulated channel. Cell-attached recordings from VMH neurons have demonstrated that increasing extracellular $[K^+]$ inhibited single channel events and that this activity was also reduced with increasing extracellular glucose. Through isolating patches of cell membrane, the same study demonstrated that the K+ channels in VMN neurons were also sensitive to inhibition by intracellular ATP (Ashford et al., 1990a, Ashford et al., 1990b, Lee et al., 1999, Spanswick et al., 1997, Treherne and Ashford, 1992). In a similar study, K+ channels in VMN GE neurons were found to be sensitive to inhibition by sulphonylureas, tolbutamide and glibenclamide, and activation by the potassium channel opener, diazoxide. Removal of extracellular glucose or dialysing the cell with ATP-free solution caused a cessation of firing, membrane hyperpolarisation and a reduction in input resistant due to activation of single K+ channel events with outward currents of 3.3 pA and reversal potential of -60 to -80 mV, similar to the $K_{ATP}$ channel characteristics observed in the pancreatic $\beta$-cell (Lee et al., 1999), (Ashcroft et al., 1984). Single-cell reverse transcription (RT)-multiplex polymerase chain reaction (PCR) analysis of VHM GE neurons has demonstrated that they do indeed express the same sulphonylurea receptor as the $\beta$-cell, SUR1, although in this particular study Kir6.1 was found to be expressed instead of Kir6.2. Non-GE VMH neurons were found to be absent of KATP channel subunits (Lee et al., 1999). However, other studies have shown widespread expression of Kir6.2 in the hypothalamus, the
Deletion of which renders mice glucose intolerant with a blunted counterregulatory response to systemic hypoglycaemia and neuroglucopenia, in the form of an absent glucagon response and increased food intake (Kefaloyianni et al., 2013), (Kang et al., 2004), (Miki et al., 2001).

As mentioned in Chapter 3, repeated exposure to hypoglycaemia results in deterioration of the sympathetic and behavioural responses to subsequent hypoglycaemia in the form of a defective CRR. Hypothalamic $K_{\text{ATP}}$ channel activation, in response to a fall in circulating glucose levels, has been shown to be required for initiating an appropriate CRR to hypoglycaemia (Miki et al., 2001). In vivo rodent studies have shown that intracerebroventricular (ICV) infusion of sulphonylureas, glibenclamide and tolbutamide, into the brain results in an attenuation of the CRR during both systemic hypoglycaemia and cerebral glucopenia, in the form of blunted glucagon and adrenaline secretion. Direct microinjection of tolbutamide into the VMH also reduced the hormonal response to systemic hypoglycaemia (Evans et al., 2004). Alternatively, bilateral ventromedial hypothalamic microinjections of potassium channel openers (KCOs), diazoxide and NN414, just prior to hypoglycaemia amplified epinephrine and glucagon CRRs during hyperinsulinaemic-hypoglycaemic clamp studies. VMH microinjection of diazoxide was also able to amplify the CRRs in a rodent model of defective CRR, following recurrent hypoglycaemia (McCrimmon et al., 2005).

While diazoxide is capable of activating both SUR2 and SUR1 expressing $K_{\text{ATP}}$ channels, NN414 is a SUR1 selective agonist and has been shown to have a more potent effect in terms of $K_{\text{ATP}}$ channel (Kir6.2/SUR1) activation. The $EC_{50}$ for NN414 was calculated to be much lower at 0.45 µM, compared to 31 µM for diazoxide, when applied to isolated inside-out membrane in the presence of 100 µM MgATP (Dabrowski et al., 2003). Interestingly, the actions of NN414 are dependent on intracellular MgATP but not MgADP, unlike diazoxide.
which will activate the channel in the presence of both (Larsson et al., 1993). NN414 was found to be ineffective at activating SUR2A/B containing $K_{ATP}$ channels, when applied intracellularly. In fact, NN414 applied to the intracellular domain of SUR2A/B $K_{ATP}$ channels, expressed in oocytes, produced an inhibitory effect when these channels were active in the absence on nucleotides (Dabrowski et al., 2003).

As the $K_{ATP}$ channel is fundamental for metabolic dependent glucose-sensing, it may potentially be a therapeutic target for type 1 diabetes. For patients with type 1 diabetes, the major obstacle in the obtaining good glycaemic control is recurrent hypoglycaemia leading to an impaired CRR to further hypoglycaemia (Ovalle et al., 1998). As demonstrated by the rodent studies mentioned previously, KCOs appear to have good therapeutic potential at normalising defective CRR, when applied centrally, in rodent models. However, it still remains to be determined whether these drugs are most effective when administered acutely during a hypoglycaemic episode, or given chronically as a preventative therapy.

Taken together, the aforementioned studies provide strong evidence that $K_{ATP}$ channels (Kir6.2/SUR1) are present and responsible for glucose-sensing in GE neurons and are partially responsible for mediating the CRRs to hypoglycaemia. The current study is based on the observation that continuous, central delivery of NN414, during antecedent hypoglycaemia and euglycaemia, produced defective CRRs in response to further hypoglycaemia in rodents (Fig 4.1). In an attempt to examine the molecular mechanisms underlying this phenomena, the GT1-7 GE cell line was used.
4.2. Results

4.2.1 Continuous NN414 blunts glucose counter regulation

The following *in vivo* studies were carried out by Dr Xiaoning Fan, under the supervision of Professor Rory McCrimmon, at Yale University, New Haven, US. Animal care and experimental protocols were reviewed and approved by the Yale Institutional Animal Care and Use Committee.

In order to investigate whether continuous $K_{ATP}$ activation during antecedent/recurrent hypoglycaemia could prevent defective CRR, male Sprague-Dawley rats were fitted with osmotic mini-pumps, which were cannulated into the third ventricle of the brain. NN414, or vehicle (NaOH) was then infused at a rate of 25 $\mu$l/h over 8 days. Rats were subjected to the recurrent insulin-induced hypoglycaemia (10 U/kg insulin) or euglycaemia protocol (saline injection) on days 5, 6 and 7 after surgery. Rats were divided into four groups in total: recurrent euglycaemia with chronic ICV vehicle (Control); recurrent hypoglycaemia with chronic ICV vehicle (RH); recurrent hypoglcaemia with chronic ICV NN414 (RH + NN414); recurrent hypoglycaemia with chronic ICV NN414 which was stopped 24 hours before the hypoglycaemic clamp study (RH + NN414 + washout) (Fig 4.1A). Hyperinsulinaemic–hypoglycaemic clamps were carried out on day 8 of the study. Hypoglycaemia (~2.8 mmol/l) was induced using a 90 minute constant infusion of human insulin (Eli Lilly & Co, Indianapolis, IN, USA) at a rate of 20 mU kg$^{-1}$ min$^{-1}$ using a variable-rate 20% dextrose infusion. Blood samples were taken at −30, 0, 60 and 90 min for analysis of adrenaline, glucagon and insulin. Plasma glucose was measured by the glucose oxidase method (Analox, Lunenburg, MA, USA), and catecholamine analysis was by HPLC using electro-chemical detection (ESA, Acton, MA, USA). Plasma insulin and glucagon were measured by RIA (Linco, Saint Charles, MO, USA).
Three days of antecedent hypoglycaemia resulted in an attenuated CRR during the hyperinsulinaemic-hypoglycaemic clamps, which presented as an increased glucose infusion rate (GIR) compared with saline controls (13.1 ± 1.5 vs 8.7 ± 1.5 mg kg\(^{-1}\) min\(^{-1}\); Fig 4.1C), although this did not reach statistical significance (n = 10–14). Continuous NN414 exposure during RH (RH + NN414) did not protect against the effects of RH and instead significantly increased GIR more so than just RH (20.3 ± 3.1 vs 13.1 ± 1.5 mgkg\(^{-1}\) min\(^{-1}\); p < 0.05; Fig 4.1C). Halting infusion of NN414 on day 7 (RH + NN414 + washout) reduced GIR by 40% (p < 0.05) compared to the RH + NN414 group, bringing GIR down to a level between control and RH animals (Fig 4.1C).

RH animals also displayed an attenuated mean (60–90 min) adrenaline responses during the hyperinsulinaemic-hypoglycaemic clamp study compared to the control group (5.5 ± 1.1 nmol/l vs 11.1 ± 1.3 nmol/l, respectively; p < 0.050; Fig 4.1D). Continuous NN414 during RH went against the hypothesis and showed a trend for an increased, rather than decreased, adrenaline response to hypoglycaemia (3.5 ± 0.9 nmol/l, p > 0.05 vs RH controls; Fig 4.1D). Washout of NN414 allowed partial recovery of the adrenaline response, which was significantly different from RH with continuous NN414 (p < 0.005) but not from control or RH. There was no significant difference in the glucagon response during the hyperinsulinaemic-hypoglycaemic clamp between the four groups (Fig 4.1D).

**4.2.2 Acute NN414 application hyperpolarises GT1-7 cells**

In order to investigate the impact that chronic K\(_{\text{ATP}}\) agonism may be having on the function and subsequent responsiveness of the K\(_{\text{ATP}}\) channel and potentially other cellular processes, the GT1-7 hypothalamic cell line was used for all further studies in this chapter.

To examine the effects of acute NN414 application on membrane potential and K\(_{\text{ATP}}\) activation of GT1-7 cells, perforated patch-clamp recordings were performed (n = 5). The
extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). 25-40 μg/ml amphotericin B was also present in the pipette in order to allow perforation of the cell membrane and gain access to the electrical activity of the cell. No current was injected during perforated patch-clamp recordings. Once a stable membrane potential was established in 2.5 mM glucose NS (2-3 minutes), it was recorded for a further 10 minutes to ensure stability before 5 μM NN414 was superfused. Addition of this SUR-1 selective agonist hyperpolarised the membrane potential (Fig 4.2A) from an average of -43.4 ± 2.5 mV, in 2.5 mM glucose NS, to -72.0 ± 2.2 mV (n = 5, P < 0.005; Fig 4.2B). This effect was reversible upon washout with 2.5 mM glucose NS, which depolarised the membrane back to -46.0 ± 4.0 mV (n = 2, P < 0.005; Fig 4.2B) which was comparable to the membrane potential at the start of the recording.

4.2.3 Continuous NN414 attenuates $K_{\text{ATP}}$ channel conductance, in GT1-7 cells.

The timescale of the in vivo experiment examining continuous NN414 infusion was replicated in the GT1-7 cell line. Cells were exposed in culture to 24, 48, 72, 96, 120, 144, 168 hours of 5 μM NN414 in culture in plating media containing 2.5 mM glucose (n = 4-10). There was also an additional group of cells that were exposed to 144 hours of NN414 plus a 24 hour washout period before $K_{\text{ATP}}$ channel function was examined (Fig 4.3). In order to measure the maximum availability of the $K_{\text{ATP}}$ channel to open, whole cell patch-clamp configuration was used to dialyse out the intracellular ATP from the cell, alleviating all nucleotide dependent channel inhibition. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). Currents were evoked by a voltage-clamp protocol from -160 to -40 mV steps which were 400 milliseconds in duration and 20 milliseconds apart (Fig 4.3Aii, Bii and Cii). This was applied when the cell was first ruptured and during the period when intracellular ATP had been dialysed out of the cell (run-up) in order to obtain the maximum conductance.
density. Run-up was defined when the membrane had reached a steady state hyperpolarisation at ~15 minutes after the cell membrane had been ruptured. The results in regards to maximum conductance density for this experiment have been summarized in Table 4.1. At each time point, NN414 treated cells exhibited severely reduced maximum conductance density, inferring that chronic $K_{\text{ATP}}$ agonism reduces the availability of the channel to activate maximally under ATP-free conditions.

<table>
<thead>
<tr>
<th>Exposure Time (Hours)</th>
<th>500 μM NaOH</th>
<th>5 μM NN414</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>1.08 ± 0.29</td>
<td>0.31 ± 0.06 **</td>
</tr>
<tr>
<td>48</td>
<td>1.45 ± 0.27</td>
<td>0.30 ± 0.04 ***</td>
</tr>
<tr>
<td>72</td>
<td>1.17 ± 0.22</td>
<td>0.24 ± 0.03 ***</td>
</tr>
<tr>
<td>96</td>
<td>1.38 ± 0.17</td>
<td>0.32 ± 0.11 ***</td>
</tr>
<tr>
<td>120</td>
<td>1.79 ± 0.34</td>
<td>0.29 ± 0.04 **</td>
</tr>
<tr>
<td>144</td>
<td>1.42 ± 0.36</td>
<td>0.21 ± 0.05 *</td>
</tr>
<tr>
<td>168</td>
<td>1.39 ± 0.33</td>
<td>0.34 ± 0.11 **</td>
</tr>
<tr>
<td>144 + 24 Hour Washout</td>
<td>1.27 ± 0.19 *** (compared to 168 hours NN414)</td>
<td></td>
</tr>
</tbody>
</table>

Table 4.1. Maximum $K_{\text{ATP}}$ channel conductance densities following chronic NN414 or vehicle treatment.
Analysed by students unpaired t-test * $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$ compared to corresponding vehicle controls

**4.2.4 Short-term NN414 exposure attenuates $K_{ATP}$ channel conductance, in GT1-7 cells**

In an effort to establish how quickly continuous agonism attenuates $K_{ATP}$ channel conductance, GT1-7 cells were exposed to 5 μM NN414, or 500 μM NaOH vehicle control, for 3 hours in culture in plating media containing 2.5 mM glucose ($n = 5$). Whole cell patch-clamp configuration was used to dialyse out the intracellular ATP from the cell, alleviating channel inhibition. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). Currents were evoked from the voltage clamp protocol, as mentioned previously, when the cell membrane was first ruptured and at maximal run-up. The maximum conductance density at run-up was significantly reduced compared to the vehicle controls (1.41 ± 0.47 vs 3.33 ± 0.57 pS/pF respectively; $P < 0.05$; Fig 4.4C). This result indicates that down regulation of $K_{ATP}$ channel activity occurs relatively quickly in the presence of agonist.

**4.2.5 Continuous NN414 exposure attenuates hypoglycaemia sensing, in GT1-7 cells.**

As the $K_{ATP}$ channel is the main channel involved in regulating the membrane potential in response to fluctuations in ambient glucose levels and metabolism, the glucose-sensing ability of the GT1-7 cells were investigated following continuous NN414 exposure.

GT1-7 cells were incubated with 5 μM NN414, or 500 μM NaOH vehicle control, in plating media with 2.5 mM glucose for 24 hours ($n = 4-8$). Perforated patch-clamp recordings were performed in order to monitor the response of the membrane potential to low glucose without disrupting the intracellular metabolism of the cell. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). 25-40 μg/ml amphotericin B was also present in the pipette in order to perforate the cell membrane and no current was injected during the recordings. A stable
recording was obtained for ~10 minutes at 2.5 mM glucose NS before 0.7 mM glucose was superfused. This concentration of glucose chosen to test the response of GT1-7 cells to low glucose as this is a concentration at which the cells have been shown to hyperpolarise in response to (Beall et al., 2012). 0.7 mM is in the middle of the glucose-sensing range for GT1-7 cells (respond to glucose dose-dependently 0-2.5 mM) and so was used to try and decipher if there was a rightward shift in the glucose dose response of cells exposed to chronic K$_{ATP}$ agonism which might not be apparent at lower concentrations which cause near maximal K$_{ATP}$ activity. In response to 0.7 mM glucose, the membrane potential of cells exposed to vehicle control hyperpolarised on average by $-16.2 \pm 2.5$ mV (Fig 4.5A) compared to $-9.4 \pm 2.0$ mV in cells exposed to continuous NN414 (Fig 4.5B). However, this trend for attenuated low glucose sensing at 0.7 mM glucose following 24 hours NN414 incubation was not significant (Fig 4.5C) The experiment was repeated using 0.5 mM glucose as this produced a more reproducible hyperpolarisation in untreated GT1-7 cells. At this glucose concentration, cells treated with NaOH vehicle control for 24 hours hyperpolarised from 2.5 to 0.5 mM glucose on average by $-14.8 \pm 2.8$ mV (Fig 4.6A) whereas this was significantly attenuated following 24 hours NN414 with an average response of $-1.7 \pm 0.8$ (Fig 4.6B) ($n = 4-6$, respectively; $P < 0.001$; Fig 4.6C).

### 4.2.6 Short-term NN414 exposure attenuates hypoglycaemia sensing, in GT1-7 cells.

In an effort to investigate if 3 hour NN414 exposure also attenuated glucose sensing, along with K$_{ATP}$ conductance, GT1-7 cells were incubated with 5 µM NN414, or 500 µM NaOH for vehicle controls, for 3 hours in plating media containing 2.5 mM glucose. Perforated patch-clamp recordings were used to examine glucose sensing. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). 25-40 µg/ml amphotericin B was also present in the pipette in order to perforate the cell membrane and no current was injected during the recordings. As before,
a stable recording was obtained in 2.5 mM glucose before 0.5 or 0.7 mM glucose NS was superfused. GT1-7 cells treated with NaOH vehicle control for 3 hours hyperpolarised from 2.5 to 0.7 mM glucose by an average of -9.0 ± 1.8 mV (Fig 4.7A) whereas this response was attenuated following 3 hours NN414 exposure, -4.3 ± 1.8 mV (Fig 4.7B) (n = 6). However, the trend for blunted glucose-sensing was not significant at 0.7 mM glucose so the experiment was repeated in a separate batch of cells, testing the response at 0.5 mM glucose. GT1-7 cells incubated with NaOH vehicle control for 3 hours hyperpolarised from 2.5 to 0.5 mM glucose on average by -12.40 ± 1.77 mV (Fig 4.8A) compared to -0.8 ± 0.5 mV following 3 hours NN414 treatment (Fig 4.8B) (n = 5; P < 0.001; Fig 4.8C).

4.2.7 Antecedent NN414 exposure attenuates hypoglycaemia sensing in GT1-7 cells but does not suppress $K_{\text{ATP}}$ channel conductance.

As the deleterious effects of continuous NN414 exposure for 7 days on $K_{\text{ATP}}$ channel conductance could be reversed upon washout, the effects of antecedent NN414 on glucose-sensing were examined by exposing GT1-7 cells to 3 hours of 5 μM NN414, or 500 μM NaOH vehicle on day 1. At the end of the incubation period, cells were washed with PBS and placed into plating media containing 2.5 mM glucose and assayed 24 hours later. Perforated patch-clamp recordings were used to examine glucose sensing. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). 25-40 μg/ml amphotericin B was also present in the pipette and no current was injected during the recordings. As before, a stable recording was obtained in 2.5 mM glucose before 0.7 mM glucose NS was superfused. GT1-7 cells treated with antecedent NaOH vehicle control for 3 hours hyperpolarised from 2.5 to 0.7 mM glucose by an average of -8.7 ± 1.2 mV (Fig 4.9A) whereas this response was significantly attenuated following NN414 exposure, -3.6 ± 0.9 mV (Fig 4.9B) (n = 6-7 respectively; $P<0.005$; Fig 4.9C). This experiment was repeated in a separate batch of cells to test if
glucose sensing was further attenuated at the lower concentration of 0.5 mM glucose. GT1-7 cells treated with antecedent NaOH vehicle control for 3 hours hyperpolarised from 2.5 to 0.5 mM glucose on average by -14.3 ± 1.4 mV (Fig 4.10A) whereas this response was significantly attenuated following NN414 exposure, -3.8 ± 1.7 mV (Fig 3.10B) (n = 4-6 respectively; P<0.005; Fig 4.10C).

Next, the experiment was repeated again in a separate batch of cells which were subjected to whole-cell patch-clamp recordings in order to test whether the attenuated glucose-sensing following short-term antecedent NN414 exposure was due to attenuated K\textsubscript{ATP} channel conductance. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). Currents were evoked from the voltage clamp protocol, mentioned previously, when the cell membrane was first ruptured and at run-up. The maximum conductance density at run-up in cells treated with antecedent NaOH did not differ significantly from those incubated with NN414 (n = 4-5; 3.0 ± 0.9 vs 4.0 ± 0.7 pS/\rho F respectively; Fig 4.11C).

To summarise the findings so far with continuous NN414 exposure, chronic activation of the K\textsubscript{ATP} channel in GT1-7 cells attenuates hyperpolarisation of the cell membrane in response to low glucose. This occurs at the same time as a reduction the availability of the channel to open maximally in response to the removal of intracellular ATP. Removal of NN414 for 24 hours appears to reverse the inhibition of K\textsubscript{ATP} channel conductance; however the glucose-sensing remains impaired. In an attempt to decipher if this effect could be replicated with chronic application of other KCOs, Diazoxide was next applied continuously to the cells.

### 4.2.8 Continuous Diazoxide exposure reduces K\textsubscript{ATP} channel conductance, in GT1-7 cells.

Diazoxide was applied continuously to GT1-7 cells in culture for 3 and 24 hours. Whole-cell patch-clamp recordings were performed where the extracellular bath solution contained 2.5
mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). Currents were evoked from the voltage clamp protocol, mentioned previously, when the cell membrane was first ruptured and at run-up. The maximum conductance density at run-up in cells treated with 200 µM Diazoxide for 24 hours was significantly attenuated compared to 0.1% DMSO vehicle controls (n = 5; 0.46 ± 0.11 vs 3.43 ± 0.46 ρS/ρF respectively, P<0.001; Fig 4.12C).

Whole-cell patch-clamp recordings were also performed on GT1-7 cells exposed to short-term (3 hours) Diazoxide incubation in order to investigate if this KCO acted as rapidly as NN414 to attenuate K$_{\text{ATP}}$ conductance. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). Currents were evoked from the voltage clamp protocol, mentioned previously, when the cell membrane was first ruptured and at run-up. The maximum conductance density at run-up in cells treated with 200 µM Diazoxide for 3 hours was significantly attenuated compared to 0.1% DMSO vehicle controls (n = 5-6; 1.65 ± 0.28 vs 3.25 ± 0.44 ρS/ρF respectively, P<0.05; Fig 4.13C).

These data strengthen the hypothesis that continuous K$_{\text{ATP}}$ activation results in some form of negative feedback system where the conductance of the channel becomes downregulated. In an attempt to investigate if this is due to hyperpolarisation of the cell membrane, the concentration of extracellular K$^+$ was increased to a concentration which chemically clamped the membrane potential at -55 mV in the presence of NN414.

**4.2.9 Chronic NN414-associated attenuated K$_{\text{ATP}}$ channel conductance is not due to membrane potential hyperpolarisation.**

In order to determine whether the attenuated K$_{\text{ATP}}$ channel conductance associated with continuous NN414 exposure was due specifically to a direct effect on K$_{\text{ATP}}$ or an effect of
prolonged membrane hyperpolarisation, GT1-7 cells were exposed to 5 μM NN414, or 500 μM NaOH vehicle control, for 24 hours in plating media containing 2.5 mM glucose and 13.5 mM KCl in order to prevent NN414 hyperpolarising the membrane down to near $E_K$. Using the Nernst equation, 13.5 mM KCl was calculated as the concentration required in order to chemically clamp the membrane potential at -60 mV. This membrane potential was chosen because the resting membrane potential of GT1-7 cells at 2.5 mM glucose is between -40 to -60 mV, with an average of -50 mV (Beall et al., 2012). The more hyperpolarised end of this range was chosen in order to avoid problems with calcium toxicity as voltage-gated L-type calcium channels would be active and influx calcium at more depolarised membrane potentials (Arkhammar et al., 1987). Firstly, perforated patch-clamp recordings were performed on naive GT1-7 cells in order to test the membrane potential that would be achieved when NN414 was applied acutely in the presence 13.5 mM KCl. The extracellular bath solution contained NS with 2.5 mM glucose, plus 13.5 mM KCl, and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). 25-40 μg/ml amphotericin B was also present in the pipette and no current was injected during the recordings. A stable recording was obtained in 2.5 mM glucose before 5 μM NN414 was superfused. The membrane potential hyperpolarised from $-46.3 \pm 3.2$ mV at 2.5 mM glucose to $-51.3 \pm 3.5$ mV, on average, when 5 μM NN414 was superfused (Fig 4.14A) ($n = 3$; Fig 4.14B). Although this was not the theoretical value of membrane potential that was calculated from the Nernst equation, this is due to the fact that the cell membrane is not just selective for $K^+$ and so the movement or other cations and anions will alter the calculated value. Therefore, 13.5 mM KCl was used to prevent hyperpolarisation during continuous NN414 exposure.

Next, whole-cell patch-clamp recordings were performed in order to test whether membrane hyperpolarisation contributed to attenuated $K_{ATP}$ channel conductance following continuous
NN414 exposure. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). Currents were evoked from the voltage clamp protocol, mentioned previously, when the cell membrane was first ruptured and at run-up. The maximum conductance density at run-up in cells treated with 24 hour NN414 was significantly attenuated compared to vehicle controls (n = 5; 0.15 ± 0.01 vs 1.39 ± 0.29 pS/pF respectively, P<0.005; Fig 4.15C).

Whole-cell patch-clamp recordings were performed on cells exposed to short-term (3 hours) NN414 exposure was also repeated in order to see if the reduced K\textsubscript{ATP} channel conductance observed in this experiment was attributed to membrane hyperpolarisation. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). Currents were evoked from the voltage clamp protocol, mentioned previously, when the cell membrane was first ruptured and at run-up. The maximum conductance density at run-up in cells treated for 3 hours with NN414, in 13.5 mM KCl, was significantly attenuated compared to vehicle controls (n = 11-12; 1.26 ± 0.33 vs 2.73 ± 0.35 pS/pF respectively, P<0.005; Fig 4.16C).
Figure 4.1 Continuous NN414 leads to higher GIRs after RH mediated by further suppression of adrenaline secretion.

(a) Study protocol; black line represents vehicle (NaOH) and grey line NN414 protocols. Insulin-induced hypoglycaemia (IIH) or saline (Sal) was given to vehicle and NN414 treated rats on days 5, 6 and 7. Hyperinsulinaemic–hypoglycaemic clamp was performed on day 8. (b) Plasma glucose during the hyperinsulinaemic–hypoglycaemic clamp on day 8. Black circles, RH; white squares, NN414 + RH (N+RH); white circles, NN414 + RH + washout (N+RH+W); black squares, vehicle control. (c) GIRs during the hyperinsulinaemic–hypoglycaemic clamp. Values are mean ± SEM (n=8/9). (d) Adrenaline secretion. Values are mean ± SEM (n=8/9). (e) Glucagon secretion. Values are mean ± SEM (n = 8–13)
Figure 4.2 NN414 activates $K_{ATP}$ channels and hyperpolarises the cell membrane in GT1-7 cells

(A) Perforated-patch current clamp recording of a GT1-7 cell in 2.5 mM glucose NS. When 5 μM NN414 is superfused, the membrane potential is hyperpolarised. (B) Pooled membrane potential data (n = 2-5). Statistical significance was analysed by one-way ANOVA (***, P < 0.001).
Figure 4.3 8 days of NN414 exposure reduces availability of $K_{ATP}$ channel to activate maximally in response to dialysis of cell with 0 ATP

(Ai) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 168 hours 500 μM NaOH as it is dialysed with 0 ATP. (Aii) Corresponding families of currents evoked by the voltage-clamp protocol stated in "Methods". Whole-cell macroscopic currents were examined immediately after the cell membrane was ruptured (control), after the cell had been dialysed with 0 ATP (Run-up) ($n = 8$). (Bi) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 168 hours 5 μM NN414 as it is dialysed with 0 ATP. (Bii) Corresponding families of currents ($n = 10$). (Ci) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 144 hours 5 μM NN414 + 24 hour washout in media containing 500 μM NaOH, as it is dialysed with 0 ATP. (Cii) Corresponding families of currents ($n = 10$).

(D) Pooled conductance densities at maximum run-up of GT1-7 cells exposed to NN414, or NaOH vehicle, for 8 days and tested every 24 hours ($n = 4-10$). Maximum conductance density was significantly reduced in cells exposed to NN414 as early as 24 hours, compared to NaOH vehicle control. Statistical significance was analysed in panel C by student’s unpaired t-test (* $P < 0.05$, ** $P < 0.005$, *** $P < 0.001$).
Figure 4.4 3 hour NN414 exposure reduces availability of KATP channel to activate maximally in response to dialysis of cell with 0 ATP

(Ai) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 500 μM NaOH for 3 hours, as it is dialysed with 0 ATP. (Aii) Corresponding families of currents evoked by the voltage-clamp protocol stated in “Methods”. Whole-cell macroscopic currents were examined immediately after the cell membrane was ruptured (control), after the cell had been dialysed with 0 ATP (Run-up) and after 200 μM Tolbutamide was superfused (n = 5). (Bi) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 5 μM NN414 for 3 hours, as it is dialysed with 0 ATP. (Bii) Corresponding families of currents (n = 5). (C) Pooled conductance densities at run-up, which were significantly reduced in cells exposed to 3 hours NN414 compared to NaOH vehicle control. Statistical significance was analysed in panel C by student’s unpaired t-test (* P < 0.05).
Figure 4.5 24 hour NN414 exposure results in a trend for attenuated glucose-sensing at 0.7 mM glucose.

(A) Representative perforated-patch current clamp recordings from a GT1-7 cell exposed to 500 μM NaOH for 24 hours. Superoxide of 2.5 mM glucose depolarised the cell membrane where as substitution with 0.7 mM glucose or addition of 5 μM NN414 induced hyperpolarisation. Pooled membrane potential data for 24 hours NaOH (n = 4). (B) Representative current clamp recordings from a GT1-7 cell exposed to 5 μM NN414 for 24 hours. Pooled membrane potential data for 24 hours NN414 (n = 8). For illustrative purposes, the change in membrane potential from 2.5 to 0.7 mM glucose is shown in panel C. Statistical significance was analysed by one-way ANOVA in panels A and B and student’s unpaired t-test in panel C.
Figure 4.6 24 hour NN414 exposure causes significantly attenuated glucose-sensing at 0.5 mM glucose.

(A) Representative perforated-patch current clamp recordings from a GT1-7 cell exposed to 500 μM NaOH for 24 hours. Superoxide of 2.5 mM glucose depolarised the cell membrane whereas substitution with 0.5 mM glucose or addition of 5 μM NN414 induced hyperpolarisation. Pooled membrane potential data for 24 hours NaOH (n = 4). (B) Representative current clamp recordings from a GT1-7 cell exposed to 5 μM NN414 for 24 hours. Under these conditions, the cells displayed a significantly attenuated hyperpolarisation to superoxide of 0.5 mM glucose and 5 μM NN414. Pooled membrane potential data for 24 hours NN414 (n = 6). For illustrative purposes, the change in membrane potential from 2.5 to 0.5 mM glucose is shown in panel C. Statistical significance was analysed by one-way ANOVA in panels A and B and student’s unpaired t-test in panel C (\( P < 0.05 \), \( P < 0.005 \), \( P < 0.001 \)).
Figure 4.7 3 hour NN414 exposure results in a trend for attenuated glucose-sensing at 0.7 mM glucose.

(A) Representative perforated-patch current clamp recordings from a GT1-7 cell exposed to 3 hours 500 μM NaOH. Superfusion of 2.5 mM glucose depolarised the cell membrane where as substitution with 0.7 mM glucose or addition of 5 μM NN414 induced hyperpolarisation. Pooled membrane potential data for 3 hours NaOH (n = 6). (B) Representative perforated-patch current clamp recordings from a GT1-7 cell exposed to 5 μM NN414 for 3 hours. Under these conditions, the cells displayed a slightly attenuated hyperpolarisation to superfusion of 0.7 mM glucose and 5 μM NN414. Pooled membrane potential data for 24 hours NN414 (n = 6). For illustrative purposes, the change in membrane potential from 2.5 to 0.7 mM glucose is shown in panel C. Statistical significance was analysed by one-way ANOVA in panels A and B and student’s unpaired t-test in panel C (** P < 0.001)
Figure 4.8 3 hour NN414 exposure causes significantly attenuated glucose-sensing at 0.5 mM glucose.

(A) Representative current clamp recordings from a GT1-7 cell exposed to 500 μM NaOH for 3 hours. 2.5 mM glucose depolarised the cell membrane whereas substitution with 0.5 mM glucose or addition of 5 μM NN414 induced hyperpolarisation. Pooled membrane potential data for 3 hours NaOH (n = 5). (B) Representative current clamp recordings from a GT1-7 cell exposed to 3 hours 5 μM NN414. Under these conditions, the cells displayed a significantly attenuated hyperpolarisation to superfusion of 0.5 mM glucose and 5 μM NN414. Pooled membrane potential data for 24 hours NN414 (n = 5). For illustrative purposes, the change in membrane potential from 2.5 to 0.5 mM glucose is shown in panel C. Statistical significance was analysed by one-way ANOVA in panels A and B and student's unpaired t-test in panel C (* P < 0.05, ** P < 0.005, *** P < 0.001).
Figure 4.9 Antecedent NN414 results in attenuated glucose-sensing at 0.7 mM glucose.

(A) Representative perforated-patch current clamp recordings from a GT1-7 cell exposed to 500 μM NaOH for 3 hours on day 1 before electrical activity was examined on day 2. Superfusion of 2.5 mM glucose depolarised the cell membrane where as substitution with 0.7 mM glucose or addition of 5 μM NN414 induced hyperpolarisation. Corresponding pooled membrane potential data (n = 6). (B) Representative current clamp recordings from a GT1-7 cell exposed to 5 μM NN414 for 3 hours on day 1 before electrical activity was examined on day 2. Under these conditions, the cells displayed an attenuated ability to hyperpolarise to superfusion of 0.7 mM glucose. Corresponding pooled membrane potential data (n = 7). For illustrative purposes, the change in membrane potential from 2.5 to 0.7 mM glucose is shown in panel C. Statistical significance was analysed by one-way ANOVA in panels A and B and student’s unpaired t-test in panel C (* P < 0.05, ** P < 0.001).
Figure 4.10 Antecedent NN414 results in attenuated glucose-sensing at 0.5 mM glucose.

(A) Representative perforated-patch current clamp recordings from a GT1-7 cell exposed to 500 μM NaOH for 3 hours on day 1 before electrical activity was examined on day 2. Superfusion of 2.5 mM glucose depolarised the cell membrane where as substitution with 0.5 mM glucose or addition of 5 μM NN414 induced hyperpolarisation. Corresponding pooled membrane potential data (n = 4).

(B) Representative perforated-patch current clamp recordings from a GT1-7 cell exposed to 5 μM NN414 for 3 hours on day 1 before electrical activity was examined on day 2. Under these conditions, the cells displayed an attenuated ability to hyperpolarise to superfusion of 0.7 mM glucose. Corresponding pooled membrane potential data (n = 6). For illustrative purposes, the change in membrane potential from 2.5 to 0.7 mM glucose is shown in panel C. Statistical significance was analysed by one-way ANOVA in panels A and B and student’s unpaired t-test in panel C (* P < 0.05, *** P < 0.001).
Figure 4.11 Antecedent NN414 exposure does not affect the availability of $K_{ATP}$ channel to activate maximally in response to dialysis of cell with 0 ATP.

(Ai) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 500 μM NaOH for 3 hours on day 1, before electrical activity was examined on day 2, as it is dialysed with 0 ATP. (Aii) Corresponding families of currents evoked by the voltage-clamp protocol stated in “Methods”. Whole-cell macroscopic currents were examined immediately after the cell membrane was ruptured (control), after the cell had been dialysed with 0 ATP (run-up) and after 200 μM Tolbutamide was superfused (n = 4). (Bi) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 5 μM NN414 3 hours on day 1, before electrical activity was examined on day 2, as it is dialysed with 0 ATP. (Bii) Corresponding families of currents (n = 6). (C) Pooled conductance densities at run-up. Statistical significance was analysed in panel C by student’s unpaired t-test.
Figure 4.12 24 hour Diazoxide exposure reduces availability of KATP channel to activate maximally in response to dialysis of cell with 0 ATP

(Ai) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 0.1% DMSO for 24 hours, as it is dialysed with 0 ATP. (Aii) Corresponding families of currents evoked by the voltage-clamp protocol stated in “Methods”. Whole-cell macroscopic currents were examined immediately after the cell membrane was ruptured (control) and after the cell had been dialysed with 0 ATP (run-up) (n = 5). (Bi) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 250 μM Diazoxide for 24 hours, as it is dialysed with 0 ATP. (Bii) Corresponding families of currents (n = 5). (C) Pooled conductance densities at run-up, which were significantly reduced in cells exposed to 3 hours Diazoxide compared to DMSO vehicle control. Statistical significance was analysed in panel C by student’s unpaired t-test (*** P < 0.001).
Figure 4.13 3 hour Diazoxide exposure reduces availability of KATP channel to activate maximally in response to dialysis of cell with 0 ATP.

(Ai) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 0.1% DMSO for 3 hours, as it is dialysed with 0 ATP. (Aii) Corresponding families of currents evoked by the voltage-clamp protocol stated in “Methods”. Whole-cell macroscopic currents were examined immediately after the cell membrane was ruptured (control) and after the cell had been dialysed with 0 ATP (run-up) (n = 6). (Bi) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 250 μM Diazoxide or 3 hours, as it is dialysed with 0 ATP. (Bii) Corresponding families of currents (n = 5). (C) Pooled conductance densities at run-up, which were significantly reduced in cells exposed to 3 hours Diazoxide compared to DMSO vehicle control. Statistical significance was analysed in panel C by student’s unpaired t-test (* P < 0.05).
Figure 4.14 High external [K+] prevents membrane potential reaching $E_K$ in response to acute NN414 activation

(A) Current clamp recording of a GT1-7 cell in 2.5 mM glucose NS containing 13.5 mM KCl. When 5 μM NN414 is superfused, the membrane potential does not hyperpolarise to the same degree as shown previously. (B) Pooled membrane potential data ($n = 3$). Statistical significance was analysed in panel B by student’s unpaired t-test.
Figure 4.15 24 hour NN414-associated reduced \( K_{\text{ATP}} \) is not a result of membrane hyperpolarisation

(Ai) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 500 \( \mu \text{M} \) NaOH, in 13.5 mM KCl, for 3 hours, as it is dialysed with 0 ATP. (Aii) Corresponding families of currents evoked by the voltage-clamp protocol stated in “Methods”. Whole-cell macroscopic currents were examined immediately after the cell membrane was ruptured (control), after the cell had been dialysed with 0 ATP (Run-up) and after 200 \( \mu \text{M} \) Tolbutamide was superfused (n = 11). (Bi) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 5 \( \mu \text{M} \) NN414, in 13.5 mM KCl, for 3 hours, as it is dialysed with 0 ATP. (Bii) Corresponding families of currents (n = 12). (C) Pooled conductance densities at run-up, which were significantly reduced in cells exposed to NN414 compared to NaOH vehicle control. Statistical significance was analysed in panel C by student’s unpaired t-test (** \( P < 0.005 \)).
Figure 4.16 3 hour NN414-associated reduced $K_{\text{ATP}}$ is not a result of membrane hyperpolarisation

(Ai) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 500 μM NaOH, in 13.5 mM KCl, for 3 hours as it is dialysed with 0 ATP. (Aii) Corresponding families of currents evoked by the voltage-clamp protocol stated in “Methods”. Whole-cell macroscopic currents were examined immediately after the cell membrane was ruptured (control), after the cell had been dialysed with 0 ATP (Run-up) and after 200 μM Tolbutamide was superfused ($n = 11$). (Bi) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 5 μM NN414, in 13.5 mM KCl for 3 hours as it is dialysed with 0 ATP. (Bii) Corresponding families of currents ($n = 12$). (C) Pooled conductance densities at run-up, which were significantly reduced in cells exposed to NN414 compared to NaOH vehicle control. Statistical significance was analysed in panel C by student’s unpaired t-test (*** $P < 0.005$).
4.2.10 Continuous NN414 exposure does not alter glucose metabolism, in GT1-7 cells

Due to the observation that antecedent NN414 exposure results in an attenuated hyperpolarisation response to low glucose, in the absence of a reduction in $K_{\text{ATP}}$ channel conductance, glucose metabolism following continuous NN414 was investigated as alterations in cellular metabolism may go towards explaining the changes seen in this metabolically regulated channel. The NN414 treatment condition chosen at which to examine glucose metabolism was 24 hours continuous exposure. This length of agonist exposure was shown to produce the most robust attenuation in glucose sensing coupled with reduced $K_{\text{ATP}}$ channel activity, and therefore it was hypothesised that if any changes were going to be seen in glucose metabolism it would be with this approach.

Glucose uptake was examined using the 2-deoxy-d-$[^3\text{H}]$glucose (non-metabolisable analogue of glucose) uptake assay. Assays were performed within the time frame that glucose uptake is linear in GT1-7 cells (15/20 minutes), as determined previously. Different unlabeled concentrations of glucose were added to the assay buffer containing 2-deoxy-d-$[^3\text{H}]$glucose in order to set the rate of substrate uptake. Increasing the substrate pool with unlabelled glucose increases the competition for uptake with $[^3\text{H}]2DG$ for the transporter and therefore will decrease the radioactivity that is taken up into the cell. The hypothesis was that perhaps GT1-7 cells had increased glucose uptake following chronic exposure to NN414, resulting in increased metabolism and ATP production which would keep the channel closed. If this was true then for any given unlabelled glucose concentration it would produce a greater suppression of $[^3\text{H}]2DG$ uptake than then vehicle treated cells. However, no change in glucose uptake was observed following 24 hour NN414 incubation compared to vehicle controls at physiological brain glucose concentrations of 0, 0.1, 0.5 and 2.5 mM ($n = 4$; $6.04 \pm 0.55$ vs $6.44 \pm 0.54$; $5.32 \pm 0.32$ vs $5.75 \pm 0.28$; $3.8 \pm 0.33$ vs $3.84 \pm 0.23$; $1.83 \pm 0.16$ vs $1.74 \pm 0.19$ pmol/min/mg, respectively; Fig 4.17).
Next, glucose oxidation was examined using a radiolabeled glucose (\(^{14}\)C-glucose) oxidation assay. This assay involves the metabolism of \(^{14}\)C-glucose resulting in the production of \(^{14}\)CO\(_2\) which can be captured and taken as a measure of glucose oxidation. Glucose which has been incorporated into the cell through anabolic processes can also be investigated through measurement of \(^{14}\)C the cell lysate. The assay was performed over 4 hours at 37°C in assay buffer that contained \(^{14}\)C-glucose and a concentration of unlabelled glucose at which to set the rate of oxidation. The hypothesis behind this experiment was that, following continuous NN414 exposure, GT1-7 cells might be becoming more efficient at using lower levels of glucose which would produce more ATP to keep the K\(_\text{ATP}\) channel closed during low glucose exposure. However, no change in glucose oxidation was observed following 24 hour NN414 incubation compared to vehicle controls at physiological brain glucose concentrations 0.1, 0.5 and 2.5 mM (\(n = 3\); 5.29 ± 1.24 vs 5.19 ± 0.71; 17.92 ± 1.82 vs 17.93 ± 1.30; 39.38 ± 9.99 vs 35.60 ± 8.02 pmol/min/mg, respectively; Fig 4.18A). There was also no change in the glucose incorporation observed at 0.1, 0.5 and 2.5 mM glucose (\(n = 3\); 61.16 ± 16.61 vs 62.86 ± 11.69; 158.62 ± 39.65 vs 167.27 ± 38.79; 213.49 ± 34.27 vs 230.35 ± 51.18 pmol/min/mg, respectively; Fig 4.18B).

Despite there being no change in glucose oxidation between chronic NN414 and vehicle treated cells, it could be possible that GK activity is altered following continuous K\(_\text{ATP}\) agonism, which in turn increases the rate of glycolytic ATP production and inhibits the channel. Therefore, GK activity was examined using a fluorometric assay which indirectly measures GK activity via the appearance of β-NADPH from the conversion of glucose-6-phosphate (produced from the phosphorylation of glucose by GK) to 6-phospho-D-gluconate by glucose-6-phosphate dehydrogenase (Meakin et al., 2007). [This assay was kindly performed by Dr Craig Beall]. Figure 4.19 demonstrates that increasing glucose increases GK activity in a concentration dependant manner, displaying Michaelis-Menten kinetics,
reaching Vmax between 0.5 and 2.5 mM glucose for both continuous NN414 treated cells and vehicle controls (7.15 ± 0.48 vs 6.19 ± 0.34 mU/mg, respectively). There appeared to be a trend for increased hexokinase activity but overall there was no significant difference in activity following 24 hour exposure to NN414, compared to vehicle controls, at 0, 0.1, 0.25, 0.5, 1, 2.5 or 5.0 mM glucose (n = 4; 0.24 ± 0.91 vs 0.11 ± 0.07; 4.28 ± 0.48 vs 4.19 ± 0.39; 5.68 ± 0.65 vs 5.33 ± 0.41; 6.65 ± 0.83 vs 6.33 ± 0.60; 5.98 ± 1.32 vs 4.80 ± 0.97; 7.26 ± 0.85 vs 6.31 ± 0.45; 7.15 ± 0.90 vs 6.38 ± 0.46, respectively; Fig 4.19).

Due to no apparent change in glucose uptake, oxidation or rate of phosphorylation, following continuous NN414 exposure, mitochondrial function and real-time analysis of cellular respiration were then investigated using the Seahorse XF-24 extracellular flux analyser (Seahorse Bioscience). GT1-7 cells were seeded at 30,000 cells/well in a 24-well culture microplate and incubated with 5 μM NN414, or 500 μM NaOH for vehicle controls, for 24 hours in 2.5 mM glucose plating media. Cells were placed in to serum free seahorse media 1 hour before the assay, which contained 2.5 mm glucose as the only fuel source. No NN414 was present during the assay in order to replicate the conditions during electrophysiological investigations. Cells were then placed in the XF-24 and OCR was measured in real-time. Inhibitors of specific electron transport chain complexes were injected in to some wells in order to assess the amount of oxygen contributing to certain mitochondrial processes. Wells that received no drugs were lysed in order to normalise basal OCR to protein. The OCR of GT1-7 cells exposed to NN414 for 24 hours was unaltered from vehicle control cells (n = 5-6; 10.31 ± 0.58 vs 10.37 ± 1.65 pmoles O₂/min/μg, respectively; Fig 4.20)

Next, activity of mitochondrial ATP generation was examined by applying the Oligomycin to inhibit ATP-synthase. The reduction in OCR (which has been normalised to 100% at baseline as wells injected with drug were not normalised to protein in case cell death occurred with addition of the drug) after Oligomycin is applied represents the percentage oxygen
contributing towards mitochondrial ATP generation. There was no change in the suppression of OCR (and hence the activity) of ATP-synthase following continuous NN414 exposure compared to vehicle controls (n = 5-7; 47.47 ± 2.26 vs 51.32 ± 1.54 % change in OCR, respectively; Fig 4.21B). Once the Oligomycin response had stabilised, Rotenone and Antimycin A were co-applied to the wells in order to inhibit mitochondrial complexes I and III, respectively, which will inhibit the functioning of the electron transport chain. Complexes I and III are responsible for facilitating the extrusion of protons from the mitochondria and generating a protonmotive force across the inner membrane of the organelle, which is then dissipated by re-entry of protons back into the matrix via ATP-producing (ATP-synthase) and non ATP producing processes (Divakaruni and Brand). Because ATP-synthase is already inhibited at the point of Rotenone and Antimycin A addition, the change in OCR observed is indicative of non-ATP producing mitochondrial leak. There was no change in the mitochondrial leak (analysed by the change in % suppression of OCR) in GT1-7 cells incubated with NN414 for 24 hours compared to vehicle controls (n = 6-7; 35.09 ± 2.79 vs 30.71 ± 2.79 % change in OCR, respectively; Fig 4.21B).

The spare respiratory capacity of the GT1-7 cells was examined using the mitochondrial uncoupler (FCCP), in order to determine if the mitochondria were in a less uncoupled state following chronic NN414. As mentioned previously, reduced uncoupling of the mitochondria may increase cytosolic ATP concentration which could inhibit the K\textsubscript{ATP} channel. FCCP was titrated into the wells in increments of 0.2 μM (in order to establish a dose that would uncouple the mitochondria without causing cell death) with a maximum final concentration being 0.6 μM (Fig 4.22A). There was no significant difference observed in the spare respiratory capacity of GT1-7 cells incubated with 5 μM NN414 for 24 hours compared to vehicle controls, when 0.6 μM FCCP was applied (n = 4; 115.70 ± 24.55 vs 95.80 ± 5.62, respectively; Fig 4.22B).
These results indicate that continuous KATP channel agonism does not feedback to alter cellular glucose respiration or mitochondrial function in GT1-7 cells.

4.2.11 Continuous NN1414 exposure does not alter the ATP/ADP ratio, in GT1-7 cells.

In a final attempt to investigate if continuous $K_{\text{ATP}}$ agonism affected cellular metabolism in GT1-7 cells, ATP/ADP ratio was examined using a bioluminescence assay (Abcam). GT1-7 cells were seeded at 1000 cells/well in a 96-well plate with 5 μM NN414, or 500 μM NaOH as vehicle controls, in 2.5 mM glucose plating media for 24 hours before the assay. Fresh media, containing NN414 or vehicle, was applied to the wells first thing on the day of the assay. Cells were placed into 2.5 mM glucose NS for 30 minutes before being lysed and assayed. However, following 24 hours NN414 incubation there was no apparent change in the ATP/ADP ratio compared to vehicle controls ($n = 9$; 0.62 ± 0.02 vs 0.64 ± 0.02, respectively; Fig 4.23).

As there appeared to be no adaptations in glucose metabolism which would explain the attenuated glucose-sensing following chronic NN414 exposure, the focus of the study was brought back to the $K_{\text{ATP}}$ channel. As the deleterious effects of continuous NN414 were not due to membrane potential hyperpolarisation, the effect of $K_{\text{ATP}}$ channel opening were investigated by co-applying Tolbutamide in order to keep the channel closed in the presence of NN414.
Figure 4.17 Glucose uptake is not altered by chronic NN414 exposure.

2DG uptake was measured in GT1-7 cells following a 20 minute incubation in a range of unlabelled glucose concentrations. There was no change in 2DG uptake between cells exposed to NaOH (n = 4) or NN414 (n = 4) in replicates of 10 for 24 hours.
Figure 4.18 Glucose oxidation is not altered by chronic NN414 exposure.

Glucose utilisation was measured in GT1-7 cells following a 4 hour incubation in $^{14}$C-Glucose and a range of unlabelled glucose concentrations. There was no change in glucose oxidation (A) or Incorporation (B) between cells exposed to NaOH (n = 3) or NN414 (n = 3) for 24 hours.
Figure 4.19 Hexokinase activity in GT1-7 cells was unaltered following chronic NN414 exposure.

Hexokinase activity was measured in GT1-7 cells at 0, 0.1, 0.25, 0.50, 2.50 and 5 mM glucose concentrations. Hexokinase activity was unaltered following 24 hour exposure to 500 μM NaOH (n = 4) or 5 μM NN414 (n = 4).

Data courtesy of Dr Craig Beatt.
Figure 4.20 Cellular oxygen consumption is unaltered following chronic NN414 exposure, in GT1-7 cells.

Real-time analysis of the oxygen consumption rate (OCR) was performed, using the XF-24 Seahorse Extracellular Flux Analyser, on intact GT1-7 cells. 2.5 mM glucose was the only fuel substrate present during this test. Therefore, there was no change in whole cell glucose oxidation between cells exposed to 5 μM NN414 (n = 6) or 500 μM NaOH vehicle control (n = 5) for 24 hours. Statistical significance was analysed using unpaired student's t-test.
Figure 4.21 Efficiency of ATP synthesis was unaltered following chronic NN414 exposure, in GT1-7 cells

Real-time analysis of the oxygen consumption rate (OCR) was performed, using the XF-24 Seahorse Extracellular Flux Analyser, on intact GT1-7 cells exposed to 24 hours 5 µM NN414 or 500 µM NaOH. 2.5 mM glucose was the only fuel substrate present during this test. The OCR has been normalised to 100% before mitochondrial inhibitors were applied in order to examine the amount of oxygen contributing to specific mitochondrial processes. (A) Display of entire assay protocol and results. ATP-synthase was inhibited by addition of Oligomycin, followed by Rotenone and Antimycin A to inhibit processes which facilitate proton leak across the mitochondrial membrane (n = 7). For illustrative purposes, the % change in OCR from before and after Oligomycin and Rotenone plus Antimycin A were applied are displayed in panels B and C, respectively. The % OCR left after Rotenone plus Antimycin A was applied is displayed in panel D and represents non-mitochondrial oxygen consumption. Statistical significance was analysed using unpaired student's t-test.
Figure 4.22: Spare respiratory capacity was unaltered following chronic NN414 exposure, in GT1-7 cells.

Real-time analysis of the oxygen consumption rate (OCR) was performed, using the XF-24 Seahorse Extracellular Flux Analyst, on intact GT1-7 cells exposed to 24 hours 5 μM NN414 or 500 μM NaOH. 2.5 mM glucose was the only fuel substrate present during this test. The OCR has been normalised to 100% before mitochondrial inhibitors were applied in order to examine the amount of oxygen contributing to specific mitochondrial processes. In panel A, cells were exposed to increasing concentrations of FCCP in order to uncouple the mitochondria (n = 4). For illustrative purposes, the % change in OCR from before and after 0.6 μM FCCP was applied is displayed in panel B. Statistical significance was analysed using unpaired student’s t-tests.
Figure 4.23 ATP/ADP ratio was unaltered following chronic NN414 exposure, in GT1-7 cells.

ATP/ADP ratios were measured using a luminescence assay and carried out at 2.5 mM glucose. There was no difference in the ATP/ADP ratio following 24 hour exposure to 500 μM NaOH (n = 9) or 5 μM NN414 (n = 9).

Data courtesy of Dr Craig Beall.
4.2.12 Tolbutamide prevents chronic NN414-associated attenuated $K_{ATP}$ channel conductance.

To ensure that Tolbutamide could indeed block the actions of NN414, perforated patch-clamp recordings were performed on naive GT1-7 cells. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). 25-40 μg/ml amphotericin B was also present in the pipette and no current was injected during the recordings. A stable recording was obtained in 2.5 mM glucose before 200 μM tolbutamide was superfused. The membrane potential depolarised from $-54.5 \pm 1.5$ mV at 2.5 mM glucose to $-46.5 \pm 1.5$ mV when 200 μM tolbutamide was superfused (Fig 4.24A,B) (n = 2). After perfusion had stopped and a stable membrane potential was obtained for ~5 minutes, 5 μM NN414 was co-applied with 200 μM tolbutamide. The membrane potential depolarised slightly to $-49.5 \pm 0.5$ mV (Fig 4.24A,B) (n = 2). Therefore, co-application of tolbutamide with NN414 was used to block $K_{ATP}$ channel activation during chronic exposure to the agonist.

Next, GT1-7 cells were treated in culture with 200 μM tolbutamide (which was applied 1 hour before NN414) and 5 μM NN414 for 24 hours. The vehicle controls were incubated with 0.1% DMSO (applied first) and 500 μM NaOH. Whole-cell patch-clamp recordings were performed in order to examine the effect of blocking NN414 action on the $K_{ATP}$ channel, chronically, on the $K_{ATP}$ channel conductance. The extracellular bath solution contained 2.5 mM glucose NS and the electrode solution contained 140 mM KCl with 0 mM ATP (pipette solution A). Currents were evoked from the voltage clamp protocol, mentioned previously, when the cell membrane was first ruptured and at run-up. The maximum conductance density at run-up in cells treated for with 24 hours with tolbutamide and NN414 was not attenuated and comparable to vehicle controls (n = 5-7; $2.49 \pm 0.38$ vs $2.36 \pm 0.56$ pS/pF respectively; Fig 4.25).
Figure 4.24 Acute Tolbutamide application prevents NN414 activation of KATP channel

(A) Representative perforated-patch current clamp recording of a GT1-7 cell in 2.5 mM glucose NS. 200 μM Tolbutamide was superfused before co-application with 5 μM NN414, which prevented NN414-associated membrane hyperpolarisation. (B) Pooled membrane potential data (n = 2).
Figure 4.25. Tolbutamide prevents chronic NN414-associated attenuation of K\textsubscript{ATP} channel conductance.

(Ai) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 500 μM NaOH and 0.1% DMSO for 24 hours, as it is dialysed with 0 ATP. (Aii) Corresponding families of currents evoked by the voltage-clamp protocol stated in “Methods”. Whole-cell macroscopic currents were examined immediately after the cell membrane was ruptured (control) and after the cell had been dialysed with 0 ATP (run-up) (n = 7). (Bi) Representative whole-cell current clamp recording of a GT1-7 cell exposed to 5 μM NN414 and 200 μM Tolbutamide for 24 hours, as it is dialysed with 0 ATP. (Bii) Corresponding families of currents (n = 5). (C) Pooled conductance densities at run-up. Statistical significance was analysed in panel C by student’s unpaired t-test.
Although the exact binding domain of NN414 has yet to be identified, both Tolbutamide and NN414 bind to SUR1 of the $\text{K}_{\text{ATP}}$ channel (Dabrowski et al., 2003, Ashfield et al., 1999). The above results suggest that Tolbutamide is either physically blocking NN414 from binding to the channel or NN414 is still binding but the inhibitory action of Tolbutamide is maintaining the channel in a closed state. Nevertheless, this study shows that chronic activation of the $\text{K}_{\text{ATP}}$ channel directly by NN414 is responsible for the consequential attenuated $\text{K}_{\text{ATP}}$ channel conductance.

4.2.13 Continuous NN414 exposure reduces $\text{K}_{\text{ATP}}$ channel sensitivity to activation by MgADP and acute NN414.

Although the availability of the $\text{K}_{\text{ATP}}$ channel to activate maximally is attenuated following continuous NN414 exposure, this did not alter the mRNA expression of the channel subunits Kir6.2 ($\text{Kcnj11}$) and Sur1 ($\text{Abcc8}$) or the level of fully assembled $\text{K}_{\text{ATP}}$ channel at the plasma membrane (data not shown but published here (Beall et al., 2013)). Therefore single channel recordings were performed on isolated inside-out membrane patch which was isolated from GT1-7 cells exposed to 5 μM NN414 or 500 μM NaOH vehicle control, for 24 hours. Recordings were made in symmetrical 140 mM KCl, with the bath containing External Solution B and the electrode solution containing Pipette Solution B. +50 mV was injected during the recording in order to clamp the membrane potential at -50 mV. A stable recording was obtained for at least 2 minutes following patch excision in the presence of 0 nucleotides (control). Channel activity was analysed by averaging the 90-120 seconds of the recording just before MgADP, NN414 or trypsin was added or when the recording was stable following perfusion. Note that channel activity (inward current) is represented as downward deflections. N,P(o) was calculated, as previously described, where N = the number of functional channels in the patch and P(o) = the open state probability which was determined by measuring the total time spent at each unitary current level and expressed as a proportion of the total time of
the segment of recording that was analysed. The N.P(o) control value in cells exposed to 24 hours NN414 (Fig 4.26B) was significantly reduced compared to vehicle treated cells (Fig 4.26A) \((n = 11-15; 0.00 \pm 0.00 \text{ vs } 0.012 \pm 0.005; P < 0.005; \text{Fig 4.26C})\). This indicates that the intrinsic activity of the \(K_{\text{ATP}}\) channel is reduced following continuous NN414 treatment. After 2 minutes of stable recording from patch isolation, 100 \(\mu\text{M}\) MgATP was perfused into the bath in order to inhibit single channel events. This was only applied to vehicle treated cells, as channel activity was only observed in this treatment group, where the N.P(o) was reduced to \(0.008 \pm 0.004\) \((n = 10; \text{Fig 4.26C})\). However, MgATP has been shown to activate the \(K_{\text{ATP}}\) channel and upon reflection MgATP should also have been applied to NN414 treated cells in order to examine if channel activity could be stimulated. Although at a concentration of 100 \(\mu\text{M}\), MgATP is predominantly inhibitory (Gribble et al., 1998).

Once MgATP perfusion had stopped and the trace had stabilised, a recording was made for at least 2 minutes before 200 \(\mu\text{M}\) MgADP was applied (in the absence of ATP). There was a reduction in the sensitivity of cells exposed to NN414 for 24 hours to activation by MgADP compared to vehicle treated cells \((n = 4; 0.00 \pm 0.00 \text{ vs } 0.098 \pm 0.067 \text{ respectively; Fig 4.26C})\).

Next, 100 \(\mu\text{M}\) NN414 was applied in order to evaluate if \(K_{\text{ATP}}\) channels were still sensitive to acute application of this agonist when it was applied to the intracellular domain of the channel, following chronic exposure. 100 \(\mu\text{M}\) was the concentration chosen to test with as this has been shown previously to elicit \(K_{\text{ATP}}\) channel activation when co-applied with 100 \(\mu\text{M}\) MgATP to the intracellular domain of the channel (Dabrowski et al., 2003). There was a significant reduction in the activation of \(K_{\text{ATP}}\) channels from cells that had been incubated with NN414 for 24 hours in response to acute NN414 application (Fig 4.27B), compared to vehicle treated cells (Fig 4.27A) \((n = 5; 0.00 \pm 0.00 \text{ vs } 0.095 \pm 0.037 \text{ respectively; } P<0.05; \text{Fig 4.27C})\).
Due to an almost complete loss of single channel events observed in response to control conditions (0 nucleotides), MgADP and NN414 following chronic NN414 exposure, the proteolytic enzyme Trypsin was applied to the excised patches in an attempt ‘awaken’ channel activity. Trypsin has been shown to activate $K_{ATP}$ channels, even once run down, resulting in an increased N.P(o) without affecting the single channel conductance, ionic selectivity or rectification of the channel (Lee et al., 1994). The mechanism through which trypsin activates $K_{ATP}$ channels appears to involve cleavage or conformational change of SUR1 as the EC$_{50}$ of sulphonylurea inhibition is increased following Trypsin application to the intracellular domain of the channel, along with a reduction in radiolabelled sulphonylurea binding (Lee et al., 1994). 100 µg/ml Trypsin application to the intracellular side of the $K_{ATP}$ channel greatly increased activity in inside-out patches from both chronic NN414 (Fig 4.28B) and vehicle treated cells (Fig 4.28A) ($n = 3-8$; $0.265 \pm 0.161$ vs $0.318 \pm 0.199$ respectively; Fig 4.28C). This result confirms that following chronic NN414 exposure, functional KATP channels (Kir6.2/SUR1) are still present at the plasma membrane but inactive possibly due to NN414 inducing a conformational change which results in a stable inactive state. It is unlikely that the inhibition is due to the NN414 molecules being permanently bound to the channel, preventing further binding and activation by NN414 and MgADP, as the acute application of NN414 to naïve cells was readily reversible (Fig 4.2).
Figure 4.26 24 hour NN414 exposure reduces $K_{\text{ATP}}$ channel sensitivity to activation by MgADP.

(A) Single channel recordings from an inside-out membrane patch isolated from a GT1-7 cell exposed to 24 hours 500 μM NaOH. Single channel currents were recorded in symmetrical 140 mM KCl at a membrane potential of -50 mV. The top trace displays channel activity directly after the patch is pulled, the middle trace shows that channel activity is markedly reduced when 100 μM ATP is applied to the intracellular side of the patch, and the bottom trace demonstrates that channel activity is stimulated by applying 200 μM ADP to the intracellular side of the patch ($n = 4-11$). (B) Single channel recordings from an inside-out membrane patch isolated from a GT1-7 cell exposed to 24 hours 5 μM NN414. The top trace displays reduced channel activity directly after the patch is pulled and the bottom trace demonstrates that there is an attenuated ability to stimulate channel activity when 200 μM ADP to the intracellular side of the patch ($n = 4-15$) 100 μM ATP was not applied to cells following chronic NN414 treatment as there was no channel activity, when the patch was first isolated, to inhibit. (C) Pooled NP% data. Statistical significance was analysed by student's unpaired t-test ($^* P < 0.005$).
Figure 4.27 24 hour NN414 exposure reduces $K_{ATP}$ channel sensitivity to activation by acute application of NN414.

(A) Single channel recordings from an inside-out membrane patch isolated from a GT1-7 cell exposed to 24 hours 500 µM NaOH. Single channel currents were recorded in symmetrical 140 mM KCl at a membrane potential of -50 mV. Channel openings are indicated by downward deflections. The top trace displays channel activity directly after the patch is pulled, the middle trace shows that channel activity is markedly reduced when 100 µM ATP is applied to the intracellular side of the patch, the bottom trace demonstrates that channel activity is stimulated by co-applying 100 µM ATP and 100 µM NN414 to the intracellular side of the patch ($n = 5-11$). (B) Single channel recordings from an inside-out membrane patch isolated from a GT1-7 cell exposed to 24 hours 5 µM NN414. The top trace displays reduced channel activity directly after the patch is pulled and the bottom trace demonstrates that there is an attenuated ability to stimulate channel activity when 100 µM ATP and 100 µM NN414 is applied to the intracellular side of the patch ($n = 5-15$). 100 µM ATP was not applied to cells following chronic NN414 treatment as there was no channel activity when the patch was first isolated, to inhibit. (C) Pooled NP(0) data. Statistical significance was analysed by student’s unpaired t-test (* $P < 0.05$, ** $P < 0.005$).
Figure 4.28 KATP channels silenced by 24 hour NN414 exposure are still present at the plasma membrane.

(A) Single channel recordings from an inside-out membrane patch isolated from a GT1-7 cell exposed to 24 hours 500 μM NaOH. Single channel currents were recorded in symmetrical 140 mM KCl at a membrane potential of -50 mV. Channel openings are indicated by downward deflections. The top trace displays channel activity directly after the patch is pulled, the middle trace shows that channel activity is markedly reduced when 100 μM ATP is applied to the intracellular side of the patch, the bottom trace demonstrates that channel activity is stimulated by applying 100 μg/ml Trypsin to the intracellular side of the patch (n = 3-11). (B) Single channel recordings from an inside-out membrane patch isolated from a GT1-7 cell exposed to 24 hours 5 μM NN414. The top trace displays reduced channel activity directly after the patch is pulled and the bottom trace demonstrates that activity can be restored when 100 μg/ml Trypsin is applied to the intracellular side of the patch (n = 8-15). 100 μM ATP was not applied to cells following chronic NN414 treatment as there was no channel activity, when the patch was first isolated, to inhibit. (C) Pooled NP(o) data. Statistical significance was analysed by student’s unpaired t-test (** P<0.005).


4.3 Discussion

Contrary to the original hypothesis that continuous VMH infusion of the SUR1-selective KCO, NN414, during antecedent hypoglycaemia would protect against the development of defective CRR, this actually led to worsening of the CRR, in rats (Fig 4.1). GIR rates were higher following chronic NN414 delivery in conjunction with RH, compared to RH alone. This coincided with a suppression of adrenaline release during hyperinsulinaemic-hypoglycaemic clamps. However, rats that received continuous NN414 for 7 days followed by withdrawal of the drug for 24 hours, prior to hyperinsulinaemic-hypoglycaemic clamp studies, displayed lower GIR rates compared to animals that had been subjected to RH alone. This result was accompanied by a partial restoration of the adrenaline response, despite RH on days 5-7. These results suggest that chronic activation of K\textsubscript{ATP} during antecedent hypoglycemia may be partially protective against the development of defective CRR, following removal of NN414 for 24 hours.

Fully functioning K\textsubscript{ATP} channels are required for the glucose-sensing properties of GE VMH neurons, which have a role in maintaining whole body glucose homeostasis, and so it was hypothesised that chronic NN414 infusion into the hypothalamus may be preventing channel activation during hypoglycaemia (Miki et al., 2001, Evans et al., 2004). Due to the lack of availability of reporter mouse models to identify pure glucose-sensing populations of neurons, the mouse hypothalamic GE GT1-7 cell line was utilised in order to elucidate the molecular mechanisms of chronic hypothalamic K\textsubscript{ATP} channel agonism. The chronic NN414 in vivo study was replicated in vitro, and NN414 was applied to GT1-7 cells for 8 days (Fig 4.3). A subset of cells was exposed to continuous NN414 for 7 days before the drug was washed out, by placing cells into drug free media. 8 days of NN414 exposure resulted in a reduction in the availability of the K\textsubscript{ATP} channel to open maximally in response to 0 ATP, which was concluded through the examination of whole-cell currents following dialysis of
intracellular ATP. As observed in the in vivo study, the deleterious effects of continuous NN414 exposure were reversible. Cells exposed to 7 days of NN414 followed by a 24 hour washout period of the drug displayed maximal whole-cell macroscopic currents that were comparable to vehicle controls. In an effort to elucidate how quickly NN414 was able to inhibit the $K_{\text{ATP}}$ channel, conductance was checked every 24 hours over the 8 days, again using whole-cell current clamp electrophysiology. Attenuated $K_{\text{ATP}}$ channel conductance presented as quickly as 24 hours and so the incubation period was shortened to 3 hours, which also produced a significant reduction in the maximal conductance of the channel (Fig 4.4). These results demonstrate that $K_{\text{ATP}}$ channel inactivation occurs rapidly in response to continuous agonism, which persists in the presence of the drug, and is reversible when NN414 is withdrawn.

Due to the principal role of the $K_{\text{ATP}}$ channel to couple intracellular metabolism to the electrical activity of the plasma membrane, the glucose-sensing ability of GT1-7 cells exposed continuously to NN414 was examined using perforated-patch clamp electrophysiology. This allowed continuous monitoring of the membrane potential whilst maintaining intracellular metabolism. Chronic exposure of GT1-7 cells to NN414 (24 and 3 hours) resulted in an attenuated hyperpolarisation response to both 0.5 and 0.7 mM glucose (Figs 4.5, 4.6, 4.7 and 4.8). Such an effect, in vivo, could account for the suppression of the CRR to hypoglycaemia. Although terminating NN414 administration in the animal studies restored the CRR to hypoglycaemia and the maximal $K_{\text{ATP}}$ channel conductance in GT1-7 cells after 24 hours, it did not recover the attenuated glucose-sensing associated with chronic NN414 exposure in the cells (Figs 4.9 and 4.10). This may be due to the inhibitory effect of chronic NN414 on the $K_{\text{ATP}}$ channel being partially dependent on the presence of intracellular ATP and/or ADP which is dialysed out of the cell in whole-cell configuration. The discrepancies between the washout effects in the GT1-7 cells and the animals may be due to
better removal of NN414 from the body during washout compared to the cells due to enzymatic degradation. Alternatively, glucose-sensing neurons in the animals may be more sensitised to detecting hypoglycaemia which was insulin-induced \textit{in vivo}, as this may facilitate GLUT4 translocation to the membrane of GE neurons. Finally, the degree of hypoglycaemia in the animals may have been greater than the 0.5 mM glucose used to test the response of the GT1-7 cells, which may have only displayed an attenuated response at submaximal $K_{\text{ATP}}$ channel activating concentrations of glucose and not at near maximal channel activating concentrations, such as 0.1 mM (Beall et al., 2012). Nevertheless, as chronic NN414 appeared to cause defective glucose-sensing in GT1-7 cells, in the absence of a reduced $K_{\text{ATP}}$ conductance following the washout period, adaptations in glucose metabolism were investigated to try and determine if this could be the reason for attenuated glucose-sensing. Glucose metabolism was examined following 24 hours of continuous NN414 exposure, rather than following the washout period as it was hypothesised that any changes would be most prominent when both the glucose-sensing and the channel conductance were reduced. Had changes in metabolism been observed under these conditions, metabolism would have also been investigated following NN414 washout. No significant changes were observed in glucose uptake and oxidation, cellular OCR, mitochondrial efficiency or intracellular ATP:ADP ratio following 24 hour NN414 exposure (Figs 4.17, 4.18, 4.19, 4.20, 4.21, 4.22, 4.23). There appeared to be a slight trend for increased hexokinase activity, which theoretically should increase cytosolic ATP:ADP ratio through glycolytic flux resulting in inhibition the $K_{\text{ATP}}$ channel (Dunn-Meynell et al., 2002) (Fig 4.19). As this difference was not significant and, coupled with no change in glucose uptake or OCR, it begs the question if there is any functional impact of a slight increase in hexokinase activity here?

Due to the fact that chronic NN414 did not appear to alter glucose metabolism in GT1-7 cells, the focus of the study was brought back to the function of the $K_{\text{ATP}}$ channel in order to
explain the attenuated glucose-sensing along with the reduced channel conductance. In order to determine if other KCOs were capable of producing similar effects to NN414 when given chronically, Diazoxide was applied to cells for 3 and 24 hour hours (Figs 4.12 and 4.13). As observed with NN414, Diazoxide also reduced the availability of the $K_{\text{ATP}}$ channel to open maximally after 3 hours continuous application. Although the impact of this on the glucose-sensing ability of the cells was not tested, nor was the reversibility examined, these results suggest that NN414 and Diazoxide are producing their chronic effects through similar mechanisms. Indeed, both of these KCOs require the presence of intracellular MgATP, or MgADP in the case of Diazoxide, which suggests that hydrolysis of MgATP at NBD1 of SUR1 is required for the action of NN414 (Gribble et al., 1997, Larsson et al., 1993, Shyng et al., 1997, Dabrowski et al., 2003, Kozlowski and Ashford, 1992). Although the exact binding domain of NN414 has not been identified, evidence in the literature strongly suggests that the site of action is on SUR1 as currents are not evoked when Kir6.2 is expressed in the absence of SUR1 (Kir6.2ΔC36) or in Kir6.2/SUR2A/B channels (Dabrowski et al., 2003). In the case of Diazoxide, activation of the channel requires hydrolysis of MgATP at NBD1. Mutation of a lysine residue within the Walker A motif of NBD1 (K179A) resulted in a lack of activity of recombinant Kir6.2/SUR1 channels in response to Diazoxide in the presence of MgATP and inhibition in the presence of MgADP. However, mutation in the corresponding lysine residue of the Walker A motif in NBD2 (K1384M) still enabled channel activation in response to Diazoxide in the presence of MgATP, which was comparable to the activation achieved in wild type Kir6.2/SUR1 channels. A slight increase in current could also be evoked in response to Diazoxide in the presence of MgADP, in K1384M mutant channels, although overall activity was still significantly reduced compared to wild type channels. When the lysine mutations were present in both NBDs, no channel activation was observed in response to Diazoxide in the presence of MgATP or MgADP (Gribble et al., 1997). This suggests that
the normal nucleotide sensitivity of both NBDs is required for Diazoxide action, although
NBD1 rather than NBD2 may be more important for MgATP hydrolysis. This was confirmed
in studies from the same group, again using transgenic recombinant Kir6.2/SUR1 channels.
The fact that ATP is a more potent inhibitor of \( K_{ATP} \) channels than MgATP suggests that
MgATP has both stimulatory and inhibitory effects (Gribble et al., 1998). An ATP inhibitory
site has been located to a glycine residue on Kir6.2 and mutation of this residue (R50G) leads
to increased \( K_{ATP} \) channel activity in response to MgATP. This result, coupled with the fact
that MgATP does not show produce a less potent inhibition than ATP when applied to \( K_{ATP} \)
channels that only express Kir6.2 (Kir6.2ΔC36) indicates that the stimulatory site for MgATP
is on SUR1. The same NBD lysine residue mutations used in the previous study were used to
test the ability of MgATP to activate Kir6.2/SUR1 channels containing the R50G mutation.
MgATP inhibited channels containing the R50G mutation plus either a K179A or K1384M
lysine mutation, to a similar degree as wild type channels exposed to ATP in the absence of
Mg\(^{2+}\) (Gribble et al., 1998). This suggests that the lysine residues in the Walker A motifs may
facilitate Mg\(^{2+}\) binding and that normal function of both of these residues is required for the
stimulatory action of MgATP. Aspartate residues in the Walker B motifs of NBD1 (D853N)
and NBD2 (D1505N) have also been implemented in the activation of \( K_{ATP} \) MgATP (Gribble
et al., 1998). The intrinsic together, these data suggest that changes in the binding and/or
action of Mg-nucleotides at SUR1 following chronic NN414 and Diazoxide exposure may be
facilitating the lack of channel conductance observed. Interestingly, the whole-cell
macroscopic of recombinant \( K_{ATP} \) channels expressing the K179A and/or K1384M lysine
mutations were reduced in the absence of intracellular nucleotides, similar to the result
produced by chronic NN414 and Diazoxide.

In an attempt to investigate if the effects of chronic NN414 were membrane potential
dependent, high extracellular K\(^+\) (13.5 mM KCl) was used to chemically clamp the
membrane potential at -51 mV in the presence of NN414 (Fig 4.14). Following 3 or 24 hours NN414 exposure, the availability of the $K_{\text{ATP}}$ channel to open maximally, in response to the removal of intracellular ATP during whole-cell voltage clamp, was still attenuated (Figs 4.115 and 4.16). Therefore it was concluded that the effects of chronic NN414 were not directed through a membrane potential dependent process and perhaps direct activation and/or binding of NN414 to the channel was the driving the mechanism. To address this question, Tolbutamide was applied to GT1-7 cells in the presence of NN414. Acutely, the inhibitory effects of Tolbutamide predominated over the stimulatory action of NN414 and prevented the membrane potential from hyperpolarising (Fig 4.24). 24 hours of NN414 plus Tolbutamide exposure prevented the reduction in whole-cell macroscopic currents observed with continuous NN414 exposure (Fig 4.25). However, as the binding site of NN414 has not been identified, it cannot be concluded whether the presence of Tolbutamide is blocking NN414 from binding to the channel or if NN414 is still binding but Tolbutamide is maintaining inhibition of the channel. The binding site of Tolbutamide has been located to transmembrane domains 14-16 at the C-terminus of SUR1, with mutation of a tyrosine residue (S1237Y) in this domain resulting in attenuated inhibition of Kir6.2/SUR1 in response to sulphonylureas (Ashfield et al., 1999). If Tolbutamide is preventing NN414 binding to the channel, either through a conformational change or competitive inhibition, then this would suggest that NN414 binds to a similar region of SUR1. However, radio-ligand binding studies in addition to mutagenesis of certain residues on SUR1 would have to be performed in order to determine this.

Finally, single channel activity was assessed following chronic NN414 exposure, in excised patches of membrane using inside-out patch clamp configuration. There was a significant reduction in the intrinsic activity of the $K_{\text{ATP}}$ channel (0 nucleotides) following chronic NN414 compared to channels from vehicle treated cells (Fig 4.26). 100 µM MgATP was
used to inhibit channel activity in control cells but was not applied to patches treated with chronic NN414, as there was no activity to inhibit. However, in hindsight this should have been done as MgATP not only inhibits \( K_{\text{ATP}} \) channels but also has a stimulatory component, as mentioned previously. MgATP is also capable of “refreshing” rundown \( K_{\text{ATP}} \) channels as demonstrated by Ohno-Shosaku et al. who showed that channel activity was greater following inhibition by MgATP compared to before MgATP was applied to the patch, in the presence of 0 nucleotides. The “refreshing” of channel activity was dependent on Mg\(^{2+}\) and hydrolysis of MgATP (Ohno-Shosaku et al., 1987). However, the site of action for MgATP-associated channel refreshment differs slightly from activation of the channel by MgATP, as mutations in the lysine residues of the Walker A motifs of NBD1 (K719A) and NBD2 (K1384M) did not prevent this effect (Gribble et al., 1997). If MgATP had refreshed \( K_{\text{ATP}} \) channels in the patches of chronic NN414 treated cells, it may have provided more information to suggest if there was disruption to MgATP binding/hydrolysis or Mg\(^{2+}\) binding. Indeed, there appeared to be an alteration in the binding or hydrolysis of 200 \( \mu \)M MgADP, as the channel activation was attenuated in the current study following chronic NN414 (Fig 4.26). However, this result did not quite reach significance. It would have been interesting to see if the channel activity could have been stimulated further following chronic NN414 if MgADP had been applied in the presence of MgATP. If channel activity had increased from just applying MgADP alone, this may have indicated that NN414 was disrupting nucleotide action at NBD2, as this is thought to be the site of action for MgADP but not MgATP (Gribble et al., 1997). Chronic NN414 also significantly attenuated the ability of acutely applied NN414, in the presence of MgATP, to stimulate \( K_{\text{ATP}} \) channels (Fig 4.27). This result correlates with the observation from experiments performed in the perforated patch-clamp configuration, that chronic NN414 attenuates the hyperpolarisation response to acute NN414 (Figs 4.6, 4.7 and 4.8). Again, this result implies that there could be disruption to the MgATP
binding/hydrolysis at SUR1 following chronic NN414. In a final attempt to activate $K_{\text{ATP}}$ channels, silenced by chronic NN414, trypsin was applied to the intracellular domain of the patch, as this proteolytic enzyme has been shown to potently activate run-down $K_{\text{ATP}}$ channels (Lee et al., 1994). Trypsin activated channels inhibited by chronic NN414 to the same degree as channels from vehicle treated cells, as determined by the N.P(0) value (Fig 4.28). This result indicates that chronic NN414 does not affect $K_{\text{ATP}}$ channel trafficking to the plasma membrane and that the channels are still functional. Trypsin prevents $[^3\text{H}]$-glibenclamide binding to SUR1, as do transgenic mutations which render the $K_{\text{ATP}}$ channel insensitive to Tolbutamide inhibition (S1237Y tyrosine mutation and replacing SUR1 transmembrane domains 13-16 with those from SUR2A), and reduces Tolbutamide inhibition of Kir6.2/SUR1 (Lee et al., 1994, Ashfield et al., 1999). Taken together, these results indicate that trypsin is cleaving off the binding domain of Tolbutamide from SUR1 which may also be the same site that NN414 is binding to. Therefore, if NN414 is still bound to that site following chronic exposure or if that is where a conformational change in the channel has occurred then trypsin may be cleaving that part of the channel off and alleviating inhibition of the channel.

In summary, chronic NN414 attenuates the ability of GT1-7 cells to hyperpolarise to low glucose and acutely applied NN414. This coincides with reduced availability of the channel to open maximally, in response to dialysis of intracellular ATP, and attenuated single channel activation to MgADP and NN414. However, this is not due to a reduction in the number of $K_{\text{ATP}}$ channels present in the plasma membrane as mRNA expression of SUR1 and Kir6.2 at the plasma membrane was unchanged following chronic NN414 and the N.P(0) value obtained from trypsin application to the intracellular domain of the channel was comparable to that calculated from control cells (Beall et al., 2013). The chronic NN414-associated reduction in $K_{\text{ATP}}$ channel conductance density was reversible after 24 hours, as was the
ability of acute NN414 to hyperpolarise the cell membrane. However, the hyperpolarisation response to 0.5 mM glucose was still blunted following drug washout. As stated above, this could be due to disruption of MgATP action at NBD1 of SUR1. Na-azide reduces intracellular ATP content through inhibition of the mitochondrial electron transport chain and has been shown to hyperpolarise intact oocytes expressing recombinant Kir6.2/SUR1 channels (Gribble et al., 1997). However, mutation of the lysine residue in NBD1 (K719A), but not NBD2 (K1384M), attenuates the activation of the $K_{\text{ATP}}$ channel to this type on metabolic-dependent process. Therefore, there may still be some form of modification at NBD1, which confers metabolic-dependent activation of the channel, that is preventing the membrane potential from hyperpolarising to a submaximal $K_{\text{ATP}}$ channel metabolism-dependent stimulation, even when NN414 has been washed out.

As mentioned previously, ADP produces differing effects on $K_{\text{ATP}}$ channel activity in the form of stimulation when it is bound at the NBDs of SUR1 in the presence of Mg$^{2+}$ and inhibition when bound to Kir6.2 in the absence of Mg$^{2+}$ (Shyng et al., 1997, Proks et al., 2010). Therefore it could be hypothesised that, following continuous exposure, NN414 may be blocking the MgADP binding site on SUR1 and so during low glucose exposure, when metabolism falls, MgADP can only act through the inhibitory site on Kir6.2, preventing hyperpolarisation of the membrane potential.
Chapter 5

Final Conclusions
5.1 Final conclusions

It is well established that the brain is involved in regulating whole-body glucose homeostasis, including the CRR to hypoglycaemia (Borg et al., 1997, Borg et al., 1994, Borg et al., 1995). A body of evidence now exists within the literature to support the idea that adaptations occur within the brain following RH, leading to defective CRR (Alquier et al., 2007, Fioramonti et al., 2013, Herzog et al., 2008, Kang et al., 2008, Song and Routh, 2006). Glucose-sensing neurons, astrocytes and tanycytes have all been implicated in modulating the hormonal and neuronal responses to hypoglycaemia. Changes in the normal response of these cells to fluctuations in ambient glucose levels have been demonstrated following RH. However, it is still unclear whether the adaptations that occur in glucose-sensing neurons following RH are cell autonomous or if they are influenced by glial cells, through processes such as the lactate-shuttle hypothesis, or signals from the periphery. One of the aims of the current study was to determine if changes occur in an in vitro model of a homogenous population of GE neurons in response to RH, in isolation of astrocytic influences. Utilising the GT1-7 hypothalamic mouse neuronal cell line as an in vitro model of GE neurons, the current study has demonstrated that recurrent low glucose exposure reprograms intracellular metabolism towards a “hypometabolic state”.

As the $K_{ATP}$ channel is inhibited by a rise in intracellular ATP, the most popular hypothesis in the literature is that metabolic flux is enhanced following RH through increased GK activity, glucose uptake or lactate utilisation from astrocytes (Kang et al., 2008, Herzog et al., 2013, Simpson et al., 1999). However, in the GT1-7 model, where glucose was the only fuel source available during the electrophysiological and biochemical metabolic investigations, a reduction in glucose metabolism was observed. Theoretically this metabolic state should have activated the $K_{ATP}$ channel whereas this study has demonstrated that the opposite occurs. It is logical that following repeated glucose/energy deprivation that the bioenergetics of the cell
would be modified to pre-empt further energy deprivation so that the cell can continue to function. The energy that the neuron conserves may be directed towards neurotransmitter release. Perhaps the adaptations that occur in glucose-sensing neurons in response to RH are determined by the additional physiological roles of the neuronal population in question. For example, GT1-7 cells are derived from hypothalamic GnRH neurons which are required for successful reproductive function. (Roa, 2013). Therefore, the evolutionary purpose of glucose-sensing GnRH neurons becoming desensitised to hypoglycaemia may be so that these neurons remain active when fuel sources are scarce, which may help to preserve fertility and avoid a fall in the population.

Due to the fact that this study has utilised an in vitro GE neuron model, in isolation of inputs for astrocytes or the periphery, it cannot be established from this model how much of a role alternate fuels play in the development of attenuated glucose-sensing following RH. A co-culture system of GT1-7 cells and astrocytes may help elucidate the answer to this question. As astrocytes produce both lactate and ketone bodies, perhaps the preference of these neurons for glucose would switch in this system following RH (Blazquez et al., 1999, Chih and Roberts Jr, 2003). Indeed, GT1-7 cells are depolarised in response to lactate application, although ketone application (β-hydroxybutatrate) ceases action potential firing whilst having no effect on the membrane potential (data not shown) (Beall et al., 2012). Although an increase in VMH ketone concentration following high fat feeding correlates with suppressed food intake in rats, the reduction in activity of GT1-7 cells questions whether a ketogenic diet would be of therapeutic potential in the setting of RH (Le Foll et al., 2014)? A low carbohydrate ketogenic diet has proven successful in the treatment of epilepsy and the application of the ketones to rat or mouse neurons of the substantia nigra pars reticulate reduces action potential firing. This effect was absent in Kir6.2−/− mice suggesting that it is KATP mediated (Ma et al., 2007). The hypothesised mechanism for the reduction in neuronal
firing is thought to be due to the fact that ketones are metabolised in neurons through oxidative metabolism, therefore bypassing glycolysis and reducing anaerobic ATP production, which may be inhibiting the $K_{ATP}$ channel. However, this hypothesis is yet to be proven. If ketones can silence the firing of GE neurons following RH, this may signal in the same way as hyperpolarising the cell membrane and partially rescue the CRR to hypoglycaemia. Although, the fact that an increased concentration of ketones in the VMH is associated with reduced food intake would indicate that the behavioural responses to hypoglycaemia, in terms of food foraging, would not be recovered in an individual with hypoglycaemia unawareness.

Another possible therapeutic avenue for RH which could be explored with this model is the effect of restoring PDH activity, either during or following RH, on the functional outputs of the cell. One way in which PDH may be upregulated is with α-lipoic acid, which is produced endogenously and a synthetic version is also commercially available. α-lipoic acid is a co-factor for the activation of the PDH complex and also facilitates increased activity of glutathione reductase which generates more reduced glutathione to scavenge ROS (Maczurek et al., 2008, Jordan and Cronan, 1997). The therapeutic potential of synthetic α-lipoic acid has been investigated for the treatment of dementia, where increased oxidative stress and reduced glucose utilisation occur in the brain of these patients (Maczurek et al., 2008). Therefore, the electrical and metabolic effects of preserving or upregulating PDH activity in the setting of RH could be investigated using the GT1-7 model. This may provide more information as to whether the changes in oxidative metabolism are translated in the electrical phenotype observed following RH.

There is an indication from in vivo studies and the current study that RH causes increased oxidative stress in glucose-sensing neurons (Fioramonti et al., 2013). What can be confirmed is that acute low glucose exposure increases ROS production within GT1-7 cells. If this
recurrent oxidative stress is producing post-translational modifications at the $K_{\text{ATP}}$ channel, resulting in attenuated activation to low glucose, then this could potentially be a therapeutic target for the treatment of hypoglycaemia unawareness. Indeed, this is the first study to demonstrate that RH causes adaptations to the function of $K_{\text{ATP}}$ channels in GE neurons, in terms of a reduced sensitivity to activation by MgADP. However, mass spectrometry and/or experiments where the RH protocol is adapted to include antioxidants, in order to identify any post-translational modifications and prevent possible oxidative stress, would have to be performed in order to confirm this.

Taking the above $K_{\text{ATP}}$ channel result into account, applying pharmacological compounds to directly activate the channel during hypoglycaemia would seem like a logical therapeutic intervention, especially as the hyperpolarisation response to NN414 was maintained in GT1-7 cells following RH. However, as demonstrated, continuous application of the SUR1-selective KCO NN414 inhibited the CRR to hypoglycaemia and $K_{\text{ATP}}$ channel activity, in vivo and in vitro respectively. While this result has provided very strong evidence against administering KCOs chronically, as $K_{\text{ATP}}$ channel activity was also reduced in response to chronic Diazoxide, this study has shown for the first time that $K_{\text{ATP}}$ channels can adopt a state of stable inactivation through continuous stimulation. However, it cannot be concluded from this study if this is directly through the channel being continuously open or if it is actually through NN414 binding to the channel and causing a conformational change whereby NN414 or MgADP can no longer activate the channel. It would be interesting to repeat these experiments on recombinant channels that have gain-of-function mutations, seen in infants with neonatal diabetes, as these mutations occur on Kir6.2 and alter the ability of ATP to inhibit the channel (Hattersley and Ashcroft, 2005). If chronic NN414, or Diazoxide, inhibited these mutated channels then that would further suggest that the site of action for the deleterious effects of continuous KCOs is located on SUR1.
These results suggest that chronic activation of the $\text{K}_{\text{ATP}}$ channel leads to the induction of a negative feedback mechanism to reduce channel activity. This may be in an attempt to maintain neuronal membrane potential within a physiological range. These results also suggest that inactivation of the $\text{K}_{\text{ATP}}$ channel through continuous agonism is partially protective against the detrimental effects of RH in glucose-sensing neurons as animals which received continuous ICV NN414, in conjunction with RH, displayed better CRRs to hypoglycaemia following removal of the drug than those animals that had received RH alone. Therefore activation of central $\text{K}_{\text{ATP}}$ channels during antecedent or recurrent hypoglycaemia may be directly driving the resulting defective CRR. However, adaptations in metabolism following RH may also be altering the function of central $\text{K}_{\text{ATP}}$ channels.
Chapter 6

Appendices
Appendix Figure 1 Glycolysis is more stable in response to 5 and 25 mM glucose in GT1-7 cells

Real-time analysis of the glycolysis (extracellular acidification rate = ECAR) was performed, using the XF-24 Seahorse Extracellular Flux Analyser, on intact GT1-7 cells. Cells were starved in 0 mM glucose containing media supplemented with 1 mM L-glutamine and 2.5 mM Na-pyruvate to order to shut off glycolysis and maintain cell energetics through oxidative metabolism. (A) 5 or (B) 25 mM glucose was injected into the wells to stimulate glycolysis. Oligomycin was applied in an attempt to obtain maximal glycolysis. Finally 2-deoxyglucose (2DG) was added to inhibit glucose uptake and metabolism.
Appendix Figure 2 Results of glucose metabolism gene array performed on GT1-7 cells following RH

Results are displayed as fold change following RH compared to gene expression in the control sample.
Chapter 7

References


