EFFECTS OF SOMATOSTATIN RECEPTOR 2 INHIBITION ON GLUCAGON COUNTERREGULATION AND C-PEPTIDE LEVELS IN HYPOGLYCEMIA CONDITIONED MALE SPRAGUE-DAWLEY RATS

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ABSTRACT

The counterregulatory hormone glucagon normally prevents hypoglycemia (low blood glucose); however, recurrent hypoglycemia attenuates glucagon counterregulation to subsequent bouts of hypoglycemia. This attenuated response of glucagon with repeated hypoglycemic events may explain why patients with diabetes mellitus are at increased risk for more severe hypoglycemic events with time. As somatostatin normally inhibits glucagon secretion, we hypothesize that a somatostatin receptor type II antagonist (SSTR2a), PRL-2903, improves glucagon responses attenuated by recurrent hypoglycemia. Healthy male Sprague-Dawley rats (n=22) were made hypoglycemic on three consecutive days (blood glucose 1.7-2.2 mmol/L for ~2 h) via insulin bolus administration (10-, 8- and 5-U/kg of Humulin-R on days 1-3, respectively). Glucagon levels during hypoglycemia (blood glucose [BG] ≤ 3.5 mmol/L) on day 3 were significantly lower than on day 1 (107±10.27 vs. 168.30±15.22 [mean±SEM] pg/mL; p=0.0035). On day 4, rats were treated with either SSTR2a (PRL-2903, 10 mg/kg IP; n=13) or vehicle (10 mg/kg IP; n=9) 1 h prior to the induction of hypoglycemia with 5 U/kg of R-insulin. Glucagon levels during hypoglycemia were 2.5-fold higher (109.38±15.37 vs. 44±8.70 pg/mL; p=0.004) compared to vehicle, and time to reach hypoglycemia was 3.2-fold longer (63.85±12.48 vs. 20±3.23 min; P=0.001), with SSTR2a pre-treatment. Also, C-peptide levels were lower (p=0.01) with SSTR2a (0.35±0.06 ng/mL) compared to vehicle (0.63±0.07 ng/mL). Hepatic glycogen levels were also lower with SSTR2a pre-treatment compared to vehicle (11.77±1.41 vs. 20.66±2.34; p=0.001). In conclusion, our data suggests that SSTR2a improves glucagon responsiveness and increases hepatic glycogen breakdown during a hypoglycemic event in animals previously exposed to recurrent hypoglycemia. These findings are important in understanding the potential therapeutic benefit of drugs that can block somatostatin receptor 2 action in patients living with diabetes who are prone to recurrent hypoglycemia.

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LIST OF ABBREVIATIONS

BG	Blood Glucose
ANS	Autonomic Nervous System
T1D	Type I Diabetes
SST	Somatostatin
PP Cells	Pancreatic Polypeptide Cells
GLUT	Glucose Transporter
K_{ATP} Channel	ATP sensitive K^+- Channel
GABA	Gamma Aminobutyric Acid
GHB	Gamma Hydroxybutyric Acid
CNS	Central Nervous System
GI	Gastrointestinal Tract
GH	Growth Hormone
SD	Sprague-Dawley
SSTR	Somatostatin Receptor
GLP-1	Glucagon Like Peptide-1
GIP	Glucagon Dependent Insulinotropic Polypeptide
EGP	Endogenous Glucose Production
VHM	Ventro-Medial Hypothalamus
BBD	Biobreeding
STZ	Streptozotocin
HAAF	Hypoglycemia Associated Autonomic Failure
SSTR2a	Somatostatin receptor type II antagonist
IP	Intraperitoneal
HPA	Hypothalamic, pituitary, adrenal axis

INTRODUCTION 1

Blood glucose (BG) regulation relies on an interconnected system of multiple organs and biochemical pathways. Both the central (hypothalamic) and peripheral (hormonal) counterregulatory pathways operate in a synchronized manner to maintain BG levels within a tight normal range (i.e. 4.0-7.0 mmol/L; 72-126 mg/dL) even during exercise, with short term fasting, and within a few hours after feeding in healthy organisms (1,2). Various sites in the body, including but not limited to the brain, portal vein, liver, intestines, carotid sinus, and the pancreas are sensitive to changes in BG levels and respond in a concerted fashion to preserve glucose homeostasis (1). This facilitates fast and effective whole body detection of variations in BG levels at multiple locations, which is required for stimulation of appropriate hormonal, and neuronal responses involved in glucose metabolism. In other words, in the healthy state, multiple interactions between nutrients, metabolites, hormones, and neurotransmitters allow for maintenance of BG homeostasis. Therefore, when studying normal and abnormal glucose homeostasis, using an in vivo animal model has advantages over cell lines or in vitro preparations, since the former involves the complex interactions between various biological and physiological systems. In studies of glucose homeostasis, rodent are commonly used due to better availability and accessibility of samples (blood, tissues, etc.) and because all mammals tend to have similar physiologic systems to preserve glucose homeostasis (3).

Focusing on the hormonal aspect of BG regulation, in healthy, non-diabetic individuals, the pancreas produces insulin and glucagon, which are the primary regulators of BG. A balance between these two hormones is essential to maintain one's BG within

a normal range in the fasting or post-prandial (i.e. post meal, and after the macronutrients have largely been absorbed and assimilated) state (4,5).

Such balance is maintained by interactions between direct and indirect glucose regulatory mechanisms which involve glucose sensing cells/receptors, the endocrine system and the autonomic nervous system (ANS) (sympathetic and parasympathetic systems) (6).

One situation where BG concentrations fluctuate markedly from the normal range is with type 1 diabetes (T1D). T1D is an autoimmune disorder in which the body attacks its own β-cells, thereby inhibiting the pancreas from producing insulin (7–9). Even with exogenous insulin therapy, in T1D, the body is unable to regulate BG levels due to the inability to perfectly emulate endogenous insulin secretion, in response to various physiological stimuli (feeding, fasting, exercise) and because individuals living with the disease often develop impairments in the glucagon secretion during hypoglycemia (typically defined as a BG ≤ 3.5 mmol/L) (10,11). In other words, even with intensive insulin therapy (i.e. 3-4 insulin injections per day, or with the use of a constant insulin infusion device, with frequent glucose monitoring), significant abnormalities in BG homeostasis exist, not only because insulin delivery is not perfectly normalized, but also because the glucose counterregulatory system appears to be lost with time (12). The exact biological mechanism(s) behind the loss of counterregulation is not fully known, but glucagon appears to be a key player in the pathophysiology of counterregulatory failure (9,13,14). In addition to insulin and glucagon, various neurotransmitters, and other hormones such as somatostatin (SST), growth hormones, glucocorticoids, and catecholamines (epinephrine and norepinephrine) are important players in glucose metabolism whose functions may be also be impacted by diabetes.

LITERATUR REVIEW 2

2.1 Overview of the pancreas

The pancreas is an organ with both endocrine and exocrine functional properties. The exocrine pancreas is composed of the acinar, centroacinar, and ductal cells that are responsible for production and release of variety of enzymes into the duodenum where they are involved in digestion (15). The endocrine pancreas makes up about 1-2% of the entire pancreas, containing clusters of various cells, termed islets of Langerhans, that are responsible for the production of a number of hormones involved in glucose regulation and metabolism. Insulin, the hormone most widely studied, is produced by the beta (β) cells (16), and is released as BG levels rise to initiate glucose disposal into various insulin-sensitive tissues (muscle, liver, adipose), whereas glucagon is produced by the alpha (α) cells, and opposes the action of insulin to increase BG levels during hypoglycemia (1,3,15,17,18). Paradoxically, somatostatin, a hormone released by the delta (δ) cells, that make up ~5% of islet cells in humans, inhibits the release of both insulin and glucagon, although the action of this hormone on these other pancreatic hormones may be glucose dependent with hypoglycemia and hyperglycemia (high BG), respectively (1,2,16,19) (Fig.1). Additionally, a less common cluster of pancreatic cells, called pancreatic polypeptide (PP) cells (1-2% of pancreatic cells in humans), are responsible for the production of pancreatic polypeptides which are also involved in metabolism, albeit to a lesser extent (3,16,18,20). Epsilon (ε) cells are the least frequent islet cell type (<1% in humans), that produce ghrelin which is an appetite hormone (21).The release of these hormones is modulated by direct effects of

glucose and other nutrients, as well as paracrine factors released by neighboring islet cells, circulating hormones, and neurotransmitters released by intra-islet nerve endings.

2.1.1 Pancreatic architecture: human vs. rodent models

As mentioned earlier, pancreatic studies are commonly carried out using a rodent model. For scientific studies to be clinically relevant, it is essential to consider overall similarities and differences between a rodent and a human pancreas. Although pancreases from both species contain all pancreatic cell types mentioned above, there are differences in composition, distribution, organization, overall architecture, density, and size of the islet cells (22). For example, in rodents, the pancreas contains more ß-cells (60-80% of islet cells) than in humans (50-70% of islet cells). As such, the α-cell composition of the pancreas in rodents tends to be lower (10-20% of islet cells) than in humans (20-40% of islet cells) (18,23). Moreover, organization of human islet cells appears to be scattered, where ~70% of ß-cells are in direct contact with non ß-cells, increasing the possibility of paracrine signaling involving intercellular diffusion of hormones (17,18,23). Unlike, in rodents, islet cells are more localized and have distinct patterns. In vitro studies show that α-cells are mainly concentrated around the periphery of the islets, ß-cells are localized in the core, and δ-cells appear to be scattered between the layers of α- and ß-cells (3,17,23,24). Additionally, looking at the neuronal composition of the two pancreases, there is less neural influences on activities of the cells of human islets as they are less innervated compared to that of in rodents (6). With regards to blood circulation, in human islets, there are no clear anatomical subdivisions between different cells of the islets; thus, blood flow appears to be along the layers of the cells. Unlike in

rodents, blood flow is from the inside of the islet with centrally located ß-cells out toward the periphery where the other non ß-cells reside (16,23,25).

2.1.2 The endocrine pancreas: main pancreatic hormones

2.1.2.1 Insulin

Insulin is a hypoglycemic agent and an anabolic hormone that triggers storage of excess glucose in form of glycogen in the liver and skeletal muscle, and triacylglycerol in adipose tissues for later use during exercise and/or the fasted state. An increase in circulating insulin levels post-meal is followed by an increase in muscle-capillary blood flow to help increase glucose disposal into the liver, muscles, and other tissues, thus preventing hyperglycemia (4). Insulin also inhibits glucagon action, a hyperglycemic agent, resulting in inhibition of glucose production by the liver, and in turn maintenance of BG homeostasis (1) (Fig.1).

Endogenous insulin is stored in secretory granules and released by the pancreatic β-cells. Normally, secretion rates and circulatory levels are elevated in response to a meal, particularly if carbohydrate is consumed, or in response to a rise in BG from any cause (26). A rise in BG levels is detected by glucose sensing cells present in various sites including the brain and the portal vein (the vein that directly delivers blood from the pancreas to the liver), stimulating an increase in insulin production which helps lower BG to normoglycemia through stimulation of glucose uptake (6,27). In the post-prandial state, glucose enters β-cells by means of glucose transporters (GLUT), mainly the glucose transporter II (GLUT-2) in humans and rodents, where its metabolism is initiated by glucokinases. Glucokinases phosphorylate glucose to generate glucose-6-phosphate,

regulating the entry of glucose into its subsequent metabolic pathways to generate ATP (28). A rise in intracellular ATP level inhibits activity of ATP-sensitive K^+(K_{ATP}) channels, resulting in depolarization of β-cells. The K_{ATP} channels are normally active/open at low levels of BG, maintaining a negative/hyperpolarized state, whereas as rise in BG concentrations induces closure of K_{ATP} channels causing cell membrane potential to be more positive, leading to depolarization (17). Depolarization leads to influx of calcium into β-cells through voltage gated L-type Ca^{2+} channels, and in turn secretion of insulin due to a rise in cytoplasmic Ca^{2+} concentrations.

Other than the direct regulatory effect of plasma glucose levels, intra-islet paracrine factors and circulating hormones also modulate insulin secretion. In an immunoneutralization study using an anti-glucagon antibody, insulin secretion is significantly stimulated under conditions of both low and high BG (29), thereby suggesting that glucagon has a paracrine effect to inhibit insulin secretion. Other than glucagon (24), SST produced by δ-cells (16,17), epinephrine (30,31), galanin (32) , ghrelin (18,21,33), and leptin can also inhibit insulin secretion, particularly under conditions of hypoglycemia (6,18,24,28,34–36).

Several in vivo and in vitro studies (11,27,37) suggest that insulin is not released at a constant rate, it is rather released in an oscillatory/pulsatile manner in which a complex network of factors, both inside and outside of the endocrine pancreas, are involved in regulation of the hormone secretion (27,37). Pulsatile secretion ensures proper timing and amount of secretion, and likely helps to balance the release of pancreatic hormones through various feedback mechanisms. As a result, insulin production varies based on how much insulin is circulating in the blood, and how much

more insulin is needed to maintain BG homeostasis (27,37). In accordance with the pulsatile theory, insulin secretion appears to be in phase with SST, but out of phase with glucagon with a lag of ~ 2minutes (24). Therefore, insulin and SST levels are normally elevated, while glucagon levels are suppressed during hyperglycemia. Unlike, under conditions of hypoglycemia, insulin and SST levels tend to be lowered, while glucagon levels are elevated (26,34,38). The similarity between insulin and SST in secretion, and inhibitory effects of both on glucagon release is possibly due to the fact that β-cells and δ-cells share an immediate common progenitor cell (17).

2.1.2.2 Glucagon

Glucagon is a hyperglycemic agent and a catabolic hormone. It is the primary counterregulatory hormone that increases BG concentration by opposing insulin's action on the liver (9,13,39). Thus, unlike insulin, plasma glucagon concentration is reduced after a meal and increased during fasting and exercise (1,26,28). In other words, insulin and glucagon are secreted in an anti-synchronized fashion where glucagon production is at its maximum when insulin secretion is minimal (26). During exercise and/or insulin induced hypoglycemia, a rise in glucagon counters insulin action by stimulating glucose production via gluconeogenesis (i.e. de novo glucose production) and glycogenolysis (i.e. the breakdown of glycogen into glucose) to maintain BG levels within a normal range (~4-7 mmol/L) (Fig.1). It is important to note that multiple mechanisms are likely to be involved in glucagon release, with some potential mechanisms still being examined (40).

Glucagon secretion, at least in vitro, is modulated by a direct effect of intrinsic glucose sensing receptors on the α-cells (26,39,41). Like insulin, glucagon secretion is regulated by changes in plasma glucose levels, except that high plasma glucose levels

inhibits glucagon release via increased ATP production from glycolysis and the inactivation of various voltage gated ion channels (28,42). Under conditions of hyperglycemia, glucose uptake by α-cells results in elevation of cellular ATP levels that inactivate K_{ATP} channels, which are predominantly inhibited even in euglycemia (i.e. normal BG concentration). Depolarization followed by inactivation of K_{ATP} channels, result in closure of T-type Ca^{2+} and Na^{+} channels, reducing amplitude of action potentials which leads to inhibition of P/Q Ca^{2+} channels, and preventing Ca^{2+} influx, therefore glucagon release. In contrast, under conditions of hypoglycemia, activation of voltage gated T-type Ca^{2+} and Na^{+} channels result in opening of high threshold P/Q Ca^{2+} channels, generating high amplitude action potentials, and in turn glucagon release (6). It is believed that in healthy humans and in rodents, glucagon release during low glucose concentrations (0-7 mmol/L) is mainly regulated by intrinsic properties of α-cells themselves and positive feedback system, whereas glucagon inhibition during hyperglycemic state is mainly through inhibitory paracrine signaling involving pancreatic factors such as insulin and SST (6,39,43).

Intra-islet circulation, defined as blood flow in the pancreas itself and between the pancreatic islets, appears to traverse from β cells to α- and δ-cells, according to in vivo studies in rodents (19,41,44) Thus, a rise in intra-islet insulin concentration acts as a paracrine inhibitory signal to downregulate glucagon secretion (44). Binding of both insulin and SST to their receptors on α-cells activates K_{ATP} channels resulting in membrane hyperpolarization and closing of voltage-gated Ca^{2+} channels (28), thereby inhibiting glucagon release and reducing glucose production during hyperglycemia (26). An in vitro study using BG clamping techniques in an isolated human pancreas shows

that there is a significant inhibition of glucagon secretion during low-glucose exposure (perfusion), but significant stimulation of glucagon secretion during high-glucose perfusion (29). These results indicate the existence of regulatory feedback loop between α- and β-cells where insulin promotes either a rise or a reduction in glucagon release during hypoglycemia and hyperglycemia, respectively. Consequently, insulin appears to have a glucose-dependent regulatory role in the secretion of glucagon.

Other than SST (17,39,43) and insulin, other paracrine inhibitors of glucagon include co-secreted factors from β-cells such as Zn^{2+} ions, γ-aminobutyric acid (GABA) (35,45) and γ-hydroxybutyric acid (GHB) (46), both of which are also inhibitory neurotransmitters produced in the brain (6,16,35,37). On the other hand, The autonomic nervous system and in particular the stimulatory effects of epinephrine produced by the adrenal medulla increase glucagon secretion (6,47).

2.1.2.3 Somatostatin

Somatostatin (SST), a polypeptide with a very short half-life (30 seconds to 1 minute) in circulation, was originally isolated from the hypothalamus, and exists in two main forms, SST14 and SST28, which are made up of 14 and 28 amino acids, respectively (17,24). Both forms are derived from the precursor pre-prosomatostatin, cleaved into pro-somatostatin, which undergoes translational modifications to produce SST14 and 28 (17). Although both forms are produced in the hypothalamus, neuronal cells of the central nervous system (CNS), gastrointestinal (GI) tract (43,48), and the pancreas (18,43,48), SST28 is mainly produced in the GI tract; whereas SST14 (5-10% of circulating SST) is predominantly produced by the δ-cells of the pancreas (17). SST is an inhibitory regulator of various systems throughout the body (7,49), and acts as a

neurotransmitter in the CNS (8,17,43). SST is a major inhibitor of growth hormone (GH) secretion in the pituitary (50) , and as mentioned above, is a paracrine inhibitor of insulin and glucagon under conditions of hypo- and hyperglycemia, respectively (49) (Fig.1). SST can also alter secretion of α- and β-cells indirectly by means of various proteins, enzymes, and hormones such as epinephrine and cortisol (25).

In the pancreas, SST14 and SST28 are produced and stored by the δ-cells, and have inhibitory effects on biochemical pathways, specifically targeting glucose regulation (7,49). It appears that SST14 is more common in terms of its inhibitory effects on glucagon secretion, whereas SST28 is more potent in the inhibition of insulin (51). In rodents, α-cells are generally more sensitive to the inhibitory effects of SST than β-cells (6,49), which could possibly be due to closer spatial association between δ-cells and α-cells, which creates a more direct cell-cell contact (6). It is important to note that it is not yet fully known if pancreatic SST exerts whole-body effects. This is because pancreatectomy (pancreas removal) has no impact on circulating levels of SST, likely because most circulating SST is derived from the intestines (17).

Just like insulin and glucagon, SST is produced in a pulsatile fashion with its secretion in phase with insulin, but asynchronous to glucagon (24,52). The fact that SST release is out of phase with glucagon suggests that the hormone has a stronger inhibitory effect on glucagon secretion rather than insulin secretion. An in vitro study using anti-SST antibody to neutralize endogenous SST in human pancreata, shows an increase in insulin release in the presence of the antibody, and under conditions of both low and high glucose infusions, suggesting inhibitory role of SST in insulin secretion. Surprisingly, inhibition of

SST tends to increase glucagon secretion, but under low glucose infusion only, suggesting limited/tonic inhibitory effects of SST on glucagon production (29).

Another in vitro study used a SST knockout model ($sst^{-/-}$) to look at stimulus-induced insulin and glucagon levels in the absence of neuronal, gastrointestinal, and/or pancreatic sources of SST. Results show significant differences in hormonal levels with both insulin and glucagon being lower in the presence of pancreatic SST specifically (49). Also, administration of exogenous SST is shown to inhibit both glucagon and insulin (26,29,53,54). A study in male Sprague-Dawley (SD) rats shows, following a rise in BG levels, SST is initially and very briefly suppressed, causing a simultaneous increase in both insulin and glucagon. Then, after ~2 minutes, SST along with insulin rise exponentially to reduce glucagon release, preventing hyperglycemia (24)(Fig.1). Consequently, it is evident that SST regulates secretion of both hormones with a profound impact in the presence of a stimulus like glucose.

Pancreatic SST production itself is regulated by different factors. Similar to α- and β-cells, SST producing δ-cells use glucose sensing mechanisms to detect changes in BG levels (34). Direct stimulatory impact of glucose on hormone secretion is observed when specific glucose transporters on δ-cells, GLUT-1 and GLUT-3, facilitate the uptake of glucose into the cells where it is metabolized to produce ATP, increasing the ATP to ADP ratio. Like β-cells, a rise in intracellular ATP levels alters the activity/status of K_{ATP} channels. More specifically, a rise in extracellular BG levels elevates ATP production and closes/inactivates K_{ATP} channels, thereby initiating membrane depolarization causing an influx of Ca^{2+} through voltage gated Ca^{2+} channels, which in turn increases SST secretion (17). Paracrine factors such as glucagon and glutamate (an excitatory factor co-released

with glucagon) (16,19,25,42), various amino acids such leucine and arginine, GABA, and Urocrtin 3 which is a peptide co-released with insulin from β-cells (17,55), all stimulate SST release. The role of insulin itself in regulation of SST is not fully understood yet. Some studies suggest that insulin has both stimulatory and inhibitory effects of on SST secretion (17).

Both SST14 and SST28 bind to five different somatostatin receptor (SSTR) subtypes of inhibitory G-protein coupled receptors (i.e. SSTR1-5) expressed on various organs, such as the pancreas, brain, liver, intestines, stomach, and the kidneys targeting various biological pathways (43,48,51,56). Activities of SST receptors are modulated by voltage gated K^+ and Ca^{2+} channels, a variety of substrates, and receptors endocytosis and trafficking (53). SST binding to its receptors on α-and β-cells suppresses the electrical activity and exocytosis of glucagon and insulin granules, respectively (17). In rodents, somatostatin receptor type II (SSTR2) is specifically expressed in glucagon producing α-cells whereas somatostatin receptor type V (SSTR5) is mainly expressed in insulin producing β-cells (48,53,54,57–59). Therefore, SST binding to SSTR2, and SSTR5 inhibits glucagon and insulin secretion, respectively. Pancreatic β-cells in humans express all five receptors subtypes with type I being the most predominant type, followed by type V which is expressed by about 87% of β-cells, and type II expressed by 47% of β-cells, while type III and IV are poorly expressed (53). In humans, unlike in rodents, SSTR2 appears to be a major SST receptor subtype expressed in both α and β-cells (53). Interestingly, perfusion of isolated human pancreases with SSTR2 agonist show significant reduction in insulin levels under conditions of both hypo- and hyperglycemia,

highlighting the role SST and SSTR2 in insulin regulation in humans (25,53,57). If these and other mechanisms exist in vivo are unclear.

2.2 Other (pancreatic and non-pancreatic) blood glucose regulatory hormones

Glucocorticoids (i.e. cortisol also known as corticosterone in rodents), GH, and catecholamines (i.e. epinephrine and norepinephrine) are all involved in modulating glucose metabolism, either directly, or by regulating the release of other hormones such as glucagon (60). For example, hypoglycemia leads to a rise in circulating cortisol and epinephrine resulting in inhibition of insulin and SST secretion (17), a reduction in hepatic and peripheral insulin sensitivity, and in turn reduced glucose uptake in the muscles and other tissues in an effort to increase BG levels back to euglycemia (61). Epinephrine binding to its receptors on α-cells causes cellular depolarization (28), an influx of Ca^{2+} through opening of Ca^{2+} channels and a stimulation of glucagon release (6). The increase in circulating glucagon facilitates renal and hepatic glucose production/mobilization during hypoglycemia (30,61,62). Interestingly, one study suggests that epinephrine's stimulatory effects on glucagon release may be weak or absent in humans (28).

Ghrelin is a metabolic hormone that is mainly produced in the GI tract but is also produced by the ε-cells of the pancreas, hypothalamus, kidneys, and pituitary glands (63). Ghrelin regulates food intake, body weight, and the release of GH, insulin, glucagon, SST and pancreatic polypeptide (18,19,33,50). In both humans and rodents, a rise in ghrelin levels during the fasted state inhibits Ca^{2+} mediated insulin release, and stimulates food intake (Fig2). Therefore, ghrelin appears to aid with counterregulatory responses to hypoglycemia through reduction of circulating insulin levels and by increasing the drive

for feeding (18,50,63). However, ghrelin is normally inhibited under conditions of insulin induced hypoglycemia, or hyperinsulinemia; therefore, it is not an adequate counterregulatory agent by itself (64). Leptin is a lipid-derived appetite hormone that regulates plasma glucose levels independent of actions on food intake and energy expenditure. Leptin directly contributes to BG homeostasis through its actions as an inhibitory regulator of both insulin and glucagon, and a modulator of glucose uptake in the peripheral tissues (65). Galanin, a neuropeptide with multiple physiological activities including metabolism, is released from intrapancreatic nerve endings, and inhibits insulin secretion, facilitating recovery from hypoglycemia (32). Pancreatic polypeptide, a hormone produced by the PP cells (or F cells) of the pancreas, is involved in regulation of satiety and metabolism as well. PP production is regulated through neuronal signals, mainly cholinergic factors, and arginine levels (20,46). Like insulin, PP levels rise after food intake, inhibiting glucagon production under conditions of high BG levels (18,46). GABA, a factor co-released with SST from δ-cells, stimulates SST secretion, thereby regulating insulin and glucagon secretion through feedback mechanisms (28). Incretin hormones are hypoglycemic agents that stimulate insulin release but inhibit glucagon secretion in response to meals. The two most widely studied incretins are glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP). GLP-1 is made in the endocrine cells of small intestine and colon, while GIP is made by cells in the upper small intestine, both regulating appetite and food intake. A rise in GLP-1 and/or GIP levels result in enhancement of insulin and SST secretion, suppression of glucagon, and inhibition of gastric emptying, thus contributing to maintenance of BG homeostasis (17,66,67).

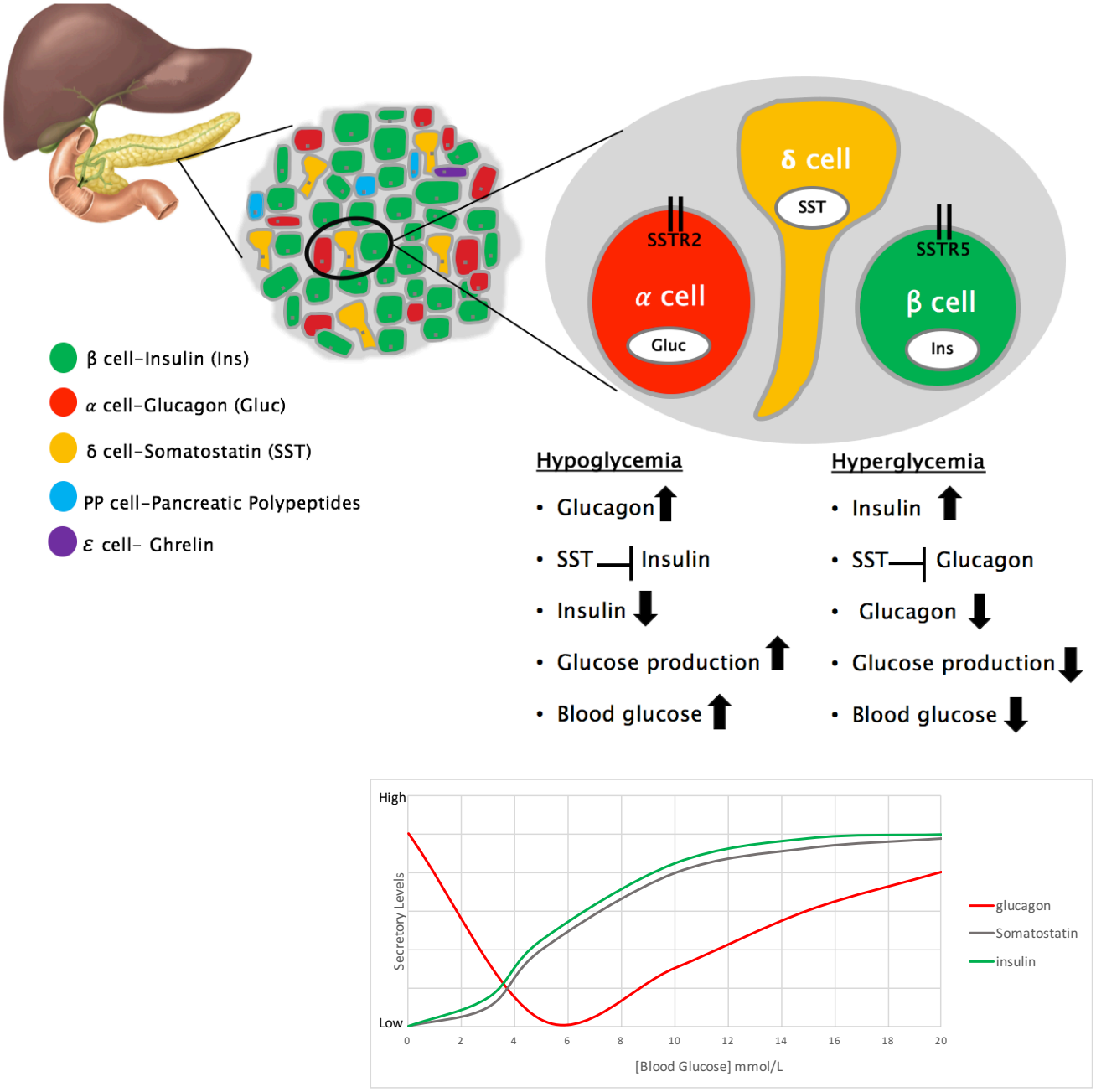

Figure 1. The role of pancreatic hormones in blood glucose regulation Insulin produced by the β-cells, glucagon produced by the α-cells, and somatostatin produced by the δ-cells are the main pancreatic hormones. Insulin functions to lower blood glucose levels during hyperglycemia, whereas glucagon counters the action of insulin to increase blood glucose levels during hypoglycemia. Somatostatin is an inhibitory regulator of both. A balance between these hormones is required for maintenance of blood glucose homeostasis. Inset: Insulin, glucagon, and somatostatin secretion during various blood glucose levels (Adapted from Rorsman and Huising, 2018).

2.3 The liver's role in blood glucose homeostasis

The liver is a major metabolic organ, that regulates energy metabolism through interactions with skeletal muscle, the GI tract, adipose tissue, and the brain. In the post-prandial state, excess plasma glucose enters the liver via the portal vein to be stored in form of glycogen, the main form of carbohydrate storage in mammals, for later use (68). The portal vein not only carries nutrients and hormones from the pancreas to the liver; it also contains neurons and glucose sensors (64) that are sensitive to changes in plasma glucose levels. Deviations from euglycemia, and the rate at which BG levels fluctuate, result in appropriate responses involving activation and firing of glucose sensors in the portal vein. Activation of glucose sensors results in functional modifications of tissues such as the liver, hypothalamus, brain, and the endocrine pancreas to maintain BG homeostasis through regulation of food intake and hepatic glucose uptake (64). Liver glycogen concentrations are controlled by many factors, but mainly the rate of portal vein delivery of glucose to the liver and portal vein insulin levels (62,69). Both high glucose and insulin levels in the portal blood after food intake normally reduce glucose production and stimulate glycogen synthesis by the liver (69). It is important to note that BG levels are normally higher in the portal vein than in peripheral circulation. This creates a concentration gradient from the liver to peripheral tissues, facilitating glucose delivery to peripheral tissues. Pancreatic hormones (i.e. insulin and glucagon) are also released into the portal vein, extracted by the liver, and later delivered into the peripheral circulation. The relative concentrations of insulin and glucagon, as the main BG regulatory hormones, help determine whether the liver functions as an organ of fuel storage or fuel production (23).

The liver is the main organ of glucose production through glycogenolysis and gluconeogenesis when BG levels drop below the normal range, and muscle glycogen stores are low after exercise (1,38). Hormonal and neuronal signals, plus availability of nutrients dictate which source of energy is dominating at a given time (69). Glycogenolysis is the main source of endogenous glucose production (EGP) during early stages of fasting and/or hypoglycemia, whereas gluconeogenesis is the predominant method of glucose production during prolonged fasting and/or hypoglycemia. When hepatic glycogen levels are low, the liver produces glucose in a *de novo* fashion using pyruvate, glycerol, and amino acids as precursors (62,69). Glucagon and epinephrine are two major counterregulatory hormones that stimulate glycogenolysis and gluconeogenesis (62). Other hormones such as growth hormone, and glucocorticoids also employ various pathways and mechanisms to induce glycogen break down and/or gluconeogenesis to increase circulating glucose levels in the fasted state and during exercise (69)

2.4 Skeletal muscle and blood glucose regulation

Skeletal muscle is the main target for insulin-induced glucose uptake, and the most important tissue in limiting glucose uptake during counterregulatory responses to hypoglycemia (68). During conditions of hyper- and hypoglycemia, insulin-induced glucose uptake in skeletal muscles increases and decreases, respectively, to maintain BG homeostasis, and spare glucose for the brain. In humans, most glycogen (~500 g in total in the average adult male) is stored in skeletal muscle, and can be used during high intensity exercise (68). However, hepatic glycogen remains the main source for EGP via glycogenolysis and is essential for maintenance of whole-body BG

homeostasis. Glycogen breakdown in skeletal muscle can only provide energy locally during exercise, as the glucose doesn't enter the systemic circulation. Lactate, an alternative fuel for glucose and a byproduct of muscle glycogen breakdown, can be transported to the liver where it is used a major substrate required for gluconeogenesis during hypoglycemia (30,68).

In accordance with their metabolic and mechanical properties, age, sex, and species, skeletal muscles are composed of different muscle fibers, or basically a combination of them (70). Diversity in fiber type and muscle function is due to presence of different myosin isoforms (71). Glycolytic and oxidative are the two main fiber types. Glycolytic fibers (Type IIb fibers) are fast twitch, fatigue rapidly, have very low mitochondria content and rely on glucose as the main source of energy. Fast twitch oxidative (Type IIa) fibers are moderately resistance to fatigue, and use glucose as the main source of ATP production. In contrast, slow twitch (Type I) fibers, have high mitochondrial content, and use oxidative phosphorylation as the main method of ATP synthesis (70,72,73).

In rodents, just like with other mammals, skeletal muscles have different fiber composition based on metabolic and contractile function. For example, in lower limb, tibialis anterior and gastrocnemius are composed of a mixture of type IIa and IIb fibers (70,73), extensor digitorum longus and plantaris are mainly made of type IIb fibers (71,74) whereas soleus predominantly contains type I (71,74).

2.5 Glucose regulation in type I diabetes

Due to absence of endogenous insulin, chronic and intensive insulin treatment is required to manage BG levels in individuals with T1D. Poorly controlled glycaemia is associated with major complications such as renal failure, cardiovascular diseases, neuropathy, blindness, amputations, and stroke (75). However, even intensive insulin use has some negative side effects, the most catastrophic being severe hypoglycemia (loss of consciousness, possibly convulsions and death). Excessive insulin administration over years in diabetes appears to cause significantly dysregulated glucagon secretion during hypoglycemia in insulin-treated diabetes (11,26,34,76). Additionally, blunted epinephrine and cortisol responses due to maladaptation in the central and peripheral nervous systems, that result in delayed response activation (i.e. higher activation threshold), and reduced magnitude (14). All of these factors contribute to defective counterregulatory responses to hypoglycemia and/or recurrent hypoglycemia, which worsens with time and/or age (76,77).

Glucagon is known to play a key role in the pathophysiology of attenuated counter-regulation as it is the first line of defense against hypoglycemia in healthy individuals (61). Under the healthy conditions, when BG levels fall below 4.4 mmol/L (75), counterregulatory responses including a rise in glucagon and a reduction in insulin secretion, increase glucose production, and decrease insulin-induced glucose uptake in peripheral tissues, mainly in skeletal muscle, to maintain whole body BG homeostasis. In T1D, as plasma glucose levels drop, there is an attenuation in glucagon secretion along with impaired autonomic and adrenomedullary (reduced catecholamine)

responses all of which result in increased depth and duration of hypoglycemia (10,26,35).

Although glucagon counterregulation involves multiple pathways and factors, some studies suggest that the insulin “switch-off” by the ß-cells is the most important stimulus for glucagon secretion; thus, activation of glucagon counterregulatory responses (26). The switch-off theory involves intra-pancreatic interactions between different islet cells, deemed essential for glycemic homeostasis, and a normal counterregulatory system. It is defined as downregulation/termination of intra-pancreatic factors such as insulin and SST, that exert inhibitory effects on glucagon secretion during low BG (6,26).Therefore, absence of endogenous insulin in T1D, and in turn the loss in switch-off by the ß-cells when BG falls, is thought to contribute to the defective glucagon counterregulation observed in patients living with T1D (11,26,34,38,76). In support of the switch-off theory, a rodent study in which insulin was infused into the pancreas, but was not turned off during hypoglycemia, shows suppression of glucagon counterregulatory responses (38).

Other rodent studies of T1D (11,26,76) suggest that, chronic insulin use results in high basal glucagon levels (hyperglucagonemia), possibly due to absence of intra-islet insulin, therefore lack of post-prandial glucagon suppression. The lack of initial rise in insulin levels, after a meal intake, as an inhibitory signal for glucagon production, leads to continuation of glucagon production even though BG levels are still rising (11,23). This failure of glucagon to drop with a meal in T1D likely contributes to post-prandial hyperglycemia. This increase in basal intra-islet glucagon release after meals reduces the difference between basal and peak levels (i.e. during hypoglycemia and/or fasting),

thus lowering the amplitude of glucagon secretory pulses (11). In other words, the higher basal glucagon levels are, the less efficient glucagon responses would be during hypoglycemia. Consequently, some investigators have proposed that a reduction in basal glucagon levels in diabetes might lead to a significant improvement in glucagon responsiveness to hypoglycemia (11,26). Indeed, therapeutic agents such as leptin and GLP-1 that inhibit glucagon production may improve glucagon counterregulation during hypoglycemia, even in absence of insulin signaling (11,78,79). Interestingly, the administration of exogenous SST in patients with hyperglucagonemia tends to improve glycemic management and lower exogenous insulin needs (17).

In addition to altered glucagon secretion in diabetes (i.e. elevated levels post meal and blunted release during hypoglycemia), animal and human studies suggest negative alterations in glycogen synthesis and metabolism in T1D that appear to be aggravated by insulin management. Poorly managed TID results in reduced glycogen production and breakdown, likely because of low insulin and high glucagon post feeding, which may contribute to insufficient EGP and defective counterregulatory responses during hypoglycemia (62). Even with insulin therapy, the lack of hepatic glycogen breakdown during hypoglycemia in subjects with T1D persists, and it is likely due to a significantly lower concentration of basal, or fasting hepatic glycogen content even after insulinization (62,69). Lack of change in hepatic glycogen content in the T1D subjects during hypoglycemia suggests that gluconeogenesis (not glycogenolysis) is the predominant source of EGP when BG levels fall (62). Thus, it is likely that the underlying mechanisms that impair EGP during hypoglycemia include a failure in the

drop in portal insulin levels, a blunted glucagon response, impaired catecholamine release and a low level of liver glycogen content (62,69).

Taking into consideration what is discussed above, a combination of chronic insulin use, disruption of islet architecture and intra-islet cross-talk (i.e. absence of intraislet insulin which is the stimulatory signal for activation of α-cells during hypoglycemia) (26,35,76), glucose blindness of α-cells (i.e.inability to detect changes in glucose levels), autonomic neuropathy (desensitized autonomic responses), and alteration in glycogen metabolism may explain why people living with T1D often develop a loss of glucagon conterregulation over time (13,35,38,75).

2.6 Hypoglycemia

To avoid long-term health complications associated with sustained elevations in glycaemia (i.e. heart disease, kidney failure, nerve damage, vision impairments and blindness), control of high BG concentrations with insulin is a primary clinical objective for T1D. However, with intensive insulin therapy, impaired counterregulatory responses, and with exercise, the risk for hypoglycemia increases markedly (77,80,81). Hypoglycemia can lead to physical and/or psychosocial impairments, neuroglycopenia, cognitive dysfunction, and if left untreated can lead to brain damage, seizures, and in severe cases, death. About 4-10% of deaths in T1D has been associated with hypoglycemia (17,74,79,81). On the other hand, chronic hyperglycemia can cause toxicity leading to β-cell dysfunction and a host of other co-morbidities (1). If one could better titrate insulin dosage upward, without causing hypoglycemia, T1D treatment and glycemic management would dramatically improve, reducing occurrences of associated complications.

As mentioned earlier, a defective counterregulatory mechanism is associated with hypoglycemia. Studies show that, defects in glucagon counterregulation is associated with at least a 25-fold increased risk of severe hypoglycemia in T1D (10,30,80), in part due to inadequate glucagon counterregulation (38,61,80,82). Moreover, antecedent insulin induced, and/or exercise induced, hypoglycemia plays a major role in impaired glucagon counterregulation in T1D, thus increasing the chances of future hypoglycemic episodes (75,81,83,84). It is important to note that hyperglycemia itself inhibits glucagon, but hypoglycemia on its own is not sufficient for an adequate glucagon counterregulatory response. Therefore, other regulatory factors are involved in providing protection against hypoglycemia (26).

In healthy individuals, the neuroendocrine system provides protection against hypoglycemia. Detection of low BG in the brain, stimulates the activation both autonomic and endocrine responses to downregulate insulin secretion and increases hormones that counter insulin when BG levels drop (i.e. glucagon, catecholamines, GH, and cortisol) (11). The brain is the main site for detection of hypoglycemia (64). In rodents, the ventromedial hypothalamus (VMH) appears to be the main trigger site for counterregulatory responses such as glucagon and epinephrine. GABA is an inhibitory neurotransmitter that acts within the VMH to modulate the magnitude of both the glucagon and epinephrine responses to hypoglycemia in nondiabetic rats (35,85). Both human and rodent studies suggest that, GABA functions to downregulate glucagon counterregulatory responses to hypoglycemia (35). Studies using two different animal models of T1D, the Biobreeding (BBD; a diabetic rat model that simultaneously develops T1D) and Streptozotocin (STZ) rodent models, show that basal GABA levels

in the VMH are abnormally high in T1D. Observations indicate that in response to an insulin-induced hypoglycemic challenge, VMH GABA levels decrease in control animals which corresponds with activation of both the glucagon and epinephrine responses. In contrast, VMH GABA levels remain elevated during hypoglycemia in both diabetic models, and this phenomenon is associated with absent glucagon and attenuated epinephrine responses (35,85). Therefore, elevated levels of GABA in VMH in T1D may be an important contributor to counterregulatory failure in diabetic rats exposed to hypoglycemia.

In addition, activation of sympathetic system generates responses to hypoglycemia that result in hypoglycemia awareness which is associated with signs and symptoms such as sweating, dizziness, hunger, lack of concentration, anxiety, and nausea (75). Hypoglycemia awareness gives the patient the opportunity to take appropriate actions necessary for recovery from hypoglycemia before it becomes life threatening. Research suggests that responses to hypoglycemia depend on the rate of fall in BG with higher sympathoadrenergic responses during a slower fall in comparison with a rapid fall (64). However, such lifesaving mechanisms are impaired in individuals living with T1D. In addition to causing hormonal imbalances, hypoglycemia can cause defects in sympathetic and adrenergic responses making it more difficult for detection by the patient; therefore contributing to hypoglycemia unawareness, also knowns as hypoglycemia associated autonomic failure (HAAF) (75,81,85). Inability to detect early warning signs and symptoms of hypoglycemia over time can result in a reduction in magnitude and intensity of sympathetic, adrenal, and glucagon counterregulatory responses, which then are only generated at a significantly lower BG, putting diabetic

patients in danger of severe and/or recurrent, or simply repeated, hypoglycemia (14,75,81,83). One rodent study suggests that repeated hypoglycemia causes structural changes in glucose sensing regions of the brain, thus significantly increasing likelihood of future episodes (77). Additionally, upregulation of glucose metabolism in the brain through increased expression of glucose transporters, and in turn glucose uptake as a result of cellular adaptation to repeated hypoglycemia, can ultimately shut down sympathoadrenal responses. In short, downregulation of neuronal and hormonal responses with recurrent hypoglycemia leads to hypoglycemia unawareness (75,77). Patients with T1D, on average, experience 1-3 episodes of severe hypoglycemia per year, and hypoglycemia unawareness increases incidence of severe hypoglycemia by 6-fold (75,77). The risk increases over time with duration of diabetes, and intensity of insulin therapy for strict glycemic control. Therefore, the fear of hypoglycemia appears to be the limiting factor in glycemic management of diabetes (39,77,80). Antagonism of autonomic responses to hypoglycemia such as epinephrine by itself does not impair counterregulatory response suggesting pancreatic regulation of BG plays a major role in glycemic control and hypoglycemia prevention (10). An in vitro study of denervated non-diabetic human pancreas (86) shows a reduction and an increase in intra-islet insulin and glucagon, respectively, in response to hypoglycemia. Unlike, in presence of an intact autonomic system, but an impaired pancreatic counterregulatory system, recovery from hypoglycemia becomes a challenge.

In summary, decreased hormonal counterregulation makes future episodes of hypoglycemia not only more likely, but also more severe due to decreased sympathoadrenal responses and reduced hypoglycemia awareness (35,75,81,85).

Although the role of recurrent hypoglycemia in counterregulatory impairments is reasonably well established, the underlying mechanisms are not fully understood. It is not known whether it is the hyperinsulinemia induced hypoglycemia itself and/or the exposure to associated factors that leads to the development of impaired glucose counterregulation (83).

2.7 Somatostatin receptor type II antagonist (SSTR2a)

The over-production of SST in diabetes may be one factor contributing to a blunted glucagon response to hypoglycemia. In both healthy, nondiabetic humans and animals, where β-cells are intact, SST may have minimal inhibitory effects on glucagon producing α-cells because of its dominant role in β-cell (insulin) inhibition, whereas in T1D, SST paracrine inhibition of α-cells rises due to absence of β-cells (7,80,82). In addition to absence of β-cells in T1D, the number of δ-cells, plasma SST, pancreatic pro-somatostatin mRNA, and SST protein levels are also increased causing increased suppression of glucagon producing α-cells, and a reduced counterregulatory response under conditions of hypoglycemia (8,43,49,58).

Since elevated SST levels in diabetes leads to increased inhibition of glucagon responses under conditions of hypoglycemia, researchers have shown that a somatostatin receptor type 2 antagonist (SSTR2a) can improve glucagon counterregulatory responses to low BG levels (7–9,17) (Fig.2). In a series of in vivo experiments on rodents, it was shown that that a newly developed compound, PRL-2903 which is a SSTR2a, results in an improved glucagon response to hypoglycemia induced by insulin administration or by exercise (7–9,87). Additionally, a SSTR2 knockout model

in mice has shown a two-fold increase in glucagon secretion (58,88). Although SSTR2a is shown to improve the blunted glucagon response during hyperinsulinemia/hypoglycemia in T1D, the pathophysiology behind this effect is not fully understood.

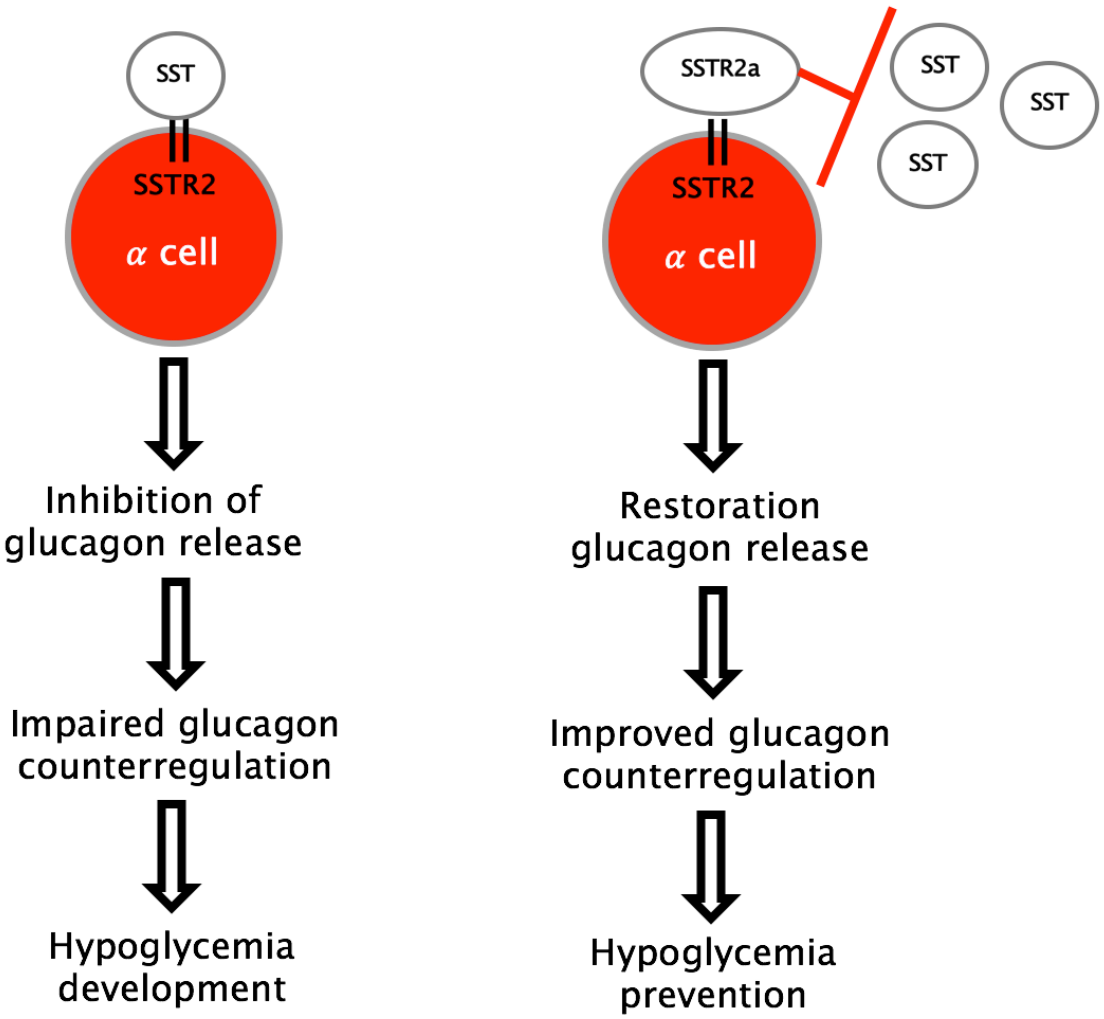

Figure 2. The role of somatostatin in hypoglycemia development and effects of SSTR2a on hypoglycemia prevention. Somatostatin (SST) is a major paracrine inhibitory regulator of glucagon secretion. Therefore, abnormal inhibition of glucagon secretion by STT can impair counterregulatory responses to low blood glucose which leads to hypoglycemia development. As somatostatin receptor type II is mainly expressed on α-cells, a somatostatin receptor type II antagonist (SSTR2a) may improve glucagon counterregulatory responses to hypoglycemia by blocking inhibitory effects of SST on glucagon secretion.

2.8 Summary

In summary, BG regulation and homeostasis requires a complex network of biochemical pathways involving a variety of biological factors including nutrients like glucose, hormones such as insulin and glucagon, and neurotransmitters like GABA. In non-diabetic individuals, the pancreas produces three major BG regulatory hormones, insulin, glucagon, and SST secreted by α-, ß-, and δ-cells, respectively. Insulin and glucagon are the primary regulators of BG where insulin functions to lower BG levels, and glucagon opposes the action of insulin by increasing BG levels. A balance between these hormones is essential to maintain one's BG within a normal range at all times. SST is an inhibitory regulator of both insulin and glucagon under conditions of hypo- and hyperglycemia, respectively. Secretion of these hormones is directly affected by glucose and other nutrients, as well as paracrine and autocrine mechanisms (Fig.3). Defects in production, secretion, and/or functions of one or some of these hormones and factors lead to loss of BG homeostasis in T1D. Unfortunately, with T1D, the body is unable to regulate BG levels due to the loss of endogenous insulin, and impairments in the insulin-glucagon dynamic, which leads to the loss of BG counterregulation. A defective counterregulatory system is associated with a dangerous, life-threatening, low circulating glucose condition called hypoglycemia, and glucagon appears to be a key player in the pathophysiology of this condition. Glucagon prevents hypoglycemia; however, a defective glucagon counterregulatory system, and recurrent hypoglycemia attenuates counterregulatory responses to a greater extent, increasing vulnerability to subsequent bouts of hypoglycemia. As SST normally inhibits glucagon secretion and its levels are upregulated in T1D, a somatostatin receptor type II antagonist (SSTR2a), PRL-2903, may improve glucagon responses attenuated by recurrent hypoglycemia.

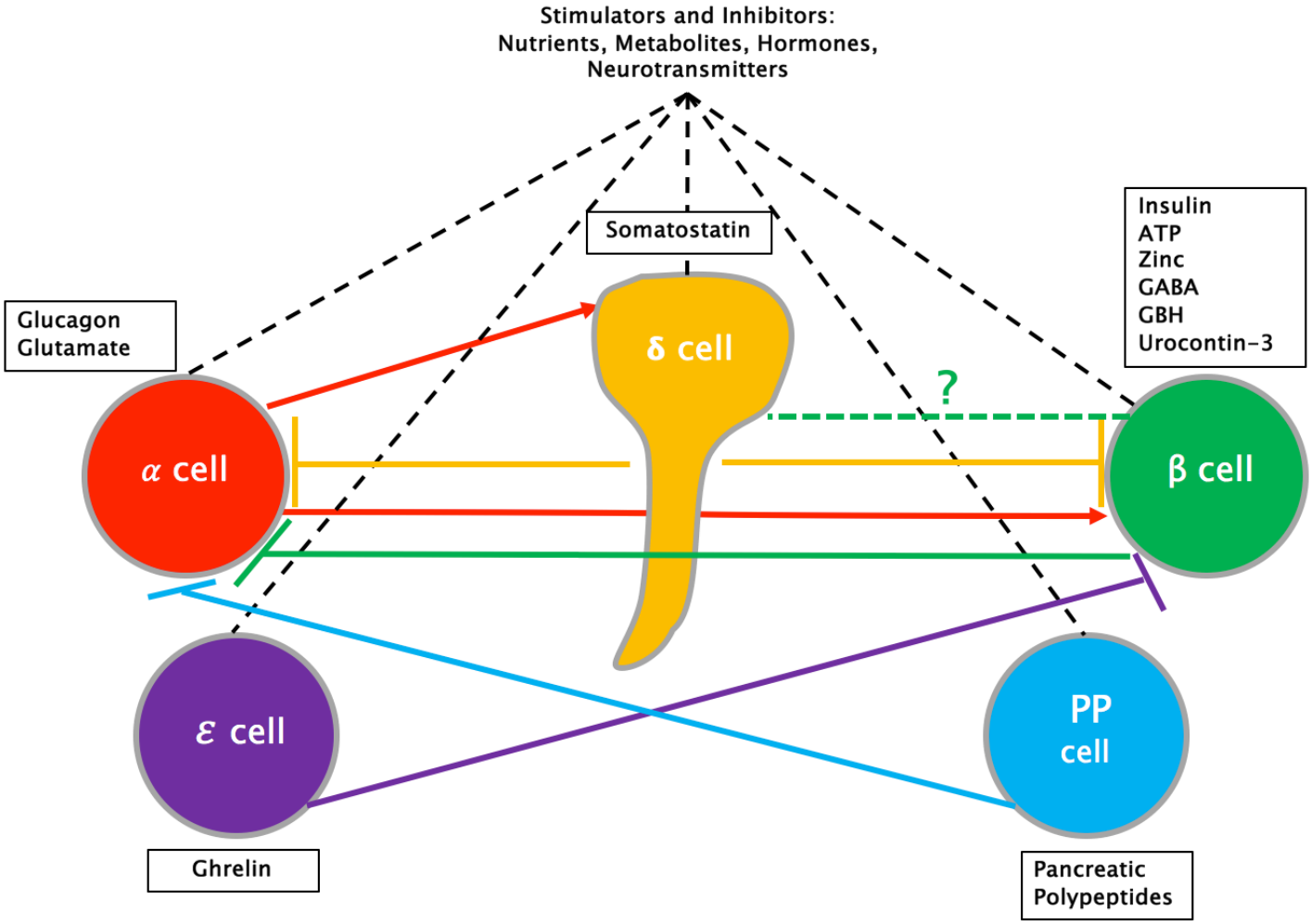

Figure 2. Summary: Regulation of pancreatic hormones involves complex intrasilet interactions. Regulation of blood glucose regulatory hormones involves both intracellular and extracellular factors. A complex instraislet communication network incorporating feedback mechanisms and paracrine signaling ensures hormonal balance and maintenance of blood glucose homeostasis in the healthy state.

RATIONALE AND OBJECTIVE 3

3.1 Rationale

T1D is a metabolic disorder in which defects in both insulin and glucagon production/secretion play a role. Chronic use of exogenous insulin while life sustaining in T1D, combined with a defective counterregulatory system, creates a serious complication called hypoglycemia. Although the role of recurrent hypoglycemia, or simply repeated hypoglycemic episodes, in counterregulatory impairments is reasonably well established, the underlying mechanisms are not fully understood. It is not known if it is the hyperinsulinemia induced hypoglycemia itself and/or the exposure to associated factors that leads to the development of impaired glucose counterregulation that is behind the high rates of the complication in this disease state (83). SST production by the δ-cells of the pancreas exerts inhibitory effects on glucagon secretion, and elevated SST levels in T1D likely cause a greater suppression of glucagon producing α-cells under conditions of hypoglycemia (7–9,17,43,58,80). Since SST is known to inhibit glucagon, a somatostatin receptor type II antagonists (SSTR2a) may be able to restore the dysregulated glucagon counterregulation during hypoglycemia as the somatostatin receptor type II is specifically expressed in glucagon producing α-cell (7–9,17,53,58). In a series of in vivo experiments on rodents, it was shown that that a newly developed compound PRL-2903, which is a highly selective SSTR2a, results in an improved glucagon response to hypoglycemia induced by insulin administration or exercise (7–9,87). Additionally, a SSTR2 knockout model in mice has shown a two-fold increase in glucagon secretion at euglycemia (58,88).

Therefore, SSTR2a could be a novel therapeutic agent which would be used in form of combination therapy with insulin, enabling better glycemic control with intensive insulin therapy without the fear of hypoglycemia.

Although SSTR2a is shown to improve the blunted glucagon response during hyperinsulinemia/hypoglycemia in T1D, the pathophysiology behind this effect is not fully understood. It is unclear whether the effectiveness of SSTR2a is dependent on diabetes, or diabetes related factors (i.e. elevated SST levels), or because of antecedent hypoglycemia, the latter of which can occur in non-diabetic organisms. In other words, we do not know if the SSTR2a would be able to improve impaired glucagon responses to hypoglycemia in absence of diabetes. Therefore, in this thesis, we designed a nondiabetic rodent model, instead of a diabetic model, that mimics the counterregulatory failure in T1D. Our goal was to eliminate the diabetes-related variables, except for recurrent hypoglycemia, to better understand the functional properties of the antagonist.

3.2 Objectives

The objectives of this study were to develop a rodent model of recurrent hypoglycemia that rapidly develops a weakened glucagon counterregulatory response to hypoglycemia, and determine if a SSTR2a would be able to improve glucagon counterregulation in this model. In this study, we specifically examined the effects of PRL-2903, a SSTR2a, on glucagon and insulin secretion during hypoglycemia, in our newly established rodent model of recurrent hypoglycemia that mimics counterregulatory failure in T1D. The primary goal of this study was to determine the

impact of the antagonist on circulating insulin and glucagon levels, and in turn glucose production via glycogenolysis in such abnormal (compromised counterregulatory system) condition.

3.3 Hypotheses

We hypothesized that the antagonism of SSTR2 on α-cells relieves the inhibitory effects of SST on glucagon secretion thereby improving the compromised glucagon response. Since in our non-diabetic model β-cells are still intact, endogenous insulin secretion exists, but we expect no significant impact on insulin release in presence of the SSTR2a, as the antagonist used, PRL-2903, is specific for the type II receptor which is mainly expressed on the α-cells in rodents. We also anticipated that hepatic, but not skeletal muscle glycogen content may be lower in the SSTR2a group compared to controls animals because improved glucagon responses in the treatment group leads to increased glycogenolysis, and in turn glucose production in an attempt to recover from hypoglycemia.

MANUSCRIPT 4

SOMATOSTATIN RECEPTOR II ANTAGONISM IMPROVES GLUCAGON COUNTERREGULATORY RESPONSES TO RECURRENT HYPOGLYCEMIA IN MALE SPRAGUE-DAWLEY RATS

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Keywords: Blood glucose (BG), counter regulation, Glucagon, Hypoglycemia,

Somatostatin (SST), SSTR2a, C-peptide, Glycogen

Figure: 10

CONTRIBUTION BY THE AUTHORS

This thesis project was designed by myself, Dr. Erin R. Mandel, Dr. Owen Chan, and Dr. Michael Riddell. I carried out all the experiments including repeated hypoglycemia, hypoglycemia challenge, saphenous vein and portal vein blood samplings for glucagon and C-peptide assays, liver and muscle collection for glycogen assay, data analysis, and creation of all the figures. Dr. Erin R. Mandel, Caylee Greenberg, Trevor Teich, and Efa Pasieka assisted with data collection during all phases of the study. Dr. Richard Liggins provided our laboratory with SSTR2a (PRL-2903) and vehicle compounds, and provided input on the interpretation of data. Dr. Michael Riddell is the principal investigator and supervisor of this project.

Introduction

Blood glucose (BG) regulation relies on an interconnected network of multiple organs and biochemical pathways. Both the central (hypothalamic) and peripheral (hormonal) counterregulatory pathways operate in a synchronized manner to maintain BG levels within a normal range (i.e. 4.0-7.0 mmol/L) during exercise, fasting, and feeding states (1,2). Focusing on the hormonal aspect of BG regulation, in healthy, non-diabetic individuals, the pancreas produces the opposing hormones, insulin and glucagon, which are the primary regulators of BG. Insulin functions to lower BG levels, whereas glucagon counters the action of insulin by elevating BG concentrations. A balance between these two hormones is essential to maintain one's BG concentration within a normal range in the fasted or post-prandial states (~4-7 mmol/L) (4,5). One situation where BG levels fluctuate markedly from the normal range is with type 1 diabetes (T1D). T1D is an autoimmune disorder in which the body attacks its own β-cells, thereby preventing the pancreas from producing insulin (7–9,77). Because of this loss, patients with T1D have difficulty regulating their BG levels. To avoid long-term health complications associated with sustained elevations in glycaemia (i.e. heart disease, kidney failure, nerve damage and eye disease), tight control of glucose remains the primary clinical objective for patients living with T1D. However, even with intensive insulin therapy via multiple daily insulin injections or by continuous subcutaneous insulin infusion devices (i.e. insulin pumps), significant abnormalities in BG homeostasis exist, not only because insulin delivery is not perfectly normalized, but because the glucose counterregulatory system is also impaired to some degree. Specifically, prolonged diabetes is associated with loss of glucagon secretion and imbalances in the insulin-to-glucagon dynamic, and loss of

glucose counterregulation when blood glucose levels drop below a normal range (i.e. <3.9 mmol/L). The exact biological mechanism(s) behind the loss of counterregulation is not fully known, but glucagon appears to be a key player in the pathophysiology of counterregulatory failure (9,13,14,26). Possible explanations as to why people living with T1D often develop a loss of glucagon counterregulation over time include chronic insulin use, disruption of islet architecture, absence of intra-islet insulin (11,26,35,76), glucose blindness of α-cells, blunted epinephrine and cortisol responses, autonomic neuropathy, and alteration in glycogen metabolism (62,69,75,77).

A defective glucagon counterregulatory system in T1D is associated with a number of serious health complications, the most catastrophic being severe hypoglycemia where the patient either loses consciousness or requires assistance. Hypoglycemia itself, even in milder forms (a BG<3.9 mmol/L), can lead to physical and/or psychosocial impairments, neuroglycopenia, cognitive dysfunction, and if left untreated can lead to brain damage, seizures, and in severe cases, death. About 4-10% of deaths in T1D has been associated with hypoglycemia (17,75,80,82). Studies have shown that, defects in glucagon and catecholamine secretion in response to hypoglycemia is associated with at least a 25-fold increased risk of severe hypoglycemia in T1D (38,61,80,82). In healthy individuals, the detection of low BG in the brain and peripheral tissues, initiates the activation of a series of autonomic and endocrine responses that protect against hypoglycemia, including a decrease in insulin secretion and increases in glucagon, catecholamines, and cortisol secretion which counter insulin action (11). However, these lifesaving responses are impaired in individuals with T1D.

Furthermore, antecedent hypoglycemia in T1D, caused by excessive insulin administration or by prolonged aerobic exercise, plays a major role in impairing the counterregulatory responses even more; thus, increasing the chances of future hypoglycemic episodes. Although the association of recurrent hypoglycemia, or simply repeated hypoglycemic episodes, and the development of counterregulatory impairments is well established, the underlying mechanisms are not fully understood.

It has been shown that the over-production of SST in diabetes results in a significant reduction in circulating glucagon levels that prevent effective recovery from hypoglycemia (8,43,49,58). Hence somatostatin receptor type II antagonists (SSTR2a) are an attractive therapy to improve glucagon secretion during hypoglycemia (7–9,17,57,58,87). In a series of in vivo experiments on rodents, it is shown that a newly developed SSTR2a, PRL-2903, improves the glucagon response to hypoglycemia induced by either insulin or exercise (7–9,87). Additionally, basal glucagon secretion are two-fold higher in SSTR2 knockout mice (58,88). Although SSTR2a have been shown to improve the blunted glucagon response to hypoglycemia in T1D, the underlying mechanisms are not well understood.

The primary goal of this study is to determine the impact of a SSTR2a on plasma insulin and glucagon levels, and whether improvements in glucose production are associated with increased glycogenolysis in a non-diabetic model of counterregulatory failure. We hypothesize that the antagonism of SSTR2 on α-cells relieves the inhibitory effects of somatostatin on glucagon producing α-cells, thereby improving glucagon secretion and facilitating recovery from insulin-induced hypoglycemia.

Methods

Ethics Statement

This study is carried out in accordance with the recommendations of the Canadian Council for Animal Care guidelines and has been approved by the York University Animal Care Committee (Protocol # 2017-7).

Rodent Treatment and Experimental Design

For this protocol, we used a total of 35 healthy, non-diabetic, male Sprague-Dawley (SD) rats (Charles River Laboratories, ~250g body mass post weaned) age ~ 9-10 weeks old. Rats were housed in a light controlled (12-hour light/dark cycle) room with humidity of 50-60%, and temperature of 22-23^{o}C. Rats had ad-libitum access to standard rodent chow (Purina Labdiet,5012, St. Louis, Missouri) and water. Animals were habituated to the vivarium for seven days and following this, body weight, BG, and food intake were monitored daily. After vivarium habituation, six rats were deeply anaesthetized with isoflurane prior to liver and muscle (tibialis anterior and extensor digitorum longus) sample collections. Then rats were subsequently euthanized via exsanguination.

The remainder of the animals (n=29) underwent three consecutive days of recurrent insulin-induced hypoglycemia (BG target 1.7-2.2 mmol/L) to induce counterregulatory failure. Hypoglycemia was induced daily via intraperitoneal (IP) injection of insulin (Humulin-R, Lilly, Canada). It should be noted that all recurrent hypoglycemia events throughout the study occurred without an overnight fast, and with food temporarily removed prior to insulin administration (i.e. hypoglycemia induction).

The insulin dose was reduced with each day of treatment (10U/kg, 8U/kg, 5U/kg). This step-down reduction in insulin administration is performed in order to account for the loss in counterregulatory response that occurs even in healthy rodents with exogenous insulin treatment (89). Two recent studies have shown that a single 2-hour episode of mild hypoglycemia (3.1 mmol/L) reduces counter-regulatory endocrine responses to subsequent hypoglycemia up to 18-24 hours later (14,84). During the experimental phase of the protocol, BG concentrations were measured from a tail prick, using a hand-held glucometer (AlphaTRAK, Abbott). BG was closely monitored every 10 minutes in all rats for 120 minutes post-insulin injection to make sure they stayed within the desired glycemic range of 1.7-2.2 mmol/L. However, if animals did not exhibit the expected fall in BG levels to hypoglycemia (< 3.0 mmol/L) after 60 minutes of the initial dose of insulin, they received another full dose of insulin. Alternatively, if animals dropped below the target range (1.7-2.2 mmol/L), 35% oral dextrose was given in small amounts (0.1-1.0 mL) to bring BG levels back within the target range. Oral dextrose was administered either with a syringe for those rats that drank voluntarily, or through a feeding tube (oral gavage). If severe hypoglycemia developed (BG <1.0 mmol/L) or animal showed signs of distress such as seizures and convulsion, they received 1-2 ml dextrose (IP). In case of unsuccessful recovery (i.e. animal continued to showed signs of distress and/or BG stayed below the desired range), the experiment was stopped, and the animal was provided with food and/or sugar water to help recover blood glucose to a safe level (BG>4.5mmol/L). Upon completion of each hypoglycemic challenge, food was reintroduced (Fig.1).

The day following the recurrent hypoglycemia phase of the protocol, and prior to the last episode of hypoglycemia (i.e. hypoglycemia challenge), liver, tibialis anterior muscle, and extensor digitorum longus muscle samples from seven rats were collected under isoflurane anesthesia to investigate the impact of recurrent hypoglycemia on glycogen concentrations. Rats were then euthanized via exsanguination.

On final day of the study (Day4), a subset of animals (n=22) were randomly assigned to receive either vehicle treatment (10mg/kg, 2% glycerol in water, IP) (n=9), or SSTR2a treatment (10mg/kg in 2 mL of vehicle solution of PRL2903, CDRD, Vancouver, Canada, IP) (n=13) one hour (t=-60 min) before hypoglycemia induction via insulin administration (5 U/kg Humulin R insulin, IP) (t= 0 min). BG was measured every 10 minutes after insulin administration until the target BG of ≤3.5 mmol/L (i.e. clinical hypoglycemia) was reached. Rats were then anaesthetized with isoflurane for portal vein blood, liver, and muscle sample collection, before being euthanized via exsanguination (Fig.2)

Plasma Analysis

On the first and last days of recurrent hypoglycemia (Days 1 and 3, respectively), we performed repeat saphenous vein blood sampling just before insulin injection (t=0 min), when BG reached 3.5 mmol/L, and at 60 minutes post-insulin treatment (t=+60 min). On the final day of the experiments (Day 4), blood samples were collected from a saphenous vein bleed once before drug/vehicle injections (t=-60 min), 1 hour later just prior to insulin injection (t=0 min), and immediately before euthanasia (at BG≤3.5 mmol/L) which was when the portal vein sampling also occured.

All blood samples were collected in potassium-EDTA coated microvette capillary tubes (Sarstedt, Des Grandes Prairies, Montreal, Québec, Canada, Cat #16.444.100), centrifuged at 12,000 rpm for 5 minutes and aliquoted into polyethylene tubes. All samples were preserved at -80^{o}C to be used for the measurement of glucagon (Mercodia AB, Sweden, Cat# 10-1271-01), and insulin (C-peptide) (Crystal Chem, Downer's Grove, Cat# 90055).

Tissue Analysis

Skeletal muscle (tibialis anterior and extensor digitorum longus) and liver samples were collected at baseline (right after vivarium habituation), as well as before and after the hypoglycemia challenge, either with or without the SSTR2a treatment for purpose of evaluating glycogen content (62,90).

Statistical Analysis

It should be noted that, one animal from the vehicle group was excluded from the data analysis as it was deemed to be resistant to hypoglycemia on the final day (hypoglycemia challenge) of the protocol. All data is analyzed using an appropriate t-test, and one-way ANOVA with a criterion of $p<0.05$. All significant differences for ANOVA testing will be evaluated using a Tukey post-hoc test (GraphPad Prism version 7.0). All data are mean ± SEM.

Results

Glucagon counterregulation is attenuated by Day 3 of recurrent hypoglycemia.

Basal and peak/hypoglycemic glucagon levels on the first and last days of recurrent hypoglycemia (Days 1 and 3), and on the final experimental day (Day 4) are shown in Figure 3. No differences were observed in basal glucagon levels between Day 1 (21.63±2.86pg/ml) and Day 3 (20.62±2.12 pg/ml) of recurrent hypoglycemia, nor between the vehicle (11.59±1.78 pg/ml; n=9) and the SSTR2a (12.70±2.36 pg.ml; n=13) treated groups on experimental Day 4. Peak glucagon level during the recurrent hypoglycemic challenge on Day 3 was significantly lower than the values observed on Day 1 (107.49±10.27 pg/mL vs. 168.30±15.22 pg/mL; mean±SEM; p=0.0035), thereby confirming a significant loss in glucagon counterregulation over the three days of repeated exposure to hypoglycemia.

The SSTR2a (PRL-2903) treatment improves glucagon counterregulation following three days of recurrent hypoglycemic.

Peak glucagon levels during hypoglycemia on Day 4 in the vehicle group (44.38±8.70 pg/ml) were significantly lower than on Day 1 (168.30±15.22 pg/ml; $p<0.0001$) and Day 3 (107±15.10.27 pg/ml; p=0.01), suggesting a further decline in glucagon counterregulation with time. In contrast, on Day 4, peak glucagon levels in the SSTR2a-treated group (109.38±15.37 pg/ml) were ~ 2.5-fold higher than in the vehicle group ($P<0.01$), similar to values observed on Day 3 of recurrent hypoglycemia (Fig.3).

SSTR2a pre-treatment delays the onset of hypoglycemia following an insulin overdose.

Figure 4 shows the blood glucose concentrations on Days 1 and 3 of recurrent hypoglycemia and on experimental Day 4. Analysis of whole BG measurements using a handheld glucometer every 10 minutes post-insulin injection during the hypoglycemia challenge shows that SSTR2a-treated rats took ~3 times longer to develop hypoglycemia (i.e. BG≤3.5 mmol/L) as compared to controls (63.85±3.46 min vs. 20±1.08 min, respectively, p=0.0098). Change in BG over the course of time for all fours days of the study is depicted in supplementary figure 1, which clearly illustrates the delayed hypoglycemia development in the experimental group.

C-peptide levels during hypoglycemia are lower with SSTR2a treatment.

C-peptide (pro-insulin connecting peptide) is a by-product of insulin biosynthesis and is produced in a 1:1 ratio with insulin, thereby giving a metric of endogenous insulin secretion in animals with functioning ß-cells (91). Figure 5 shows basal and hypoglycemic C-peptide values during Days 1 and 3 of recurrent hypoglycemia and on experimental Day 4. There were no significant differences in hypoglycemic C-peptide levels during Day 1 of recurrent hypoglycemia (0.85±0.11 ng/mL) and Day 3 (0.90±0.14 ng/mL), however; levels dropped in the SSTR2a group on Day 4 (0.31±0.05 ng/mL) as compared to Day 1 (p=0.017) or Day 3 (p=0.008). Values in the vehicle group also tended to decline on Day 4 (0.63±0.07 ng/mL), as compared to the recurrent hypoglycemia phase, but this drop failed to reach statistical significance.

Recurrent hypoglycemia has no impact on skeletal muscle glycogen stores, but SSTR2a significantly increases hepatic glycogen depletion.

Figure 6 shows no difference in hepatic glycogen levels between baseline (Day 1) and after 3 days of recurrent hypoglycemia (i.e. just before the fourth hypoglycemic challenge). However, hepatic glycogen levels were significantly lower with the SSTR2a treatment compared to vehicle (Glycogen: 11.77±1.41μmol; n=13 vs. 20.66±2.34 μmol; p=0.001). No significant difference was observed in tibialis anterior muscle and extensor digitorum longus muscle glycogen levels throughout the study (Supplementary Fig.2-3).

Corticosterone levels are not likely to be impacted by SSTR2a treatment.

Corticosterone was measured only in a subset of animals. Corticosterone levels during hypoglycemia on Day 4 were similar between SSTR2a treated (536.65±60.50 ng/ml; n=6) and vehicle treated (673±58.15 ng/ml; n= 3) rats (Supplementary Fig.4).

Discussion

The association between recurrent hypoglycemia, and failure of the endocrine and sympathoadrenal responses to hypoglycemia in T1D has been well established (82–84,88). In the current study, we showed that glucagon counterregulatory responses in healthy, non-diabetic rats can be markedly attenuated with each hypoglycemia event in as little as 3-4 days. These findings reiterate the negative consequences of hypoglycemia per se on α-cells function with regards to insulin-to-glucagon dynamics, and in turn, glucose production which appears to be independent of diabetes itself. We also showed that SSTR2a can partially restore the glucagon response to hypoglycemia (BG≤ 3.5 mmol/L) in recurrently hypoglycemic animals with overt counterregulatory failure. This improvement in glucagon secretion was accompanied by reduced insulin secretion (i.e. lower C-peptide levels), and hepatic glycogen levels which likely translates into greater hepatic glucose output, in the SSTR2a treated group compared to the vehicle.

A balance between the main glucoregulatory hormones insulin and glucagon is required to maintain blood glucose levels within a narrow range during periods of fasting, feeding, and physically activity (1). Dysregulation of either insulin or glucagon, as is the case in T1D, causes metabolic dysregulation that ranges from severe hyperglycemia when there is relative hypoinsulinemia and hyperglucagonemia, to severe hypoglycemia which stems from relative excess insulin in the face of counterregulatory failure (lack of glucagon and epinephrine). Hypoglycemia is associated with 4-10% of deaths in T1D (17,74,79,81), and episodic and recurrent hypoglycemia in T1D is a major clinical barrier to maintaining good glycemic control in

diabetes as it sets the stage for a severe or catastrophic event. Defects in glucagon secretion with prolonged diabetes are associated with at least a 25-fold increased risk of severe hypoglycemia (10,30,80). This phenomenon is observed in both rodents (34,60,92) and humans (12,30,80,81,84) with diabetes, but is not necessarily unique to diabetes (83,92). Glucagon responses were reduced by 84% in non-diabetic rats made recurrently hypoglycemic over the course of four weeks (92). Results from our study show that, this recurrent hypoglycemia protocol can effectively impair the glucagon response to hypoglycemia in healthy, non-diabetic rats. Analysis of plasma glucagon concentrations from saphenous vein samples show that, peak glucagon levels on the final day (Day 3) of recurrent hypoglycemia are 57% lower compared to the first day (Fig.3); with no differences observed in basal glucagon levels. Hence, repeated episodes of insulin-induced hypoglycemia can cause short-term imbalances in glucagon-insulin dynamics that can negatively impact glucagon counterregulation, even in healthy animals.

Additionally, our data demonstrates that PRL-2903, a SSTR2a, can improve the blunted glucagon response in recurrently hypoglycemic animals and delay the onset of hypoglycemia. Somatostatin secreted from pancreatic δ-cells is a major paracrine inhibitor of insulin and glucagon under conditions of hypo- and hyperglycemia, respectively (29,49). In rodents, α-cells are generally more sensitive to the inhibitory effects of SST than β-cells (6,49), which could possibly be due to closer spatial association between δ-cells and α-cells which creates a more direct cell-cell contact (6). Studies suggest that over-production of SST in diabetes may be one factor contributing to a blunted glucagon response to hypoglycemia (8,43,49,58). In both healthy, non-

diabetic humans and animals where β-cells are intact, SST may have minimal inhibitory effects on glucagon producing α-cells because of its dominant role in β-cell (insulin) inhibition, whereas in T1D, SST paracrine inhibition of α-cells arises from the absence of β-cells (7,80,82).

In rodents, PRL-2903 improves the glucagon response to hypoglycemia induced by insulin administration or by exercise (7–9). Additionally, glucagon secretion is two-fold greater in SSTR2 knockout mice, suggesting SSTR2 may be an effective therapeutic target to augment glucagon secretion (58). This is consistent with our data showing SSTR2a treatment increases glucagon responses by ~2.5-fold which likely contributed to delaying the development of hypoglycemia by 3.2-fold in recurrently hypoglycemic animals. Improvements in glucagon secretion with SST antagonism also increased the mobilization of glucose into circulation as evidenced by the reduction of liver glycogen content which was likely responsible for delaying the onset of hypoglycemia (69). We thought that the inability to recover from hypoglycemia in both diabetic and non-diabetic models could be due to depletion of glycogen stores as a result of repeated exposure to hypoglycemia (53, 60). Surprisingly, analysis of hepatic glycogen content in this study shows that, recurrent hypoglycemia does not affect hepatic glycogen storage levels. This may be the result of impairments in glycogenolytic capacity in the setting of counterregulatory failure (62). Alternatively, the lack of difference in hepatic glycogen levels during recurrent hypoglycemia could be due to its full restoration to normal levels due to plasma increased glucose and insulin levels after food intake which stimulate glycogen synthesis. However, hepatic glycogen levels in the SSTR2a treated group are 47% lower than the vehicle group after hypoglycemia exposure on the final (Day 4)

experimental day. This novel finding indicates that, lower hepatic glycogen levels in the PRL-2903-treated group is possibly the result of a more robust glucagon response that enhances glycogenolytic capacity (62). Also, SSTR2a may be indirectly suppressing insulin secretion and hence, less effective glycogen deposition. No differences were observed in tibialis anterior and extensor digitorum longus glycogen levels either with or without SSTR2a treatment. This is likely due to the lack of glucagon receptors in muscle. Mobilization of muscle glycogen stores is predominantly mediated by epinephrine during exercise, and the produced glucose is used locally, not systematically. Therefore, even though most glycogen is stored in skeletal muscle (68), muscle glycogen breakdown mainly provides energy locally during high intensity exercise (30,68). However, no differences in muscle glycogen levels is an important observation, as it highlights the specific effects of SSTR2a on glucagon responses specifically, and not other counterregulatory mechanism such as improvement in epinephrine secretion.

Another novel finding of this study is the observation of lower C-peptide levels in the SSTR2a group compared to the vehicle group during hypoglycemia on experiment Day 4. The C-peptide results contradict our hypothesis which anticipated no significant difference in insulin secretion with or without SSTR2a treatment. Interestingly, C-peptide levels are significantly lower in the SSTR2a treated group compared to vehicle during hypoglycemia on Day4. Studies have shown that, although SST inhibits both glucagon and insulin; it predominantly inhibits insulin secretion when β-cells are intact (7,80,82). Therefore, in the presence of SSTR2 antagonist, there may be more free SST available for binding to SSTRs on β-cells which could explain the lower insulin levels in the SSTR2a treated group. Moreover, higher glucagon levels during hypoglycemia in the

treatment group could also result from lower insulin levels as glucagon is a paracrine inhibitor of insulin secretion under conditions of hypoglycemia (1,20,93). Therefore, inhibitory actions of SST along with higher levels of glucagon (i.e. better glucagon counterregulation) leads to a greater inhibition on insulin secretion, and in turn lower insulin levels with SSTR2a treatment during hypoglycemia.

Hypoglycemia activates the hypothalamo-pituitary-adrenal (HPA) axis which regulates the secretion of glucocorticoids (76). Glucocorticoids aid in the recovery from more prolonged and/or more severe hypoglycemia (83,84,92). In rats, corticosterone is the active glucocorticoid. In general, recurrent insulin-induced hypoglycemia activates the HPA (hypothalamic, pituitary, adrenal axis) which regulates secretion of glucocorticoids and epinephrine (76). Thus, hypoglycemia induced increase in corticosterone levels can facilitate recovery from hypoglycemia (2,85,94).We looked at corticosterone levels in about half of the animals (n=10), but there was no significant difference between the SSTR2a and the vehicle group. This may be an indication that the antagonist only targets the endocrine pancreas, and more specifically, the α- and ß-cells, rather than whole body counterregulatory systems. However, studies looking at effects of SSTR2a on counterregulatory responses to hypoglycemia in STZ diabetic models show improvement in both glucagon and corticosterone counterregulatory responses (8,9)

Conclusion

In conclusion, we showed that SST antagonism with PRL-2903 may be an effective way to improve glucagon secretion following recurring exposure to hypoglycemia. This improvement appears to be associated with a reduction in insulin secretion and increased hepatic glycogenolysis. As somatostatin normally inhibits glucagon and its levels are abnormally high in T1D diabetes, SSTR2a treatment may be a useful therapeutic approach to improve glucagon responses to insulin and/or exercise induced hypoglycemia in patients with T1D. This treatment would facilitate a better glycemic control for patients with T1D without the fear of hypoglycemia.

Figures

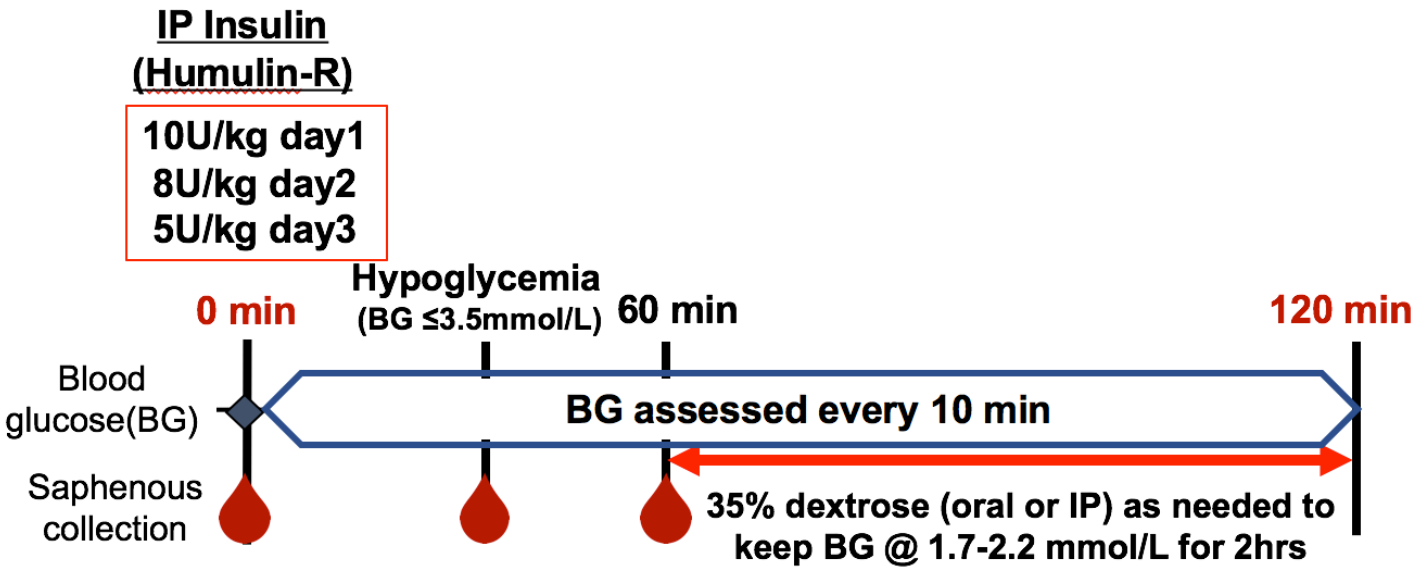

Figure 1. Experimental timeline: Recurrent hypoglycemia period (Days 1-3). Twenty-two (n=22) male Sprague-Dawley rats underwent three consecutive days of repeated insulin-induced hypoglycemia with 10-, 8-, and 5-U/kg of Humulin-R insulin on Days 1-3, respectively, to induce moderate to severe hypoglycemia (1.7-2.2 mmol/L) for up to 120 minutes. Blood samples were collected from the saphenous vein at baseline (time=0 min), at BG ≤ 3.5 mmol/L (i.e. clinical hypoglycemia), and 60 minutes post insulin injection for the determination of plasma glucagon and C-peptide levels.

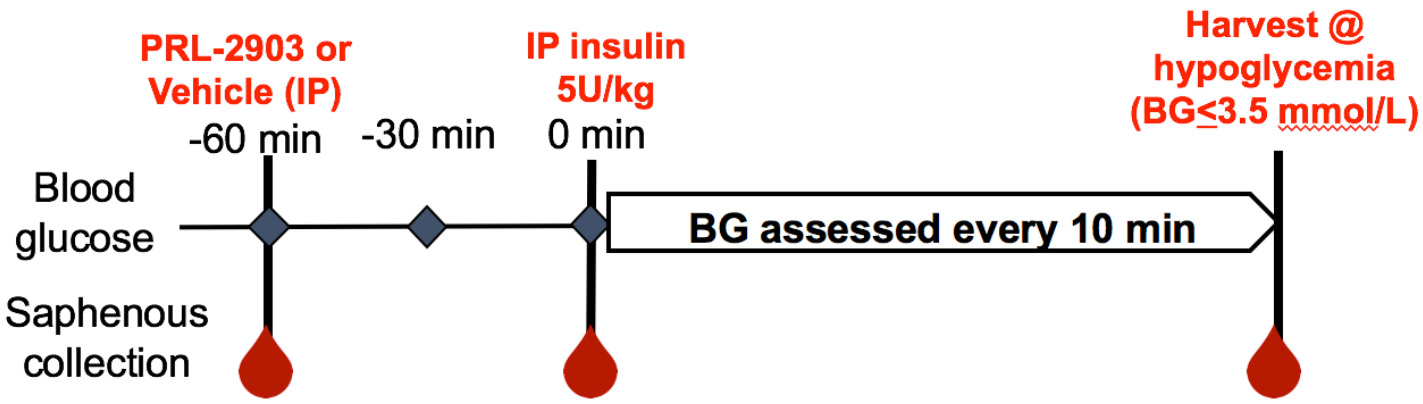

Figure 2. Experimental timeline: Experimental hypoglycemic challenge with or without SSTR2a treatment (Day 4). One hour prior to the last episode of insulin-induced hypoglycemia (time= -60 min), animals were randomly assigned to the SSTR2a treatment group (n=13) or a vehicle control group (n=9). All animals received an IP injection of Humulin-R insulin (5U/kg) 60 minutes after drug or vehicle administration (time=0 min). Blood glucose levels were assessed at baseline (time=-60 min, just before drug or vehicle administration), 30-minutes post drug/vehicle administration (time=-30 min), just prior to insulin administration (time=0 min), and every 10 minutes after that until the end of the protocol (defines asBG≤ 3.5 mmol/L). Saphenous vein samples were collected at baseline, prior to insulin administration, and at the onset of hypoglycemia for the determination of plasma glucagon, C-peptide and corticosterone levels.

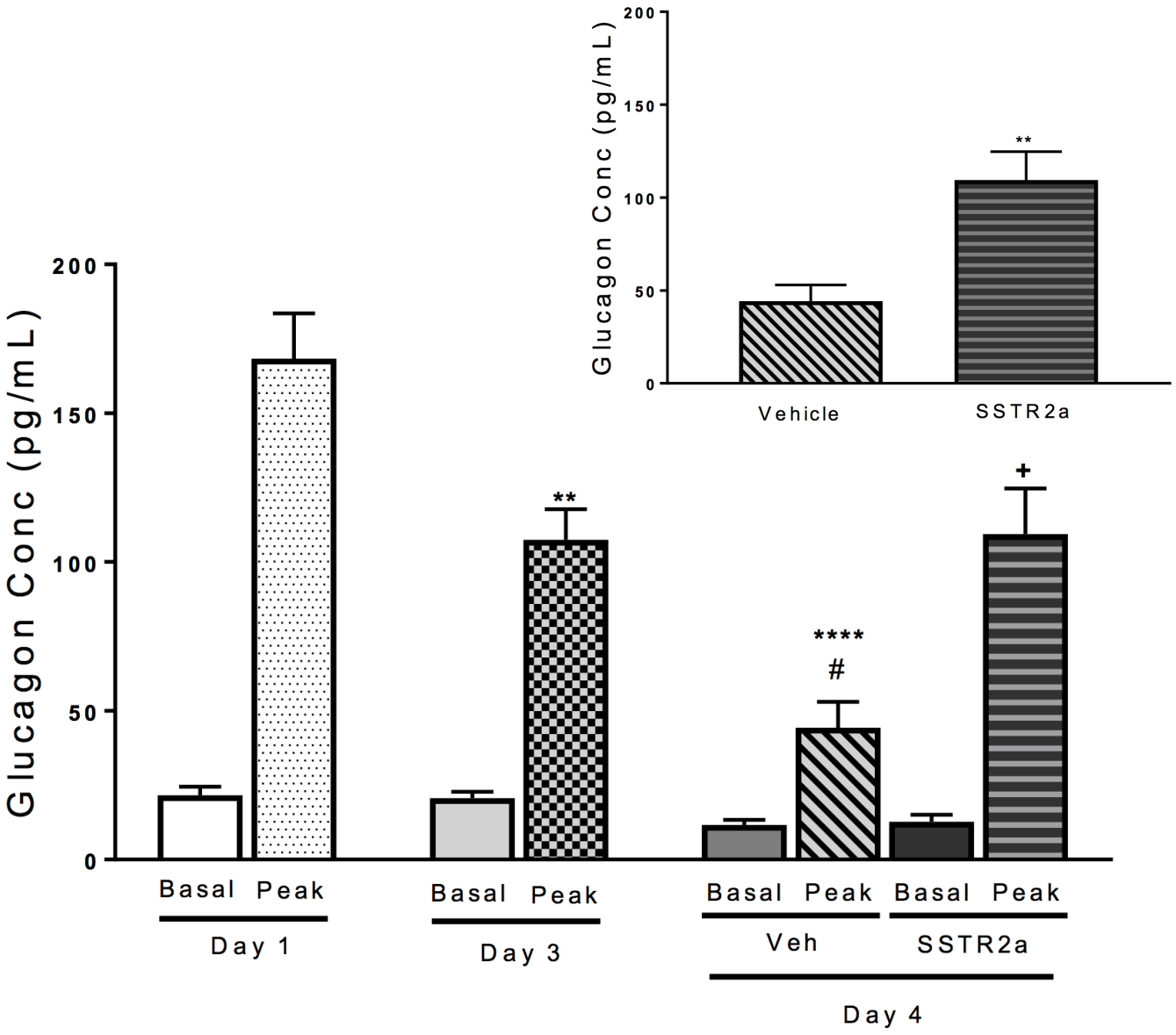

Figure 3. Baseline and peak (hypoglycemic) glucagon levels. Basal vs hypoglycemic glucagon concentrations (pg/mL) during the recurrent hypoglycemia phase (Day 1 and Day 3; n=22), and challenge day (Day 4) either with (n=13) or without (n=9) the SSTR2a (PRL2903) pre-treatment. Note: *,**,***,**** indicates significance compared to Peak/D1 P<0.05, 0.01, 0.001, 0.0001 respectively; #, indicates significance compared to Peak/D3 ; +, indicates significance compared to Vehicle. Inset: Comparison of peak glucagon responses during the hypoglycemia challenge on Day 4. All data is presented as mean±SEM.

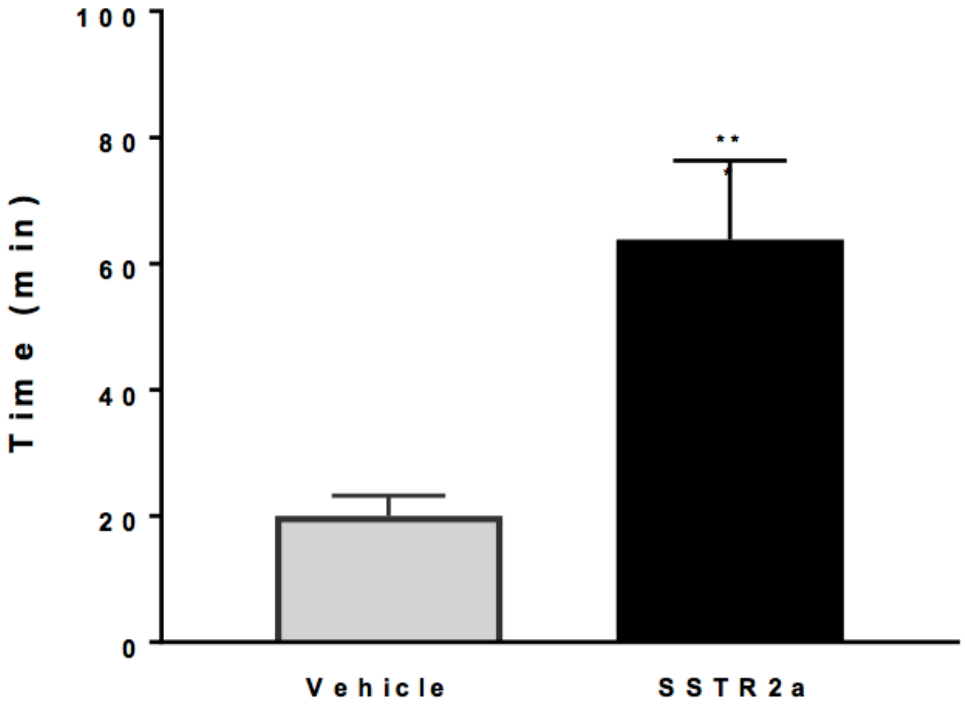

Figure 4. Time to reach hypoglycemia with and without SSTR2a treatment. Time (min) to development of hypoglycemia (BG≤3.5 mmol/L) for the SSTR2a (n=13) the vehicle groups (n=9) (p=0.001). Data is presented as mean ± SEM.

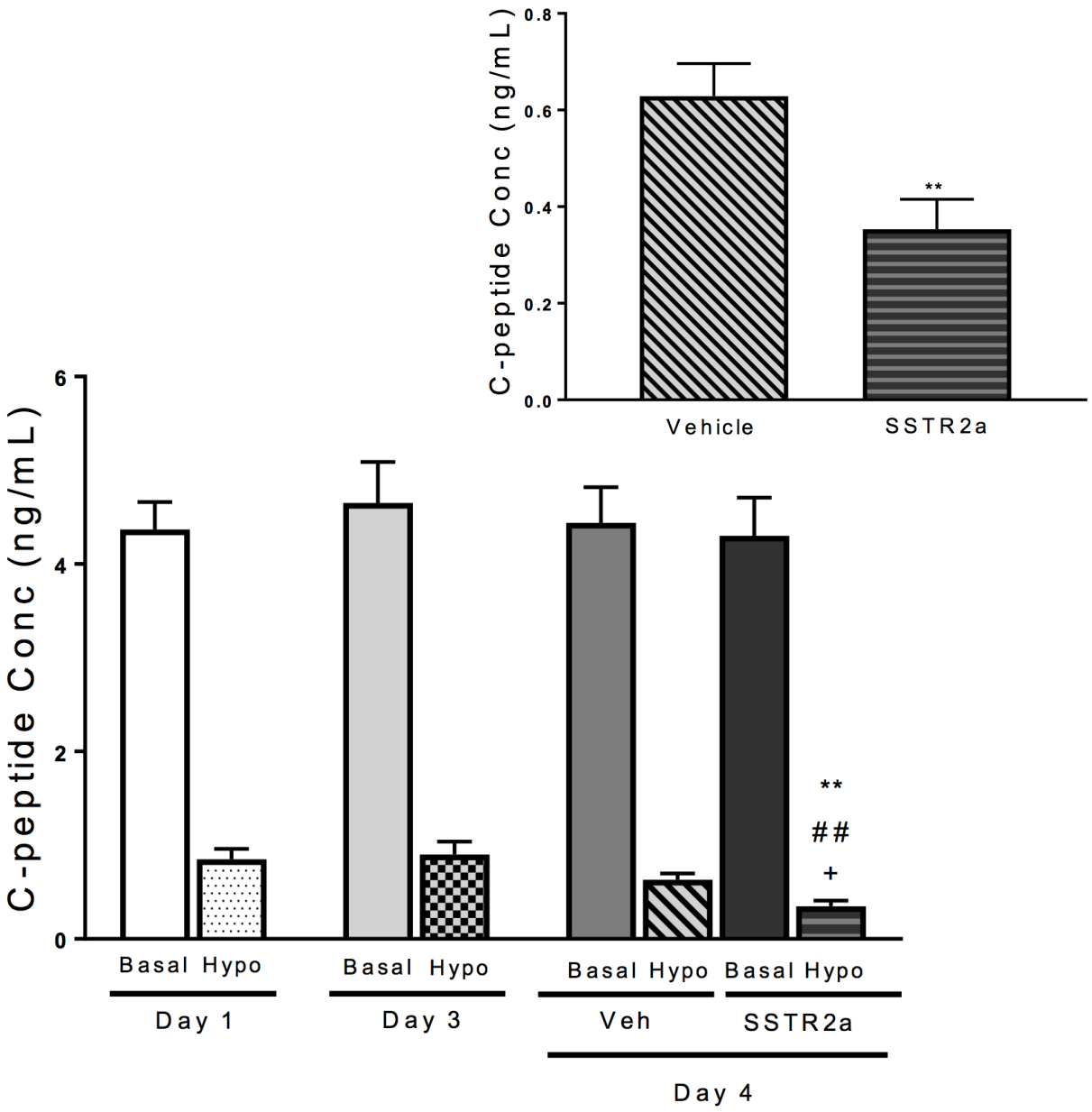

Figure 5. Baseline and nadir (hypoglycemic) C-peptide levels. Basal vs nadir (hypoglycemic) C-peptide concentrations (pg/mL) during the recurrent hypoglycemia phase (n=22), and challenge day with (n=13) or without (n=9) the SSTR2a (PRL2903) pre-treatment. Note: *,**,***,**** indicates significance compared to Peak/D1 P<0.05, 0.01, 0.001, 0.0001 respectively; #, indicates significance compared to Peak/D3 ; +, indicates significance compared to Vehicle. Inset: Comparison of C-peptide levels during the hypoglycemia challenge on Day 4. Data is presented as, mean ± SEM.

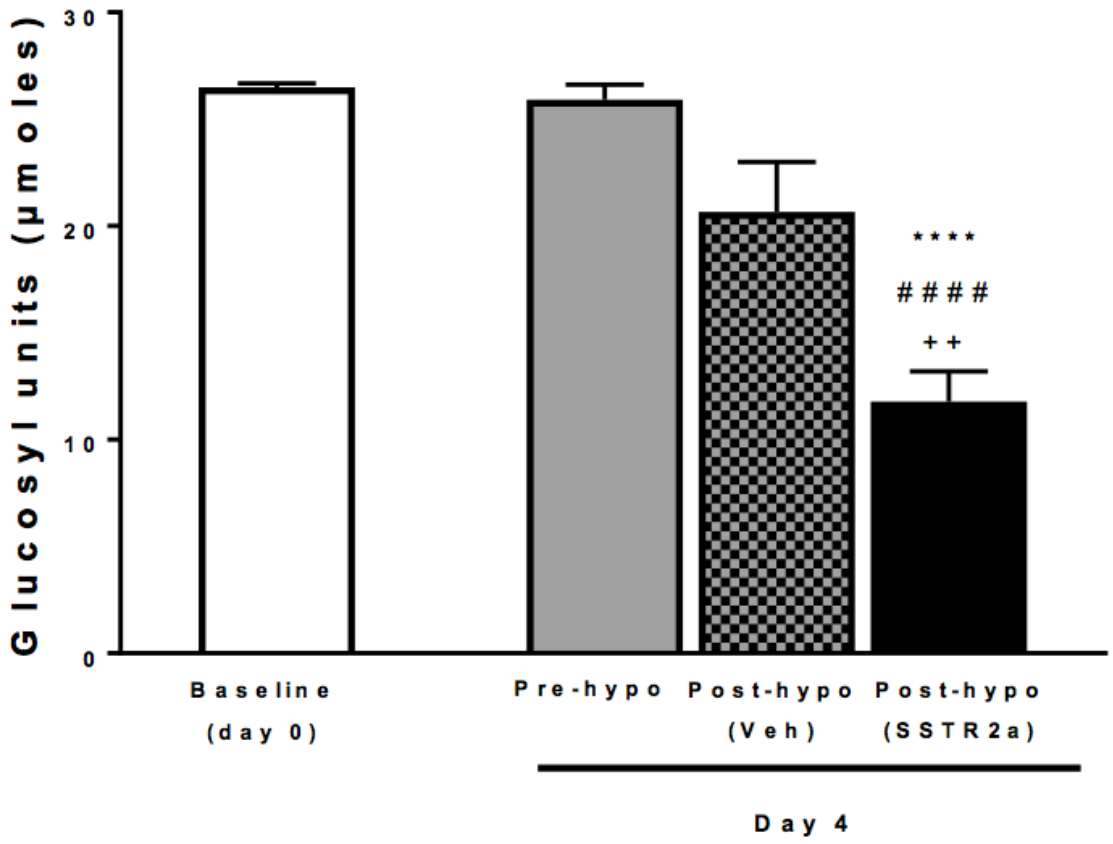

Figure 6. Hepatic (liver) glycogen levels. Baseline (day 0) represents normal hepatic glycogen levels in healthy rats that were sacrificed right after the habituation period (i.e. no hypoglycemia exposure). Pre-hypo is after three days of recurrent hypoglycemia but no hypoglycemia challenge. Post hypo, Vehicle and SSTR2a is after hypoglycemia challenge on day 4. Note: *,**,***,**** indicates significance compared to baseline (day 0); P<0.05, 0.01, 0.001, 0.0001 respectively; #, indicates significance compared to pre-hypo ; +, indicates significance compared to post-hypo, Vehicle. Data is presented as, mean ± SEM.

Summary

In summary, we show in this study that three days of recurrent hypoglycemia dramatically attenuates the glucagon response to hypoglycemia. We see that the administration of a SSTR2a can improve glucagon counterregulation, lower endogenous insulin secretion and increase hepatic glycogen breakdown during hypoglycemia, and dramatically increase the time before hypoglycemia onset following exogenous insulin administration in healthy male Sprague-Dawley rats. These findings support a growing body of evidence that SSTR2a treatment may have clinical benefit for patients living with diabetes who have frequent episodes of hypoglycemia caused by insulin therapy and or regular physical actively.

Limitations and Future direction

In this study, we wanted to investigate the impact of SSTR2a on glucagon and other counterregulatory pathways/factors to learn more about the pathophysiology of the drug. Therefore, we looked at corticosterone as a non-pancreatic regulator of BG and counterregulatory responses. Due to inadequate amount of plasma samples, we were not able to look at corticosterone levels in all animals, and throughout the recurrent hypoglycemia phase of the study. Corticosterone was only measure in ten animals, and only during the final experimental day (Day 4). Since the corticosterone data collected from those rats didn't show any trends of difference between the SSTR2a treated and the vehicle groups, we decided to save the remainder of the plasma, from the remainder of animals, for SST measurement as it is a major inhibitory regulator of both insulin and glucagon, and plays a major role in counterregulatory responses to hypoglycemia. Having a low n-value for both the treatment and the control group could be the reason why we didn't see any trends in the corticosterone data. Other than looking at the impact of SSTR2a on corticosterone, it would be interesting to look at the effects of repeated hypoglycemia on baseline and peak corticosterone levels.

SST levels were measured using ELISA assays, but the data is excluded from the study as the assays used had gone bad because of improper handling during shipment, so the collected data is not reliable.

All hormones, including glucagon, insulin, SST, and corticosterone were measured in peripheral (saphenous) as well as portal circulation, but portal vein data is excluded due to use of expired EDTA for portal vein collection. The data is significantly

variable, and unreliable. It would be interesting to compare hormonal levels in portal and peripheral circulation as pancreatic hormones bypass the liver before entering the peripheral circulation. Additionally, the antagonist treated animals show an interesting pattern of blood glucose fluctuations overtime. However, we don't clearly know what exactly happens to plasma glucagon and insulin levels during Day 4 as we only measured insulin and glucagon at the beginning and at the end of the experiment (i.e. at baseline and at the onset of hypoglycemia). Whether or not the changes in peripheral insulin and glucagon concentrations during the final hypoglycemic event reflect the same pattern as plasma glucose remains a question.

In the future, we are hoping to replicate this study addressing all the limitations mentioned above, and introduce more variables such as diabetes, sex and age differences, and method of substance (insulin/SSTR2a) administration (IP vs Subcutaneous) to the current model to amplify the importance of our current findings, and hopefully make them clinically applicable.

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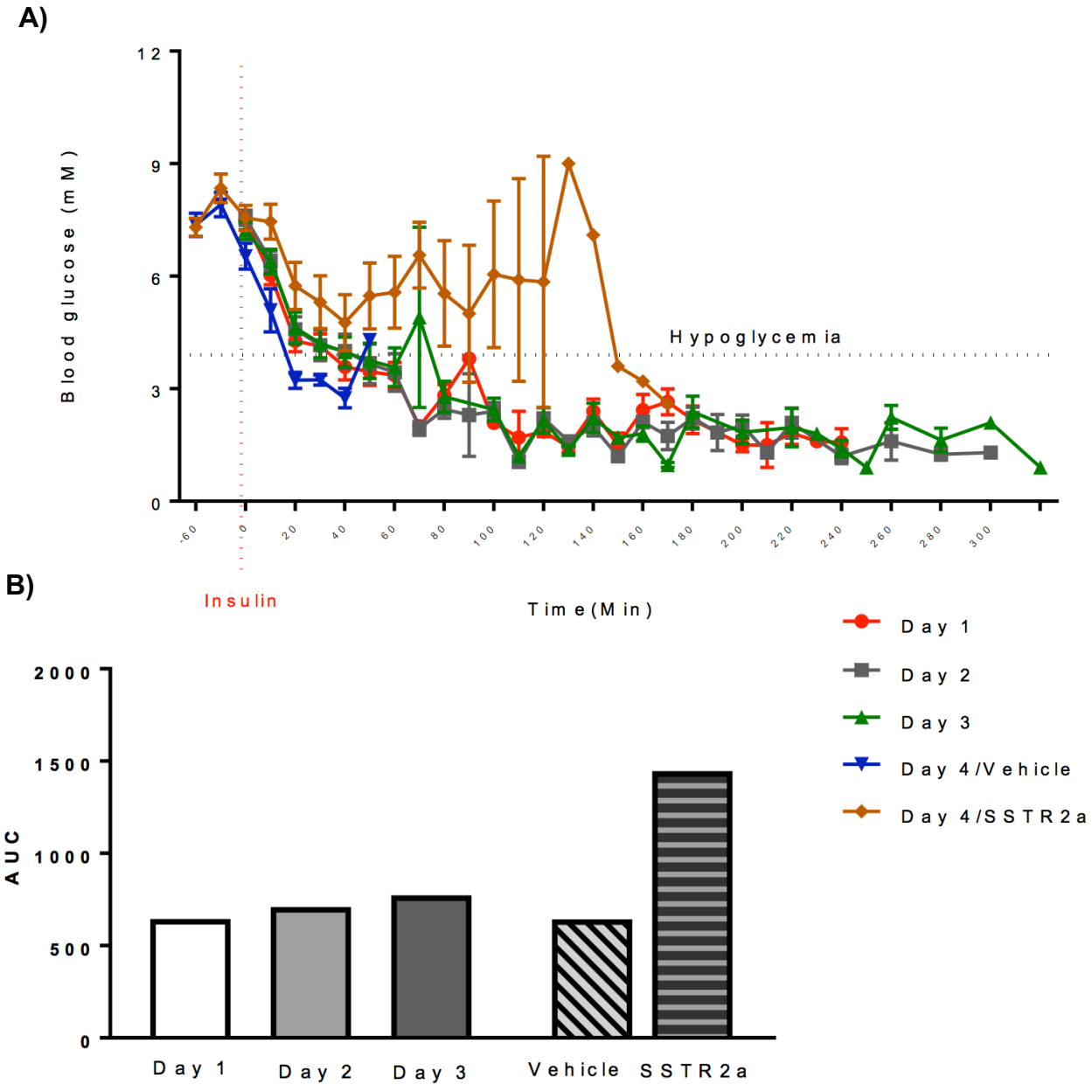
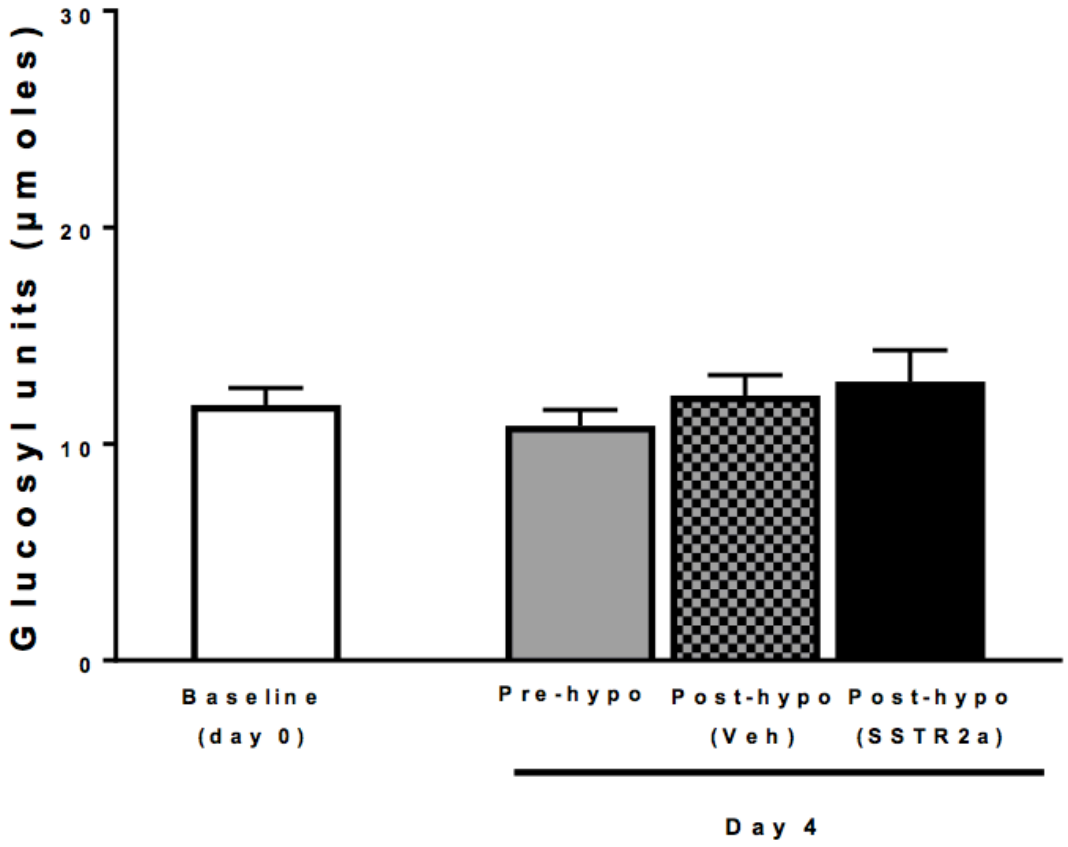
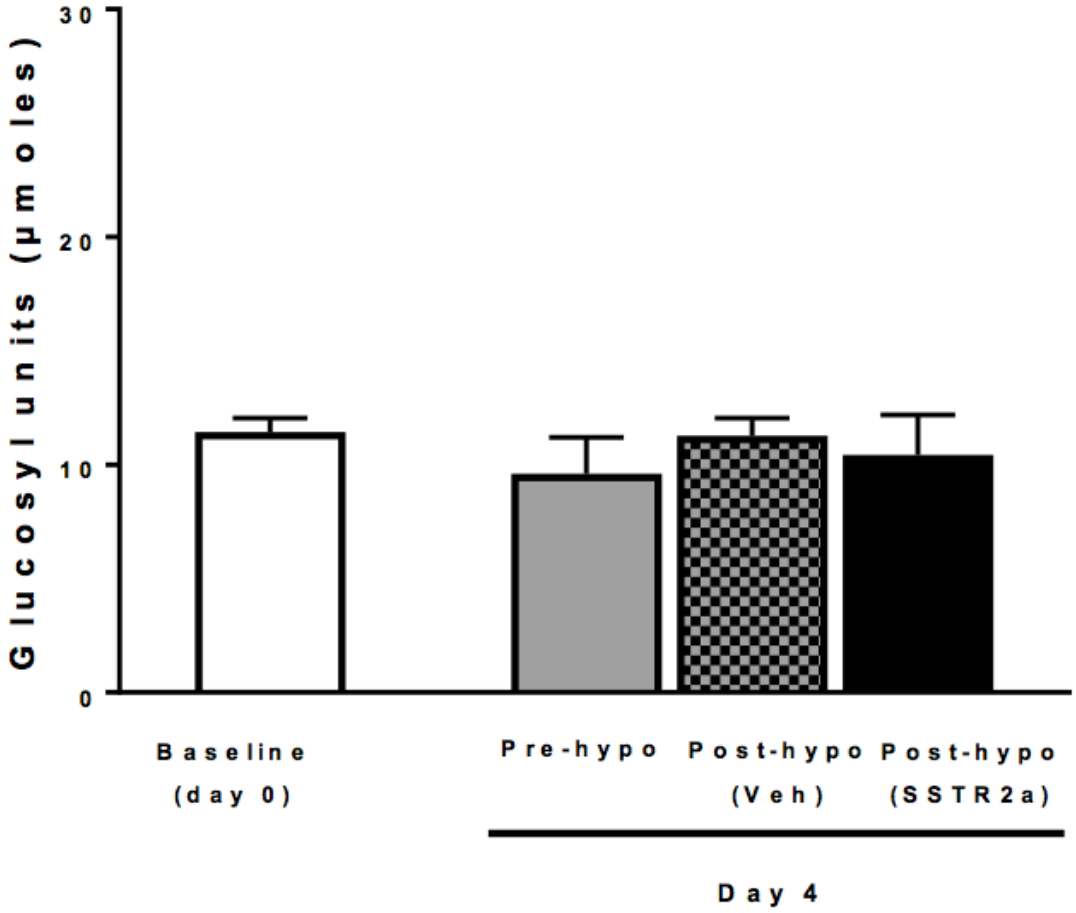
Appendix A: Supplementary Figures



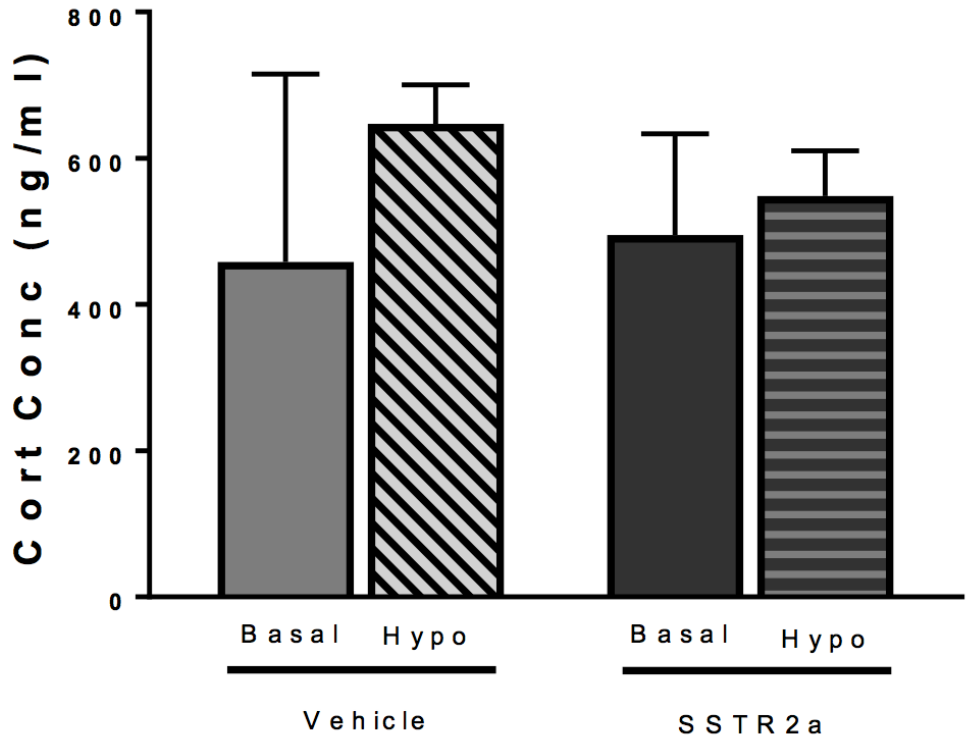
Supplementary Figure 1. A) Change in blood glucose over time. Blood glucose changes for all animals (n=22) during recurrent hypoglycemia, and hypoglycemia challenge with (n=13) and without (n=9) SSTR2a treatment. **B)** Area under the curve (AUG) for the pooled blood glucose change over time. Data is presented as, Mean±SEM.



Supplementary Figure 2. Tibialis anterior glycogen levels. Baseline (day 0) represents normal skeletal glycogen levels in healthy rats that were sacrificed right after the habituation period (i.e. no hypoglycemia exposure). Pre-hypo is glycogen levels after three days of recurrent hypoglycemia but no hypoglycemia challenge, and Post hypo, Vehicle and SSTR2a, is after hypoglycemia challenge on day 4. Data is presented as, mean ± SEM.



Supplementary Figure 3. Extensor Digitorum Longus glycogen levels. Baseline (day 0) represents normal skeletal glycogen levels in healthy rats that were sacrificed right after the habituation period (i.e. no hypoglycemia exposure). Pre-hypo is glycogen levels after three days of recurrent hypoglycemia but no hypoglycemia challenge, and Post hypo, Vehicle and SSTR2a, is after hypoglycemia challenge on day 4. Data is presented as, mean ± SEM.



Supplementary Figure 4. Corticosterone concentrations with and without SSTR2a treatment. Corticosterone levels on hypoglycemia challenge day (i.e. final day of insulin-induced hypoglycemia) at baseline (before treatment administration; time=-60 min), and at hypoglycemia (BG≤3.5 mmol/L) in saphenous vein collections. Data is presented as, mean ± SEM.