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# Catecholamines And Catecholamine Resistance In Animal Models Of Metabolic Syndrome

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# CATECHOLAMINES AND CATECHOLAMINE RESISTANCE IN ANIMAL MODELS OF METABOLIC SYNDROME

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PATHOBIOLOGY, PHARMACOLOGY, AND PHYSIOLOGY

## ABSTRACT

The sympathetic nervous system is a key regulator of energy homeostasis, in part, through the control of white adipose tissue lipolysis. Sympathetic nerves modulate adipose tissue physiology through the release of catecholamines, particularly norepinephrine, onto alpha- and beta-adrenergic receptors (αAR; βAR). Lipolysis refers to the breakdown of triglycerides into glycerol and free fatty acids, often in response to activation of β2/3-AR. Beta adrenergic activation of lipolysis is widely known to be impaired in patients with metabolic syndrome and animal models thereof (catecholamine resistance). Recent studies have demonstrated that catecholamine regulation of adipokine release is also disrupted, suggesting that catecholamine resistance may contribute to the reduced levels of insulin sensitizing hormones such as adiponectin observed in metabolic syndrome. Unsurprisingly, catecholamine resistance is a prospectively identified risk factor for development of glucose intolerance and type 2 diabetes, making further study of considerable value to preventative medicine.

Remarkably, in the decades since catecholamine resistance was first identified, comparatively little work has examined whether it might be mechanistically driven by excessive receptor stimulation. When receptors are chronically stimulated by their ligands, they often undergo desensitization and downregulation. Much data show that

sympathetic nerve activity is elevated in metabolic syndrome, a phenomenon which has been widely explored in clinical research, particularly in areas such as hypertension. This present body of work fills two key gaps in this area by 1) assessing the physiology and neurochemistry of sympathetic nerves in adipose tissue under normal diet and high fat diet (in both visceral and subcutaneous fat) and 2) testing the hypothesis that central manipulations which modulate sympathetic nerve activity could recapitulate catecholamine resistance in animal models.

Keywords: Adipose tissue, norepinephrine turnover, sympathetic nervous system, catecholamines, lipolysis

# DEDICATION

For Gwen, everything.

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Maria, you made a lab like an untamed garden. In that space, I was occasionally (okay, often) stung by a hidden thorn. However, I also took joy in uncovering a layer of order which lies beneath nature's seeming chaos. I do think there was a method to the madness, but I also think you revel a little bit in the madness. It takes all types, and you are one of a kind. You have left an indelible presence in my life.

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## DEPLETING HYPOTHALAMIC SOMATOSTATINERGIC NEURONS RECAPITULATES DIABETIC PHENOTYPES IN MOUSE BRAIN, BONE MARROW, ADIPOSE TISSUE, AND RETINA



## ELEVATED CIRCULATING AND TISSUE LEVEL CATECHOLAMINES ARE ASSOCIATED WITH CATECHOLAMINES RESISTANCE IN MALE, BUT NOT FEMALE MICE

# LIST OF ABBREVIATIONS

| | |
|---|---|
| a-methyl-p-tyrosine | AMPT |
| Adipose triglyceride lipase | ATGL |
| Alpha-adrenergic receptor | α-AR |
| Beta 3-adrenergic receptor | β3-AR |
| Body mass index | BMI |
| cAMP response element binding protein | CREB |
| Catecholamine-o-methyltransferase | COMT |
| Comparative gene identifier-58 | CGI-58 |
| De novo lipogenesis | DNL |
| Dihydroxyphenylglycol | DHPG |
| Diphtheria toxin | DTA |
| Dopamine beta-hydroxylase | DBH |
| Exchange protein directly activated by cAMP | EPAC |
| G-protein coupled receptors | GPCR |
| Hormone Sensitive Lipase | HSL |
| Interleukin-6 | Il-6 |
| Monoacylglycerol lipase | MAGL |
| Metabolic Syndrome | MetS |
| 3-methoxy 4-hydroxyphenylglycol | MHPG |
| Monoamine oxidase A | MAO-A |

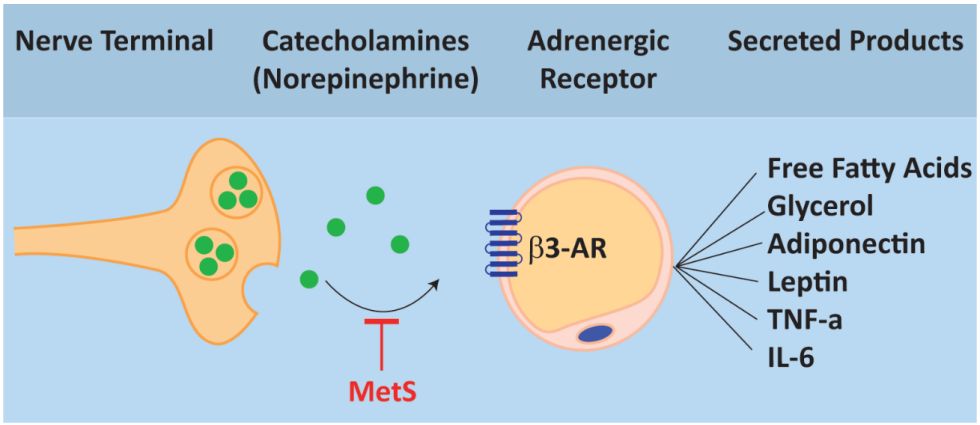
| | |
|---|---|
| Muscle sympathetic nerve activity | MSNA |
| Non-esterified free fatty acids | NEFA |
| Norepinephrine transporter | NET |
| Norepinephrine turnover | NETO |
| Normetanephrine | NMN |
| Paraventricular nucleus of the hypothalamus | PVN |
| Protein kinase A | PKA |
| Perilipin 1 | PLIN1 |
| Phosphatidylinositol 3-kinase | PI3K |
| Rostral ventrolateral medulla | RVLM |
| Sympathetic nervous system | SNS |
| Somatostatin | SST |
| Transient Receptor Potential Vanilloid 1 | TRPV1 |
| Tumor necrosis factor alpha | TNF-α |
| Tyrosine hydroxylase | TH |

# INTRODUCTION

## *The Sympathetic Paradox*

Metabolic syndrome (MetS) is a cluster of clinical findings which drive excess risk of all-cause mortality and cardiovascular mortality. Adiposity (increased body weight) and increased activity of the sympathetic nervous system[1], which mediates the fight or flight response, are commonly observed features of MetS. The co-occurrence of these two phenomena is puzzling because the sympathetic nervous system typically *decreases* fat stores through the activation of β-adrenergic receptors in the process of lipolysis. However, in MetS, elevated sympathetic nerve activity is associated with *increased* fat stores[2]. This dissertation suggests this paradox may be explained by adipose catecholamine resistance, in which adipose tissue loses its capacity to respond to catecholamines, the bioactive molecules released by sympathetic nerves.

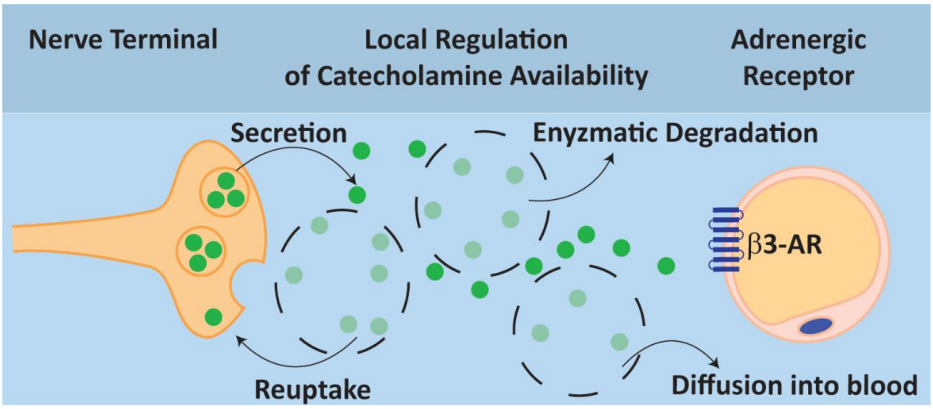
Figure 1: The Sympathetic-Adipocyte Interface in Metabolic Syndrome



The overarching hypothesis of this work is that chronic stimulation of adipose tissue β-adrenergic receptors contributes to catecholamine resistance. Findings in support of this hypothesis would be significant because they propose novel interventions to restore catecholamine sensitivity. Restored catecholamine sensitivity should improve the regulation of lipolysis and elicit favorable effects on several adipose secreted hormones, including leptin[3] and adiponectin[4], and cytokines, such as interleukin-6[5, 6].

Thus, insights into the physiology of sympathetic nerves in the adipose tissue of MetS animal models may improve the ability to rationally modulate these targets in support of metabolic health. In Chapter 2 of this dissertation, a "circuit" viewpoint of sympathetic nerves is employed, in which the relationship between catecholamines and adipose tissue is studied in the context of an upstream manipulation in brain regions regulating peripheral nerve activity. However, catecholamines are not only regulated at their point of secretion, but also by their reuptake into nerve terminals and degradation (Figure 2). The net effect of norepinephrine release, reuptake, and clearance determines the amount of stimulation that adrenergic receptors receive[7]. Chapter 3 focuses on the local regulation of catecholamines in adipose tissue, through biochemical and

Figure 2: Local Regulation of Catecholamines



pharmacologic manipulation in the context of high fat diet.

The remainder of Chapter 1 introduces key concepts in this area, beginning with the clinical context of metabolic syndrome and its associations with sympathetic nerve hyperactivity, then transitioning to a summary of mechanistic insights previously generated into the origin of sympathetic nerve dysfunction in metabolic syndrome. I then review the biochemistry and enzymes of lipolysis, which regulate the adipocyte response to sympathetic nerves, followed by a section on the relationship of lipolytic dysfunction to whole organism metabolic dysfunction. Because the secretion of adipokines is also regulated by sympathetic nerves and disrupted by catecholamine resistance, the next section discusses the role of adiponectin, the prototypical adipokine, in whole organism metabolic dysfunction. I then review the evidence showing that sympathetic nerves are the major source of catecholamines regulating adipose tissue, followed by four sections detailing the neuroanatomy and neurochemistry of sympathetic nerves, with special attention to their relationship to adipose tissue and metabolic disease. Having established this background, I conclude the chapter by reviewing clinical and mechanistic insights into catecholamine resistance, as well as relevant gaps in the study of sex differences, and restating my overarching hypothesis.

*Metabolic Syndrome: from Epidemiology to Molecular Mechanisms*

The prevalence of metabolic syndrome, the illness cluster linking hyperlipidemia, hypertension, hyperglycemia, and central adiposity[8] increased by 35% over the last twenty years.[9] MetS currently affects approximately a third of all Americans[10], almost half of Americans over age 60[10] and is associated with increased risk of cardiovascular[11], cerebro-vascular[12], hepatic[13], cancer[14] and all-cause

mortality[15], as well as life-limiting disabilities[16]. In brief, many people with metabolic syndrome are living shorter and sicker lives, and there are many more of them than in the recent past. Given its high prevalence and adverse associations, much work has investigated mechanisms linking the underlying elements of the metabolic syndrome cluster. One major area of investigation is the relationship between metabolic syndrome and autonomic nervous system dysfunction.

The autonomic nervous system is the branch of the nervous system which promotes homeostasis by controlling breathing, heart rate, blood pressure, digestion, body temperature, blood glucose, and all other core physiologic functions. The autonomic nervous system is divided into two branches: the parasympathetic “rest and digest” branch and the sympathetic “fight or flight” branch. The peripheral nerves of the parasympathetic nervous system act through acetylcholine on muscarinic acetylcholine receptors, and the peripheral nerves of the sympathetic nervous system act through norepinephrine on adrenergic receptors. In depth functional, anatomic, and biochemical features of the sympathetic nervous system are reviewed in later sections of this chapter.

Dysfunction in the autonomic nervous system is a well-recognized risk factor for developing MetS and therapeutic target for treating MetS. Specifically, exaggerated activity of the sympathetic nervous system (SNS) is strongly associated with several components of MetS[17]. The following paragraphs examine the link between SNS hyperactivity and each of the defining components of MetS: hyperglycemia and insulin resistance, hypertension, hyperlipidemia, and excess adiposity.

Several lines of evidence link sympathetic nervous system hyperactivity and disruptions in glucose homeostasis. Clinically, co-morbid disorders such depression[18], post-traumatic stress disorder[19], and chronic stress[20], each of which are associated

with increased SNS activity, are risk factors for development of insulin resistance, impaired glucose homeostasis, and type 2 diabetes. Interventions to reduce SNS activity[21]) improve insulin resistance in a manner comparable or superior to the frontline diabetes medication, metformin[22]. Convergent clinical validation of the link between SNS hyperactivity and metabolic disease comes from the observation that patients with tumors secreting catecholamines develop insulin resistance which resolves upon tumor resection[23], a strong independent line evidence showing that, in excess, molecules released by sympathetic nerves induce insulin resistance.

Hyperactivity of the sympathetic nervous system is widely recognized as a causative factor in hypertension, another diagnostic element of MetS. Meta-analyses demonstrate that muscle sympathetic nerve activity (MSNA) is consistently higher in patients with hypertension[24]. Several therapeutic approaches for hypertension inhibit sympathetic nerve activity[25, 26]. The relationship between sympathetic nerve hyperactivity and hypertension is sufficiently strong that ablation of renal sympathetic nerves is an efficacious therapeutic option in treatment resistant hypertension[27].

Sympathetic nerve dysfunction is also associated with dyslipidemia, a core component of MetS. In meta-analyses including more than 1400 patients, muscle sympathetic nerve activity is strongly positively correlated to plasma triglycerides, total cholesterol, low density lipoprotein (LDL), total cholesterol[28]. Conversely, inhibition of sympathetic tone using central nervous system acting drugs such as moxonidine in hypertensive patients has beneficial off-target effects on circulating lipids, including reducing LDL and raising high density lipoproteins (HDL) [29]. Comparable effects have been observed in animal models, specifically reduction of circulating triglycerides and free fatty acids[30].

The relationship between sympathetic nerve activity and adiposity is complex. Direct electrophysiologic recordings of muscle sympathetic nerves show hyperactivity in individuals with greater abdominal adiposity[28], even when normotensive. However, many therapies to decrease adiposity rely on activating the sympathetic nervous system or adrenergic receptors. Phentermine, an amphetamine derivative, is a clinically used example of the former approach, which has been shown to increase urinary catecholamines and cardiac sympathetic drive[31]. In the latter approach, adrenergic receptor agonists such as mirabegron are used due to their action on the β-3 adrenergic receptor, which promotes lipolysis. Mirabegron was recently shown to improve glucose homeostasis and HDL cholesterol in patients in two randomized clinical trials [32, 33] As noted above, these seemingly contradictory findings constitute the sympathetic paradox—increased nerve activity (or drugs that activated adrenergic receptors) typically improves many metabolic markers, but basal nerve activity appears elevated compared to healthy controls.

Finally, some criteria require adiposity for diagnosis of metabolic syndrome, whereas the "harmonized definition" only requires three of the five criteria established above[8]. Individuals who exhibit hypertension, hyperlipidemia, and impaired glucose homeostasis without adiposity exhibit significant excess cardiovascular mortality as compared to lean individuals without these risk factors[15]. Understanding the role of the sympathetic nervous system in metabolic syndrome without increased adiposity is an important goal for research but is not addressed in the present body of work.

*Causes of Sympathetic Nerve Hyperactivity in MetS*

This section outlines several causes of sympathetic hyperactivity in MetS: endocrine dysfunction, inflammatory/immune dysfunction, dysfunction in the sensory branch of the autonomic nervous system, as well as social and behavioral factors.

Endocrinologic dysfunction may contribute to sympathetic nerve hyperactivity. Sympathetic nerve activity is higher in non-diabetic offspring of type 2 diabetic parents than non-diabetic offspring of non-diabetic parents and is tightly correlated to plasma insulin in these subjects[34, 35]. Peripheral infusions of insulin increase sympathetic nerve firing rates in humans by up to 50%, independent of changes in glycemia[36]. Infusions of insulin into the brain also increase sympathetic nerve activity in animals[37]. Conversely, sympathetic outflow can suppress insulin secretion through alpha-2 adrenergic receptors[38]. Thus, the sympathetic nervous system regulates the secretion of insulin and is also regulated by insulin. This feedback loop highlights the potential for endocrine dysfunction to promote a state of chronic sympatho-excitation.

Another endocrine hormone, leptin, has long been identified as factor driving sympathetic nerve hyperactivity[39, 40]. Leptin also promotes satiety, however resistance to this function has long been identified in MetS[41]. Interestingly, leptin resistance is selective, in that sympatho-excitatory functions are maintained in genetic and diet induced models MetS[42, 43]. Leptin is secreted by adipocytes and its circulating levels are strongly positively correlated to adiposity[42, 43]. The ability of leptin to promote sympathetic nerve activity has led to extensive interest as an underlying mechanism of hypertension[41-43]. Sympathetic nerves also regulate leptin synthesis and secretion[3, 44, 45]. Thus, just as with insulin, leptin exists within a feedback loop in the sympathetic nervous system.

Neuroinflammation may also contribute to sympathetic nerve hyperactivity. Sympathetic nerves are regulated by many sites in the brain, and inflammation in these sites typically produces elevated sympathetic nerve activity. Hypothalamic inflammation increases sympathetic nervous system activity, particularly in the paraventricular nucleus (PVN)[46-48]. PVN inflammatory cytokine expression is upregulated within days of initiating stress induced hypertension[49], as well as intermittent hypoxia induced SNS hyperactivity[50]. Direct causal evidence also exists for the relationship between neuroinflammation and sympathetic nerve activity: infusion of TNF-α or Il-1β into the PVN dose-dependently increases renal SNS activity[51]. High fat diet exposure drives hypothalamic inflammation beginning in the arcuate nucleus within days[52], as well as increases in sympathetic nerve activity within days of diet initiation[53, 54]. Loss of endogenous anti-inflammatory factors in the hypothalamus, including somatostatin, have been identified previously[55] and in published work contained within this dissertation[56] which have been theorized to provoke increased sympathetic nerve activity.

Sympathetic nerve function is heavily modulated by the immune system. Macrophages express enzymes such as monoamine oxidases which break down norepinephrine[57], thus modulating sympathetic signaling without acting directly on sympathetic nerves. Other immune cells modulate the growth of sympathetic nerves into peripheral organs, including adipose tissue; for example, eosinophils produce nerve growth factors essential for axonal growth, as do gamma-delta T-cells[58, 59]. Immunologic dysfunction often contributes to other factors already discussed in this section. For example, monocyte invasion of brainstem and hypothalamic regions regulating sympathetic drive produce long lasting neuroinflammation[60, 61].

Local dysfunction of the autonomic circuity in adipose tissue may also contribute to sympathetic nerve hyperactivity. Adipose tissue sensory nerves also regulate sympathetic nerve activity. Adipose tissue becomes hypoxic under high fat diet[62]. In-vitro, hypoxia sensitizes transient receptor potential vanilloid 1 (TRPV1) channels on sensory nerves [63]. Sensory nerve activation can elicits increases in sympathetic nerve activity. Direct infusion of capsaicin, a TRPV1 agonist, into adipose tissue activates sympathetic nerve outflow[64]. Again, in relation to previously discussed mechanisms of sympathetic excitation, this reflex depends on the hypothalamic paraventricular nucleus [64] and the magnitude of the sympathetic response is increased by local PVN neuroinflammation[46].

Beyond mechanistic studies, attention to social and behavioral factors also elucidates potential causes of sympatho-excitation. Weight cycling, or “yo-yo dieting”, in which individuals rapidly gain and lose large volumes of weight, has been shown to increase markers of sympathetic nerve hyperactivity[65], as well as predict adverse cardiometabolic outcomes[66]. Weight cycling has no broadly agreed upon definition, however, weight regain is an extremely common outcome of dieting[67], and dieting is an extremely common behavior: approximately 70% of women and 60% of men with a BMI over 30 report dieting in a given year[68]. Not all of these individuals will exhibit weight cycling, however, the potential pool of patients who could weight cycle is nevertheless very large. Additionally, stress associated with discrimination against people at higher body weights (weight stigma) is an increasingly recognized factor in poor health outcomes[69, 70], and psychosocial stress of all kinds is well-known to increase sympathetic nerve activity[71].

In summary, a complex confluence of dietary, molecular, and social factors may produce increased sympathetic nerve activity in MetS. Many of these factors center on adipose tissue, making it critically important to understand the cell biology and physiology of interface between sympathetic nerves and adipose tissue. Such investigations are expected to improve mechanistic insights and facilitate rational drug development and repurposing to alleviate metabolic disease.

*The Biochemistry and Enzymes of Lipolysis*

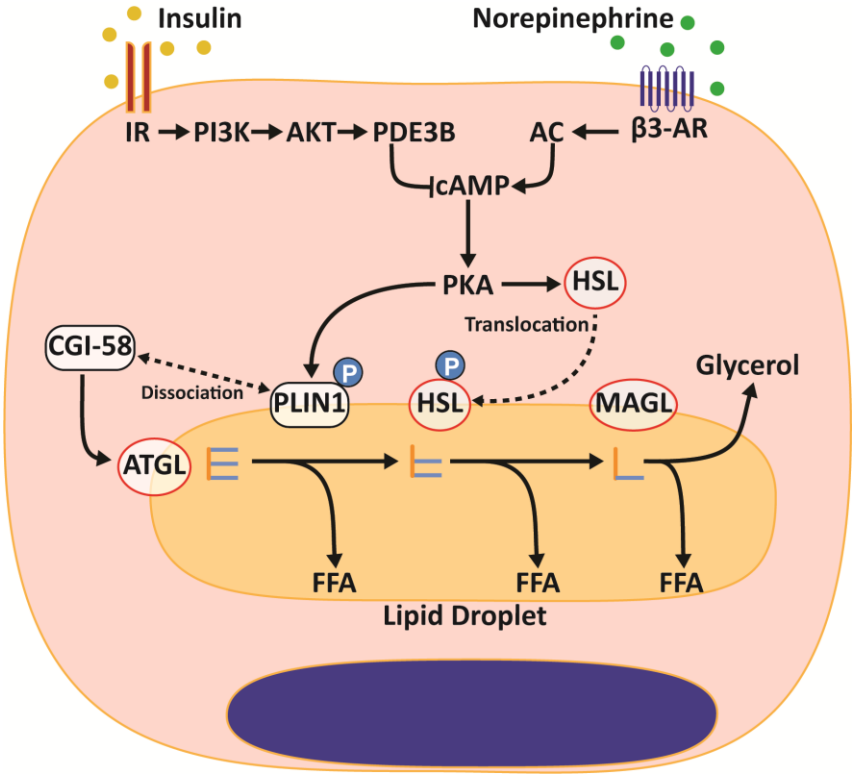
Sympathetic nerves control adipose tissue lipolysis via β-adrenergic receptors (Figure 3). In this section, I will detail the intracellular signal transduction of this process. Norepinephrine activates beta-adrenergic receptors (predominantly β2-AR in human[72], β3-AR in mouse[72]), which are predominantly coupled to $G_{as}$. $G_{as}$ signaling acts through adenylyl cyclase to generate cyclic adenosine monophosphate (cAMP), which in turn activates protein kinase A (PKA). PKA phosphorylates perilipin 1 (PLIN1), a lipid droplet associated protein, which drives the dissociation of perilipin and comparative gene identifier 58 (CGI-58)[73]. CGI-58 activates adipose triglyceride lipase (ATGL), increasing its activity 20-fold[73]. At this stage, the breakdown of triglycerides stores begins. The hydrolysis of fatty acids from the glycerol backbone dependent is upon their stereospecific position (sn-1 through sn-3)[74].

ATGL initiates the breakdown of triglycerides. ATGL cleaves the sn-1 fatty acid from the triglyceride molecule, generating one free fatty acid and a diacylglycerol[73]. PKA also phosphorylates hormone sensitive lipase (HSL), promoting its translocation from the cytosol to the surface of the lipid droplet[75]. Once translocated, HSL cleaves the sn-2 position free fatty acid from the diacylglycerol, generating

monoacylglycerol[76]. Monoacylglycerol lipase (MAGL) cleaves the final fatty acid, leaving free glycerol[74]. In summary, lipolysis in adipocytes proceeds through the steps outlined in this section. A description of anti-lipolysis is found in the following paragraph.

Insulin is a major inhibitor of lipolysis. Insulin signals through the tyrosine kinase insulin receptor (IR), which in turn sequentially activates insulin receptor substrates and phosphatidylinositol 3-kinases (PI3K)[77]. In turn PI3K activates AKT/protein kinase B[77]. The anti-lipolytic role of insulin is driven by AKT, which activates

Figure 3: Control of Adipocyte Lipolysis




IR insulin receptor; PI3K phosphatidylinositol kinase, AKT protein kinase B; PDE3B phosphodiesterase 3B; β3-AR beta-3 adrenergic receptor; AC adenylyl cyclase; cAMP cyclic adenosine monophosphate; PKA protein kinase A; HSL hormone sensitive lipase; PLIN1 perilipin 1; P phosphate; CGI-58 comparative gene identifier 58; ATGL adipose triglyceride lipase; MAGL monoacyl glycerol lipase; FFA free fatty acid.

phosphodiesterase 3b (PDE3B) to promote the breakdown of cAMP. cAMP breakdown prevents the sequence of lipolytic events outlined in the paragraph above[78]. Insulin also regulates lipolysis through α/β-hydrolase domain-containing 15 (ABHD15), which stabilizes PDE3B to promote cAMP breakdown[78]. There are several reports of AKT-independent mechanisms of insulin anti-lipolysis, which is believed to work through P13K mediated control of perilipin phosphorylation[79, 80]. Recent work has also shown that insulin also indirectly inhibits lipolysis via glycolysis dependent lactate production[81]. Lactate is a major metabolic fate of glucose in adipocytes, and insulin drives glucose uptake and lactate production in these cells[81]. In turn, adipocytes express a $G_{ai}$ coupled lactate receptor, GPR81[82], which inhibits lipolysis by blocking adenylyl cyclase mediated cAMP production. While insulin is a major antilipolytic hormone, there are other pathways inhibiting lipolysis, such as adenosine signaling. Adenosine inhibits lipolysis via the adenosine A1 receptor[83]. The adenosine A1 receptor is $G_{ai}$ coupled; activation of its receptor inhibits adenylyl cyclase, preventing cAMP formation[83]. Thus, control of intracellular cAMP, whether by production or breakdown, is a conserved means of regulating lipolysis.

*Control of Lipolysis in Metabolic Disease*

Adipocytes tune their physiology to nutrient availability. Under states of negative energy balance, a coordinated neuroendocrine response involving the suppression of insulin release and the activation of sympathetic nerves promotes the release of free fatty acids. In healthy individuals, circulating free fatty acids are low in non-fasting states and rise under physiologic stressors such as fasting[84] and exercise[85]. The mobilization of NEFA provides a critical nutrient source for many organs, especially the heart, which

consumes more than 80% of NEFA which are taken up from circulation[86]. Lipolysis also generates glycerol, which acts as a substrate for hepatic gluconeogenesis; this process provides glucose for organs that preferentially utilize that substrate, such as brain[87]. While the prior examples illustrate beneficial aspects of lipolysis, in metabolic syndrome, lipolysis plays a key role in metabolic dysregulation.

Free fatty acids play a central role in insulin resistance, and adipose tissue lipolysis is the major source of circulating free fatty acids[88]. Free fatty acid signaling rapidly promotes insulin resistance in hepatocytes and skeletal muscle[89], two key organs for regulation of glucose homeostasis. In these tissues, free fatty acids increase intracellular diacylglyercol and long-chain fatty CoA levels, which inhibit downstream insulin signal transduction through phosphorylation of insulin response substrate 1 [90]. Free fatty acids provide ATP for gluconeogenesis, and glycerol is a gluconeogenic precursor for hepatic glucose production, promoting hyperglycemia[91]. Free fatty acid levels are elevated in systemic circulation[92], as well as in the portal vein[93-95], which drains visceral adipose tissue and is believed to underlie the strong relationship between visceral adipose tissue accumulation and hepatic insulin resistance[94].

Why are free fatty acids elevated? Elevations in free fatty acids are widely believed to reflect the loss of regulation over lipolysis, although impaired fatty acid oxidation may also contribute to elevated levels[96]. As illustrated in the prior section, suppressing lipolysis is a major role of insulin; however, adipocytes are insulin resistant in MetS[97, 98]. Conversely, stimulating lipolysis is a major role of catecholamines; however, adipocytes are catecholamine resistant in MetS[99, 100]. In combination, this dual loss of regulation promotes an uncontrolled release of free fatty acids through an

increase in cell autonomous basal lipolysis[101]—a steady drip of free fatty acids untethered to neuroendocrine control.

As such, interventions which inhibit free fatty acid release promote improvements in metabolism. Pharmacologic inhibition of adipose triglyceride lipase using acipimox, which decreases circulating free fatty acids, decreases insulin resistance in patients [102], an effect which was replicated in high fat diet induced insulin resistance in mice using a separate pharmacologic inhibitor of lipolysis, atglistatin, as well as genetic ablation of triglyceride lipase[103]. In summary, abundant preclinical and clinical evidence shows that adipose tissue lipolysis mediated increases in free fatty acids is directly linked to whole organism metabolic dysfunction.

*Control of Adipokine Secretion in Metabolic Disease*

Beyond energy storage, adipocytes are major regulators of whole organism energy homeostasis by virtue of the many chemical messengers, or adipokines, that they release. Key adipokines include adipose specific signaling molecules such as leptin and adiponectin, as well cytokines such as interleukin 6 (Il-6) and tumor necrosis factor alpha (TNF-$\alpha$)[104, 105]. Each of these hormone and cytokines are also regulated by cAMP and thus by catecholamines released from sympathetic nerves[3, 5, 6, 44, 106]. Catecholamine resistance blunts the ability of sympathetic nerves to regulate these factors. For example, in adipocytes from healthy animals, catecholamine stimulation increases adiponectin secretion, an effect which is lost in high fat diet fed animals [4, 107, 108]. The following paragraph uses the example of adiponectin to illustrate the effect of impaired regulation of adipokine secretion on metabolic disease.

Reduced adiponectin levels are associated with core features of MetS: loss of glucose homeostasis, low grade inflammation, and hypertension, as outlined below. Adiponectin acts through its cognate receptors adipoR1 and adipoR2 to promote hepatic insulin sensitivity and whole organism glucose homeostasis[109-111], in part via degradation of intracellular ceramides[110, 112]. Adiponectin is also immunomodulatory and promotes human and murine adipose tissue macrophage polarization towards an anti-inflammatory phenotype. Specifically, adiponectin suppresses expression of TNF-α and reduces reactive oxygen species[113] in macrophages. This finding is important for metabolic syndrome because adipose tissue macrophages are known to regulate whole body metabolism[114, 115]. Adiponectin can also promote vascular health to oppose hypertension. For example, adiponectin promotes vasorelaxation in vitro [116], as well as in-vivo[117]. Conversely, adiponectin knockout mice are hypertensive, and delivery of adiponectin resolves the hypertension[118]. Adiponectin levels are typically low in metabolic syndrome and predict a wide variety of negative outcomes from diabetes incidence[119, 120] to cardiovascular disease to fracture risk[119].

In summary, in this section, and the prior section, the relationship between the secreted products of adipocytes and metabolic dysfunction is established. As shown above, free fatty acids and adipokines such as adiponectin are regulated by the same intracellular signaling, namely the production of cAMP. In turn, cAMP is regulated by catecholamines, and as such the following section focuses attention on catecholamines.

*Sympathetic Nerves Regulate Adipose Tissue Through Catecholamines*

One major group of ligands which activate $G_{as}$ signaling in adipocytes are catecholamine neurotransmitters, which include norepinephrine, epinephrine, and

dopamine. Epinephrine and norepinephrine have been recognized as pro-lipolytic agents since the late 1950's[121, 122]. Dopamine is by far the least well studied catecholamine in adipose tissue, though it appears to be pro-lipolytic through $G_{as}$ coupled dopamine D1 receptors (DRD1)[123, 124].

The quantitative contribution of each of the different catecholamines to adipocyte $G_{as}$/cAMP signaling under physiologic stressors has been assessed through careful loss of function studies. Fasting[125] and cold exposure[126] robustly activate lipolysis via sympathetic nerve action. In contrast, adrenalectomy, the surgical removal of the adrenal glands, the source of circulating epinephrine, does not change the lipolytic response to cold exposure[126]. Sympathectomy has also long been recognized as ablating the lipolytic response to fasting[128]. In otherwise healthy animals at thermoneutral conditions, sympathectomy increases adipose tissue catecholamine sensitivity, an effect not observed from adrenalectomy[127]. These studies support the conclusion that adipose tissue is principally regulated by norepinephrine released by sympathetic nerves.

Occasionally, data has emerged which contradicts this conclusion. One such study showed that octreotide, a somatostatin receptor agonist which broadly inhibits secretory function of many cell-types[129], inhibited exercise induced lipolysis[130]. This approach was made under the assumption that octreotide would selectively inhibit adrenal gland secretion of epinephrine and would not affect sympathetic nerve function. However, somatostatin receptor signaling inhibits sympathetic nerve activity at sites in the brain[131, 132], spinal cord[133], and peripheral nerves[134, 135], rendering the conclusions of this study unclear. In summary, a large body of work suggests that norepinephrine is the major catecholamine regulating adipocyte physiology.

Consequently, understanding sympathetic nerves, the dominant source of norepinephrine, is of critical importance to understanding adipocyte physiology.

*Somatostatinergic Neurons are Upstream Regulators of Sympathetic Nerves*

A helpful way to understand sympathetic neuroanatomy is to begin at the end-organ and trace the nerve's pathway into the central nervous system. Individual sympathetic neurons interface with their end-organs primarily via noradrenergic nerve endings (nerve endings which secrete norepinephrine). Sympathetic nerves that interface with an end-organ are also known as post-ganglionic nerves, because they emerge from sympathetic ganglia (collections of neural cell bodies) which run parallel to the spinal cord[136]. Each post-ganglionic sympathetic neuron is regulated by a preganglionic neuron through a cholinergic synapse[136]. Thus, sympathetic nerves communicate with their target organs through norepinephrine, but are activated by preganglionic release of acetylcholine onto nicotinic acetylcholine receptors. In turn, the cell bodies of preganglionic neurons are localized within the spinal intermediolateral cell column and project their axons through the ventral horns of the spinal cord to their final ganglionic synapse[137]. Preganglionic neurons are in turn regulated by descending pathways from the hypothalamus and brainstem, including the rostral ventrolateral medulla (RVLM) and paraventricular nucleus of the hypothalamus (PVN)[138]. Manipulation of PVN[46, 51, 139, 140] and RVLM[140] circuitry modulates sympathetic tone across many organ systems.

Within the PVN, somatostatinergic (SST) neurons are excellent candidate targets for control of sympathetic nerves. Hypothalamic SST neurons secrete SST as well the inhibitory neurotransmitters GABA and glycine[141]. SST inhibits neural activity

through cognate inhibitory g-protein coupled receptors SST-$R_{1-5}$ [142-145]. Intracerebroventricular infusion of somatostatin analogues increases immediate early gene expression in many brain regions, with the highest effects observed in the PVN[146]. Neural SST synthesis is triggered by stressors ranging from repeated footshock (a model of post-traumatic stress disorder)[147] to seizures[148]. SST neurons [23], and SST[131-135, 149] decrease the activity of many different sympathetic nerves. Importantly, SST also negatively regulates central leptin signaling[150]. Leptin is a major cause of sympathetic nerve hyperactivity in METS[39]. In summary, there are many lines of evidence which suggest that SST neurons, and SST itself, are reflexively activated to oppose stress responses, including sympathetic nerve activity.

SST neurons also reduce neuroinflammation, an important case of sympathetic nerve hyperactivity previously established in the section *Causes of Sympathetic Hyperactivity in MetS*. SST acts as an anti-inflammatory paracrine factor, inhibiting prostaglandin synthesis in microglia[151] and interleukin-6 secretion by astrocytes[152]. SST also maintains brain endothelial tight junction expression under lipopolysaccharide challenge[153].

Importantly, somatostatin has many well-known endocrine effects in the periphery. Specifically, circulating SST, secreted by gastrointestinal D-cells as well as pancreatic delta cells, has many endocrine effects, including the suppression of insulin secretion and inhibition of gastric acid secretion[154-156]. These elements of somatostatin physiology are independent of brain somatostatin, which signals through paracrine and synaptic mechanisms in the central nervous system[157].

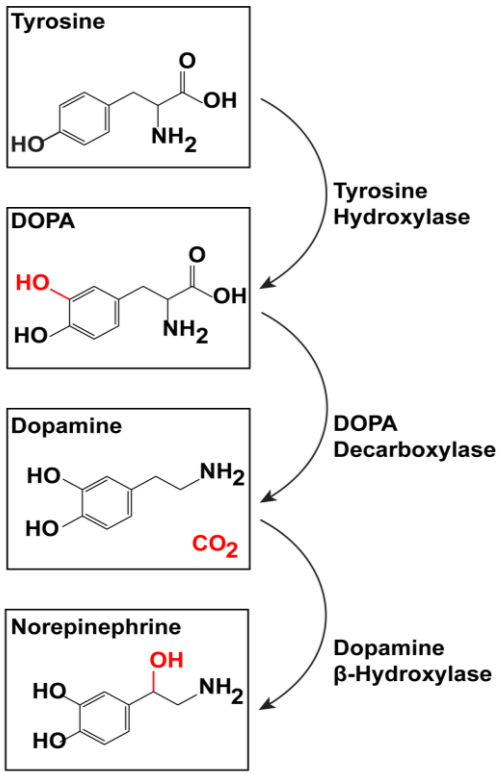
Finally, SST is a useful therapeutic target because its FDA approved analogue,

octreotide, clinically targets hypothalamic SST-$R_{2,3,5}$ by intranasal delivery [158, 159]. Specifically, intranasal octreotide has been used for treatment of acromegaly, a rare condition in which the pituitary gland overproduces growth hormone. Thus, research on SST is translatable through drug repurposing. The implications of this approach are explored in the second chapter of this dissertation.

*Catecholamine Biosynthesis*

Beyond neuroanatomy, understanding the biochemistry of norepinephrine synthesis greatly facilitates empirical studies of the sympathetic-adipose tissue interface. All catecholamines, including norepinephrine, are synthesized via a stepwise series of biochemical reactions which begin with the amino acid tyrosine (Figure 4). Tyrosine is converted into L-DOPA via the enzyme tyrosine hydroxylase (TH)[160]. The resultant structure is the core catechol group (1,2-dihydroxybenzene) conjugated to an amino acid tail. This reaction is the rate limiting step of catecholamine biosynthesis and requires co-factors of O2 and tetrahydrobiopterin [161, 162]. Importantly, tyrosine hydroxylase also hydroxylates phenylalanine to form tyrosine[163]. Thus, TH can

Figure 4: Catecholamine Biosynthesis

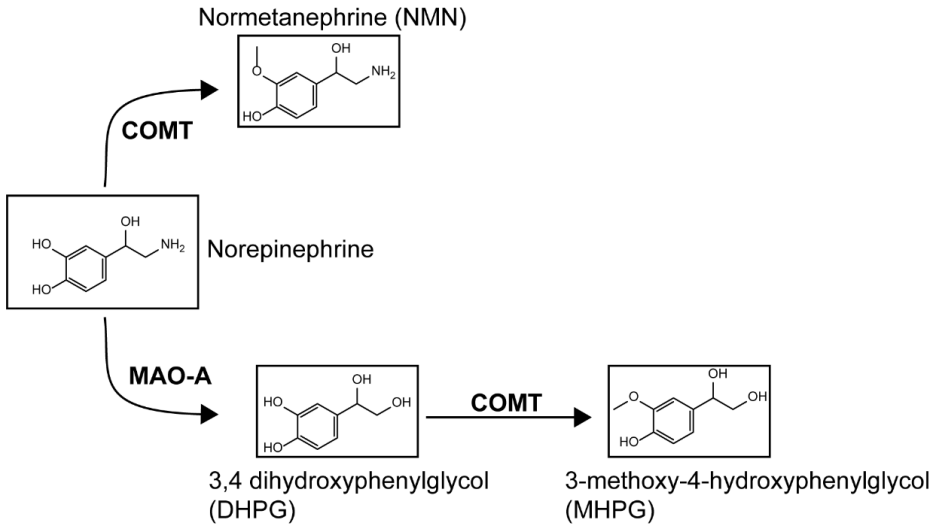


regulate the availability of substrate for conversion to L-DOPA via formation of tyrosine, as well as the conversion of tyrosine itself into L-DOPA. L-DOPA is converted into the first true catecholamine, dopamine, by removal of the carboxylic acid tail under the control of the enzyme DOPA decarboxylase, which requires pyridoxal phosphate as a co-factor[160]. Dopamine is, in turn, converted into the next catecholamine, norepinephrine, by a hydroxylation step controlled by dopamine beta-hydroxylase (DBH), with co-factors of oxygen and ascorbic acid[164]. Epinephrine, the last catecholamine in the biosynthetic pathway, is formed by phenylethanolamine-N-methyltransferase in the presence of s-adenosylmethionine, which methylates the nitrogen of the amine group in norepinephrine[160].

*Catecholamine Degradation*

Norepinephrine is not merely synthesized and released. Norepinephrine is degraded via multiple enzymes, outlined below (Figure 5). Norepinephrine, like many

Figure 5: Catecholamine Degradation



neurotransmitters, is transported back into nerve terminals (reuptake). The reuptake of norepinephrine occurs via the norepinephrine transporter (NET). Following reuptake, it is either recycled into dense core synaptic vesicles via the vesicular monoamine transporter 2 (VMAT2) or degraded into the metabolite dihydroxyphenylglycol (DHPG) by the enzyme monoamine oxidase (MAO)[165]. The final step in DHPG degradation occurs under the control of catecholamine-o-methyltransferase (COMT) into 3-methoxy 4-hydroxyphenylglycol (MHPG). Norepinephrine which does not enter the re-uptake pathway is degraded by parenchymal cells, including adipocytes[166] themselves and macrophages[57, 167] into the metabolite normetanephrine (NMN) via COMT.

Interventions which modulate norepinephrine clearance invariably produce alterations in adrenergic signaling within adipose tissue (as well as other organs), with concomitant increases or decreases in lipolysis depending on whether clearance is increased or reduced. Specifically, increased lipolysis has been observed after treatment with atomoxetine, a NET inhibitor[168], as well as after electroacupuncture, which has been shown to reduce monoamine oxidase mediated norepinephrine clearance[169], and via genetic ablation of the organic cation transporter 3, a high affinity adipocyte uptake mechanism for norepinephrine clearance[166]. Pharmacologic inhibition and genetic ablation of COMT have been shown to aggravate glucose intolerance and adipose tissue weight gain induced by high fat diet, though measurements of lipolysis were not undertaken in this study[170].

Changes in norepinephrine clearance may also be impacted by genetic variation. For example, the rs4680 polymorphism in COMT results in a valine to methionine substitution which reduces enzyme activity by three to four fold[171]. Low COMT activity was associated increased blood pressure, heart rate, and waist-to-hip ratio[171].

Strikingly, subjects with high activity COMT polymorphisms exhibit lower A1C in Type 2 diabetes and lower diabetes incidence[172]. It is tempting to interpret these findings exclusively from the lens of the peripheral, sympathetic nervous system, however, catecholamines in central nervous system are comparably degraded by these enzymes, and sex hormones such as estradiol are also degraded through COMT dependent pathways[170], rendering interpretation of genome wide association studies complex.

Understanding the neurochemistry of catecholamine degradation also permits useful measurements of sympathetic nerve function. For example, increased production of DHPG was observed in kidney and adipose tissue of spontaneously hypertensive rats as well as in plasma[173]. Interestingly, in this study, plasma norepinephrine was not elevated at early timepoints, even when interstitial norepinephrine was increased. However, plasma DHPG was consistently elevated when interstitial norepinephrine was elevated. These data raise the intriguing possibility that DHPG could be a more sensitive proxy for tissue norepinephrine levels than plasma norepinephrine. In other studies, the ratio of norepinephrine to DHPG in the central nervous system has also been used as an index of central noradrenergic drive[174]. Decreased DHPG was also detected in the circulation of patients undergoing chronic caloric restriction[175], which reduces sympathetic nerve activity in many tissues (with the notable exception of adipose tissue). Thus, in peripheral blood, DHPG, and potentially other catecholamine degradation metabolites such as normetanephrine, may usefully provide an index of whole-body sympathetic tone, even if they are not able to elucidate more fine-grained variation in tone across different organs. Circulating norepinephrine is highly and rapidly labile in response to handling stress in research animals[176], thus, examining degradation metabolites may provide helpful additional insights into sympathetic function.

*Measuring Sympathetic Function via Norepinephrine Turnover*

The amount of ligand available to stimulate adrenergic receptors is controlled by at least two factors: the rate of action potentials and the rate of norepinephrine clearance, which poses a challenge for the accurate assessments. Classic direct nerve recordings only capture the first of these two factors by assessing the rate of compound action potentials over time in a single nerve bundle (i.e. the contribution of action potentials across many single sympathetic nerve axons)[177]. However, in isolation, such assays leave entirely uncharacterized the latter of these two factors, the rate of norepinephrine clearance.

Norepinephrine turnover (NETO) is the principle assay which measures both of the above factors[178]. NETO quantitates the decline in tissue content of norepinephrine following inhibition of its synthesis. The underlying principle of NETO is as follows: when sympathetic nerves fire, the released norepinephrine is either taken back up into the terminal or cleared, as outlined above. Thus, each nerve firing elicits some loss of neurotransmitter, in a manner proportional to the reuptake and clearance within the nerve terminal and within parenchymal cells of the tissue of interest—in this case, adipose tissue. In order to maintain steady state pools of norepinephrine, more neurotransmitter must be synthesized, proportional to the loss from prior signaling[165, 179].

NETO assays measure *the gap* between supply and demand for norepinephrine by inhibiting norepinephrine synthesis and monitoring the rate of decline in tissue content of neurotransmitter. A-methyl-p-tyrosine (AMPT) is the most commonly used norepinephrine synthesis inhibitor[178]. AMPT has long been recognized to competitively inhibit tyrosine hydroxylase and result in declining tissue concentrations of

norepinephrine[178, 180]. If monitoring NETO in peripheral blood, or another source that may be repeatedly sampled such as urine, a baseline sample is taken from the research subject, followed by dosing with AMPT. Additional samples are taken following the AMPT dose over a period of hours, which show decreasing concentrations of norepinephrine[179]. When monitoring NETO in tissue, different animals must be used for baseline norepinephrine content and for norepinephrine content following AMPT treatment. This is an unavoidable challenge, as the norepinephrine content of an organ can only be measured post-mortem. Care should be taken that animals in the baseline condition are as similar as possible to animals treated with AMPT, for example by ensuring that they are matched for body weight, and that they do not come from different environments, such as different cages or animal facilities.

Norepinephrine turnover may also be measured by isotope dilution, in which a defined quantity of radiolabeled norepinephrine is injected intravenously. Sympathetic nerves will uptake the radiolabeled neurotransmitter via the norepinephrine transporter, as outlined above, which equilibrates with endogenous stores in vesicles[181]. Quantification of the decline in tissue specific activities over time acts as a comparable measurement of norepinephrine flux[182, 183]. Use of this approach is less common; however, similar values have been obtained for turnover rates when comparing it to AMPT[183, 184].

Increased norepinephrine turnover has been observed in animal models of heart failure[185] and hypertension[186], which are pathologic contexts in which sympathetic nerve activity is increased. Increased NETO has likewise been observed in adipose tissue under chronic caloric restriction[187], acute fasting[188], cold exposure[126], and glucoprivation[188]. These are physiologic contexts in which sympathetic outflow is well

known to be elevated. Thus, NETO is a rational biochemical assay for tracking the total exposure of adrenergic receptors to norepinephrine.

*Increased Norepinephrine Turnover in Adipose Tissue from MetS Animal Models*

Dietary models of metabolic syndrome exhibit rapid induction of increased norepinephrine turnover in adipose tissue. Such effects have been observed in dietary challenges with high fructose and glucose[183], low protein and high carbohydrates[184], or cafeteria diet[189] (high fat/high sucrose), though diet durations have only ranged from one to three weeks. Diet induced increases in norepinephrine turnover have been shown in retroperitoneal [184, 189] and epididymal[189] via the AMPT method, as well as in retroperitoneal fat via isotype dilution[183]. In one study, dietary changes which shift macronutrient ratios but are isocaloric do not change NETO in white adipose tissue[190], raising the intriguing possibility that increased NETO is linked directly to positive energy balance, regardless of the macronutrient source. To date, no dietary challenge has been shown to reduce norepinephrine turnover in white adipose tissue.

Direct nerve recordings in adipose tissue under dietary challenge are relatively uncommon. This may reflect the challenge of recording from a consistent site across dietary challenge and control groups, especially because adipose tissue expands significantly under dietary challenge. Nerve recordings have been used to compare sympathetic nerve activity within a dietary challenge cohort (such as high fat diet) across two different treatment conditions[191, 192], which, while valuable, does not assess the difference in nerve activity between dietary challenge and control groups.

The above evidence-base may seem to contradict considerable data showing that increasing sympathetic nerve activity or activating β-adrenergic receptors in adipose

tissue elicits weight loss and also improves many metabolic markers[32, 33, 193]. The effect of exogenously increasing adrenergic signaling, is not, however, proof that sympathetic nerve activity, or norepinephrine turnover more broadly, is suppressed in white adipose tissue of animal models or patients with metabolic syndrome as compared to healthy controls.

By analogy, insulin therapy successfully reduces blood glucose in type 2 diabetes. In the early twentieth century, such findings historically proposed the hypothesis that all hyperglycemia, whether in Type 1 or Type 2 diabetes, is the result of suppressed insulin levels, but that hypothesis was discarded in the face of clear evidence indicating the existence of an insulin resistant state. In the earliest work on insulin resistance, a 1936 publication in *The Lancet*, Harold Himsworth put forward the prediction that "the commonest type of diabetes mellitus will eventually prove to be that which is not essentially due to insulin deficiency" [194]. In a comparable manner, this dissertation, and the supporting literature cited above, put forward the framework that metabolic syndrome is a hyper-catecholaminergic state. The rational next question, therefore, is whether MetS is a catecholamine resistant state, just as MetS is an insulin resistant state.

*Clinical Aspects of Catecholamine Resistance*

Impaired responses to epinephrine, norepinephrine, and pharmacologic analogues which act on β-adrenergic receptors, such as isoproterenol, have long been recognized in the adipose tissue of patients[85, 101, 195] and animal models of MetS[4, 99, 100, 107, 196, 197]. This impaired response is referred to as "catecholamine resistance". Catecholamine resistance has been identified using a variety of approaches which stimulate or mimic stimulation of sympathetic nerves: acute exercise[85], in-vivo

microdialysis with pharmacologic agonists[198], in-vivo stimulation of sympatho-excitatory adipose sensory nerves[64], and ex-vivo stimulation of adipose tissue biopsies using pharmacologic agonists[101]. In keeping with the broad regulatory actions of catecholamines on adipocyte physiology, catecholamine resistance has been shown to impair not only lipolysis[195] and free fatty acid homeostasis[85], but also to disrupt the sympathetic control of adipokine secretion, including the anti-inflammatory, anti-hypertensive, insulin-sensitizing hormone adiponectin[4, 107, 116].

Though uncommon, long-term prospective studies highlight that adipose catecholamine resistance is a major predictive factor for worsening metabolic health. Patients with baseline impairments in catecholamine induced lipolysis are about five times more likely to be diagnosed with Type 2 diabetes and increase weight gain, and ten to twenty times more likely to exhibit worsening insulin resistance, at ten year follow up, as compared to patients with catecholamine sensitive adipocytes at baseline[101]. In summary, multiple lines of evidence indicate that adipose tissue exhibits catecholamine resistance, that this resistance dysregulates key pathways linking adipose tissue physiology to whole organism metabolic health, and that this phenomenon has major prognostic value as indicator of later in life metabolic risks.

*Mechanistic Studies of Catecholamine Resistance*

Given the above findings, significant work has been undertaken to determine the mechanistic basis for catecholamine resistance, of which a key focus has been on the role of low-grade inflammation[99, 197]. This focus is concordant with well recognized changes in adipocyte[199] and resident immune cell[200] production of pro-inflammatory cytokines in both patients and animal models under dietary challenge. In-

vitro data convincingly show that even in adipocyte cell lines such as 3T3L1 cells, acute stimulation with TNF-a is sufficient to elicit β-adrenergic downregulation and catecholamine resistance[99, 197]. Thus, studies of low-grade inflammation are valuable because they link other known features of adipose tissue physiology with desensitization of adrenergic receptors and are robust across several model systems. Because these studies examine the effect of activating one receptor (e.g. the receptor for TNF-a; TNFR1) on the sensitivity of another receptor (in this case, β-adrenergic receptors), they illustrate the role of *heterologous* receptor downregulation in catecholamine resistance. In contrast to the above work, however, studies have shown that weight loss completely restores catecholamine induced lipolysis but is accompanied by a profound increase in pro-inflammatory adipose tissue macrophages[201], suggesting other mechanisms of catecholamine resistance should be considered.

Other mechanistic inquiries into catecholamine resistance have focused on *homologous* receptor downregulation, a classic finding in receptor pharmacology by which chronic stimulation of a receptor drives its own desensitization through arrestin[202] and g-coupled protein receptor kinases[203, 204] pathways, as well as transcriptional downregulation[197]. Indeed, the β3-AR is principally controlled transcriptionally, as it lacks phosphorylation sites for arrestin and receptor kinase dependent mechanisms[205]. Regardless, cAMP is a key upstream factor in driving receptor downregulation, whether by transcriptional or post-translational mechanisms. As such, recent studies on catecholamine resistance have focused on the effect of factors such as cAMP response element binding (CREB) coactivator Crtc3[206] and exchange protein directly activated by cAMP/Ras-related protein (EPAC/RAP)[197] on

catecholamine resistance under high fat diet. These targets are downstream of adrenergic signaling through the $G_{as}$/cAMP pathway.

The focus on the role of targets directly downstream of receptor stimulation is important because they suggest the possibility that homologous receptor downregulation is a major cause of catecholamine resistance. Adipose tissue, like many other tissues, undergoes adrenergic receptor downregulation on protein and transcriptional levels and develops catecholamine resistance in response to chronic exogenous stimulation with agonists of β3-AR such as Cl316,243[197, 207] or catecholamines themselves[197, 208-210]. Conversely, 2 weeks post-chemical denervation of sympathetic nerves, upregulation of β adrenergic receptors is observed in adipose tissue, demonstrating that receptor expression is plausibly dynamically linked to and regulated by nerve function [127]. Receptor desensitization has also been observed in human studies by physiologic sympatho-excitatory stimuli such as acute exercise[211]. In this study, microdialysis infusions of norepinephrine into subcutaneous adipose tissue were performed before and after exercise, followed by analysis of interstitial glycerol. Following exercise, less glycerol was released in response to norepinephrine infusion as compared to the pre-exercise infusion. In a no-exercise control study, sequential norepinephrine infusions did not result in differing glycerol release. This study illustrates that adipose tissue adrenergic receptor sensitivity may be regulated by endogenous sympathetic nerve signaling. Indeed, at least one empirical study observed catecholamine resistance simultaneously with increased NETO under a brief (3 week), low protein, high carbohydrate challenge[184]. It has been proposed that chronically elevated SNS activity under states of positive energy balance, could, instead of driving energy expenditure, actually depress beta-adrenergic responses by driving receptor desensitization and downregulation [105, 212].

Such a framework could explain the sympathetic paradox, by which elevated sympathetic tone is associated with increased fat stores.

*Adipose Tissue Phenotypes in Female Animals and Patients*

Sex differences in adipose tissue are well recognized. Because Chapter 3 of this work explores sex as a biological variable (SABV), this section will examine several features of sex differences in adipose tissue. Notably, sex and gender may differentially impact adipose tissue, but preclinical studies are not well poised to study the latter, and as such, this section details findings relevant to biological sex differences corresponding to different chromosomal complements (XX vs XY) and gonads (testes vs ovaries). This section details differential deposition of adipose tissue, the modulatory role of hormones on lipolysis, and briefly reviews work on norepinephrine turnover and clinical data on catecholamine sensitivity.

Female patients preferentially deposit subcutaneous fat, as compared to visceral fat[213]. Several potential mechanisms explain this difference. Estrogen, acting through estrogen receptor alpha (ERα) increases in expression of anti-lipolytic adrenergic receptors, such as alpha2-AR[214]. Conversely, visceral adipose tissue expression of aromatases is positively associated with increasing visceral fat mass[215]. This finding is important because aromatases generate estrogen by breaking down testosterone, a pro-lipolytic sex hormone [215]. Direct infusion of progesterone, another sex hormone, inhibits the synthesis of lipolytic enzymes, and functionally impairs lipolysis, specifically in subcutaneous, but not visceral fat of female mice[216]. Thus, several different hormones act to maintain differential fat deposition in females.

As reviewed in greater detail in other sections of this introduction, catecholamines are major modulators of lipolysis. Intravenous infusion of norepinephrine and epinephrine drive lipolysis to a greater extent in healthy women as compared to healthy men, suggestive of increased adrenergic sensitivity[217]. During exercise of varying intensities, women also exhibit greater release of free fatty acids and glycerol (measured in plasma) as compared to men, again suggestive of increased adrenergic sensitivity[218]. Strikingly, in these same studies, female circulating catecholamines were lower than those of men, despite exhibiting greater lipolysis [218]. Thus, convergent lines of evidence suggest enhanced catecholamine sensitivity in females.

Hormonal modulation of catecholamine secretion may explain observed differences in catecholamine levels in females compared to males. Post-menopausal women receiving estrogen replacement therapy exhibited lower epinephrine responses and lower sympathetic nerve firing rates in response to experimental hypoglycemia, as compared to age and BMI matched post-menopausal women not on estrogen[219].

In contrast, prior preclinical work has not sufficiently examined adrenergic sensitivity or catecholamine release in female animals. All prior preclinical work on catecholamine resistance is in male animals[4, 99, 107, 197, 201], with the exception of two studies which did not describe the sex of experimental animals[100, 206]. Finally, whereas an abundance of preclinical work on norepinephrine turnover[183, 184, 188, 189, 220-224] has been performed in male animals, no work to date has studied female animals. This dissertation fills critical gaps in several of these areas, contributing to the study of sex as a biological variable in the area of adipose tissue and metabolic disease.

*Summary and Statement of Hypothesis*

Many lines of evidence indicate that catecholamine resistance co-occurs with increased norepinephrine turnover, and that chronic endogenous activation of adrenergic receptors could be sufficient to cause catecholamine resistance. Surprisingly, little data has assessed whether catecholamine resistance could be alleviated by targeting endogenous upstream regulators of sympathetic nerve activity or norepinephrine turnover such as hypothalamic somatostatin. This approach constitutes Chapter 2 of the dissertation.

Alternatively, alleviating pathologic overactivity of the sympathetic nervous system could focus on a more downstream target: the enzymes which regulate norepinephrine synthesis or degradation. Pursuing this strategy requires a study to determine the underlying biochemical basis of increased norepinephrine turnover. As outlined in the section on norepinephrine turnover, two key factors which may alter turnover are increased nerve activity or increased neurotransmitter clearance. Thus, an ideal study to assess these factors would simultaneously assess global norepinephrine turnover, as well as the flux in norepinephrine degradation metabolites such as normetanephrine and dihydroxyphenylglycol. Chapter 3 of the dissertation directly utilizes this approach, to define, for the first time, the endogenous drivers of adrenergic signaling in white adipose tissue under chronic high fat diet exposure.

## Statement of Hypothesis

Increased norepinephrine turnover, whether by centrally mediated increases in sympathetic tone, or peripherally mediated increases in adrenergic signaling, elicits catecholamine resistance.

# DEPLETING HYPOTHALAMIC SOMATOSTATINERGIC NEURONS RECAPITULATES DIABETIC PHENOTYPES IN MOUSE BRAIN, BONE MARROW, ADIPOSE TISSUE, AND RETINA

by

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**Abstract**

*Aims/hypothesis*

Hypothalamic inflammation and sympathetic nervous system hyperactivity are hallmark features of the metabolic syndrome and type 2 diabetes. Hypothalamic inflammation may aggravate metabolic and immunological pathologies due to extensive sympathetic activation of peripheral tissues. Loss of somatostatinergic (SST) neurons may contribute to enhanced hypothalamic inflammation.

*Methods*

The present data show that leptin receptor-deficient (*db*/*db*) mice exhibit reduced hypothalamic SST neurons, particularly in the periventricular nucleus. We model this finding, using adeno-associated virus delivery of diphtheria toxin subunit A (DTA) driven by an SST-cre system to deplete these neurons in $Sst^{cre/gfp}$ mice (SST-DTA). *Results* SST-DTA mice exhibit enhanced hypothalamic c-Fos expression and brain inflammation as demonstrated by microglial and astrocytic activation. Bone marrow from SST-DTA mice undergoes skewed haematopoiesis, generating excess granulocyte-monocyte progenitors and increased proinflammatory (C-C chemokine receptor type 2; $CCR2^{hi}$) monocytes. SST-DTA mice exhibited a ‘diabetic retinopathy-like’ phenotype: reduced visual function by optokinetic response (0.4 vs 0.25 cycles/degree; SST-DTA vs control mice); delayed electroretinogram oscillatory potentials; and increased percentages of retinal monocytes. Finally, mesenteric visceral adipose tissue from SST-DTA mice was resistant to catecholamine-induced lipolysis, displaying 50% reduction in isoprenaline (isoproterenol)-induced lipolysis compared with control littermates. Importantly, hyperglycaemia was not observed in SST-DTA mice.

*Conclusions/interpretation*

The isolated reduction in hypothalamic SST neurons was able to recapitulate several hallmark features of type 2 diabetes in disease-relevant tissues.

**Abbreviations**

| | |
|---|---|
| AAV | Adeno-associated virus |
| ACK | Ammonium chloride potassium |
| CNS | Central nervous system |
| CCR2 | C-C chemokine receptor type 2 |
| DIO | Double-floxed inverse orientation |
| DTA | Diphtheria toxin subunit A |
| ERG | Electroretinogram |
| GFAP | Glial fibrillary acidic protein |
| GFP | Green fluorescent protein |
| GMP | Granulocyte-monocyte progenitor |
| HFD | High-fat diet |
| Iba-1 | Ionised calcium binding adaptor molecule 1 |
| iWAT | Inguinal fat |
| mWAT | Mesenteric fat |
| OP | Oscillatory potential |
| PVN | Paraventricular nucleus |
| SNS | Sympathetic nervous system |
| SST | Somatostatinergic |

## Introduction

Hypothalamic inflammation and sympathetic nervous system (SNS) hyperactivity are important upstream causes of metabolic and immunological pathologies in type 2 diabetes and the metabolic syndrome. Both genetic and dietary models of these diseases elicit hypothalamic inflammation and elevations in sympathetic outflow, often early in the disease course[1-3]. Emerging evidence shows that inflammation also sensitises the hypothalamus to ascending sensory innervation, enhancing sympathetic outflow[4]. Thus, inflammation, sympathetic nerve hyperactivity and metabolic disease are redundantly linked. In keeping with the theory that hypothalamic dysregulation is a primary event driving the metabolic syndrome, exogenous induction of hypothalamic inflammation recapitulates many features of the metabolic syndrome, whereas central nervous system (CNS)-penetrating anti-inflammatory agents ameliorate many features of the metabolic syndrome [5, 6]. Centrally acting sympatholytic agents comparably improve hypertension, hyperlipidaemia and insulin resistance [7]. Strikingly, many studies have induced central inflammation using dietary, genetic or pharmacological approaches. However, comparatively few studies have modulated endogenous protective mechanisms, especially those which are damaged by the underlying disease process.

In this regard, the somatostatinergic (SST) system is a particularly attractive therapeutic target because it both negatively regulates sympathetic outflow[8-12] and frequently inhibits inflammatory cytokine secretion [13, 14]. Exogenous somatostatin preserves the brain endothelial tight junctions against lipopolysaccharide challenge[15], inhibits prostaglandin $E_2$ synthesis in microglia [16] and reduces IL-6 secretion from astrocytes [17]. However, conflicting studies exist [18, 19], and most current data relies

on in vitro models, highlighting the need for further study. Most critically, hypothalamic SST neurons are depleted in preclinical models of the metabolic syndrome and type 2 diabetes [20], suggesting that dysfunction in an endogenous anti-inflammatory, sympatholytic neuronal population could contribute to hypothalamic inflammation and SNS hyperactivity.

Because the hypothalamus is a master regulator of homeostasis, hypothalamic inflammation may induce profound dysfunction across many organ systems. Perhaps nowhere is this more concerning than in the immune system, because impaired neuroimmune regulation could initiate positive feedback loops sustaining hypothalamic inflammation. Specifically, altered sympathetic outflow to lymphoid organs may increase granulocyte-monocyte progenitor (GMP) production, driving the myeloidosis observed in diabetic individuals and animal models [21, 22]. These immune cells may in turn infiltrate the hypothalamus, further dysregulating autonomic outflow, as has recently been shown in neurogenic hypertension [23]. Thus, hypothalamic dysfunction has the capacity to induce downstream injury directly through hyperactivation of sympathetic nerves, as well as indirectly through the altering of immune homeostasis and initiation of systemic inflammation. For example, bone marrow neuropathy induced increases in proinflammatory monocytes precedes diabetic retinopathy in preclinical models [20, 24].

Other disruptions in neural regulation may result from direct nerve hyperactivity. For example, noradrenaline (norepinephrine) turnover (NETO) in adipose tissue is rapidly increased under dietary challenge [25, 26]. Additionally, adipose tissue exhibits a blunted lipolytic response to sympathetic nerve input in humans and in animal models[27-30] but little data explore whether this dysfunction is caused by the aforementioned increase in sympathetic nerve activity.

The primary objective of this study was to test the hypothesis that SST neuronal ablation is sufficient to induce hypothalamic inflammation, hypothalamic neuronal hyperactivity, bone marrow myeloidosis, proinflammatory blood monocytes and retinal dysfunction. A secondary endpoint of the study was to examine additional metabolic effects of SST neuronal ablation, particularly in visceral adipose tissue.

## Methods

### Mice

All protocols were approved by the University of Alabama at Birmingham (UAB) IACUC. SST reporter mice ($SST^{cre/gfp}$) were bred on a C57Bl6 background by Jax Laboratories (USA) in their animal facility by crossing *Sst*$^{tm2.1(cre)Zjh}$ (Jax Strain 013044) to the cre-dependent reporter line B6.Cg-*Gt(ROSA)26Sor*$^{tm6(CAG-ZsGreen1)Hze}$ (Jax Strain 007906). Mice that were heterozygous at both genomic insertions were used, with 24 mice per group, equal numbers of males and females. Injections (100–200 nl per side) were performed by a trained operator at UAB. All mice were given presurgical buprenorphine and anaesthetised with isoflurane. The following stereotactic coordinates were used in mice aged 8–10 weeks: anteroposterior −0.5; mediolateral ±0.220; dorsoventral −4.8). The control adeno-associated virus (AAV) was a double-floxed inverse orientation (DIO) vector, hSyn-DIO-GFP-AAV (SST-GFP). Cre-dependent expression of the diphtheria toxin subunit A (DTA) was achieved via AAV delivery of the DIO vector hSyn-DIO-DTA-mCherry-AAV, provided by P. M. Fuller [31, 32]; SST-DTA *db*/*db* mice were purchased from Jax Laboratories along with heterozygous *db/m* littermates(Jax Strain 000697). Mice were group-housed in specific-pathogen-free environments and fed chow diet ad libitum under a 12 h light–dark cycle at 22°C.

Experimenters were masked to treatment conditions. Male and female mice were used in all studies.

**Immunohistochemistry**

Brains were fixed in 4% paraformaldehyde, cryoprotected in 30% sucrose and embedded in optimal cutting temperature media, followed by sectioning at 14 μm thickness and storage at −20°C. Brain sections were washed twice in PBS for 10 min and blocked in 1% BSA in 0.3% Triton-X for 30 min at room temperature. The following primary antibodies were incubated overnight: rat anti-somatostatin (Abcam, USA; M09204; 1:100), rabbit anti-NeuN (Thermo Fisher Scientific, USA; PA5-78499; 1:100), rabbit anti-c-Fos (Abcam; ab90289; 1:100), rabbit anti-ionised calcium binding adaptor molecule 1 (Iba-1; Wako, Osaka, Japan; 019-19741; 1:200) and rabbit anti-glial fibrillary acidic protein (GFAP) (Abcam; Rb, ab33922; 1:100). Sections were then transferred to species-specific secondary antibodies conjugated with either AlexaFluor 594 (1:200) or AlexaFluor488 (1:100), both from Invitrogen-Molecular Probes (Carlsbad, CA, USA) for 1.5 h at room temperature. Following two additional PBS washes, sections were reacted with DAPI (Invitrogen by Thermo Fisher Scientific; D3571; 1:10,000) for 10 min and mounted in antifade mounting medium (Vector Labs, USA). Somatostatin visualisation required antigen retrieval in citrate buffer (Sigma Aldrich; C8532; pH 6.0) for 30 min prior to blocking. Liver Nile Red (Thermofisher Scientific; N1142; 12 μg/ml) staining was performed for 2 h at room temperature. All sections were examined using fluorescence microscopy with a Zeiss Axio Imager Z2 (Carl Zeiss Microscopy; USA). Cell counting was performed in ImageJ (Version 1.8.0; National Institutes of Health, USA).

**Adipose tissue lipolysis**

Pieces of mesenteric and subcutaneous inguinal fat pads (50–100 mg) were incubated in basal lipolysis media (low-glucose DMEM with 4% fatty-acid-free BSA) at 37°C with 4% $CO_2$ for 1–3 h, then transferred to induced lipolysis media (low-glucose DMEM with 4% fatty-acid-free BSA, 10 μmol/l Triacsin C to inhibit fatty acid re-esterification and 10 μmol/l isoprenaline [isoproterenol]) for 1 h. Following lipolysis, media was stored at 4°C until assayed for free glycerol (Sigma Free Glycerol Kit; MAK 117) with detection using a Biotek Epoch microplate spectrophotometer at 550 nm. Lipolysis was calculated as the ratio of free glycerol in induced media to the free glycerol in basal media, normalised for duration of basal lipolysis [28].

**Fatty acid and triacylglycerol measurements**

Plasma NEFA was measured using Wako Diagnostics Non-Esterified Free fatty acid kit (Wako, Japan; 999-34691). Triacylglycerols were measured using the T2449 Triglyceride Kit (Sigma Aldrich, USA)

**Flow cytometry**

**Peripheral blood flow cytometry**

Blood was collected under isoflurane anaesthesia via cheek bleed into heparinised capillary tubes. Blood was centrifuged at 3000 rpm at 4°C for 7 min, plasma was collected, packed cells were resuspended in cold ammonium chloride potassium (ACK) lysis buffer and lysed for 15 min on ice, then centrifuged at 400 *g* at 4°C for 5 min. Cells were washed twice in 5 ml of sterile, filtered BEP (2% fatty-acid-free BSA, 1% EDTA,

in PBS), and centrifuged at 400 *g* for 5 min. Following washes, cells were stained (see electronic supplementary material [ESM] Table 1 for flow cytometry panels) for 20 min at room temperature in the dark, then washed twice. Flow cytometry was performed using a BD Celesta and BD FACSymphony. Analysis was performed using FlowJo version 10.7.1 (FlowJo, USA). All antibodies are listed in ESM Table 1. Gating schema are shown in ESM Figs 1 and 2.

**Bone marrow flow cytometry**

Whole femurs were cleared of musculature using sterile gauze. The distal and proximal epiphysis were resected and bone marrow was flushed using 6 ml of sterile FEB buffer (Fisher Scientific, USA; 10-082-147; 2% fetal bovine serum and 1 mmol/l EDTA in PBS). The single-cell suspension was stored on ice until centrifuged. Erythrocyte lysis was achieved with ACK lysis buffer for 15 min on ice and arrested with 10 ml FEB buffer before centrifugation, washing and staining; flow cytometry was performed as outlined above.

**Retina flow cytometry**

Retina was isolated and stored on ice until digestion with 1 ml RPMI containing 5% FBS supplemented with collagenase D and DNase at 37°C for 1 h with periodic vortexing. The resulting suspension was filtered through a 70 μm cell strainer with sterile FEB buffer and centrifuged. Cell pellets were resuspended into 200 μl of FEB, and stained; flow cytometry was performed as outlined above.

**Electroretinogram**

Electroretinograms (ERGs) were recorded using the LKC Bigshot ERG apparatus. Mice were dark-adapted overnight, anaesthetised with ketamine and xylazine in sterile 0.9% saline (80 mg/kg and 15 mg/kg, respectively), and then pupils were dilated with eye drops of phenylephrine (2.5%; Paragon Bioteck, USA). Contact lens electrodes were placed with Gonak (hypromellose 2.5%; Akorn, USA) , as well as a steel reference electrode (head), and a steel ground electrode (foot). Mice were exposed to five full-field white light flashes at 0.25 and 2.5 cd.s/$m^2$ (scotopic), light-adapted for 5 min and exposed to 10–15 full-field white light flashes at 10 and 25 cd.s/$m^2$ (photopic).

**Optokinetic tracking responses**

A trained observer-operator, masked to treatment condition, assessed visual acuity by observing reflexive head tracking movements (optomotor response) using a virtual optomotor system (OptoMotry; Cerebral Mechanics, Canada) with custom software (OptoMotry HD software version 2.0.0[4907]). The mice were placed on a platform inside a four-walled chamber made of computer monitors displaying gratings ranging from 0.2 to 0.5 cycles per degrees rotating at 12.0 degrees/s shown under photopic conditions at 100% contrast. Visual acuity was defined as the highest grating frequency at which optomotor response could be reliably detected.

**Noradrenaline content and plasma hormones**

Whole fat pads were homogenised in ice-cold perchloric acid (0.4 mol/l) with reduced glutathione (5 mmol/l). Noradrenaline was measured by HPLC-electrochemistry at the Vanderbilt Hormone Assay Core. The supernatant fraction was extracted with 10 mg of acid-washed alumina, eluted, preconcentrated, and injected via an ESA model 542

autosampler (ESA Biosciences, USA) onto an ESA HR-80, reverse phase, 3 micron column and an ESA model 5200A Coulochem II electrochemical detector. Results were analysed using EZ Chrom software 3.2.0 (Agilent, USA). Adiponectin was measured via ELISA (MRP300; R&D Systems, USA), as was insulin (Alpco, USA; 80-INSMR-CH10).

**Quantitative PCR**

Adipose samples (40 mg) were rapidly dissected and snap frozen in DNAse/RNAse free tubes over liquid nitrogen and stored at −80°C until homogenisation with Qiagen TissueLyser LT in Trizol. RNA was purified (DirectZol RNA Miniprep; ZymoResearch, USA; R2051) with on-column DNAse treatment followed by reverse transcription (Superscript IV VILO; Thermofisher; 11756050). cDNA (1 μl of a 1:10 dilution) was used in a 10 μl reaction volume as follows: □3-AR transcripts were amplified (forward primer TGACTCCTGAAACAAGCGGG, reverse primer GTGGGAATTGGAGGGTGGAG) and compared with cyclophilin A (forward primer CAGACGCCACTGTCGCTTT, reverse primer TGTCTTTGGAACTTTGTCTGCAA) via SYBR Green (Biorad, USA;1725271). Specificity was measured via melting curve and confirmed via amplicon size in agarose gel electrophoresis.

**Statistical analyses, data and resource availability**

All statistical analyses were conducted using GraphPad Prism 8.2.0 (GraphPad, USA). Two-sample *t* tests were used to detect significant differences ($p<0.05$) between SST-DTA and SST-GFP groups. Welch's *t* tests were used if variance between groups was unequal. Data are presented as means ± SD unless otherwise noted. Data in dot plots

represent individual replicates. The only criterion for the exclusion of data was early death or a wound (e.g. ulcerative dermatitis). No difference in event rates was observed between groups. One sample (of ten) from the control group was discarded from the insulin analysis on the basis of a Grubb's extreme Studentised deviate analysis at $p<0.05$.

## Results

### Aged *db*/*db* hypothalamus exhibits a loss of SST neurons

Previous data indicated a reduction in hypothalamic SST neurons in BBZ/WoR rat hypothalamus[20]. We tested the hypothesis that this phenomenon was a general finding in models of the metabolic syndrome and type 2 diabetes by examining SST neuron density in the leptin-receptor-deficient *db*/*db* mouse model of the metabolic syndrome and type 2 diabetes. Serial coronal sections of the paraventricular nucleus (PVN) and periventricular nucleus were examined using immunohistochemistry for somatostatin (Fig.1a). Sections were oriented with reference to the Allen Brain Atlas. SST neuron density varied systematically between the PVN and periventricular nucleus, with an overall higher abundance of SST neurons in the periventricular nucleus (Fig. 1b,c). A strong rostral–caudal gradient was observed in the periventricular nucleus, with the highest densities of SST neurons between Bregma coordinates −0.3 to −1.1 in hypothalamus from *db*/m control mice. A significant reduction in the abundance of SST neurons was observed at most Bregma levels in the periventricular nuclei of *db*/*db* mice compared with *db*/m control mice (Fig. 1b), whereas PVN SST neuron density exhibited reductions at only one Bregma level (Fig. 1c).

### AAV-DTA treatment depletes SST cells and induces hypothalamic inflammation

To mimic the observed reduction in the abundance of SST neurons, *Sst*$^{cre/gfp}$ mice received stereotactic injections of AAV containing a cre-driven α subunit of diphtheria toxin into the hypothalamus (SST-DTA), which is sufficient to induce neural cell death [31, 32]. Control mice received a virus containing a green fluorescent protein (GFP) construct (SST-GFP). At 1 week post-treatment, a robust reduction in the abundance of SST neurons was observed in the hypothalamus of SST-DTA mice but not in neighbouring brain regions such as the ventromedial thalamus (Fig. 2a–c). At 8 months post-DTA exposure, SST neuronal density remained suppressed (Fig. 2d,e). A smaller reduction in the overall abundance of NeuN-positive cells was observed (Fig. 2f), indicating that SST neurons were preferentially eliminated by this approach. This finding is strengthened by the observation that relativising SST density to overall NeuN density still demonstrated a significant loss in SST-DTA mice (Fig. 2g).

Concomitant with these findings, at 8 months, SST-DTA mice exhibited increased abundance of Iba1$^+$ microglia (Fig. 3a) and GFAP$^+$ astrocytes (Fig. 3b). These data suggest that depleting hypothalamic SST neurons is sufficient to induce hypothalamic neuroinflammation, which persists long after the initial DTA-mediated cell death. SST neurons often act as local inhibitors of neural activity[8]; as such, their ablation should induce neural hyperactivity and increase sympathetic outflow. To test this hypothesis, immunohistochemistry for c-Fos positivity in hypothalamic sections (a commonly utilised proxy for recent cell activation [33]) was undertaken. Hypothalamuses of SST-DTA mice displayed significantly higher densities of c-Fos$^+$ cells than those of control mice (Fig. 3c).

**SST-DTA mice do not exhibit hyperglycaemia, hyperinsulinaemia or hyperlipidaemia**

No difference in weight was observed in SST-DTA vs control mice (Fig. 4a), nor were any differences observed in random blood glucose measurements (Fig. 4b), insulin levels (Fig. 4c), triacylglycerol levels (Fig. 4d) or adiponectin levels (Fig. 4e). No gross lipid deposits were observed in livers of SST-DTA mice, as measured by Nile Red staining (Fig. 4f,g), though small lipid droplets were observed in mice under both conditions.

**SST-DTA mice exhibit increased granulocyte precursors in bone marrow and increased inflammatory state of bone marrow and circulating monocyte pool**

Flow cytometry of $lin^-$, $sca1^-$, $c\text{-}kit^+$ cells from SST-DTA and control mice showed an increased percentage (relative to all $lin^-$, $sca1^-$, $c\text{-}kit^+$ cells) of GMP cells ($CD34^+$, $CD16/32^+$) (Fig. 5a,b). In contrast, a decreased percentage of megakaryocyte-erythroid progenitors was observed in SST-DTA mice ($CD34^-$, $CD16/32^-$) (Fig. 5c). Relative monocyte increases were observed in SST-DTA mice (Fig. 5d,e), as well as increased percentages of proinflammatory C-C chemokine receptor type 2 (CCR2)-positive monocytes (Fig. 5f). Examining monocyte subsets by Ly6C expression revealed no changes in $Ly6c^{lo}$ (Fig. 5g) or $Ly6C^{inter}$ populations (Fig. 5h), although proinflammatory $Ly6C^{hi}$ monocytes were elevated in SST-DTA mice (Fig. 5i). Previously, we reported that two different dysfunctions in the neural axis, hypothalamic inflammation and bone marrow neuropathy, precede retinal microvascular disease and reduced retinal function concomitant with retinal monocytosis [6, 21]. To assess whether hypothalamic SST neuron depletion recapitulated these findings, retinal flow cytometry

was performed at 8 months post-treatment (9 months of age). SST-DTA mice exhibited higher levels of retinal monocytes than control mice (Fig. 5j).

**SST-DTA mice exhibit increased retinal monocytes and reduced retinal function**

To assess retinal function in the SST-DTA mouse model, we performed ERG and optomotor tracking. The ERG assays the electrical response of the retina to light (Fig. 6a). The ERG is summed field potential containing a photoreceptor driven A-wave and a neural retina (retinal bipolar cell) dominant B-wave. Dim light scotopic ERGs reflect rod photoreceptor activity, whereas photopic ERGs isolate cone photoreceptor activity by delivering a chronic background light to suppress rod photoreceptor activity. Cone photoreceptors are then stimulated by a superimposed bright light stimulus. In addition to A- and B-waves, ERGs exhibit oscillatory potentials (OPs), which have complex, incompletely described origins in the neural retina. Importantly, delayed OP onset occurs early in diabetic retinal dysfunction, prior to frank retinopathy.

SST-DTA mice exhibited disruptions in photopic B-waves (Fig. 6b), but not photopic A-waves or scotopic B-waves (Fig. 6c,d). Delays in the onset (implicit) time of each OP were detected in SST-DTA mice (Fig. 6e–g). Finally, in a second measure of retinal function, we examined optomotor tracking, a behavioural measure of visual acuity. SST-DTA mice exhibited profound reductions in spatial acuity (Fig. 6h) from 0.4 cycles/degree in control mice to 0.25 cycles per degree in SST-DTA mice.

**SST-DTA mice exhibit adipose catecholamine resistance in mesenteric fat**

An important implication of the above studies is that SST neuronal loss could induce pathology in many tissues. One key tissue to consider in this regard is white

adipose tissue. In rodents, visceral, mesenteric fat (mWAT) is richly connected to the hypothalamus, particularly the PVN [34]. Because sympathetic nerves drive lipolysis through catecholamine signalling in adipose tissue, we hypothesised that mWAT might have impaired lipolytic function in SST-DTA mice. To measure catecholamine-induced lipolysis, we established a dose–response curve of mWAT and subcutaneous inguinal fat (iWAT) in response to isoprenaline, a β-adrenergic receptor agonist, finding that in wild-type mice, both tissues exhibited comparable NEFA and glycerol release (Fig. 7a,b).

Using this method, we measured catecholamine sensitivity in *db*/*db* mice. Compared with their heterozygote littermate controls, 6- to 12-month-old *db*/*db* mice exhibited catecholamine resistance in both mWAT and iWAT (Fig. 7c,d), as well as significant $\beta_3$-adrenergic receptor downregulation in both tissues (Fig. 7e,f). In contrast, iWAT from SST-DTA mice exhibited similar catecholamine sensitivity to iWAT from control mice but catecholamine-induced lipolysis was suppressed by 50% in SST-DTA mWAT (Fig. 7g,h). However, SST-DTA mice did not exhibit receptor downregulation at the level of mRNA (Fig. i,j). Because chronic release of noradrenaline could drive desensitisation to catecholamines, we examined the noradrenaline concentration in iWAT and mWAT, finding significantly higher levels in mWAT than in iWAT in wild-type mice (Fig. 7k).

To further examine the sympathetic nerve–adipose circuit, which drives NEFA release, we examined plasma NEFA in SST-DTA mice. At 8 months of age, SST-DTA mice exhibited a 40% reduction in plasma NEFA (Fig. 7l). Examining NEFA content of archived plasma samples from monthly blood draws showed a striking pattern of early elevations in NEFA content at 1 month post-DTA, followed by comparable NEFA levels at 5 months post-DTA, culminating in the aforementioned reduction at 8 months (Fig. 7l).

## Discussion

The current study adds to a growing literature indicating that hypothalamic inflammation is an important site of pathology in metabolic disease. For example, mice subjected to high-fat diet (HFD) show hypothalamic inflammation after 24 h, particularly in astrocytes and microglia [3]. After months of HFD, the hypothalamus exhibits apoptotic cell death [35]. Genetic models of the metabolic syndrome recapitulate the sequence of hypothalamic inflammation and cell death [36]. The present data inform our understanding of hypothalamic SST neurons in this process. While somatostatin uniformly suppresses cytokine release in the periphery, information on its anti-inflammatory action in the brain is more conflicting and largely derived from cell culture studies [16, 18, 19]. The data presented here suggest that SST neurons repress CNS inflammation. In addition to their anti-inflammatory function, SST neurons also secrete the classically inhibitory neurotransmitters GABA and glycine in brainstem and cortex [8], though recent data indicate that some hypothalamic SST neurons are glutamatergic [37]. In brief, the molecular heterogeneity of SST neurons requires functional characterisation; our c-Fos activation data suggest that SST neurons exhibit local inhibitory roles in the hypothalamus. The loss of these neurons could have an extensive impact on the many tissues under hypothalamic regulation through the SNS.

Sympathetic nerves regulate haematopoiesis, and in diabetes, dysfunction in this circuit increases short-term repopulating haematopoietic stem cells and suppresses long-term repopulating haematopoietic stem cells, biasing haematopoiesis towards myeloid progenitors and increasing blood monocytes [6, 21, 38-41]. We previously found that models of type 1 and type 2 diabetes show decreased bone marrow tyrosine hydroxylase

and CGRP, indicating frank neuropathy [21, 24]. However, loss of hypothalamic SST neurons was theorised to hyperactivate the bone marrow circuit prior to neuropathy [20]. Indeed, SST-DTA mouse bone marrow demonstrated a haematopoietic bias towards GMPs, recapitulating findings recently published in *db*/*db* mice[43]. Additionally, peripheral blood monocytes were increased and preferentially proinflammatory $CCR2^{hi}$ and $Ly6C^{hi}$. These data support the hypothesis that SST neuronal dysfunction plays a role in diabetic bone marrow.

Retinal dysfunction, mediated by vascular injury and immune infiltrate, is a common feature of diabetes. We observe increases in retinal monocytes in SST-DTA mice, and this finding was accompanied by delays in the implicit times of ERG OP, which is the most common ERG dysfunction identified in diabetic individuals [44]. In mouse models, delayed OP implicit time precedes retinal vascular damage in chronically HFD-fed mice, suggesting a step-wise pathophysiology linking inflammation to retinal dysfunction and eventual vascular damage [45]. We confirmed the functional importance of the above pathologies with the finding that SST-DTA mice exhibit reduced spatial acuity.

Sympathetic nerves also regulate white adipose tissue. Dysfunction in this tissue is of vital importance to diabetes and the metabolic syndrome, especially in visceral fat. mWAT, the only true visceral fat pad in rodents[46], is richly represented at the hypothalamic PVN [34]. We observe a well-characterised hallmark of metabolic disease in SST-DTA mouse mWAT: adipose catecholamine resistance. We hypothesise that hyperactivity of adipose sympathetic nerves (and resultant catecholamine excess) drives adipose catecholamine resistance. In support of this view, we assessed local concentrations of noradrenaline in mWAT and iWAT, finding markedly elevated

concentrations in the former. However, our data cannot rule out other potential causes.

For example, in vitro incubation of adipocytes with TNF-α reduces lipolysis in response to β-adrenergic agonists [47], suggesting that inflammation could aggravate catecholamine resistance. Adipose tissue releases NEFA during lipolysis and the only plasma metabolite that differed between SST-DTA mice and controls were NEFA, which showed dynamic differences: an early increase followed by a late decline. Increased NEFA in individuals with type 2 diabetes are driven by increased basal lipolysis; indeed, even non-diabetic individuals with family histories of diabetes exhibit enhanced basal lipolysis [27]. In contrast, we observe a deficit in induced lipolysis. We speculate that in the late stages of our mouse model, reduced plasma NEFA are a plausible correlate of isolated catecholamine resistance. Adipose catecholamine resistance is an early finding in disease progression [30] and predicts type 2 diabetes and worsening insulin resistance [28], although we did not observe signs of insulin resistance in these mice. Placing SST-DTA mice on an HFD may lead to greater insights regarding the role of SST neurons in metabolism.

A relative weakness of our study is that brain inflammation was principally assessed by cellular mediators of inflammation: astrocytes and microglia. Prior data indicate that somatostatin negatively regulates prostaglandin-$E_2$ synthesis in microglia and IL-6 secretion in astrocytes. As such, future studies in this model should assess cytokine mediators of inflammation. Microglial polarisation towards an M1 phenotype is often observed in the disease state and evaluation of cell-surface markers such as CD16/32 and CD206 would likewise be fruitful in future studies. An additional limitation is that neuronal function was principally assessed by c-Fos measurements and static noradrenaline content. An important future direction would be to measure nerve activity

or noradrenaline turnover. Additionally, while our studies indicate that SST neuronal loss induces several pathologies, future studies may examine the effect of specifically antagonising somatostatin itself (e.g. by RNA interference). Finally, our reporter line exhibits somatostatin knockdown in homozygous mice [48]. Though we only used heterozygote mice, another future direction would be to confirm these results in a different mouse line.

In summary, depleting hypothalamic SST neurons recapitulated many early phenotypes found in diabetes that precede frank neuropathy or vascular insult. The significance of this finding is that this small but significant population of neurons impacts many target tissues of diabetic complications including the brain, bone marrow, retina and visceral adipose tissue. Pathology resulting from the loss of SST neurons can potentially be corrected by pharmacological strategies, including the use of somatostatin analogues such as octreotide, delivered intranasally, to target the hypothalamus.

**Data availability**

The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

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**Authors' relationships and activities**

The authors declare that there are no relationships or activities that might bias, or be perceived to bias, their work.

**Contribution statement**

HC managed all experiments and data collection and edited the manuscript. RFR collected metabolic data and wrote the manuscript. RB performed the stereotactic surgery. PH performed the tissue processing and immunohistochemistry. YAA performed optokinetic and ERG recordings, and CPV and ALFL assisted with flow cytometry and bone marrow function assays. GML, PMF and KLG aided in experimental design. All authors contributed to the acquisition and analysis of the data. All the authors revised the manuscript critically for important intellectual content and approved the final version of the manuscript. MBG conceptualised the experiment, obtained funding, designed experiments, assisted with data interpretation, edited the manuscript and is the guarantor of all data in the manuscript.

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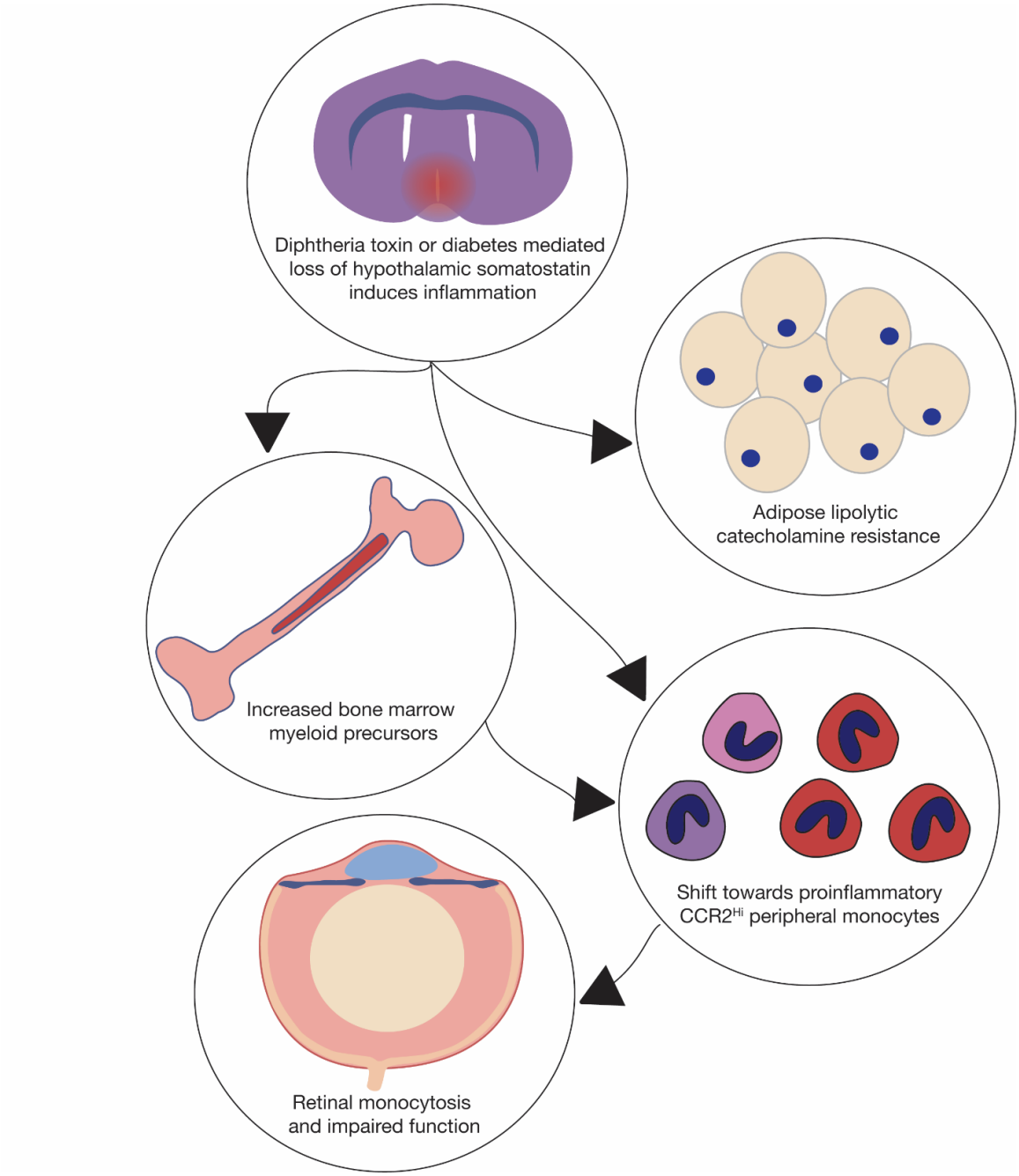


**Graphical Abstract**

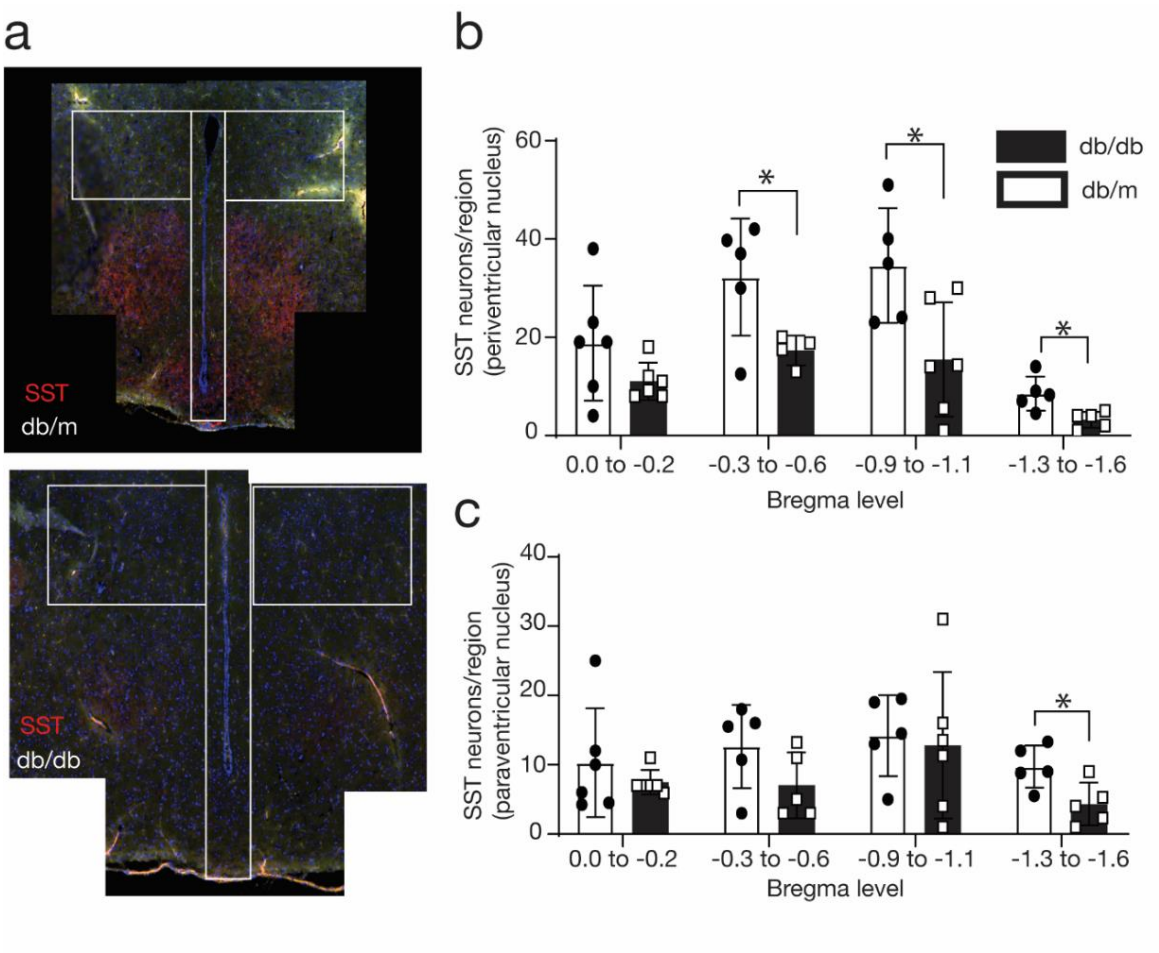


**Fig. 1** Reduced somatostatin immunofluorescence in db/db mouse paraventricular and periventricular hypothalamus.
(**a**) Coronal sections of hypothalamus from *db*/*db* mice at 12 months of age exhibited a reduction in immunofluorescent somatostatin staining, compared with heterozygote littermate control (*db*/m) mice. (**b**, **c**) Cell counts of somatostatin-positive nuclei in the periventricular nucleus (**b**) and paraventricular nucleus (**c**) from Bregma 0.0 to Bregma −1.6. $n$=5 *db*/*db*, $n$=6 *db*/m *$p$<0.05 for indicated comparison

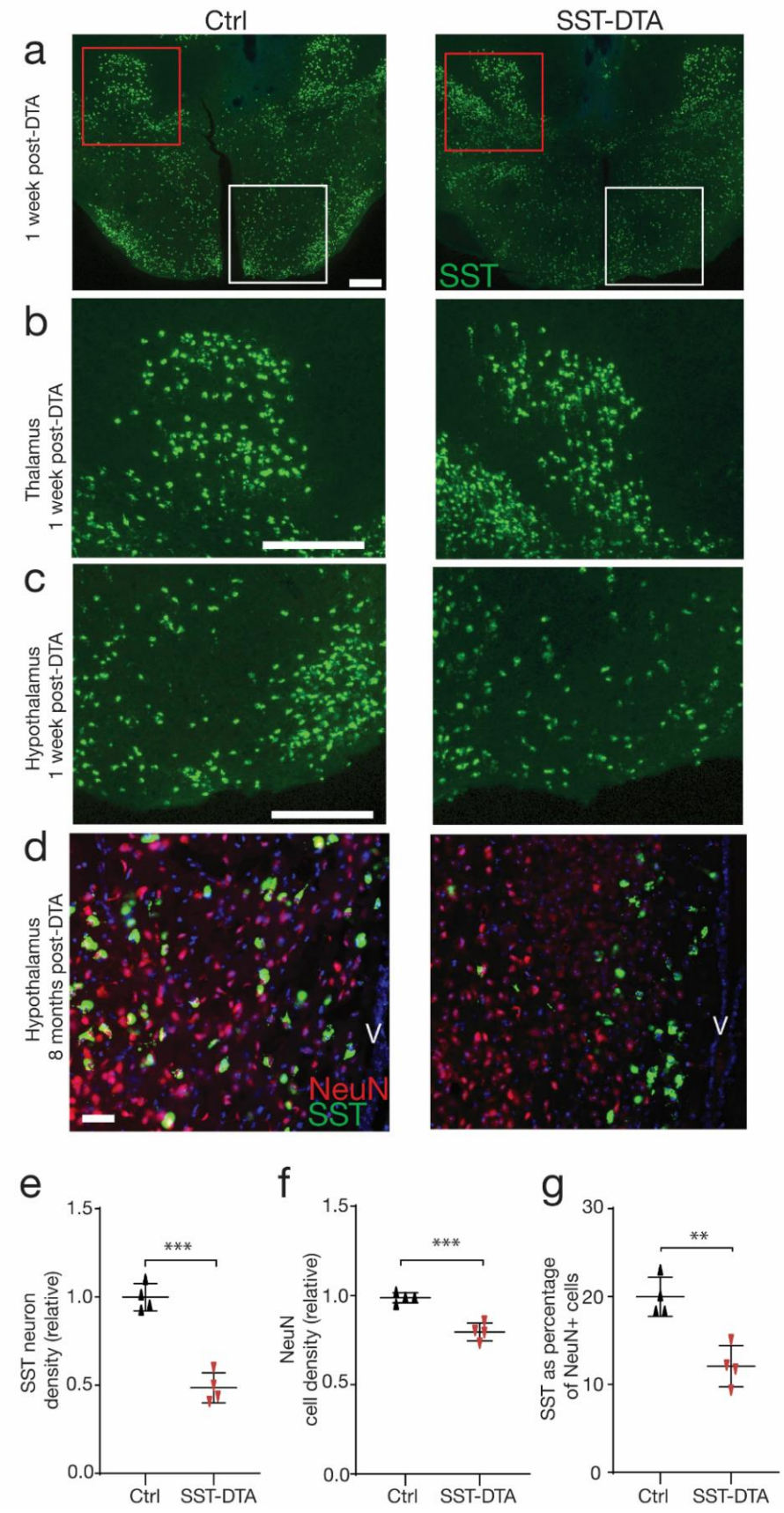
Ctrl
SST-DTA
a
1 week post-DTA
SST
b
Thalamus
1 week post-DTA
c
Hypothalamus
1 week post-DTA
d
Hypothalamus
8 months post-DTA
V
NeuN
SST
e
SST neuron density (relative)
1.5
1.0
0.5
0.0
***
Ctrl
SST-DTA
f
NeuN cell density (relative)
1.5
1.0
0.5
0.0
***
Ctrl
SST-DTA
g
SST as percentage of NeuN+ cells
30
20
10
0
**
Ctrl
SST-DTA

**Fig. 2** AAV-DTA delivery reduces the abundance of hypothalamic SST neurons in mice. (**a**) At 1 week post-injection, a large reduction in the abundance of SST-GFP$^+$ neurons was observed in the hypothalamus (white box) but not the adjacent brain regions (such as the ventromedial thalamus; red box) of SST-DTA mice. Scale bar, 100 μm. (**b**, **c**) Higher magnification images indicate comparable abundance of SST neurons (SST-DTA vs SST-GFP control mice) in the thalamus (**b**) but not the hypothalamus (**c**). Scale bar, 100 μm. (**d**) At 8 months post-DTA, SST-DTA mice exhibited sustained loss of SST neurons, with small changes in NeuN-positive neurons in red. (**e**) Quantification of the reduction in SST neurons in DTA-treated mice compared with AAV-GFP mice; cell densities were calculated per field and relativised to control mice. (**f**, **g**) Quantification of reduction in NeuN in SST-DTA mice showed the loss was significant (**f**); however, relativising SST density to NeuN density indicated significant SST loss (**g**). $n$=4 SST-DTA, $n$=4 control. **$p$<0.01 and ***$p$<0.001 for indicated comparison. Ctrl, control; V, third ventricle of the brain

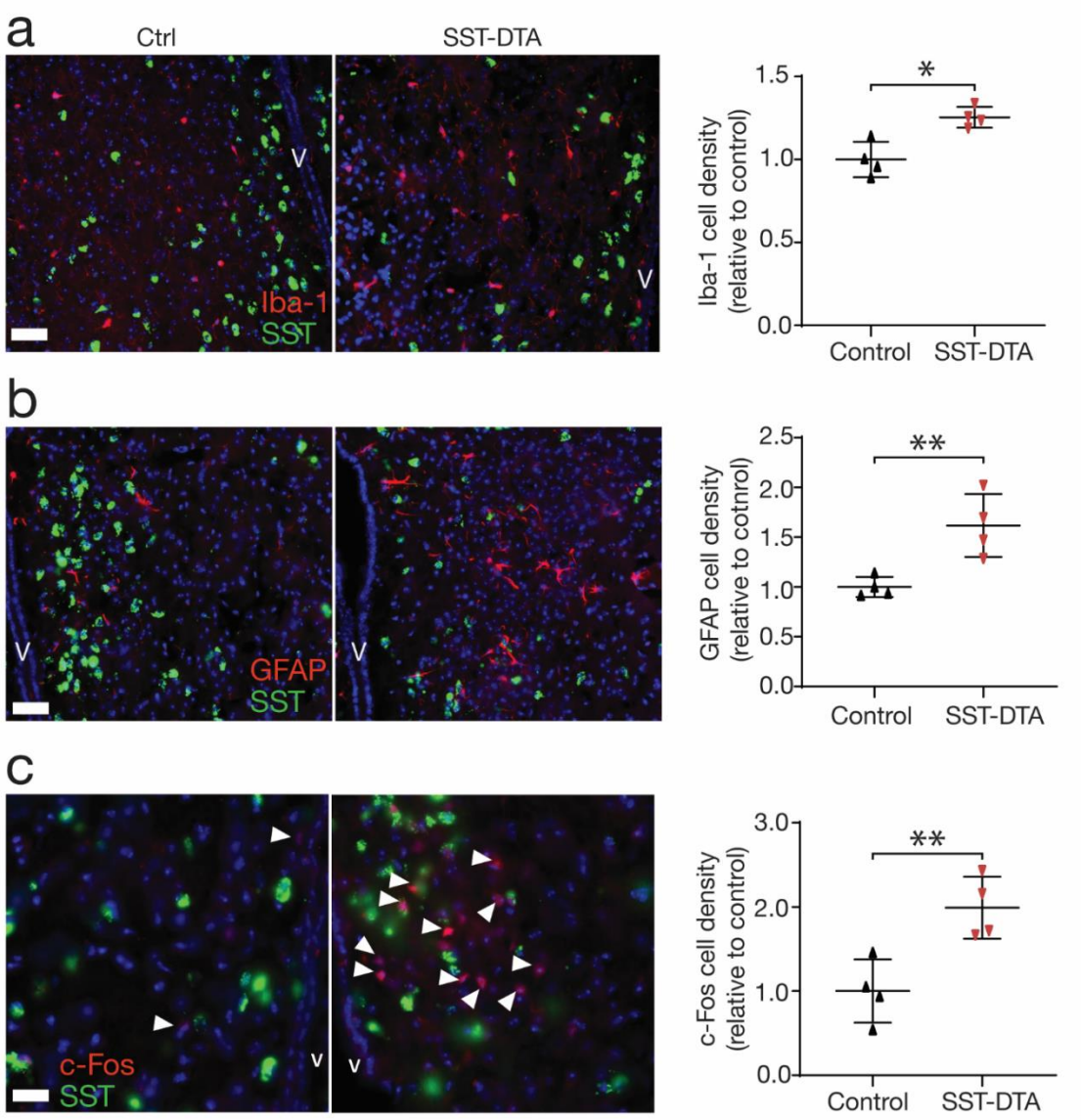


**Fig. 3** SST-DTA mice exhibit hypothalamic inflammation and hyperactivity. (**a**–**c**) Representative immunofluorescent images of Iba1$^+$ cells (red, **a**), GFAP$^+$ cells (red, **b**) and c-Fos$^+$ cells (red, **c**), labelled for somatostatin (green, GFP) and nuclei (blue, DAPI). Graphs show cell densities calculated per microscopic field and relativised to control mice. $n$=4 SST-DTA, $n$=4 *$p$<0.05 and **$p$<0.01 for indicated comparison. Ctrl, control; V, third ventricle of the brain

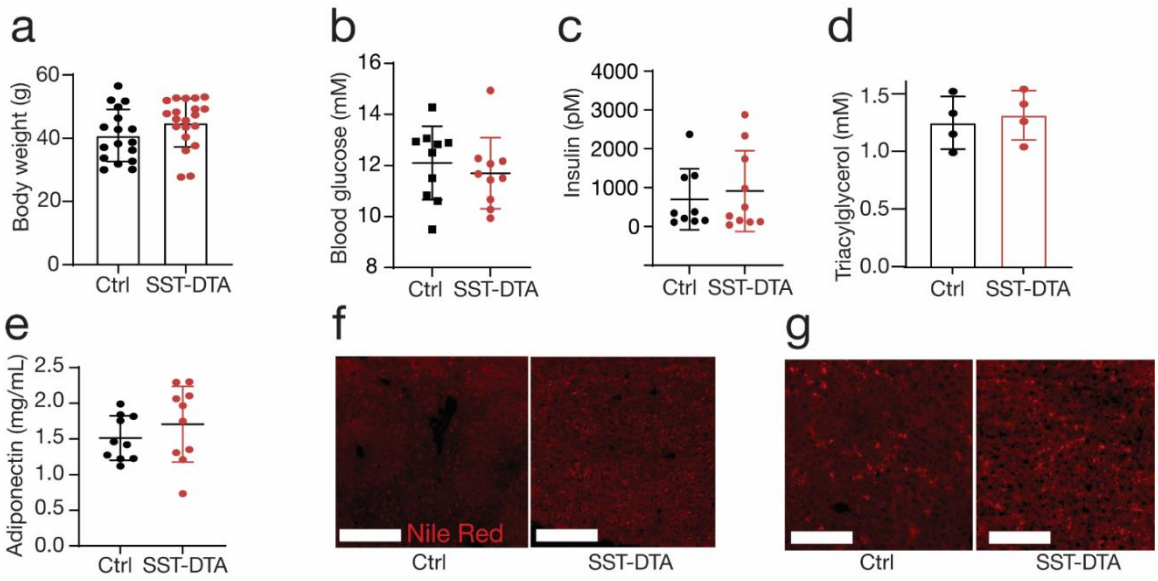


**Fig. 4** SST-DTA mice do not exhibit hyperglycaemia, hyperinsulinaemia or hyperlipidaemia. (**a**–**d**) Body weight measurements at 8 months of age showed no overall trend (**a**) (*n*=20/group), nor did blood glucose measurements (**b**) or insulin (**c**) (*n*=10/group), or triacylglycerol (**d**; *n*=4/group). (**e**) Plasma adiponectin was not depleted in DTA mice (*n*=10/group). (**f**, **g**) Nile Red neutral lipid staining of livers from SST-DTA and control mice did not show gross lipid deposits at 10× magnification (**f**; scale bar, 50 μm); higher resolution images (**g**; 20×; scale bar, 25 μm) showed small lipid droplets within a normal range. Ctrl, control.

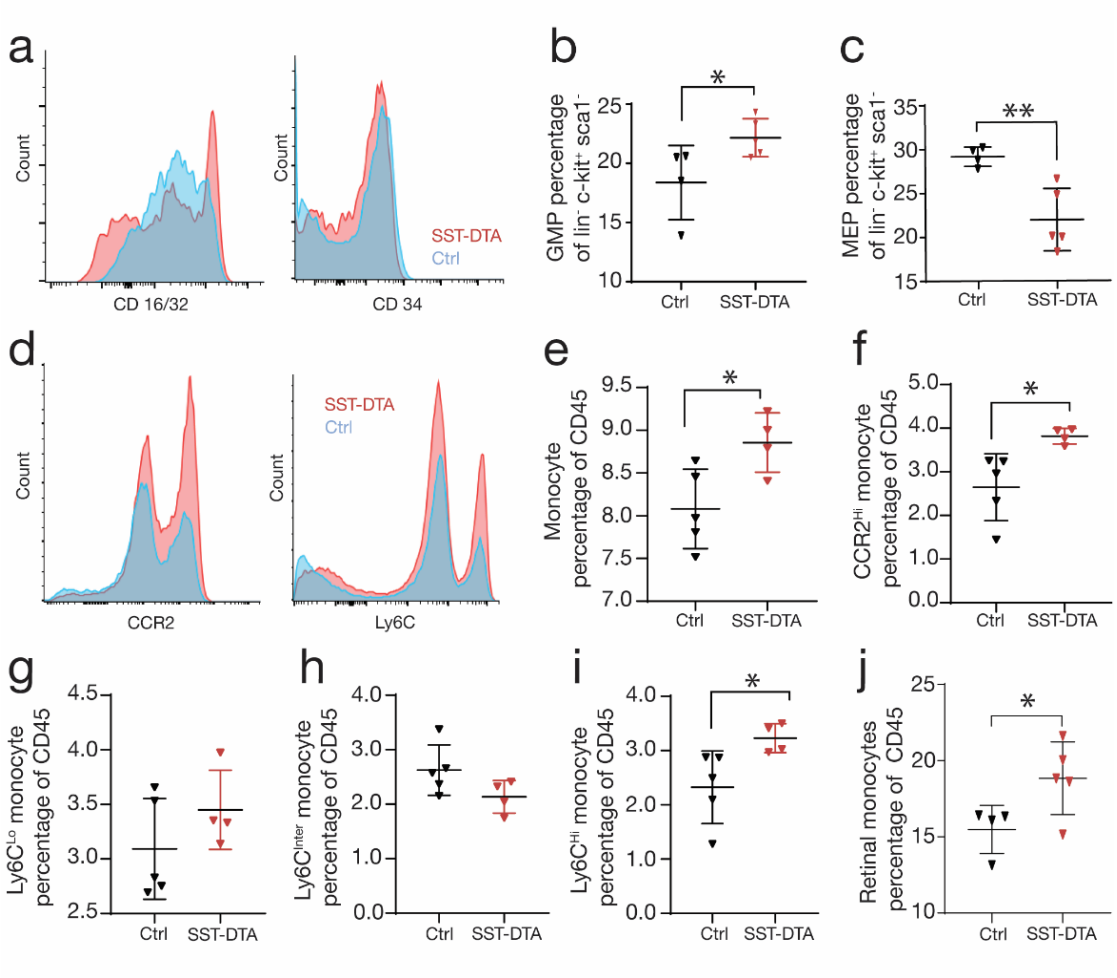


**Fig. 5** SST-DTA mice exhibit altered haematopoiesis and circulating monocytes. (**a**) Histograms showing counts of SST-DTA (red) and control (blue) $CD16^{+}$, $CD32^{+}$ and $CD34^{+}$ cells. (**b**, **c**) SST-DTA mice show increases in bone marrow GMPs (**b**), with concomitant decreases in bone marrow megakaryocyte-erythroid progenitors (MEPs) (**c**). (**d**) Histograms for monocytes expressing CCR2 and Ly6C. (**e**, **f**) DTA-treated mice exhibited a relative monocytosis (**e**) and an increase in classical $CCR2^{hi}$ monocytes (**f**). (**g**–**i**) Plots of the three circulating monocyte pools, split by Ly6C expression, showing that SST-DT mice exhibited no differences in $Ly6C^{lo}$ or $Ly6C^{inter}$ monocytes (**g**, **h**) and an increase in $Ly6C^{hi}$ monocytes (**i**). (**j**) Flow cytometry showed increases in retinal monocytes in DTA-treated mice. *n*=4 or 5/group. **p*<0.05 and ***p*<0.01 for indicated comparison. Ctrl, control; MEP, megakaryocyte-erythroid progenitor

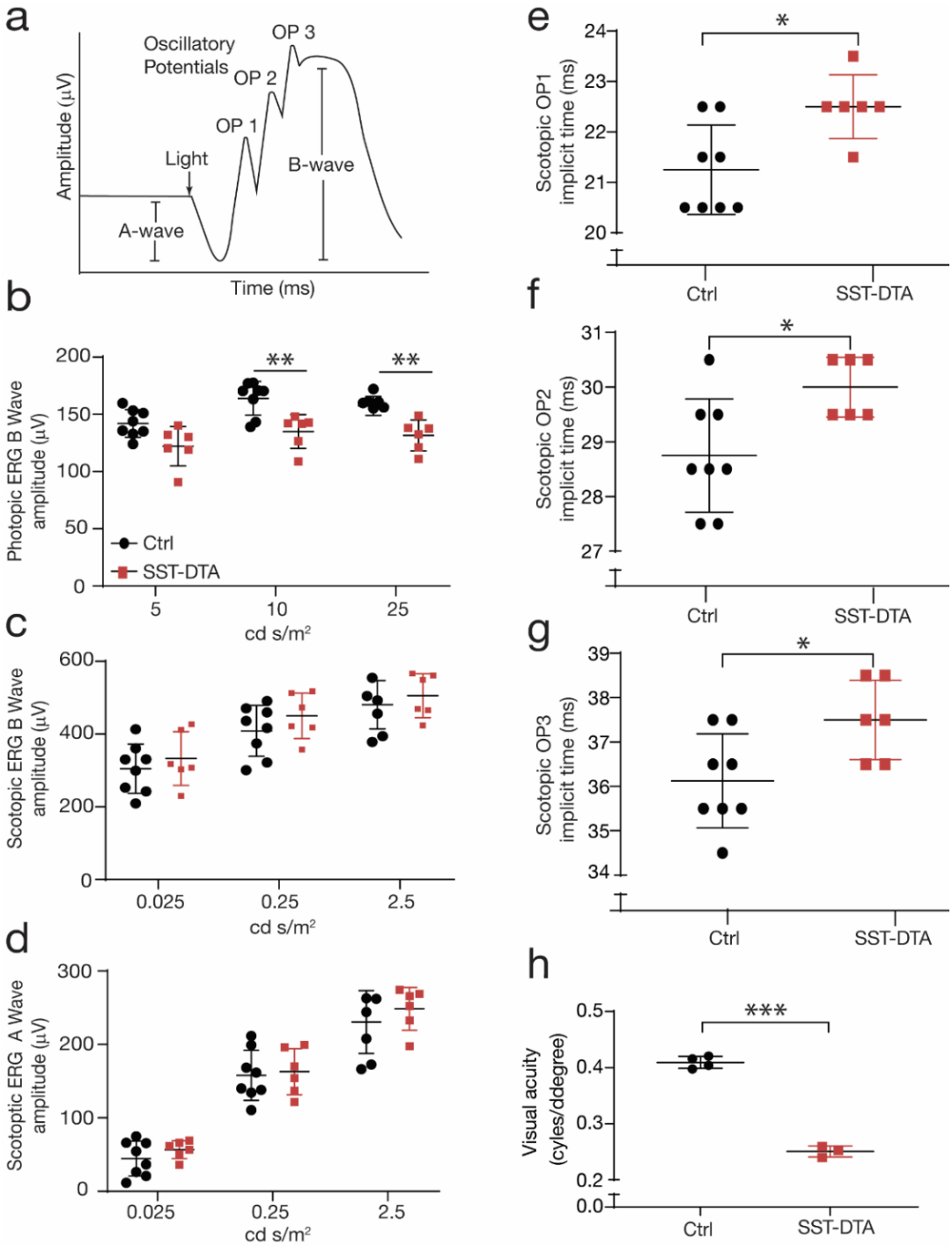


**Fig. 6** SST-DTA mice exhibit changes in retinal function. (**a**) ERG model diagram showing A-wave, B-wave and OP. (**b**, **c**) Recordings from SST-DTA mice exhibit deficits in B-wave responses at photopic intensities (**b**) but not at lower, scotopic light intensities (**c**) (*n*=8 control, *n*=6 SST-DTA). (**d**) A-wave responses are likewise comparable at scotopic light intensities. (**e**–**g**) OP onset time is delayed for OP1–3. (**h**)

Optomotor tracking data indicated reduced visual acuity in SST-DTA mice (*n*=4/group). **p*<0.05 and ****p*<0.001 for indicated comparison. Ctrl, control

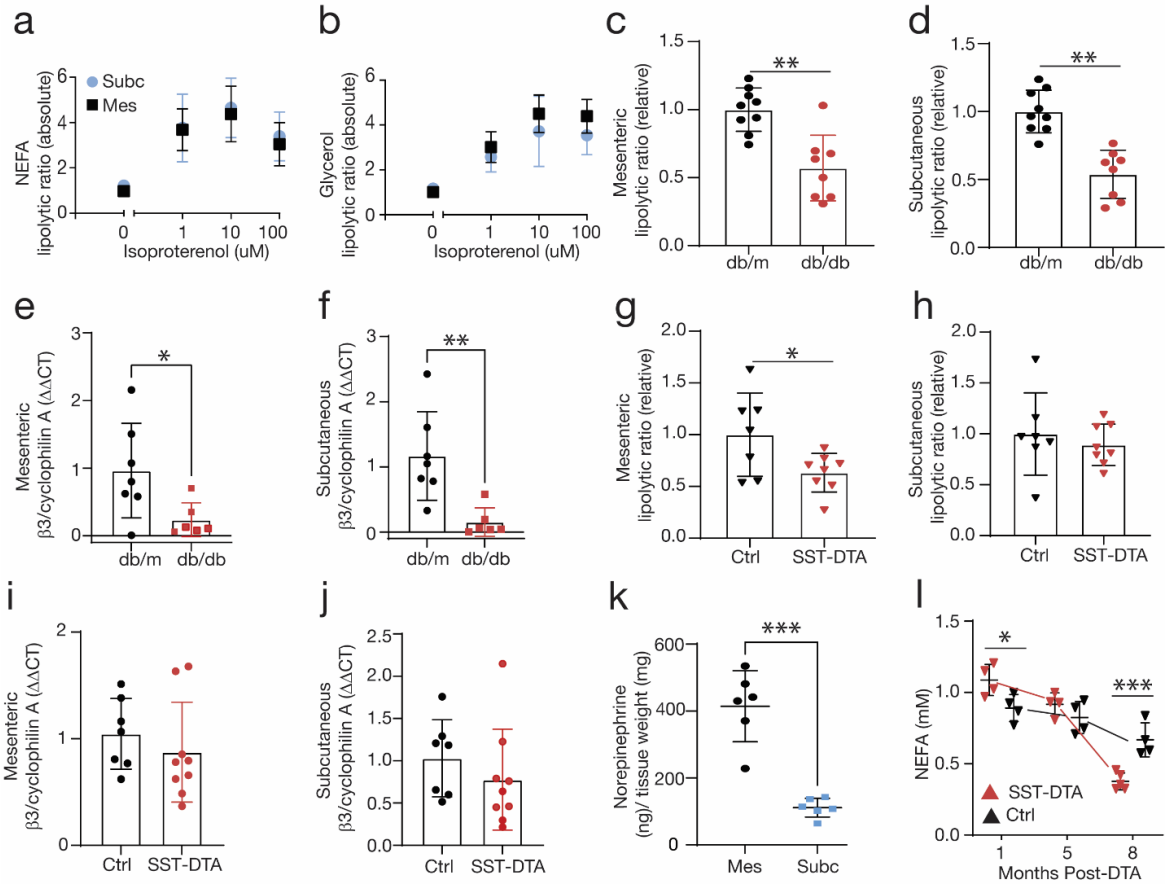


**Fig. 7** SST-DTA mice exhibit visceral fat catecholamine resistance. (**a**, **b**) Isoprenaline dose-dependently drives the release of NEFA (**a**) and glycerol (**b**) from acute subcutaneous and mesenteric fat explants. (**c**, **d**) Plots of ex vivo lipolysis in mesenteric (**c**) and inguinal (**d**) subcutaneous fat from 12-month-old *db*/*db* mice show lipolytic catecholamine resistance in both fat pads. (**e**, **f**) Quantitative PCR data show $\beta_3$-adrenergic receptors are significantly downregulated in mesenteric (**e**) and subcutaneous (**f**) fat from *db*/*db* mice. (**g**, **h**) SST-DTA mice exhibit lipolytic catecholamine resistance in mesenteric (**g**) but not subcutaneous (**h**) adipose tissue. (**i**, **j**) Quantitative PCR data show $\beta_3$-adrenergic receptors are not significantly downregulated in mesenteric (**i**) and subcutaneous (**j**) fat from SST-DTA mice. (**k**) Noradrenaline concentration in mesenteric and subcutaneous fat from wild-type mice, measured by HPLC and electrochemical detection, indicates higher basal noradrenaline content in mesenteric fat. (**l**) Serial plasma NEFA measurements in SST-DTA and control mice show dynamic alterations in fatty acid homeostasis in the SST-DTA model (*n*=7/group). **p*<0.05, ***p*<0.01 and ****p*<0.001 for indicated comparison. Ctrl, control; Mes, mesenteric; Subc, subcutaneous

# ELEVATED CIRCULATING AND TISSUE LEVEL CATECHOLAMINES ARE ASSOCIATED WITH CATECHOLAMINE RESISTANCE IN MALE, BUT NOT FEMALE MICE

by

ROBERT F. ROSENCRANS, EDGAR L. READY, ANGELA HARBOUR, JASON B. FLOYD, MARIA B. GRANT

In preparation for submission
Format adapted for dissertation

## Abstract

*Background:* The sympathetic nervous system regulates white adipose tissue function through the catecholamine neurotransmitter, norepinephrine, which stimulates lipolysis. However, in metabolic syndrome, adipose tissue downregulates β-adrenergic receptors and becomes catecholamine resistant. In many receptor-ligand systems, chronic stimulation induces desensitization, but the relationship between endogenous catecholamine flux and catecholamine resistance has not been adequately explored. Additionally, no preclinical studies have investigated whether catecholamine resistance occurs in female mice. This gap is important not only because sex differences may have important implications for treatment, but because female patients often lack the increased sympathetic nerve activity observed in male patients with metabolic syndrome. The goal of this study was to test the association between circulating and tissue catecholamines and catecholamine resistance across visceral mesenteric fat and subcutaneous fat in male and female mice.

*Methods:* We assessed circulating and adipose tissue catecholamines via mass spectroscopy. We measured catecholamine signaling at the level of second messengers, protein phosphorylation, and lipolytic products under receptor dependent (isoproterenol) and receptor independent (forskolin) stimulation paradigms.

*Findings:* Male and female mice exhibit weight gain under high fat diet, but circulating catecholamines are elevated in male, but not female mice. Female mice show consistent signs of catecholamine resistance in subcutaneous fat, but not in visceral fat, whereas male mice consistently show catecholamine resistance in all adipose tissues and endpoints studied. Elevated norepinephrine turnover is observed in male mice, without any change in the concentration of the norepinephrine breakdown product,

dihydroxyphenylglycol (DHPG), suggestive of impaired neurotransmitter clearance. Taken together, these data suggest that elevated catecholamines could be causally related to catecholamine resistance and suggest that a novel approach to reversing catecholamine resistance might involve partial inhibition of chronic receptor stimulation.

Keywords: Sympathetic nervous system, catecholamines, adrenergic, visceral adipose tissue, sex differences, metabolic syndrome

## Introduction

The sympathetic nervous system (SNS) is a key regulator of white adipose tissue physiology through the catecholamine neurotransmitter, norepinephrine. Norepinephrine acts on g-protein coupled beta-adrenergic receptors (β-AR1-3) to initiate lipolysis. Adrenergic signaling also controls the release of many cytokines and adipokines: adiponectin[1, 2], leptin[3, 4], resistin[5, 6], and tumor necrosis factor alpha[7] (TNF-a) and interleukin-6[8, 9]. Sympathetic nerve function has been extensively studied under physiologic stressors such as fasting or cold exposure[10-12], but comparatively less attention has been paid to the behavior of sympathetic nerves under hypercaloric diets. However, several studies indicate that norepinephrine turnover, a widely used proxy for sympathetic nerve activity, increases under brief (1 week to 3 week) dietary manipulations[13-15]. No study has examined adipose tissue norepinephrine turnover under longer term dietary exposures more relevant to real world settings.

The co-occurrence of increased norepinephrine turnover and weight gain is paradoxical, as increased sympathetic nerve activity is widely associated with reduced fat stores[16]. Catecholamine resistance, the impaired ability of adipocytes to undergo lipolysis in response to beta-adrenergic stimulation, may explain this association. Long term prospective studies show that catecholamine resistance is associated with greatly increased long term odds of developing worsening insulin resistance and diabetes[17]. Thus, discerning the drivers of catecholamine resistance is important. All prior preclinical work on catecholamine resistance is in male animals[1, 2, 18-20], with the exception of two studies which did not describe the sex of experimental animals[21, 22]. As such, the

possibility of sex differences in these phenotypes has been understudied, which hampers the development of specific therapeutics for female patients.

In a prior publication, we showed that catecholamine resistance can be induced by manipulation of the paraventricular nucleus of the hypothalamus, a major central nervous system control hub for sympathetic nerve activity[23]. In this work, centrally induced catecholamine resistance occurred in norepinephrine enriched adipose tissue depots such as mesenteric fat[23]. These data raise the possibility that increased norepinephrine turnover could drive catecholamine resistance. Indeed, adipose tissue, like many other organs, has been shown to desensitize and downregulate adrenergic receptors in response to chronic stimulation with catecholamines[24] or pharmacologic agonists[25]. Drawing on this work, we hypothesized that chronic adrenergic stimulation contributes to adipose catecholamine resistance. Findings in support of this hypothesis would suggest a novel therapeutic approach: reversing catecholamine resistance through the partial reduction of adrenergic signaling. Such approaches, using beta blockers, have been shown to improve cardiac adrenergic sensitivity in heart failure[26], another chronic state of elevated sympathetic nerve activity.

In the present study, we expand on our prior observations to demonstrate that 1) increased circulating catecholamines and increased tissue norepinephrine turnover are associated with catecholamine resistance in visceral and subcutaneous fat of male mice 2) female mice exhibit catecholamine resistance in subcutaneous fat and preferentially expand this fat depot over mesenteric fat, which remains catecholamine sensitive 3) increases in norepinephrine turnover are not linked to increases in norepinephrine degradation, which raises the possibility that impaired norepinephrine clearance

contributes to catecholamine resistance. Together, these data expand our understanding of the relationship between catecholamines and catecholamine resistance.

## Methods

### *Mice*

Male and female C57Bl6 mice were bred in animal facilities at the University of Alabama at Birmingham in accordance with approved protocols from the Institutional Animal Care and Use Committee. Beginning at 8 weeks of age, mice were continued on chow diet or switched to 60% high fat diet (Research Diets; D12492) for 8 weeks. Mice were group housed on a 12-hour light/12-hour dark cycle (lights on from 0600 to 1800 hours) at 22°C and constant humidity. Mice had *ad libitum* access to food and water.

### *Ex-vivo lipolysis*

Ex-vivo lipolysis was assessed using standard approaches[17]. Specifically, mice were euthanized using isoflurane, inguinal subcutaneous and mesenteric adipose tissue were dissected, washed in phosphate buffered saline, and incubated in low glucose DMEM (Corning #10-014-CV) for 3 hours (basal lipolysis) prior to stimulation with 10 µM isoproterenol (Sigma Aldrich #I6504; induced lipolysis). Media was supplemented with 2% bovine serum albumin and 5 µM Triacsin C to prevent fatty acid re-esterification (Cayman Chemical, #10007448500). Following stimulation, samples were frozen at -20°C. Media glycerol was measured (Neogen free glycerol kit, #KC-GRL), and lipolysis was quantified as millimoles of glycerol released per mg tissue per hour.

### *Quantitative real time polymerase chain reaction (qRT-PCR)*

Adipose samples (40mg) were rapidly dissected and snap frozen in DNAse/RNAse free tubes over liquid nitrogen and stored at −80°C until homogenization with Qiagen TissueLyser LT in Trizol. RNA was purified (DirectZol RNA Miniprep; ZymoResearch, USA; R2051) with on-column DNAse treatment followed by reverse transcription (Superscript IV VILO; Thermofisher; 11756050). cDNA was diluted at 1:40 with RNAse/DNAse free water, and 4 µL of diluted cDNA was used in a 10 µl reaction volume as follows: β3-AR transcripts were amplified (forward primer TGACTCCTGAAACA AGCGGG, reverse primer GTGGGAATT GGAGGGTGGAG) and compared with cyclophilin A (forward primer CAGACGCC ACTGTCGCTTT, reverse primer TGTCTTTGGAACTTTGTCTGCAA) via SYBR Green (Biorad, USA; 1725271). Specificity was measured via melting curve and confirmed via amplicon size in agarose gel electrophoresis.

*Norepinephrine turnover*

Mice were handled daily for a minimum of 1 week prior to NETO studies in order to reduce stress responses. Mice were dosed via intraperitoneal route with a-methyl-p-tyrosine methyl-ester (AMPT; M3281, Sigma Aldrich) at 250mg/kg at time zero and again after 2 hours at 150 mg/kg. AMPT was freshly dissolved in phosphate buffered saline (PBS) at a concentration of 30mg/mL. Mice were euthanized 4 hours after the first dose of AMPT and tissues were collected over wet ice with ample ice-cold sterile PBS. Due to catecholamine lability, dissections were performed under dim light. Samples were immediately snap frozen over liquid nitrogen and stored at -80 °C until analysis. Analysis of tissues following moclobemide treatment (5 mg/kg; intraperitoneal injection) followed the same protocol outlined above, although with a single injection and euthanasia at two

hours post-injection. Catecholamines and their metabolites were analyzed at the Vanderbilt Neurochemistry Core as detailed below.

*Tissue Extraction for Catecholamines*

Tissues were homogenized, using a tissue dismembrator, in 3 mL of 0.1M TCA, which contained 10-2 M sodium acetate, 10-4 M EDTA, and 10.5 % methanol (pH 3.8). Ten µL of homogenate was used for protein quantification. Samples were spun in a microcentrifuge at 10,000 g for 20 minutes at 4°C. The supernatant was removed for LC/MS analysis.

*Plasma Collection for Catecholamines*

Whole trunk blood was anti-coagulated with EDTA (4 µmol per mL blood) and catecholamines were preserved using reduced glutathione (10 µg per mL blood) in 50 mL conical vials over ice, shielded from light. Samples were transferred to 1.5 mL Eppendorf tubes and centrifuged at 1000 relative centrifugal units for 7 minutes, followed by plasma collection and snap freezing over liquid nitrogen. Plasma was stored at -80C and underwent no freeze-thaw cycles. Plasma was shipped on dry ice to Vanderbilt Neurochemistry Core for derivatization and analysis.

*Benzoyl Chloride Derivatization and LC/MS Analysis*

Analytes in tissue extract supernatant were quantified using liquid chromatography/mass spectrometry (LC/MS) following derivatization with benzoyl chloride (BZC)[27]. 5 µL of supernatant was then mixed with 10 µL each of 500mM NaCO3 (aqueous) and 2% BZC in acetonitrile in an LC/MS vial. After two minutes, the

reaction was stopped by the addition of 10 µL internal standard solution. LC was performed on a 2.1 x 100 mm, 1.6um particle CORTECS Phenyl column (Waters Corporation, Milford, MA, USA) using a Waters Acquity UPLC. Mobile phase A was 0.1% aqueous formic acid and mobile phase B was acetonitrile with 0.1% formic acid. MS analysis was performed using a Waters Xevo TQ-XS triple quadrupole tandem mass spectrometer. The source temperature was 150°C, and the desolvation temperature was 400°C.

*Protein assay*

Protein concentration was determined using the BCA Protein Assay Kit (Thermo Scientific, Waltham, MA USA) in a 96-well plate format. 10µL of tissue homogenate was mixed with 200 µl of mixed BCA reagent per manufacturer instructions. The plate was incubated at 23°C for two hours before absorbance was measured by the plate reader (POLARstar Omega).

*cAMP Analysis*

cAMP was measured in single cell suspensions of adipocytes (50 µL packed adipocytes). Single cell suspensions were generated using established tissue digestion protocols. Briefly, freshly dissected adipose tissue was minced and resuspended in a tissue digestion cocktail containing 1 mg/mL collagenase IV and 15 Kunitz units per mL DNase dissolved in low glucose DMEM, supplemented with 9.67 mM calcium chloride for optimal collagenase and DNase performance. Following 1.5 hours of gentle rocking at 37°C, cells were filtered through 300 µM filters, washed and aliquoted into 0.5mL Eppendorf vials for stimulation.

Adipocyte were stimulated with 200 µL of the same isoproterenol buffer as in lipolysis experiments for 15 minutes. Stimulation media was removed, and cells were snap frozen on liquid nitrogen and stored at -80°C until lysis and analysis using the Promega cAMP-Glo assay following kit instructions. Luminescence was measured using a Biotek Cytation 5 (Agilent).

*Western Blots*

Tissue explants from mesenteric and inguinal fat of male and female mice on high fat and control diet were stimulated for 15 minutes in the same 10 µM isoproterenol media used for lipolysis, and in separate samples with 100 µM forskolin (the higher concentration was based on an empirical dose response curve for forskolin demonstrating weaker lipolytic activity; data not shown).

Samples were snap frozen and stored at -80°C until lysis using the Qiagen Tissue Lyser LT in ice cold RIPA buffer (50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1:33 Halt Phosphatase and Protease Inhibitors (ThermoFisher 78440; containing sodium fluoride, sodium orthovanadate, β-glycerophosphate, sodium pyrophosphate, aprotinin, bestatin, E64, and leupeptin. Exact concentrations are proprietary). Lysates were stored on wet ice for 1 hour post-lysis, then spun at 20,000 relative centrifugal force for 10 minutes at 4°C. Infranatant was carefully collected to eliminate the lipid fraction supernatant and analyzed via for protein via BCA as above. Samples were mixed with Licor Biosciences 4X Loading Buffer, boiled at 95°C for 7 minutes, centrifuged and loaded into Biorad Mini-Protean TGX Gels (5%-15% polyacrylamide). Electrophoresis was performed on ice for 1.5 hours at 130V and gels were transferred using the TransBlot Turbo system

onto 0.2µM nitrocellulose. Blots were blocked using Licor Intercept, stained overnight at 4°C using Cell Signaling Technologies antibodies (#9624, 1:1000) raised against the consensus protein kinase A phosphorylation site and visualized using AlexaFluor 700 secondary antibodies (ThermoFisher A-21038). Actin was used as a loading control (Sigma Aldrich, A3854). Blot quantifications were performed using Image Studio (Licor Biosciences v. 5.5); densitometry of the phosphorylated PKA consensus site was normalized to actin.

### *Statistics*

All data was analyzed in GraphPad Prism (version 9; GraphPad Software Inc.). Normally distributed data was analyzed using two-tailed T-tests or with Welch's unequal variances t-test or, where appropriate, analysis of variance or analysis of covariance. Nonparametric data were analyzed using the Mann-Whitney *U* test. All data are presented as means ± standard deviation. Norepinephrine turnover rates and variances were calculated using the classic approach outlined by Taubin and Landsberg[28], such that the standard error of the regression slope and the standard error of baseline norepinephrine content were multiplied to generate the standard error of the turnover rate.

## Results

### *High Fat Diet Differentially Impacts Weight Gain in Male and Female Mice*

Eight weeks of high fat diet increased body weight in male and female mice (Figure 1A, Figure 1B). The masses of both mesenteric and inguinal subcutaneous fat were increased in male (Figure 1C) and female (Figure 1D) mice. Clinical data suggest that female patients accrue less visceral fat and preferentially expand subcutaneous

adipose tissue depots[29]. We tested the hypothesis that the ratio of subcutaneous to visceral fat would be higher in female mice as compared to male mice on a high fat diet using paired samples from each mouse (Figure 1E). We found a dramatic difference in this ratio, such that both males and females had subcutaneous to mesenteric ratios near 1 under normal diet but responded to high fat diet by skewing this ratio upwards in females (disproportionate subcutaneous fat) and downwards in males (disproportionate mesenteric fat).

*High Fat Diet Increases Circulating Norepinephrine in Male Mice but Not Female Mice*

Prior human data show a strong positive association between muscle sympathetic nerve activity and visceral fat mass[30], but to our knowledge, no such relationship has been explored in preclinical models. In male mice, plasma norepinephrine was elevated in high fat diet fed animals compared to normal diet fed animals (Figure 2A), but no such elevation was observed in female mice (Figure 2B). Epinephrine was similarly elevated in male mice on high fat diet, but not female mice (Supplemental Figure 1A, B). No differences were observed in the circulating levels of the epinephrine and norepinephrine precursor, dopamine, in male or female mice (Supplemental Figure 1C,D). In normal diet fed male (Figure 2C) and female (Figure 2D) mice, no relationship was found between plasma norepinephrine and the mass of either inguinal fat or mesenteric fat. In contrast, in male mice on high fat diet, a strong, positive correlation was observed between tissue mass and plasma norepinephrine (Figure 2E). We failed to detect a significant correlation in female mice, albeit with a potentially underpowered study (Figure 2F). These data indicate that circulating norepinephrine is positively correlated with both visceral and subcutaneous fat mass in high fat diet fed male mice.

*High Fat Diet Elicits Transcriptional Downregulation of β3-AR and Functional Impairments in Lipolysis*

To test whether elevated catecholamines co-occurred with reduced catecholamine sensitivity, β3-AR mRNA and lipolytic responses to the beta-adrenergic agonist, isoproterenol, were assessed in mesenteric and subcutaneous fat of male and female mice on high fat and control diet. β3-AR was transcriptionally downregulated in adipose tissues of high fat diet male mice as compared to normal diet fed control mice (Figure 3A), a finding that was replicated in adipose tissue of female mice (Figure 3B). Control samples processed without reverse transcriptase had dramatically elevated CT values or never amplified (CT>40), confirming that the overwhelming majority of signal was from mRNA (Supplemental Figure 2A). Glycerol release induced by isoproterenol, a measurement of lipolysis, was lower in both mesenteric and subcutaneous adipose tissue explants of high fat diet fed male mice, as compared to normal diet fed control mice (Figure 3C). In female mice, two-way ANOVA revealed a main effect of diet ($p<0.01$), but Sidak multiple comparisons failed to detect significant differences between tissues (Figure 3D). Collectively, these data replicate previously established findings[2, 19] that adipose tissue from male mice at 8 weeks of high fat diet exhibits transcriptional downregulation of the β3-AR and extend these findings to female mice. These data show that male mice on high fat diet exhibit impaired lipolysis in both mesenteric and subcutaneous fat, but we fail to detect a significant difference in female mice.

*High Fat Diet Impairs Phosphorylation of Downstream PKA Targets in a Sex and Tissue Dependent Manner*

To further assess catecholamine signaling and resistance, we probed isoproterenol induced phosphorylation of consensus PKA phosphorylation sites via western blot. We first established the linear range of protein quantification (Supplemental Figure 4A). We found, in keeping with prior investigators[22, 31], that adipose tissue exhibits several major bands between 60 and 70 kDa and a relative paucity at other molecular weights. Male mice on high fat diet exhibited attenuated phosphorylation in mesenteric and subcutaneous fat (Figure 4 A,B). We failed to detect differences in mesenteric fat of female mice (Figure 4C) but observed reduced phosphorylation in subcutaneous fat of female mice (Figure 4D). To determine whether these findings were recapitulated by a stimulation approach which bypasses beta-adrenergic receptors, we utilized the adenylyl cyclase agonist, forskolin. Remarkably, forskolin rescued the attenuated phosphorylation in mesenteric and subcutaneous fat of male mice (Figure 4E, F). Mesenteric fat from female mice showed no differences in forskolin mediated phosphorylation across normal and high fat conditions, as was observed in isoproterenol stimulated samples (Figure 4G). Subcutaneous fat in female mice exhibited non-significant differences in phosphorylation (Figure 4H). Because of the conflicting findings in female mice, we next assessed the second messenger immediately downstream of the beta-adrenergic receptor, cAMP, finding that isoproterenol mediated production of cAMP was preserved in mesenteric fat, but not subcutaneous fat, under high fat diet in females (Supplemental Figure 5). Altogether, these data suggest that male mice exhibit receptor dependent catecholamine resistance irrespective of adipose tissue depot, whereas female mice consistently exhibit greater catecholamine resistance in subcutaneous, as compared to mesenteric, fat.

*High Fat Diet Increases Norepinephrine Turnover in Adipose Tissue of Male Mice*

Because female mice failed to show elevated circulating catecholamines, and because the catecholamine resistance phenotype of female mice was inconsistent across transcriptional, lipolytic, and phospho-proteomic levels, we focused subsequent studies on males. We utilized a-methyl-p-tyrosine (AMPT) mediated synthesis inhibition to assess norepinephrine turnover (NETO), an approach which has been used to measure sympathetic drive under fasting, cold exposure, caloric restriction, and glucoprivation [11, 12, 32]. As in prior studies, norepinephrine turnover was assessed over four hours, a standard time window for adipose tissue over which norepinephrine decline has been shown to be linear (Figure 5A, C). Turnover rates were higher in both mesenteric and subcutaneous fat from mice on high fat diet (Figure 5B, D), as compared to normal diet fed controls. We confirmed that our NETO paradigm did not alter circulating homovanillic acid, a catecholamine metabolite, as compared to a ringer saline injection (Supplemental Figure 6A).

*High Fat Diet Does Not Alter Degradation Products of Norepinephrine in Adipose Tissue of Male Mice*

Classically, increased norepinephrine turnover has been interpreted to reflect increased sympathetic nerve firing rates and concurrently increased secretion. However, an alternative explanation for increased turnover is the enhanced degradation of norepinephrine via monoamine oxidases (MAO-A) or catecholamine-O-methyltransferases (COMT). We observed that despite increased norepinephrine turnover, the tissue content of the MAO-A norepinephrine metabolite,

dihydroxyphenylglycol (DHPG), is unaltered in either visceral or subcutaneous fat of male mice on high fat diet (Figure 6A, B). We also found that DHPG is the dominant degradation metabolite in adipose tissue, such that normetanephrine, the product of COMT, is largely undetectable (Figure 6C,D). DHPG is converted to 3-methoxy-4-hydroxyphenylglycol (MHPG) via catecholamine-O-methyltransferases, and we observed no difference in the plasma content of MHPG in high fat diet fed male mice (Figure 6E). Our confidence in the relative absence of catecholamine-O-methyltransferase activity in adipose tissue is further supported by studies using the MAO-A inhibitor, moclobemide. Moclobemide failed to elicit a change in the adipose tissue abundance of its downstream catecholamine-O-methyltransferase product, MHPG, albeit in female mice (Supplemental Figure 7). In summary, these data are consistent with a model in which MAO-A is the dominant route of norepinephrine disposal in adipose tissue. No increased DHPG, in combination with increased norepinephrine turnover, suggests impaired clearance.

*Discussion*

The main findings of this study are that in male mice, circulating catecholamines are associated with increasing fat mass in both visceral and subcutaneous fat, with a disproportionate deposition of mesenteric fat. Male mice exhibit catecholamine resistance at level of transcription, lipolytic function (glycerol release), and phosphorylation in both mesenteric and subcutaneous fat. We also observed that norepinephrine turnover was elevated in these same tissues. We show that the tissue concentration of norepinephrine's major breakdown product, DHPG, is unchanged, despite increased turnover. These data suggest that impaired clearance of norepinephrine is a previously unrecognized factor in the regulation of sympathetic tone in adipose tissue

of high fat diet fed mice. We observed that elevation in tone is temporally coincident with catecholamine resistance. The solution proposed in this work, though paradoxical, is to re-sensitize adrenergic receptors and reverse catecholamine resistance by reducing norepinephrine turnover.

While neither circulating norepinephrine or epinephrine levels were elevated in female mice, female mice on high fat diet were catecholamine resistant. Subcutaneous adipose tissue more consistently exhibited catecholamine resistance compared to visceral mesenteric fat. We also show that female mice disproportionately accrue subcutaneous fat as compared to mesenteric fat, raising the possibility that catecholamine resistance may play a role in this difference. In this instance, catecholamine resistance could theoretically act as a protective factor, diverting excess energy to a less metabolically deleterious site (as compared to visceral fat). In humans, estrogen increases expression of the anti-lipolytic, $G_{\alpha i}$-coupled alpha2-adrenergic receptor in subcutaneous fat[33]. In animals, progesterone treatment induces lipolytic resistance to agonists such as forskolin, to a greater degree in subcutaneous fat than visceral fat[34]. While we did not assess circulating hormones in the present study, our forskolin data suggest that post-receptor mechanisms such as those observed under progesterone infusion do not account for differential catecholamine sensitivity in mesenteric and subcutaneous fat under high fat diet. Thus, our present results contribute to the body of literature examining mechanisms which could explain preferential expansion of subcutaneous fat in female mice and patients.

Conversely, in male mice, we showed a disproportionate allocation of fat to mesenteric depot. Despite this tendency, male mice exhibit catecholamine resistance at transcriptional, functional lipolytic (glycerol release), and protein phosphorylation levels

in both tissues. Thus, whereas differential sensitivity could drive the difference in fat deposition in female mice, our data argue against this mechanism for male mice. However, our studies were not powered to detect subtle differences between fat depots within male mice on high fat diet, and over time, such differences could theoretically produce the differential accrual of mesenteric fat observed in male mice. Future studies should address this gap.

A puzzling finding of this study is that mesenteric fat from female mice exhibits receptor downregulation at the transcriptional level but preserved signaling at the level of second messengers and protein phosphorylation. While divergence in transcriptional and protein abundance is not an uncommon finding, prior studies have shown good agreement for these endpoints for the β3-AR in adipose tissue, albeit in male animals[19]. These findings may reflect a female-specific, mesenteric-specific mechanism preserving receptor expression and/or coupling to downstream signaling effectors despite transcriptional downregulation. Notably, in studies using β3-AR specific agonists, female mice exhibited comparable beige adipocyte recruitment as compared to male mice in subcutaneous fat, but exhibited far greater sensitivity in gonadal fat[35], a visceral fat pad, illustrating the potential for sex and location based variance in adrenergic sensitivity. The complexity of these findings supports the need to focus greater attention on sex differences in metabolic disease, as the assumption that male physiology is replicated in females appears increasingly inappropriate.

In this study, neither circulating norepinephrine, nor epinephrine, was elevated in female mice. These data contribute preclinical context to the body of literature showing that female patients are protected against sympatho-excitation and associated complications, such as hypertension[36]. Do these results suggestive that catecholamine

signaling is dispensable for the development of catecholamine resistance in female patients and animals? Due to the complexity of catecholamine resistance phenotype in female mice, we did not perform additional tissue-based assessments of catecholamine homeostasis. However, it remains possible that local elevations of norepinephrine turnover could occur in the absence of changes to circulating catecholamines in females. Furthermore, the absence of changes in circulating catecholamines could point to an efficient norepinephrine clearance or reuptake mechanism which prevents spillover into blood of female mice, in contrast to the findings in male mice. Alternatively, elevations in turnover could be highly specific to adipose tissue, and consequently the spillover of excess norepinephrine could be diluted in blood against a background of relatively low norepinephrine turnover in other tissues. Additional studies to address these questions could include direct sampling of the venous drainage of adipose tissue, which would facilitate an understanding of whether turnover was upregulated in a specific and local manner. Alternatively, assessing, as we did in male mice, total tissue content of catecholamine degradation metabolites, would be valuable.

A major finding of this study is that norepinephrine turnover increases in the adipose tissue of male mice under high fat diet challenge under longer time courses than previously established. Numerous research groups over the last three decades have shown that hypercaloric dietary challenges elicit adipose tissue catecholamine resistance [1, 2, 19, 21]. However, to our knowledge, only one prior group has simultaneously assessed norepinephrine turnover and functional measurements of catecholamine resistance. In their short-term study, exposure to low protein, high carbohydrate diets for 2 weeks was sufficient to increase NETO by 50% and dramatically blunt the lipolytic response to norepinephrine in ex-vivo isolated adipocytes[14]. Our data suggest this could be a

consistent finding throughout longer exposures to hypercaloric diets. However, dietary challenges often provoke dynamic responses, such that findings which occur in early phases may not be maintained through longer exposures. Our data show that norepinephrine turnover and catecholamine resistance co-occur at 8 weeks of high fat diet, a common time point at which catecholamine resistance has been measured[1]. However, it remains to be seen whether norepinephrine turnover is still increased at other durations of high fat diet at which catecholamine resistance has been studied, such as 16 weeks[19, 21] or 24 weeks[18].

Increasing attention has been paid to the complexity of norepinephrine handling after its release from nerve terminals, resulting in the discovery of novel transporters[37, 38] and roles for resident immune cells[39] in the process of norepinephrine clearance, each of which could modulate lipolysis. Dihydroxyphenylglycol (DHPG) is a major metabolite of norepinephrine that is generated from the monoamine oxidase A pathway, which was previously believed to reflect exclusively neuronal breakdown of norepinephrine following reuptake in the nerve terminal[40]. It has become increasingly clear that adipocytes and resident macrophages express monoamine oxidases and play a key role in the regulation of adrenergic stimulation[38, 39, 41, 42]. To date, only one prior publication has examined the adipose tissue content of DHPG[43], a study performed in spontaneously hypertensive rats, and no data have examined DHPG in adipose tissue of humans or animals on high fat diet.

We made the novel observation that the tissue concentration of DHPG is unchanged under high diet, despite increased turnover. These data suggest that impaired clearance of norepinephrine is a previously unrecognized factor in the regulation of sympathetic tone in adipose tissue of high fat diet fed mice. A lack of change in DHPG,

coupled to significant alterations in norepinephrine turnover, may reflect impaired clearance. In other settings, such as heart failure, impaired clearance has been shown to cause excess norepinephrine spillover into blood, raising plasma levels of norepinephrine[44]. Additionally, impaired norepinephrine clearance may partly underlie the cardiac adrenergic desensitization observed in heart failure[26]. Our data suggest a comparable phenomenon could be occurring in adipose tissue under high fat diet.

An important limitation of this study is that normetanephrine, an additional breakdown product of norepinephrine, was not detectable. Measuring the tissue content of all catecholamine breakdown products, which commonly occur in the picogram range, as in this study, is an important future direction for research. Given the challenges of assessing low abundance metabolites in whole tissue preparations, an alternative approach would be to acutely culture tissue resident macrophages, which express MAO-A[39, 41] or adipocytes, which express norepinephrine transporters and MAO-A[37, 38, 45] from high fat diet fed mice, then stimulate them with radiolabeled norepinephrine. In such controlled conditions, isotopologues could be assessed, such as DHPG and normetanephrine, that could provide greater clarity on whether and how norepinephrine clearance is modulated under high fat diet exposure.

An important question raised by these findings is why, if norepinephrine turnover is increased, adipocytes from mice on high fat diet fail to show any of the metabolically favorable signatures usually associated with adrenergic stimulation, such as beiging[38]. The simplest explanation is that the amount of endogenous norepinephrine turnover, even if increased relative to normal diet fed mice, is not sufficient to stimulate the intracellular signaling which drives beiging. Put simply, catecholamine resistance may abrogate the effects of increased turnover in a vicious cycle. The solution proposed in this work,

though paradoxical, is to re-sensitize adrenergic receptors and reverse catecholamine resistance by reducing norepinephrine turnover. Alternatively, the coincident action of heterologous factor, such as low grade inflammation or hyperinsulinemia, may further inhibit the beiging action of catecholamines in adipose tissue under high fat diet challenge.

How do our findings regarding norepinephrine turnover comport with recent studies of catecholamine resistance focusing on low-grade inflammation as a stimulus driving downregulation of beta-adrenergic receptors[18, 19]? We note with interest than animal models of weight loss show even greater adipose tissue inflammation than actively high fat diet fed animals but show completely resolved catecholamine resistance[20]. Thus, we suggest that our research into alternative mechanisms driving catecholamine resistance fills an important gap left by these data.

If excess sympathetic drive, or impaired clearance, contributes to catecholamine resistance, increasing clearance or decreasing nerve activity should facilitate adrenergic re-sensitization. Numerous pharmacologic candidates exist for such an approach, whether central sympatholytics such as clonidine or moxonidine, or orphan drugs such as nepicastat, a dopamine-β-hydroxylase inhibitor which was clinically well-tolerated in initial trials for heart failure[46]. Indeed, some central sympatholytics have shown promise not only for alleviating hypertension, the primary indication for which they are prescribed, but also for improving insulin resistance[47-49], illustrating strong potential for drug repurposing.

The results of the current study are significant because they add to the body of literature showing that excess norepinephrine turnover occurs in adipose tissue and co-occurs with catecholamine resistance. This finding suggests that reducing norepinephrine

turnover could rescue catecholamine resistance, restore sympathetic control not only of lipolysis but also of the secretion of many adipokines and cytokines, and ultimately promote improvements in whole body metabolism.

## Acknowledgments

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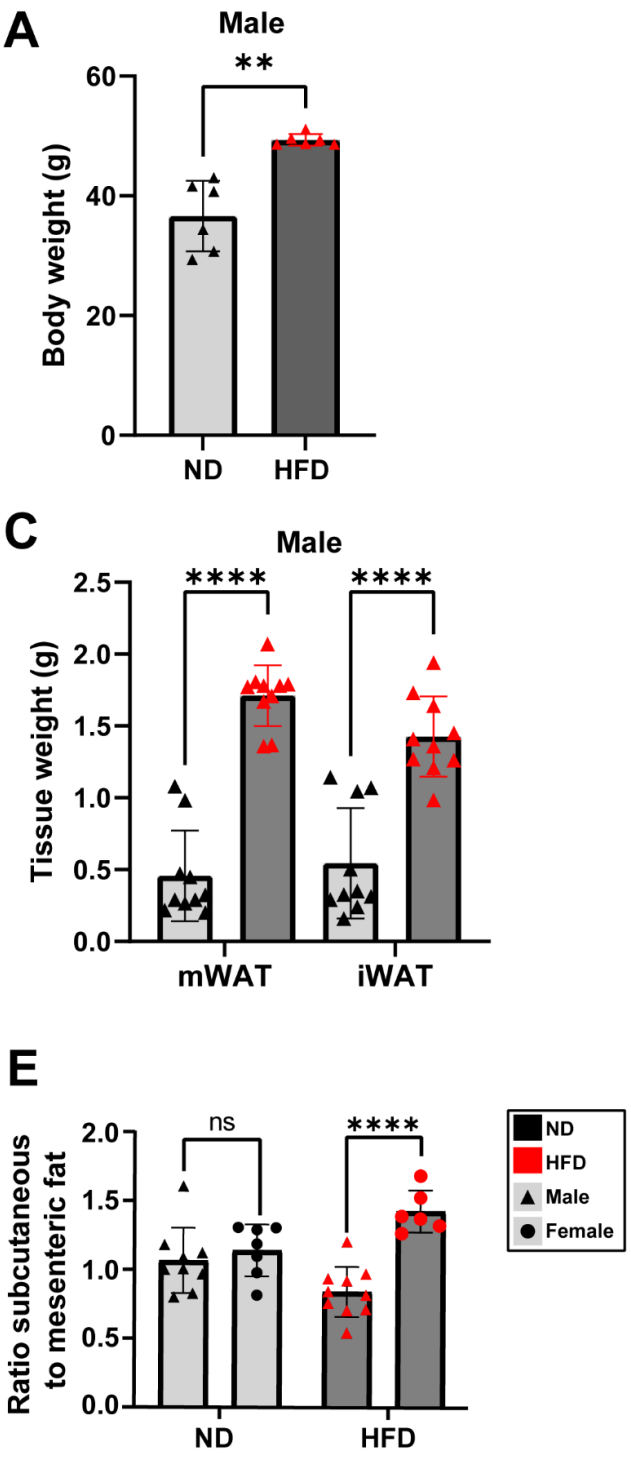


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Figure 1: High Fat Diet Differentially Impacts Weight Gain in Male and Female Mice
A) Body weights of male mice on eight weeks of 60% high fat diet and control diet. B) Body weights of female mice on 8 weeks of 60% HFD and control diet. C) Weight of inguinal subcutaneous and mesenteric fat in male mice on 8 weeks of high fat diet and control diet. D) Weight of inguinal subcutaneous and mesenteric fat in female mice on 8 weeks of high fat diet and control diet. E) Ratio of subcutaneous fat weight of mesenteric fat weight in males and females on control and high fat diet. mWAT mesenteric white adipose tissue; iWAT inguinal white adipose tissue; HFD high fat diet; ND normal diet; g grams. Each dot represents a single biological animal. Error bars represent standard deviation. *$p<0.05$; **$p<0.01$, ***$p<0.001$, ****$p<0.0001$. Welch's T-test for unequal variances (A,B); two-way ANOVA (B,C,D)

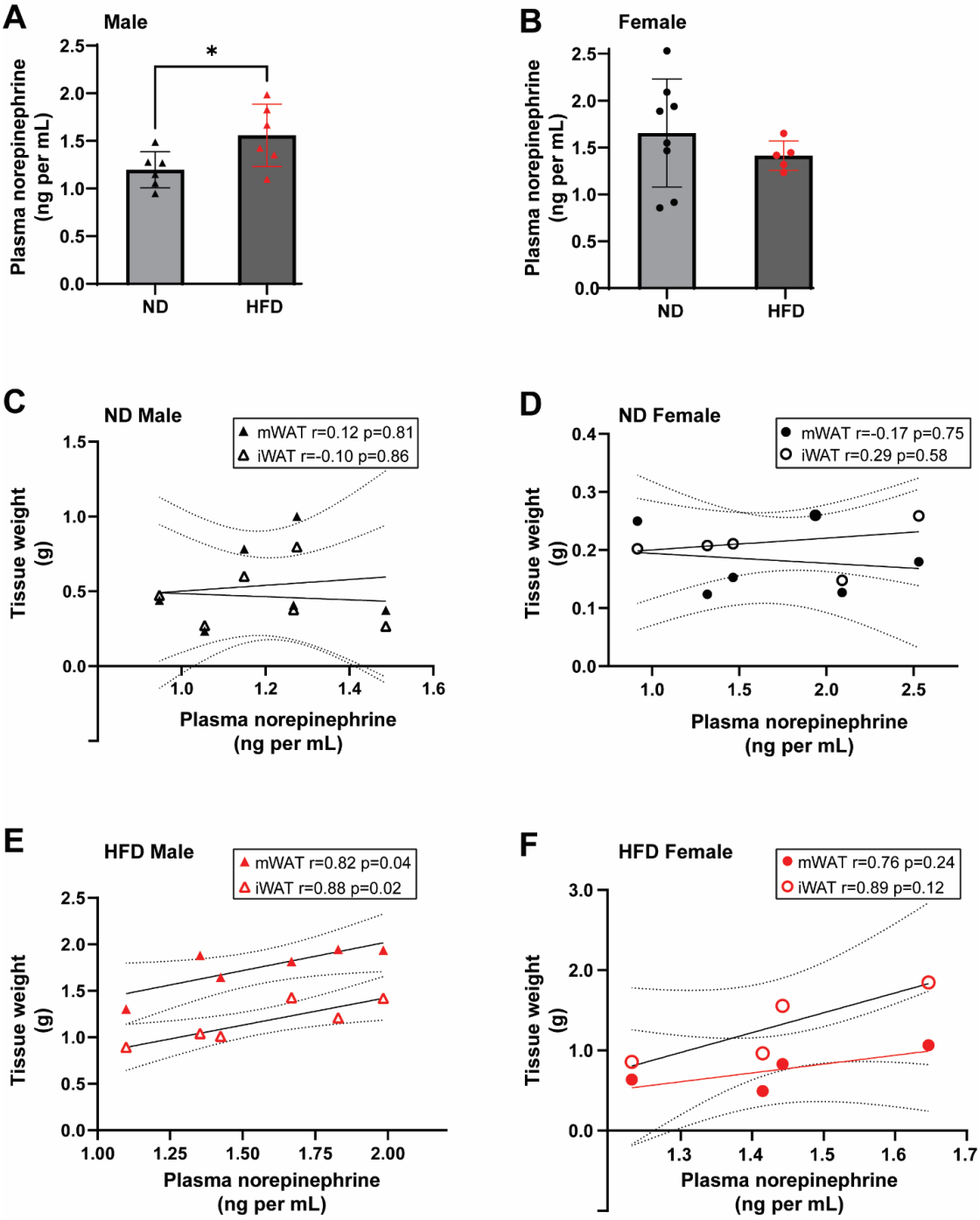



Figure 2: High fat diet differentially impacts circulating norepinephrine in male and female mice

A) and B) Norepinephrine levels in plasma of male mice and female mice, respectively, following 8 weeks of high fat diet and normal diet. C) and D) Pearson's correlation of plasma norepinephrine with tissue masses in normal diet fed male and female mice, respectively. E) and F) Pearson's correlation of plasma norepinephrine with tissue masses in high fat diet fed male and female mice, respectively. mWAT mesenteric white adipose

tissue; iWAT inguinal white adipose tissue; HFD high fat diet; ND normal diet; g grams. r=Pearson's correlation Each dot represents a single animal. Error bars represent standard deviation. *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001. Student *t* test (A); Pearson's correlation coefficient (C-E).

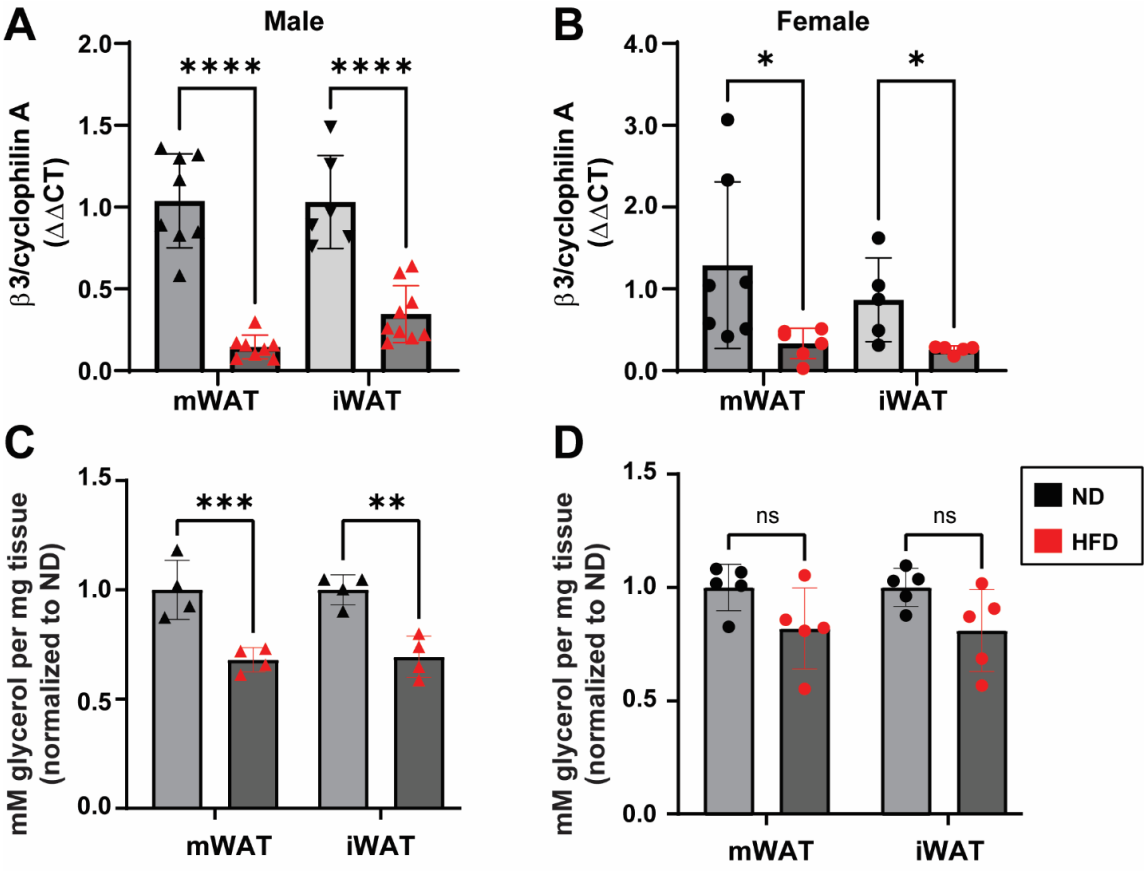



Figure 3: Transcriptional and functional measurements of adipose β3-AR under high fat diet. Real time quantitative PCR of β3-AR in subcutaneous and visceral fat of A) male and B) female mice following 8 weeks of high fat or control diet. Glycerol release stimulated by isoproterenol in subcutaneous and visceral fat of C) male and D) female mice following the same high fat diet challenge. mWAT mesenteric white adipose tissue; iWAT inguinal white adipose tissue; HFD high fat diet; ND normal diet; mM millimolar; ΔΔCT delta-delta cycle threshold. Each dot represents a single biological replicate. Error bars represent standard deviation. *p<0.05; **p<0.01, ***p<0.001, ****p<0.0001. Two way ANOVA (A-D).

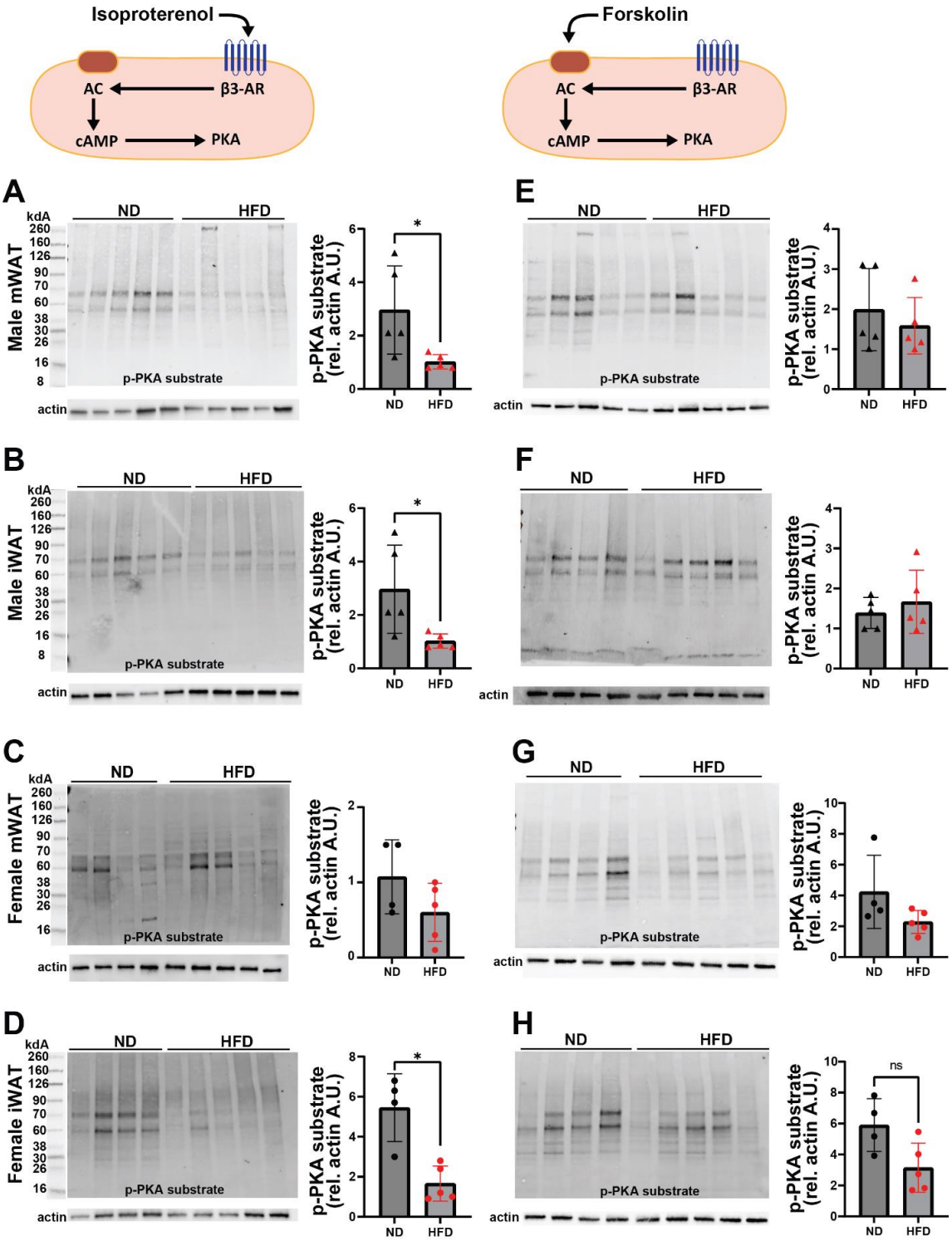



Figure 4: Bypassing B3-AR rescues downstream phosphorylation in adipose tissue from high fat diet fed male and female mice

Western blot targeting phosphorylated PKA substrate (RRXS*/T* consensus sequence in isoproterenol stimulated adipose tissue from high fat diet and normal diet fed mice.

Western blots and quantifications of A) male mesenteric adipose tissue and B) male inguinal adipose tissue quantified for the dominant bands at 60 and 70 kDa show differential phosphorylation, whereas C) female mesenteric adipose tissue exhibits non-significant differences in phosphorylation, but D) female inguinal adipose tissue shows defects in adrenergic mediated phosphorylation. Forskolin stimulation alleviates phosphorylation defects in E) male mesenteric adipose tissue and F) male inguinal adipose tissue. Non-significant differences are seen in G) female mesenteric adipose tissue and H) rescue of phosphorylation is observed in female subcutaneous adipose tissue. β3-AR beta-3 adrenergic receptor; mWAT mesenteric white adipose tissue; iWAT inguinal white adipose tissue; HFD high fat diet; ND normal diet; AC adenylyl cyclase; PKA protein kinase A; cAMP cyclic adenosine monophosphate; AU arbitrary units; rel relative. Each lane represents a unique animal. Error bars represent standard deviation. $p<0.05$; Mann Whitney *U* test (A-H).

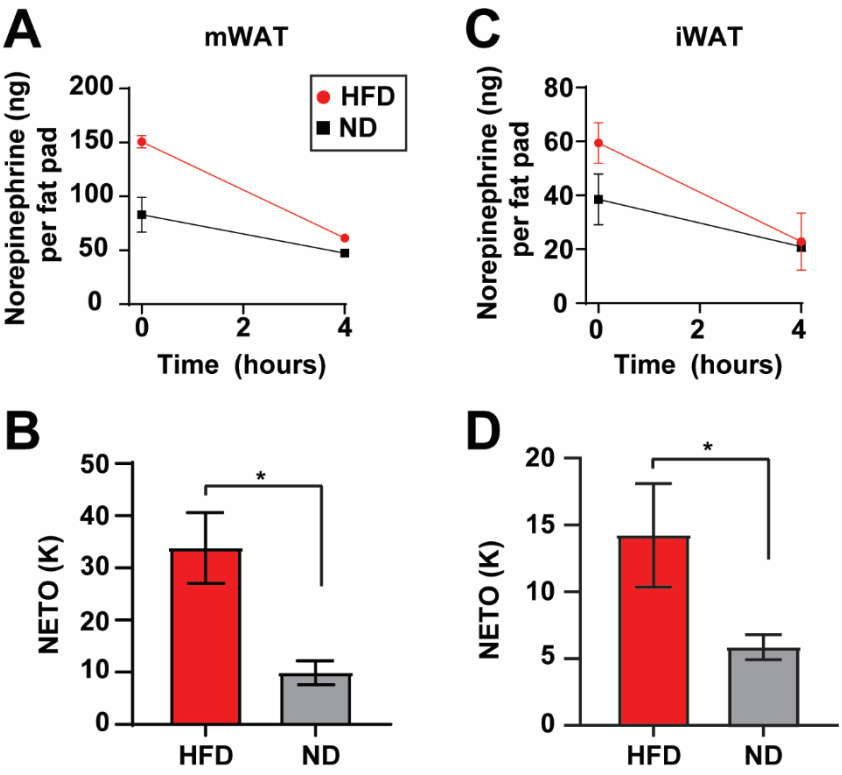



Figure 5: Adipose tissue norepinephrine turnover is elevated in high fat diet fed male mice.

A) Norepinephrine turnover and B) fractional turnover rate (k) in mesenteric and (C, D) subcutaneous adipose tissue in male mice high fat diet challenge assessed by mass spectroscopy of whole adipose tissue beds at baseline and following treatment with a-methyl-p-tyrosine. mWAT mesenteric white adipose tissue; iWAT inguinal white adipose tissue; HFD high fat diet; ND normal diet; NETO norepinephrine turnover. * $p<0.05$ Student's *t* test. Error bars represent standard deviation. n=6 to 8 per diet

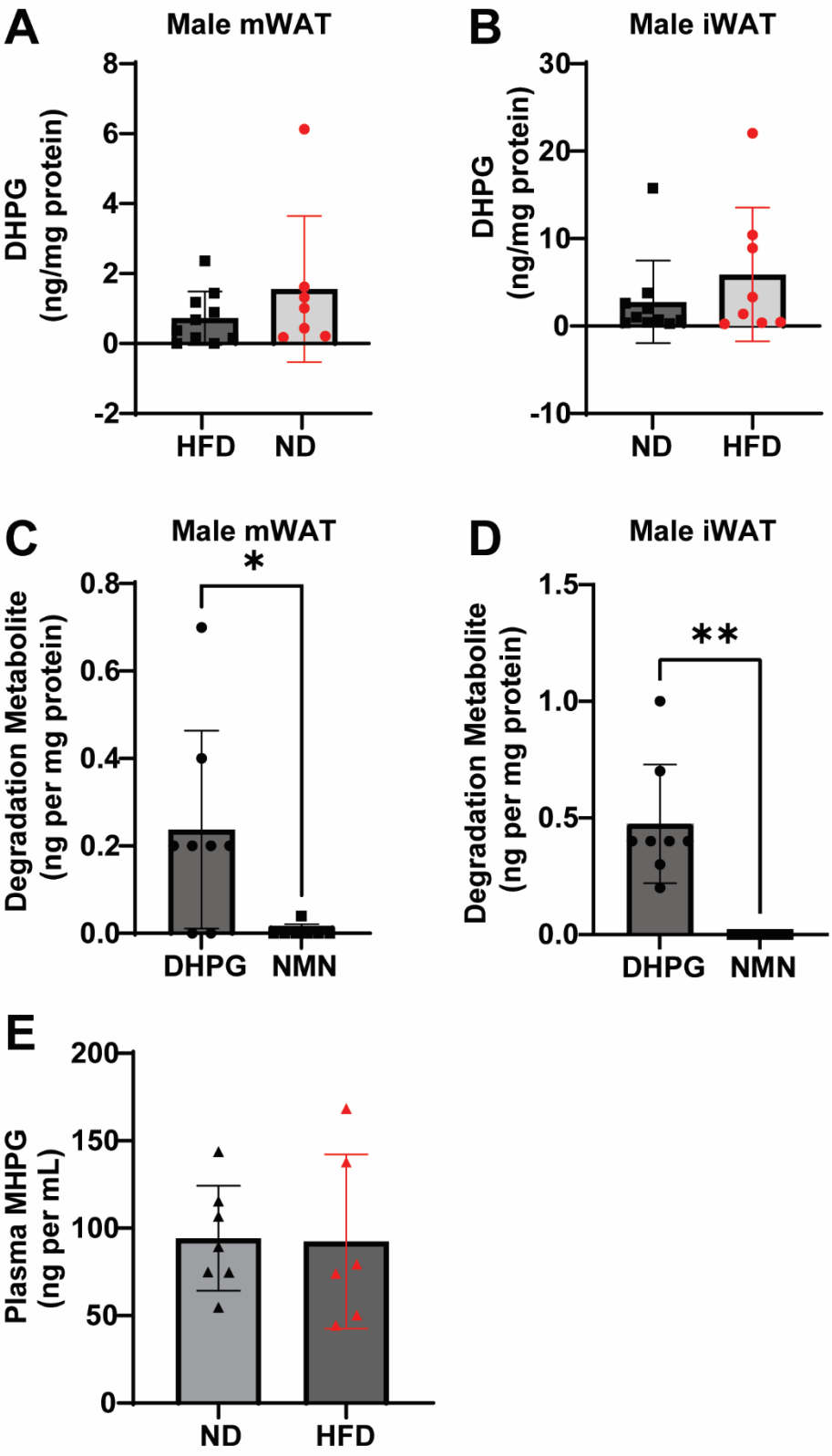
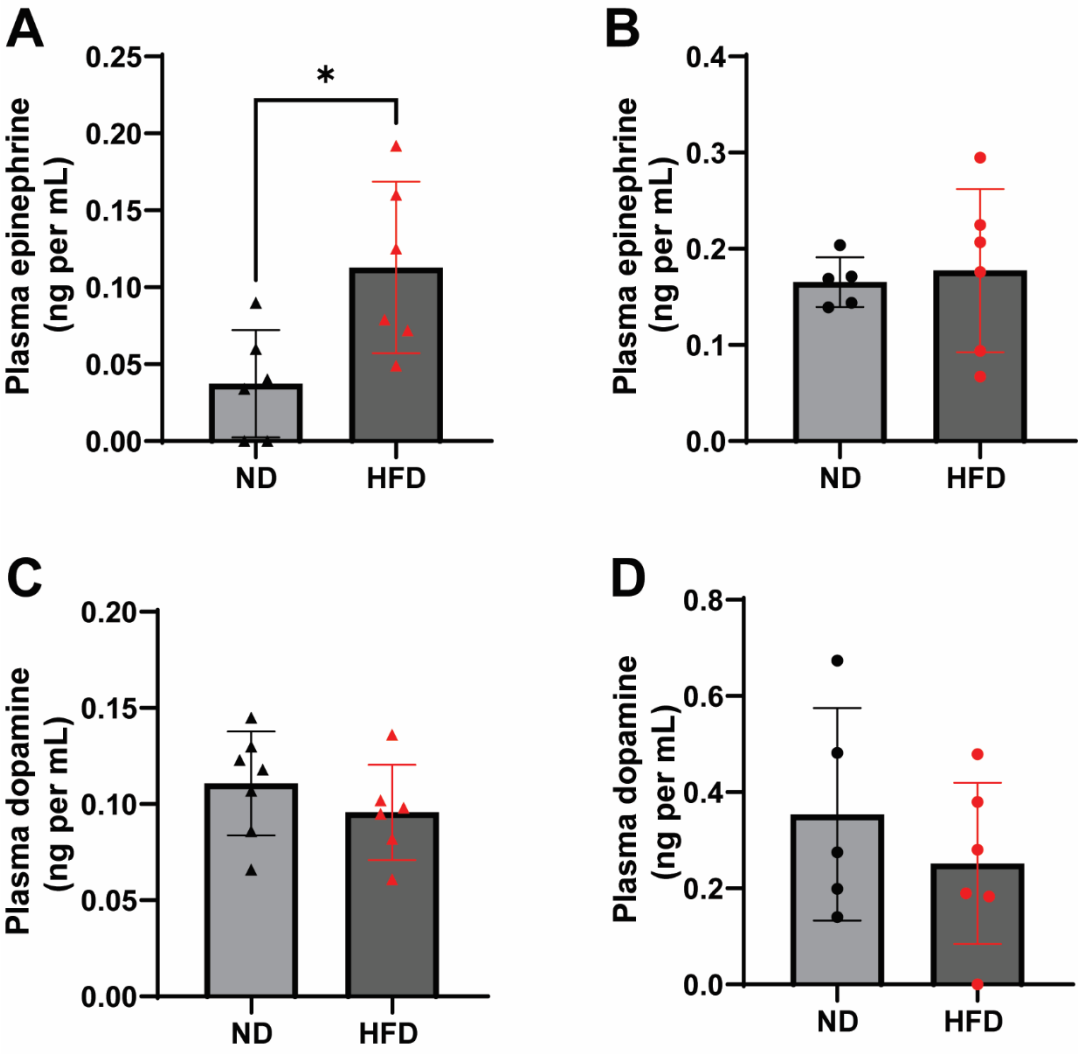
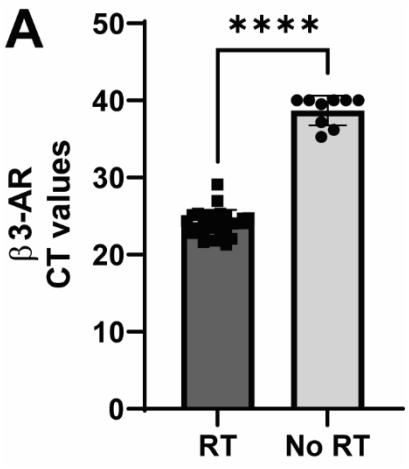



Figure 6: Tissue content of norepinephrine degradation products does not differ under high fat diet in male mice.

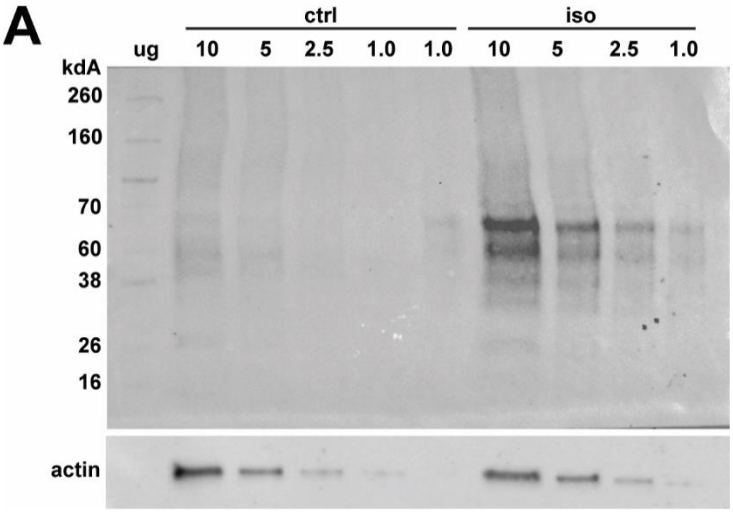
Tissue concentrations of dihydroxyphenylglycol (DHPG) in whole adipose tissue beds of A) mesenteric and B) subcutaneous fat following 8 weeks of high fat diet or control diet in male mice. C) Concentrations of DHPG compared to the catecholamine o-methyltransferase metabolite, normetanephrine, in C) mesenteric and D) subcutaneous fat from normal diet fed mice. E) Plasma content of the catecholamine o-methyltransferase metabolite, MHPG, in high fat diet and normal diet fed male mice. mWAT mesenteric white adipose tissue; iWAT inguinal white adipose tissue; HFD high fat diet; ND normal diet; DHPG dihydroxyphenylglycol; MPHG 3-Methoxy-4-Hydroxyphenylglycol; NMN normetanephrine. *$p<0.05$; **$p<0.01$, Mann Whitney *U* test. Each dot represents a single animal. Error bars represent standard deviation.



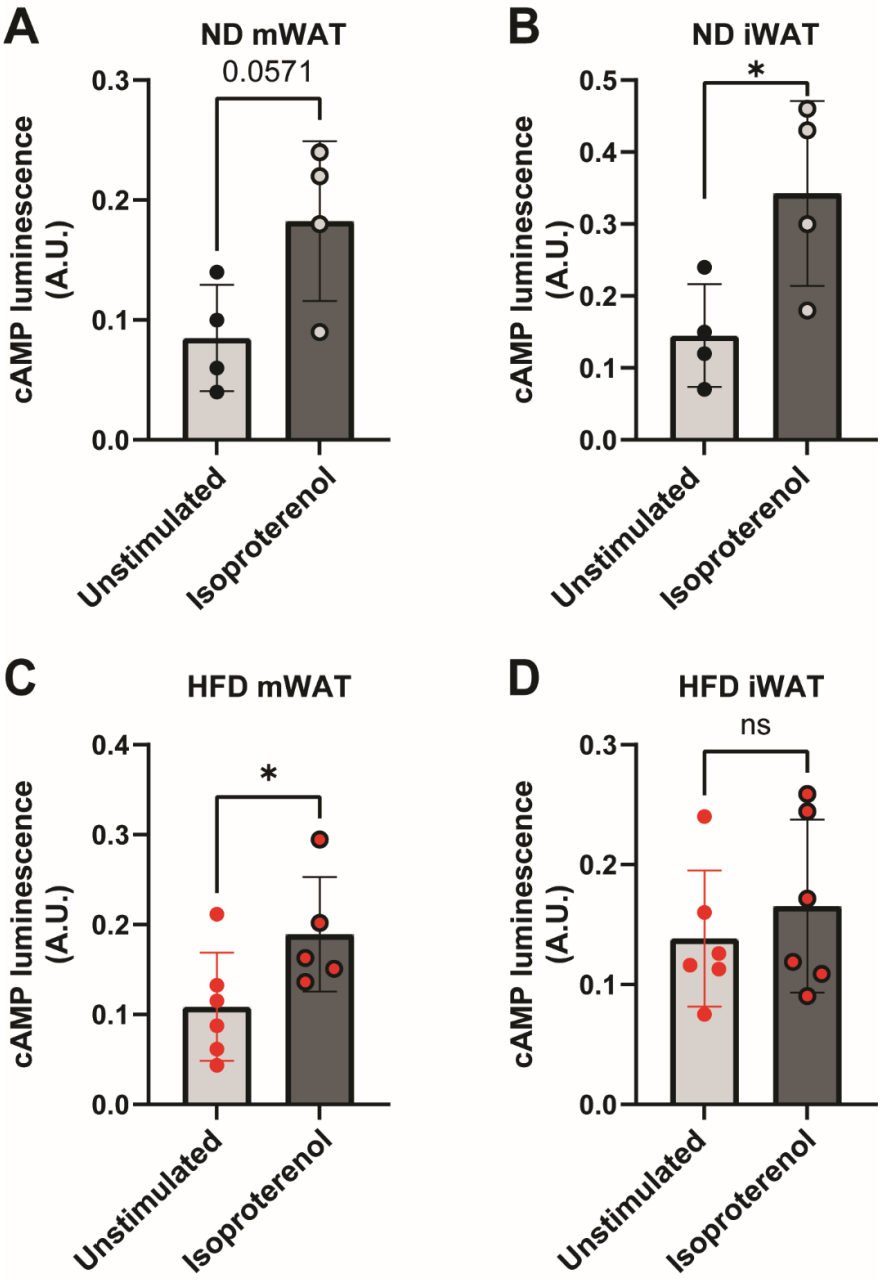

Supplemental Figure 1: Plasma epinephrine, but not its precursor, dopamine, is elevated in male, but not female mice under high fat diet challenge. Mass spectroscopy analysis of plasma content of epinephrine shows elevated levels in A) male but not B) female mice under high fat diet. Plasma content of dopamine in C) male and D) female mice under high fat diet does not differ as compared to normal diet controls. ng nanogram; mL milliliter; HFD high fat diet; ND normal diet; each dot represents a single biological replicate. *$p<0.05$ Mann Whitney *U* Test. Each dot represents a single animal. Error bars represent standard deviation.



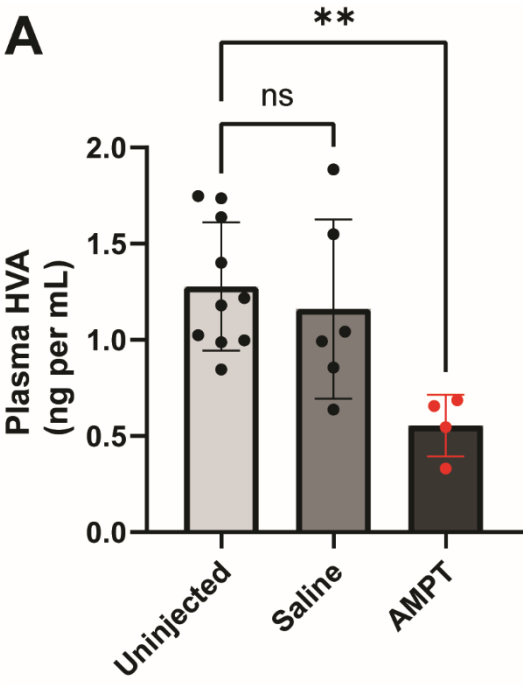

Supplemental Figure 2: No reverse transcriptase control for β3-AR qPCR data. A) Raw CT values of adipose qPCR data from studies performed with and without reverse transcriptase. ****$p<0.0001$. Student's *t*-test Each dot represents a single technical replicate. Error bars represent standard deviation.



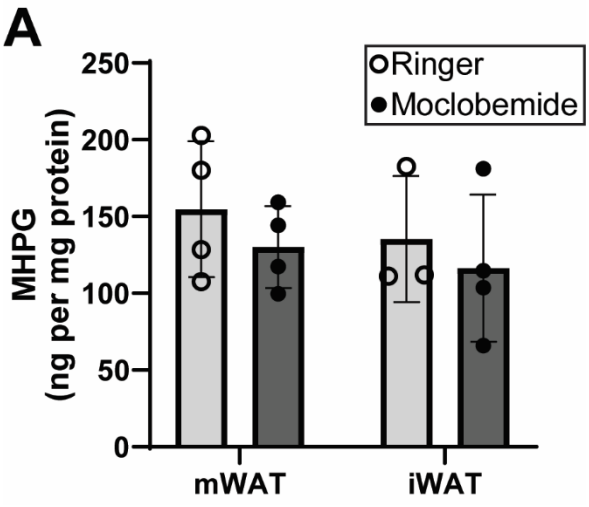

Supplemental Figure 3: Western blot validation. A) Western blot of phosphorylated protein kinase A substrates under control and isoproterenol stimulation following loading of a range of micrograms of total protein.




Supplemental Figure 4: Female mice exhibit preserved second messenger production in mesenteric but not subcutaneous fat under high fat diet challenge. cAMP mediated luminescence under unstimulated and isoproterenol stimulated conditions in single-cell suspensions of adipocytes from A) mesenteric fat and B) subcutaneous fat in normal diet fed female mice and C) mesenteric fat and D) subcutaneous fat in high fat diet fed female mice. *p<0.05; Mann Whitney *U* test. Each dot represents a single biological replicate. Error bars represent standard deviation.




Supplemental Figure 5: Specificity of a-methyl-p-tyrosine inhibition. A) Plasma homovanillic acid, a dopamine metabolite, does not differ between uninjected and saline injected normal diet fed mice, but is depleted by a-methyl-p-tyrosine treatment. AMPT a-methyl-p-tyrosine; HVA homovanillic acid; ns not significant. **p<0.01. One way ANOVA. Each dot represents a single animal. Error bars represent standard deviation.




Supplemental Figure 6: Tissue content of MPHG, the catecholamine O-methyltransferase metabolite, is not impacted by monoamine oxidase inhibition A) MHPG levels in mesenteric and subcutaneous fat following moclobemide or ringer saline injection. MHPG 3-Methoxy-4-Hydroxyphenylglycol; mWAT mesenteric white adipose tissue; iWAT inguinal white adipose tissue. Each dot represents a single biological replicate. Error bars represent standard deviation.

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# DISCUSSION

## *Catecholamine Resistance: from Circuits to Catecholamine Metabolism*

The work presented herein makes several major contributions to the understanding of sympathetic nerves and catecholamine resistance. Prior literature largely fails to account for the endogenous functioning of sympathetic nerves in adipose tissue in metabolic syndrome, focusing instead on the role of low-grade inflammation in blunting adipocyte lipolytic responses to catecholamines[99, 197]. My work examines sympathetic nerves from a circuit point of view in Chapter 2, showing for the first time that catecholamine resistance can be induced by manipulation of brain nuclei which regulate sympathetic nerve activity. I went on to show that the adipose tissue depots most impacted by this manipulation were enriched for norepinephrine. These studies also provided valuable negative data, demonstrating that catecholamine resistance could be induced in the absence of hyperinsulinemia. In Chapter 3, I extend these findings, showing that circulating catecholamines and tissue norepinephrine turnover are associated with the expansion of adipose tissue and development of catecholamine resistance in high fat diet fed male animals. I also showed that norepinephrine turnover is not linked to increased norepinephrine degradation via the monoamine oxidase pathway. These data support the novel prediction that impaired norepinephrine clearance could contribute to the development of catecholamine resistance. I also contribute valuable negative data, demonstrating that mesenteric fat from female mice remains functionally catecholamine sensitive, whereas subcutaneous adipose tissue from female mice is

preferentially expanded and catecholamine resistant. No prior investigation has defined the female catecholamine resistance phenotype under high fat diet. Each of these contributions is discussed at greater length in the following sections.

### *Targeting Paraventricular Somatostatinergic Neurons Yields Novel Insights into Central Control of Catecholamine Resistance*

Chapter 2 of this work defines a potentially novel therapeutic target in the upstream control of catecholamine resistance: somatostatinergic neurons in the paraventricular nucleus (PVN) of the hypothalamus. This target was derived from significant extant literature indicating that somatostatin was a key regulator of neuroinflammation[152, 153, 225, 226] as well as sympathetic tone[131, 133-135, 149, 227, 228]. Importantly, prior data from our laboratory also indicated that somatostatin immunoreactivity was reduced in the PVN of BBZ/Wor rats[55], a polygenic model of type 2 diabetes, suggesting this endogenous anti-inflammatory, sympatholytic pathway could be abrogated in the disease state. Hypothalamic inflammation has been repeatedly shown to increase sympathetic nerve activity, particularly in the paraventricular nucleus[46, 48-50, 229]. These data converged with the overarching hypothesis of the dissertation because chronically increased sympathetic signaling could cause catecholamine resistance, just as chronic stimulation with exogenous catecholamines or pharmacologic agonists elicits adipose catecholamine resistance in humans[209], canines[210], rodents[207], and adipocytes in cell culture[197].

Chapter 2 utilized a loss of function approach in which PVN SST neurons were selectively depleted via diphtheria toxin (SST-DTA). I studied the effect of this loss on the local inflammatory milieu of the PVN, as well as downstream effects on adipose

tissue lipolysis, expression of adrenergic receptors, and circulating non-esterified free fatty acids. The three key hypotheses of this project were 1) that the loss of hypothalamic somatostatin is a common feature of animal models of metabolic disease 2) that diphtheria toxin induced ablation of hypothalamic SST neurons (SST-DTA) would result in local neuroinflammation in the paraventricular nucleus 2) that SST-DTA mice would exhibit adipose catecholamine resistance through altered sympathetic nerve function.

To test hypothesis 1, I utilized an immunohistochemical approach and found that in leptin receptor deficient db/db mice, PVN SST immunoreactivity was also reduced, as in BBZ/Wor rats. Thus, I confirmed that this is a general finding across multiple animal models of T2D and MetS, albeit both genetic, not dietary. To test hypothesis 2, I examined astrocyte and microglial density in the PVN of SST-DTA mice. I observed reactive astrocytosis and microgliosis, consistent with in a neuroinflammatory state, as had previously been identified in db/db mice[230]. These data supported the first two hypotheses of Chapter 2: that SST neurons are an endogenous anti-inflammatory population in the PVN which is lost in states of metabolic disease. To assess hypothesis 3, that SST loss would result in catecholamine resistance in a sympathetic nerve dependent manner, I assessed catecholamine resistance across two different adipose tissue beds: mesenteric and subcutaneous adipose tissue. Mesenteric adipose tissue was found to contain two to three folder greater static concentrations of norepinephrine than subcutaneous adipose tissue. These data add to existing literature in which mesenteric adipose tissue is strongly neuroanatomically connected to the PVN via viral nerve tracing studies[231] and exhibits significantly greater increases in norepinephrine turnover under central leptin infusion[221], indicating that there may be a functionally specialized pathway connecting mesenteric fat to the PVN. Thus, these two tissues were good test

cases for the theory that loss of SST neurons might differentially impact adipose tissue by virtue of its sympathetic innervation. Remarkably, adipose tissue explants from these tissues in SST-DTA mice exhibited different degrees of lipolysis as compared to tissue from control mice. Subcutaneous adipose tissue from SST-DTA mice was as catecholamine sensitive as subcutaneous fat from control mice, whereas mesenteric adipose tissue demonstrated catecholamine resistance, providing significant support for our hypothesis.

In an attempt to generate an in-vivo corollary to ex-vivo studies of lipolysis, serum non-esterified free fatty acids (NEFA) were assessed in SST-DTA and control mice over several months following the depletion of SST neurons. These data showed an interesting pattern in which NEFA were elevated at one-month post-depletion in SST-DTA mice, not significantly different at 4 months post-depletion, and reduced at 8 months post-depletion. These results may reflect dynamic changes in the catecholamine sensitivity of adipose tissue. For example, at the 1-month time point, increased sympathetic tone onto relatively catecholamine sensitive adipose tissue may have caused the increased NEFA I observed. At four months post-depletion, if sympathetic tone remained chronically elevated, the tissue may have become catecholamine resistant, and the same increased tone would elicit less lipolysis and subsequent NEFA release. This explanation matches our observation that mesenteric adipose tissue explants from SST-DTA mice underwent less lipolysis than that of control mice. Alternatively, the amount of sympathetic drive may have changed over the time-course of SST-depletion, such that initially increased tone eventually subsided, resulting in a relative reduction in lipolysis and resultantly lower NEFA levels over time. However, such an explanation does not

account catecholamine resistance seen in mesenteric explants at the 8-month timepoint, making it less probable.

It is important to note that in MetS, free fatty acids are consistently elevated, albeit with circadian variation [92], and do not show the variation we observed in the SST-DTA model. (All SST-DTA samples were taken at the same point in the circadian cycle). An additional factor to consider in this regard is that as adipose tissue becomes catecholamine resistant, basal lipolysis, the steady, spontaneous leak of free fatty acids, typically increases in MetS, and contributes significantly to circulating NEFA levels[88]. Increased basal lipolysis is clinically linked to hyperinsulinemia[88], and SST-DTA mice were not hyperinsulinemic. Thus, the variation of NEFA we observed likely reflects the effect of isolated catecholamine resistance in the absence of hyperinsulinemia.

As an additional endpoint, β3-AR mRNA was assessed in mesenteric and subcutaneous adipose tissue from SST-DTA and control mice, under the prediction that mesenteric fat would show transcriptional downregulation. Such data would match the functional deficit in lipolysis. Puzzlingly, mesenteric adipose tissue from SST-DTA mice did not exhibit transcriptional downregulation of β3-AR. In contrast, adipose tissue from db/db mice exhibited both functional catecholamine resistance and receptor downregulation. Two technical explanations for this discrepancy are worth considering: 1) transcriptional and protein abundances are not always tightly correlated, though prior data show good relationships between these endpoints for β3-AR[197]; 2) immune cell infiltration into adipose tissue may skew these data, as hypoxia has been shown to upregulate β3-AR on immune cells[232] and large changes in circulating immune cells (specifically, $CCR2^{hi}$ monocytes) were observed in the SST-DTA model. In this sense, an

important limitation of Chapter 2 was the absence of adipose tissue flow cytometry to assess whether infiltrating immune populations mirrored changes in circulating immune cells and if the infiltrating cells were β3-AR positive. Alternatively, bulk RNAseq or qPCR of purified adipocyte populations would have improved the rigor of this study by eliminating any signal from resident immune and other stromal vascular cells.

What broader mechanistic insights can be made into catecholamine resistance by comparing findings from db/db and SST-DTA models? Both mesenteric and subcutaneous adipose tissue from db/db mice exhibited catecholamine resistance, whereas only mesenteric fat from SST-DTA mice was catecholamine resistant. This may reflect distinct causes of catecholamine resistance in visceral and subcutaneous fat, such that visceral fat catecholamine resistance is driven by excess sympathetic outflow, whereas subcutaneous adipose tissue catecholamine resistance is caused by other factors.

Insulin signaling could explain the divergence in phenotypes between SST-DTA mice and db/db mice in subcutaneous fat. SST-DTA mice did not exhibit changes in plasma insulin, whereas hyperinsulinemia is an early and sustained feature of the db/db model[233]. Insulin treatment in-vitro drives downregulation of adrenergic receptor expression in adipocytes[234]. Notably, subcutaneous fat is more insulin sensitive than mesenteric fat[97], which provides an additional mechanistic basis for the divergence in phenotypes between visceral and subcutaneous fat being potentially related to insulin signaling.

Leptin signaling may likewise drive differences between the db/db model and the SST-DTA model. db/db mice are genetically leptin resistant, and leptin drives sympathetic nervous system activation[41, 43, 221] which is itself pro-lipolytic, thus linking changes in leptin to lipolytic function. Leptin secretion, plasma levels, and

functional measurements of leptin signaling, such as Stat-3 phosphorylation after leptin treatment, were not examined in SST-DTA adipose tissue or hypothalamic preparations, limiting insight from this line of inquiry. Importantly, somatostatin signaling has previously been shown to oppose leptin signaling in various hypothalamic nuclei including the PVN[150], and central leptin infusions disproportionately drive norepinephrine turnover in mesenteric fat[221]. Thus, it is possible that SST-DTA mice exhibited mesenteric catecholamine resistance due to relatively unopposed central leptin action. However, this hypothesis would require that catecholamine resistance in db/db mesenteric fat have a separate mechanism than that of SST-DTA mice, as db/db mice are genetically deficient in the long form leptin receptor[235].

Finally, a key limitation of Chapter 2 is that norepinephrine turnover, a functional measurement which reflects the net effect of sympathetic nerve release of norepinephrine and norepinephrine clearance, was not assessed. The approach taken to predict which adipose tissue beds would exhibit catecholamine resistance was based on static, or baseline, catecholamine levels. Thus, in Chapter 3 of my dissertation, a far more detailed assessment of norepinephrine homeostasis was undertaken. Rather than pursue these studies in a complex model reliant on neurosurgery to deliver viral vectors to the PVN, I elected to utilize a simpler and more common model: high fat diet exposure.

### *Increased Norepinephrine Turnover Without Increased Degradation Under High Fat Diet*

In Chapter 3, I tested the hypothesis that adipose tissue catecholamine resistance elicited by 8 weeks of high fat diet is associated with increased circulating and tissue catecholamines. I also define sex differences in catecholamine resistance which

correspond to sexually dimorphic fat deposition patterns, for the first time. Endogenous catecholamine flux, measured by norepinephrine turnover, is increased under hypercaloric diets[183, 184]. These studies were exclusively conducted using very brief dietary exposures, ranging from 1 to 3 weeks. Additionally, norepinephrine turnover is controlled not only by changes in sympathetic nerve activity, but also by modulation of the rate of reuptake and clearance of norepinephrine following its release[165], variables not accounted for in the existing literature on adipose tissue under high fat diet. Thus, in Chapter 3, I phenotype circulating catecholamines, adipose tissue catecholamine resistance, adipose tissue norepinephrine turnover, and norepinephrine degradation in adipose tissue. This study fills several crucial gaps in the study of catecholamine resistance: 1) All prior preclinical studies of catecholamine resistance have been performed in male mice[4, 17, 22, 23, 49, 55], 2) Only one prior study has simultaneously assessed the correlation of a catecholamine endpoint concurrently with functional measurements of catecholamine resistance[184] 3) Only one prior study has examined the neurochemistry of norepinephrine degradation in adipose tissue, in a spontaneously hypertensive rodent model[41], despite ample interest in targeting the sympathetic-adipose tissue interface for the treatment of metabolic disease.

Male mice on 8 weeks of high fat diet increased total body weight and adipose tissue weight, with disproportionate expansion of mesenteric fat as compared to subcutaneous fat. In addition, adipose tissue explants showed functional resistance to catecholamine stimulation, as measured by media glycerol following stimulation with isoproterenol. Findings of catecholamine resistance were further supported by decreased transcription of the dominant rodent lipolytic receptor, β3-AR, as well as impaired phosphorylation downstream of adrenergic stimulation. These data suggested that male

mice on high fat diet exhibited catecholamine resistance, making them a fruitful model in which to carefully characterize adipose tissue catecholamine biochemistry.

To test the hypothesis that norepinephrine turnover was increased in adipose tissue of male mice on high fat diet, I first utilized the classic synthesis inhibition approach using a-methyl-p-tyrosine (AMPT). Our data show that norepinephrine turnover comparably increases in subcutaneous and mesenteric fat under prolonged dietary stimuli (8 weeks of high fat diet). These data contribute to the literature showing that norepinephrine turnover increases in hypercaloric conditions. However, measurement of changes in norepinephrine content under synthesis inhibition does not, on its own, illuminate which processes drive increased NETO (i.e. increased nerve firing or decreased neurotransmitter reuptake and degradation). Thus, assessment of norepinephrine degradation metabolites was undertaken, to test the hypothesis that chronically impaired norepinephrine clearance was associated with catecholamine resistance.

Measurements of tissue dihydroxyphenylglycol (DHPG), a major degradation metabolite produced by the monoamine oxidase A pathway (MAO-A) did not differ between high fat diet and control fed mice, in either mesenteric or subcutaneous fat. A lack of change in DHPG, while norepinephrine turnover was significantly elevated, suggests the presence of an impairment in norepinephrine clearance. To gain further traction on this question, I attempted to define the tissue content of normetanephrine, the norepinephrine degradation metabolite produced through the catecholamine-o-methyltransferase (COMT) pathway. Unfortunately, normetanephrine levels were undetectable in my tissue preparations. Inhibition of MAO-A using moclobemide permitted detection of normetanephrine, suggesting that I had the technical capacity to

detect it, but that at steady state conditions, it is of very low abundance. I also measured the COMT metabolite MHPG, which is derived from DHPG, and showed that it was unchanged, increasing my confidence that COMT is not a dominant pathway for norepinephrine disposal in adipose tissue. However, it is possible that norepinephrine is not adequately cleared by MAO-A and is instead being cleared by increased flux through the COMT pathway. This scenario makes the prediction that tissue normetanephrine should be elevated in adipose tissue under high fat diet challenge. Alternatively, there may be a comparable lack of increase in normetanephrine, which would suggest that norepinephrine is being inadequately cleared. Impaired clearance could result in overstimulation of adrenergic receptors and could contribute to catecholamine resistance, in keeping with my overarching hypothesis.

These results raise important questions about how high fat diet (and potentially other hypercaloric challenges) change tissue handling of norepinephrine, an increasing focus of recent literature on sympathetic nerve function in adipose tissue. Specifically, both adipocytes and macrophages have been shown to produce MAO-A and COMT, as well as norepinephrine transporters such as Slc6a2 and Oct3[166]. However, the current literature investigates the effect of *manipulating* these transporters and enzymes, not phenotyping their baseline function under dietary challenge. Manipulations which decrease norepinephrine clearance by blunting macrophage norepinephrine uptake have many metabolic benefits: increased lipolysis, increased thermogenesis, and weight loss in *ob/ob* (leptin deficient) mice[167]. Such manipulations also reduce weight gain in high fat diet fed mice[167]. Ablation of adipocyte machinery for norepinephrine uptake, such as the *Oct3* norepinephrine transporter, likewise increases thermogenesis in response to cold challenge in high fat diet fed (male) mice, as well as increased free fatty acid release,

indicating increased lipolysis[166]. Importantly, neither of the above studies examine the *basal* phenotype of norepinephrine handling in high fat diet fed (or *ob/ob*) mice in either the macrophage or adipocyte compartment. Specifically, tissue content of normetanephrine or DHPG were not assessed in these studies, nor was the biochemical fate of infused norepinephrine tracers assessed. Thus, the above studies comport with the broader literature showing that increasing adrenergic signaling (in this case, by altering clearance) has major metabolic benefits, but do not provide insight into the baseline functioning of norepinephrine reuptake or clearance under dietary challenge. My studies fill this gap by contributing the first ever characterization of adipose tissue DHPG under dietary challenge.

Utilizing an ex-vivo approach to assess norepinephrine handling in adipose tissue under high fat diet may help resolve important questions about how high fat diet alters the clearance of norepinephrine. Isolating adipocytes and macrophages from adipose tissue and acutely treating them with radiolabeled norepinephrine would permit a rigorous assessment of the isotopologues generated by these differing cell populations under control and high fat diet conditions. Indeed, recent advances have made it possible to culture primary adipocytes for days to weeks[236], and as such, this approach would also allow for chronic treatment with norepinephrine, followed by acute treatment with radiolabeled norepinephrine, to assess the effect of chronic stimulation on norepinephrine handling. The results of my whole tissue preparations predict that degradation via MAO-A would be impaired under this research design as well.

*Sex Differences in Catecholamine Resistance*

Studies of catecholamine resistance[100, 197] and norepinephrine turnover[183, 184] have exclusively focused on male mice and rats. However, increasing focus on sex as a biological variable is necessary, as distinct mechanisms may be at play in the development of metabolic disease. Indeed, rising rates of MetS and its various components are not distributed equivalently across the population. Overall, men are more likely to have Type 2 diabetes (T2D) than premenopausal women, but multiple prospective studies in both the United States[237] (Bogalusa Heart Study) and Middle East (Tehran Lipid and Glucose Study)[238] have demonstrated that women who go on to develop T2D carry greater risk profiles (elevated blood pressure, reduced HDL) than men who go on to develop T2D. Studies in Europe[239] show that men with impaired glucose homeostasis tend accrue more visceral adiposity than women. In summary, studies across multiple ecological and cultural contexts suggest that, while core risk factors are the same, MetS has different developmental trajectories in men and women, some of which directly implicate adipose tissue function.

Studies of catecholamine resistance may have examined male mice in part due to their exaggerated weight gain compared to female mice. The bias towards male mice leads to a notable mismatch between clinical and preclinical literature, as clinical literature has examined adipose tissue biopsies from female patients and clearly shows that female patients exhibit catecholamine resistance in subcutaneous fat[101, 195], a finding that was not reflected in preclinical literature until the studies within this dissertation were undertaken.

In women, in contrast to men, BMI is not correlated with indices of sympathetic nerve activity such as muscle sympathetic nerve firing rates[240]. However, sympathetic

activity in adipose tissue could differ from that observed in skeletal muscle. Also, as established throughout this work, catecholamine flux is regulated not only by nerve firing but also by norepinephrine clearance. Thus, an important future direction is to ascertain whether norepinephrine turnover increases in adipose tissue of female mice on high fat diet, and to determine whether comparable changes are seen in the degradation pathway of norepinephrine. In the present studies, I observed that female catecholamine resistance occurs without elevations in plasma norepinephrine. Thus, it remains possible that catecholamine resistance in female mice is not related to increased turnover and has entirely separate mechanism as compared to catecholamine resistance in males, and future studies should determine whether or not this is the case.

Finally, catecholamine resistance is largely conceptualized as a pathologic phenomenon. My studies in female mice raise an interesting alternative viewpoint, in which selective catecholamine resistance in subcutaneous fat could help to divert storage away from visceral, mesenteric fat. Indeed, I make this observation in this preclinical data, showing that female mice disproportionately store subcutaneous fat. Future studies should measure the effect of selectively inducing catecholamine resistance in female mesenteric fat, or selectively relieving catecholamine resistance in female subcutaneous fat, to discern the mechanistic contribution of resistance in these differing sites to whole body metabolism.

*Synthesizing Insights into Catecholamine Resistance Across Animal Models*

The work in this dissertation examines catecholamine resistance in four animal models: db/db mice, SST-DTA mice, and high fat diet fed male and female mice. The following paragraphs review insights into catecholamine resistance, catecholamines, and

the central nervous system in these models, with particular attention to how Chapter 2's focus on somatostatinergic neurons could inform future investigations into the high fat diet model studied in Chapter 3.

In each animal model, catecholamine resistance was measured in mesenteric and subcutaneous fat across multiple endpoints. Lipolytic responses to isoproterenol were concurrently investigated with transcriptional abundance of the β3-AR. db/db mice exhibited impaired lipolytic responses to isoproterenol as well as reduced β3-AR transcript levels in mesenteric and subcutaneous fat. In contrast, SST-DTA mice exhibited impaired lipolytic responses to isoproterenol in mesenteric fat, but not subcutaneous fat, and β3-AR was not transcriptionally downregulated in mesenteric or subcutaneous fat of SST-DTA mice. Finally, impaired lipolytic responses and receptor transcriptional downregulation were observed in male mice on high fat diet, in both mesenteric and subcutaneous fat. Male high fat diet fed mice also showed impaired protein phosphorylation following isoproterenol treatment, again in both tissues. In female mice, transcriptional downregulation diverged from second messenger and protein phosphorylation measurements of adrenergic signaling in mesenteric fat, which appears to be catecholamine sensitive, in contrast to subcutaneous fat in female mice. Collectively, these data indicate that catecholamine resistance occurs in both visceral and subcutaneous fat in db/db males and high fat diet male mice. SST-DTA and female high fat diet fed mice show opposing patterns of catecholamine resistance, with SST-DTA mice showing specific mesenteric resistance, and female HFD mice showing specific mesenteric sensitivity. These results suggest that catecholamine resistance does not

necessarily arise via common mechanisms across all adipose tissue depots, a novel insight.

The approaches examining catecholamines in each chapter differ substantially. In Chapter 2, I made the observation that static catecholamines are elevated in mesenteric fat, as compared to subcutaneous fat, but norepinephrine turnover studies and degradation studies were not performed. In Chapter 3, norepinephrine turnover studies were performed concurrently with studies of norepinephrine degradation metabolites. These studies raised the possibility that impaired degradation of norepinephrine could lead to chronic receptor stimulation in males on high fat diet. In female high fat diet fed mice, neither circulating norepinephrine, nor epinephrine, were elevated.

Finally, while the central nervous system phenotype of SST-DTA and db/db mice was studied, no such studies were performed on high fat diet fed mice. My studies raise the possibility that somatostatinergic neurons are comparably depleted in high fat diet fed mice, as in db/db and BBZ/Wor rats. Such loss could contribute to the neuroinflammation observed under high fat diet[52]. Assessing this possibility is an important future direction. New insights into somatostatin loss have emerged from the study of chronic stress which shed light on this possibility. Recent work shows that stress induced overactivation of SST neurons elicits downregulation of the somatostatin peptide [241]. Under high fat diet, a stimulus leading to overactivation of SST neurons could be the well-known neuroinflammatory state evoked by that dietary challenge[52]. My data and others show that SST neurons, and SST itself, are anti-inflammatory in the central nervous system[56, 152, 153, 242]. Thus, it is possible that chronic diet induced neuroinflammation activates SST neurons as a reflexive response to the inflammatory state, and that the chronic activation of this anti-inflammatory reflex provokes SST

downregulation. Such a finding would provide further mechanistic support for clinical approaches which seek to restore hypothalamic somatostatin which are outlined in the following section.

*Implications for Bioelectronic Medicine*

Novel non-invasive therapeutic approaches using focused ultrasound or via trans-auricular vagus nerve stimulation have permitted unprecedented control of autonomic nerves in an organ specific manner. These approaches constitute the burgeoning field of bioelectronic medicine. Bioelectronic medicine addresses autonomic dysfunction on its native scale by directly engaging and modulating specific neural circuities. For example, focused ultrasound in the spleen in humans reduces inflammation, likely via activation of sympathetic nerves[63, 64], focused hepatic ultrasound in insulin resistant mice, rats, and swine improves glucose homeostasis[65], and trans-auricular vagus nerve stimulation in patients with MetS improves endothelial and monocyte function [66]. Each of these approaches relies on activating sensory nerves, whether by direct electric currents in the ear or by ultrasound on specific organs. In adipose tissue, many sensory nerves have recently been shown to be sympatho-inhibitory[67], and thus, a novel therapeutic approach to reducing catecholamine resistance could be to transiently stimulate sensory nerves via focused ultrasound to reduce sympathetic nerve tone. As with the pharmacologic approaches described in prior sections, the prediction of this approach is that transiently, intermittently reducing sympathetic tone could partly alleviate catecholamine resistance. Thus, on “off” days, when focused ultrasound is not in use, increased adiponectin, decreased leptin, and an appropriately elevated fatty acid response to fasting are predicted, in keeping with improved catecholamine sensitivity, though more

invasive biopsy approaches could be taken to directly assess adrenergic receptor expression and sensitivity, or using the microdialysis approach outlined above. This approach is more temporally and spatially controlled than global pharmacologic modulation which targets many organs simultaneously.

*Clinical Implications and Summary*

The significance of the studies in this dissertation is that they point to a novel, and unorthodox approach to treating catecholamine resistance: reducing sympathetic signaling to recover adrenergic sensitivity. Current therapeutic approaches rely on the activation of a relatively depressed receptor array, via β3 adrenergic agonists such as mirabegron[53, 54]. If excess adrenergic signaling drives catecholamine resistance, a paradoxical but rational therapeutic strategy would be to partially reduce this signaling. Such approaches, using beta blockers, have been shown to improve cardiac adrenergic sensitivity in heart failure[55], another chronic state of elevated sympathetic nerve activity. In a comparable manner, antibody mediated neutralization of leptin has recently been shown to alleviate leptin resistance associated with hyperleptinemia[56], and pharmacologic inhibition of insulin secretion via relatively specific agents, such as diazoxide, has also been shown to alleviate insulin resistance associated with hyperinsulinemia[57-59]. Thus, my findings fit within a broader pattern of pharmacotherapies to reverse various forms of impaired signaling.

Tremendous clinical effort has been undertaken to stimulate adrenergic receptors in pursuit of increased lipolysis and adipose tissue beiging[53, 54]. The premise of the work within this dissertation is that efforts to directly stimulate adrenergic receptors are impeded by a core feature of the pathology in MetS: catecholamine resistance. A

therapeutic approach which restored endogenous adrenergic sensitivity would allow the sympathetic nervous system to activate β adrenergic receptors in an adipose tissue specific manner, under the correct physiologic triggers, such as fasting and exercise. This degree of specificity is not plausibly achieved using pharmacology. Indeed, in current clinical studies, four times the maximal approved dose of mirabegron is needed to activate human β3- AR and exhibits off-target effects by raising blood pressure and heart rate[60].

How might adrenergic re-sensitization be achieved, based on the findings of the two papers presented herein? This work makes the prediction that *partial* reductions in sympathetic tone should alleviate chronic stimulation of adrenergic receptors, reducing downregulation and desensitization and restoring sensitivity to endogenous catecholamines. Many central sympatholytic agents exist, including clonidine and moxonidine, however, no currently utilized pharmacologic approach simultaneously targets central control of sympathetic tone as well as brain inflammation. Octreotide, a somatostatin receptor agonist, exhibits both these functions, and is available for direct CNS delivery via intranasal spray[61]. The work in Chapter 2 of this dissertation makes the prediction that intranasal octreotide should resolve hypothalamic inflammation in MetS, reduce sympathetic tone, and potentially reverse catecholamine resistance. An important step prior to this repurposing would be to perform human post-mortem neuropathology studies to confirm that patients with MetS show the same downregulation of somatostatin as observed in BBZ/Wor rats and db/db mice.

An alternative pharmacologic approach reduces sympathetic tone at a more downstream site: neuronal synthesis of norepinephrine itself. Dopamine beta-hydroxylase inhibitors, such as nepicastat and etamicastat, rapidly reduce norepinephrine synthesis.

These drugs were initially developed for heart failure and hypertension[62]. In these trials, nepicastat was determined to be safe and well tolerated in humans but was not brought to market for these indications, making it an appealing target for repurposing. This approach could comparably alleviate chronic adrenergic overstimulation and restore endogenous sensitivity.

With both octreotide and nepicastat, as with most drugs, an important consideration is the timing and duration of dosing. Intermittent dosing, for example on an alternate day basis, could produce a desirable combination of alleviating overstimulation while still permitting activation of receptors on non-drug days. The prediction based on this approach is that lipolysis should be increased alongside increased adiponectin and suppressed leptin on non-drug days, due to enhanced adipocyte responses to endogenous stimulation. This hypothesis could be tested non-invasively using repeated peripheral blood sampling before and after drug insufflation (in the case of octreotide) or dosing via the oral route (in the case of nepicastat). Alternatively, repeated microdialysis studies could investigate these hormones at their site of secretion, potentially following direct infusion of adrenergic agonists, as has been performed previously in subcutaneous adipose tissue in human volunteers. The latter approach would have the advantage of directly activating adrenergic receptors, which would permit more robust conclusions regarding any potential changes in tissue catecholamine sensitivity.

Taken together, the work presented in this dissertation contributes to a body of literature suggesting that aberrant activation of the sympathetic nervous system contributes to pathology in MetS, potentially including the development of catecholamine resistance, and develops novel therapeutic concepts in the restoration of catecholamine sensitivity.

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# APPENDIX

*IACUC Approval*

THE UNIVERSITY OF ALABAMA AT BIRMINGHAM

**MEMORANDUM**

**DATE:** 18-Aug-2021

**TO:** Grant, Maria Bartolomeo

**FROM:** Robert A. Kesterson, Ph.D., Chair

Institutional Animal Care and Use Committee (IACUC)

**SUBJECT: NOTICE OF APPROVAL**

The following application was approved by the University of Alabama at Birmingham Institutional Animal Care and Use Committee (IACUC) on 18-Aug-2021.

| | |
|---|---|
| **Protocol PI:** | Grant, Maria Bartolomeo |
| **Title:** | Somatostatin Blockade Of CNS Autonomic Hyperactivity For Treatment Of Diabetic Retinopathy |
| **Sponsor:** | National Eye Institute/NIH/DHHS |
| **Animal Project Number (APN):** | IACUC-21291 |

This institution has an Animal Welfare Assurance on file with the Office of Laboratory Animal Welfare (OLAW), is registered as a Research Facility with the USDA, and is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

This protocol is due for full review by 17-Aug-2024.

**Institutional Animal Care and Use Committee (IACUC)**

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