

**INTERLEUKIN-6 ENHANCES GLUCAGON SECRETION:  
AMPLIFICATION VIA THE PANCREAS AND BRAIN**

By

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## **DEDICATION**

For the teachers along the way who inspired me more  
than they could ever know, without your passion  
and encouragement, I would not be where I am today.

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## CHAPTER I: INTRODUCTION

Inflammatory diseases such as sepsis or type 2 diabetes have more in common than some might imagine. First, both diseases represent huge costs in United States health care. In 2008, \$14.6 billion was spent for the treatment of sepsis (48), and in 2012, \$245 billion was the estimated total economic cost of diabetes (2). While diabetes and acute or chronic inflammation are vastly different in etiology and pathology, both settings are associated with an elevation in plasma pro-inflammatory cytokines such as interleukin-6 and feature profound alterations in glucose homeostasis. In both cases, controlling glycemia can improve patient morbidity and mortality (135). Much of glucoregulatory research has focused on either ameliorating the insulin resistance that develops in tissues such as liver, muscle, and adipose. However, a story that has been less explored until recent years is that of glucagon. In light of recent work, the field is beginning to shift to a more “glucagonocentric” view of managing glycemia (136).

Septic patients often exhibit hyperglucagonemia (85), and in diabetics, the ratio of glucagon:insulin or the absolute glucagon concentration in plasma is often elevated. Recent work has pointed to a critical role of glucagon in contributing to the hyperglycemia observed in rodent models of diabetes. Hyperglycemia associated with insulin deficiency does not manifest in the absence of glucagon-dependent signaling (73). While this work was done in models of type 1 diabetes rather than type 2 diabetes, it should be mentioned that both disease states are associated with elevated plasma glucagon and elevated pro-inflammatory cytokines. Therefore, new strategies to limit hyperglycemia by reducing glucagon secretion could make vast advancements in

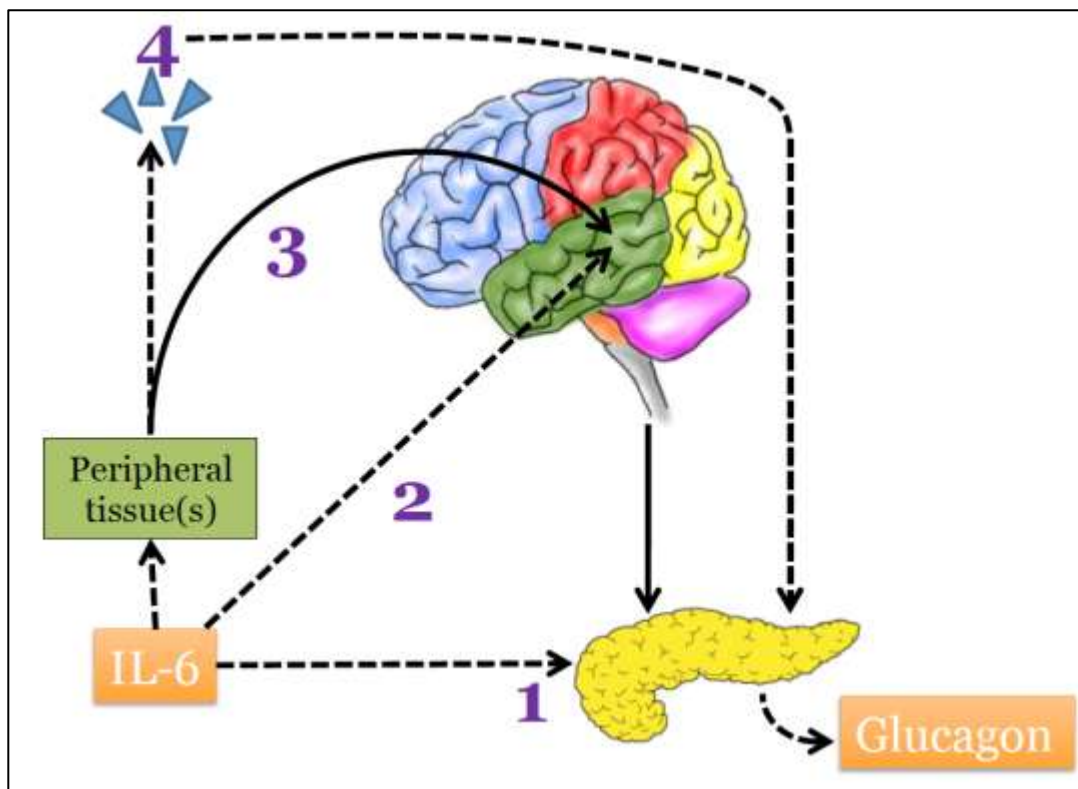
maintenance of glucose homeostasis and improve patient morbidity and mortality for multiple inflammatory diseases.

Current therapeutic strategies for the management of glycemia in type 2 diabetes are mostly focused on either enhancing insulin secretion or increasing insulin sensitivity. Common treatments for managing type 2 diabetes are not typically used for individuals with sepsis. Currently, the most common method to manage the hyperglycemia in septic individuals is via exogenous insulin administration. However, hypoglycemia is a common complication in these patients as clinicians try to regulate glycemia with insulin, and incidents of hypoglycemia also increase patient morbidity and mortality. An alternative (or perhaps an additional) approach to current therapies in the treatment of diabetes or sepsis may be to limit glucagon secretion by reducing inflammation.

Research from the McGuinness lab and others has demonstrated that the inflammatory cytokine, interleukin-6 (IL-6) can regulate the secretion of glucagon from alpha cells of the pancreatic islets of Langerhans (6, 34, 133). Moreover, others have demonstrated that inhibition of IL-6 signaling improves HbA1c levels in diabetic individuals with rheumatoid arthritis (97). While Ellingsgaard and colleagues found that IL-6 can regulate glucagon secretion in a slow, transcriptional-dependent manner (34), work from the McGuinness lab has demonstrated that IL-6 can also act more rapidly to increase plasma glucagon (133). Therefore, the site by which IL-6 acts to enhance glucagon secretion is not yet clear. The primary goal of my thesis work was to determine the route by which IL-6 enhances glucagon secretion.

Given that the IL-6 receptor subunit (IL-6R $\alpha$ ) is expressed rather ubiquitously throughout the body, there are several conceivable sites of action for circulating IL-6 to

enhance glucagon secretion from alpha cells. First of all, IL-6 could directly act on islet alpha cells (in a manner faster than what was previously characterized) (34) (**Figure 1.1**). Alternatively, IL-6 could be acting within the brain to modulate autonomic drive to the islet. IL-6 can cross the blood-brain barrier (5) as well as be produced within the brain (36), and IL-6 receptors are expressed rather ubiquitously throughout the brain, including within critical glucose-sensing brain regions (137). Peripheral tissues such as the liver are exquisitely sensitive to IL-6. It is possible that IL-6 acts through peripheral tissues to either stimulate release of an endocrine factor or modulate vagal afferents, which, in turn, modulate autonomic drive to the pancreas.



**Figure 1.1: Ways in which IL-6 may stimulate glucagon secretion.** IL-6 may either (1) act directly on the alpha cells, (2) act via brain to modulate autonomic drive to the pancreas, or act on peripheral tissues to either (3) modulate vagal input or (4) promote endocrine factor secretion to modulate glucagon secretion.

The body of work in this dissertation focused on discovering whether IL-6 could modulate glucagon secretion by 1) directly acting on islets and/or 2) directly acting within the brain to modulate autonomic drive. Given that the McGuinness lab observed that IL-6 elicited a rapid glucagon secretory response in their studies (133), my hypothesis was that IL-6 does not act directly on islets; the interaction of IL-6 with islets has previously been characterized to be a slow, transcriptional-dependent process (34). Instead, I hypothesized that IL-6 acts in a more rapid manner by modulating autonomic drive through direct actions within the brain. I focused on determining the role of central IL-6 on glucagon secretion in two known settings normally associated with both elevated plasma pro-inflammatory cytokines and elevated plasma glucagon, 1) endotoxemia (using lipopolysaccharide) and 2) hypoglycemia (using hyperinsulinemic/hypoglycemic clamps).

The findings of this work suggest that IL-6 is a central player in glucagon secretion in multiple inflammatory settings. IL-6 acts within the brain to augment glucagon secretion both following a lipopolysaccharide challenge as well as during insulin-induced hypoglycemia. Interestingly, central IL-6 has no effect on glucagon secretion alone; rather it only amplifies glucagon secretion in known settings of elevated adrenergic tone. In contrast to my initial hypothesis, IL-6 was not only found to act via the brain to augment glucagon secretion, but it also modulated glucagon secretion from isolated islets. As in the brain, IL-6 alone did not have an effect on glucagon secretion. However, IL-6 augmented epinephrine-stimulated glucagon secretion. Given that autonomic drive and plasma epinephrine increase following lipopolysaccharide challenge as well as during insulin-induced hypoglycemia, these results suggest that IL-

6 can not only enhance neural drive to the islet in settings of stress, but it can also increase the effectiveness of epinephrine at islets to amplify glucagon secretion.

## **Lipopolysaccharide as a model for glucagon secretion**

### *Characteristics of lipopolysaccharide*

There are three stages involved in pathogenesis of infection. First is the release of bacterial toxins. In the case of Gram-negative bacterial infection, lipopolysaccharide (LPS) is one of those toxins released. The second phase in the pathogenesis of infection is the innate immune system's response. Resident macrophages primarily in the liver release cytokines and drive the overall response to the pathogen. Both pro- and anti-inflammatory cytokines are normally released in the response, and this response helps to mobilize the immune system to attack and destroy the pathogen. However, if the cytokine response is too robust (stage 3), and if the pro-inflammatory mediators far exceed the anti-inflammatory mediators, sepsis can develop. Sepsis is the body's response to systemic infection and is defined as an infection accompanied by two or more of the following: hypo or hyperthermia, tachycardia, tachypnea, and drastically altered leucocyte profiles (leukocytosis or leukopenia) (82). The resultant vascular and metabolic responses can ultimately lead to organ system collapse and death.

LPS interacts with the innate immune system via the Toll-like receptor 4 (TLR4) to activate macrophages. Toll-like receptors are expressed by select cells of the innate immune system (such as macrophages and dendritic cells), and they normally recognize specific regions of common pathogens called pathogen-associated molecular

patterns (PAMPs). Signaling through TLR4 results in a cascade of events that will ultimately drive the destruction of the PAMP-containing (LPS-harboring) invaders (bacteria in this case). Demonstrating the importance of TLR4, animals lacking TLR4 are protected from the negative metabolic and cardiovascular effects of LPS (39, 57). Further, various single nucleotide polymorphisms on the TLR4 gene have been identified that are associated with increased susceptibility of individuals to Gram-negative bacterial infection (76, 91, 113).

Administration of LPS (in the absence of Gram-negative bacteria) can mimic many of the cardiovascular and metabolic responses that are observed with exposure to Gram-negative bacteria. Thus, LPS has been used as a model system to understand the complex interaction between the pathogen, the immune system, and metabolism. In response to high doses of LPS, there is systemic vasodilation, cardiac dysfunction and a robust activation of the neuroendocrine system. With even higher doses, organ dysfunction can manifest (as is observed clinically during severe sepsis). Because the dose of LPS can be easily manipulated, investigators can control the extent of the magnitude of the activation. Importantly, low doses of LPS (1 mg/kg) were used in the studies presented within this dissertation; this dose is associated with neither organ dysfunction nor cardiovascular complications and allowed us to focus solely on the metabolic response to LPS.

### *The metabolic response to lipopolysaccharide*

In response to bacterial invasion, the host activates an inflammatory response that alters both the cardiovascular and metabolic systems. The cardiovascular responses include marked vasodilation, impaired cardiac function, a redistribution of

organ blood flow, and, depending upon the severity of the insult, either an increase or decrease in cardiac output. These cardiovascular alterations are also associated with robust metabolic and neuroendocrine responses that interact to have profound effects on metabolism, ultimately leading to the acceleration of glucose, fat (increased lipolysis), protein (muscle proteolysis), and amino acid flux. The net effect is a loss of protein coupled to hyperglycemia (85).

A net release of glucose from the liver is observed early with a high-dose (10 mg/kg) challenge of LPS (70). Meinz demonstrated that this early elevation is most likely due to increased glycogenolysis rather than gluconeogenesis (88), while others showed that increased gluconeogenesis becomes more important with longer exposure time (85). Glycemia is briefly elevated despite concurrently elevated plasma insulin (22). Work conducted by Clemens and colleagues suggests that the LPS-induced insulin resistance occurs both at the liver (sustained hepatic glucose production in spite of elevated circulating insulin) as well as the muscle and other peripheral tissues (decreased insulin-stimulated glucose utilization) (22). During this time, there is also elevated plasma lactate, increased amino acids, and elevated plasma glucagon (25, 58). It has been previously demonstrated that substrate supply alone does not facilitate gluconeogenesis; animals treated with the gluconeogenic precursors alanine or lactate do not increase production of glucose (24, 28). However, elevated fatty acids in the presence of gluconeogenic precursors promotes gluconeogenesis (86). Acute inflammation increases adipose tissue lipolysis and thus increases availability of fatty acids (146); thus, this increased gluconeogenic precursor supply combined with the elevated supply of fatty acids could explain the increased gluconeogenesis.



Depending on the dose of LPS, either hyperglycemia or hypoglycemia can develop. High-dose LPS is often used in animal models to mimic some of the immunologic complexity of sepsis that is observed clinically. Following exposure to LPS, The transient hyperglycemia as described above can be followed by moderate to severe hypoglycemia. The hypoglycemia that develops is due to both depleted glycogen stores as well as an impairment in gluconeogenesis (69). However, sometimes despite impaired liver function, gluconeogenesis can be increased due to the combined effect of the profound activation of the neuroendocrine system and the increase in gluconeogenic substrate supply (85). **Table 1.1** summarizes the metabolic and neuroendocrine responses that were found following exposure to high-dose LPS.

**Table 1.1: The metabolic and neuroendocrine response to high-dose endotoxin.**

Analyte	% Change from Basal	Metabolic Parameter	% Change from Basal
Glucose, 1 hour	↑69%	R <sub>a</sub> , 1 hour	↑70%
Glucose, 4 hours	↑19%	R <sub>a</sub> , 4 hours	↑28%
Lactate, 4 hours	↑86%	Glucose recycling, 4 hours	↑290%
Insulin, 4 hours	↓29%	Glucose clearance, 1 hour	↑17%
Glucagon, 4 hours	↑340%	Liver Glycogen, 4 hours	↓86%
Norepinephrine, 4 hours	↑750%	Muscle Glycogen, 4 hours	↓27%
Epinephrine, 1 hour	↑3300%		
Epinephrine, 4 hours	↑2500%		
Corticosterone, 4 hours	↑300%		

Adapted from Lang *et al.* (1987). *Circ Shock*. and Buday *et al.* (1986) *Circ Shock*. R<sub>a</sub> indicates endogenous glucose production.

As with high dose administration, animals treated with low-dose LPS can display transient hyperglycemia. However, treatment with low-dose LPS does not result in the profound hypoglycemia that can often be observed with high-dose treatment. Rather,

blood glucose levels return to basal levels gradually without exhibiting hypoglycemia. Lang and colleagues suggest that hepatic glucose production can be maintained in this setting to maintain a constant supply of glucose for peripheral tissue demands (70).

### *The cytokine response to lipopolysaccharide*

Activation of macrophages induces production of both pro- and anti-inflammatory cytokines. Pro-inflammatory cytokines released include TNF-alpha, interleukin-1 beta, and interleukin-6. TNF-alpha is the earliest cytokine to increase in response to systemic infection, and it has been characterized as being one of the most important cytokines in terms of its involvement in the pathophysiology of sepsis (82, 109). The pro-inflammatory cytokines promote leukocyte adhesion on endothelial cells, induce the activation of inducible nitric oxide synthase (iNOS) to generate large amounts of the vasodilator, nitric oxide, promote coagulation, and promote the release of metabolites of arachidonic acid.

Anti-inflammatory cytokines, on the other hand, work to limit the extent of inflammation promoted by the pro-inflammatory cytokines. For instance, anti-inflammatory cytokines inhibit TNF-alpha, effectively functioning in a negative feedback manner to both decrease production of pro-inflammatory mediators and moderate their effects. However, it is presumed that in cases such as sepsis, there is an imbalance between pro-inflammatory and anti-inflammatory cytokines, and this imbalance leads to pathology (109).

### The neuroendocrine response to lipopolysaccharide

LPS stimulates activation of multiple facets of the neuroendocrine system. The hypothalamic-pituitary-adrenal (HPA) axis is activated by LPS. Ultimately, this increases the production and secretion of the glucocorticoid corticosterone, which helps to limit the extent of inflammation. Next, LPS activates the autonomic nervous system. In particular, the sympathetic branch of the autonomic nervous system is intimately associated with the immune system and can function as a modulator of the immune response. Finally, LPS regulates production of endocrine hormones such as insulin and glucagon.

### The hypothalamic-pituitary-adrenal axis is activated by inflammation

A rise in pro-inflammatory cytokines in the plasma (as may occur during an inflammatory event) can activate the HPA axis. Stimulation of the HPA axis results in hypothalamic production of corticotropin releasing hormone (CRH), entry into the portal vasculature, and subsequent activation of corticotroph cells in the anterior pituitary. The corticotroph cells then release adrenocorticotropic hormone (ACTH) into the bloodstream, which stimulates cortical cells of the adrenal gland to synthesize and secrete glucocorticoids (such as cortisol in the human and corticosterone in the mouse) (122). Glucocorticoids limit the inflammatory event by reducing levels of pro-inflammatory cytokines (IL-1-beta, IL-6, TNF-alpha) and increasing the levels of anti-inflammatory cytokines (IL-10). Glucocorticoids also negatively feed back at both the hypothalamus and pituitary to limit HPA axis activation. The importance of glucocorticoid production by adrenocortical cells is illustrated by the fact that rodents with adrenalectomy have a higher mortality rate compared to sham operated controls

when injected with LPS; further, administration of physiologic levels of glucocorticoid reduced mortality following LPS administration (63).

Importantly, infusion of cortisol in the presence of other stress hormones such as glucagon, epinephrine, and norepinephrine, can lead to sustained hyperglycemia (10, 44) due to increased gluconeogenesis (87). Removing cortisol from the stress hormone infusion (i.e. infusing only glucagon, epinephrine, and norepinephrine) resulted in depleted gluconeogenesis, and this was due to a decreased substrate supply (42). In these studies, removing cortisol from the infusion mixture also led to reduced hepatic glycogen stores; consistent with this, infusion of cortisol alone increases hepatic glycogen content (46).

#### The autonomic nervous system is activated by inflammation

The autonomic nervous system (ANS) can also regulate immune system function. The sympathetic nervous system extends pre-ganglionic fibers to the spinal cord, and post-ganglionic fibers directly innervate peripheral tissues. In contrast, parasympathetic pre-ganglionic fibers extend directly to organs or tissues, and post-ganglionic fibers extend rather locally. For both the parasympathetic and sympathetic neurons, acetylcholine secreted by pre-ganglionic neurons acts on nicotinic acetylcholine receptors of post-ganglionic neurons. Sympathetic post-ganglionic neurons secrete norepinephrine, which can effect changes in peripheral tissues through actions on adrenergic receptors. In contrast, post-ganglionic neurons of parasympathetic neurons secrete acetylcholine, which acts via muscarinic acetylcholine receptors present on target tissues. The pancreas receives sympathetic fibers from the

splanchnic nerve and parasympathetic fibers from the vagus nerve, and at least the vagal fibers appear to enter the pancreas through blood vessel walls (9).

The sympathetic nervous system in particular communicates with the immune system. Immune system organs such as the spleen, thymus, and lymph nodes are highly innervated by sympathetic fibers. Further, lymphocytes express adrenergic receptors, and activation of these immune cells by either norepinephrine or epinephrine can alter production of both pro- and anti-inflammatory cytokines. Different populations of immune cells are differentially affected by adrenergic signaling. In some cases, norepinephrine and epinephrine decrease the production of pro-inflammatory cytokines and increase the production of anti-inflammatory cytokines, although this is not always the case (37). In effect, the autonomic nervous system can work with the glucocorticoids of the HPA axis to modulate the inflammatory environment during inflammation.

Besides its effect on immune function, activation of the sympathetic nervous system has been demonstrated to be important in the early glucose response to infection. In particular, hepatic glucose production is stimulated by high-dose endotoxin, and this is prevented with combined alpha- and beta-adrenergic blockade (49). The findings of this work suggest that the increased hepatic glucose production is not due to a change in plasma glucagon. However, others have demonstrated that adrenergic stimulation can modulate glucagon secretion from the alpha cell (45, 67). Data presented in this dissertation also points to a role of adrenergic signaling in promoting the glucagon response to LPS.

### Secretion from the endocrine pancreas is modulated by inflammation

The pancreatic islets of Langerhans make up about 1-2% of the entire pancreas and represent the endocrine portion of the pancreas. Within islets are specific cell subtypes, including glucagon-secreting alpha cells, insulin-secreting beta cells, somatostatin-secreting delta cells, and pancreatic polypeptide-secreting epsilon cells. In mouse, the alpha cells surround a core of primarily beta cells (with few delta, gamma, and epsilon cells scattered within the beta cell core). In humans, the islet cell subtypes are more scattered, and alpha cells can make up upwards of 50% of the islet, demonstrating a potentially more important role of alpha cells in humans.

#### *The pancreatic beta cell*

The endocrine pancreas is exquisitely responsive to changes in metabolism and the inflammatory state. Most explored within the islet has been the regulation of the insulin-secreting beta cell. The primary mechanism by which the beta cell is modulated is glucose. When glucose concentrations rise (as might occur following a meal), increased glucose metabolism within the beta cell results in an increase in the ratio of ATP:ADP. This triggers a closing of potassium-sensitive ATP channels ( $K_{ATP}$ -channels), which then depolarizes the plasma membrane to activate voltage-dependent calcium channels. Calcium influx then triggers insulin secretion by the exocytosis of insulin granules (93). This mechanism is thought to dominate the first-phase insulin response, while other mediators may facilitate sustained second-phase insulin secretion (53, 92). Still, other channels have recently been identified to play a role in glucose-mediated insulin secretion, including the two-pore-domain acid-sensitive potassium channel

(TASK-1) (27), indicating that the original picture for glucose-stimulated insulin secretion is more complex than previously imagined.

Beta cells are also regulated by a number of neuroendocrine factors, including by the incretin factor, glucagon-like peptide-1 (GLP-1). GLP-1 increases the activity of adenylate cyclase, which increases intracellular cyclic adenosine monophosphate (cAMP). It is possible that one of the ways by which GLP-1 stimulates insulin secretion is through increased cAMP, which has been suggested to modulate insulin in both protein kinase A (PKA)-dependent and independent mechanisms (32, 115). One of the calcium channels inhibited by GLP-1 in beta-cells is the voltage-dependent potassium channel ( $K_v$ ). Stimulation of  $K_v$  channels results in repolarization of the plasma membrane, which inhibits calcium influx. Thus, GLP-1 promotes insulin secretion by inhibiting activation of  $K_v$  channels (80, 115).

The autonomic nervous system can also modulate secretion of insulin from beta-cells. The parasympathetic nervous system stimulates insulin secretion. Vagotomized (i.e. lacking parasympathetic input) rodents exhibit a diminished insulin response to a glucose challenge (125). As mentioned above, parasympathetic post-ganglionic fibers transmit acetylcholine into the synapse. Mouse pancreatic islets have been demonstrated to stain densely positive at the core (mainly beta cells) for vesicular acetylcholine transporter (vAChT), which is a marker for cholinergic neurons and axons. Interestingly, however, human islets expressed sparse amounts of vAChT, suggesting a potentially different involvement of the parasympathetic nervous system on islets (107). In contrast, the sympathetic nervous system can actually inhibit or stimulate insulin secretion; alpha-2 adrenergic agonists inhibit glucose-stimulated insulin secretion *in*

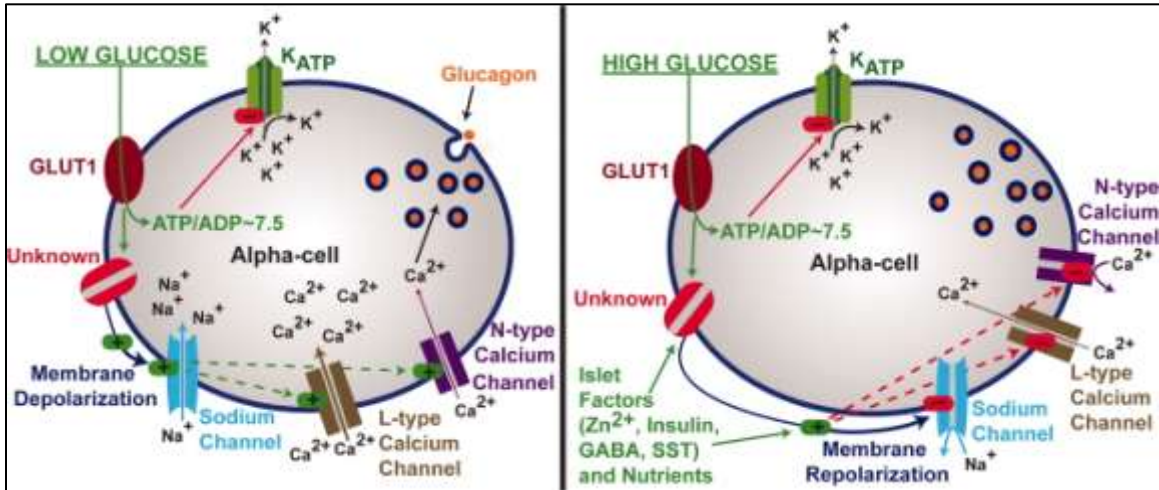
*vivo* (102), while beta-2 adrenergic agonists stimulate insulin secretion (1). *In vivo*, the net effect of sympathetic stimulation is inhibitory (74, 105).

During inflammation, insulin levels are elevated, even in the presence of hyperglycemia and elevated norepinephrine spillover from nerve termini as well as circulating epinephrine (85). One of the proposed mediators of the insulin secretion is GLP-1. Recent work from Nguyen *et al.* demonstrated that with low-dose LPS (2 mg/kg), the increase in plasma insulin correlates with GLP-1 (0.709). Further, wild-type mice administered the dipeptidyl peptidase-4 (DPP4) inhibitor, sitagliptin (effectively preventing the breakdown of plasma GLP-1) exhibited a decreased glucose excursion following an oral glucose tolerance test. Conversely, treatment with the GLP-1 receptor antagonist exendin-9 increased the glucose excursion, suggesting that signaling through GLP-1 may increase insulin secretion in settings of inflammation. It should be noted, however, that plasma insulin levels were not assessed in the glucose tolerance tests (94). GLP-1 also can act via other tissues (such as brain) in addition to the beta cell to modulate glucose tolerance (16). Others have also demonstrated that either low-dose LPS (0.1 mg/kg) or administration of individual pro-inflammatory cytokines such as TNF-alpha, IL-1 beta, and IL-6 increases plasma GLP-1. Interestingly, they found that IL-6 is both sufficient and necessary for the increase in plasma GLP-1 following LPS exposure (61). Work from Ellingsgaard and colleagues suggests that GLP-1, which is normally produced in L cells of the intestine, may also be produced directly within islets to act as a paracrine factor (35).



### *The pancreatic alpha cell*

Like beta cells, Alpha cells are a highly regulated (if not perhaps even more regulated) cell population. The mechanism for glucose-dependent glucagon secretion is slightly more complicated than in the beta cell. While alpha cells also have  $K_{ATP}$ -channels, the closing of these channels (as would happen with a high ATP:ADP ratio and high glucose), inhibits glucagon secretion. Why does closing  $K_{ATP}$ -channels facilitate insulin secretion in one cell population and inhibit glucagon secretion in another? The reason lies in the presence of additional ion channels present in the alpha cell (**Figure 1.2**). In high-glucose conditions, blocking  $K_{ATP}$ -channels depolarizes the membrane potential beyond a range where voltage-dependent channels can trigger calcium influx (79). In contrast, in low glucose,  $K_{ATP}$ -channels are moderately active, allowing the membrane potential of the alpha cell to open voltage-dependent L- and N-type calcium channels as well as voltage-dependent sodium channels. The net result is an action potential, which triggers the influx of calcium and glucagon granule exocytosis (59, 103).



**Figure 1.2: Schematic of glucose-regulated glucagon secretion from the alpha cell.** (Figure from Jacobson *et al.* (2009). *Diabetes*.) Shown are conditions in which depolarization and calcium influx is permissive (low glucose; left panel) and conditions in which depolarization and calcium influx do not occur (high glucose; right panel). The figure in orange marked “unknown” represents a glucose-dependent pathway that facilitates repolarization of the plasma membrane. The islet factors depicted regulate the alpha cell independently of glucose. SST, somatostatin.

The complex regulation of the alpha cell extends far beyond glucose. Regulation of alpha cells can be divided into three categories: intra-islet regulation, hormonal/nutrient regulation, and neural regulation. These forms of regulation may even occur (and likely do occur) simultaneously, meaning that the alpha cell must integrate the regulatory signals to determine whether to enhance or suppress glucagon production and/or secretion.

Within the islet, several factors can influence glucagon secretion. First, insulin can inhibit glucagon secretion. Kawamori *et al.* demonstrated that alpha-cell-specific knock-out of the insulin receptor resulted in glucose intolerance and mild hyperglycemia accompanied by hyperglucagonemia in the fed state (64). Glucagon secretion is also inhibited by somatostatin (124). Cabrera *et al.* found that glutamate, which is co-

secreted within glucagon-containing secretory granules, can act in a positive feedback manner to promote glucagon secretion in low glucose conditions (17). Co-secreted from insulin granules is zinc, which Gyulkhandanyan *et al.* suggested may have a modulatory effect on glucagon secretion (47). Additionally, glucagon itself can enhance glucagon secretion (77).

In terms of circulating factors that modulate glucagon secretion, amino acids such as L-arginine are also potent stimulators of glucagon secretion. Circulating fatty acids such as palmitate have also been found to stimulate glucagon secretion. In response to food intake, the incretin hormone GLP-1 (discussed above) is secreted from intestinal L-cells, and it acts to promote insulin secretion and suppress glucagon secretion. Another incretin, glucose-dependent insulinotropic polypeptide (GIP), stimulates both glucagon and insulin secretion (103).

As mentioned previously, islets are innervated by both parasympathetic and sympathetic fibers (107), and both branches of the autonomic nervous system can stimulate glucagon secretion (i.e. adrenergic and cholinergic signaling).  $\gamma$ -Amino-butyric acid (GABA) is synthesized from glutamate and acts as an inhibitory neurotransmitter in the central nervous system. Islets receive neural input from GABAergic neurons (120), and thus it is possible that this neurotransmitter may act to inhibit alpha cell function. Interestingly, the GABA-A receptor is recruited to the plasma membrane via an insulin-dependent pathway (147), suggesting collaborative negative feedback on the alpha cell in settings where insulin is secreted and glucagon secretion is suppressed.

In the presence of inflammation, hyperglucagonemia is often observed in spite of the elevation of several factors known to inhibit glucagon secretion: hyperglycemia,

hyperinsulinemia, and elevated GLP-1. With high adrenergic drive from the sympathetic nervous system, perhaps elevated circulating catecholamines, inflammatory mediators, and elevated fatty acids drive glucagon secretion in this setting. Work presented within this dissertation suggests that the central nervous system is required for the glucagon response to LPS; in the absence of neural input (achieved through ganglionic blockade), the glucagon response to LPS is completely abrogated. I also found that the pro-inflammatory cytokine, interleukin-6 (IL-6) augments glucagon secretion following LPS by acting within the brain to augment autonomic drive and increase the effectiveness of epinephrine directly at the islet.

Clearly, the regulation of the alpha cell is complex and appears to be dependent on the milieu of factors present at the islet at any given time. Ultimately, alpha cells integrate endocrine, paracrine, and neural input. How the integration occurs may be context-dependent, making the study of the alpha cell rather complicated.

#### *The periphery signals to the brain during inflammation*

In addition to signaling from the brain outward to modulate the inflammatory response, it is also important to consider signaling from the periphery to the brain in cases of inflammation. How do cytokines produced in the periphery signal to the brain? There are multiple ways in which communication may occur. One manner is via entrance directly into the brain through “leaky” regions where the blood-brain-barrier (BBB) is less intact. Alternatively, the cytokines may be directly taken up into the brain, as has been demonstrated with the cytokine, IL-6 (5). Still, other possibilities include indirect communication via the vagus or via second messengers such as nitric oxide or prostaglandins (37). Researchers have demonstrated that communication of peripheral

inflammation to the brain is necessary for a full inflammatory event to occur. For example, mice that undergo vagotomy have reduced the expression of pro-inflammatory cytokine mRNA in the brain following an LPS injection. Furthermore, the effect of LPS (in this case, sleep) was lost with vagotomy (150).

*In sum, inflammation results in profound metabolic, neuroendocrine, and immunologic changes. Preventing hyperglycemia in this setting can improve patient morbidity and mortality. Controlling hyperglucagonemia may represent a viable therapeutic option for management of glycemia. However, the response to systemic inflammation is complex, and there is still much to be learned regarding the regulation of the alpha cell in settings of inflammation.*

### **Inflammation is not the only system that relies on glucagon secretion for normal function**

Other settings beyond that of infection or endotoxemia are associated with elevated glucagon secretion. Two well-characterized settings include exercise and hypoglycemia. Further, in type 2 diabetes, patients often present with hyperglucagonemia. Interestingly, not only are all three of these settings associated with elevated plasma glucagon, but they are also associated with elevated plasma pro-inflammatory cytokines, specifically interleukin-6 (7, 30, 100).

Wasserman *et al.* demonstrated that glucagon regulates upwards of 65% of the increase in hepatic glucose production observed during exercise. Glucagon contributes to both elevated glycogenolysis and gluconeogenesis in this setting (144). Further, in healthy human subjects, Lavoie and colleagues demonstrated that suppression of

glucagon ablated the rise in hepatic glucose production (71). Exercise, like acute inflammation, is a setting associated with elevated autonomic drive and increased plasma interleukin-6 (100). While plasma norepinephrine and epinephrine increase during exercise, the relevance of direct neural input to the pancreas during exercise remains controversial (23).

As in exercise and acute inflammation, glucagon is a key player in the response to hypoglycemia. Further, while the factors controlling the glucagon response to inflammation have not been extensively examined, they have been carefully studied with regard to the response to hypoglycemia. Besides glucose-regulated glucagon secretion (discussed above), a critical factor in driving glucagon secretion is central autonomic drive to the pancreas.

### **Hypoglycemia as a model for glucagon secretion**

During hypoglycemia, the body must respond quickly to plummeting glycemia to ensure that the brain continues to receive the required amount of glucose to maintain normal processes. Glucose-sensing neurons within critical brain regions (mostly within the hypothalamus; see below) detect plummeting glycemia and respond ultimately by increasing autonomic drive, stimulating glucagon secretion from the islet and epinephrine secretion from the adrenal gland. Sites of glucoregulation as well as examples of central modulation of this so-called “counter-regulatory response” are shown below. A focus has been placed on a well-characterized glucoregulatory region, the ventromedial hypothalamus, as it appears to have a critical role in the glucagon

secretory response to hypoglycemia. Incidentally, this region also expresses receptors for interleukin-6 (138).

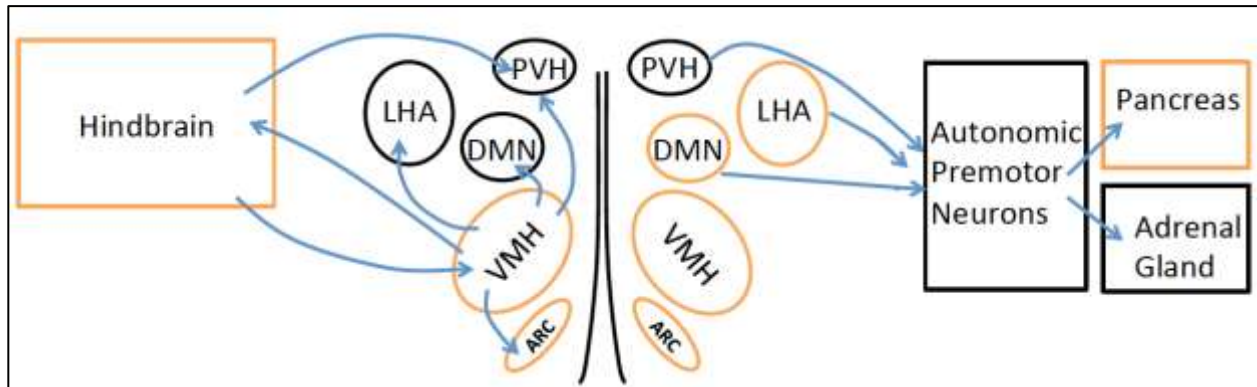
### Sites of glucoregulation within the nervous system

There are three steps involved in the neural control of blood glucose: first, glucose levels must be detected. Next, information regarding the state of blood glucose must be integrated within the brain. Finally, the brain must effect an autonomic response to the change in blood glucose.

Glucose-sensitive neurons are found throughout the body, including in the mouth, gut, portal vein, carotid body, and in specific regions of the brain (discussed below). There are two types of glucose responsive neurons: glucose-excited and glucose-inhibited. As the names suggest, these neurons either increase or decrease their firing when blood glucose increases. When blood glucose drops, the system reverses; glucose-excited neurons cease firing, and glucose-inhibited neurons increase firing. One can imagine how this glucose-sensing system could facilitate careful modulation of glucose homeostasis.

In general, the anatomical regions of the brain that are involved in glucose sensing and regulation exist in the hindbrain and in the hypothalamus. However, as mentioned above, glucose-sensitive neurons are also found outside of the central nervous system. For instance, the vagal afferents that project from the portal/mesenteric vein to the hindbrain and the hypothalamus are part of the neural reflex that controls first-phase insulin secretion from the pancreas. Regions of the brain containing glucose-sensing neurons include the lateral hypothalamic area (LHA), the dorsomedial nucleus (DMN) of the hypothalamus, the ventromedial hypothalamus (VMH), the

arcuate nucleus (ARC), and the hindbrain. These regions are shown in orange below in **Figure 1.3**.



**Figure 1.3: The neural network for glucose-sensing.** The ventromedial hypothalamus (VMH) extends projections to the dorsomedial nucleus (DMN), the lateral hypothalamic area (LHA), the arcuate nucleus (ARC), and to the paraventricular nucleus (PVH), an area that integrates glucose sensing. Information is integrated in the hypothalamus and hindbrain, which then provides input to motor neurons that send input to both the pancreas and adrenal gland. Glucose-sensing regions are highlighted in orange. Modified from Beall *et al.*, 2011, *AJP*.

*The ventromedial hypothalamus: a well-characterized site of glucagon regulation*

Starting from the beginning of the 1970's, research has pointed out that the ventromedial nucleus of the hypothalamus (VMH) is an important glucoregulatory site. Frohman and Bernardis showed in 1971 using electrical stimulation of the VMH that hyperglycemia occurs almost immediately during a 3 minute stimulation (41). This effect did not occur when stimulation was applied to the cerebral cortex or the lateral hypothalamus. Increases in plasma growth hormone also occurred following stimulation, although the response was delayed 2-5 minutes compared to the rise in plasma glucose. Frohman and Bernardis hypothesized that two responses may result from the same neurons. Further, adrenalectomy did not ablate the hyperglycemic response to



electrical stimulation of the VMH, suggesting that the rise in plasma glucose was not epinephrine-mediated. However, removal of the adrenal did shorten the duration of hyperglycemia. Insulin secretion was suppressed following VMH electrical stimulation. However, the suppression was lost following adrenalectomy, suggesting a major role for epinephrine and not direct sympathetic stimulation at islets in insulin suppression.

In further support of a positive role of the VMH in glucoregulation, the Schulman lab demonstrated in Sprague Dawley rats that ablation of neurons in this region using ibotenic acid blunted the glucagon as well as the epinephrine and norepinephrine response during insulin-induced hypoglycemia (using a hyperinsulinemic/hypoglycemic clamp)(13). These results were observed in both mild hypoglycemia (3.0 mM glucose) and severe hypoglycemia (2.5 mM glucose). This effect was not demonstrated when ibotenic acid was applied to the frontal lobe, the lateral hypothalamus, or in sham-operated rats. Taken together with early work by Frohman and Bernardis, it was possible that the rise in plasma glucose following electrical stimulation was due to early rises in plasma glucagon, epinephrine, and norepinephrine.

Borg and colleagues later demonstrated that application of 2-deoxyglucose to the region produced a counter-regulatory (glucagon, epinephrine) response in the absence of peripheral glucopenia (14). This study was nicely complemented by another study from the same laboratory in which peripheral hypoglycemia (hyperinsulinemic/hypoglycemic clamp) was achieved, and glucose was introduced to the VMH (12). By administering glucose at the VMH, the counter-regulatory hormone response was prevented. This occurred in a dose-dependent manner (more suppression at 100 mM vs. 15 mM or 0 mM glucose). Further, the glucose infusion rate increased upon glucose

delivery to the VMH, which is consistent with a failure to secrete counter-regulatory hormones.

Later studies by Beverly *et al.* demonstrated using microdialysis during insulin-induced hypoglycemia (hyperinsulinemic/hypoglycemic clamp) that norepinephrine is elevated in both the VMH as well as the periventricular nucleus of the hypothalamus (11). GABA concentrations also increased in the VMH in a similar manner to norepinephrine release. The norepinephrine release occurred bimodally—during the first 10 minutes and 20-30 minutes following insulin administration. Extracellular norepinephrine concentrations in the lateral hypothalamic area were slightly lower, and GABA levels remained at baseline. The increases in norepinephrine and GABA in the VMH were absent during euglycemic clamp; however, norepinephrine in the periventricular nucleus of the hypothalamus still increased, reflecting a direct response to hyperinsulinemia.

Ritter and colleagues demonstrated possible projections to paraventricular nucleus of the hypothalamus were responsible for the epinephrine response to insulin-induced hypoglycemia (104). Ritter injected saporin into the paraventricular nucleus of rats. This drug selectively lesions brain epinephrine and norepinephrine-expressing neurons in a retrograde manner, meaning the catecholaminergic projecting neurons to the hypothalamus were lesioned. These neurons were responsive to insulin-induced hypoglycemia, and lesioning reduced the epinephrine response. Glucagon was not assessed in these studies (104).

More recent work from the Sherwin lab has demonstrated a role of adrenergic signaling in the counter-regulatory response to insulin-induced hypoglycemia. As

previously mentioned, Beverley *et al.* demonstrated a rise in norepinephrine in the VMH during insulin-induced hypoglycemia (11). Szepietowska and colleagues examined the role of activating various adrenergic receptors known to be present in the VMH (127). Specifically, it was demonstrated that activation of the beta-2 adrenergic receptor via VMH bilateral injection of the agonist formoterol increased the counter-regulatory (glucagon and epinephrine) response to insulin-induced hypoglycemia. Further, antagonism of the beta-2 adrenergic receptor using the compound ICI-118,551 reduced the glucagon and epinephrine responses. These findings were paralleled by changes in the glucose infusion rate (GIR); agonism of the beta-2 adrenergic receptor decreased the GIR, while antagonism increased the GIR. These are consistent with improved and reduced counter-regulatory responses, respectively.

The above findings demonstrate the important role of the VMH in glucagon secretion during insulin-induced hypoglycemia in Sprague-Dawley rats. Not only does this site play a critical role in glucagon and epinephrine production, but it is also regulated locally by catecholamines. Activation of adrenoceptors, particularly the beta-2 adrenergic receptor, appears to play a role in how great the counter-regulatory response can be during an episode of hypoglycemia. Whether or not this region is also important in facilitating the glucagon response to other stimuli such as systemic inflammation remains an unanswered question.

## Interleukin-6: A role in glucagon secretion

### Interleukin-6: A cytokine with many metabolic roles

Interleukin-6 (IL-6) is a cytokine affecting a wide range of physiological functions, including those listed above. First characterized by two independent groups as B-Cell Stimulatory Factor (BSF) or T-Cell Replacing Factor (TRF), IL-6 was initially found to influence immune system function (62). Since its discovery and eventual isolation, this cytokine has been found to elicit a vast number of effects across several organs in addition to the immune system. Below are a few established examples of the range of IL-6-mediated effects.

**Table 1.2: The many roles of IL-6 in physiology**

Organ/Tissue /Cell Type	Response to IL-6
Liver	↑Glucose output, ↓insulin action on glycogen synthesis
Muscle	↑Glucose uptake, fat oxidation, insulin action on glucose transport
Adipose Tissue	↑Lipolysis, ↓insulin action on glucose transport and lipogenesis
Brain	↑Fever, cachexia
Pancreas	↑Insulin and glucagon secretion
Immune: B & T <sub>cyt</sub> cells	↑Antibody expression and cytotoxic activity

IL-6 is a cytokine with a variety of physiologic roles (**Table 1.2**). IL-6 stimulates glucose production from liver and inhibits insulin action on glycogen synthesis (114). In muscle, IL-6 stimulates fat oxidation and glucose uptake. Further, IL-6 acts in adipose tissue to stimulate lipolysis and inhibit insulin action on glucose transport and

lipogenesis. IL-6 has also been well established as both a pyrogen (via HPA axis activation) and a stimulator of the acute-phase protein response via the liver. Plasma IL-6 is elevated in physiologic and pathophysiologic settings where glucagon is also elevated: exercise (100), diabetes (7), and inflammatory stress (55). Furthermore, IL-6 has become implicated in metabolic disease as a predictive factor in the development of type 2 diabetes (121).

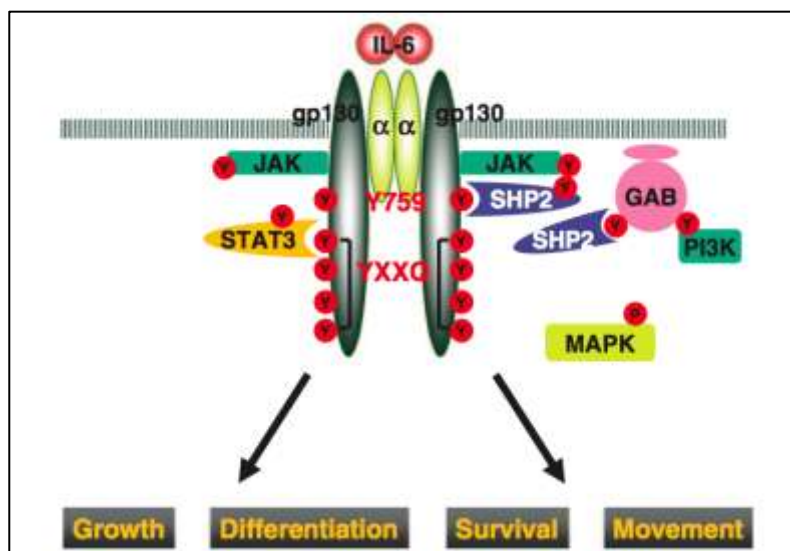
Prior work from the McGuinness lab demonstrated that IL-6 deficient (IL-6 knock-out) mice have a blunted glucagon response to acute inflammation (lipopolysaccharide, LPS) compared to their wild-type littermates. The glucagon response is completely rescued by intravenous replacement of IL-6 (133). In those studies, other pro-inflammatory cytokines (e.g. TNF-alpha and IL-1 beta) increased normally following LPS administration, suggesting a specific role for IL-6 in modulating the acute effect of LPS on glucagon secretion. IL-6 may also have longer-acting effects on  $\alpha$ -cells; human islets incubated for 4 or 24 hours in the presence of IL-6 exhibited increased glucagon secretion and *proglucagon* expression compared to untreated controls (34). In contrast, the glucagon response that was observed in studies performed by the McGuinness lab was much more rapid. Therefore, IL-6 may modulate glucagon secretion in two different manners; a slower, transcriptional manner at the islet and a more rapid, yet uncharacterized manner. The primary goal of this dissertation was to define the site from which IL-6 acts to facilitate this rapid glucagon secretion.

### Interleukin-6 signaling

IL-6 signals through the IL-6 receptor (IL-6R). Studies performed from Toshio Hirano's laboratory in the late 1980s demonstrated that the IL-6R consists of two

subunits. The first subunit, IL-6R $\alpha$  (also known as gp80 or CD126), is necessary for IL-6 binding (148). Upon binding of IL-6 to IL-6R $\alpha$ , the second subunit, gp130, is recruited (with stoichiometry 1 IL-6R $\alpha$ : 2 gp130) (54, 129). The long cytoplasmic tail of gp130 functions in signal transduction (54) and is utilized by a number of other interacting receptor subunits (termed the IL-6 or gp130 family of cytokine receptors) (62). The expression of these two subunits differs across cells. While IL-6R $\alpha$  expression is more restricted, gp130 is more ubiquitously expressed (110). In fact, nine other IL-6 family member cytokines have been identified and all use gp130 as a receptor and signal transducer, including oncostatin M, LIF, CNTF, CT-1, IL-11, and IL-27 (55).

IL-6 is thought to signal primarily through two different signaling pathways. First, the JAK2/STAT3 pathway is regulated via the YxxQ motif on gp130. The second signaling pathway IL-6 employs is the SHP2/Gab/Ras/Erk/MAPK pathway, and this is regulated by the cytoplasmic Y759 residue of gp130 (90).



**Figure 1.4: Schematic of Interleukin-6 signaling.** IL-6 signals through two major signal transduction pathways, JAK2/STAT3 and SHP2/Gab/MAPK signaling. (Figure from Hirano (2010), *Proc Jpn Acad Ser B Phys Biol Sci.*)

While IL-6R $\alpha$  is mostly found as an intramembrane subunit, two known soluble forms (sIL-6R $\alpha$ ) have been identified. The first form results from proteolytic cleavage, while the second results from alternative splicing of its mRNA. When cells do not express membrane-bound IL-6R $\alpha$ , sIL-6R $\alpha$  can act agonistically with gp130 to transduce signals (called the receptor conversion model; (56)). This opens up the possibility for IL-6 to act across multiple areas of the body, even when the cells do not (at least initially) express IL-6R $\alpha$ .

#### *Sites of IL-6 production*

While several of the innumerable sites of IL-6 action have been defined above, it must be stated that IL-6 is produced by a vast assortment of tissues under a wide array of physiologic or pathophysiologic settings. Although IL-6 mRNA expression has been found in the brain to be increased during inflammation (150), the main site of plasma IL-6 production is thought to be macrophages (109). In contrast, in type 2 diabetes, which is often coupled to obesity, the source of circulating IL-6 is adipose tissue (38). In exercise, myocytes secrete IL-6 proportionally to the duration and intensity of the exercise (40). The fact that IL-6 is synthesized by different tissues depending on the setting points to the important modulatory role of this cytokine.

#### *Clinical significance of IL-6 inhibition*

As previously mentioned, IL-6 has become implicated in a wide variety of physiological functions. As one might expect, the cytokine has also been demonstrated to have a role in pathophysiology. Patients with rheumatoid arthritis (RA) have elevated levels of plasma IL-6 (126). One common co-treatment for patients with RA is the drug,

tocilizumab (brand name Actemra). This drug is a humanized monoclonal antibody against both the soluble and membrane-bound IL-6R. RA patients that also were diabetic were treated with tocilizumab, HbA1c levels were improved in this population of subjects (97). Investigators did not measure glucagon levels in this population, but one possible explanation is that IL-6 signaling inhibition reduced glucagon levels in this population, which assisted in the reduction of HbA1c.



## Specific Aims and Hypothesis

The overall goal for the body of this work was to determine the site(s) of action by which interleukin-6 (IL-6) works to augment glucagon secretion. Previous work generated from the McGuinness lab demonstrated that IL-6 rescues the glucagon secretory response to the endotoxin, lipopolysaccharide (LPS) in IL-6 knock-out mice (133). Pro-inflammatory cytokines such as TNF-alpha and IL-1 beta rose similarly to IL-6 knock-out controls in the absence of a peripheral infusion of IL-6, suggesting that IL-6 specifically enhances glucagon secretion in settings of inflammation. Other research groups have demonstrated that incubation of isolated islets with IL-6 enhances *proglucagon* gene expression as well as glucagon secretion after 4 and 24 hour incubations (34). These data suggest that IL-6 has a slow effect on glucagon secretion by its direct actions on islets. Therefore, we hypothesized that IL-6 may enhance the more rapid glucagon response to LPS by an alternative manner. Knowing that IL-6 can cross the blood-brain barrier (5) as well as be produced within the brain (36), and that IL-6 receptors are expressed rather ubiquitously throughout the brain (137), we hypothesized that the rapid glucagon secretory response to LPS was due to direct actions within the brain, which in turn augmented autonomic drive to the islet. To assess the action of IL-6 on glucagon secretion via the brain and pancreas, the following Specific Aims were designed:

Specific Aim 1: To determine whether central IL-6 alone can augment glucagon secretion

Specific Aim 1a: Previous studies did not determine whether IL-6 alone had an effect on glucagon secretion in IL-6 knock-out mice. Therefore, it was unclear whether

IL-6 alone (in the absence of inflammation) can stimulate glucagon secretion or whether IL-6 sustains glucagon secretion only in the presence of an inflammatory stressor. Given that our overarching hypothesis is that IL-6 enhances glucagon secretion via the brain, Specific Aim 1a sought to determine whether central IL-6 alone could enhance glucagon secretion in the absence of an inflammatory stressor.

Specific Aim 1b: The second objective of this aim was to determine whether administration of central IL-6 alone was able to elevate glucagon secretion in the presence of LPS.

Specific Aim 1c: The third objective of this aim was to determine whether central IL-6 could augment glucagon secretion during an alternative physiologic stress, hypoglycemia.

For all sub aims of Specific Aim 1, it was critical that endogenous IL-6 not be produced; this could confound data interpretation. Therefore, mice lacking IL-6 (IL-6 knock-out mice) were utilized in Specific Aim 1. The results of these studies, therefore, would suggest that in the absence of a peripheral rise in IL-6 (i.e. IL-6 is only introduced to the brain and not the periphery), central IL-6 alone is sufficient to stimulate glucagon secretion, presumably by augmenting autonomic tone to the pancreas or perhaps to the adrenal gland.

Specific Aim 2: To determine the role of the autonomic nervous system in the response to lipopolysaccharide

Specific Aim 1 determined that central IL-6 does, in fact, modulate glucagon secretion, but only in the presence of a stressor such as LPS or hypoglycemia. The role of the autonomic nervous system (ANS) has been examined in depth during insulin-

induced hypoglycemia. However, the ANS's role in the glucagon response to inflammation (LPS) had not yet been assessed. Importantly, if one branch of the ANS predominated in mediating the glucagon response to LPS, this may provide further insight as to how central IL-6 could be interacting to enhance glucagon secretion in states of stress.

Specific Aim 2a: The objective of this sub aim was to determine whether the sympathetic or parasympathetic nervous system predominated in the glucagon response to LPS. Pharmacologic blockade of the sympathetic (combined adrenergic receptor blockade) or parasympathetic (muscarinic cholinergic receptor blockade) nervous system was employed, and the glucagon response to LPS was assessed.

Specific Aim 2b: Given that results from Specific Aim 2a suggested that both sympathetic and parasympathetic input has an effect on LPS-stimulated glucagon secretion, Specific Aim 2b sought to determine whether combined blockade of both the sympathetic and parasympathetic nervous system (ganglionic blockade) would have a synergistic effect on reducing the glucagon response to LPS.

Both sub aims of Specific Aim 2 were conducted in wild-type mice.

Specific Aim 3: To determine whether IL-6 can modulate a rapid glucagon response directly on islets

Although we found that central IL-6 alone was sufficient to augment glucagon secretion during settings of stress, this did not rule out the possibility that IL-6 could also acutely modulate glucagon directly from islets. Therefore, the objective of Specific Aim 3 was to determine whether central IL-6 alone or in the presence of other known secretagogues was sufficient to stimulate or augment glucagon secretion.

Specific Aim 3a: The objective of this sub aim was to determine whether IL-6 alone or in the presence of known glucagon secretagogues is sufficient to enhance glucagon secretion from islets. Islets were isolated from IL-6 knock-out mice (thus avoiding endogenous IL-6 production) and underwent pancreatic perfusion studies to determine whether IL-6 could augment glucagon secretion alone or in the presence of arginine or epinephrine, two known (and potent) glucagon secretagogues. Importantly, epinephrine is elevated under various physiological or pathophysiological settings of stress. While we did not look at the direct impact that LPS might have on IL-6-mediated glucagon secretion, it is important to realize that elevated epinephrine is one index of the impact of stress on the islet.

Specific Aim 3b: Specific Aim 3a demonstrated that IL-6 augments epinephrine-stimulated glucagon secretion. Therefore, the objective of this sub aim was to determine whether IL-6 enhances epinephrine-stimulated glucagon secretion via a calcium-dependent mechanism.

### Hypothesis

Although others have previously demonstrated that IL-6 can act directly on the pancreas to increase glucagon transcription and secretion, the effects of this cytokine were found to be slow and happened over several hours. In studies previously published from the McGuinness lab, IL-6 infusion had a rapid effect on modulating the glucagon response to lipopolysaccharide. Therefore, the overarching hypothesis for this work was that IL-6 enhances glucagon secretion predominantly via the brain.

## CHAPTER II: MATERIALS AND METHODS

### Animal Care and Usage

#### Ethics Statement

All animal procedures were performed with approval of the Institutional Animal Care and Usage Committee of Vanderbilt University. The NIH guidelines for the care and use of laboratory animals were followed.

#### Animal Care and Husbandry

Mice were group housed in micro-isolater cages in a temperature and humidity-controlled room with a 12:12 hour light/ dark cycle. At the time of surgery, mice were individually housed in clean cages. Mice were maintained on a standard rodent chow diet purchased from Research Diets, Inc. (New Brunswick, NJ) and were fed *ad libitum* with free access to water. All *in vivo* studies were performed on mice that were handled extensively on a daily basis from the time of initial surgery to reduce the contribution of stress to these studies. Mice that were cannulated also were prepared for the morning of study by loosening and tightening the dummy cannula for two days leading up to the experiment (see *Cannulation* below).

#### Mice Used in Lateral Ventricle Injection Studies and Perfusion Studies

Male IL-6<sup>-/-</sup> (IL-6 knock-out) mice on a C57Bl/6 background (B6.129S2-*Il6*<sup>tm1Kopf</sup>/J) were purchased from Jackson Laboratories (Bar Harbor, ME) at 10 weeks of age. All surgeries and experiments were performed on 12-14 week old mice. IL-6

knock-out mice at this age do not become obese and have not yet developed the obesity-associated complications characterized in older mice (141).

#### Mice Used in Pharmacologic Blockade Studies

These studies used wild-type (C57Bl/6) mice bred in our in-house colony (originally from Jackson Laboratories, Bar Harbor, ME). Mice were between 12-14 weeks of age at the time of the experiment. At three weeks of age, these mice were weaned and separated by gender and subsequently fed standard rodent chow.

#### Mice Used in Alpha Cell Calcium Studies

For the islet calcium imaging studies, mice (C57Bl/6 background) expressing tandem dimer red fluorescent protein tdRFP under control of the *proglucagon* promoter (72) were used.

### **Surgical Procedures**

All surgeries were performed in 12-14 week-old mice. At the time of surgery, mice were separated and maintained in micro-isolator cages on a 12-h light/dark cycle with free access to food and water. Mice were handled extensively on a daily basis following surgery to minimize stress on the day of the experiment.

#### Cannulation

Cannulation surgeries were performed one week prior to catheterization surgery to allow mice to fully recover between surgeries. Surgical setups as well as the surgeries themselves were performed aseptically. Mice were anesthetized using

isoflurane and prepped for surgery by shaving the head and washing the area with ethanol.

The mouse was connected to a Stoelting Stereotaxic Frame (Kiel, WI), using ear bars and incisor bar to properly set the cranium in place for cannulation. In this stereotaxic setup, the incisor bar fits into a nose cone that allows for isoflurane to flow through the system to maintain anesthesia throughout the procedure. A single incision was made down the center of the skull from ~0.5 cm posterior to the eyes to lambda on the mouse skull. This incision is large enough to access the required area as well as set screws in place but small enough that minimal suturing is necessary. The area was rinsed with warm saline, and the meninges were pushed sideways to expose the skull.

Following exposure of the skull, proper orientation in the stereotaxic frame was assessed; ear bar platforms were raised or lowered to assure that the skull was in a level position. Before placing the guide cannula, skull screws (PlasticsOne, Roanoke, VA) that would ultimately anchor the guide cannula were placed. First, a small hand drill (PlasticsOne) was used to make 3 separate holes to house the screws. The holes were placed away from capillary beds and away from the area where the guide cannula would be placed. Any bleeding was stopped by pressing a sterile cotton applicator against the affected area (Note: bleeding was usually very minimal). The screws were carefully screwed in place; they were not screwed in completely to avoid cortical damage.

Following screw implantation, the guide cannula (held in place by the stereotaxic frame) was carefully lowered to meet Bregma. The cannula (PlasticsOne) was then raised and moved to the desired location for cannulation (coordinates: 0.6 mm posterior

to bregma, 1.5 mm lateral to midline). A Dremel drill with a sterile drill bit was used to make a small hole at this location; any minimal bleeding was stopped again using a cotton applicator). The guide cannula was lowered to the surface of the skull and then slowly and carefully lowered to the desired depth (1.4 mm below the surface of the skull).

Once the guide cannula was lowered, FujiCEM glass ionomer cement (Instech, Plymouth Meeting, PA) was applied to the area to secure cannula placement. The cement was spread around all the skull screws as well as around the guide cannula. The cement was allowed to dry, and a dummy cannula (PlasticsOne) was subsequently screwed in place. The mouse was sutured to close any exposed region of the skull and given an intraperitoneal bolus of ketoprofen per Vanderbilt Institutional Animal Care and Use Committee guidelines for analgesia. Following surgery, mice were individually housed in clean cages. Successful cannulation was assessed following the experiment by cryosection or using methylene blue dye injection.

Post-operative measures were taken to ensure that each rodent recovered following surgery. Included in the care was monitoring the daily body weight of the mouse following surgery (this was done at the same time handling was done) as well as maintaining the mouse on a heating pad for a minimum of two days following surgery. Mice were also observed for any abnormal behavior cues that would suggest discomfort/complications following the surgery.



### Catheterization

Mice were first anaesthetized with isoflurane. The setup for surgery as well as the surgical process utilized aseptic technique. While under anaesthesia, mice were prepared for surgery by shaving around the surgical site. The area was subsequently washed with ethanol and betadine scrub.

An incision was first made 5 mm cephalic to the sternum, and the left sternomastoid muscle was exposed. The sternomastoid muscle was reflected, to reveal the left carotid artery. The connective tissue and vagus nerve were then separated from the vessel. A silk suture was tied at the cephalic end and another loosely knotted on the caudal end of the carotid. The artery was clamped with micro-serrefine and cut just below the ligated end and the catheter was inserted until the tip reached the aortic arch. The ligatures were secured and catheter sampling confirmed.

An incision was made 5 mm to the right of the midline and 2 mm caudal to the incision for the carotid catheter. The jugular vein was isolated and ligated at the cephalic end with a loose knot at the caudal end similar to the carotid catheter. The catheter for the jugular vein was inserted after a small incision and flushed to ensure patency.

A third incision was made between the shoulder blades of each mouse and a 14-gauge needle was tunneled under the skin. The catheters (carotid and jugular) were threaded through the needle to tunnel out of the back of the mouse. The ventral incisions were closed with a nylon suture. The arterial catheter was clamped with micro-serrefine at the incision site between the shoulder blades. The catheter was cut 1 cm above the clamp and connected to the MASA™ with a silk suture and repeated for the either the venous or gastric catheter. The dorsal incision was closed with silk sutures.

The animal was individually housed in a clean cage after this surgical procedure.

Post-operative measures were similar to those described following cannulation surgery. Surgical procedures as well as the post-operative care regimen are published and described within the Vanderbilt Mouse Metabolic Phenotyping Center website ([www.vanderbilt.edu/mmpc](http://www.vanderbilt.edu/mmpc)), and a video by Ayala and colleagues is also available (4).

### ***In vivo* Metabolic Experiments**

All *in vivo* metabolic experiments were performed following a ~5-day post-operative recovery period. Animals were studied only if they were within 10% of their pre-surgery body weight. Conscious, unrestrained mice were placed in ~1-L plastic container lined with bedding (7:00 am) and fasted for 5 hours to ensure that all mice were in the post-absorptive phase during study. Approximately 2 hours prior to the experiment, animals were connected to a Dual Channel Stainless Steel Swivel (Instech Laboratories, Plymouth Meeting, PA.) to allow for free movement, while being able to simultaneously infuse via the jugular vein and sample through the carotid artery, as well as microinfuse into the lateral ventricle. To prevent a fall in hematocrit due to sampling, an infusion of donor red blood cells was begun 1 hour prior to the experiment and continued for the duration of the study. The hyperinsulinemic-hypoglycemic clamp and LPS studies utilized IL-6 knock-out mice (Jackson Labs) and were performed in catheterized and cannulated mice, while wild-type mice used in the ganglionic blockade and sympathetic/parasympathetic antagonist studies were catheterized only. All mice were handled extensively to minimize stress on the day of the experiment. At the end of all studies, mice were sacrificed via a lethal dose of sodium pentobarbital (IV).

### Central IL-6 and Lipopolysaccharide

Twenty minutes prior to the start of the experiment ( $t = -20$ ), a baseline arterial blood sample was drawn for measurement of blood glucose, hematocrit, plasma insulin, and plasma glucagon. At  $t = -10$  min, a microinjection of 2  $\mu\text{L}$  of bacteriostatic saline (vehicle control) or 200 ng recombinant mouse IL-6 (Biomyx, San Diego, CA) was administered (100 ng/ $\mu\text{L}$  IL-6 at 1  $\mu\text{L}/\text{min}$  for 2 min) via the lateral ventricle cannula using a microinfusion pump and a Hamilton microinfusion syringe (Hamilton, Reno, NV). At  $t = 0$  min, either saline or 1 mg/kg lipopolysaccharide (LPS, Sigma Aldrich, St. Louis, MO) dissolved in saline was administered as a bolus via the jugular vein. Arterial blood was sampled at 10-minute intervals to assess the glucose response. Plasma was taken at  $t = 60$  and 120 min for glucagon measurement and at  $t = 120$  for insulin. A basal and terminal plasma sample was also taken for plasma catecholamines; however, the samples were compromised and lost in the assay procedure.

### Sympathetic/Parasympathetic Blockade and LPS

Thirty minutes prior to the start of the experiment ( $t = -30$ ), a baseline arterial blood sample was drawn for measurement of glucagon and at  $t = -25$  min for baseline plasma insulin. Ten minutes prior to LPS bolus, ( $t = -10$ ), mice were given either 1) phentolamine and propranolol (2 mg/kg priming bolus for both followed by a 0.02 mg/kg/min intravenous infusion for the duration of the study) to block alpha and beta adrenergic receptors, respectively, 2) atropine (1 mg/kg prime followed by 0.01 mg/kg/min intravenous infusion for the duration the study) to block muscarinic cholinergic receptors, or 3) saline. At  $t = 0$  min, 1 mg/kg LPS was given as a bolus via

the jugular vein. For all groups of mice, arterial blood was sampled at 20-minute intervals to assess the glucose response. Plasma was taken at t= 60 and 100 min for glucagon measurement and at 120 min for insulin. Basal and terminal plasma was also sampled for catecholamines; however, the samples were also compromised and lost in the assay procedure.

### *Ganglionic Blockade and LPS*

A baseline sample was taken for assessment of glucagon (t= -20 min). At t= -30 min, mice were given a bolus of 12 mg/kg chlorisondamine diiodide (Sigma Aldrich, St. Louis, MO) or saline as a control. At t= 0 min, 1 µg/g LPS was given as a bolus via the jugular vein. For both groups of mice, arterial blood was sampled at 20-minute intervals to assess the glucose response. Plasma was taken at t= 60 and 120 min for glucagon measurement. Since chlorisondamine decreases blood pressure, the carotid artery catheter was connected to a blood pressure monitor (via a Y connector at the carotid artery catheter) to ensure efficacy of the chlorisondamine bolus; successful ganglionic blockade reduced mean arterial pressure by ~50 mmHg.

### *Hyperinsulinemic-Hypoglycemic Clamp*

Prior to onset of hyperinsulinemia or microinjection into the lateral ventricle, baseline arterial plasma samples were taken for assessment of glucagon (t= -30 min), insulin (t= -25 min), or catecholamines (t= -20 min). Ten minutes prior to the start of hypoglycemia, a 2 µL microbolus of either bacteriostatic saline (vehicle control) or 200 ng recombinant mouse IL-6 was infused at a rate of 1 µL/min for 2 minutes (as described above). At t= 0, a constant jugular vein infusion of insulin ( $10 \text{ mU}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$ )

was begun, and a variable amount of glucose (20% dextrose) was administered to target or “clamp” the blood glucose at approximately 60 mg/dl. Blood glucose was monitored at 10-minute intervals. Arterial plasma was taken at t= 30, 60, and 120 min to assess plasma glucagon. A terminal arterial sample was also taken to assess plasma insulin and catecholamines (t= 120 min).

### **Plasma Collection and Measurements**

For all *in vivo* studies, arterial blood was drawn from catheterized mice and placed into EDTA-containing microcentrifuge tubes. The tubes were subsequently spun 1 minute at 12,000 r.p.m. Plasma was collected into tubes and stored at -80°C until analysis. The Vanderbilt Hormone Assay and Analytical Services core assayed glucagon, insulin, and catecholamines. Immunoreactive insulin and glucagon were assayed using a Rat Radioimmunoassay kit (EMD Millipore, Billerica, MA) using a double antibody method. Catecholamines were assessed using HPLC with electrochemical detection following alumina extraction and subsequent elution (3, 78). Plasma IL-6 was assessed using ELISA (Millipore, Billerica, MA). For collection of blood for glucagon analysis, 2 µL trypsinol was put into EDTA tubes prior to blood collection. For collection of blood for catecholamine analysis, 2 µL of glutathione/EGTA was put into EDTA tubes prior to blood collection.

## ***Ex vivo Experiments***

### ***Islet Isolation and Perifusion***

Islet isolation and perifusion were performed with the help of the Vanderbilt Islet Procurement and Analysis Core. IL-6 knock-out mice were anesthetized via intraperitoneal injection of a mixture of ketamine and xylazine (80 and 20 mg/kg, respectively), and islets were isolated as previously described (15). Briefly, islets were separated from exocrine tissue by density gradient centrifugation (Histopaque and RPMI, Sigma-Aldrich, St. Louis, MO). Purified islets were then hand-picked and maintained free-floating in islet culture medium: RPMI with added D-glucose (concentrations varied as described below), L-glutamine (2mM; Sigma-Aldrich), ascorbic acid (0.7 mg/mL; Sigma-Aldrich), benzyl penicillin (100 U/mL, Roche Diagnostics), streptomycin (0.1 mg/mL), and 10% (volume/volume) fetal calf serum (Sigma-Aldrich). The ability of IL-6 to modulate glucagon secretion was examined in the dynamic cell perifusion system (15). One hundred islet equivalents were placed in a chamber and washed with baseline media (5.6 mM glucose) for 30 minutes prior to the experiment. Islets were perfused for 9 minutes with multiple secretagogues of glucagon, preceded each time by a 9-minute washout period with 5.6 mM glucose-containing buffer. Islets were perfused with secretagogues in the following order: 1) 1.7 mM glucose, 2) 1.7 mM glucose + 200 ng/ml recombinant mouse interleukin-6 (IL-6; Biomyx, San Diego, CA), 3) 1.7 mM glucose + IL-6 + 1  $\mu$ M epinephrine, and finally 4) 1.7 mM glucose + IL-6 + 20 mM arginine. The effluent fractions were collected at 3-minute intervals using an automatic fraction collector. The glucagon content of each fraction was assessed by radioimmunoassay.

### Islet Isolation and Calcium Imaging

Islets were isolated and imaged from mice expressing tdRFP under control of the *proglucagon* promoter as previously described (72). Islets were incubated with 5  $\mu$ M Fluo4-AM for 30 minutes at 2.8 mM glucose. After washing, islets were placed in a microfluidic device (106) and allowed to equilibrate on the microscope stage for 15 minutes in the same solution. Islets were exposed to low glucose (1.7 mM), low glucose + epinephrine (1  $\mu$ M), low glucose + IL-6 (200 ng/ml), or low glucose + IL-6 + epinephrine. Fluo-4 was excited at 488 nm and detected between 495 and 545 nm. Red alpha cells were localized by recording tdRFP fluorescence excited by 561 nm. Time courses were collected with an LSM780 (Carl Zeiss) using a Fluar 40x/1.3NA lens and a 2 Airy unit pinhole. Mean intensities were normalized to the first 5 frames of data 8 minutes following reagent change.

### Statistical Analysis

SigmaPlot 12.0 software was used for all statistical analyses. All data are presented as mean  $\pm$  SEM. All *in vivo* data were analyzed using two-way repeated measures ANOVA followed by Bonferroni post-hoc test as appropriate. Perfusion data and calcium imaging data were analyzed using Student's t-test. The significance level was set at  $p < 0.05$ .

### CHAPTER III: INTERLEUKIN-6 ENHANCES GLUCAGON SECRETION VIA THE BRAIN IN SETTINGS OF STRESS AND VIA THE PANCREAS IN SETTINGS OF ELEVATED ADRENERGIC TONE

(Adapted from Barnes et al. (2014). *Am J Physiol Endocrinol Metab.* Nov 15: 307 (10): E896-905.)

#### Aims

The primary goal of Chapter III was to determine whether interleukin-6 (IL-6) could modulate glucagon secretion via the brain either alone (Specific Aim 1a) or during a setting of inflammatory stress (Specific Aim 1b). Previous work from the McGuinness lab demonstrated that IL-6 deficient (IL-6 knock-out) mice have a blunted glucagon response to acute inflammation (lipopolysaccharide, LPS) compared to their wild-type littermates. The glucagon response is completely rescued by intravenous replacement of IL-6 (133). In those studies, other pro-inflammatory cytokines (e.g. TNF-alpha and IL-1 beta) increased normally following LPS administration, suggesting a specific role for IL-6 in modulating the acute effect on glucagon secretion. IL-6 may also have longer-acting effects on  $\alpha$ -cells; human islets incubated for 4 or 24 hours in the presence of IL-6 exhibited increased glucagon secretion and *proglucagon* expression compared to untreated controls (34). The glucagon response that was observed in our previous studies was much more rapid. Therefore, knowing that IL-6 can cross the blood-brain barrier (5) as well as be produced within the brain (36), and that IL-6 receptors are expressed rather ubiquitously throughout the brain (137), we hypothesized that the rapid glucagon secretory response to LPS was due to direct actions within the brain, which in turn augmented autonomic drive to the islet.



To address this hypothesis, we utilized IL-6 knock-out mice and followed an approach based on our historic literature; mice were catheterized and given an intravenous bolus of LPS to stimulate glucagon secretion (6). The novelty in our approach was that mice were also cannulated in the lateral ventricle. In previous studies, IL-6 was administered intravenously, whereas in these studies, IL-6 was administered only into the lateral ventricle. Thus, the results of these findings would demonstrate whether IL-6 stimulates glucagon secretion when it is administered only centrally. Because we had not yet characterized the effect of central IL-6 alone (in the absence of inflammation), we also studied the effect of central IL-6 injection alone in another cohort of mice. Additionally, to determine if central IL-6 plays a role in an alternative setting of stress (hypoglycemia), an additional cohort of mice underwent hyperinsulinemic-hypoglycemic clamps (Specific Aim 1c). Utilizing IL-6 knock-out mice allowed for us to rule out production of endogenous IL-6, which could complicate the interpretation of our results. These studies would function to determine whether central IL-6 alone was sufficient to augment the glucagon response to LPS and whether central IL-6 had an effect on glucagon secretion in the absence of inflammation.

Further studies were also conducted in wild-type mice to pharmacologically determine the contribution of the sympathetic and parasympathetic branches of the autonomic nervous system (ANS) to the glucagon response to LPS (Specific Aim 2). The intent for these studies was to determine if one branch of the ANS may predominate in modulating glucagon secretion following LPS; if one branch was found to be unnecessary for the glucagon response, then perhaps this branch is not the one through which central IL-6 may operate to enhance glucagon secretion.

Importantly, demonstrating that a central effect of IL-6 does not rule out that IL-6 modulates glucagon secretion from the islet in a more rapid manner than previously observed (34). A series of *ex vivo* studies examined the role of IL-6 on acute glucagon secretion from islets to determine if IL-6 alone or in a setting of simulated stress (elevated epinephrine) could augment glucagon secretion (Specific Aim 3).

## **Introduction**

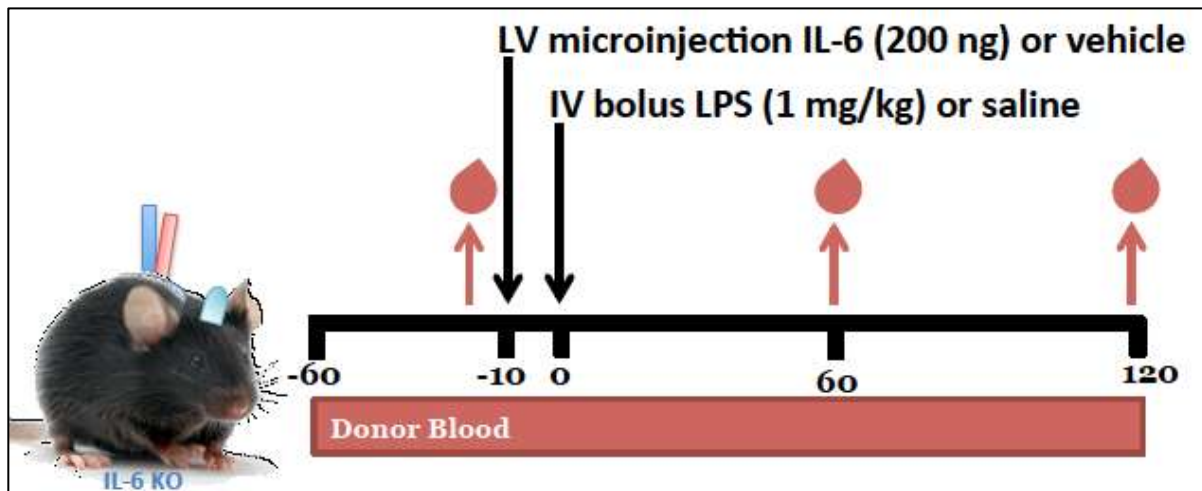
Inflammatory disease causes profound alterations in glucose homeostasis and accompanying insulin resistance. Exquisite control of insulin and glucagon secretion from the pancreas maintains euglycemia in healthy individuals (143). However, this balance is altered in inflammatory disease. The bulk of glucoregulatory research has focused on the insulin side of the story for some years, while less research has focused on therapies to attenuate glucagon secretion or action. Demonstrating the importance of glucagon, hyperglycemia associated with insulin deficiency does not manifest in the absence of glucagon-dependent signaling in rodent models of diabetes (73). Moreover, in models of type 2 diabetes, inhibition of glucagon also improves glucose homeostasis (119). While diabetes and acute or chronic inflammation are vastly different in etiology and pathology, they have some similarities: hyperglycemia is associated with either a rise in absolute levels of glucagon or in the ratio of glucagon-to-insulin as well as in pro-inflammatory cytokines. Moreover, improving glycemic control improves patient morbidity and mortality (60, 85, 139). Therefore, understanding the mechanisms that cause inappropriate glucagon secretion is critically important for developing treatment strategies to improve glucose homeostasis in inflammatory disease.

IL-6 is a cytokine with a variety of physiologic roles. IL-6 acts in adipose tissue to stimulate lipolysis and inhibit insulin action on glucose transport and lipogenesis. In muscle, IL-6 stimulates fat oxidation and glucose uptake. Further, IL-6 stimulates glucose production from liver and inhibits insulin action on glycogen synthesis (114). IL-6 has also been well established as both a pyrogen (via HPA axis activation) and a stimulator of the acute-phase protein response via the liver. Previous work has demonstrated a role of the pro-inflammatory cytokine, interleukin-6 (IL-6) in modulating glucagon secretion. Plasma IL-6 is elevated in physiologic and pathophysiologic settings where glucagon is also elevated: exercise (100), diabetes (7), and inflammatory stress (55). Furthermore, IL-6 has become implicated in metabolic disease as a predictive factor in the development of type 2 diabetes (121). Our previous data demonstrated that IL-6 acutely modulates glucagon secretion *in vivo* during acute inflammation. Herein, we tested whether IL-6 exerts its actions via the brain, islets, or both. In this dissertation, I have demonstrated that central IL-6 augments glucagon secretion, but it does so only in the presence of an accompanying stressor, be it acute inflammation or hypoglycemia. These data suggest a potent modulatory role for this cytokine. Contrary to my original hypothesis, I found that IL-6 also amplifies acute glucagon secretion from isolated islets in the presence of an accompanying stressor. These data provide insight into the dual role of IL-6 in amplifying neural drive to the pancreas and increasing the effectiveness of adrenergic signaling at the islet to stimulate glucagon secretion.

## Results

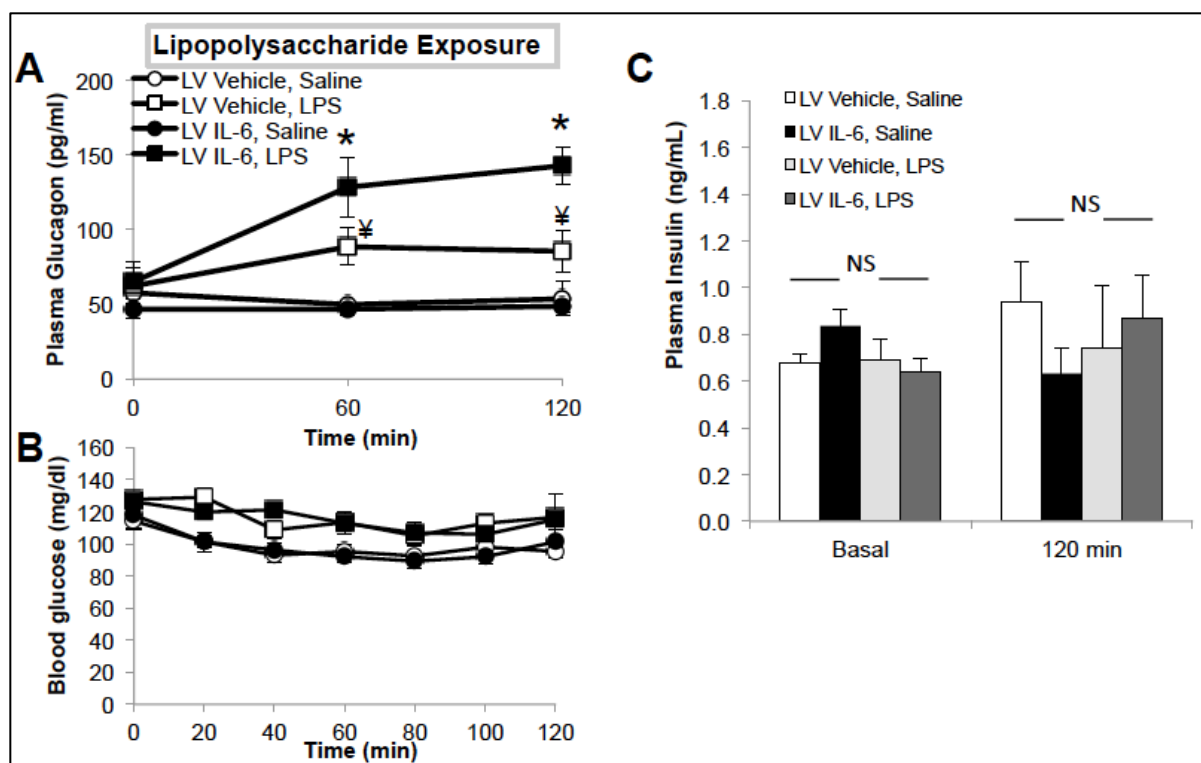
### Central Interleukin-6 enhances glucagon secretion during acute inflammation in IL-6 knock-out mice

To determine whether central IL-6 signaling modulates glucagon secretion, chronically catheterized, cannulated IL-6 knock-out mice were microinjected with IL-6 or vehicle (Veh) via the lateral ventricle. As acute inflammation is a potent stimulator of glucagon secretion, separate cohorts of mice were exposed to intravenous lipopolysaccharide (LPS; 1 mg/kg, IV) to determine whether central IL-6 could augment LPS-stimulated glucagon secretion (**Figure 3.1**). IL-6 knock-out mice were used to avoid endogenous production of IL-6 that might confound interpretation of these studies.



**Figure 3.1: Time-course of IL-6 lateral ventricle injection study.** Mice that were 5 h fasted were administered a microbolus of IL-6 or vehicle 10 minutes prior to an IV bolus of either LPS or saline. To compensate for loss of blood, mice were administered donor blood beginning 60 minutes prior to the start of the experiment and continuing throughout the study. Arterial blood was collected over time to monitor changes in glucose, and plasma was collected over time to monitor changes in glucagon and insulin.

IL-6 knock-out mice experienced no change in plasma glucagon in response to IL-6 microinjection alone ( $54\pm 11$  vs.  $49\pm 5$  pg/ml at 120 minutes; Veh vs. IL-6 injection) (**Figure 3.2A**). In contrast, central IL-6 microinjection nearly doubled circulating glucagon by 120 minutes when mice were administered LPS ( $85\pm 14$  vs.  $143\pm 12$  pg/ml; Veh vs. IL-6 injection). LPS administration slightly elevated plasma glucose levels throughout the period of study (**Figure 3.2B**), but central IL-6 did not further modulate the glucose response. There were no differences observed between groups in plasma insulin prior to or 2 hours following either LPS/saline injection (**Figure 3.2C**). IL-6 did not escape the central nervous system, as circulating IL-6 concentrations were undetectable following central IL-6 administration. Consistent with the data found here in IL-6 knock-out mice, in a small cohort of wild-type mice, central IL-6 injection alone did not enhance glucagon secretion at any time point observed (t= 0 min,  $56\pm 6$  vs.  $64\pm 6$ ; t= 30 min,  $64\pm 14$  vs.  $59\pm 6$ ; t= 60 min,  $50\pm 0$  vs.  $68\pm 7$  pg/ml; Veh vs. IL-6 injection). These results provide evidence in IL-6 knock-out mice that IL-6 acutely modulates glucagon secretion via the brain, but interestingly, it does so only in the presence of an accompanying stressor such as LPS.

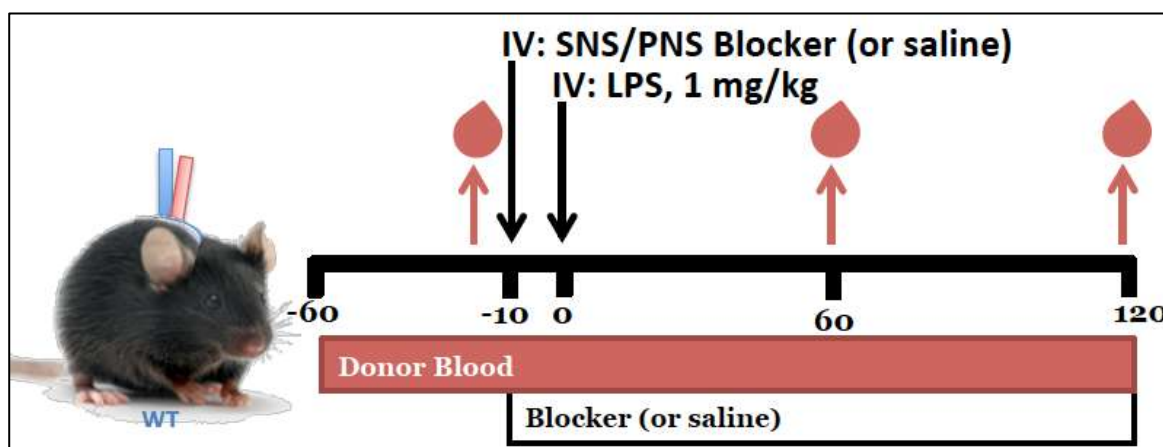


**Figure 3.2: Central IL-6 augments the glucagon response to endotoxin.** Chronically catheterized, cannulated IL-6 knock-out mice were administered either IL-6 or vehicle (Veh) into the lateral ventricle (LV) prior to IV injection of lipopolysaccharide (LPS) or saline. Plasma glucose (Panel A), GCG (Panel B), and insulin (Panel C) were assessed over time. n= 5-7 mice/group. \* = p < 0.05 relative to both LV Veh, Saline and LV Veh, LPS treatments. ‡ = p < 0.05 relative to LV Veh, Saline.

*The sympathetic and parasympathetic nervous systems drive LPS-stimulated glucagon in wild-type mice*

While data support the involvement of the autonomic nervous system (ANS) in the glucagon response to hypoglycemia (50, 51, 128), the role of the ANS in the glucagon response to LPS is yet to be elucidated. Activation of both the sympathetic and parasympathetic nervous system can stimulate glucagon secretion. Therefore, understanding if one branch predominates in the response to LPS could inform our grasp of how central IL-6 signaling influences autonomic drive. To determine the role of each branch of the ANS in the glucagon response to LPS, chronically catheterized, 5-

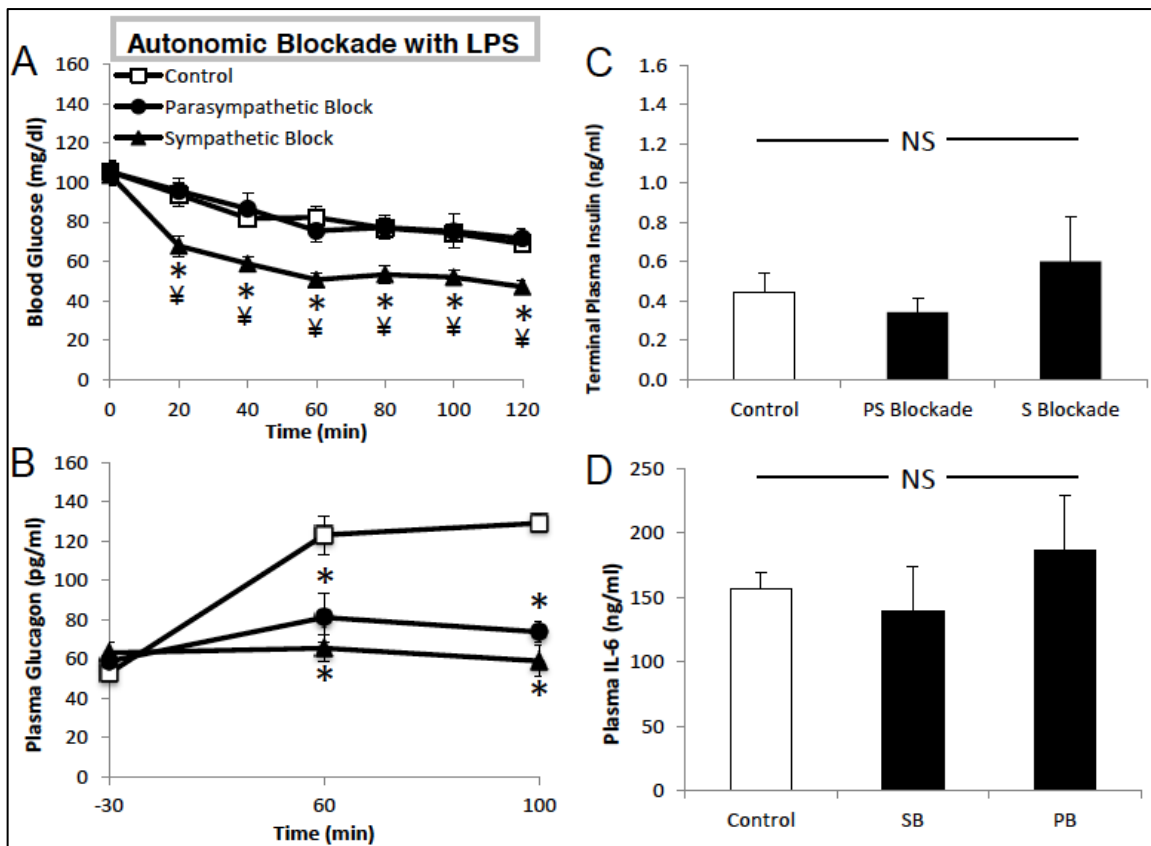
hour fasted wild-type male mice were pre-treated with pharmacologic antagonists of either the sympathetic nervous system or parasympathetic nervous system followed by exposure to LPS (1 mg/kg, IV). To target the sympathetic nervous system (SNS), combined alpha and beta-adrenergic receptor blockade with phentolamine and propranolol, respectively, was employed. The parasympathetic nervous system (PNS) was targeted using muscarinic cholinergic receptor blockade with atropine (**Figure 3.3**).



**Figure 3.3: Time-course of adrenergic blockade study.** Mice that were 5 h fasted were administered a primed continuous infusion via the jugular catheter of pharmacologic antagonists of the sympathetic nervous system (combined adrenergic blockade with propranolol and phentolamine) or of the parasympathetic nervous system (atropine). After beginning the infusion (10 min), a bolus of LPS was administered IV. To compensate for loss of blood, mice were administered donor blood beginning 60 minutes prior to the start of the experiment and continuing throughout the study. Arterial blood was collected over time to monitor changes in glucose, and plasma was collected over time to monitor changes in glucagon, insulin, and IL-6.

Pharmacologic blockade of the SNS but not PNS resulted in a rapid fall in blood glucose in response to LPS administration (**Figure 3.4A**). Interestingly, however, the LPS-induced glucagon response was blunted following both sympathetic blockade (SB) and parasympathetic blockade (PB) ( $129 \pm 4$  vs.  $59 \pm 8$  vs.  $74 \pm 6$  pg/ml at 120 min; control

vs. SB vs. PB) (**Figure 3.4B**). No differences in terminal plasma insulin were observed (**Figure 3.4C**). Terminal plasma was also assessed for IL-6 levels. Neither adrenergic blockade nor cholinergic blockade had an effect on terminal plasma IL-6 compared to controls (**Figure 3.4D**). Despite the hypoglycemic (and thus further glucagon-stimulating) environment in mice with SB, plasma glucagon levels were blunted even greater than mice with PB. This suggests that while both branches of the ANS mediate the glucagon secretory response to LPS, the SNS plays the predominant role.

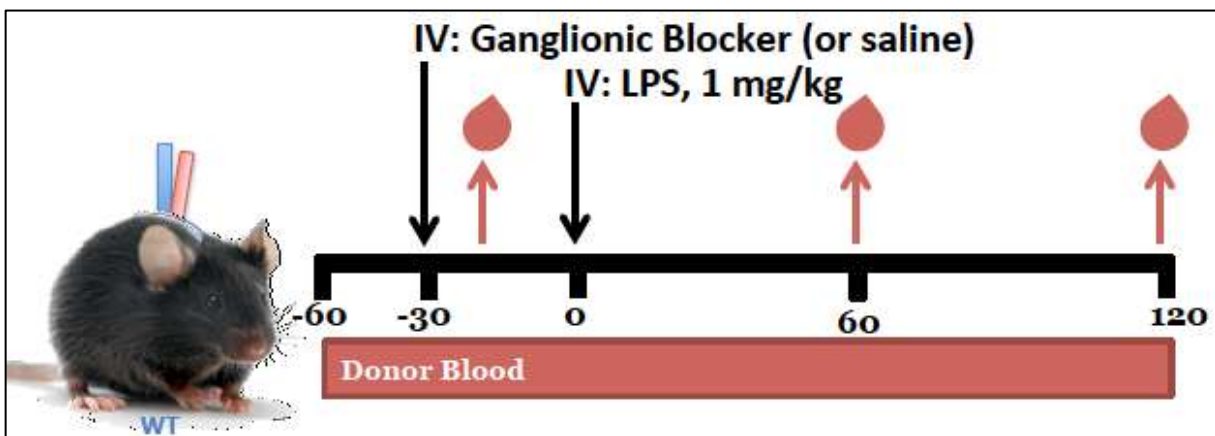


**Figure 3.4: Both the sympathetic and parasympathetic nervous systems are involved in the glucagon response to LPS.** Chronically catheterized wild-type mice were administered (IV) parasympathetic blocker (PB), sympathetic blockers (SB), or saline (Control). Mice were then administered LPS. Blood glucose (Panel A) and plasma GCG (Panel B) were assessed over time. Terminal plasma insulin (Panel C) and IL-6 (Panel D) were also assessed. n= 6 mice per group. \* = p < 0.05 relative to control. † = p < 0.05 relative to PB.



Neural drive is necessary for the glucagon response to LPS in wild-type mice

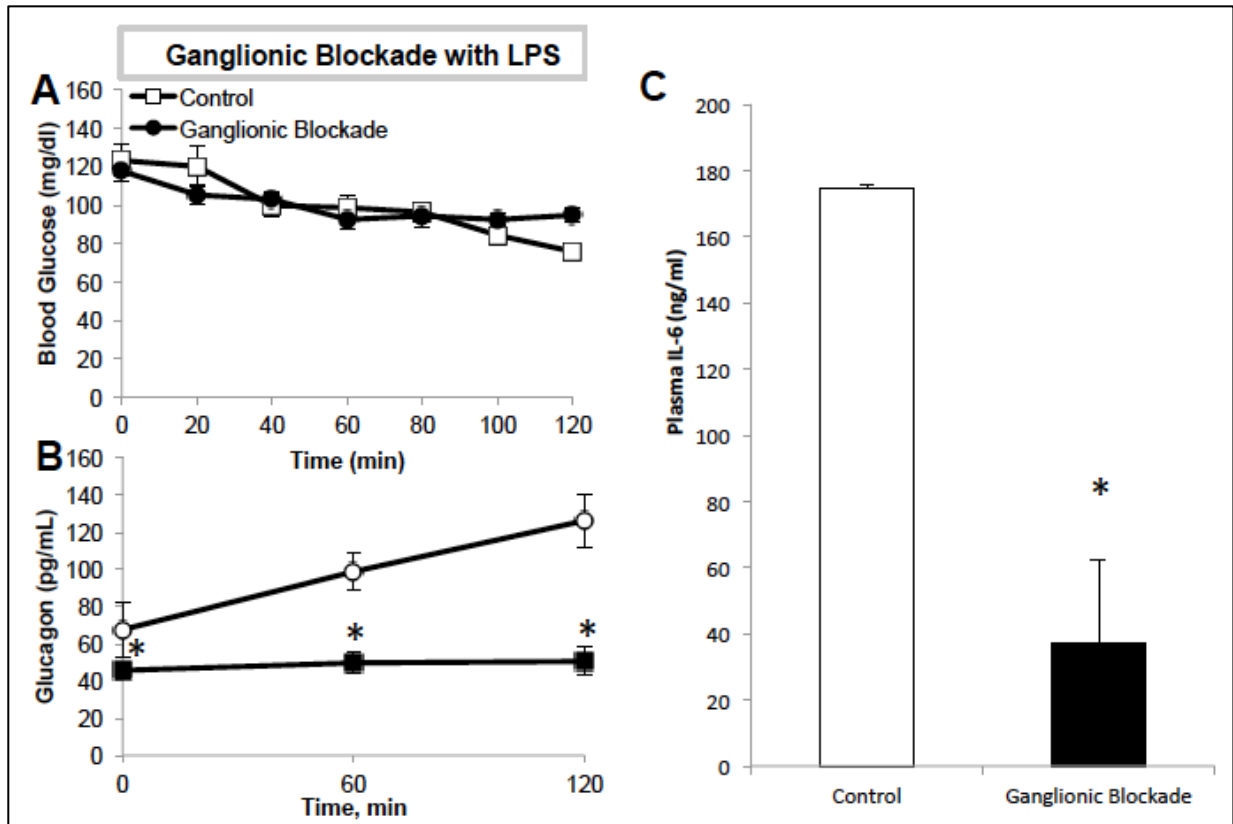
To further determine the role of neural drive in the glucagon response to LPS, chronically catheterized, 5 hour fasted wild-type mice were exposed to ganglionic blocker (12 mg/kg chlorisondamine, IV) or saline control and subsequently treated with LPS (1 mg/kg) (**Figure 3.5**).



**Figure 3.5: Time-course of ganglionic blockade study.** Mice that were 5 h fasted were administered a jugular bolus of the ganglionic blocker, chlorisondamine (or saline as a control). After the bolus (30 min), LPS was also administered via the jugular vein. To compensate for loss of blood, mice were administered donor blood beginning 60 minutes prior to the start of the experiment and continuing throughout the study. Arterial blood was collected over time to monitor changes in glucose, and plasma was collected over time to monitor changes in glucagon, insulin and IL-6.

No significant difference in blood glucose levels was observed throughout the study between treatments (**Figure 3.6A**). Mice treated with ganglionic blocker (GB) failed to increase glucagon in response to LPS (**Figure 3.6B**), with the effect most pronounced at the end of the study ( $126 \pm 14$  vs.  $51 \pm 7$  pg/ml; control vs. GB). GB suppressed plasma glucagon even prior to administration of LPS ( $t = 0$ ;  $68 \pm 15$  vs.  $46 \pm 5$

pg/ml; control vs. GB), supporting the role of the ANS in modulating glucagon levels, even in the non-stimulated, resting state. Interestingly, GB also blunted the LPS-induced rise in plasma IL-6 ( $175\pm 25$  vs.  $37\pm 11$  ng/ml; control vs. GB) (**Figure 3.6C**); this was not observed with either sympathetic or parasympathetic blockade alone (**Figure 3.4D**). The IL-6 response was not abolished by ganglionic blockade, however, as IL-6 levels are  $<0.007$  ng/ml in the absence of LPS. These results suggest that neural drive is needed for not only the glucagon response, but also for a robust IL-6 response to LPS.

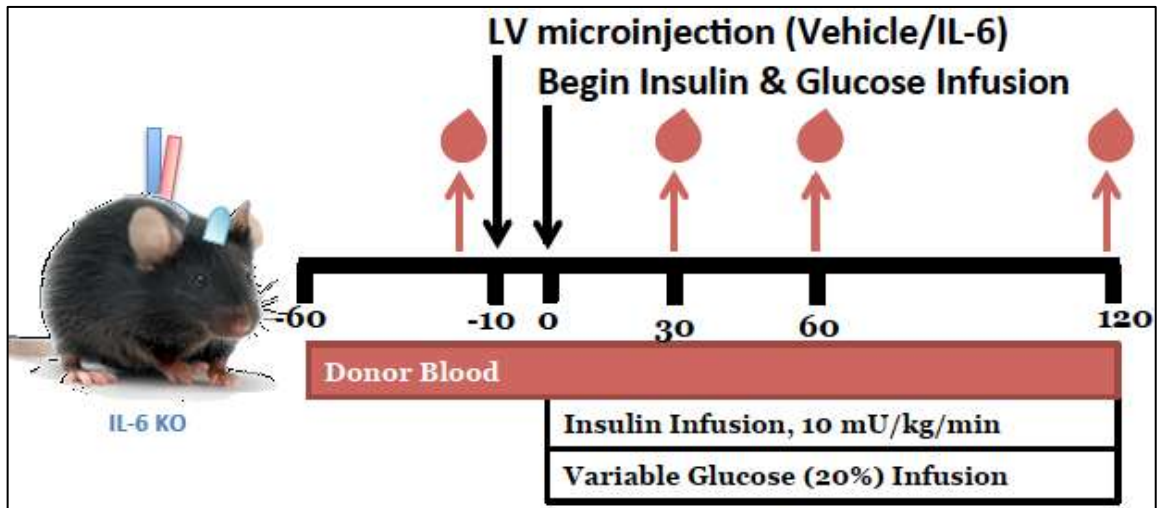


**Figure 3.6: Autonomic drive is necessary for the glucagon response to LPS.**

Chronically catheterized wild-type mice were given a jugular bolus of a ganglionic blocker (GB), or saline (Control). Mice were subsequently administered LPS. Blood glucose (Panel A) and plasma GCG (Panel B) were assessed over time. Terminal plasma IL-6 (Panel C) was also assessed.  $n = 6$  mice per group. \* =  $p < 0.05$  relative to control.

Central Interleukin-6 augments the glucagon response to hypoglycemia in IL-6 knock-out mice

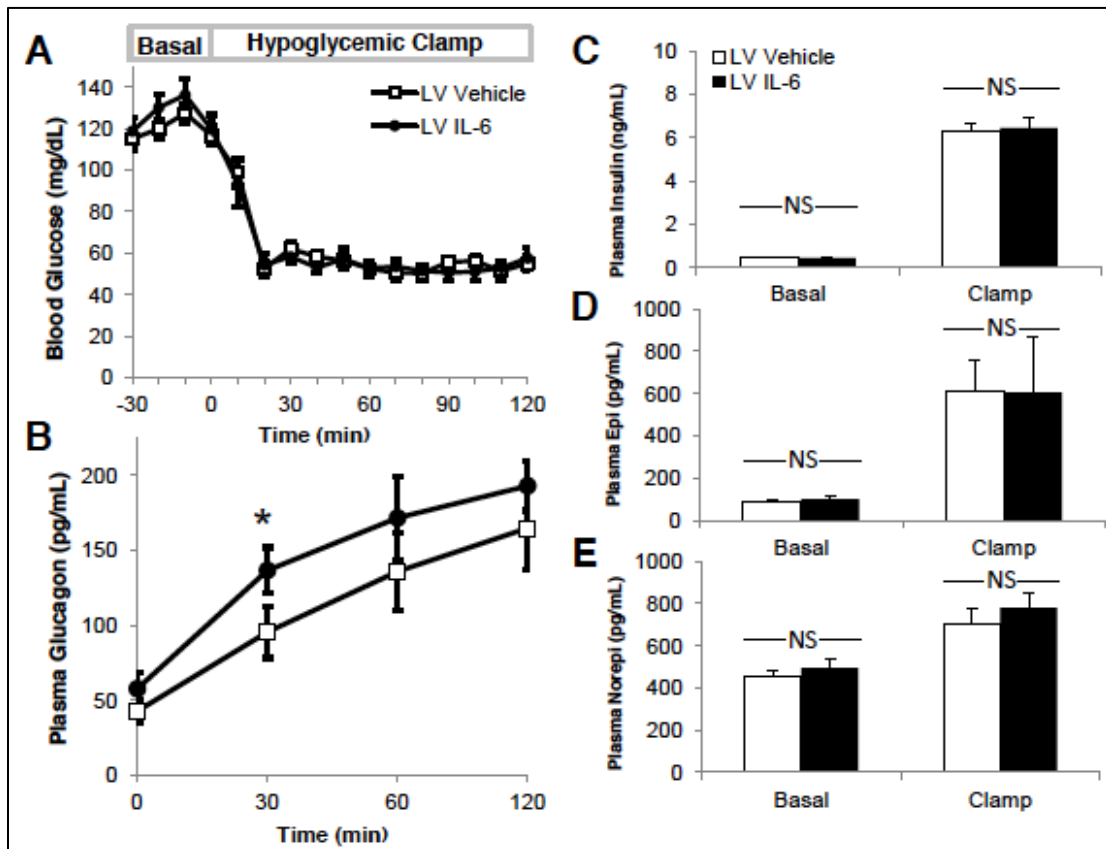
Prior work indicates the importance of the CNS for a full counter-regulatory (glucagon, epinephrine) response to hypoglycemia (127). Knowing that plasma IL-6 levels rise during hypoglycemia (30), we sought to determine whether central IL-6 could modulate glucagon secretion in response to this alternative stressor. Chronically catheterized, cannulated IL-6 knock-out mice underwent hyperinsulinemic/hypoglycemic clamps (Figure 3.7).



**Figure 3.7: Time-course of hyperinsulinemic-hypoglycemic clamp study.** Mice that were 5-hour fasted were administered a microbolus of IL-6 (or vehicle control) 10 minutes prior to the start of the hypoglycemic clamp. During the clamp, a continuous jugular infusion of insulin (10 mU/kg/min) and a variable infusion of 20% dextrose was administered to “clamp” blood glucose around 50 mg/dl. To compensate for loss of blood, mice were administered donor blood beginning 60 minutes prior to the start of the experiment and continuing throughout the study. Arterial blood was collected over time to monitor changes in glucose, and plasma was collected over time to monitor changes in glucagon, insulin, and catecholamines.

The rate of fall in blood glucose and the absolute glucose levels during hypoglycemia were well matched between vehicle (Veh) and IL-6-microinjected mice, allowing for proper comparison of counter-regulatory response (**Figure 3.8A**). By 20 minutes of hypoglycemia, the target glucose was reached and was maintained throughout the clamp period. No differences were observed in baseline glucagon (**Figure 3.8B**), but when IL-6 was administered centrally, there was a significant increase in plasma glucagon ( $t= 30$  min) in mice treated with central IL-6 ( $95\pm 15$  vs.  $137\pm 17$  pg/ml; Veh vs. IL-6). This trend was maintained throughout the clamp study. Plasma insulin concentrations were similar at baseline and rose similarly during the

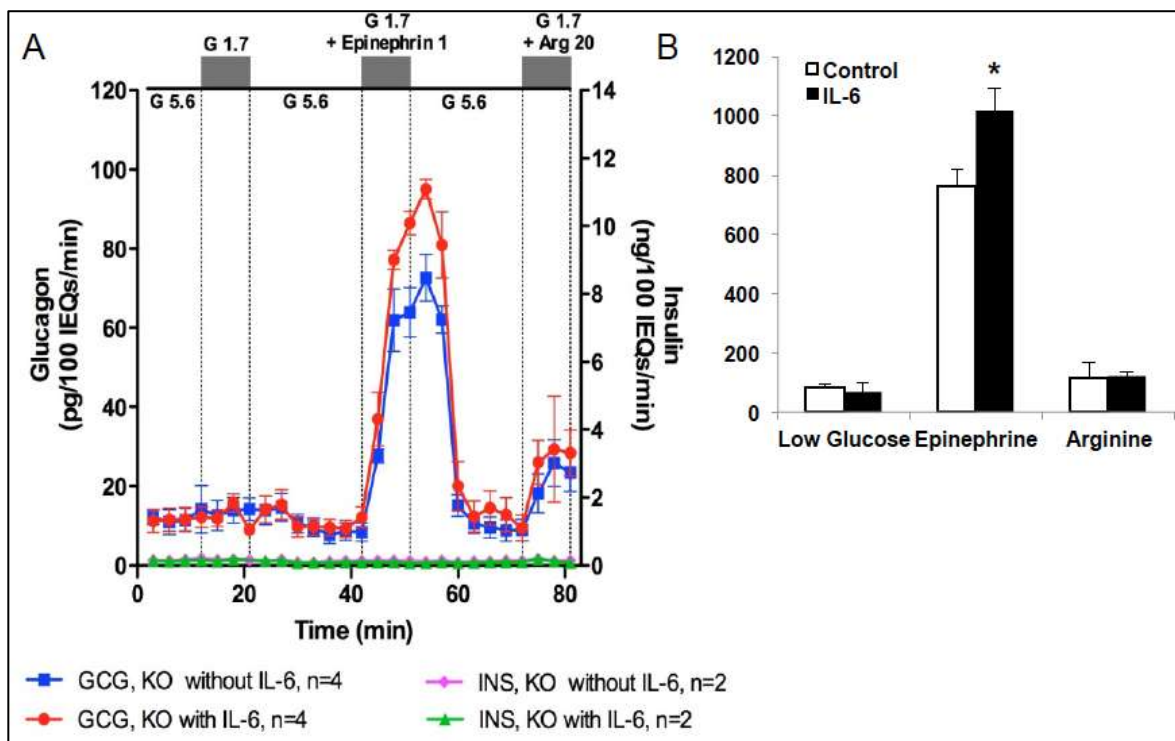
clamp; **Figure 3.8C**). Catecholamine levels were not different between treatment groups (**Figure 3.8D and 3.8E**). Thus, centrally administered IL-6 enhances the glucagon response to insulin-induced hypoglycemia without amplifying circulating epinephrine and norepinephrine. These data provide evidence that IL-6 augments glucagon secretion during multiple stressors.



**Figure 3.8: Central IL-6 augments the glucagon response to hypoglycemia.** Chronically catheterized, cannulated IL-6 knock-out mice were given either IL-6 or vehicle (Veh) into the lateral ventricle (LV) prior to onset of insulin-induced hypoglycemia (10 mU/kg/min). Blood glucose (Panel A) and plasma GCG (Panel B), insulin (Panel C), epinephrine (Panel D; Epi), and norepinephrine (Panel E; Norepi) were also assessed.  $n = 6-7$  mice per group. \* =  $p < 0.05$  relative to control.

IL-6 enhances epinephrine-stimulated glucagon secretion from IL-6 knock-out islets

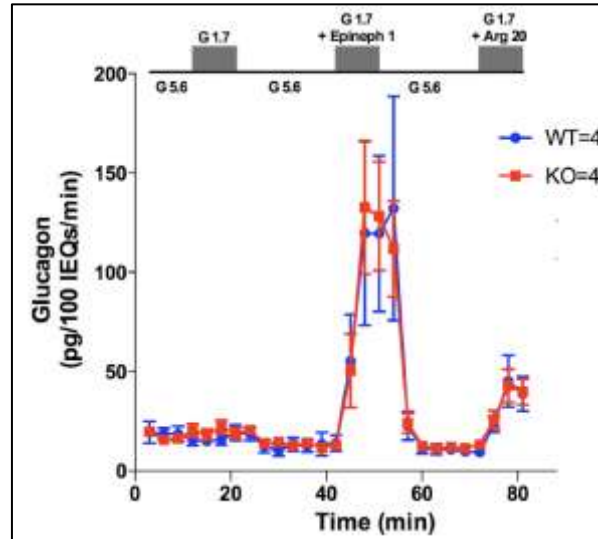
While studies have examined longer-term transcription-related effects of IL-6 on glucagon secretion, none have yet characterized the acute action of the cytokine at islets. A dynamic islet perfusion system was used to determine whether IL-6 acutely modulates glucagon secretion from the pancreas. Islets isolated from IL-6 knock-out mice were used to avoid the contribution of endogenous IL-6 to the studies. Islets were perfused with media containing 200 ng/ml IL-6 or vehicle and known glucagon secretagogues.



**Figure 3.9: IL-6 enhances the epinephrine-stimulated glucagon response in isolated islets.** IL-6 knock-out islets were isolated and perfused in a cell perfusion system with 1.7 mM glucose either alone (Lo) or with 1  $\mu$ M epinephrine (Epi) or 20 mM arginine (Arg). Half of the islets received concurrent IL-6 exposure (200 ng/mL). A 5.6 mM glucose wash was performed between secretagogue exposures. Fractions were collected over time and analyzed for glucagon and insulin. Panel A: time-course of glucagon and insulin over various secretagogue exposures. Panel B: AUC values from time-course (pg/islet equivalent). \* =  $p < 0.05$  relative to control.

IL-6 had no effect on glucagon secretion in low glucose (1.7 mM) media (**Fig. 3.9A**). Surprisingly, however, in the presence of low glucose plus 1  $\mu$ M epinephrine, IL-6 enhanced the glucagon response ( $765\pm 76$  vs.  $1017\pm 55$  pg glucagon/ islet equivalents), suggesting a potential interaction between the IL-6 and catecholamine signaling pathways to increase glucagon secretion. IL-6 did not enhance arginine-stimulated glucagon secretion. Since insulin can inhibit glucagon secretion, the concentration of glucose in the perfusate was kept low in these studies to inhibit insulin release; none was detectable for all treatments.

To verify that IL-6 knock-out islets have a similar glucagon-secreting capacity as wild-type islets, a separate experiment was conducted using the same secretagogues and concentrations (in the absence of IL-6). We found that glucagon secretion was similar in both wild-type and IL-6 knock-out islets in response to secretagogues (**Figure 3.10**). These data demonstrate that IL-6 modulates glucagon secretion from islets in the presence of adrenergic (epinephrine) signaling but not independently.

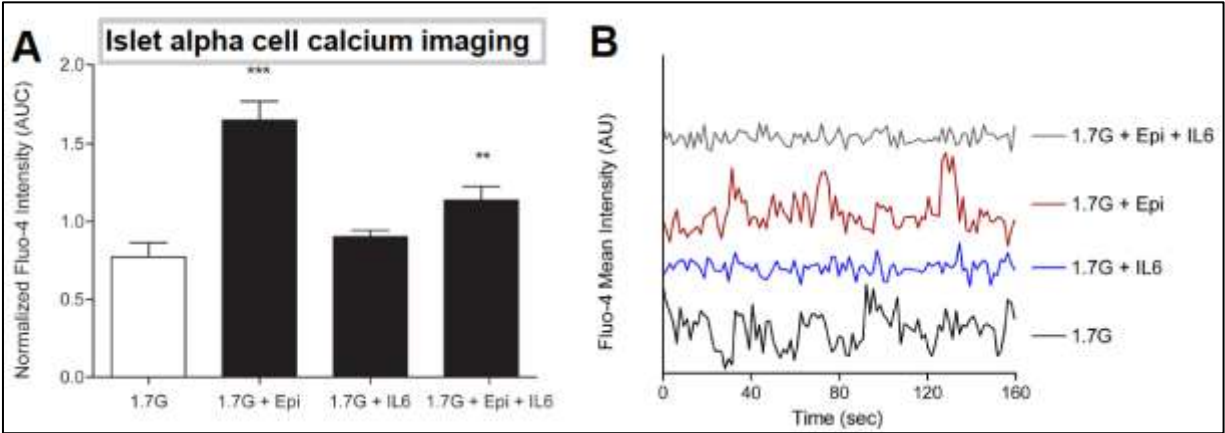


**Figure 3.10: IL-6 knock-out and wild-type islets respond similarly to known secretagogues.** IL-6 knock-out or wild-type islets were isolated and perfused with 1.7 mM glucose either alone, with 1  $\mu$ M epinephrine (Epineph 1), or with 20 mM arginine (Arg 20). A 5.6 mM glucose wash was performed between secretagogue exposures.

*Synergy between IL-6 and epinephrine signaling is calcium-independent*

To determine whether IL-6 may augment epinephrine-stimulated glucagon secretion via a global intracellular  $Ca^{2+}$  ( $[Ca^{2+}]_i$ )-dependent mechanism, islets were isolated from mice with tdRFP-expressing  $\alpha$ -cell (72), and subsequently loaded with the calcium ( $Ca^{2+}$ ) indicator dye, Fluo-4. Using a microfluidic device (106), Fluo-4 intensity was monitored over time in islets from 4 mice treated first with 1.7 mM glucose and subsequently switched into 1.7 mM glucose buffer containing IL-6 (200 ng/ml), epinephrine (1  $\mu$ M), or both.





**Figure 3.11: IL-6 enhances the epinephrine-stimulated glucagon response via a calcium-independent mechanism.** Islets isolated from GCG-tdRFP mice were incubated with the calcium indicator Fluo-4 and placed in a microfluidic device. Islets were first incubated with 1.7 mM glucose and subsequently switched to media containing 1  $\mu$ M epinephrine, 200 ng/mL IL-6, or both. Calcium oscillations were observed using confocal microscopy. Normalized fluo-4 intensity (Panel A) and representative traces (Panel B) are presented. \*\* =  $p < 0.005$  relative to control and \*\*\* =  $p < 0.0005$ .

Epinephrine induced a  $2.22 \pm 0.09$  ( $p < 0.0001$ )-fold increase relative to 1.7 mM glucose alone (**Figure 3.11A**). IL-6 alone did not alter  $[Ca^{2+}]_i$  ( $1.89 \pm 0.04$ -fold over glucose alone). Surprisingly, co-treatment of islets with IL-6 and epinephrine blunted the epinephrine-induced increase in  $[Ca^{2+}]_i$  ( $1.5 \pm 0.09$ -fold over 1.7 mM glucose;  $p = 0.0008$ ). Therefore, IL-6 is likely exerting its effect on glucagon secretion in a calcium-independent manner.

## Discussion

The results presented herein demonstrate that IL-6 enhances glucagon secretion via at least two distinct yet complimentary mechanisms in IL-6 knock-out mice. Glucagon is often elevated in inflammatory disease, despite accompanying hyperglycemia. Furthermore, inflammatory diseases often associate with autonomic

dysfunction. These studies support a synergistic link between IL-6 signaling and autonomic drive to enhance glucagon secretion. The hyperglucagonemia observed in certain disease states may result from IL-6-mediated effects on autonomic function. Indeed, acute IL-6 delivery 1) enhances neural drive to islets and 2) increases the effectiveness of epinephrine at islets to augment glucagon secretion.

In previous studies, the vascular infusion of IL-6 into IL-6 knock-out mice completely restored the glucagon response to LPS (133). We hypothesized that part of the rapid glucagon secretory response was via action of IL-6 in the brain. Indeed, infusing IL-6 into the lateral ventricle (LV) of IL-6 knock-out mice enhanced LPS-induced glucagon secretion by nearly two-fold (a physiologically significant change). Plasma IL-6 levels were undetectable following these studies, indicating that central IL-6 alone (i.e. in the absence of vascular IL-6) was sufficient to augment glucagon secretion in response to LPS. Furthermore, we found in a separate cohort of wild-type mice that central injection of IL-6 alone did not stimulate glucagon secretion, consistent with our data in knock-out mice. These data support our hypothesis that IL-6 mediates the cross talk between the brain and pancreas during inflammation. These data are consistent with work indicating that IL-6 injected into the LV of the anaesthetized rat (anesthesia is a stressor) increases splenic autonomic nerve activity (52). Others have demonstrated that pro-inflammatory cytokines in the brain acutely increase sympathetic tone in renal failure-induced hypertension (149). Although this work does not directly address the regulation of autonomic drive by IL-6, our glucagon secretion data lead us to speculate that during settings of stress, IL-6 augments autonomic tone. Unfortunately, catecholamine samples were compromised in the LPS experiments. However, based on

our previous work, peripheral IL-6 does not augment circulating epinephrine or norepinephrine during inflammatory stress (133). Thus, the central effects of IL-6 are likely via direct changes in autonomic tone to the pancreas and do not augment adrenal catecholamine secretion. Further studies to directly measure autonomic tone to the pancreas are needed to corroborate this hypothesis.

Other groups have found using *in vitro* or *ex vivo* models that IL-6 can also regulate islet beta cell function (34, 111, 116). While no differences in terminal insulin were observed between all cohorts, it is possible that central IL-6 modulated an early insulin response to LPS, which was not tested in these studies. An early rise in insulin would explain why elevated glucagon would not increase glucose levels following LPS. In our hands, we have found that there is a short, transient (~30 min) increase in insulin following initial exposure to the selected dose of LPS. Central IL-6 may enhance this transient elevation in insulin. However, we would anticipate that glucagon levels would actually be reduced in IL-6 treated mice, given that insulin is a negative regulator of glucagon secretion. Nonetheless, it is possible that central IL-6 also augmented an early insulin response to LPS.

Our studies point to a dominant role of the sympathetic nervous system in the glucagon response to LPS. Sympathetic blockade almost completely blunted the glucagon response to LPS in wild-type mice, while parasympathetic blockade did so to a lesser degree. Sympathetic blockade also decreased blood glucose levels compared to control and to parasympathetic blockade. Hypoglycemia is a potent stimulator of glucagon secretion. Despite having both LPS and hypoglycemia on board, sympathetic blockade still suppressed glucagon to a greater degree than parasympathetic blockade.

While blood glucose levels fell with sympathetic blockade, blood glucose levels following LPS treatment were similar in control, parasympathetic, and ganglionic blocker treated mice. Propranolol crosses the blood brain barrier, whereas phentolamine, atropine, and chlorisondamine do not (98). Thus, propranolol delivery may have resulted in off-target effects in the brain. Alternatively, plasma catecholamines may have increased and thus kept blood glucose levels from dropping in mice treated with parasympathetic blockade (atropine). Unfortunately, the catecholamine samples were compromised for these LPS studies, and this remains a point of speculation. These data lead us to hypothesize that centrally administered IL-6 may augment sympathetic drive to the pancreas in response to LPS.

We also found that abolishing total neural drive (ganglionic blockade) completely blunted the glucagon response to LPS in wild-type mice. In fact, ganglionic blockade lowered basal glucagon levels prior to LPS treatment, demonstrating that autonomic tone to the pancreas sustains glucagon secretion even in the resting state. Interestingly, the plasma IL-6 response to LPS was blunted in mice with ganglionic blockade. This contrasts to mice treated with sympathetic or parasympathetic blockade, where plasma IL-6 was elevated similar to control-treated mice. Other investigators have found that increased adrenergic (sympathetic) and cholinergic (parasympathetic) tone can increase local production of IL-6 (19, 89, 101). Since we found that both branches contribute to LPS-stimulated glucagon secretion, and both are thus activated in response to LPS, the fact that targeting only one branch did not decrease plasma IL-6 levels is not surprising. These data are consistent with previous work demonstrating that vagotomy impaired the pro-inflammatory cytokine mRNA response to LPS in the brain

and in the periphery; this was associated with decreased LPS-induced sleep (150). Cumulatively, the data lead us to hypothesize that an afferent signal(s) following LPS administration leads to elevated pro-inflammatory cytokines in the brain, which, in turn, enhances neural drive and amplifies the production of pro-inflammatory cytokines in the periphery.

Hypoglycemia is an alternative setting of both increased plasma IL-6 (30) and increased glucagon secretion. Increases in glucagon and autonomic nervous system activation work to limit the nadir in blood glucose levels during episodes of hypoglycemia. Our studies utilized IL-6 knock-out mice to determine whether central IL-6 signaling alone was sufficient to enhance glucagon secretion in response to insulin-induced hypoglycemia. ICV delivery of IL-6 enhanced the rapid rise in glucagon secretion. Furthermore, no significant differences in plasma epinephrine were detected during hypoglycemia with IL-6 administration. These data imply that IL-6 triggers glucagon secretion independent of adrenal function; this is consistent with the observation we previously made in the presence of LPS (133). These results are consistent with other studies that suggest that separate neural pathways may stimulate glucagon and epinephrine secretion in response to glucoprivation (99, 132).

Since insulin can inhibit glucagon secretion, an alternative inducer of hypoglycemia may have yielded more pronounced differences in glucagon secretion with central IL-6 administration. In fact, studies replacing insulin with phlorizin (83), an SGLT1 and 2 inhibitor that prevents glucose uptake in kidneys, were attempted. There were two problems with this technique. First, unlike with insulin, phlorizin does not operate over a predictable time-course. The rate of fall in glucose is inconsistent

between mice. In order to collect usable data in hypoglycemic clamps, the rate of fall in glucose should be similar between mice; this will ensure that the glucagon response operates in a similar kinetic manner and thus the glucagon measurements will be more consistent between mice. Second, the degree to which glycemia drops is variable and sometimes is not enough to yield a significant amount of glucagon secretion. Therefore, this technique was not a viable option for the desired purpose.

Collectively, these *in vivo* data indicate that central IL-6 augments glucagon secretion not only in response to LPS, but also in response to insulin-induced hypoglycemia. Further studies are required to delineate the specific site(s) of action and mechanism whereby central IL-6 enhances autonomic drive to the pancreas in these stressed states.

IL-6 not only modified neural drive but also acutely modulated glucagon secretion in isolated islets in the presence of epinephrine. Ellingsgaard *et al.* demonstrated that human islets incubated with IL-6 undergo a slow-onset increase in glucagon production: by 4 hours, there is a significant increase in *proglucagon* gene expression, and by 24 hours, there is an increase in secreted glucagon (34). Our islet perfusion experiments demonstrated that IL-6 had no acute effect on islets perfused with low glucose alone or with arginine, but IL-6 amplified epinephrine-stimulated glucagon secretion. These data suggest that the adrenergic signaling pathway may synergize with IL-6 signaling to augment glucagon secretion. I have since performed isolated islet culture experiments with the Vanderbilt Islet Procurement and Assessment Core with the goal of determining through which adrenergic receptor population IL-6 synergizes. However, due to extreme inconsistency between experiments, these studies must be redone. A likely explanation

of the inconsistency is the manner in which the islets were isolated. Since our experience, the Islet Core has found that gradient centrifugation is not an appropriate manner to isolate islets when alpha cell function is to be explored, as it damages the alpha cells that cover the periphery of mouse islets. Islets must be hand-picked to study alpha cell function. Thus, these experiments will need to be repeated.

The relationship between catecholaminergic and IL-6 signaling in the islet may be extrapolated to glucose-sensing regions of the brain. Szepietowska *et al.* showed that modulating adrenergic signaling in the glucose-sensing ventromedial hypothalamus (VMH) modulates the glucagon response to insulin-induced hypoglycemia in rats (127). Moreover, endogenous production of norepinephrine in this region increases during hypoglycemia (11). Since IL-6 receptors are expressed throughout the brain (137), adrenergic and IL-6 signaling may synergize not only in the islet, but also in glucose-sensing regions of the brain.

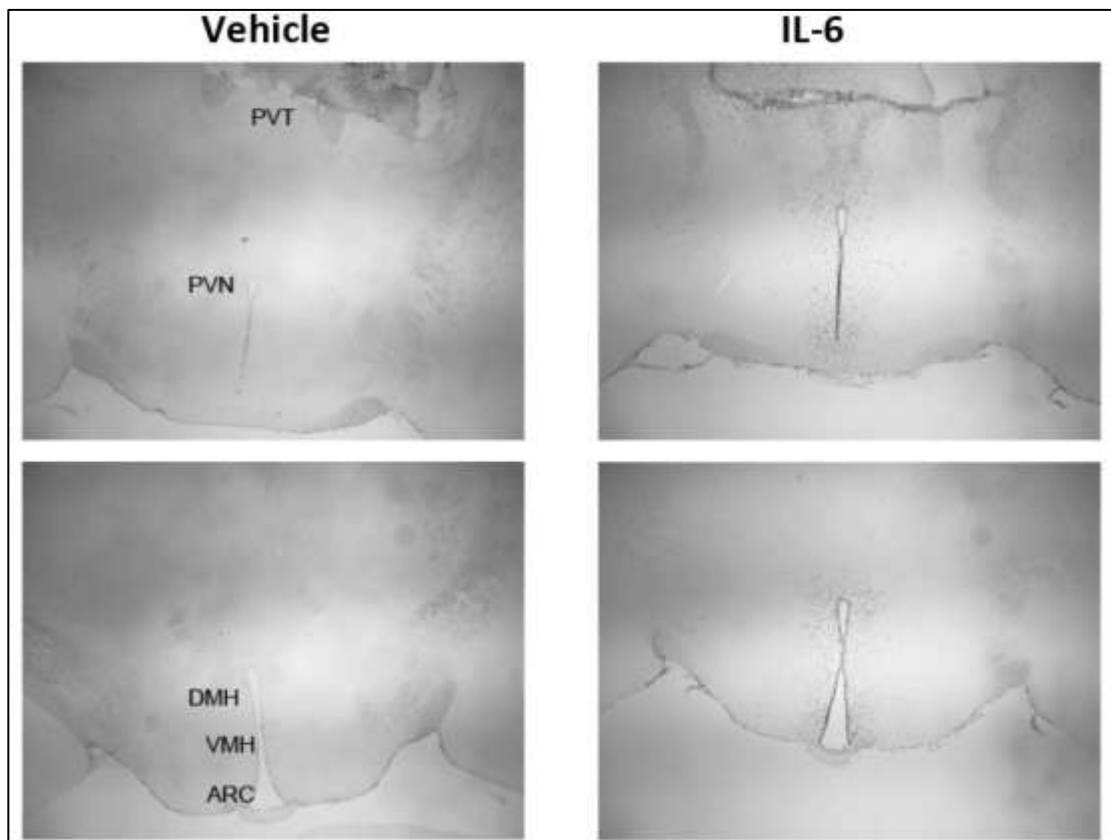
Given that calcium can modulate glucagon secretion, we determined whether IL-6 could enhance epinephrine-stimulated calcium signaling. Surprisingly, IL-6 actually reduced the epinephrine-stimulated increase in calcium oscillations while not altering basal calcium oscillations. These data suggest that epinephrine and IL-6 synergize to increase glucagon secretion through a calcium-independent mechanism. Both IL-6 and catecholaminergic signaling ( $G_q$ -coupled) may converge at the level of the MAPK/ERK signaling pathway. Moreover, ERK has been implicated as a positive effector of glucagon secretion (21). IL-6 and catecholaminergic signals may operate through the ERK signaling pathway to increase glucagon production. Further studies are needed to address this possibility.

Both our *in vivo* and *ex vivo* data suggest that IL-6 does not by itself acutely modulate glucagon secretion. It is only in the presence of an additional stressor that IL-6 augments glucagon secretion. Both LPS and hypoglycemia are associated with increased autonomic tone. Our islet perfusion data used epinephrine as a surrogate for elevated adrenergic tone at the islet. It was only with epinephrine that we saw the ability of IL-6 to amplify glucagon secretion. What occurs during this shift in autonomic tone that allows for IL-6 to presumably amplify neural drive to the pancreas and increase glucagon secretion is yet to be elucidated.

A great body of work in a critical glucose-sensing region of the brain, the ventromedial hypothalamus (VMH) (130), points to a potential mechanism by which central IL-6 may enhance glucagon secretion in settings of stress. Local induction of glucopenia in this region results in increased systemic levels of glucagon and epinephrine (EPI) (14), and delivery of glucose in the VMH during systemic glucopenia prevents the release of these hormones (12). Increasing adrenergic signaling in the VMH increases the glucagon response to hypoglycemia, while blocking adrenergic signaling decreases the response (127). Furthermore, norepinephrine levels increase in the VMH during hypoglycemia (11), and norepinephrine can increase local production of IL-6 (81, 96). These data, when combined with our findings that catecholaminergic signaling and IL-6 signaling pathways synergize in islets, lead us to speculate that a similar synergy may also happen within the VMH and potentially other glucoregulatory brain regions. IL-6 receptors are abundant throughout the hypothalamus, including within the VMH (137). In a separate cohort of mice, we also found that administration of IL-6 to the lateral ventricle activates phosphorylation of STAT3 (an index of IL-6



signaling) in regions surrounding the ventricles, including the VMH (**Figure 3.12**). Furthermore, pathophysiologic settings such as endotoxemia or physiologic settings such as hypoglycemia are also associated with elevated adrenergic tone and elevated IL-6. Therefore, it is possible that IL-6 signaling in the brain may synergize with catecholaminergic signaling to amplify the glucagon response in each of these settings.



**Figure 3.12: IL-6 increases phosphorylation of STAT-3 in regions surrounding cerebral ventricles.** Wild-type mice were cannulated in the lateral ventricle as described for IL-6 knock-out mice in the materials and methods section of this dissertation. Mice were subsequently injected with 200 ng of IL-6 (in a 2  $\mu$ l microbolus) or vehicle control, and 30 min later, mice were sacrificed. Brains were fixed, cryoprotected, and immunohistochemically assessed for an index of IL-6 signaling (phospho-STAT3, tyrosine 705). Regions surrounding the ventricles such as the paraventricular nucleus of the hypothalamus (PVN), dorsomedial hypothalamus (DMH), ventromedial hypothalamus (VMH), and arcuate nucleus of the hypothalamus (ARC) were positive for p-STAT3 following central IL-6 injection.

Clinical research has demonstrated that targeting the IL-6 pathway is of benefit to diabetic patients. Tocilizumab is a humanized IL-6 receptor (IL6R $\alpha$ ) antibody used clinically to treat rheumatoid arthritis (RA). When administered to diabetics with RA, tocilizumab reduced HbA1C in type 2 diabetics compared to those without diabetes, who showed no changes in HbA1C (97). While the concurrent RA of these patients complicates this particular study, targeting the IL-6 pathway clearly shows promise in lowering HbA1C levels. We speculate that tocilizumab may suppress glucagon secretion and thus contribute to the improvement of HbA1C levels in these patients. Reducing glucagon production via IL-6 antagonism may represent an additional treatment option in diabetics with hyperglucagonemia. As chronic levels of circulating IL-6 can also contribute to reduced insulin sensitivity in adipose, muscle, and liver (18, 65, 66, 68, 95, 108), reducing IL-6 signaling could have benefits beyond controlling glucagon levels.

In summary, our data support IL-6 as an important driver of glucagon secretion. IL-6 exerts its actions via at least two mechanisms: IL-6 (1) acts centrally to enhance glucagon secretion in response to both inflammation and insulin-induced hypoglycemia, and (2) enhances epinephrine-stimulated glucagon secretion in the islet. Thus, IL-6 may modulate glucagon secretion *in vivo* by augmenting neural drive to the pancreas and amplifying the effects of circulating epinephrine or neural drive directly at the pancreas. However, future work is needed to demonstrate that both effects are happening simultaneously *in vivo* as well as to determine whether varying concentrations of IL-6 will yield similar results. IL-6 is elevated in multiple disease states, many of which associate with autonomic dysfunction. These findings implicate IL-6 in contributing to

the hyperglucagonemia that exists in multiple inflammatory settings. The IL-6 signaling pathway may represent a novel target in the treatment of glucoregulatory disorders that are also characterized by autonomic dysfunction.

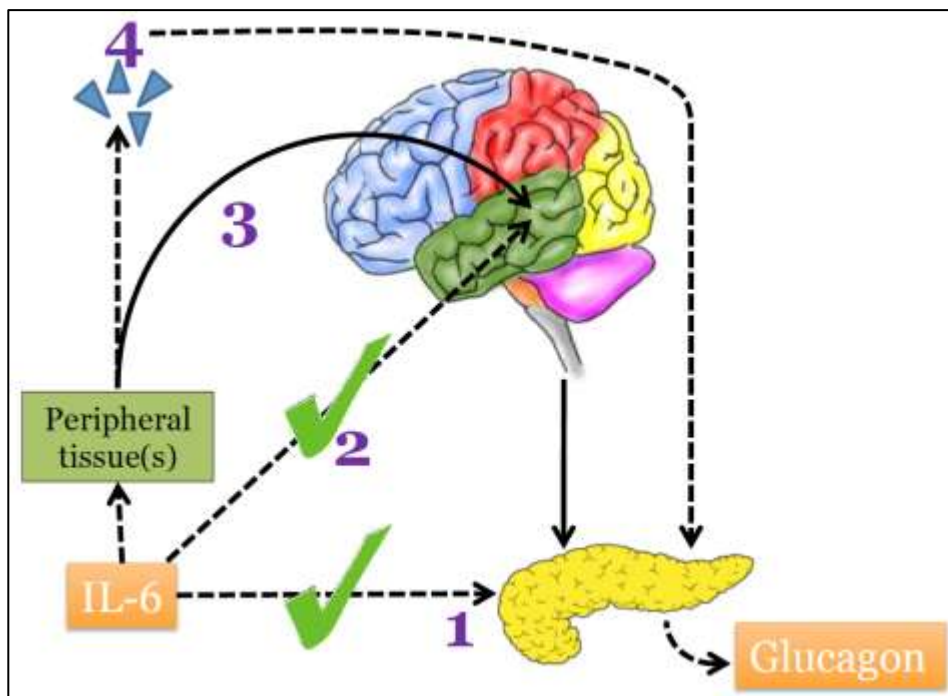
## CHAPTER IV: CONCLUSIONS

Collectively, the results presented within this dissertation point to a modulatory role of IL-6 on glucagon secretion that, contrary to the original hypothesis, is not only via its action within the brain, but also via its action within islets. Thus, IL-6 enhances glucagon secretion by at least two distinct yet complimentary mechanisms.

First, IL-6 is able to augment glucagon secretion via the brain. Central IL-6 administration alone has no acute effect on glucagon secretion in either IL-6 knock-out or wild-type mice. However, administration of the cytokine centrally enhances the glucagon response in IL-6 knock-out mice to two different settings associated with elevated autonomic drive: endotoxemia (LPS) and hypoglycemia. While these settings of enhanced glucagon secretion seem at first entirely different, they are both associated with elevated pro-inflammatory cytokines and autonomic drive. However, the body of work in this dissertation did not determine whether autonomic drive to the pancreas is similarly modulated by both stimuli. This is one potential future direction for this project (see **Chapter V**).

Second, IL-6 can act directly on islets in the presence of elevated adrenergic tone (epinephrine). Previous work has demonstrated that IL-6 has a slow, transcriptional effect on glucagon production (34). The studies within this body of work demonstrate that IL-6 does not acutely (i.e. within minutes) modulate glucagon secretion. However, IL-6 does acutely modulate glucagon secretion in the presence of epinephrine. Given that epinephrine is elevated following LPS exposure and during insulin-induced hypoglycemia, this work suggests that IL-6 can not only modulate autonomic drive to

the islet, but it can also modulate the effects of autonomic drive (i.e. epinephrine) on the islets (**Figure 4.1**).



**Figure 4.1: IL-6 stimulates glucagon secretion by direct actions on the pancreas and by actions within the brain.**

While historic literature supports the involvement of the autonomic nervous system (ANS) in the glucagon response to hypoglycemia (50, 51, 128), the role of the ANS in the glucagon response to LPS has not been characterized. The findings of this dissertation suggest that in wild-type mice, both the sympathetic and parasympathetic nervous systems are involved in the glucagon secretory response to LPS. When both branches of the ANS are inhibited via ganglionic blockade, glucagon secretion following LPS is maximally suppressed. Interestingly, so is plasma IL-6. These data suggest that the ANS is highly involved in the peripheral inflammatory response to LPS.

## CHAPTER V: FUTURE DIRECTIONS

The work presented within this dissertation has further characterized the manner by which IL-6 effects glucagon secretion. Not only can IL-6 function within the brain in settings of stress to augment glucagon secretion, but the cytokine can also enhance glucagon secretion by direct action on islets in settings of stress simulated by elevated epinephrine. While the findings of this dissertation offer insights into the nature of IL-6 in the setting of a stressor, several important questions remain.

### **Where does IL-6 signal in the brain to enhance glucagon secretion in settings of stress?**

Perhaps one of the first questions to be answered is where IL-6 functions in the brain to enhance the glucagon response during both hypoglycemia and acute inflammation (LPS). Beyond the sites of IL-6 action, is it possible that the neural drivers culminating in glucagon and epinephrine secretion in these settings are shared? To expand, when blood glucose drops (such as after a long fast or following insulin injection), our body has a normal response to limit the nadir in blood glucose, thus ensuring that the brain has an adequate supply of glucose. This normal physiologic response to hypoglycemia involves increased pro-inflammatory cytokine production (30) as well as activation of specific neural circuits that enhance autonomic drive to the pancreas and adrenal gland (8). Given that inflammation is another setting associated with enhanced autonomic drive coupled to an increase in pro-inflammatory cytokines, it is reasonable to assume that the body may utilize this existing system during inflammatory events.

Using *c-fos* as a marker for neural activation, we can speculate that there are regions that are similarly activated during both hypoglycemia and acute inflammation. Multiple groups demonstrated that intraperitoneal injection of LPS results in robust activation of *c-fos* in the paraventricular nucleus of the hypothalamus (PVH) as well as in the nucleus tractus solitarius (an area from which neural projections extend to the PVH) 3 hours following injection (138, 142). Further, Diggs-Andrews found elevated *c-fos* in the PVH of mice 2 hours following a bolus of insulin to achieve hypoglycemia (29). The PVH is an area that receives many inputs from glucose-sensing brain regions (8) as well as an area from which pre-motor neurons controlling peripheral outputs project to the pancreas, as evidenced by pseudorabies virus retrograde tracking (75). See **Figure 1.3** for a schematic of glucose-sensing circuitry.

Although these studies did not define the type of projection neurons from which these pre-motor neurons extend, others have found sympathetic fibers (fibers staining positive for tyrosine hydroxylase, the enzyme precursor to dopamine and norepinephrine) extending and making connections to alpha cells of mouse islets (20). Parasympathetic fibers (fibers staining positive for the vesicular acetylcholine transporter) were also found to extend throughout mouse islets (107). Although expression of the IL-6 receptor (IL-6R $\alpha$ ) mRNA is not detectable at rest in the PVH (112), IL-6R mRNA is increased in the PVH 3 hours following LPS injection (138). The PVH, therefore, may represent a site by which IL-6 may augment glucagon secretion in settings of stress.

While the PVN has been demonstrated to be a critical integration site for glucose homeostasis (8), the site many investigators consider to be most critical for glucose

sensing itself is the ventromedial hypothalamus (VMH). The VMH is one of many central glucose-sensing regions that projects to the PVN. The VMH is thought to indirectly elicit pancreatic responses from peripheral motor neurons based on pseudorabies tracking studies (75). Nonetheless, this particular region has become defined as critical for the glucagon and epinephrine responses to hypoglycemia. Whether this same circuitry is harnessed for the glucagon or epinephrine response to inflammatory stress remains to be determined. Unlike in the PVH, the VMH also expresses the IL-6 receptor (IL-6R $\alpha$ ) in the unstimulated state. However, Vallieres and colleagues did not observe a further increase in VMH IL-6R mRNA expression following LPS exposure (112). Moreover, they also did not observe an increase in VMH *c-fos* following LPS exposure, although more recently, others have produced data that conflicts with this finding (43). In sum, the VMH stands out as another potential site where IL-6 may augment glucagon secretion in settings of stress. In fact, it is possible that IL-6 may act via both nuclei to elicit its effect on glucagon.

The work presented within this dissertation demonstrates that IL-6 injection into the lateral ventricle is sufficient to enhance glucagon secretion not only in response to LPS, but also in response to insulin-induced hypoglycemia. Both endotoxemia (LPS) and hypoglycemia are two settings of enhanced autonomic drive. Moreover, both of these drivers of glucagon secretion are also associated with acute neuroinflammation. Therefore, it is conceivable that the same circuitry that is harnessed to facilitate the glucagon response to hypoglycemia may be the very same circuitry that is utilized in the response to LPS. Whether the response occurs in well-characterized glucoregulatory nuclei such as the PVH or VMH remains to be defined. Future studies comparing *c-fos*



activation in the brain between mice lacking IL-6 and wild-type mice during both hypoglycemia and acute inflammation may allow for initial parsing apart of what regions are affected by IL-6. An additional IL-6 knock-out + central IL-6 injection (rescue) would be an important control in this case. These studies would also help define any differences between acute inflammation and hypoglycemia in terms of activated (*c-fos*) nuclei.

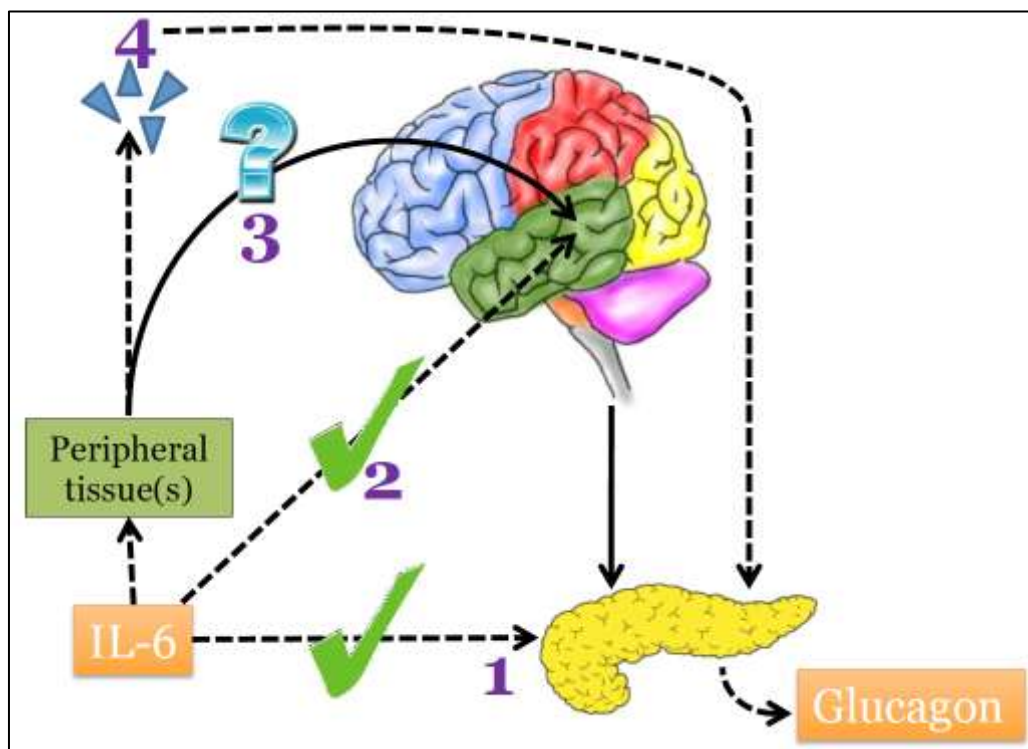
### **What is the contribution of peripheral IL-6 signaling versus central IL-6 signaling in the glucagon response to stressors?**

The studies performed for this dissertation suggest that the CNS plays a critical role in the glucagon response to inflammation. Previous data has also confirmed the crucial involvement of the brain in the counter-regulatory response to hypoglycemia. However, the work contained herein as well as previous work did not measure the relative contribution of central versus peripheral IL-6 signaling simultaneously during a stress *in vivo*. Wild-type mice were used in the pharmacologic studies to determine the role of the autonomic nervous system (ANS) in the glucagon response to inflammation. While IL-6 levels normally surge following LPS injection (and did in control mice), IL-6 levels were greatly blunted following ganglionic blockade. These data make it difficult to untangle whether the resultant loss in the glucagon secretory response following LPS administration was due to a loss of IL-6's ability to modulate central drive or a loss of plasma IL-6 interacting with epinephrine or norepinephrine that might normally occur due to the enhanced autonomic drive. The other pharmacologic studies targeting specific branches of the ANS do not provide enough information to determine the relative importance of central versus peripheral IL-6 signaling, either. Additional *in vivo*

studies need to be performed to elucidate the relative importance of IL-6 action directly on the pancreas versus IL-6 action within the brain.

A study performed by Zielinski and colleagues demonstrates the importance of vagal afferents in the response to LPS (in this case, the readout for a response to LPS was sleep). Mice administered an intraperitoneal bolus of LPS experience decreased sleep when they are vagotomized (150). Importantly, vagotomy not only reduced central neuroinflammation in response to LPS, but it also reduced plasma pro-inflammatory cytokines. This study suggests that 1) vagal afferents are critical for a full inflammatory response to LPS, and 2) vagal afferents contribute to the increased central production of pro-inflammatory cytokines in settings of stress. The liver is innervated by vagal afferents, which respond to inflammatory cytokines (26), including IL-6, which triggers production of hepatic acute phase proteins. Hepatic vagal afferents project to the nucleus tractus solitarius (NTS), which, as previously mentioned, sends projections to the PVH, an area that is activated (as indicated by *c-Fos*) following LPS administration (138, 142) and also itself upregulates expression of IL-6R during acute inflammation (138). These data lead to the question of whether peripheral IL-6 signaling in the liver indirectly contributes to the secretion of glucagon in settings of inflammation. Moreover, plasma IL-6 is elevated during hypoglycemia in healthy individuals (30) as well as in the resting state in obese individuals (33). Whether vagal IL-6 signaling via the liver contributes to glucagon secretion in these settings is an interesting question that should be further explored (**Figure 5.1**). Studies utilizing vagotomized mice in the presence or absence of IL-6 (wild-type vs. knock-out) would be an informative starting point. A stronger future direction of these studies might use either hepatocyte-specific IL-6

receptor (IL-6R $\alpha$ ) knock-out (84) or an adenoviral dominant negative IL-6 receptor (IL-6R $\alpha$ ) via tail vein injection in the presence or absence of vagotomy compared to controls. The advantage of an adenoviral injection approach is that hepatocytes as well as other hepatic cellular populations such as Kupffer cells (145), the immune cells that found interspersed among vagal fibers and also express the IL-6 receptor (26), will be targeted.



**Figure 5.1: IL-6 stimulates glucagon secretion by direct actions on the pancreas and by actions within the brain. Does it also work via vagal input to the brain?**

Previously in this dissertation, a study was discussed wherein Tocilizumab, a humanized IL-6 receptor antibody, was administered to diabetic patients with rheumatoid arthritis, and HbA1c improved upon treatment (97). Although it is common

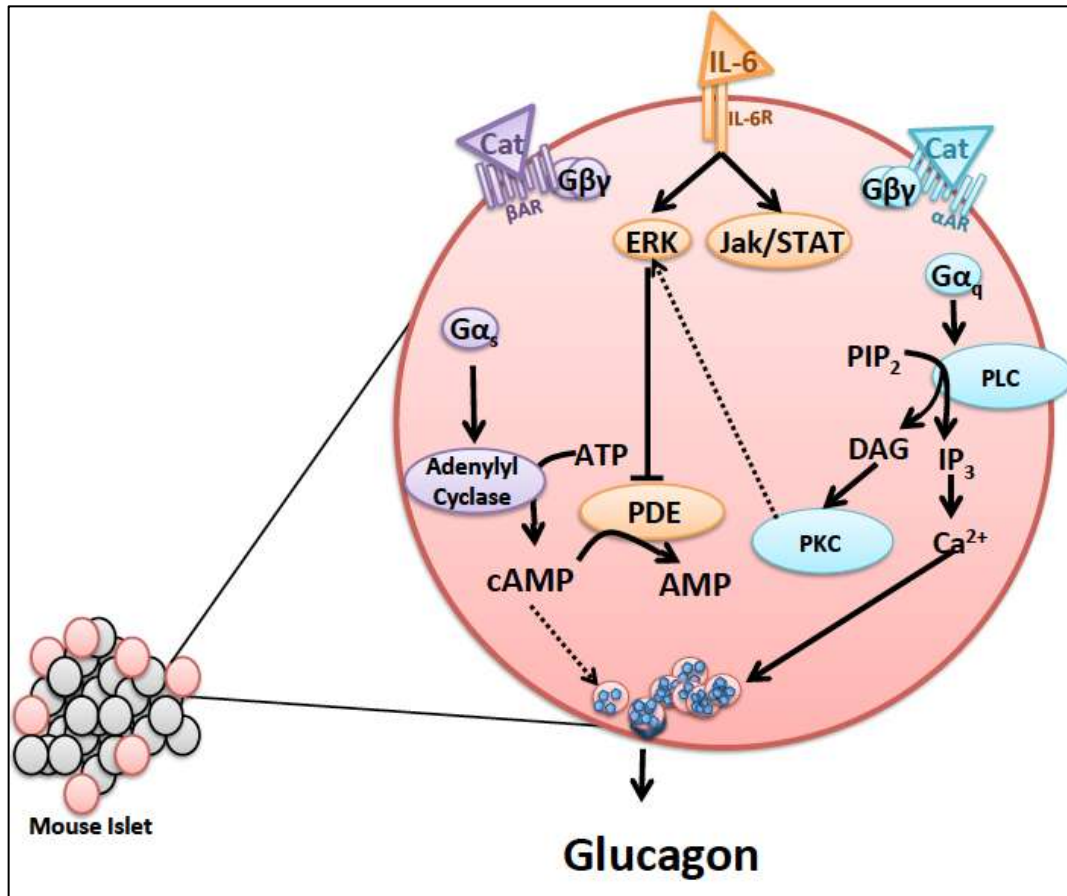
that individuals with type 1 diabetes have elevated plasma glucagon or at least an elevation in the ratio of glucagon to insulin, glucagon was not assessed in these studies. One possible way by which Tocilizumab may have reduced blood glucose is by reducing plasma glucagon. By inhibiting the ability of IL-6 to signal peripherally (not centrally, as the antibody would not cross the blood-brain barrier), vagal afferent input may have been reduced. By reducing vagal afferent input, neuroinflammation may also have been reduced, and this, in turn, may have reduced autonomic drive to the pancreas, thereby reducing glucagon production. The concept of targeting IL-6 peripherally rather than centrally makes the IL-6 pathway an exciting and viable therapeutic option.

### **How does IL-6 signaling synergize with epinephrine signaling?**

The data generated in islets suggests that IL-6 augments glucagon secretion in the presence of epinephrine (which is elevated in settings of stress) in a calcium-independent manner. Epinephrine is a nonselective agonist for adrenergic receptors and therefore could bind to either of the adrenergic receptors expressed on alpha cells. Islet alpha cells express primarily  $\alpha_1$  ( $G\alpha_q$ -coupled) and  $\beta_2$  ( $G\alpha_s$ -coupled) adrenergic receptors. As the synergy of IL-6 and epinephrine is calcium-independent, one would speculate that the synergy is likely not via  $G\alpha_q$ -coupled ( $\alpha_1$ ) signaling. However, another result of  $G\alpha_q$ -coupled signaling is activation of protein kinase C (PKC) (**Figure 5.2**). In some cell populations, activation of PKC activates the MAPK/ERK pathway (134). As described in the introduction, the IL-6 signaling pathway also shares the MAPK/ERK pathway. Moreover, ERK has been implicated as a positive effector of glucagon

secretion (21). Therefore, synergy between these pathways via  $\alpha 1$  adrenergic signaling is an interesting possibility that should be further explored.

Given that calcium signaling was not enhanced by epinephrine in the presence of IL-6, synergy between IL-6 signaling and  $\beta 2$  adrenergic signaling may be a more viable explanation. Further, pharmacologic studies have suggested that  $\beta$  adrenergic signaling may predominate in epinephrine-stimulated glucagon secretion (140). The  $\beta 2$  adrenergic receptor operates via a  $G\alpha_s$ -coupled mechanism, which results in elevated levels of cyclic adenosine monophosphate (cAMP). There are data to suggest that cAMP may also be involved in glucagon secretion (131), and experiments using forskolin (which increases cellular cAMP) or IBMX (a phosphodiesterase inhibitor which indirectly raises cAMP) point to the role of cAMP in epinephrine-stimulated glucagon secretion (31, 123). How may the IL-6 signaling pathway converge with the  $\beta 2$  adrenergic signaling pathway? One of the messengers activated by IL-6 signaling through its receptor is ERK. Although not yet demonstrated in islet alpha cells, ERK has been found in other cell populations to inhibit phosphodiesterase activity (118). Given that others have found inhibition of phosphodiesterase to result in alpha cell glucagon secretion (123), it is possible that IL-6 augments epinephrine-stimulated glucagon secretion by decreasing the activity of phosphodiesterase, thereby amplifying intracellular cAMP and stimulating glucagon secretion.



**Figure 5.2: The proposed mechanism for IL-6 to enhance epinephrine-stimulated glucagon secretion.** There are currently two hypotheses that may occur simultaneously. First, via ERK, IL-6 reduces phosphodiesterase activity, thereby enhancing the pool of cAMP during  $\beta$ -adrenergic stimulation and increasing glucagon secretion. Alternatively (or concurrently), IL-6 reduces activity of phosphodiesterase, and  $\alpha$ -adrenergic input further reduces the pool by increasing ERK phosphorylation via PKC.

Beyond understanding the signaling cascade that results in enhanced glucagon secretion under settings of stress, another question which remains is whether the synergy that happens between adrenergic and IL-6 signaling in the islet alpha cell may also happen in other electrically excitable cell populations—neurons. The similarities and differences between these signaling events represent an exciting new path of discovery.

## **What role might other cytokines have in the IL-6-mediated enhancement of glucagon secretion?**

Based on the initial studies demonstrating that IL-6 completely rescues the glucagon response to LPS while not altering concentrations of other plasma pro-inflammatory cytokines such as IL-1 beta or TNF-alpha, this led to a focus solely on IL-6 as the driver for glucagon secretion. However, it should be emphasized that during an inflammatory event, IL-6 does not exist alone. IL-6 is present amid a variety of hormones and other cytokines (both inflammatory and anti-inflammatory).

For example, others have claimed that IL-1 $\beta$  and IL-6 signaling pathways may synergize in the brain (117). In light of work suggesting IL-1 beta can signal through ERK in other cell populations (29), synergy between IL-1 beta and IL-6 would be consistent with the hypothesis that ERK signaling is responsible for augmenting catecholaminergic-stimulated glucagon secretion (see Future Direction 3 above). Alternatively, given that the earlier study observing synergy between the two pathways observed synergy during a longer-term study (food intake), IL-1 beta-mediated production of NF- kappa B could have also increased expression of IL-6. The first logical step for this project would be to assess the effect of IL-6 in the presence or absence of IL-1 $\beta$  *ex vivo* on islet catecholaminergic signaling. This would involve a series of perfusion experiments that compare the glucagon response of islets to epinephrine in the presence or absence of IL-1 beta. These studies would then be compared to studies where both IL-6 and IL-1 beta are administered to islets. If IL-6 and IL-1 beta are synergistic, the glucagon response to epinephrine will be further augmented above the degree to which IL-6 augments the response. The amount of treatment groups in this proposed experiment is large, however. A more straightforward

static incubation approach may be simpler, although kinetic data that could be gleaned from these experiments (i.e. how rapid the glucagon secretory response is) would be limited. To conclude, to fully understand the effects of IL-6, more work must be done to examine the actions of IL-6 in light of other cytokines or hormones that may also influence its action.



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