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Mechanism of Diabetes Remission Induced by the Central Action of Fibroblast Growth Factor 1

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Abstract

Mechanism of Diabetes Remission Induced by the Central Action of Fibroblast Growth Factor 1

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The rising prevalence of type 2 diabetes mellitus (T2D) is a major health concern worldwide. Growing evidence of a role for the brain in glucose homeostasis has stimulated interest in therapeutic approaches that target the brain for the treatment of T2D. Our recent finding that a single intracerebroventricular (icv) injection of fibroblast growth factor 1 (FGF1) elicits sustained diabetes remission in rodent models of T2D supports a growing consensus that the brain is a key target for diabetes drug development. The goal of the research reported in this dissertation is to identify the brain area, signal transduction pathway, and peripheral mechanisms responsible for mediating sustained diabetes remission induced by central FGF1. We identified the hypothalamic arcuate nucleus (ARC) median eminence (ME) (ARC-ME), a brain area known to participate in glucose homeostasis, as being one of only two brain areas that show robust induction of MAP Kinase / ERK (MAPK/ERK) signaling (a marker of FGF receptor activation) following icv

injection of FGF1, and that FGF1 microinjection localized to this brain area is capable of inducing sustained glucose lowering. Additionally, activation of the MAPK/ERK signaling pathway occurs in the ARC-ME for at least 24 hours post icv injection, and diabetes remission induced by the central action of FGF1 depends upon this prolonged hypothalamic MAPK/ERK signaling. Finally, in the Zucker diabetic fatty rat model of T2D, sustained glucose lowering induced by the central action of FGF1 involves both preservation of β-cell function and stimulation of hepatic glucose utilization through increased hepatic glucokinase activity. This work provides fundamental insight into mechanisms underlying the brain's capacity to induce sustained diabetes remission.

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Chapter 2 is an original manuscript published in 2019, in *Diabetes*. 'The Hypothalamic Arcuate Nucleus–Median Eminence Is a Target for Sustained Diabetes Remission Induced by Fibroblast Growth Factor 1'. Chapter 4 is an original manuscript published in 2018, in *Diabetes*. 'Peripheral Mechanisms Mediating the Sustained Anti-Diabetic Action of FGF1 in the Brain'. Chapter 5 is an original manuscript published in 2019, in *The Journal of Clinical Investigation.* 'Rethinking the role of the brain in glucose homeostasis and diabetes pathogenesis'.

DEDICATION

For my extraordinary family. My parents Layne and Betty Brown, whose encouragement, love, and support kept my spirits high the last five years. To my siblings Jonathan, Sarah, and Thomas for always looking out for their little sister. To my grandparents, Layne and Marian Brown, for your unconditional love and boxes of fresh peaches every summer and to Charles and Eunice Meuret, who now watch over me and give me the inspiration to do my best. Most importantly, to my love, Victor Robledo, for being my everything. This thesis is for you!

Chapter 1. CENTRAL FIBROBLAST GROWTH FACTOR 1 SIGNALING IN GLUCOSE AND ENERGY HOMEOSTASIS

1.1 INTRODUCTION

Type 2 diabetes mellitus (T2D) is projected to affect 578 million people by the year 2030 (Federation 2019), heightening the need for an improved understanding of the disease pathogenesis to develop better therapeutic strategies. The incidence has tripled in the last 30 years, and it now costs Americans $327 billion in annual health care costs ('Economic Costs of Diabetes in the U.S. in 2017' 2018). Accounting for 90% of all diabetes worldwide, T2D predisposes to numerous comorbidities, including cardiovascular disease, neuropathy, nephropathy, and retinopathy (Zheng, Ley, and Hu 2018). To avoid the comorbidities associated with hyper- and hypoglycemia the goal of T2D therapeutics is to maintain normal glycemia. Current approaches to T2D treatment involve daily administration of drugs that transiently lower blood glucose levels from their biologically regulated value without actually lowering the value that is defended (Edelman and Polonsky 2017). Even with the implementation of over 40 new pharmaceuticals on the market, most people fail to achieve glycemic control, and disease progression continues (Edelman and Polonsky 2017).

Growing evidence that the brain plays a key role in glucose homeostasis has stimulated interest in therapeutic approaches that target the brain for the treatment of T2D (Ruud, Steculorum, and Bruning 2017; Brüning et al. 2000; Thorens 2010). Evidence that dates back to iconic studies performed by physiologist Claude Bernard in the 1850s observed that brain manipulations could induce sugar excretion into the urine (Ruud, Steculorum, and Bruning 2017). Considering that the brain controls many homeostatic systems (Tan and Knight 2018; Lowell 2019), it is improbable that glucose homeostasis would be controlled independently of mechanisms controlled by the brain. However, the discovery of insulin shifted science and research to focus on the endocrine pancreas. Pancreatic β-cells play a crucial role in

glucose homeostasis by transducing changes of circulating glucose and other nutrients into an insulin secretory response that preserves euglycemia (Kahn, Hull, and Utzschneider 2006). The pathogenesis of T2D involves a disruption of this response system characterized by progressive deterioration of β-cell structure and function, which, when combined with insulin resistance, results in overt hyperglycemia(Kahn, Hull, and Utzschneider 2006). Despite decades of intense study, the mechanism underlying this islet dysfunction remains unknown. Research over the past thirty years is beginning to chip away at the current dogma by revealing that the pancreas is part of a more extensive homeostatic regulatory system integrated by the brain (Schwartz et al. 2013; Rosario et al. 2016b).

In response to nutrient- and hormone-related input, the brain engages neurocircuits that maintain body fuel stores while also balancing glucose production with glucose uptake to keep blood glucose levels within a narrow physiological range (Ruud, Steculorum, and Bruning 2017). Cell type-specific chemogenetic and optogenetic experiments are uncovering how the brain orchestrates the neuroregulatory networks that control feeding, peripheral insulin sensitivity, and glucose metabolism in regulatory centers that include both the hypothalamus and hindbrain (Stanley et al. 2016; Faber et al. 2018; Roh, Song, and Kim 2016). We hypothesize the pathogenesis of obesity and T2D involves defects in these respective homeostatic systems that result in a progressive increase of the biologically defended level of both long-term fuel stores (adipose tissue) and fuel available for immediate use (circulating glucose).

Among promising therapeutic agents for diabetes are members of the fibroblast growth factor (FGF) peptide family. FGF1, FGF19, and FGF21 have been linked to the regulation of energy and glucose homeostasis (Nies et al. 2015). Pharmacological doses of FGFs cause weight loss, increase energy expenditure, and improve carbohydrate and lipid metabolism

in animal models of obesity and T2D (Morton et al. 2013; Lan et al. 2017; Bookout et al. 2013). FGFs can affect these regulatory systems by binding to FGF receptors (FGFR) in the brain as well as in peripheral organs. The antidiabetic effects of the endocrine FGFs FGF19 and FGF21 are well established (Lan et al. 2017; Bookout et al. 2013). The brain is implicated in their pharmacological effects since the central administration of either peptide at doses that are ineffective when given systemically elicits transient glucose-lowering and weight loss in rodent models of obesity and T2D. Similarly, if either FGF receptor 1 (FGFR1) or beta klotho, a coreceptor, is knocked out of the brain, the metabolic effects of these peptides are attenuated (Lan et al. 2017). Unlike FGF19 and FGF21, however, a single injection of FGF1 into the brain of diabetic rodents induces sustained diabetes remission (Scarlett et al. 2016; Suh et al. 2014).

The conceptual framework that guides our approach is based on the hypothesis that hypothalamic neurocircuits involved in glucose homeostasis are dysfunctional in T2D, that this dysfunction contributes to hyperglycemia, and that FGF1 ameliorates this dysfunction. Our recent finding that sustained diabetes remission can be induced via the action of FGF1 in the central nervous system (CNS) exemplifies the fundamental therapeutic advance represented by the potential of brain-directed diabetes drug development (Gasser et al. 2017; Scarlett et al. 2016). If, indeed, the brain can effectively reset the defended level of glycemia, it follows that a brain-specific defect can potentially contribute to the defense of elevated blood glucose levels in T2D.

An essential piece of data missing is where is FGF1 acting in the brain to exert its effects on glucose homeostasis. Identifying the brain region, cell types, and signal transduction pathways will uncover key regions in the brain that can induce sustained diabetes remission. This thesis seeks to identify the specific brain area(s) and signal

transduction mechanisms through which FGF1 mediates this remarkable effect and therefore advance our understanding of the role of the brain in glucose homeostasis.

FGFs Basic Signaling transduction

Fibroblast growth factors (FGF) are a family of cell-signaling proteins that regulate a wide range of processes essential for normal development and homeostatic functions in the adult (Ornitz and Itoh 2015). Each of the 22 members of the FGF peptide family are classified as either canonical, intracellular, or endocrine, based on sequence homology and phylogenetic analysis (Ornitz and Itoh 2015). Sequence variation in the N and C terminus tails account for variations in ligand function. Intracellular FGFs are non-signaling proteins that act as cofactors (Wu et al. 2012). Canonical FGFs activate FGFRs with heparin or chondroitin sulfate proteoglycans as cofactors and act in an autocrine and paracrine manner, with FGF1 being representative of this group, whereas endocrine FGFs, such as FGF19 and FGF21, require the Klotho family as cofactors and act at distant sites.

FGFs bind to and activate a specific set of single-pass transmembrane tyrosine kinase receptors. FGFRs comprise of three extracellular immunoglobulin-like domains, a single transmembrane domain, and an intracellular tyrosine kinase domain split by an insertion domain. The mammalian FGFR family contains four Fgfr genes, ie. *Fgfr1*, *Fgfr2*, *Fgfr3*, and *Fgfr4*. FGFRs have tissue-specific alternative splicing of domain 3 that generates two main variants that result in alternative Ig-like domain referred to as b and c (Ornitz and Itoh 2015). Epithelial “b” and mesenchymal “c” splice isoforms restrict tissue expression patterns that contribute to FGFRs wide variety of biological function and limit ligand-binding specificities. This results in a total of seven receptor isoforms: FGFR1b, FGFR1c, FGFR2b, FGFR2c, FGFR3b, and FGFR3c)

Termed the universal ligand, FGF1 overcomes this limited interaction with a three amino acid sequence at the N Terminus enabling FGF1 to bind and activate all seven FGFRs. Beenken et al. determined that this FGF1 promiscuity is due to the N terminus and how it interacts with FGFRs (Beenken et al. 2012). The signal transduction pathways engaged by FGF1 binding to FGFRs include 1) mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK), 2) Janus Kinase – Signal Transducer and Activator of Transcription (Jak/Stat), 3) phospholipase C (PLCγ), and 4) phosphatidylinositol-3-OH kinase (PI3K) (Ornitz and Itoh 2015).

FGF Receptors in the Brain

Research over the last decade revealed that the brain is a primary site of action responsible for the pharmacological action of FGFs to reduce body weight and food intake and normalize blood glucose and plasma lipid levels. A variety of neuroanatomical studies have identified the location of FGFs, FGFR, and cofactors. Hultman et al. added to this body of work with a nuclei-specific and cellular resolution of FGFR expression from the mouse, nonhuman primates, and humans (Kasten, Blasingame, and Boehm). By comparing the expression pattern across multiple mammalian models, Hultman et al. conclude that the broad expression pattern of FGFR1-3 is similar in the midbrain and hindbrain across these species. Extensive expression of FGFR1-3 was observed across the entire brain, and FGFR1 has the widest distribution. In contrast, FGFR4 is expressed exclusively in the habenula and sub-commissural organ. FGFR and cofactors are expressed by both neurons and glia, including tanycytes which are located exclusively in the hypothalamus, an important site for energy and glucose homeostasis (Kaminskas et al. 2019).

Role of FGF1 in the regulation of energy homeostasis

Released after feeding and glucose administration by tanycytes into the cerebrospinal fluid (CSF), FGF1 was proposed as a glucose sensor in the brain three decades ago (Oomura, Sasaki, and Suzuki 1992). FGF1 was first isolated from pituitary extract, and its postprandial changes were first examined by studying CSF collected from rats and assayed using a hydra feeding assay. When hydra was exposed to CSF from fed rats, the hydra feeding response was inhibited to a higher degree and this effect was suggested to be mediated by FGF1 (Oomura, Sasaki, and Suzuki 1992). Both glucose administration and food intake increased the depressive activity of the CSF in the hydra experiments (Oomura, Sasaki, and Suzuki 1992) suggesting that FGF1 levels had increased in the CSF. Additionally, icv microinfusion of FGF1 reduced food intake in Wistar rats in a dose-dependent manner at picomolar doses, whereas neither heat-inactivated FGF nor peripheral administration affected food intake (Oomura, Sasaki, and Suzuki 1992; Sasaki et al. 1994). Similarly, an anti-FGF1 antibody increased feeding (Sasaki et al. 1994).

Challenging FGF1 knockout mice (FGF1-/-) with a high-fat diet revealed a physiological role for FGF1 in glucose homeostasis. It became clear these mice develop overt diabetes and have an impaired energy balance phenotype (Jonker et al. 2012). FGF1 is a critical transducer of adipose tissue remodeling, a process that fluctuates in response to nutrient availability. An imbalance of energy intake and expenditure, obesity is characterized by excess fat mass. Adipose tissue functions to expand and contract in response to changing nutrient availability. Healthy expansion occurs with appropriate angiogenic and preadipocyte recruitment, whereas pathogenic expansion is often accompanied by an insufficient angiogenic response and the expansion of existing adipocytes with the induction of fibrosis and a pro-inflammatory response (Jonker et al.

2012). FGF1-/- mice have no phenotype and develop normally. When placed on a high-fat diet, however, FGF1-/- mice showed identical body weight gain curves but they exhibited elevated fasting blood glucose, severe insulin resistance, and increased markers of adipose macrophage infiltration (Jonker et al. 2012).

Histopathology revealed a failure of appropriate adipose tissue remodeling in response to HFD stress and impaired contraction when the HFD was withdrawn. FGF1 regulation is linked to the nuclear receptor PPARc (peroxisome proliferator-activated receptor c), a master metabolic regulator (Jonker et al. 2012). PPAR expression was induced in WAT of FGF1-/- mice, and HFD elevates PPAR ligand. A luciferase assay revealed induction of the human FGF1A transcript by PPARs of with PPAR gamma being the most potent. Site-directed mutagenesis of the PPAR response element led to a complete loss of response (Jonker et al. 2012). Additionally, FGF1 protein levels increase in adipose tissue after exposure to a high-fat diet.

Role of FGF1 in the regulation of glucose homeostasis

These findings led to the question of whether supplementing with FGF1 improves glucose homeostasis, and to test this hypothesis a single peripheral injection of FGF1 induced glucose-lowering for 48 H in diabetic Lep$^{ob/ob}$ and diet-induced obese (DIO) mice (Suh et al. 2014). No glucose-lowering was achieved when compared to FGF2, 9, or 10. Importantly, FGF1 is not an insulin secretagogue or insulin-mimetic as FGF1 injection had no significant effect on glucose-stimulated insulin secretion in isolated islets and did not increase serum insulin levels in basal conditions. A hyperinsulinemic-euglycemic clamp revealed that FGF-treated mice had a ~75% higher glucose infusion rate indicating increased responsiveness to insulin. FGF1 also improved insulin's ability to suppress hepatic glucose production. Whereas FGF1 failed to reduce

fasting glucose levels in mice with severe insulin deficiency induced by the β-cell toxin streptozotocin (STZ), it improved exogenous insulin's ability to lower blood glucose (Suh et al. 2014).

Central Administration of FGF1

Sustained glucose-lowering with peripheral injections of FGF1 led Scarlett and Rojas et al. to propose a role for the brain int his effect. They showed that a single intracerebroventricular (icv) injection of FGF1, at a dose one-tenth of that required for systemic efficacy, induces sustained diabetes remission in rodent models of obesity and T2D (Scarlett et al. 2016). They further showed that this effect is not secondary to reduced food intake or body weight and that it occurs without hypoglycemia (Scarlett et al. 2016). Central FGF1 increased hepatic glucose utilization and increased glucose clearance from the bloodstream. While previous evidence suggests that FGF1 reduces blood glucose levels by acting on the HPA axis(Perry et al. 2015), no difference of corticosterone was found in $Lep^{ob/ob}$ mice either acutely or after sustained glucose lowering. The underlying mechanism requires sufficient residual function of pancreatic islets to generate an intact basal insulin signal (as is characteristic of T2D, but not T1D). Responsiveness to icv FGF1 is lost in animals with severe insulin deficiency induced by STZ (Scarlett et al. 2016), and the same is true of otherwise healthy mice in which diabetes is induced by systemic administration of S961, a high-affinity insulin receptor antagonist (Scarlett et al. 2016). These observations collectively suggest that in rodent models of T2D, the action of FGF1 in the brain leads to a re-setting of glycemia at a reduced level, rather than exerting a simple glucose-lowering effect, and that the effect requires residual function of pancreatic islets.

Potential sites of FGF1 action on glucose homeostasis

The mediobasal hypothalamus (MBH) contains nuclei including the arcuate nucleus (ARC) and median eminence (ME) that are implicated in brain control of both energy and glucose homeostasis. In the hypothalamus, the ARC is located at the base of the third ventricle just above the ME, a circumventricular organ with a leaky blood brain barrier (Banks 2006). Neurons in the ARC send axon projections to several hypothalamic nuclei, including the ventromedial hypothalamic nucleus (VMN), dorsal nucleus of the hypothalamus (DMH), the paraventricular nucleus (PVN) and the lateral hypothalamic area (LHA). Many axon terminals from ARC neurons project to the ME, an area with fenestrated capillaries, specialized ependymal cells (tanycytes), oligodendrocytes, astrocytes, and neurons. The ARC-ME is a critical site that integrates peripheral signals and relays information to the other nuclei in the hypothalamus. Of note in humans and rodent models, obesity and impaired glucose homeostasis are associated with neuronal injury, inflammation, gliosis, localized to the ARC-ME area, changes that are associated with synaptic remodeling (Schwartz et al. 2013; Kalin et al. 2015; Thaler JP1 2011).

Therefore, we hypothesized that the hypothalamic ARC is a key brain area for the effect of FGF1 to induce sustained diabetes remission and proposed the following specific aims that constitute the basis of my thesis:

Specific Aim 1: To determine if FGF1 signaling in the ARC-ME is sufficient to explain diabetes remission induced by icv injection of FGF1.

Specific Aim 2: To identify the signal transduction mechanisms underlying sustained glucose lowering induced by icv injection of FGF1.

Specific Aim 3: To determine if icv injection of FGF1 prevents progressive deterioration of β-cell function in ZDF rats.

Chapter 2. THE HYPOTHALAMIC ARCUATE NUCLEUS-MEDIAN EMINENCE IS A TARGET FOR SUSTAINED DIABETES REMISSION INDUCED BY FIBROBLAST GROWTH FACTOR 1

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2.1 ABSTRACT

In rodent models of type 2 diabetes (T2D), sustained remission of diabetic hyperglycemia can be induced by a single intracerebroventricular (icv) injection of fibroblast growth factor 1 (FGF1). To identify the brain area(s) responsible for this effect, we first used immunohistochemistry to map the hypothalamic distribution of phosphoERK (pERK1/2), a marker of MAP kinase-ERK signal transduction downstream of FGF receptor activation. Twenty minutes after icv FGF1 injection in adult male Wistar rats, pERK1/2 staining was detected primarily in two hypothalamic areas: the arcuate nucleus and adjacent median eminence (ARC-ME), and the paraventricular nucleus (PVN). To determine whether an action of FGF1 localized to either the ARC-ME or the PVN is capable of mimicking the sustained antidiabetic effect elicited by icv FGF1, we microinjected either saline vehicle or a low dose of FGF1 (0.3 μg/side) bilaterally into either the ARC-ME area or PVN of Zucker Diabetic Fatty (ZDF) rats, a model of T2D, and monitored daily food intake, body weight, and blood glucose levels over a 3-week period. Whereas bilateral intra-arcuate microinjection of saline vehicle was without effect, remission of hyperglycemia lasting >3 wk was observed following bilateral microinjection of FGF1 into the ARC-ME. This antidiabetic effect cannot be attributed to leakage of FGF1 into cerebrospinal fluid and subsequent action on other brain areas, since icv injection of the same total dose was without effect. Combined with our finding that bilateral microinjection of the same dose of FGF1 into the PVN was without effect on glycemia or other parameters, we conclude that the ARC-ME area (but not the PVN) is a target for sustained remission of diabetic hyperglycemia induced by FGF1.

2.2 INTRODUCTION

Several members of the fibroblast growth factor (FGF) family of peptides have been shown to have antidiabetic properties when administered either systemically or centrally in rodent models of type 2 diabetes (T2D). Among these are the two "endocrine FGFs" – FGF19 and FGF21 – and the tissue growth factor FGF1 (Morton et al. 2013; Sarruf et al. 2010; Scarlett et al. 2016; Lan et al. 2017). The centrally-mediated antidiabetic effect of FGF1 is unique in that it lasts for weeks or months following a single icv injection in both mouse (*ob/ob*, *db/db*) and rat (Zucker diabetic fatty (ZDF)) models of T2D (Scarlett et al. 2016; Scarlett et al. 2018).

To better understand how FGF1 elicits this effect, we sought in the current work to identify the brain area(s) involved. To this end, we first mapped the central nervous system (CNS) distribution of phosphoERK (pERK1/2), a cellular marker of FGF receptor activation, following icv injection FGF1 in normal rats. This work revealed robust FGF1-induced pERK1/2 staining in only two brain areas, both of which are known to express FGF receptors and is implicated in glucose homeostasis: the hypothalamic arcuate nucleus-median eminence (ARC-ME) and the paraventricular nucleus (PVN) (Roh, Song, and Kim 2016). To determine whether an action of FGF1 limited to either area is sufficient to recapitulate the sustained anti-diabetic effect of FGF1 following icv administration, we microinjected into either the ARC-ME or PVN either saline vehicle or a dose of FGF1 5-fold below what is needed for efficacy following icv injection in separate cohorts of ZDF rats, and monitored daily levels of food intake, body weight, and blood glucose for 3 wk. We report that whereas sustained remission of hyperglycemia is elicited by FGF1 microinjection into the ARC-ME, microinjection of the same low dose of FGF1 into the PVN of ZDF rats was without effect, despite robustly inducing pERK1/2 in this brain area. Moreover, the pERK1/2 immunoreactivity observed in the ARC-ME following icv FGF1 is concentrated in glial

cells (tanycytes and astrocytes) rather than neurons, raising the possibility that neuronal responses to FGF1 be secondary to its action on glial cells. These findings collectively identify the ARC-ME as a key target for the sustained anti-diabetic effect of FGF1.

2.3 RESULTS

Icv FGF1 injection activates pERK1/2 signaling in the ARC-ME and PVN

As a first step to identify candidate brain regions involved in the anti-diabetic action of FGF1, we assessed the regional distribution of pERK1/2 immunoreactivity in serial coronal sections collected from optic chiasm to hindbrain in normal Wistar rats 20 min after icv injection of either FGF1 (3 µg) or saline vehicle. pERK1/2 immunoreactivity was detected primarily in the PVN and along the ventral surface of the third ventricle, extending into the parenchyma of the ARC-ME along its entire rostro-caudal axis (Table 1 and Fig 1). These findings are consistent with the known distribution of FGF receptors in ARC-ME and PVN (Belluardo et al. 1997), and they identify these hypothalamic areas as potential targets for sustained remission of diabetic hyperglycemia induced by centrally-administered FGF1. Although pERK signal intensity was also high in the supraoptic and suprachiasmatic nuclei, this was true for both vehicle- and FGF1-treated animals. Thus, these two brain areas do not stand out as being FGF1-responsive, although we cannot exclude the possibility that the high basal pERK precluded our ability to detect a response.

Table 1 Distribution of pERK1/2 immunofluorecence in the rat brain after icv injection of FGF1.

Brain region	Nucleus	Abbreviation	Treatment	
			icv Vehicle	icv FGF1
Hypothalamas	Suprachiasmatic	SCN	***	***
	Preoptic area	POA	*	*
	Supraoptic	SON	*****	******
	Paraventricular	PVN	***	*****
	Retrochiasmatic area	Rch	*	***
	Ventromedial hypothalamic n.	VMN	*	*
	Dorsomedial hypothalamic n.	DMN	*	**
	Arcuate hypothalamic n.	ARC	**	******
	Mammillary n.	MM	-	-
Pineal gland Pituitary	Median eminence	ME	***	******
	Infundibular Stock	InfS	**	******
Thalamas	Paraventricular thal n. Ant	PVA	-	-
	Anteroventral thalamic n.	AV	-	*
	Anteroventral medial n.	AM	-	-
	Paratenial n.	PT	-	-
	Mediodorsal n.	MD	-	-
	Paraventricular thal n.	PV	-	-
	Paraventricular thal n. post	PVP	-	-
	Centromedial thalamic n.	CM	-	-
	Reuniens n.	Re	-	-
Amygdala	Medial Amygdaloid n.	MeA	-	-
	Cortical amygdaloid n.	CoA	-	-
	Basolateral amygdaloid n.	BLA	-	-
	Basomedial amygdaloid n.	BMA	-	-
	Central Amygdaloid n.	CeA	*	-
	Amygdalostriatal transition	Ast	-	-
Hippocampus	CA1 of the Ammon's horn	CA1	-	*
	CA2 of the Ammon's horn	CA2	-	-
	CA3 of the Ammon's horn	CA3	-	-
	Dentate Gyrus	DG	-	*
Hind Brain	Raphe pallidus nu	Rpa	**	**
	N. of the Solitary tract	NTS	-	-
	Area postrema	AP	-	**
	Hypoglossal n.	12N	*	*
	Spinal n. of trigeminal nerve	sp5	**	**

Positive signal density in an area or nucleus defined by a strong signal and a signal that occupies the entire region (******), moderate signal and it occupies most of the region (****), little signal and it occupies a small region of the nucleus(**) and (-) no signal.

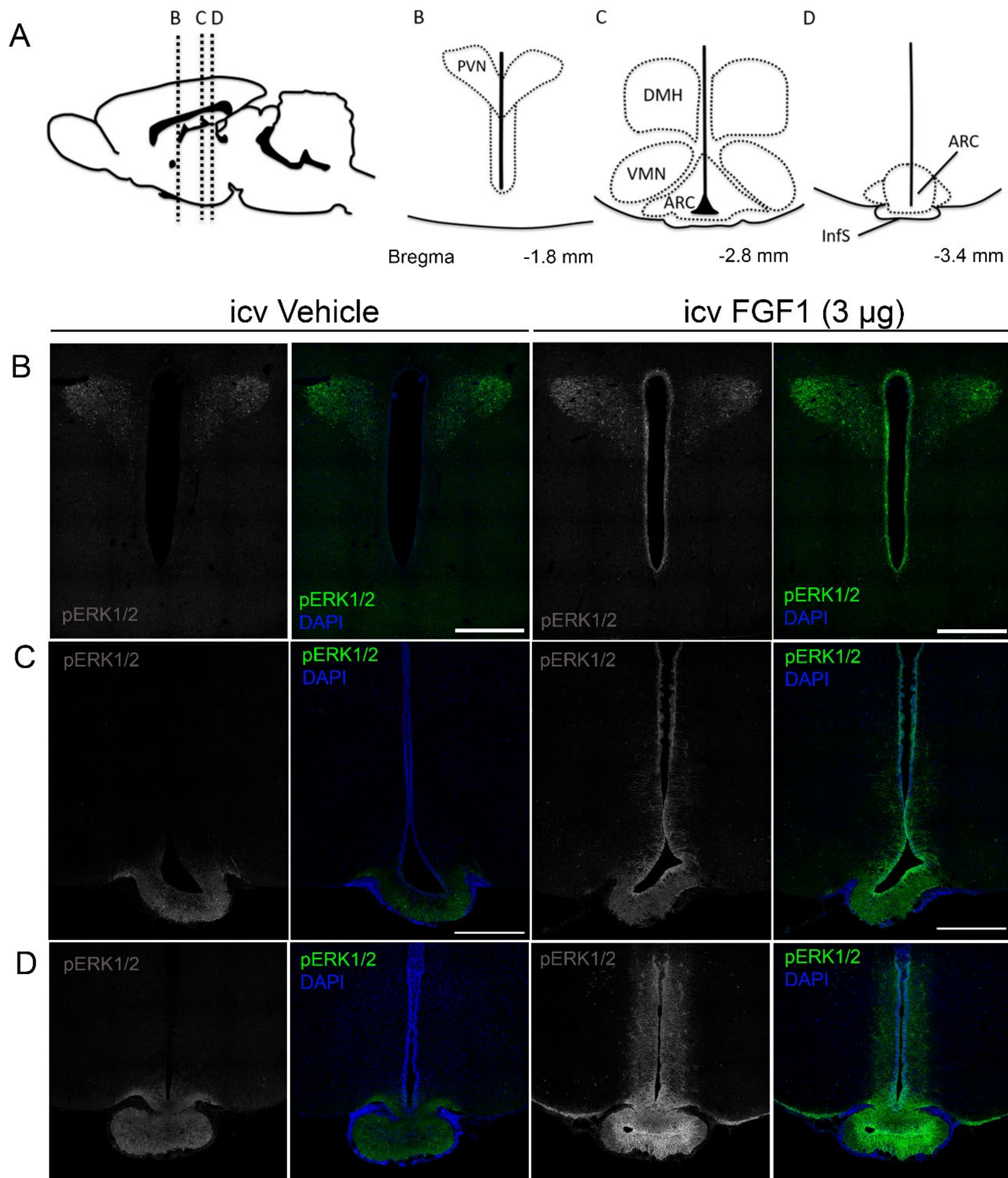

Figure 2-1 Regional activation of MAPK/ERK1/2 signaling in the hypothalamus after icv FGF1.

A: Diagram at top shows sagittal view of the rat brain (left) and inserts of the mediobasal hypothalamus (right) showing orientation of panel images of the PVN, ARC, ME, ventromedial nucleus (VMN), dorsomedial nucleus (DMN), and infundibular stalk (InfS). B–D: Confocal images of coronal sections showing pERK1/2 (green) and DAPI (blue) obtained 20 min after icv injection of either vehicle or FGF1 (3 mg). Images are taken from the PVN (B), the ARC-ME (C), and InfS (D). Scale bars: 500 mm (B and C). 3V, third ventricle.

Effect of Intra-ARC-ME FGF1 microinjection in Zucker Diabetic Fatty Rats

Based on evidence that the sustained glucose-lowering effect induced by icv FGF1 requires a dose of 3.0 µg ((Scarlett et al. 2016; Scarlett et al. 2018) and unpublished data), we asked whether this effect can be recapitulated by delivery of a much lower dose directly into the ARC-ME of ZDF rats. A dual-guide cannula (Fig 2A) was used to microinject into this brain area either vehicle or a dose of FGF1 5-fold below that necessary for efficacy when given icv (0.3 µg/side given bilaterally). We report that this intervention induced reductions of body weight and food intake (Fig 2C and D) comparable to what is observed following the higher dose of FGF1 given icv (Scarlett et al. 2018). Moreover, mean blood glucose levels fell by nearly 50% (from 300 ± 22 mg/dl to 158 ± 12; $p<0.05$), whereas no effect was observed in vehicle-injected ZDF controls (Fig 2B). This glucose-lowering effect was apparent within 48 h after intra-ARC-ME injection and persisted for ~3 wk, mimicking the effect of higher-dose icv injection. In contrast, the same low dose of FGF1 (0.3 µg/side) did not reduce levels of blood glucose (Fig 2E), food intake or body weight (data not shown), following icv injection (Fig 2E).

Having established that FGF1 action limited to the ARC-ME is sufficient to elicit sustained normalization of glycemia in ZDF rats (at a dose below that needed for efficacy when given icv), we sought to identify the cellular elements involved in this response. To this end, we used confocal microscopy to colocalize pERK1/2 with glial and neuronal markers in the ARC-ME of Wistar rats 20 min after icv injection of either FGF1 or vehicle. Given the prominent pERK1/2 activation induced by icv FGF1 in cells lining 3rd ventricle, we anticipated many of these cells would be tanycytes, and this was confirmed by our finding of extensive co-localization of pERK1/2 and vimentin immunostaining (Fig 2F and G). While we also observed colocalization of pERK1/2 with GFAP(+) astrocytes, colocalization with the neuronal marker NeuN was not observed (Fig 2I).

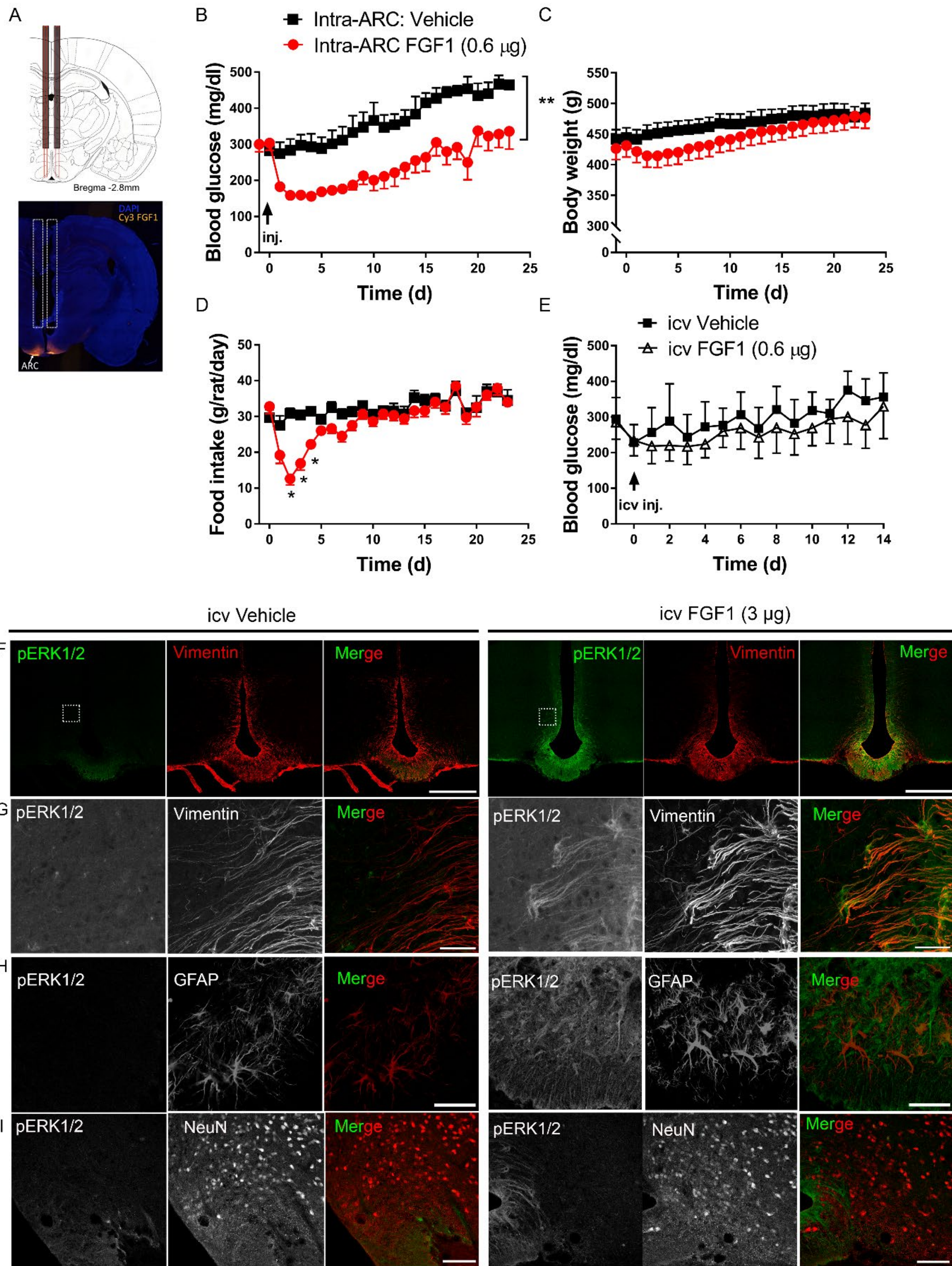

Figure 2-2 Effect of intra-ARC microinjection of FGF1 in ZDF rats.

A: Scale diagram of bilateral intra-ARC guide cannula (top panel) and representative image of Cy3-labled FGF1 injectate spread following microinjection into the ARC area (bottom panel). Daily blood glucose (B), body weight (C), and food intake (D) from ZDF rats after a single bilateral intra-ARC microinjection of either vehicle (n = 7) (black squares) or FGF1 (0.3 mg/side, for a total of 0.6 mg; n = 8) (red circles). E: Daily blood glucose levels in ZDF rats after a single icv injection of either vehicle (n = 4) or FGF1 at a dose equal to that given by microinjection into the ARC (0.6 mg; n = 4). Significance determined by linear mixed-model analysis. Confocal images of coronal sections of the ARC-ME costained with antibodies to pERK1/2 (green) and markers of tanycytes (vimentin), astrocytes (GFAP), or neurons (NeuN) 20 min after icv injection of either vehicle (left panels, F–I) or FGF1 (3 mg) (right panels, F–I) in Wistar rats (n = 5/group). G: Higher magnification view of images shown in inset in F. Scale bars: 500 mm (F), 50 mm (G and H), 100 mm (I). Colocalization of GFAP and pERK1/2 denoted by white arrowheads in (H). Data are mean6SEM. ***P,0.001 vs. intra-ARC vehicle. 3V, third ventricle; d, days; inj., injection.

Effect of FGF1 microinjection into the PVN

We next sought to determine if the same low dose of FGF1 (0.6 μg) elicits sustained glucose lowering following microinjection into the PVN, rather than the ARC-ME, of ZDF rats (Fig 3A). Unlike the potent effects on food intake, body weight, and blood glucose levels observed following FGF1 microinjection into the ARC area, microinjection into the PVN was without effect on these parameters (Fig 3B-E). As anticipated, intra-PVN FGF1 microinjection robustly induced pERK1/2 in the PVN, but not in the ARC/ME (Fig 3F), confirming that FGF1 signaling was limited to the former area.

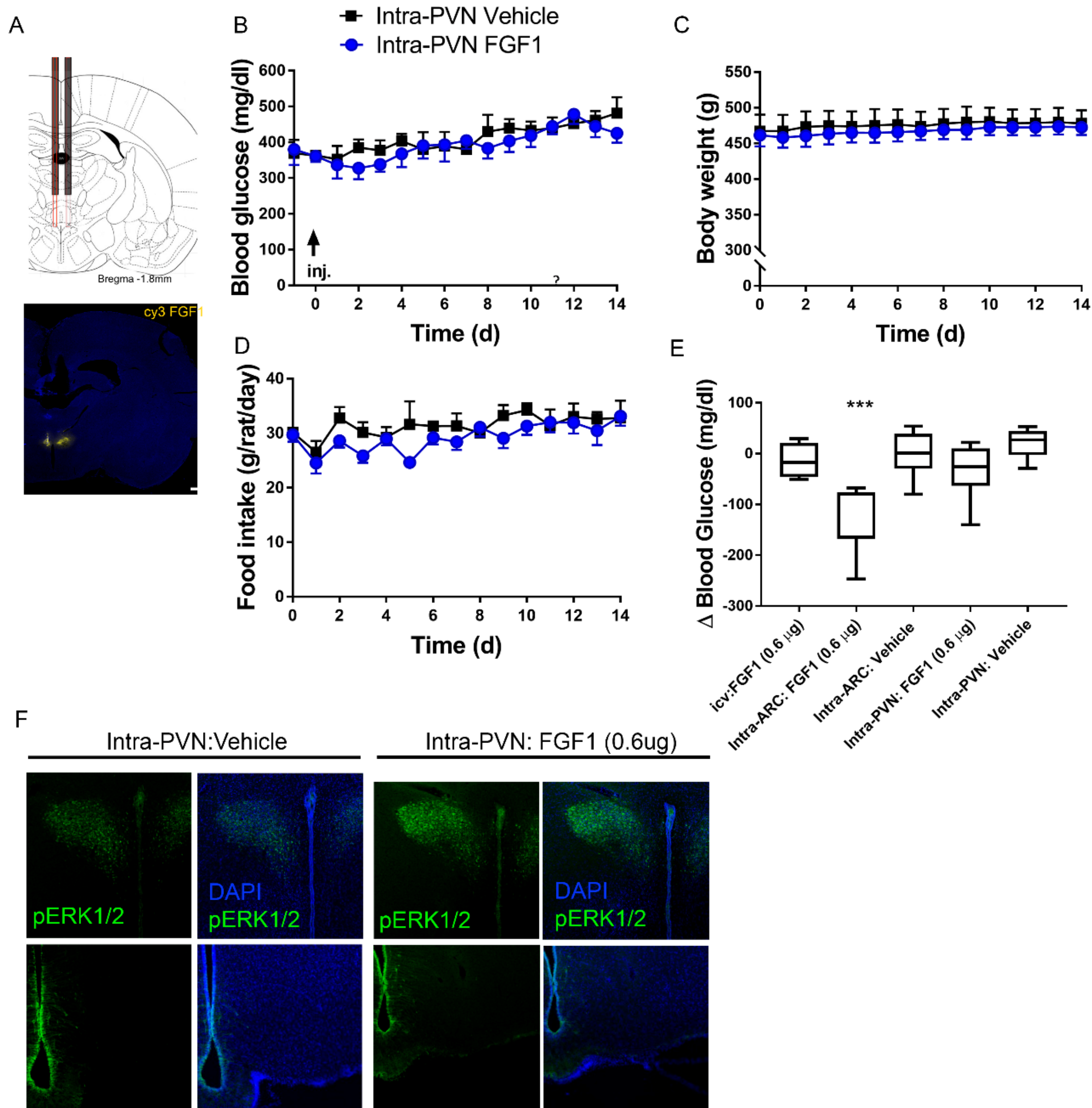

Figure 2-3 Effect of intra-PVN FGF1 microinjection on blood glucose levels in ZDF rats.

A: Scale diagram of bilateral intra-PVN guide cannula placement (top panel) and representative image of Cy3 FGF1 injectate spread following intra-PVN microinjection (bottom panel). Scale bar: 1,000 mm. Daily levels of blood glucose (B), body weight (C), and food intake (D) of ZDF rats after a single bilateral microinjection into the PVN of either vehicle (n = 5) or FGF1 (0.3 mg/side, for a total of 0.6 mg; n = 6). No significant differences in group mean values of any of these parameters were observed. Significance determined by linear mixed-model analysis. E: Change of blood glucose levels measured 48 h after icv, intra-ARC, or intra-PVN injection of either vehicle or FGF1 in the same animals. ***P , 0.05 vs. the other four groups by one-way ANOVA with Tukey post hoc test. F: Confocal images of pERK1/2 and DAPI immunostaining in coronal sections collected 20 min after an intra-PVN microinjection of either vehicle or FGF1 (0.3 mg/side; n = 3/group) at the level of either the PVN (top panel) or ARC-ME (bottom panel) in Wistar rats. Data are mean 6 SEM. d, days.

2.4 DISCUSSION

Based on activation of MAP Kinase/ERK activation downstream of FGF receptors (measured by pERK1/2 immunostaining), we report that the hypothalamic ARC-ME and PVN are the principal brain areas acutely engaged by FGF1 after icv administration. We further demonstrate that in the ZDF rat model of T2D, the effect of icv FGF1 to normalize diabetic hyperglycemia in a sustained manner is recapitulated by local delivery of FGF1 to the ARC-ME, but not to the PVN. Since the dose of FGF1 used for microinjection in these studies was below the threshold for inducing detectable glucose lowering when given icv, the effects cannot be attributed to leakage into CSF and subsequent action elsewhere in the brain. Together, these findings identify the ARC-ME area as a target for the sustained anti-diabetic action of FGF1 in the brain.

As a first step toward understanding the cellular basis for this FGF1 response, we colocalized FGF1-induced pERK1/2 with markers of tanycytes (vimentin), astrocytes (GFAP), and neurons (NeuN) in the ARC-ME. Interestingly, whereas a cellular response to FGF1 was readily detected in tanycytes and astrocytes, pERK1/2 induction was not observed in neurons. Although we cannot rule out the possibility that alternative intracellular signaling pathways were activated in neurons, these findings support a model in which effects of FGF1 on ARC-ME neurocircuit activity occur indirectly as a consequence of actions on glial cells, rather than via a direct effect on neurons. This notion is consistent both with evidence implicating tanycytes and astrocytes in brain glucose sensing (Benford et al. 2017; Elizondo-Vega et al. 2015; Rogers et al. 2018), and with the known role played by astrocytes to regulate neuronal function (Covelo and Araque 2018). Future investigation is warranted into the role of glial cells as mediators of the neuronal response to FGF1.

In light of growing evidence of a key role for ARC-ME neurocircuits in the regulation of glucose homeostasis (Bentsen, Mirzadeh, and Schwartz 2018; Deem et al. 2017; Meek et al. 2016), our finding that sustained glucose lowering can be induced by FGF1 action limited to this brain area is not completely unexpected. As a circumventricular organ, the ME is characterized by the absence of a blood-brain barrier, thereby providing glia and neurons in the medial aspect of the ARC with access to circulating nutrients and hormones crucial to metabolic homeostasis. The ARC-ME has also been linked to the pathogenesis of obesity and T2D by virtue of the reactive gliosis involving activation of microglia and astrocytes that develops in this area during high-fat diet feeding (Thaler JP1 2011). Indeed, recent evidence suggests that this gliosis is both necessary and sufficient for obesity to develop on this diet (Valdearcos et al. 2018). Future studies are warranted to determine how FGF1 action on glial cells in this brain area impacts circuits involved in the control of energy balance as well as glucose homeostasis.

While our data highlights the role of the ARC-ME in the sustained remission of diabetic hyperglycemia induced by FGF1, we cannot exclude contributions made by any brain area other than the PVN. Another potential limitation pertains to our decision to map FGF1-responsive brain areas in normal rats, while performing the microinjection studies in diabetic ZDF rats, as it is conceivable that the response differs between these two rat strains. Offsetting this concern is the need to establish the pattern of brain FGF1 responsiveness in normal animals and to then using this information to guide our work, as we have done. Future studies are warranted to investigate whether and how obesity and diabetes might affect brain responsiveness to FGF1. Last, our work also does not address whether an action of FGF1 in the ARC-ME is *necessary* as well as *sufficient* to explain this effect. Ongoing studies seek to answer these questions and to clarify the cellular

and molecular mechanisms involved in the sustained remission of diabetes induced by the central action of FGF1.

2.5 Research Design and Methods

Animals

All procedures were performed according to the NIH Guidelines for Care and Use of Animals and were approved by the Animal Care Committee at the University of Washington. Animals were individually housed under specific-pathogen free conditions in a temperature-controlled room with a 12:12h light: dark cycle and provided with ad libitum (ad-lib) access to water and standard chow unless otherwise stated. Male, 6-week-old Zucker Diabetic Fatty rats (ZDF) (ZDF-Leprfa/Crl) were purchased from Charles River Laboratories (Wilmington, MA), and were provided ad-lib access to Purina 5008 chow (Animal Specialties, Inc., Hubbard, OR). Studies were performed on 8-week-old rats when blood glucose levels obtained under ad-lib fed conditions are typically >250 mg/dl (Etgen and Oldham 2000). For each study, groups receiving icv Veh or FGF1 injection were matched for body weight and blood glucose levels. Male, 8-week-old Wistar rats were purchased from Harlan Laboratories (Madison, WI) to assess immunofluroecent (IF) pERK1/2 activation in the hypothalamus after icv FGF1 injection.

Intra-parenchymal and lateral ventricle guide cannula implantation

Rats were placed in a stereotaxic frame (Koph 1900; Cartesian Research Inc., Sandy, OR) and underwent surgical implantation of an indwelling bilateral cannula directed to the ARC or PVN (Plastics One, Roanoke, VA) under isoflurane anesthesia using stereotaxic coordinates: for ARC, –2.8 mm posterior to bregma; 0.5 mm lateral, and 8 mm depth; for PVN, -1.8 mm posterior to bregma ±0.5 mm lateral, and 6.0 mm posterior to bregma below the skull surface. The cannula

was secured to the skull with stainless steel screws and dental cement. To confirm cannula targeting, we microinjected Cy3-labeled FGF1 into the ARC-ME or PVN and assessed the distribution of the Cy3 label postmortem. In other animals, a single cannula was implanted in the lateral ventricle (LV) for icv injection as previously described (Scarlett et al. 2018). Animals were allowed to recover for at least 7 d before studied.

Intra-parenchymal and Intracerebroventricular (icv) injections

Rats were anesthetized under isoflurane anesthesia and placed in a stereotaxic device. Bilateral intraparenchymal injections of either saline vehicle or recombinant rat FGF1 (FGF1; Prospec-Tany TechnoGene Ltd, East Brunswick, NJ) dissolved in sterile water at a concentration of 1 μg/μl were microinjected over 3 minutes in a volume of 300nl bilateral (total dose = 0.6 μg) using a Hamilton syringe (80030) with a 33g needle that projected 1.5 mm beyond the tip of the cannula, at a rate of 75 nL/min (Micro4 controller, World Precision Instruments, Sarasota, FL) followed by a 5-min delay before needle withdrawal. Icv injection of either saline or FGF1 was performed at 1 wk after cannulation of the lateral ventricle (LV). Injections were given over 1 min using a 33-ga needle extending 2 mm beyond the tip of the LV cannula, as previously described (Scarlett et al. 2018).

Immunofluorescence

To detect FGF1-responsive brain areas, pERK1/2 (which reflects activation of the MAP Kinase/ERK pathway) was detected by immunohistochemistry (IHC) in habituated overnight-fasted Wistar rats. Twenty minutes following icv injection of either vehicle or rFGF1 (3ug), rats were anesthetized with ketamine and xylazine and perfused with PBS followed by 4% paraformaldehyde in 0.1M PBS, after which brains were removed. Anatomically-matched free-

floating coronal sections (35 µm thickness) were collected from –1.2 mm bregma to –14.7 mm bregma, washed in phosphate-buffered saline at room temperature, permeabilized in NP40 1%, 1.0% BSA for 10 min, blocked in freshly prepared 5% normal goat serum (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania) and incubated overnight at 4°C with rabbit anti-pERK1/2 antibody (1:1000; #4370S, Cell Signaling, Danvers, Massachusetts). To detect pERK (a marker of FGF receptor activation (Zakrzewska et al. 2013; Wu et al. 2011)) in tanycytes, neurons or astrocytes, sections were incubated with chicken anti-vimentin (1:1000; ab24525, Abcam, Eugene, Oregon), mouse anti-NeuN (1:1000, MAB377, Millipore Sigma, Burlington, Massachusetts) or mouse GFAP-cy3 (1:1000; C9205, Millipore Sigma, Burlington, Massachusetts), respectively. After incubation at 4°C for 6 h with goat anti-rabbit Alexa 488 (secondary antibody) diluted 1:1000, sections were washed overnight in PBS and mounted on slides. pERK1/2 immunofluorescence density was assessed in a semi-quantitative manner as follows: ******/***** denotes a strong signal that occupies the entire region/nucleus, ****/*** a moderate signal that occupies most of the region, and **/* denotes minimal signal occupying a small region. Immunofluorescence images were captured using a Leica SP8X Scanning Confocal microscope (Buffalo Grove, IL) with a HC FLUOTAR L 25X/0.95 W objective.

Statistical Analysis

All results are expressed as mean ± SEM and statistical comparisons performed using R (Team 2013) with the interface R Studio (Team 2016). Longitudinal data were analyzed using a linear mixed model including fixed effect of treatment and day and random effects of animal. Linear mixed models were conducted with the R statistical package "nlme" (Jose Pinheiro 2013), one way ANOVA or the equivalent nonparametric test using the R statistical package "nparLD" (Konietschke 2012). Probability values of less than 0.05 were considered significant.

Chapter 3. ROLE OF HYPOTHALAMIC MAPK/ERK SIGNALING IN DIABETES REMISSION INDUCED BY THE CENTRAL ACTION OF FIBROBLAST GROWTH FACTOR 1

3.1 SUMMARY

Sustained remission of hyperglycemia in rodent models of type 2 diabetes (T2D) can be achieved by a single intracerebroventricular (icv) injection of fibroblast growth factor 1 (FGF1), and the hypothalamus has been identified as a key target for this effect. Here, we show that icv FGF1 induces sustained signaling by extracellular signal-regulated kinases 1 and 2 (ERK1/2), members of the mitogen-activated protein kinase (MAPK/ERK) family, in the hypothalamus. We report that pharmacologic blockade of MAPK/ERK signaling in the hypothalamus using the selective MAPK inhibitor U0126 abolished the anti-diabetic effect of icv FGF1 in diabetic $Lep^{ob/ob}$ mice. Further, icv injection of R50E FGF1, a FGF1 mutant that activates FGF receptors but does not induce sustained MAPK/ERK signaling, recapitulates the transient anorexia and weight loss induced by native FGF1, but fails to elicit sustained diabetes remission. Combined, our data implicate sustained hypothalamic MAPK/ERK signaling in diabetes remission induced by icv FGF1.

3.2 INTRODUCTION

Type 2 diabetes (T2D) is among the most common and costly chronic diseases affecting human health(Zheng, Ley, and Hu 2018; Federation 2019). Treatment outcomes for affected patients with T2D have not improved in over a decade; indeed, less than half of patients currently meet therapeutic targets, despite the introduction of over 40 new drugs since 2005 (Edelman and Polonsky 2017). Therefore, an urgent need for new, more effective treatment options exists, and strategies targeting the brain have the opportunity to meet this need(Gasser et al. 2017; Brown, Scarlett, and Schwartz 2019).

We recently reported that a single intracerebral ventricular (icv) injection of fibroblast growth factor 1 (FGF1) can induce remission of diabetic hyperglycemia in both mice (Lep$^{ob/ob}$ and LepR$^{db/db}$) and rat (Zucker Diabetic Fatty (ZDF)) models of T2D (Scarlett et al. 2016; Scarlett et al. 2019). Further, in ZDF rats, the effect of icv FGF1 to normalize diabetic hyperglycemia in a sustained manner is recapitulated by microinjection of FGF1 to hypothalamus, identifying the hypothalamus as a target for the sustained antidiabetic action of FGF1 in the brain (Brown et al. 2019). These studies also revealed robust FGF1-induced extracellular signaling-regulated kinase 1 and 2 (ERK1/2) staining in the hypothalamus (Brown et al. 2019). The current work was undertaken to investigate the role of hypothalamic MAPK/ERK signaling in the anti-diabetic effect of icv FGF1.

Among multiple intracellular signaling cascades that are activated by FGF1-FGF receptor (FGFR) signaling is the mitogen-activated protein kinase (MAPK)/ERK pathway (Zhao et al. 2019; Raju et al. 2014). Hypothalamic MAPK/ERK signaling is implicated in the anti-diabetic effects of FGF signaling in the brain as the endocrine FGF, FGF19, activates ERK1/2 signaling in the arcuate nucleus (ARC) of diabetic mice, and this ERK1/2 activation is required for the acute glucose-lowering effects (Marcelin et al. 2014; Morton et al. 2013). Similar to FGF19, we observed in mice that icv administration FGF1 activated ERK1/2 in both tanycytes and astrocytes the ARC (Brown et al. 2019). However, unlike FGF19 which induces only a transient increase in hypothalamic ERK1/2 signaling (Marcelin et al. 2014), FGF1 was found to induce a more durable response lasting >24 h.

To establish a mechanistic link between hypothalamic MAPK/ERK signaling and the sustained anti-diabetic effect of icv FGF1, we sought to determine if disrupting FGF1-induced hypothalamic MAPK/ERK activation would block the anti-diabetic effect of FGF1.

We show here that the sustained anti-diabetic response to icv FGF1 in Lep$^{ob/ob}$ mice is blocked by co-administration of the MEK1/2 inhibitor U0126. Further, we observed that R50E FGF1, a FGF1 mutant that activates FGF receptors and induces transient, but not sustained MAPK/ERK signaling(Mori et al. 2008; Mori et al. 2013), fails to elicit sustained diabetes remission. Collectively, our data support a mechanism whereby icv FGF1 activates MAPK/ERK signaling in the hypothalamus; that this activation is sustained for 24 hours; and that this prolonged activation is required for FGF1 to induce sustained remission of diabetic hyperglycemia.

3.3 RESULTS

Quantitative analysis shows hypothalamic ERK1/2 activation is sustained for 24 hours.

Consistent with our previous observations (Brown et al. 2019), we found that a single icv injection of FGF1 (3 μg) induced phosphorylation of ERK1/2 (pERK1/2) in the MBH within 20 minutes relative to vehicle-treated controls in overnight-fasted mice as assessed using quantitative western blot (Fig. 1A). To our surprise, not only did icv FGF1 induce acute activation of ERK1/2, this effect was sustained for up to 24 H (Fig. 1 B and C). Notably, the effect seen at 24 H had returned to control levels by 48 H (Fig. 1 D). These data suggest that the central administration of FGF1 activates the MAPK/ERK pathway the mediobasal hypothalamus in prolonged manor.

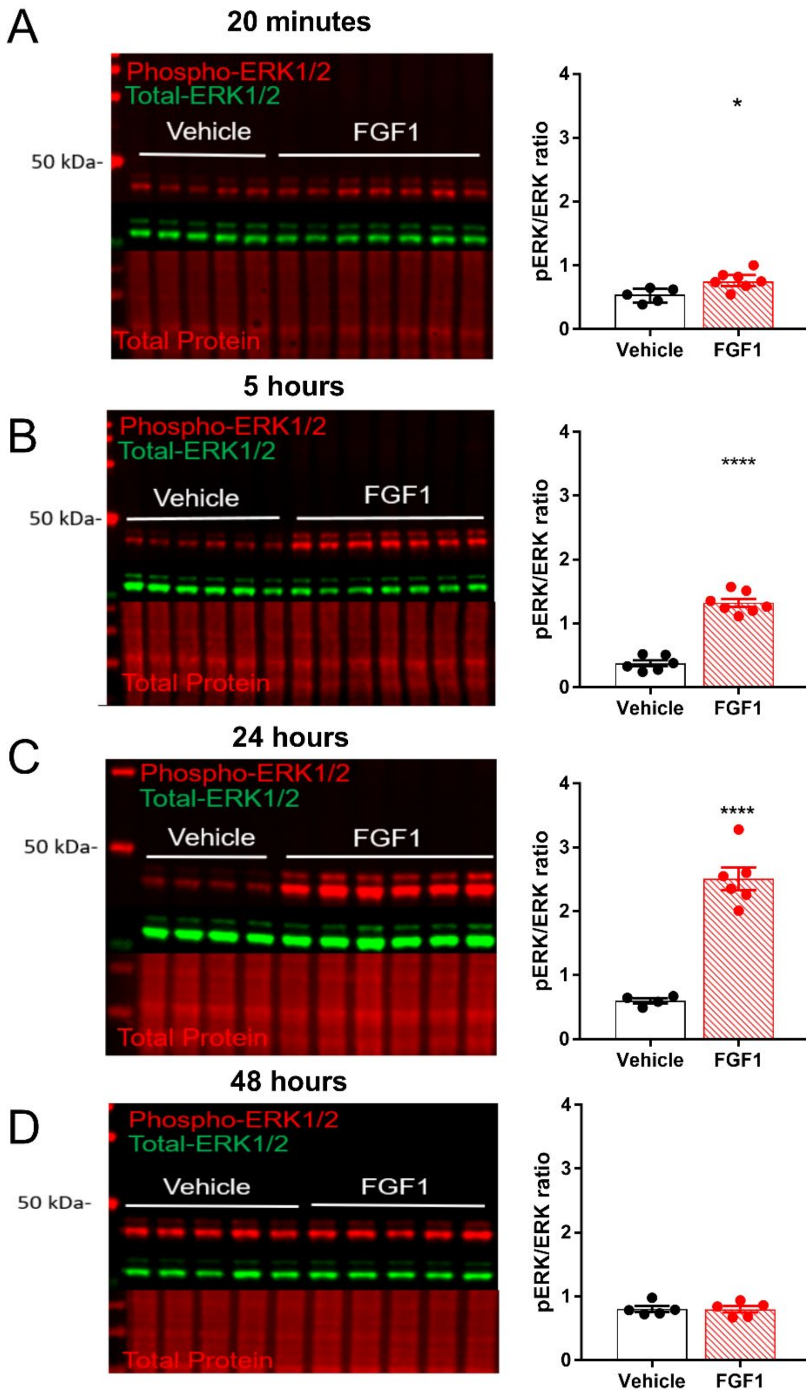

Figure 3-1 Quantitative analysis shows hypothalamic ERK1/2 activation is sustained for 24 hours.
Representative western blot (left panel) and quantitative comparison (right panel) of phosphorylated and total ERK1/2 and total protein from hypothalamic punches from adult male C57Bl6J mice at A) 20 minutes B) 5 C) 24, and D) 48 hours after a single icv injection of either vehicle or 3 ug of FGF1 (n=4-7 per group). *P<0.05, ****P<0.0001

Diabetes remission induced by central FGF1 requires sustained hypothalamic ERK1/2 activation.

To directly test whether sustained diabetes remission induced by icv FGF1 in diabetic Lep$^{ob/ob}$ mice requires prolonged MAPK/ERK signaling, we generated four groups of these mice. Each group received three separate icv treatments: a pre- icv injection loading dose of either the MEK inhibitor, U0126, or vehicle, followed by an icv injection of FGF1 or Vehicle, followed immediately by continuous icv infusion of the same pre icv injection compound for 24 hours (Figure 2a). We found that in order to inhibit sustained pERK1/2 induced in the MBH by icv injection of FGF1, a continuous infusion of U0126 into the third ventricle for 24 H was required (Sup. Fig 1). Diabetic Lep$^{ob/ob}$ mice underwent indwelling cannula placement targeted at the 3V for both icv injection and icv continuous infusion. Icv FGF1 injection elicited initial reduction of food intake and sustained normalization of glycemia when it was coupled to continuous icv infusion of the vehicle. When icv FGF1 injection was combined with continuous U0126 infusion, however, remission of hyperglycemia was not sustained (Fig. 2B) and even though there was no difference of either food intake or body weight since all the treatment groups were pair-fed to the icv FGF1 vehicle osmotic pump group until the food intake returned to pre icv injection levesl (Fig. 2D). These findings collectively demonstrate that although MAPK/ERK signaling is not required for acute, transient effects of icv FGF1 injection on food intake and body weight (Sup. Fig 1), it is required for sustained diabetes remission. Because FGF1-induced MAPK/ERK signaling is limited primarily to the hypothalamus, we infer that it is in this brain area that signaling transduction via this pathway is required for FGF1-induced glucose lowering.

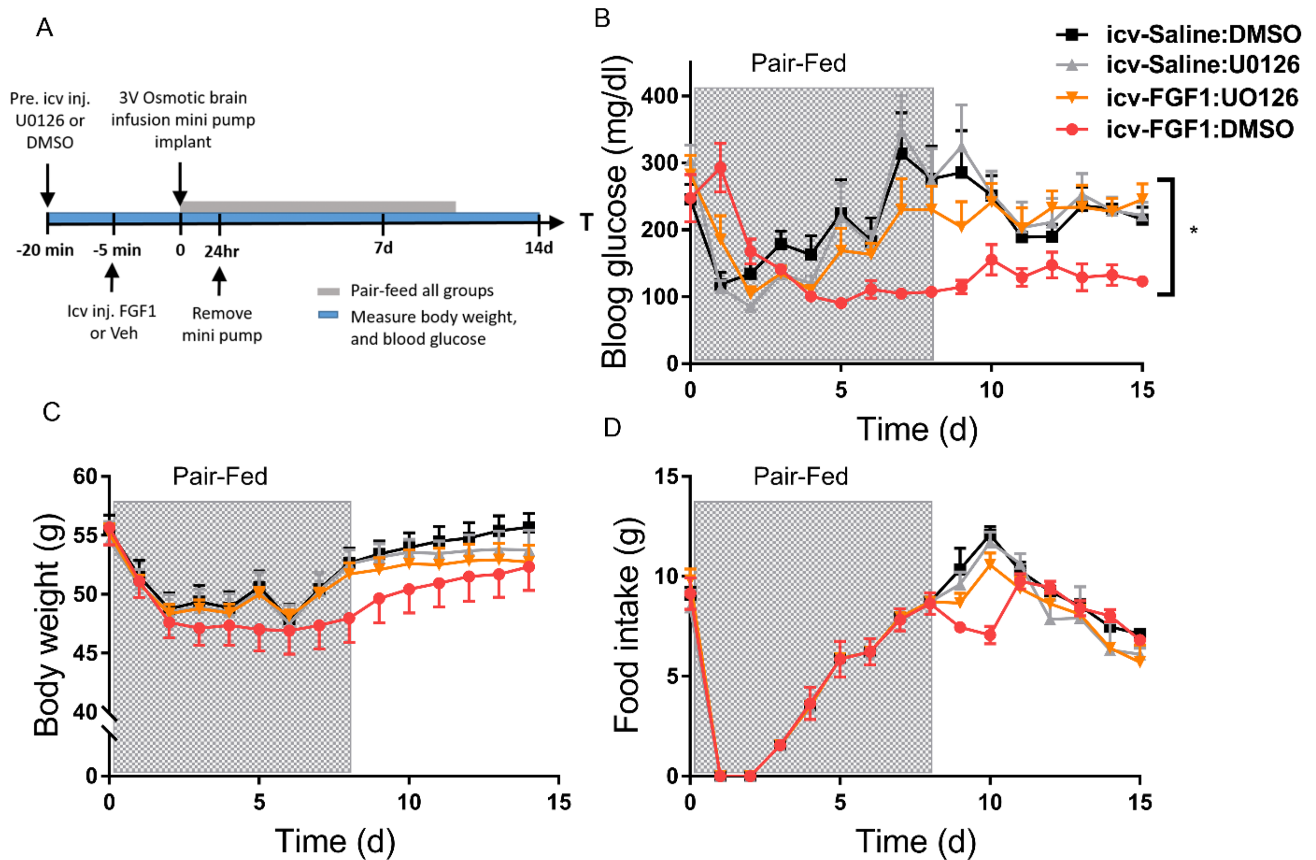

Figure 3-2 Sustained hypothalamic ERK1/2 activation is required for diabetes remission induced by central FGF1

A) Strategy for MAPK/ERK inhibition by continuous 3V infusion of U0126 or Vehicle DMSO for 24 hours followed by disconnection of osmotic pump and metabolic phenotyping. B) blood glucose, C) Body weight, and D) food intake was measured for 15 days post treatment of either an icv injection of Vehicle saline or 3ug FGF1 followed by continuous infusion of U0126 or Vehicle DMSO (n=5-8 per group) *$P<0.05$.

ERK1/2 Activation in the MBH by FGF1 Mutant R50E FGF1 is Attenuated.

Next, we next took advantage of an FGF1 mutant protein. Previous evidence suggests that a mutant FGF1, R50E FGF1, where arginine at the amino acid position 50 is substituted with glutamic acid, retains the ability to bind and activate FGFR (Mori et al. 2013; Mori et al. 2008; Yamaji et al. 2010), but fails to induce pERK1/2 in a sustained manner in cell culture. We therefore sought to determine if FGF1 R50E induces either transient or sustained ERK1/2 signaling in the MBH of C57Bl6J mice. Western blot and

histochemical analysis of the MBH revealed similar pERK1/2 activation by icv injection of FGF1 or FGF1 R50E compared to vehicle-treated controls for up to 8 hours post-injection (Fig. 3A). Importantly, similar to findings in vitro (Mori et al. 2008), R50E FGF1 failed to induce sustained phosphorylation of ERK1/2 as assessed by western blot (Fig. 3A). Histochemical analysis also revealed far less ERK1/2 activation in tanycytes and astrocytes 14 hours after icv injection of FGF1 R50E FGF1 compared to native FGF1 (Fig. 3B and Fig. 3C).

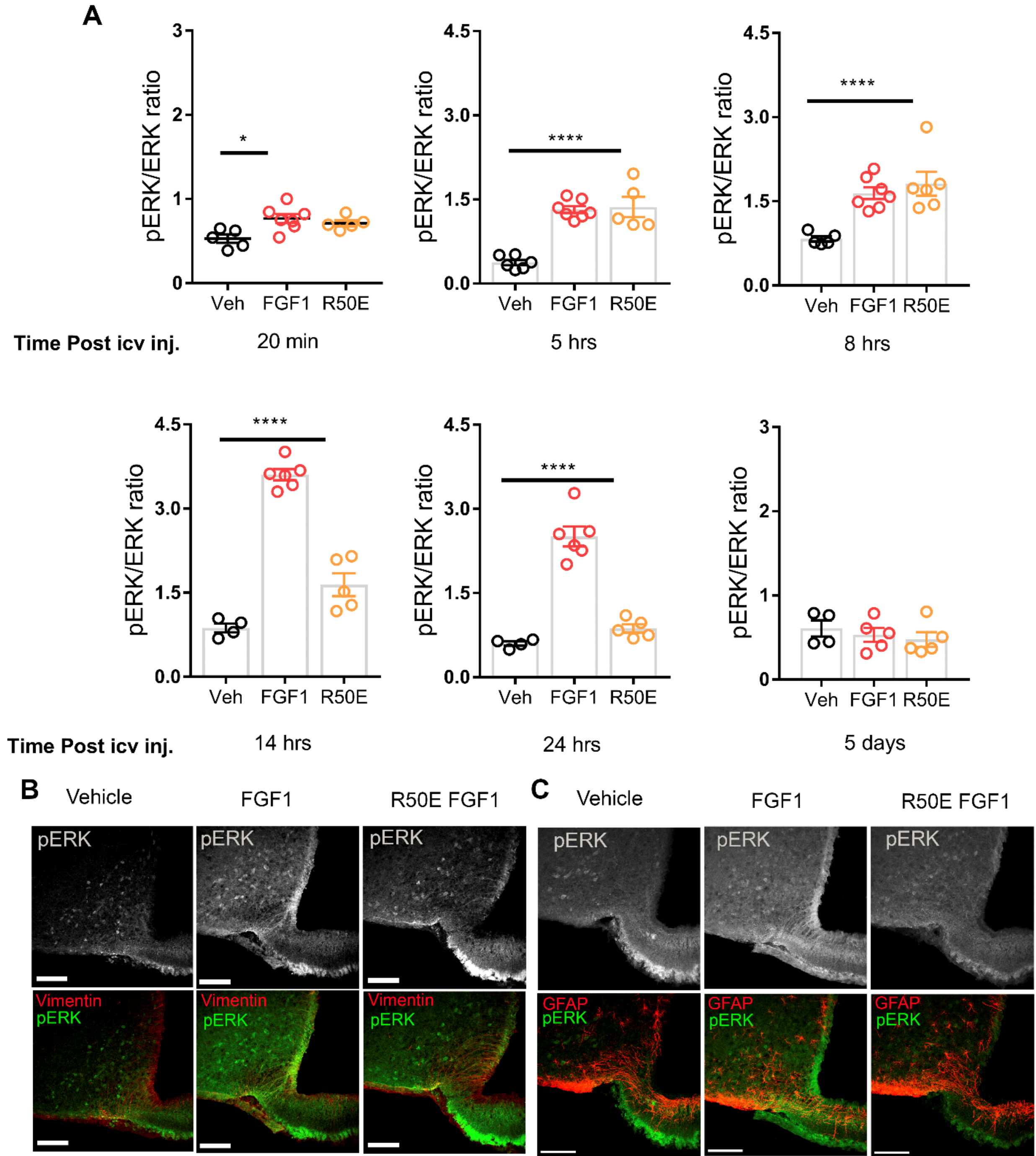

Figure 3-3 FGF1 Mutant R50E FGF1 Attenuates ERK1/2 Activation in the MBH
A) Quantitative western blot time course of ERK1/2 phosphorylation after a single icv injection of R50E FGF1 B) confocal images of coronal sections showing pERK1/2 (green) and vimentin (red) and C) pERK1/2 (green) and GFAP (red) 14 hours after a single icv injection of either vehicle, (3µg) FGF1 or (3µg) R50E FGF1. (n=4-7 per group *P<0.05, ****P<0.0001)

Sustained Glucose lowering is not induced with R50E FGF1

We next sought to determine if R50E FGF1 mimics the effect of FGF1 to induce sustained glucose lowering following icv injection in diabetic Lep$^{ob/ob}$ mice. We found that in Lep$^{ob/ob}$ mice, icv injection of FGF1 induced sustained pERK1/2 in the MBH as was observed in normal mice whereas the duration of pERK induction by FGF1 R50E was attenuated (Fig 4 A). Like the potent effect of FGF1, FGF1 R50E induced transient reductions of food intake, body weight, and blood glucose (Figure 4 B-D). However, FGF1 R50E failed to induce sustained glucose lowering (Figure 4 B). These data collectively demonstrate that although icv injection of R50E FGF1 induces acute but transient MBH pERK activation, as well as anorexia, weight loss and blood glucose lowering responses induced by native FGF1, it fails to elicit either sustained pERK activation or sustained diabetes remission.

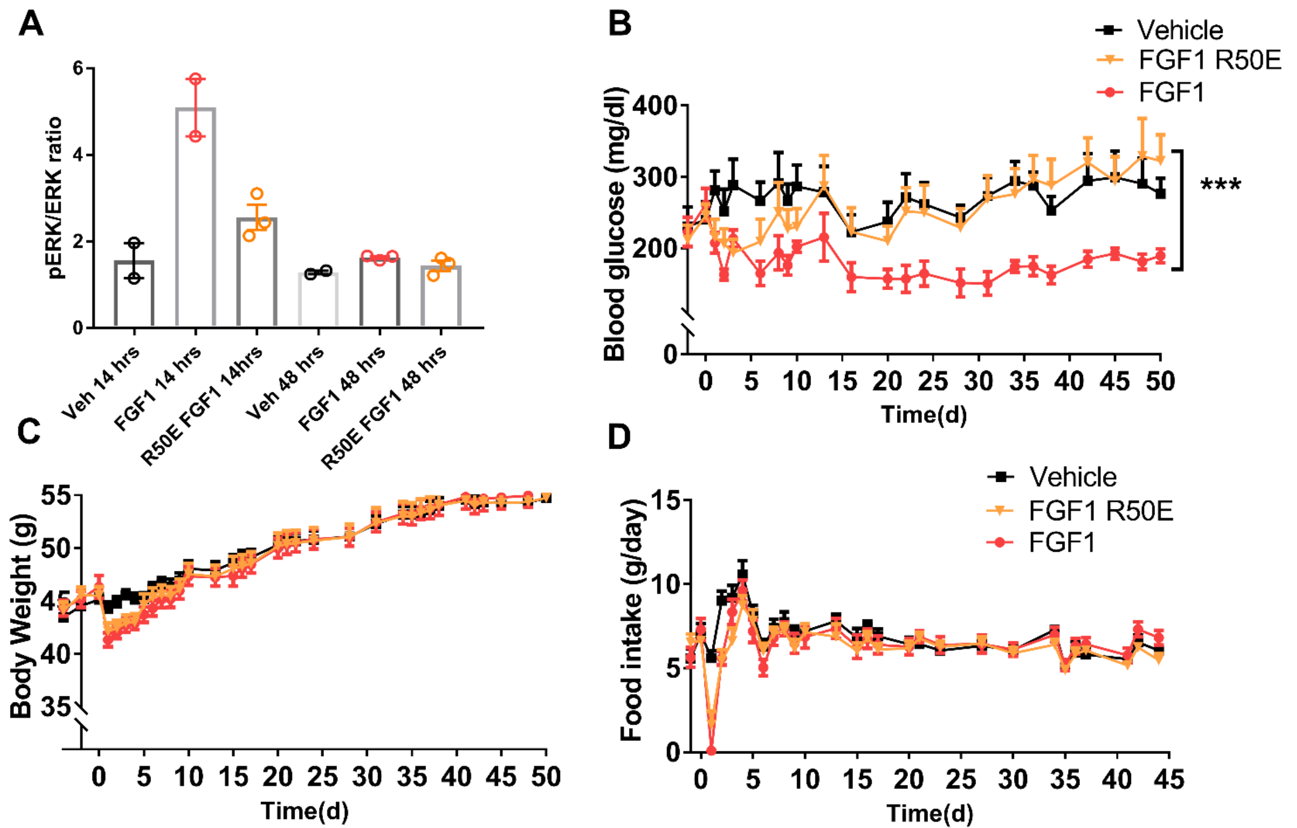

Figure 3-4 Failure of R50E FGF1 to induce diabetes remission in Lep$^{ob/ob}$ mice.
A) Quantitative western blot time course of ERK1/2 phosphorylation after a single icv injection of R50E FGF1 B) Plasma glucose levels C) Body weight and D) food intake from Lep$^{ob/ob}$ mice after a single icv injection of Vehicle, FGF1, or FGF1 R50E. (n=8-9 per group, ***P<0.001)

Unlike endocrine FGF19 FGF1 induces sustained cellular changes in the MBH after icv administration.

Astrocytes are highly responsive to icv injection of FGF1 (Brown et al. 2019), and retain prolonged MAPK/ERK signaling (Fig 3C). Our recent studies showed robust induction of glial fibrillary acidic protein (GFAP) associated with striking changes in astrocyte morphology one week after icv injection of FGF1 compared to vehicle-treated controls (In press Bentsen and Rausch et al. Nat. Commun, 2020) implicating sustain

changes in astrocytes as a mediator of the hypothalamic response to central FGF1. To compare GFAP expression between FGF1 and a related FGF peptide that is known to improve glucose homeostasis we included animals receiving FGF19, since icv administration of FGF19 induces pERK1/2, and this effect is required for it to improve glucose tolerance in Lep$^{ob/ob}$ mice (Marcelin et al. 2014). We first assessed the ability of icv injection of FGF19 to induce sustained pERK1/2 activation in the hypothalamus of wild type mice. Unlike central FGF1, FGF19-induced ERK activation is similar to icv Vehicle at 24 hours (Fig 5A). As expected, icv FGF19 injection also did not induce sustained glucose lowering in diabetic Lep$^{ob/ob}$ mice (Fig 5B and C). To evaluate if the effect of FGF1 to induce changes in astrocyte morphology is sustained for two weeks, we performed immunohistochemistry of coronal sections using antibodies against GFAP from diabetic Lep$^{ob/ob}$ mice treated with icv injection of vehicle, FGF19, or FGF1. Fourteen days post-injection GFAP+ astrocytes were increased in the ARC area after icv injection of FGF1 compared to either vehicle or icv FGF19 treatment (Fig 5D). These findings support a model in which, unlike FGF19, FGF1 induces sustained changes in astrocytes that may participate in the sustained antidiabetic action of FGF1.

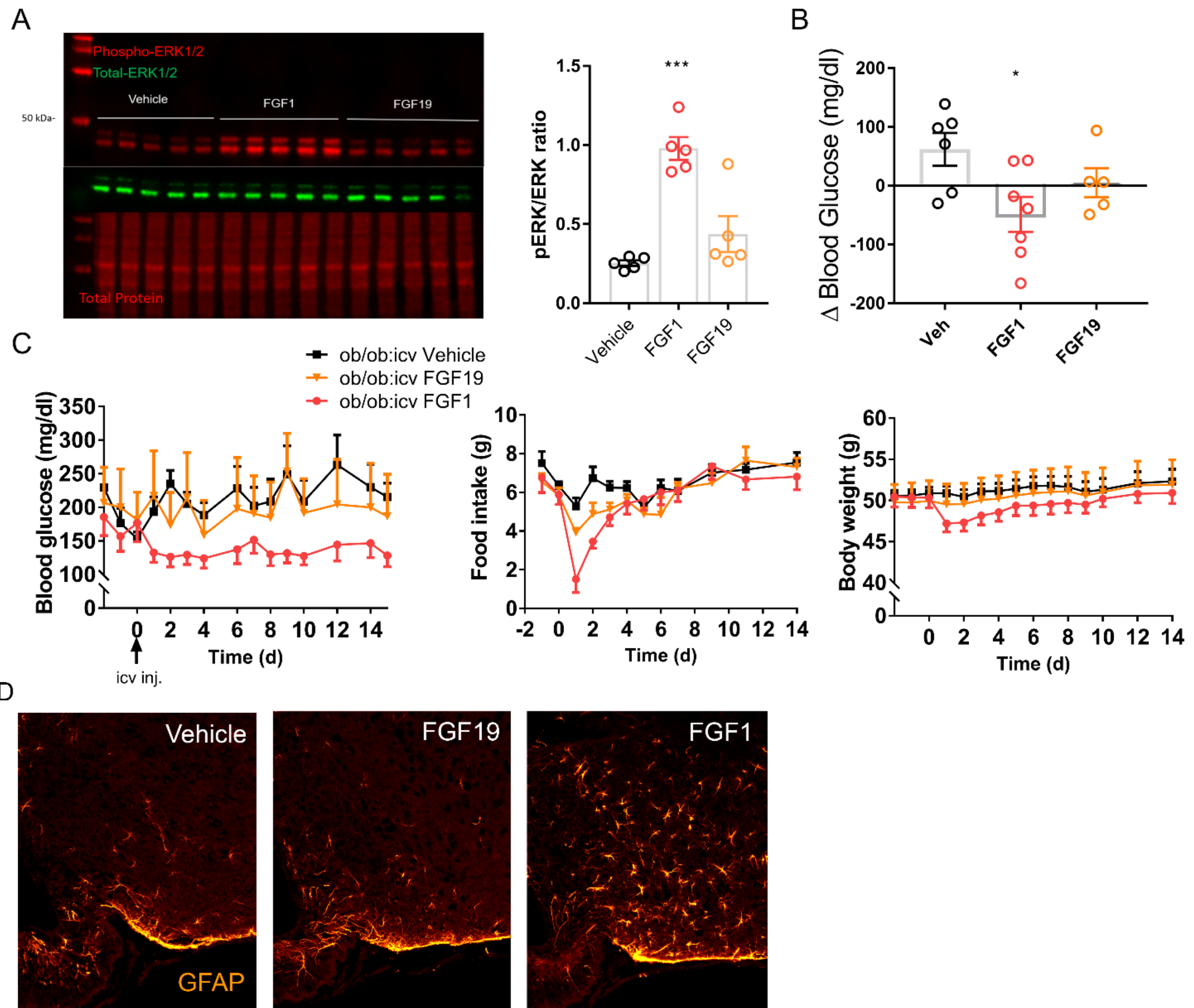

Figure 3-5 Unlike endocrine FGF19 FGF1 induces sustained cellular changes in the MBH after icv administration.
A) Quantitative western blot time course of ERK1/2 phosphorylation 24 hours after a single icv injection of FGF19 B) Change in plasma blood glucose 14 days after icv injection C) Plasma glucose levels, body weight and food intake from Lep$^{ob/ob}$ mice after a single icv injection of Vehicle, FGF1, or FGF19. D) Hypothalamic GFAP expression 14 days after icv injection. (n=5-7 per group *P<0.05 and ***P<0.001)

3.4 DISCUSSION

Based on the activation of MAPK/ERK downstream of FGF receptors (measured by pERK1/2 western blot and immunostaining), we report that icv injection of FGF1 engages prolonged activation of the hypothalamic MAPK/ERK signaling pathway. We further demonstrated that in diabetic Lep$^{ob/ob}$ mice a model of T2D, the effect of icv FGF1 to normalize diabetic hyperglycemia in a sustained manor requires prolonged MAPK/ERK signaling, since: blockade of FGF1 action with U0126, an inhibitor of MAPK/ERK signal transduction also fails to elicit sustained diabetes remission, and icv injection of a mutant FGF1 R50E FGF1 that lacks a sustained signaling capacity blocked the sustained glucose-lowering effect of central FGF1. Together these findings identify a key role for sustained hypothalamic MAPK/ERK signaling in the sustained antidiabetic action of FGF1 in the brain.

ERK activation is known to affect gene expression within hypothalamic glucoregulatory neurocircuits, and previous studies have implicated hypothalamic ERK action in the regulation of glucose homeostasis (Zhang et al. 2015; Mayer and Belsham 2009). Our findings extend this previous work. To establish a role for hypothalamic MAPK/ERK signaling in central FGF1-induced sustained glucose-lowering, we characterized the time course of hypothalamic pERK1/2 induction after icv FGF1. To our surprise, pERK1/2 was induced in the hypothalamus for up to 24 H following a single icv FGF1 injection in both wild-type and Lep$^{ob/ob}$ mice, whereas the duration of this response was reduced by ~70% following icv R50E FGF1 injection.

R50E FGF1 activates FGFRs and subsequent downstream signaling pathways but does so in a transient manner compared to native FGF1. Similarly, R50E FGF1 has transient effects

resembling those of native FGF1 on food intake, body weight, and blood glucose following icv injection, and yet sustained diabetes remission was not observed.

In addition to signal transduction downstream of the FGF receptor, FGF1 can also activate integrin signaling. Integrins are a family of cell adhesion receptors that recognize both extracellular matrix ligands and cell surface ligands (growth factors, in particular)(Mori et al. 2013). Integrin receptors are heterodimers composed of an alpha and a beta subunit that can be assembled into 24 different combinations. Whereas native FGF1 induces integrin receptor co-activation, the R50E FGF1 mutant does not, and this likely explains the shortened duration of pERK signaling in the MBH (Mori et al. 2008; Yamaji et al. 2010; Mori et al. 2013), although, Szlachcic et al. suggests that a lower stability of the mutant FGF1 leds to a shorter duration of its biological activity (Szlachcic et al. 2019). Moreover, the role of integrin signaling in FGF1-induced diabetes remission warrants further studies.

A large group of investigators in the Schwartz lab continue to focus on the question of how distinct hypothalamic cell types respond to centrally-administered FGF1 over time. Among many different technologies employed in this effort are single-cell and single-nucleus transcriptomic analysis, pharmacology, histochemistry and electron microscopy, work that is being performed in collaboration with investigators from several other institutions (In press Bentsen and Rausch et al. Nat. Commun, 2020). Among the findings revealed by this work is that in the MBH, glial cells are far more responsive to FGF1 than are neurons, leading us to hypothesize that sustained remission of hyperglycemia induced by FGF1 may involve an action on tanycytes, astrocytes or other glial cell types that in turn promote the restructuring of glucoregulatory neurocircuits. Unlike FGF1, FGF19 does not induce pERK in a sustained manner in the hypothalamus. Combined with evidence that, unlike FGF1, FGF19 does not

induce either fos in tanycytes or a reduction in basal blood glucose levels (Scarlett et al. 2016), we therefore sought to determine if the response of MBH astrocytes to FGF1 is sustained and whether it differs from an endocrine FGF, FGF19. Expanding on our previous finding, we found that icv injection of FGF1 is associated with increased astrocyte GFAP protein expression in the ARC-ME, and that this response was not observed after icv FGF19. How this astrocyte response may contribute to the sustained antidiabetic action of FGF1 is under investigation.

Taken together, we conclude that in diabetic $Lep^{ob/ob}$ mice, sustained diabetes remission induced by icv injection of FGF1 involves a robust and highly durable pERK response in the MBH and that the sustained antidiabetic action of FGF1 is ERK-dependent, whereas the transient anorexia is not. Prolonged MAPK/ERK signaling in astrocytes, and tanycytes further implicate glia cells in the glycemic benefit stemming from FGF1 action in the hypothalamus. Ongoing studies will continue to clarify the role of glia-neuron interactions in brain control of glucose homeostasis.

3.5 METHODS

Animals

Male, 8-week-old C57BL/6J (WT) and $Lep^{ob/ob}$ (B6.Cg-Lep^{ob}/J) mice were purchased from Jackson Laboratory (Bar Harbor, ME). All animals were housed individually under specific pathogen-free conditions in a temperature control environment with either a 12 h: 12h or 14 h:10 h light: dark cycle with ad libitum access to water and standard laboratory chow (LabDiet, St Louis, MO). All animal procedures were performed according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the Institutional Animal Care and Use Committee at the University of Washington.

Surgery

Lateral ventricle (LV) and third ventricle (3V) cannulation (LV;26-ga, Plastics One, Roanoke, VA) were performed under isoflurane anesthesia using the following stereotaxic coordinates for mice 3V: -1.8 mm posterior to bregma; mid-line and -4.3 mm below the skull surface; LV: -0.7 mm posterior to bregma; 1.3 mm lateral, and 1.3 mm below the skull surface. Animals received buprenorphine hydrochloride (Reckitt Benckiser Pharmaceuticals Inc., Richmond, VA) for pain relief and were allowed to recover for one week prior to the study.

Icv injections

Mean blood glucose and body weight values were matched between groups before the icv injections. Animals received a single icv injection via the LV of either saline vehicle, recombinant mouse FGF1 (FGF1; Prospec-Tany TechnoGene Ltd., East Brunswick, NJ) dissolved in sterile water at a concentration of 1.5 μg/μl, R50E FGF1 (A generous gift from Novo Nordisk) dissolved in 20 mM Tris pH 8.0, 0.5 M NaCl or human FGF19 (hFGF19; Phoenix Pharmaceuticals, Burlingame, CA) was dissolved in 0.9% normal saline at a concentration of 1.5 μg/μl and injected using a 33-gauge needle extending 0.8 mm beyond the tip of the icv cannula over 60 s for a final volume of 2 μl as previously described (Scarlett et al. 2016; Morton et al. 2013).

To determine if the effect of icv FGF1 to induce sustained remission of hyperglycemia in Lep$^{ob/ob}$ mice depends on sustained MAPK/ERK signaling, we studied four groups of diabetic *Lep*$^{o/bob}$ mice. Two groups received a single icv injection of FGF1, while the other two received icv vehicle injected into the 3V using a 33-gauge needle extending 1mm beyond the tip of the icv cannula. Each group also received a pre injection and an icv infusion of either the selective MEK inhibitor U0126 (19-147; Millipore Sigma, St. Louis, MO) dissolved in DMSO at a

concentration of 30mM or DMSO vehicle into the 3V via an micro-osmotic pump (model 1003D; Alzet, Durect Corporation, Cupertino, CA) connected to the 3V cannula for 24 hours at 1 µl per hour.

Quantitative Western Botting

Hypothalamic punches (3 mm) were collected after indicated treatment. Dissection of a single coronal section (1.5 mm) between the rostral and caudal Circle of Willis using an ice-cold brain matrix followed by a 3 mm biopunch (Harris Uni-Core, Ted Pella Inc, Redding, CA) of the MBH was flash-frozen in liquid nitrogen and stored at -80 °C until further processing. Hypothalamic punches were homogenized by sonication in lysis buffer and centrifuged at 10,000 g for 15 minutes supernatant was collected and assayed for protein concentration using a BAC assay. Lysates were mixed in the Licor protein sample loading buffer and heated to 100 °C for 5 minutes. Samples were run on a 10% Bis-Tris criterion XT gel (Bio-Rad Laboratory Inc., Hercules, CA) 200 volts for 25 minutes electrophoretic transferred at 100 V for 45 minutes. Loaded protein concentrations were determined based on primary antibodies 1:1000 rabbit anti-pERK1/2 antibody (#4370;Cell Signaling Technology, Danvers, MA), mouse anti-ERK1/2 (#9107; Cell Signaling Technology, Danvers, MA), secondary IRDye 680RD (#926-68073 Li-cor, Lincoln, NE), IRDye 800CW (#92632212; Li-cor, Lincoln, NE) and total protein Revert 700 Total Protein Stain Kit (# 926-11011; Li-cor, Lincoln, NE) combined linear range.

Immunofluorescence

Activation of the MAPK/ERK pathway at 14 hours post icv injection pERK1/2 was detected by immunohistochemistry in habituated overnight-fasted C57Bl6J mice. Fourteen hours following icv injection of either vehicle or recombinant FGF1 (3ug), mice were anesthetized as previously decided (Brown et al. 2019). Cryostat sections (30-um thick) free-floating sections

were permeabilized with 0.4% PBS-T overnight Wash in 0.4% PBS-T incubate in pERK antibody, Host: Rabbit, (add #) 1:1000 in (3%BSA+0.4%TritonX-100+ 0.2%Normal Donkey Serum in PBS-azide) for 24hr at 4C Wash sections 5× 10min with 0.4% PBS-T Incubate in Secondary Antibody (DαR 594-A21207) 1:1000 in (3%BSA+0.4%TritonX-100+ 0.2%Normal Donkey Serum in PBS azide) for overnight in 4 C Incubate in DAPI 1:10,000 in PBS for 10min at RT Wash tissue 3× 10min with PBS Mount, dry and coverslip with PVA mounting media. Immunofluorescence images were captured using a Leica SP8X Scanning Confocal microscope (Leica Microsystems, Buffalo Grove, IL) with an HC FLUOTAR L 25X/0.95 W objective.

To assess the changes in astrocytes GFAP expression in response to central FGF1 or FGF19 diabetic $Lep^{ob/ob}$ mice underwent LV cannulation. Animals received an icv injection of either veh, FGF1, or hFGF19 two weeks later, mice were anesthetized and perfused and their brains were processed for immunohistochemical analysis. Briefly, 14 um thick sections in the coronal plane throughout the region of interest were direct mounted on slides and incubated with anti-GFAP-cy3 (1:1000) (C9205; Millipore Sigma, St. Louis, MO). Slides were coverslip with PVA mounting media. Images were captured using a Nikon Eclipse E600 upright microscope (Nikon) equipped with a Diagnostic Instruments Spot RT Color digital camera.

Statistical Analysis

Data from individual experiment are shown as dot plots representing data from individual animals and average ±SEM. Statistical analyses were performed using Prism 7 (Graph Pad) and R (Team 2013). Student's t-test was used to compare the means in two groups and one-way ANOVA to compare three groups. Longitudinal data were analyzed using a linear mixed model including fixed effect of treatment and day and random effects of animal. Linear mixed models were conducted with the R statistical package "nlme"(Jose Pinheiro 2013), or the equivalent

nonparametric test using the R statistical package “nparLD” (Konietschke 2012). Probability values less than 0.05 were considered statistically significant.

Chapter 4. PERIPHERAL MECHANISMS MEDIATING THE SUSTAINED ANTI-DIABETIC ACTION OF FGF1 IN THE BRAIN.

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4.1 ABSTRACT

We recently reported that in rodent models of type 2 diabetes (T2D), a single intracerebroventricular (icv) injection of fibroblast growth factor 1 (FGF1) induces remission of hyperglycemia that is sustained for weeks. To clarify the peripheral mechanisms underlying this effect, we employed the Zucker diabetic fatty *fa*/*fa* rat model of T2D, which, like human T2D, is characterized by progressive deterioration of pancreatic β-cell function after hyperglycemia onset. We report that while icv injection of FGF1 delays the onset of β-cell dysfunction in these animals, it has no effect on either glucose-induced insulin secretion or insulin sensitivity. These observations suggest that FGF1 acts in the brain to stimulate insulin-independent glucose clearance. Based on our finding that icv FGF1 treatment increases hepatic *glucokinase* gene expression, we considered the possibility that increased hepatic glucose uptake (HGU) contributes to the insulin-independent glucose-lowering effect of icv FGF1. Consistent with this possibility, we report that icv FGF1 injection increases liver glucokinase activity by a ~2-fold. We conclude that sustained remission of hyperglycemia induced by the central action of FGF1 involves both preservation of β-cell function and stimulation of HGU via increased hepatic glucokinase activity.

4.2 INTRODUCTION

Unlike other therapies for type 2 diabetes (T2D), remission of hyperglycemia lasting for weeks or longer can be induced by a single intracerebroventricular (icv) injection of fibroblast growth factor 1 (FGF1) (Scarlett et al. 2016). While this prolonged anti-diabetic effect of icv FGF1 was observed in both *ob/ob* mice and Zucker Diabetic Fatty *fa*/*fa* (ZDF) rats, it lasted longer (>4mo) in the former than in the latter animals (~4 wk), potentially owing to the

progressive deterioration of β-cell function characteristic of ZDF rats following hyperglycemia onset (Shiota and Printz 2012). As this deterioration does not occur in *ob/ob* mice (on the C57Bl6 background), the ZDF rat model more closely approximates human T2D, which is also characterized by progressive loss of β-cell function and mass over time (Chen et al. 2017).

We therefore selected the ZDF model for the current studies, which were undertaken to identify peripheral mechanisms responsible for sustained glucose lowering induced by the central action of FGF1. Our results suggest that in these animals, icv injection of FGF1 induces remission of hyperglycemia both by delaying the onset of β-cell failure and by increasing hepatic glucose uptake (HGU) via increased hepatic glucokinase (GCK) activity.

4.3 RESEARCH DESIGN AND METHODS

Animals

All procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee at the University of Washington. Animals were individually housed under specific-pathogen free conditions in a temperature-controlled room with a 12:12h light:dark cycle and provided with *ad libitum* (*ad-lib*) access to water and Purina 5008 chow (Animal Specialties, Inc.). Male, 6-week-old ZDF (ZDF-Lepr^{fa}/Crl) and lean controls (ZDL-Lean fa/+) were purchased from Charles River Laboratories and studied once their blood glucose (BG) levels exceeded 250 mg/dl (age ~8wk). Study groups were matched for age, body weight (BW) and BG levels.

Surgery

Rats underwent surgical implantation of an indwelling lateral ventricle cannula as previously described (Scarlett et al. 2016). Both the carotid artery and the internal jugular vein were cannulated during the same surgical session using established methods (Rojas et al. 2015). Animals were allowed to recover for 7 d prior to study. Animals whose food intake (FI), BW or BG had not returned to baseline 7 d after surgery were excluded.

Intracerebroventricular (icv) injections.

Animals received a single icv injection of either saline vehicle (Veh) or recombinant rat FGF1 (FGF1; Prospec-Tany TechnoGene Ltd.), which was dissolved in sterile water at a concentration of 1 µg/µl and injected over 60 s in a volume of 3µl (total dose = 3.0 µg).

Frequently sampled intravenous glucose tolerance test (FSIGT).

Blood sampling was performed via an arterial catheter in unrestrained, conscious animals as previously described (Scarlett et al. 2016; Morton et al. 2013). After a 5 h fast, baseline fasting blood samples were drawn at –10 and 0 min. After a bolus of 50% dextrose (1 g/kg body weight) was injected intravenously (iv) over a period of 15s, blood (20 µl) was sampled for serial measurement of glucose using a GM9D Analox (Analox Instruments, UK) and for subsequent assay of plasma insulin and lactate levels.

Minimal model analysis and calculations.

Plasma insulin and BG profiles during each FSIGT were analyzed using MinMod software to quantify insulin-independent glucose disposal (referred to as glucose effectiveness

(GE) (S_G)) and insulin sensitivity (S_I), as previously described (Morton et al. 2013; Scarlett et al. 2016) and recently validated in rats (Morton et al. 2017). The acute insulin response to glucose (AIR_G) was calculated as the mean increment above basal insulin values measured between t = 0–4 min. Glucose tolerance was estimated from the incremental area under the glucose curve (AUCglucose) during the FSIGT.

Lactate kinetics and hepatic glucokinase activity calculations.

Modeled liver glucokinase activity (K_{GK}), glycolysis (K_{12}), and whole-body lactate clearance (K_{01}) were estimated by analyzing the kinetic relationship between plasma glucose and lactate levels obtained during the FSIGT, as described (Stefanovski et al. 2012). Because of the reduced sampling number taken in rodents, K_{12} was fixed to a standard value, rather than estimated, to facilitate the parameter estimation.

Direct measurement of liver glucokinase activity was performed on liver samples that were immediately frozen in liquid nitrogen and subsequently homogenized in 50 mmol/l HEPES, 100 mmol/l KCl, 1 mmol/l EDTA, 5 mM $MgCl_2$, and 2.5 mmol/l dithioerythritol (8). GK activity was measured in the supernatant fraction after centrifugation, as described (Fujimoto, Donahue, and Shiota 2004).

Intravenous insulin tolerance test (IVITT)

After a one week recovery from the FSIGT study, ZDF rats fasted for 5 h underwent an IVITT. Baseline blood samples and glucometer readings were taken at 0 min, after which animals received an iv bolus of recombinant rat insulin 2 (0.75U/kg BW; Novo Nordisk A/S). Blood samples were taken via the arterial catheter at 0, 15, 30, 45, 60, 120 and 180 min for

measurement of glucose using a handheld glucometer. The extent of insulin-induced glucose lowering was taken as a measure of whole body insulin sensitivity.

Euglycemic clamp

Two weeks following icv injection of FGF 1 (3 μg) or Veh (when BG levels were much lower in the former than the latter group), ZDF rats bearing catheters in the right jugular vein and left carotid artery were subjected to a variable insulin and glucose infusion protocol designed to ascertain the level of plasma insulin in Veh-treated ZDF rats required to match and maintain BG levels to those of icv FGF1-treated ZDF rats. After a 5 h fast, baseline blood samples and glucometer readings were taken at -10 min and 0 min and beginning at $t = 0$ min and infusions of both recombinant rat insulin (mU/kg/min; rat insulin 2 was synthesized and provided by Novo Nordisk A/S (supplemental methods)) and glucose (D50) (mg/kg/min) were initiated simultaneously with rates adjusted so as to clamp BG levels at 170 mg/dl in both icv Veh- and FGF1-treated rats. Rat insulin was selected for these studies to ensure that endogenous and infused insulin would be detected in equimolar amounts using a rat insulin ELISA. Blood samples were taken at 10 min intervals for a duration of 120 min.

Quantitation of β-cell mass

Pancreata were fixed in 10% neutral-buffered formalin for 24 h, processed in paraffin and analyzed as previously described (Paulsen et al. 2010). Paraffin blocks were cut in 3 μm sections and stained with guinea pig anti-Insulin (DAKO), and rabbit anti-Nkx6.1 antibodies (Sigma Aldrich). Insulin immunoreactivity was visualized with goat-anti-guinea pig (Invitrogen) secondary antibody and donkey-anti-goat-Alexa 488 (Jackson). Nkx6.1 immunoreactivity was

used to identify β-cells and was visualized with donkey-anti-rabbit-biotin (Jackson), Streptavidin-HRP (Perkin Elmer) and TSA-Cy3 (Invitrogen). The slides were counterstained with DAPI to detect cell nuclei and scanned on a VS120 slide scanner at 10x magnification (Olympus) and pancreatic β-cell mass was quantified using Visiomorph (Visiopharm).

Blood collection and tissue processing

Daily BG levels were measured using a handheld glucometer on blood obtained from tail capillary samples. At study completion, whole blood samples for plasma hormone measurement were collected into appropriately treated tubes (German, Thaler, Wisse, Oh-I, et al. 2011), separated into plasma, aliquoted and stored at -80°C. Plasma insulin (Crystal Chem), corticosterone (Crystal Chem), and glucagon (Mercodia) levels were measured by ELISA. Plasma lactate levels were measured using a GM9D analyzer. Plasma lipids were measured with enzymatic colorimetric assays using the following kits: Triglyceride (TG) from Raichem; non-esterified free fatty acid (FFA) from Wako Diagnostics.

RT-PCR.

Total RNA was extracted from liver using TriReagent (Sigma-Aldrich) and NucleoSpin RNA (Fischer Scientific). Levels of specific transcripts for *Gck, Pklr, Gys2, Pck1* and *G6p* were quantified by real-time PCR (ABI Prism 7900 HT; Applied Biosystems) using SYBR Green (Applied Biosystems) and results normalized to the housekeeping gene 18s. For comparative analysis, RNA ratios of the treatment group were normalized to the icv-Veh control group.

Statistical analysis

All results are expressed as mean ± SEM. Group by time designs were analyzed using linear mixed model analysis (SPSS v. 23, IBM Corp., Somers, NY) and mixed factorial analyses (GraphPad software, La Jolla, CA) or two-way ANOVA with repeated measures (mixed model) with Bonferroni post-test comparison. For pairs of data, a two-sample, unpaired Student's *t*-test was used. In all instances, probability values of less than 0.05 were considered significant.

4.4 RESULTS

Effect of icv FGF1 on levels of blood glucose, food intake and body weight in ZDF rats

As expected (Etgen and Oldham 2000), male ZDF rats fed *ad-libitum* developed hyperglycemia (Fig. 1A) between age 7-10 wk. Following a single icv injection of FGF1 (3 μg), morning BG levels dropped into the normal range (Fig. 1A) for ~30 d, after which hyperglycemia relapsed (Fig. 1A). FI was also reduced transiently (Fig. 1B) and although BW was also decreased, the effect did not achieve statistical significance (Fig. 1C). In non-diabetic ZDL controls, FI was also transiently reduced following icv FGF1 injection but BG levels remained unchanged (Fig. 1D,E), as is observed in wild-type mice (Scarlett et al. 2016).

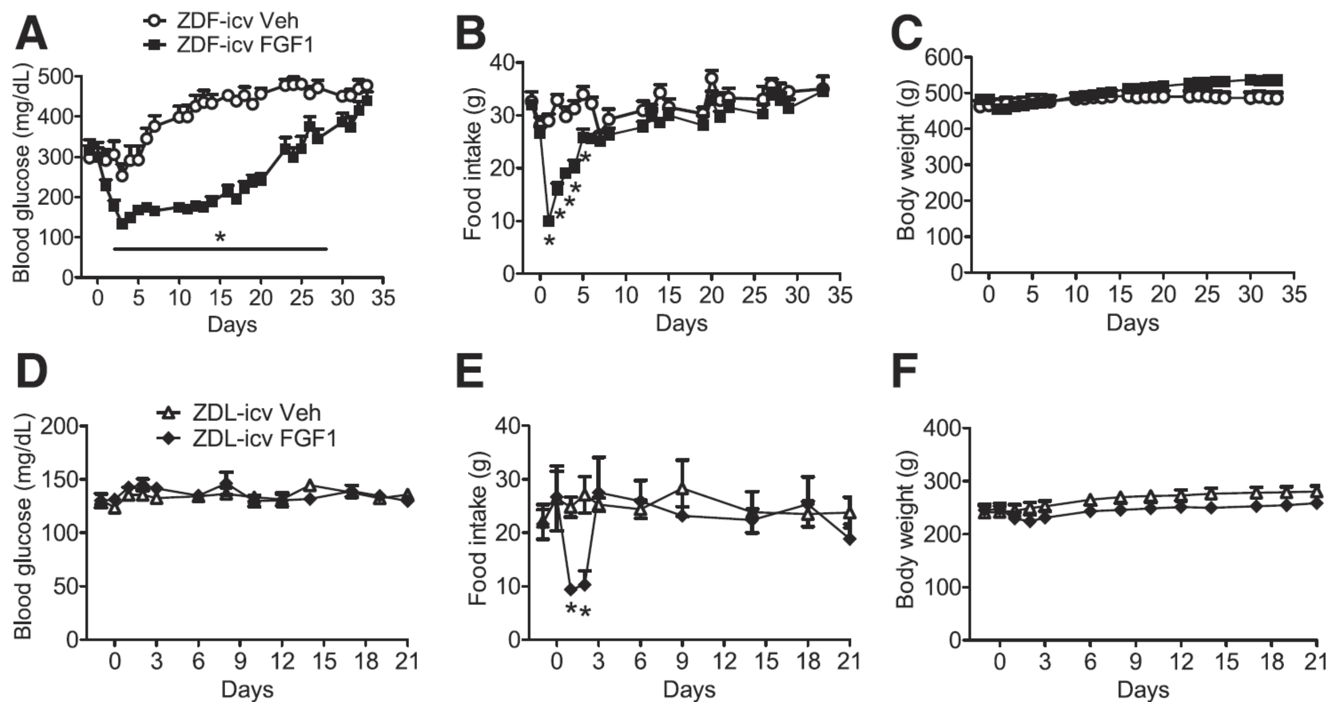

Figure 4-1 Glucose-lowering effect of a single icv FGF1 injection in ZDF and ZDL rats. Daily BG (A), FI (B), and BW (C) values from ad libitum–fed ZDF rats after a single icv injection of either Veh (n = 9) or FGF1 (3 mg; n = 9). Daily BG (D), FI (E), and BW (F) values from ad libitum–fed ZDL rats after a single icv injection of either Veh (n = 7) or FGF1 (3 mg; n = 8). Data are mean 6 SEM. *P , 0.05 vs. icv Veh.

To assess the contribution made by reduced FI to the glucose-lowering effect of icv FGF1, we performed an additional study in which icv Veh-treated ZDF rats were pair-fed to the intake of ZDF rats receiving icv FGF1. Although BG values initially declined in pair-fed controls, as expected (Etgen and Oldham 2000; Torres et al. 2009), the effect was transient such that BG levels returned to baseline by Day 13 (Fig. 2A), concurrent with the restoration of baseline levels of FI and BW (Fig. 2B, C).

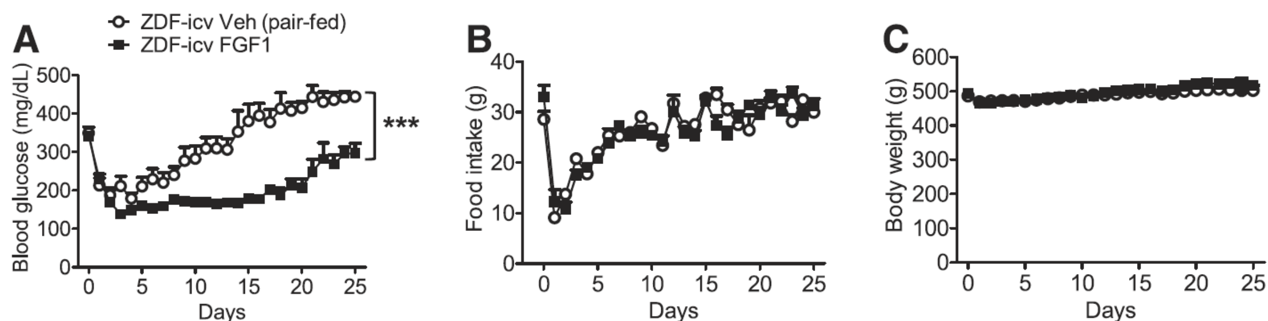

Figure 4-2 Contribution of transient anorexia to the glucose-lowering effect of icv FGF1 in ZDF rats.
Daily BG (A), FI (B), and BW (C) values from pair-fed ZDF rats after a single icv injection of either Veh (n =9) or FGF1(3mg; n =9).Dataaremean6 SEM. ***P , 0.001 vs. icv Veh.

Effect of icv FGF1 injection on hormonal and metabolic parameters in ZDF rats

In animals receiving icv Veh, basal plasma insulin levels peaked at the onset of hyperglycemia, followed by progressive decline (Fig. 3A), as previously reported (Etgen and Oldham 2000). In icv FGF1-treated ZDF rats, by comparison, insulin remained at pre-treatment levels for 3-4 wks, followed by a precipitous decline (Fig. 3A) that corresponded temporally with hyperglycemia relapse (Fig 1A). Thus, the progressive decline of pancreatic β-cell function characteristic of the ZDF rat model was delayed by ~3-4 wks following icv injection of FGF1, and it was during this interval that hyperglycemia was ameliorated. Although plasma glucagon levels were reduced 1 wk after icv FGF1 injection, subsequent values did not differ significantly from those of icv Veh-treated controls (Fig. 3B). No changes of circulating FFA or TG levels were detected (Figs. 3C, D).

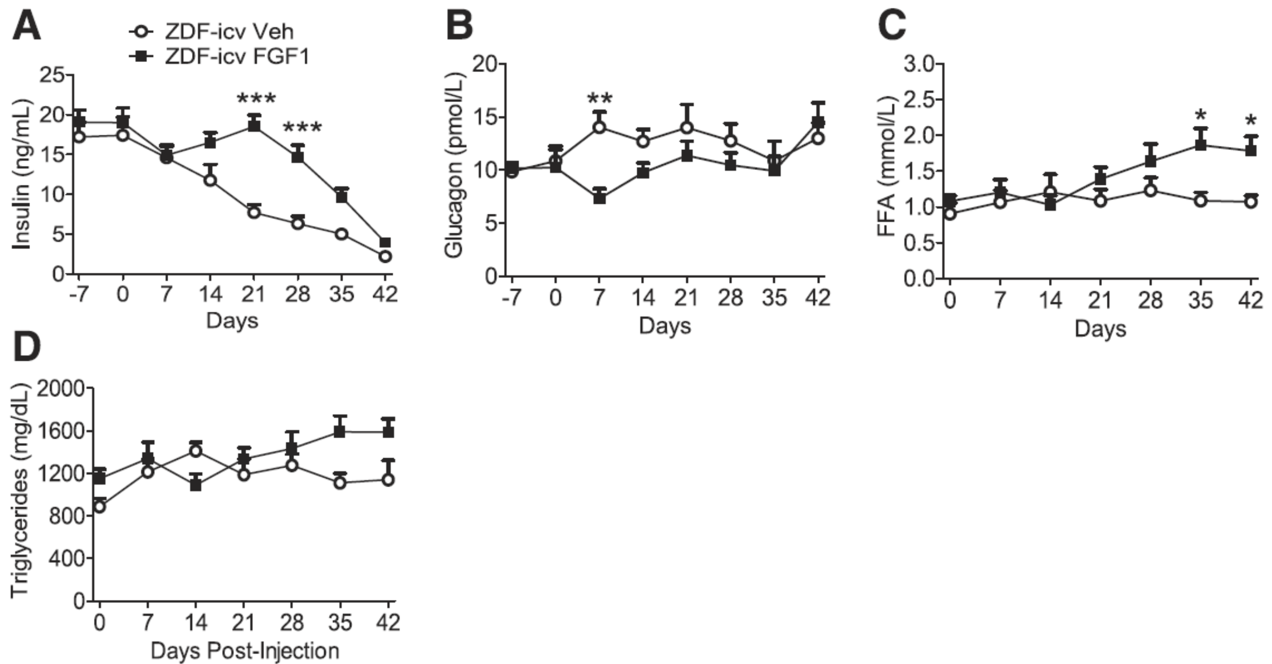

Figure 4-3 Preserved basal insulin secretion by icv FGF1 in ZDF rats. Preserved basal insulin secretion by icv FGF1 in ZDF rats.
Weekly plasma insulin A), glucagon (B), FFA (C), and TG (D) from ad libitum–fed ZDF rats after a single icv injection of either Veh (n = 9) or FGF1 (3 mg; n = 9). Data are mean 6 SEM. *P , 0.05, **P , 0.01, ***P , 0.001 vs. icv Veh.

Effect of icv FGF1 injection on β-cell mass

Histochemical analysis of pancreatic islets from icv FGF1- and Veh-treated animals (Fig. 4A-C) revealed a strong association between islet β-cell mass and basal plasma insulin levels. At the 3 wk time point following icv injection (when baseline plasma insulin levels were higher in icv FGF1- than icv Veh-treated animals (Fig. 3A)), β-cell mass was significantly increased in icv FGF1-treated animals relative baseline (Day 0) and to icv Veh-treated animals at the same 3-wk time point. However, this effect was transient such that by Week 7 after icv injection, β-cell mass had decreased by 86% to achieve values comparable to those of icv Veh-treated animals (Fig. 4D), with plasma insulin levels showing a similar drop (Fig. 3A) and BG levels returning to the diabetic range (Fig. 1A). These findings suggest that icv FGF1 injection delays the onset of β-

cell decompensation in ZDF rats, with the eventual loss of β-cell function then driving diabetes relapse.

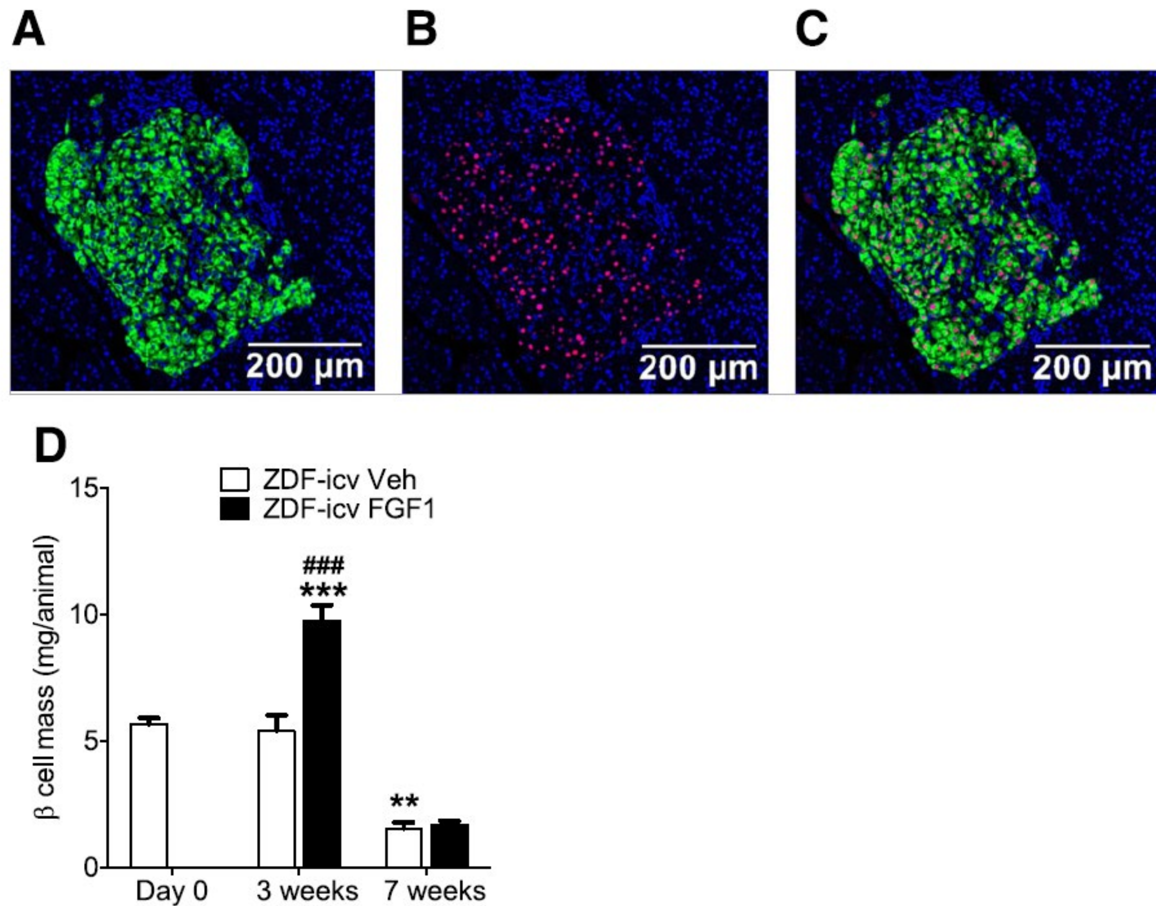

Figure 4-4 Time course of the effect of icv FGF1 on b-cell mass in ZDF rats. Representative images of staining of pancreatic islet sections from ZDF rats for insulin (A), Nkx6.1 (B), and merged images (C). Pancreatic b-cell mass at day 0 (n = 7), 3 weeks (n = 8 icv Veh vs. n = 10 icv FGF1), and 7 weeks (n = 9/group) in ad libitum–fed ZDF rats after a single icv injection of either Veh or FGF1 (3 mg) (D). Data are mean 6 SEM. **P , 0.01 vs. icv Veh at day 0 and 3 weeks; ***P , 0.001 vs. icv Veh at day 0, 3 weeks, and 7 weeks; ###P , 0.001 vs. icv FGF1 at 7 weeks.

Effect of icv FGF1 on the determinants of glucose tolerance in ZDF rats

To determine the contribution made by the three major determinants of glucose tolerance (insulin secretion, insulin sensitivity and GE) to the response to icv FGF1, we performed a FSIGT on a separate cohort of ZDF rats two weeks after icv injection of either FGF1 (3 μg) or Veh. At this time point, levels of FI and BW were comparable between groups (Fig. 1A-C)) and, by design, fasting BG levels were much lower in icv FGF1- than Veh-treated treated animals (Fig. 5A). BG levels remained lower in icv FGF1- than Veh-treated animals for the duration of the FSIGT, but after correcting for the difference in basal glucose levels, the area under the glucose tolerance curve (AUC_G) did not differ significantly between groups (Fig. 5B, C). In ZDF rats, therefore, treatment with icv FGF1 has no effect on glucose tolerance at a time when basal glucose levels are completely normalized, in agreement with our findings in *ob/ob* mice (Scarlett et al. 2016).

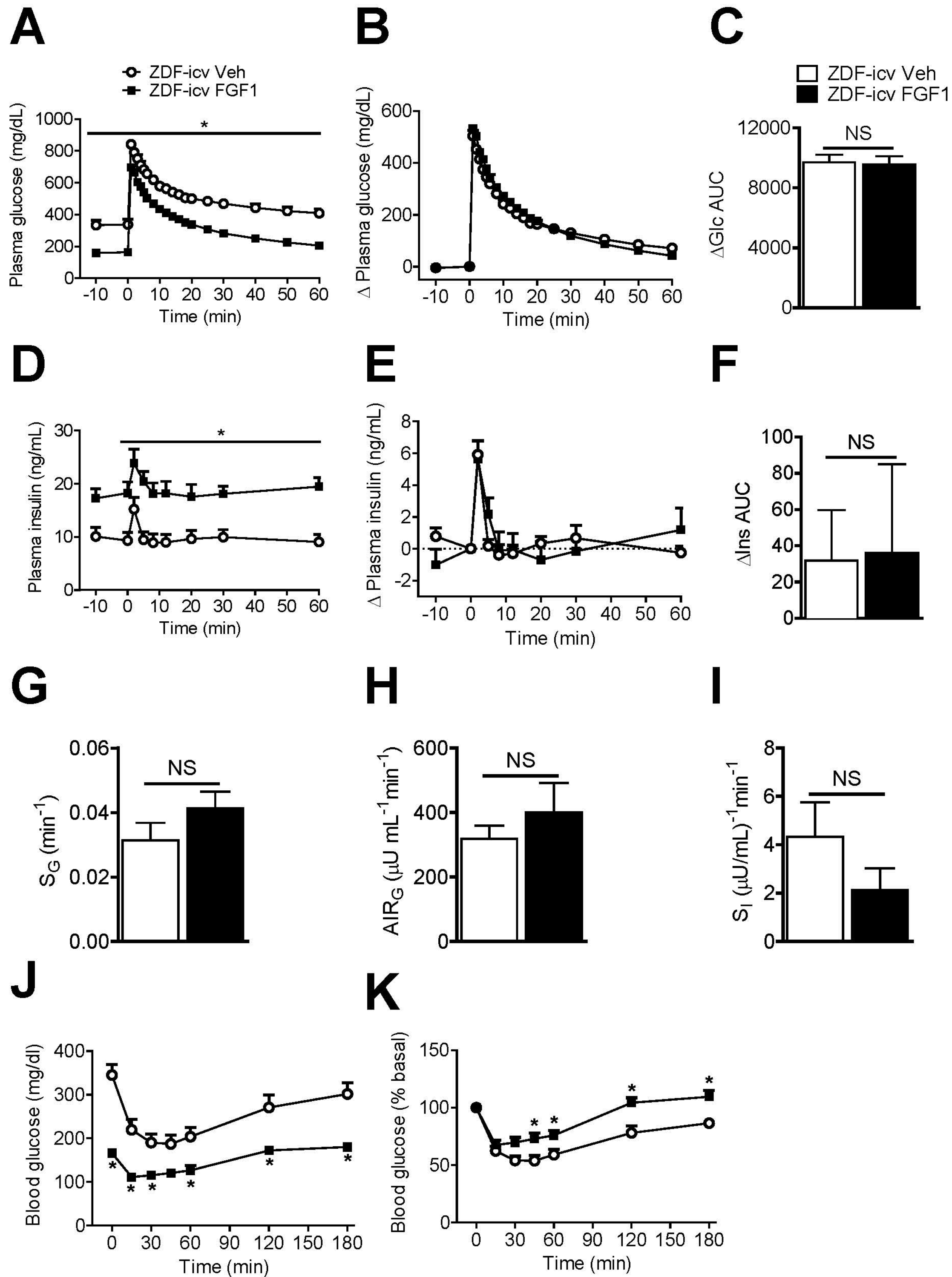

Figure 4-5 Effect of icv FGF1 on determinants of glucose tolerance in ZDF rats. Plasma glucose (A), D plasma glucose (correcting for differences in basal glucose) (B), D plasma AUCglucose (Glc AUC) (C), plasma insulin (D), D plasma insulin (correcting for differences in basal insulin) (E), D plasma insulin AUC (Ins AUC) (F), SG (G), AIRG (H),

and SI (I) during an FSIGT in ZDF rats 2 weeks after receiving a single icv injection of either Veh (n = 7) or FGF1 (3 mg; n = 7). BG (J) and percent D BG (K) during an IVITT in ZDF rats 3 weeks after receiving a single icv injection of either Veh (n = 7) or FGF1 (3 mg; n = 6). Data are mean 6 SEM. *P , 0.05 vs. icv Veh.

As plasma insulin levels decline during the first few weeks after icv injection in Veh-treated animals, but not in those receiving icv FGF1 (Fig. 3A), we expected fasting plasma insulin levels to be higher in FGF1-treated animals on Day 14 after icv injection, prior to the FSIGT (despite lower BG levels), and this proved to be the case (Fig. 5D). After controlling for differences in the basal insulin level, however, there was no difference in AIR_G between groups (Fig. 5E, F, H). Furthermore, although a trend towards increased S_G was detected in animals receiving icv FGF1 treatment, it also did not achieve statistical significance (Fig. 5G). Paradoxically, S_I was decreased by ~50% in FGF1-treated animals, although this effect did not reach statistical significance (Fig. 5I). Thus, neither glucose-induced insulin secretion nor insulin sensitivity appear to be affected by icv administration of FGF1.

To further characterize the effect of icv FGF1 on insulin sensitivity in these animals, we performed an IVITT 1 wk following the FSIGT in the same cohort of animals. As this study demonstrates directly that the glucose-lowering effect of iv insulin injection was in fact reduced in ZDF rats that received icv FGF1 *vs.* icv Veh (Fig. 5J, K), the anti-diabetic action of FGF1 in the brain cannot be attributed to an increase of insulin sensitivity.

Contribution of preserved basal plasma insulin secretion to the anti-diabetic effect of icv FGF1

To quantify the contribution made by preservation of β-cell function to remission of hyperglycemia induced by icv FGF1, we employed a modified glucose clamp protocol whereby

icv Veh-treated ZDF rats received iv insulin at a variable rate designed to reduce their BG level into the normal range, at the same level (170 mg/dl) maintained by ZDF rats treated with icv FGF1 2wk earlier (Fig. 6A). There was no difference of FI or BW between groups (Fig. 6B, C), and baseline ($t = 0$ min) plasma BG levels were higher and insulin levels lower in icv Veh- *vs.* FGF1-treated animals (Fig. 6D, E), as expected. After variable-rate iv insulin infusion for ~70 min (Fig. 6F), BG levels were effectively matched between the two groups and remained so for the duration of the study (until $t = 120$ min; Fig. 6D).

Overall, the plasma insulin level required for icv Veh-treated animals to achieve normoglycemia was ~2-fold greater (Fig. 6E) than was observed in icv FGF1-treated animals that spontaneously maintain the same BG level (Fig. 6D). This finding is made more striking by the fact that FGF1-treated animals required a variable-rate glucose infusion to prevent their BG from dropping below basal levels (Fig. 6G). At baseline, plasma glucagon levels were higher in icv Veh- than icv FGF1-treated rats (Fig. 6H), but there were no differences in plasma corticosterone (Cort) levels (Fig. 6I). During the insulin infusion period, plasma glucagon levels remained stable (Fig. 6H), whereas plasma Cort levels increased by >2-fold in response to normalization of BG among icv Veh-treated animals (Fig. 6D), whereas it did not change in the icv FGF1-treated group (Fig. 6I).

Collectively, these data suggest that although a relative increase of basal insulin secretion may contribute, it cannot fully account for icv FGF1-induced glucose lowering, nor is there evidence that this effect involves an increase of insulin sensitivity. We also note that, based on the plasma Cort response, acute normalization of BG levels appears to elicit a stress response in

diabetic, icv Veh-treated ZDF rats, an effect that is not observed in animals with diabetes remission induced by icv FGF1 injection.

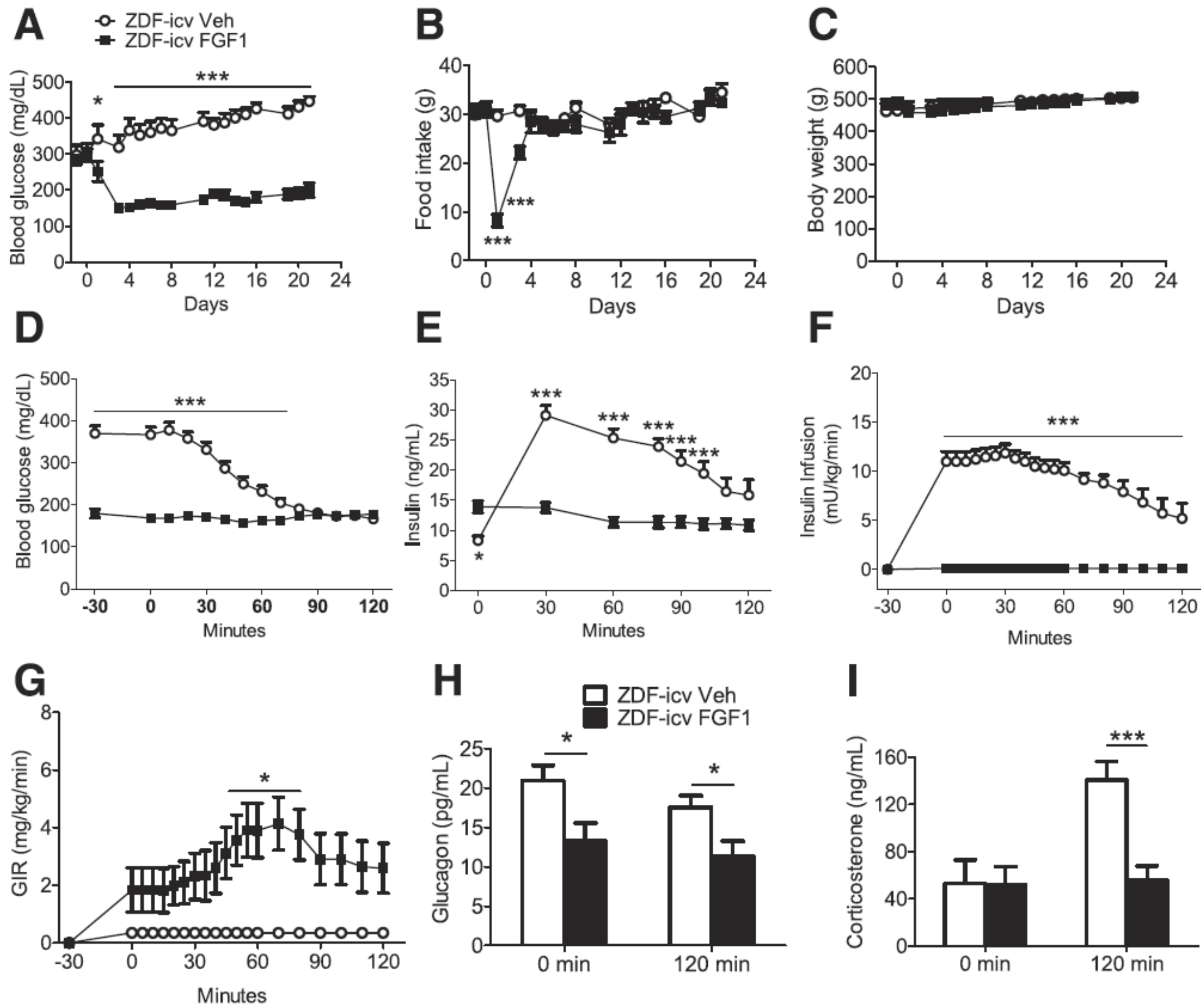

Figure 4-6 Dependence of basal insulin secretion on antidiabetic effect of icv FGF1. ZDF rats underwent matched euglycemic clamp 14 days after a single icv injection of either Veh (n = 11) or FGF1 (3 mg; n = 11). Daily morning BG (A), FI (B), and BW (C) values from ad libitum–fed ZDF rats. Plasma glucose (D), plasma insulin (E), insulin infusion rate (F), and glucose infusion rate (GIR) (G) during the matched euglycemic clamp. Plasma glucagon (H) and plasma Cort (I) at the start (t = 0 min) and end (t = 120 min) of the matched euglycemic clamp. Data are mean 6 SEM. *P , 0.05, ***P , 0.001 vs. icv Veh.

Effect of icv FGF1 injection on plasma lactate and hepatic glucokinase expression and activity

These considerations collectively suggest that an insulin-independent process must contribute to FGF1-induced remission of hyperglycemia. To test this hypothesis, we focused on HGU, a process that is largely insulin-independent, is crucial for normal glucose homeostasis and is regulated in part by the brain (Rojas and Schwartz 2014; Moore et al. 2012). Specifically, we investigated whether the mechanism underlying glucose lowering induced by icv FGF1 involves increased hepatic GCK activity, which constitutes a key rate-limiting step for HGU by phosphorylating glucose upon entry into the hepatocyte.

As an initial test of this hypothesis, we considered that the magnitude of the rise of plasma lactate levels in response to a glucose load (which reflects glucose taken up by the liver and subsequently converted by glycolysis to lactate, which is released back into circulation) can be used to estimate hepatic GCK activity in vivo (Stefanovski et al. 2012; Lovejoy et al. 1992). To this end, we performed serial measures of plasma lactate levels obtained before and during the previously described FSIGT performed 2 wk following icv injection of either Veh or FGF1 (Fig 5). We found that although basal plasma lactate levels were higher in icv Veh- than icv FGF1-treated ZDF rats (Fig. 7A), reflecting their much higher basal glucose level, the plasma lactate response to an iv glucose challenge was increased by >2-fold in the FGF1-treated group (Fig. 7B, C). Model-based estimation of the hepatic GCK activity rate constant from this lactate response (K_{GK} (Stefanovski et al. 2012)) demonstrated a similar, >2-fold increase in icv FGF1- relative to icv Veh-treated controls, a highly significant effect (Fig. 7D). Model-derived estimates of K_{GK} were well-identified, with mean (SD) normalized root square error values of

15% and 11% for icv Veh, and 6% and 5% for icv FGF1, respectively. These data, combined with our biochemical findings of a 2-fold increase in the expression of both *Gck* mRNA and GCK activity in the liver of ZDF rats treated with icv FGF1 both confirm and validate our model-based finding and offer direct evidence implicating the action of FGF1 in the brain to increase liver glucokinase activity in the associated remission of hyperglycemia (Fig. 7E).

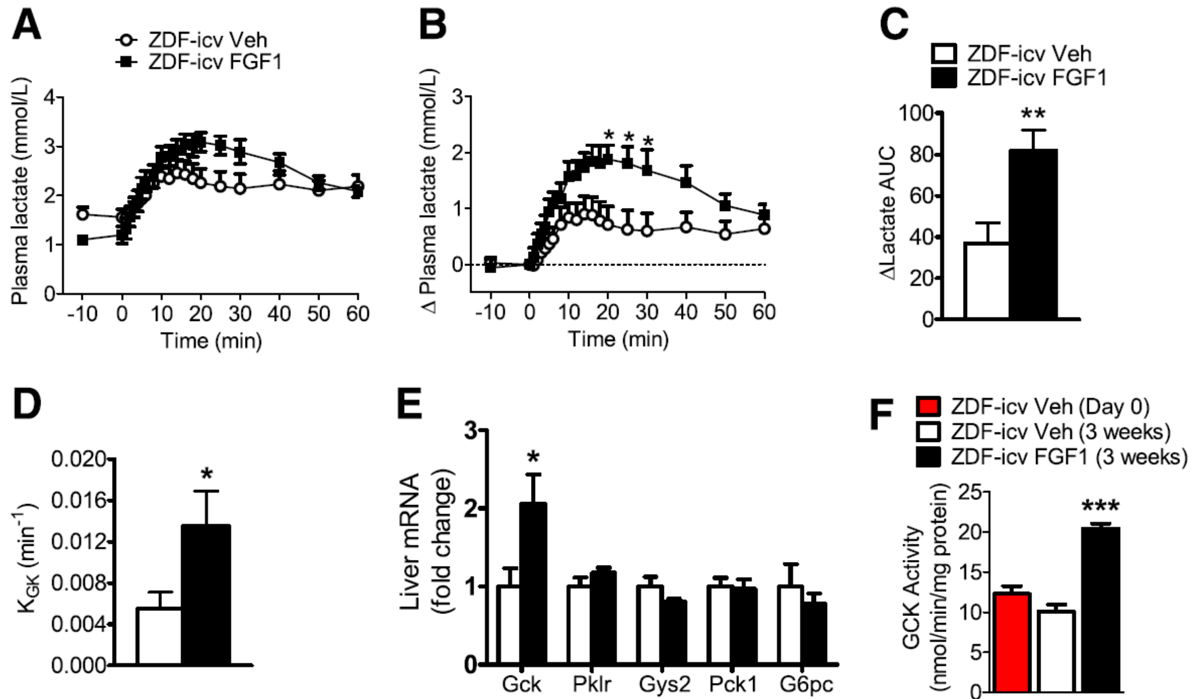

Figure 4-7 Effect of icv FGF1 on plasma lactate and hepatic GCK in ZDF rats.
Plasma lactate (A), D plasma lactate (correcting for differences in basal lactate) (B), D plasma lactate AUC (C), and calculated hepatic KGK (D) during an FSIGT in ZDF rats 2 weeks after receiving a single icv injection of either Veh (n = 7) or FGF1 (3 mg; n = 7). E: Hepatic mRNA expression of Gck, Pklr, Gys2, Pck1, and G6pc by real-time PCR in ZDF rats 3 weeks after receiving a single icv injection of either Veh (n = 11) or FGF1 (3 mg; n = 11). F: Hepatic GCK activity in ZDF **rats** at day 0 baseline (icv Veh, n = 6) or 3 weeks after receiving a single icv injection of either Veh (n = 8) or FGF1 (3 mg; n = 11). Data are mean 6 SEM. *P , 0.05, **P , 0.01, ***P , 0.001 vs. icv Veh.

4.5 DISCUSSION

We report that in the ZDF rat model of T2D, icv FGF1 injection delays the onset of progressive β-cell dysfunction, and we interpret this finding to suggest that FGF1 action in the

brain transiently suppresses pathogenic processes that drive β-cell loss in this model. However, this mechanism alone is unlikely to explain the observed normalization of glycemia, since basal plasma insulin levels never increased over pre-treatment values in animals receiving icv FGF1 injection. Instead, the rapid decline of plasma insulin levels observed in icv-Veh treated animals was transiently prevented by icv FGF1 injection over a time course that corresponds to the period of diabetes remission. With respect to other peripheral mechanisms of glucose lowering, we observed no effect of icv FGF1 on glucose tolerance, whole-body insulin sensitivity or glucose-stimulated insulin secretion, nor were circulating levels of Cort, FFAs or TGs altered. Instead, our data point to an action of FGF1 in the brain to increase basal glucose clearance via a mechanism involving increased hepatic GCK activity.

These findings extend previous evidence that the activity of glucoregulatory neurocircuits (Grayson, Seeley, and Sandoval 2013; Morton and Schwartz 2011; Schwartz et al. 2013) is sensitive to input from FGF peptides such as FGF19 and FGF21. Although glucose lowering elicited by these peptides was initially hypothesized to involve actions primarily on peripheral tissues (Xu et al. 2009; Kir et al. 2011; Kharitonenkov et al. 2005), subsequent work revealed that an action in the brain is sufficient (Fu et al. 2004; Marcelin et al. 2014; Morton et al. 2013; Perry et al. 2015; Ryan et al. 2013; Sarruf et al. 2010) and may also be necessary (Lan et al. 2017) for this effect. While the prolonged duration of glucose lowering induced by FGF1 action in the brain is unique (Scarlett et al. 2016), the duration of this effect is considerably shorter in ZDF rats than is reported in *ob/ob* mice (Scarlett et al. 2016), potentially owing to rapid loss of β-cell mass and function in the former (Shiota and Printz 2012), but not the latter animals (Lindstrom 2010). Combined with evidence that in our hands, icv FGF1 injection does not

induce glucose lowering in rodents with uncontrolled insulin-deficient diabetes (Scarlett et al. 2016; Suh et al. 2014), we interpret these data to suggest that an intact insulin signal is required for the anti-diabetic response induced by central administration of FGF1. Consistent with this hypothesis, the observed delay in onset of β-cell dysfunction corresponds closely with the period of diabetes remission induced by icv FGF1 in ZDF rats, such that diabetes relapse was accompanied by precipitous declines of both plasma insulin and β-cell mass. In the ZDF rat model, therefore, eventual relapse of hyperglycemia following icv FGF1 injection appears to be driven by the onset of severe, progressive β-cell dysfunction – an effect that does not occur in *ob/ob* mice.

Increased β-cell mass is an integral feature of the adaptation to insulin resistance in ZDF rats. At 5–7 wk of age, β-cell mass in male ZDF rats is approximately twice that of age-matched ZDL controls, and this expansion continues until 10–12 wks, after which it progressively and markedly declines (Topp, Atkinson, and Finegood 2007; Pick et al. 1998). Several potential explanations can be considered for how β-cell mass and function are preserved, albeit transiently, following central administration of FGF1. One obvious possibility is that FGF1 activates neurocircuits that directly control islet function. This hypothesis is consistent with evidence that the pancreas is richly innervated by autonomic fibers (Yoshimatsu et al. 1984; Rossi et al. 2005) and that both parasympathetic and sympathetic outflow to the pancreas can powerfully influence not only β-cell function, but if sustained, islet mass (Ahren 2000; Porte et al. 1973). Furthermore, retrograde mapping studies reveal that some of the same hypothalamic areas that respond to FGF1 (Rosario et al. 2016a) are anatomically linked to efferent circuits innervating pancreatic islets (Rosario et al. 2016a), and both β-cell mass and function can be altered by either surgical

(Kiba et al. 1996) or pharmacologic (Ando et al. 2017; Osundiji et al. 2012) manipulation of these circuits. This explanation is also consistent with the increase of β-cell mass over baseline that was observed 3 wk after icv FGF1 injection. An alternative and perhaps more straightforward explanation, however, is that FGF1-induced preservation of β-cells is primarily an indirect consequence of reduced demand for insulin secretion (owing to normalization of glycemia). These two possibilities are not mutually exclusive, and additional studies to address them are warranted.

Our finding that sustained amelioration of hyperglycemia induced by icv FGF1 in ZDF rats occurred despite no increase of either insulin levels (over baseline values) or insulin sensitivity suggests that an insulin-independent mechanism must contribute to the effect of icv FGF1 injection to increase glucose disposal. This hypothesis is strengthened by our observation that the circulating insulin level needed to normalize glycemia in icv Veh-treated ZDF rats is far higher than is observed in animals with normoglycemia induced by icv FGF1 injection.

That the brain can stimulate insulin-independent glucose disposal is well-established. In rodent models of uncontrolled diabetes induced by streptozotocin (STZ-DM), for example, hyperglycemia can be reversed by continuous icv administration of leptin, even in the face of persistent, severe insulin deficiency (German, Thaler, Wisse, Oh, et al. 2011; Hidaka et al. 2002). Similarly, increased insulin-independent glucose disposal plays a major role to mediate the anti-diabetic effect of icv FGF19 administration in *ob/ob* mice (Morton et al. 2013). Although the anti-diabetic effect of icv FGF1 has been suggested to involve via suppression of excessive hypothalamic-pituitary-adrenal axis activity (Perry et al. 2015), we observed no effect of icv

FGF1 injection on plasma Cort levels in either the current study (in ZDF rats) or in our past work (in *ob/ob* mice) (Scarlett et al. 2016). Excess glucagon secretion has also been hypothesized to drive diabetic hyperglycemia (Unger and Cherrington 2012), and plasma glucagon levels were significantly decreased in ZDF rats receiving icv FGF1 compared to icv vehicle. However, plasma glucagon levels do not increase in parallel with progressive hyperglycemia in ZDF rats (Torres et al. 2009), and diabetes induced by stretptozotocin is not ameliorated by a glucagon-neutralizing antibody (Meek et al. 2015). Based on these considerations, we suspect that the modest decline in plasma glucagon is not the primary mechanism underlying anti-diabetic effect of icv FGF1.

Glucose sequestration by the liver is a major contributor to insulin-independent glucose clearance (Best et al. 1996). After glucose enters hepatocytes via insulin-independent GLUT2 glucose transporters, it is phosphorylated by GCK to generate glucose-6-phosphate, a step that is rate-limiting for HGU. Before this can occur, GCK must be dissociated from glucokinase regulatory protein, which otherwise sequesters the enzyme in the nucleus. Glucose-6-phosphate has several fates after conversion to triose phosphates—storage as glycogen, metabolism via the TCA cycle, or conversion to lactate and export from liver into the blood (Moore et al. 2012). The latter process provides the foundation for assessment of GCK activity in vivo using a mathematical model.

This model is based on the hypothesis that glucose conversion to lactate and subsequent export into the bloodstream occurs in proportion to the rate of glucose phosphorylation (and hence, GCK activity) (Stefanovski et al. 2012). Applying this model to the dynamic relationship

between plasma glucose (input to liver) and lactate (output from liver) during an FSIGT, we report that GCK activity was estimated in animals 2 wk after either icv injection of either Veh or FGF1. We report that model-estimated liver GCK activity was effectively doubled in ZDF rats with diabetes remission induced by icv FGF1. This physiological assessment of GCK activity is in remarkable agreement with the 2-fold increases of both hepatic *Gck* gene expression and GCK enzymatic activity measured biochemically in liver homogenates in ZDF rats treated with icv FGF1. These data strongly suggest that increased hepatic GCK activity contributes to the mechanism underlying the sustained anti-diabetic action of FGF1 in the brain, a conclusion in agreement with our earlier work showing that in *ob/ob* mice, icv FGF1 increases hepatic content of both *Gck* mRNA and glycogen, while also increasing plasma lactate levels (Scarlett et al. 2016).

With respect to the mechanism whereby FGF1 action in the brain induces liver GCK gene expression and enzyme activity, we note that following icv FGF1 injection, cellular activation is concentrated in the mediobasal hypothalamus (Scarlett et al. 2016), and sympathetic connections have been identified between this brain area and liver (Uyama, Geerts, and Reynaert 2004). Moreover, sympathetic input to the liver appears to play a key role to control HGU. Specifically, studies show that the ability of a portal glucose load to stimulate HGU (by increasing hepatic GCK activity) is decreased by ~75% in a canine model of T2D induced by consuming a diet high in both fat and fructose (Coate et al. 2013; Dicostanzo et al. 2006), and this effect is reversed by denervating the sympathetic supply to the liver. Sympathetic tone to the liver in this model, therefore appears to drive the associated decrease of HGU by suppressing GCK activity. We therefore hypothesize that the anti-diabetic action of icv FGF1 in ZDF rats

involves reduced sympathetic outflow to the liver, and additional studies are planned to test this hypothesis. We acknowledge, however, that normalization of glycemia via other mechanisms may also have contributed to increased liver GCK activity induced by icv FGF1, and additional studies are needed to address this possibility as well.

In conclusion, we report that in the ZDF rat model of T2D, hyperglycemia is ameliorated in a sustained manner following central FGF1 administration, consistent with our earlier work in *ob/ob* mice. A key difference between these two models of T2D is that in ZDF rats (Scarlett et al. 2016) hyperglycemia onset is associated with progressive, severe β-cell decompensation (Shiota and Printz 2012) that is not observed in *ob/ob* mice (on the C57Bl/6 background). Although administration of FGF1 into the brain did not prevent this β-cell decompensation, its onset was delayed by ~3-4 wk via mechanisms that remain to be identified. Since BG levels were normalized during this time, transient preservation of β-cell function is likely to contribute glucose lowering induced by icv FGF1. As plasma insulin levels in animals treated with icv FGF1 never increased over baseline values, however, a major role for increased GE in the sustained normalization of glycemia is implied, and our findings suggest that this increase of GE involves activation of liver GCK, which in turn increases the rate of HGU.

Chapter 5. RETHINKING THE ROLE OF THE BRAIN IN GLUCOSE HOMEOSTASIS AND DIABETES PATHOGENESIS

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5.1 INTRODUCTION

The brain plays a major role in homeostatic processes ranging from control of body temperature and fat mass to blood pressure and volume. Tight regulation of the circulating glucose level is similarly crucial for survival, and since the brain relies almost exclusively on glucose as a fuel source, it seems counterintuitive to think that the brain doesn't also play an important role in glucose homeostasis. Based on overwhelming evidence implicating the endocrine pancreas as the primary controller of the blood glucose (BG) level, however, the notion of a key role for the brain has largely been discounted.

Support for a pancreatic islet-centered model of glucose homeostasis originated with the discovery of insulin nearly a century ago, and the seminal finding that severe diabetes can be caused by pancreas removal and is remedied by insulin replacement. Subsequent findings included: *i)* pancreatic β-cells are uniquely capable of sensing extracellular glucose concentrations and coupling them in a dose-dependent manner to insulin secretion, *ii)* insulin activation of its receptor effectively lowers blood glucose (BG) levels by promoting glucose uptake in tissues throughout the body while also blocking glucose production by the liver, *iii)* absolute and relative impairments of insulin secretion are implicated in the pathogenesis of type 1 and type 2 diabetes (T2D), respectively, and *iv)* insulin effectively lowers BG levels in all but the most rare forms of diabetes. Together, these observations would seem to leave little room for a key role played by the brain.

However, recent findings are beginning to chip away at the foundation of this prevailing, islet-centered view. This perceptual shift is being driven *not* by evidence against a role for the endocrine pancreas in glucose homeostasis, but by evidence that the endocrine pancreas is part of

a larger regulatory system, the activity of which is integrated with other critical homeostatic control systems governed by the brain.

5.2 INTEGRATION OF GLUCOSE HOMEOSTASIS WITH ENERGY HOMEOSTASIS AND THERMOREGULATION

This countervailing narrative begins with recognition that the amount of insulin secreted in response to a glucose challenge can be dynamically regulated by both humoral and autonomic inputs. Pancreatic islets are richly innervated by both sympathetic and parasympathetic fibers, with the former capable of powerfully inhibiting glucose-stimulated insulin secretion (GSIS) and the latter having the opposite effect (Rosario et al. 2016b; Thorens 2010). More importantly, growing evidence that physiologically important changes of both insulin secretion and tissue glucose utilization can occur in the absence of any change of the BG level suggests that pancreatic β-cell function can be regulated as part of a larger system for controlling glucose homeostasis.

To illustrate this concept, consider that across much of the planet, free-living mammals are confronted with swings of environmental temperature on a daily basis that pose a substantial homeostatic challenge. Meeting this challenge requires activation of diverse metabolic and autonomic responses involving three distinct homeostatic systems – glucose homeostasis, energy homeostasis and thermoregulation – that must be integrated seamlessly if body temperature, body fat stores and BG levels are to be maintained within narrow physiological limits.

In response to cold exposure, for example, heat production must increase in a rapid and sustained manner if hypothermia is to be avoided, and the sympathetic nervous system (SNS)

plays a key role to drive this process (Tan and Knight 2018; Haman et al. 2005). Specifically, activation of SNS outflow to thermogenic tissues (*e.g.,* brown and white adipose tissue, skeletal muscle), driven by thermoregulatory neurocircuits situated in the hypothalamic pre-optic area, increases heat production via a mechanism that is highly reliant on oxidation of glucose as a substrate (Tan and Knight 2018; Haman et al. 2005). Beyond the challenge of preserving core temperature, the cold-exposed animal is thus confronted with two additional challenges: *1)* how to preserve energy balance in the face of markedly increased rates of energy expenditure, and *2)* how to preserve stable glycemia in the face of markedly increased rates of glucose utilization. This is where integration across these 3 regulatory systems comes into play.

As a result of this integration, cold exposure increases food intake in a manner that – somehow – precisely offsets the increase of energy expenditure, such that energy balance and body fat mass are preserved (Kaiyala et al. 2015). At the same time, insulin secretion is reduced in a manner that precisely offsets the diversion of glucose into thermogenic tissues, thereby averting any fall of BG levels (Morton et al. 2017). Consistent with a role for the brain in this effect, pharmacological blockade of α-adrenergic receptors rapidly reverses the cold-induced inhibition of β-cell function (Morton et al. 2017), implying that it, like the thermogenic response to cold, is driven by SNS activation. Thus, the brain orchestrates highly coordinated changes across multiple homeostatic systems that collectively enable heightened thermogenic needs to be met while ensuring that body temperature, body fat mass (Kaiyala et al. 2015), and BG levels remain virtually unchanged (Morton et al. 2017) (Figure 5-1).

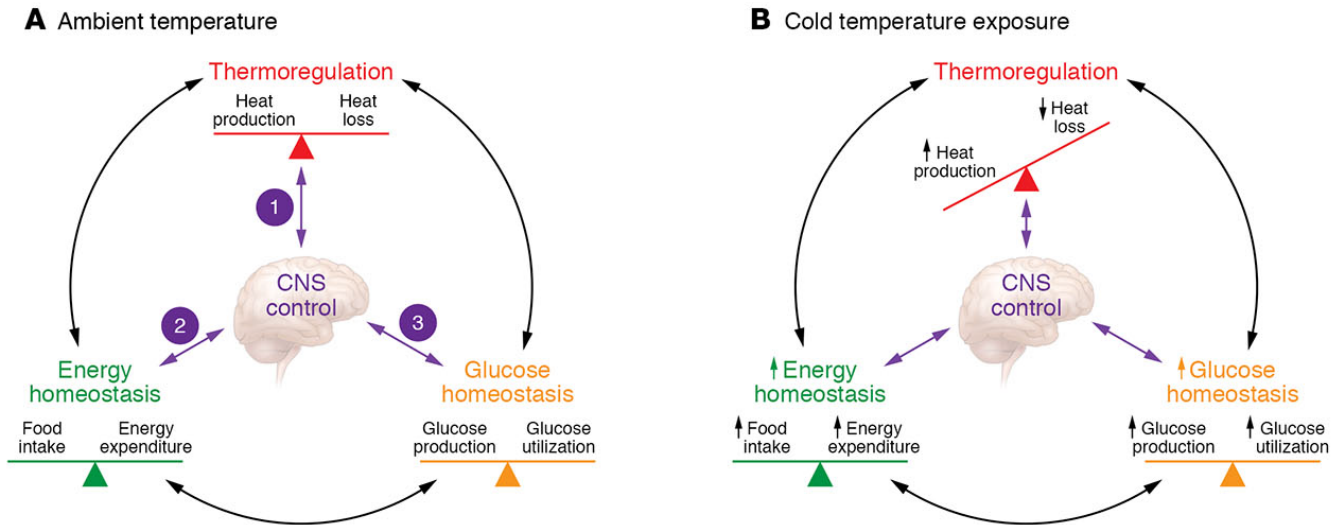

Figure 5-1 Model for integrated central control of body temperature, fat mass, and blood glucose levels.
(A) Maintenance of body temperature, body fat stores, and blood glucose levels within narrow physiological limits requires seamless integration of systems governing thermoregulation, energy homeo-stasis, and glucose homeostasis. This integration is coordinated by the brain, and it is dependent upon accurate sensing by the brain of external tempera-ture (1), body fat content (2), and the blood glucose level (3). (B) During cold exposure, the increased demand for heat production is met through markedly increased rates of glucose utilization by thermogenic tissues. Energy homeostasis is preserved by a centrally mediated increase in food intake, while glucose homeostasis preserved by centrally mediated inhibition of insulin secretion (to avert hypoglycemia). Impaired sensing of the relevant afferent input results in a compensatory increase in the defended level of the regulated variable. In the case of T2D, impaired brain glucose sensing is hypothesized to raise the defended blood glucose level into the diabetic range, with inhibition of insulin secretion playing a key role.

Although our understanding of how these homeostatic systems are integrated is far from complete, regulation via a classical negative feedback loop seems improbable, since this would require the defended variable(s) (temperature, fat mass, BG level) to change before adaptive responses could be mounted, and changes in these variables were not observed (Kaiyala et al. 2015). An alternative possibility is that these responses are governed by feed-forward control mechanisms that can be engaged rapidly in anticipation of future need so as to prevent the defended variable from changing (Lowell 2019). To our knowledge, the capacity for this type of regulation is unique to the brain.

5.3 RELEVANCE TO PATHOGENESIS OF DIABETES AND ASSOCIATED METABOLIC IMPAIRMENT

Available data indicate that at least early in the development of T2D, the BG level continues to be regulated in the usual manner, even as it rises out of the normal range (Holman and Turner 1979). Precedent for this type regulatory defect can be found in two associated and highly prevalent disorders: essential hypertension and obesity, which are characterized by increases in the defended level of blood pressure and body fat mass, respectively. That these three disorders cluster together as part of the metabolic syndrome raises the possibility of a shared underlying defect that drives elevation of the defended level of each of these biological variables.

What mechanisms drive the defense of hyperglycemia in T2D? While β-cell dysfunction clearly plays a role, an important unanswered question is whether this reflects a β-cell-autonomous defect, or is instead imposed upon β-cells by the brain (analogous to the reduction of GSIS observed during cold-exposure). Consistent with the latter notion is that sympathetic inhibition of insulin secretion is increased in patients with T2D (Robertson, Halter, and Porte 1976). But is the brain capable of inhibiting GSIS to an extent similar to what is observed in patients with T2D?

Studies in mice have identified a distinct subset of neurons in the hypothalamic ventromedial nucleus (VMN) that, when activated, not only induce diabetes-range hyperglycemia, but also completely block GSIS (Faber et al. 2018). It is not difficult to imagine, therefore, that if the brain were to perceive the BG level to be lower than it truly is, it would mount responses (including GSIS inhibition) that raise the defended level of glycemia. Indeed,

the response to experimentally-induced "neuroglucopenia" establishes this to be the case. Neuroglucopenia is induced by administration of a non-metabolizable glucose analogue (*e.g.,* 2-deoxy-D-glucose) which is transported into cells and phosphorylated (as normally occurs with glucose), but cannot be metabolized further, thereby disrupting cellular glucose metabolism in neurons and other cell types. In response, the brain raises the BG level in a manner that is so rapid and robust as to serve as a readout for whether neuroglucopenia was in fact achieved (Molina et al. 1993). Moreover, the aforementioned VMN neurons are implicated as drivers of this hyperglycemic response (Meek et al. 2016; Flak et al. 2014). These observations collectively support the feasibility of a model in which defective brain glucose sensing contributes to the pathogenesis of hyperglycemia in T2D, analogous to the effect of impaired leptin sensing to drive excessive accumulation of body fat.

The progressive nature of β-cell dysfunction in T2D, culminating in overt □-cell failure, would at first glance seem to challenge this model of disease pathogenesis, since it is not immediately clear how this progression might result from a defect that does not reside within the β-cell itself. Despite a decades-long search, however, a cell-autonomous basis for progressive β-cell failure remains to be identified. Moreover, most endocrine cell types become severely atrophic and dysfunctional if they are subjected to continuous inhibition over long time intervals. As one example, consider the inhibitory effect of chronic glucocorticoid therapy on pituitary corticotroph cells – it can take months for the profound impairment of ACTH secretion to recover once therapy is discontinued. Investigation into the contribution to β-cell dysfunction made by tonic inhibition arising from the brain, perhaps aggravated by worsening metabolic status (*e.g.,* hyperglycemia and associated glucose toxicity) and/or genetic susceptibility, is a key priority for future study.

Does T2D pathogenesis involve aberrant activity of hypothalamic glucoregulatory neurocircuits capable of raising the defended level of glycemia? Although our understanding of glucoregulatory neurocircuitry is in its infancy, available evidence indicates that *1)* fuel-sensing neurocircuits are concentrated in the mediobasal hypothalamus (MBH), and *2)* some of these circuits are overactive in rodent models of diabetes. Among these are GABAergic neurons situated in the arcuate nucleus that express both agouti-related peptide (Agrp) and neuropeptide Y (NPY) (referred to as Agrp neurons) (Lowell 2019). These neurons are physiologically-important regulators of both food intake and glycemia, and they are tonically inhibited by humoral signals that convey information regarding the status of either stored fuel (*e.g.,* leptin) or fuel available for immediate use (glucose) (Xu et al. 2018; Beutler et al. 2017). Consequently, these neurons are activated by low plasma levels of either leptin or glucose, and in otherwise normal mice, this activation is sufficient to both stimulate food intake and elevate the BG level into the diabetic range, while conversely, silencing of these neurons is sufficient to ameliorate hyperglycemia in diabetic *db/db* mice (Xu et al. 2018). That these neurons are activated across rodent models of diabetes (Schwartz et al. 1996; Havel et al. 2000; Park et al. 2005) makes them an attractive candidate mediator of the defense of hyperglycemia in T2D. The hypothesis that activation of other subsets of glucoregulatory neurons, including those in the VMN, contributes to diabetic hyperglycemia is under active investigation.

5.4 Therapeutic implications

Since Agrp neurons are activated by hypoglycemia/neuroglucopenia (Han et al. 1997), it seems paradoxical that they should also be activated in diabetic, hyperglycemic animals, and yet this is clearly the case (Schwartz et al. 1996; Havel et al. 2000; Park et al. 2005). To explain this

paradox, we hypothesize that brain sensing of glucose and other fuels is impaired in T2D, and that hypothalamic glucoregulatory neurocircuits are activated as part of a compensatory response that drives an increase in the defended BG level (in part by inhibiting GSIS). This model of T2D pathogenesis predicts that correcting the underlying defect should normalize glycemia in diabetic animals, and recent evidence supports this hypothesis. Notable in this regard is the sustained antidiabetic action induced by central administration of fibroblast growth factor 1 (FGF1) (Scarlett et al. 2016; Scarlett et al. 2018; Brown et al. 2019; Tennant et al. 2019). In rodent models of T2D, remission of hyperglycemia can be sustained for weeks or months following a single intracerebroventricular injection of FGF1. The underlying mechanism remains under active study, but instead of simply lowering the BG level, FGF1 appears to act on MBH neurocircuits to re-set the defended level of glycemia in the normal range. Such an effect would not seem possible unless *1)* the brain plays a key role to establish the defended BG level, and *2)* a defect in this system contributes to the pathogenesis of hyperglycemia in these animal models. Whether this response reflects the normalization of impaired brain glucose sensing in diabetic animals is an active area of study. Of even greater importance is the question of whether T2D in human involves a similar pathogenic sequence.

5.5 Conclusion

The notion that glucose homeostasis is governed primarily by the pancreas, rather than the brain, has come under increasing scrutiny in the wake of findings that simply cannot be explained by this model. Particularly noteworthy is evidence that in rodent models of T2D, the defended BG can be restored to normal for weeks or months by targeting brain systems that control glucose homeostasis. Fortunately, recent advances in neuroscience offer an

unprecedented ability to map and functionally characterize the relevant neurocircuits in rodent models (Scarlett et al. 2016; Scarlett et al. 2018; Brown et al. 2019; Tennant et al. 2019). We should seize upon this opportunity to advance our understanding of how glucose homeostasis is regulated by the brain, identify the contribution made by defects in this regulatory system to the pathogenesis of T2D, and determine if such defects offer novel approaches to more effective treatment of this disease.

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Zheng, Yan, Sylvia H. Ley, and Frank B. Hu. 2018. 'Global aetiology and epidemiology of type 2 diabetes mellitus and its complications', *Nature Reviews Endocrinology*, 14: 88-98.

APPENDIX A- PUBLICATIONS

Peer-Reviewed Publications

1. **Brown JM**, Scarlett JM, Matsen ME, Nguyen HT, Secher AL, Jorgensen R, Morton GJ, Schwartz MW. "The Hypothalamic Arcuate Nucleus-Median Eminence Is a Target for Sustained Diabetes Remission Induced by Fibroblast Growth Factor 1." Diabetes. 2019 May;68(5):1054-1061. Chapter 2 of this thesis

2. Scarlett JM, Muta K, **Brown JM**, Rojas JM, Matsen ME, Acharya NK, Secher A, Ingvorsen C, Jorgensen R, Høeg-Jensen T, Stefanovski D, Bergman RN, Piccinini F, Kaiyala KJ, Shiota M, Morton GJ, Schwartz MW. "Peripheral Mechanisms Mediating the Sustained Antidiabetic Action of FGF1 in the Brain." Diabetes. 2019 Mar;68(3):654-664. Chapter 4 of this thesis

3. Hultman K, Scarlett JM, Baquero AF, Cornea A, Zhang Y, Salinas CBG, **Brown J**, Morton GJ, Whalen EJ, Grove KL, Koegler FH, Schwartz MW, Mercer AJ. "The central fibroblast growth factor receptor/beta klotho system: Comprehensive mapping in Mus musculus and comparisons to nonhuman primate and human samples using an automated in situ hybridization platform." J Comp Neurol. 2019 Aug 15;527(12):2069-2085.

4. Mirzadeh Z, Alonge KM, Cabrales E, Herranz-Pérez V, Scarlett JM, **Brown JM,** Hassouna R, Matsen ME, Nguyen HT, Garcia-Verdugo JM, Zeltser LM, Schwartz MW. "Perineuronal Net Formation during the Critical Period for Neuronal Maturation in the Hypothalamic Arcuate Nucleus." Nat Metab. 2019 Feb;1(2):212-221.

5. **Brown JM,** Scarlett JM, Schwartz MW. "Rethinking the role of the brain in glucose homeostasis and diabetes pathogenesis." J Clin Invest. 2019 Jul 22;129(8):3035-3037. doi: 10.1172/JCI130904. eCollection 2019 Jul 22. Chapter 5 of this thesis

6. Scarlett JM, Rojas JM, Matsen ME, Kaiyala KJ, Stefanovski D, Bergman RN, Nguyen HT, Dorfman MD, Lantier L, Wasserman DH, Mirzadeh Z, Unterman TG, Morton GJ, Schwartz MW. "Central injection of fibroblast growth factor 1 induces sustained remission of diabetic hyperglycemia in rodents." Nat Med. 2016 Jul;22(7):800-6. Acknowledgment: **Brown JM**

Publications under Peer-Review

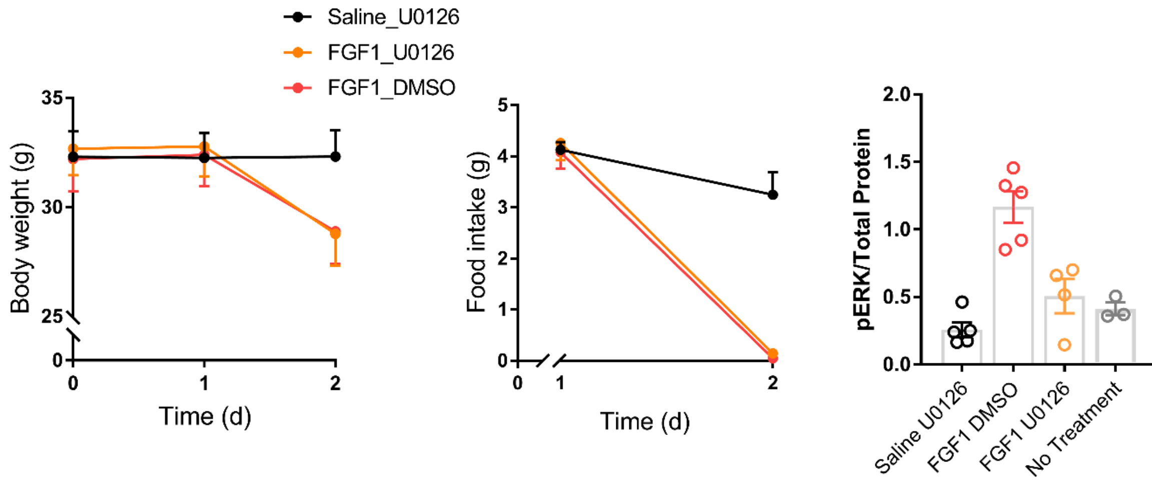
1. Bentsen MA, Rausch DM, Mirzadeh Z, Muta K, Scarlett JM, **Brown JM,** … Pers TH, and Schwartz MW. "Transcriptomic Analysis Links the Response of Diverse Hypothalamic Cell Types 2 to Sustained Diabetes Remission Induced by Fibroblast Growth Factor 1." Nat Comm. Re-submission Nov.2019

2. Alonge A, Mirzadeh Z, Scarlett JM, Logsdon AF, **Brown JM**, Cabrales E, Chan CK, Karl J. Kaiyala KJ, Bentsen MA, Banks WA, Guttman M, Wight TN, Morton G and Schwartz MW. "Hypothalamic perineuronal net assembly is required for sustained diabetes remission induced by fibroblast growth factor 1." Mat Metab.

Publications in Preparation

1. **Brown JM**, Phan B, Bentsen MA, Rausch DM, Matsen ME, Secher AL, Jorgensen R, Pers TH, Morton GJ, Schwartz MW and Scarlett JM. "Central Fibroblast Growth Factor 1 mediates diabetes remission through sustained phosphorylation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK)." Cell Reports. Chapter 3 of this thesis

APPENDIX B- SUPPLEMENTARY FIGURES



Supplementary Figure 3-2 MAPK/ERK signaling is not required for acute, transient effects of icv FGF1 injection on food intake and body weight.

Icv injection of FGF1 or Veh followed by infusion of U0126 or Vehicle DMSO for 24 hours A) Body weight, and B) food intake followed by C) quantitative western blot of hypothalamic tissue punches validating pERK1/2 inhibition after icv injection of FGF1 (n=3-5/group).

VITA

Dr. Jenny M. Brown

EDUCATION

University of Washington, Seattle, WA
PhD Pathology, UW School of Medicine
Program: Molecular Medicine and Mechanisms of Disease
Expected Defense Date: June 2020

California State University Monterey Bay, Seaside, CA
B.S. in Biology Emphasis in Molecular biology
Minor in Statistics
Graduation Date: June 2015

RESEARCH AND TRANING FUNDING

2019-2020	UW Diabetes Research Center Samuel and Althea Stroum Endowed Graduate Fellowship (1 yr of funding)
2016-2019	UW Cardiovascular Pathology Training Grant (3 yrs of funding)
2015-2016	UW Molecular Medicine and Mechanism of Disease Training Grant (1 yr of funding)
2015	Travel Grant; American Heart Association ($1500)
2015	CSU Sally Casanova Pre-Doctoral Scholar ($3000)
2015	Associated Student Capstone Grant: California State University Monterey Bay ($500)
2015	Research Grant: University of California, Monterey Bay ($1500)
2014	Travel Grant: Wadsworth Center New York Department of Health ($2000)
2014	Research and Travel Grant: California State University Monterey Bay ($2400)
2013	Research and Travel Grant: California State University Monterey Bay ($2000)
2013	Robert Embree Scholarship ($1500)

HONORS AND AWARDS

2019	Pathology and Lab Medicine Retreat Poster Presentation Award ($50)
2017	Samuel and Althea Stroum Endowed Graduate Fellowship
2015	Spotlight on Service Award "For Kids Eat Right"
2014	Outstanding Presentation ABRCMS Conference ($200)
2014	Louis Stokes Alliance for Minority Participation Student Scholar Award
2013-2015	Undergraduate Research Opportunities Center Scholar

CURRENT RESEARCH

Mechanisms of Central Fibroblast Growth Factor 1 **(March 2016-present)**
University of Washington, Department of Medicine
Principal Investigator: Dr. Michael Schwartz, MD and Dr. Gregory Morton, PhD

Thesis Project: Investigating the role of fibroblast growth factor 1 (FGF1) in the brain my goals are 1) to identify the specific brain regions that central administration of FGF1 acts to induce sustained diabetes remission 2) determine the signaling mechanisms required and 3) determine the peripheral mechanisms by which glucose lowering is achieved. In addition, I became fluent with stereotactic microinjections, cannula placement surgeries, intra-cardial perfusion fixation, and confocal microscopy imaging and

staining analyses of coronal brain sections. I found that direct microinjection of FGF1 into the arcuate nucleus was sufficient to induce glucose lowering; diabetes remission may depend on sustained MAPK/ERK signaling and is driven in part by changes in peripheral glucose metabolism including increases in hepatic glucose uptake and liver glucokinase. Since beginning my thesis research, I have honed my surgery skills, developed a protocol to target the arcuate nucleus in rats, and published two first author manuscripts with 2 more upcoming.

Relevant Skills: Experimental design, pharmacological drug administration into discrete brain regions, stereotaxic surgery in rodents, and robust statistical analysis of longitudinal data.

PUBLICATIONS

Peer-Reviewed Publications

1. **Brown JM**, Scarlett JM, Matsen ME, Nguyen HT, Secher AL, Jorgensen R, Morton GJ, Schwartz MW. "The Hypothalamic Arcuate Nucleus-Median Eminence Is a Target for Sustained Diabetes Remission Induced by Fibroblast Growth Factor 1." Diabetes. 2019 May;68(5):1054-1061.

2. Scarlett JM, Muta K, **Brown JM**, Rojas JM, Matsen ME, Acharya NK, Secher A, Ingvorsen C, Jorgensen R, Høeg-Jensen T, Stefanovski D, Bergman RN, Piccinini F, Kaiyala KJ, Shiota M, Morton GJ, Schwartz MW. "Peripheral Mechanisms Mediating the Sustained Antidiabetic Action of FGF1 in the Brain." Diabetes. 2019 Mar;68(3):654-664.

3. Hultman K, Scarlett JM, Baquero AF, Cornea A, Zhang Y, Salinas CBG, **Brown J**, Morton GJ, Whalen EJ, Grove KL, Koegler FH, Schwartz MW, Mercer AJ. "The central fibroblast growth factor receptor/beta klotho system: Comprehensive mapping in Mus musculus and comparisons to nonhuman primate and human samples using an automated in situ hybridization platform." J Comp Neurol. 2019 Aug 15;527(12):2069-2085.

4. Mirzadeh Z, Alonge KM, Cabrales E, Herranz-Pérez V, Scarlett JM, **Brown JM,** Hassouna R, Matsen ME, Nguyen HT, Garcia-Verdugo JM, Zeltser LM, Schwartz MW. "Perineuronal Net Formation during the Critical Period for Neuronal Maturation in the Hypothalamic Arcuate Nucleus." Nat Metab. 2019 Feb;1(2):212-221.

5. **Brown JM,** Scarlett JM, Schwartz MW. "Rethinking the role of the brain in glucose homeostasis and diabetes pathogenesis." J Clin Invest. 2019 Jul 22;129(8):3035-3037. doi: 10.1172/JCI130904. eCollection 2019 Jul 22.

6. Scarlett JM, Rojas JM, Matsen ME, Kaiyala KJ, Stefanovski D, Bergman RN, Nguyen HT, Dorfman MD, Lantier L, Wasserman DH, Mirzadeh Z, Unterman TG, Morton GJ, Schwartz MW. "Central injection of fibroblast growth factor 1 induces sustained remission of diabetic hyperglycemia in rodents." Nat Med. 2016 Jul;22(7):800-6. Acknowledgment: **Brown JM**

Publications under Peer-Review

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Publications in Preparation

1. **Brown JM**, Phan B, Bentsen MA, Rausch DM, Matsen ME, Secher AL, Jorgensen R, Pers TH, Morton GJ, Schwartz MW and Scarlett JM. "Central Fibroblast Growth Factor 1 mediates

diabetes remission through sustained phosphorylation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK)." Nat Metab.

2. **Brown JM**, Schwartz MW and Scarlett JM. "Fibroblast Growth Factor 1 insights into glucose and energy homeostasis". Review Article. Diab Int.

3. Alonge A, Mirzadeh Z, Scarlett JM, Logsdon AF, **Brown JM**, Cabrales E, Chan CK, Karl J. Kaiyala KJ, Bentsen MA, Banks WA, Guttman M, Wight TN, Morton G and Schwartz MW. "Hypothalamic perineuronal net assembly is required for sustained diabetes remission induced by fibroblast growth factor 1." Mat Metab.

CONFERENCE PRESENTATIONS

Invited

Brown JM. "High Stakes Scholarships", California State University Monterey Bay 2014 Undergraduate Research Week, Seaside, California (April 2014).
Brown JM. "Identification of the brain area underlying diabetes remission induced by fibroblast growth factor 1 (FGF1)", Diabetes Research Center Annual Retreat 2019, Seattle, Washington (Nov 2019).

Oral Presentations

Brown JM. "Targeting to Brain to Cure Type Two Diabetes", M3D Capstone Presentation 2019, Seattle, Washington (April 2019).
Brown JM. "Investigating a role for integrin signaling in central FGF1 induced diabetes remission", M3D Spring Retreat 2019, Seattle, Washington (June 2019).
Brown JM, Scarlett JM, Matsen ME, Acharya NK, Secher A, Nguyen HT, Morton G and Schwartz MW. "An action in the hypothalamic arcuate nucleus is sufficient to explain the sustained remission of diabetes induced by central administration of Fibroblast Growth Factor 1 (FGF1)", American Diabetes Scientific Sessions 2018, Orlando, Florida (June 2018).
Brown JM. "An action in the ARC is sufficient to explain central FGF1 induced diabetes remission", M3D Spring Retreat 2018, Seattle, Washington (June 2018).
Brown JM, Scarlett JM, Rojas JM, Matsen ME, Acharya NK, Secher A, Harvey TJ, Velasco K, Nguyen HT, Kaiyala KJ, Morton G and Schwartz MW. "Improved b-cell function may contribute to diabetes remission induced by central FGF1 in Zucker Diabetic Fatty rats", American Diabetes Scientific Sessions 2017, San Diego, California (June 2017).
Brown JM, Scarlett JM, Morton G and Schwartz MW. "Role of Insulin Signaling in the Glucose Lowering effect of Central Fibroblast Growth Factor 1", M3D Spring Rotation Talk. Seattle, Washington (May 2016).
Brown JM, Herron B, and Kay D. "Geographic Distribution of Transient Thyroid Hormone Abnormalities Across NYS", Fall 2015 Capstone Festival, Seaside, California (May 2015).
Brown JM, Herron B, and Kay D. "Geographic Distribution of Transient Thyroid Hormone Abnormalities Across New York State", Wadsworth Center REU Student Symposium. Albany, New York (August 2014).

Poster Presentations

Brown JM, Scarlett JM, Matsen ME, Nguyen HT, Secher A, Jorgensen R, Morton G and Schwartz MW. "The hypothalamic arcuate nucleus-median eminence is a target for sustained diabetes remission induced by fibroblast growth factor 1", Diabetes Day, Seattle, Washington (May 2019).

Brown JM, Scarlett JM, Matsen ME, Nguyen HT, Secher A, Jorgensen R, Morton G and Schwartz MW. "The hypothalamic arcuate nucleus-median eminence is a target for sustained diabetes remission induced by fibroblast growth factor 1", Pathology and Laboratory Medicine Joint Retreat, Seattle, Washington (September 2019). *poster award

Brown JM, Scarlett JM, Matsen ME, Nguyen HT, Secher A, Jorgensen R, Morton G and Schwartz MW. "Sustained diabetes remission induced by the action of fibroblast growth factor 1 hypothalamic arcuate nucleus", Diabetes Day, Seattle, Washington (May 2018).

Brown JM, Scarlett JM, Rojas JM, Matsen ME, Acharya NK, Secher A, Harvey TJ, Velasco K, Nguyen HT, Kaiyala KJ, Morton G and Schwartz MW. "Sustained diabetes remission induced by the action of Fibroblast Growth Factor 1 (FGF1) in the hypothalamic arcuate nucleus", American Society for Biochemistry and Molecular Biology (ASBMB) Deuel Conference on Lipids, San Diego, California (March 2018).

Brown JM, Scarlett JM, Morton G and Schwartz MW. "Sustained Glucose Lowering by the central action of Fibroblast Growth Factor 1", M3D Autumn Rotation Talk. Seattle, Washington (December 2015).

Brown JM, Hank Farr, and Dr. Lisa Maves. "Characterization of Pbx2 Pbx4 Gene Function in Danio rerio Cardiovascular Development", M3D Winter Rotation Talks. Seattle, Washington (March 2016).

Brown JM, Herron B, and Kay D. "Geographic Distribution of Transient Thyroid Hormone Abnormalities Across New York State", Annual Biomedical Research Conference for Minority Students. San Antonio, Texas (November 2014). *poster award

Brown JM, Knuder S, Abdallah S, Muturi H, and Najjar SM. "CEACAM1: is a key regulator of IRS1 phosphorylation and FAS activity", 26th Annual CSU Biotechnology Symposium. Santa Clara, California (January 2014).

Brown JM, Knuder S, Abdallah S, Muturi H, and Najjar SM. "CEACAM1: is a key regulator of IRS1 phosphorylation and FAS activity", The Undergraduate Research Week. Seaside, California (April 2014

PREVIOUS RESEARCH

Gene Function in Cardiovascular Development **(January-March 2016)**

University of Washington, Seattle Children's Hospital

Principal Investigator: Dr. Lisa Maves, PhD

Rotation Project: Characterized pbx gene function in cardiovascular development in *Danio rerio*. Tested whether pbx genes are required for cardiomyocytes differentiation using in-situ hybridization and conduct functional heart rate assays. Collected, analyzed with R- code, and interpreted data for a poster presentation.

Relevant Skills: Zebra fish husbandry, in situ hybridization and statistical analysis.

Fibroblast Growth Factor 1 effects on Hypoglycemia **(September-December 2015)**

University of Washington, Department of Medicine

Principal Investigator: Dr. Michael Schwartz, MD and Dr. Gregory Morton, PhD

Rotation Project: Investigated the effect of repeated central administration of Fibroblast Growth Factor 1 on blood glucose, body weight, and food intake and contrasted different FGF peptides effect on cellular activation in hypothalamic brain regions. Collected, compiled, and interpreted data for a poster presentation. Relevant Skills: Stereotaxic surgery, and intracerebroventricular injections.

Sodium Hydrogen Sulfide effects on Rat Cardiomyocytes **(July-August 2015)**

University of Washington, Department of Anesthesiology

Principal Investigator: Dr. Wang Wang, MD PhD

Project: Investigated the exogenous role of hydrogen sulfide on reactive oxygen species (ROS) and mitochondrial flash in primary rat cardiomyocytes. Conducted a 10-week research project funded by the Sally Casanova Scholarship. Relevant Skills: Primary cell culture and confocal microscopy.

Impact of Thyroid Hormones on Skin Angiogenesis **(June-August 2014)**

Undergraduate Researcher, The Wadsworth Center New York Department of Health, Dept. of Molecular Genetics, Albany, New York

Principal Investigator: Dr. Bruce Herron, PhD

Project: Conducted a novel skin angiogenesis assay using B6, FVB, and 129 mice to optimize the assay for testing the impact of thyroid hormones on skin angiogenesis. Relevant Skills: Tissue culture, animal handling, and UV microscopy.

Transient Thyroid Abnormalities in Newborns Screening (June-August 2014)

Undergraduate Researcher, The Wadsworth Center New York Department of Health, Dept. of Human Genetics, Albany, New York

Principal Investigator: Dr. Denise Kay, PhD

Project: Analyzed the geographic distribution of transient thyroid abnormalities across New York State and consolidated the results for a report and presentation. Relevant Skills: Large data analysis, excel power map, and sanger sequencing.

Gene Identification of Organophosphate Degradation Bacteria (November 2013)

Undergraduate Researcher, California State University Monterey Bay, California Division of Science and Environmental Policy, Seaside, California

Principal Investigator: Dr. Arlene Haffa, PhD

Project: Summarized bacteria in a treatment wetland to analyze organophosphate degradation by identifying genes in bacteria that break down pesticides.

Compared results and consolidated data sets for presentations at conferences. Relevant Skills: PCR, bacteria culture, and gene sequencing.

Protein Interactions of Insulin Resistance and Atherosclerosis (June-August 2013)

Undergraduate Researcher, University of Toledo Health Science Campus, Center for Diabetes and Endocrine Research, Toledo, Ohio

Principal Investigator: Dr. Sonia Najjar, PhD

Project: Investigated the mechanism of carcino embryonic antigen related cell adhesion molecule 1 (CEACAM1) and its regulation of fatty acid synthase (FAS), tyrosine phosphatase SHP2 and endothelial nitric oxide synthase (eNOS). Relevant Skills: Cell culture, transfection, co-immunoprecipitation, western blot analysis, and FAS activity assays.

TEACHING & MENTORING EXPERIENCE

2019 NIDDK medical student from University Southern Florida, Huzaifa Wasanwala
2017-Present Teach Annual Grant Writing Workshop (NIH F31 and F32 focus) UW, Seattle
2017 NIDDK medical student from Midwestern University, Eddie Khav
2016 Co-mentored Summer Undergraduate Whiteman College, Danielle Wieck
2016 Graduate TA, Mechanism of Disease (Path 550), UW Seattle, WA
2014 Undergraduate TA, Organic Chemistry (Chem 312), CSU Monterey Bay, CA
2013-2015 Peer-to-Peer Mentor, CSU Monterey Bay, CA
2013-2015 Student Teacher, Kids Eat Right, CHOMP Hospital, Monterey Bay, CA

LEADERSHIP EXPERIENCE

2020 Student Advocates for Graduate Education (SAGE) Working Group-Research Funding
2020 Student coordinator for Pathology Presents Speaker Series
2019-2020 Graduate and Professional Student Senate (Pathology Senator)
2017-2018 Graduate and Professional Student Senate (Pathology Senator)
2017-Present Professional Development Committee Leader Pathology
2015-2017 South Lake Union Group Professional Development Chair
2014 CHAMACOS CSUMB Campus Tour

2013 Established a Multi-plant Monitoring Site to Study Phenology CSUMB

TRAINING AND CERTIFICATES

Independent Aseptic Surgical Certification **(March 2019)**
Passed Aseptic Surgical Certification Approved by the University of Washington for working with the IACUC on Dr. Schwartz's Protocol.

Animal Use Training Sessions **(July 2015-present)**
Approved by the University of Washington for working with the IACUC on Dr. Schwartz's Protocol.

Animal Care and Use in Research and Education Certification **(August 2014)**
Approved by the Wadsworth Center AC Group for working with the IACUC on Dr. Herron's Protocol.

Hazardous Communication Training **(November 2013)**
Completed hazardous waste, human pathogen, personal protective equipment, and hazardous communications training.

Institutional Animal Care and Use Committee (IACUC) Training **(June 2013)**
Researched at the University of Toledo, Health Science Campus. A week-long course certified by IACUC to work on Dr. Najjar's Protocol.

TECHNICAL SKILLS

- Academic Writing
- Peer Review
- Teaching and Supervision
- Statistics
    - R-code

In-vivo

- Handling mice and rats
- Systemic and intracerebroventricular injections
- Energy homeostasis measures
- Metabolic studies (ITT, IPGTT)
- Stereotaxic cannula surgery
- Stereotaxic microinjection techniques
- Osmotic minipump implantation

Basic lab techniques

- RNA extraction
- Real-time PCR
- Western blot analysis
- Immunohistochemistry
- ELISA
- Microscopy
    - Confocal
    - Light
- Cell Signaling
- Cell Culture
- Transfection

Computer Skills

- MS office (Word, Powerpoint, Excel)
- Adobe Photoshop and Illustrator
- EndNote, Mendeley, Citavi
- Image J, Qqpath
- GraphPad Prism