THE LIVER-DERIVED ENDOCRINE HORMONE FGF21 ALTERS METABOLISM AND DIURNAL BEHAVIOR VIA THE NERVOUS SYSTEM

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DEDICATION

For my husband

His generous heart and superhuman patience carry me through and through.

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by

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Fuel acquisition is essential to survival. During privation, the body protects glucose concentrations acutely by glycogenolysis, and later by gluconeogenesis and ketogenesis. Additionally, animals alter daily behavioral patterns to seek food, but eventually reduce energetically costly activities (growth, reproduction, locomotion). Little is known about the mechanisms that orchestrate and coordinate these physiological and behavioral responses to starvation. The liver-derived endocrine hormone fibroblast growth factor 21 (FGF21) is induced in chronic fasting and acts as a global starvation signal. Previous studies focusing on FGF21 as an anti-diabetic drug indicate that FGF21 coordinates whole-body fat utilization and energy expenditure. However, its basic physiological role is underexplored.

Acute injection of recombinant FGF21 quickly elicits a coordinated program between tissues resulting in reduced plasma insulin and gluconeogenic and thermogenic gene expression programs in liver and brown adipose, effects that require an intact

animal. Mice with chronic FGF21 overexpression (FGF21tg) are smaller in size, females are infertile, and if fasted, they undergo torpor, an energy-conserving process. Taken together, these data suggest that FGF21 may exert some effects through the nervous system.

To explore this idea, I utilized anatomically-guided laser capture microdissection followed by quantitative, real-time PCR to profile expression of the FGF receptor/co-receptor family in specific hypothalamic nuclei of mice. Surprisingly, the FGFR1-IIIc/βKlotho complex is found in the suprachiasmatic nucleus (SCN), area postrema (AP), nucleus tractus solitarii (NTS), and nodose ganglion (cell body of vagus nerve), implicating roles in circadian and metabolic regulation.

Results of surgical, pharmacological, and genetic strategies indicate the vagus senses circulating FGF21, resulting in adrenergic efferent responses that reduce insulin secretion, while a different adrenergic site modulates liver and brown adipose gene expression.

Analyses of the effects of FGF21 on the SCN, the body's master clock, using running wheels show FGF21tg mice have dramatically altered circadian activity, likely as a consequence of inhibiting SCN output functions. Deletion of βKlotho specifically from the SCN rescues this behavior in addition to growth defects of FGF21tg mice.

To date, this is the first description of a liver-derived endocrine hormone that affects such diverse aspects of the starvation response by acting on the nervous system.

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ACC – Acetyl-Coenzyme A Carboxylase

ACTH – Adrenocorticotropin Releasing Hormone

ALS –Insulin-Like Growth Factor Binding Protein, Acid Labile Subunit

ANS- Autonomic Nervous System

AP – Area Postrema

ARC – Arcuate Nucleus

ATGL – Adipose Triglyceride Lipase

AVG – Average

AVP – Arginine Vasopressin

AVPV – anteroventral periventricular nucleus

BAT – Brown Adipose Tissue

Bmal – Brain And Muscle Aryl Hydrocarbon Receptor Nuclear Translocator-Like

BMP8 – Bone Morphogenic Protein 8

bOHB – Beta Hydroxybutyrate

CamKIIa – calmodulin kinase IIa

CCK – Cholesystokinin

cDMH – Compact Dorsal Medial Nucleus

CEL – Carboxyl Ester Lipase

CLPS – Colipase, Pancreatic

CR – Circadian Rhythm

Cre – cre recombinase

CTX – Cerebral Cortex

Cyp11a1 – Cytochrome P450, 11a1

Cyp2d9 – Cytochrome P450, 2d9

DBH – Dopamine Beta Hydroxylase

DBP – D Site Albumin Promoter Binding Protein

Dio2 – Deiodinase 2

DMV (X) – Dorsal Medial Nucleus Of The Vagus (Tenth Cranial Nerve)

dmVMH– Dorsal Medial Ventromedial Nucleus

DRG – Dorsal Root Ganglion

DUSP4 – Dual-Specificity Phosphatase 4

ELOVL3 – Elongation Of Very Long Chain Fatty Acid Elongase 3

FA – Fatty Acid

FAS – Fatty Acid Synthase

FE – Food Entrainment

FEO – Food Entrainment Oscillator

FGF15/19 – Fibroblast Growth Factor 15/19

FGF21 – Fibroblast Growth Factor 21

FGFR – Fibroblast Growth Factor Receptor

G6Pase – Glucose 6 Phosphatase

G6Pc – Glucose 6 Phosphatase

GnRH – Gonadotropin Releasing Hormone

GPCR – G-Protein Coupled Receptor

HSD11b1 – Hyroxysteroid Dehydrogenase 11b1

HSD11b2 – Hydroxysteroid Dehydrogenase 11b2

HSD3b1 – Hydroxysteroid Dehydrogenase 3b1

HSD3b5 – Hydroxysteroid Dehydrogenase 3b5

HSL – Hormone Sensitive Lipase

ICV – intracerebroventricular

IGF1 – Insulin-Like Growth Factor

IGFBP1 – Insulin-Like Growth Factor Binding Protein

IML – Interomedial Lateral

KD – Ketogenic Diet

Keto – Ketogenic Diet

KO – Knock Out

lDMH– Lateral Dorsal Medial Nucleus

LH – Luteinizing Hormone

LHA1 – Lateral Hypothalamic Area, At The Level Of The PVH

LHA2 – Lateral Hypothalamic Area, At The Level Of The Rostral DMH

lPBN – Lateral Parabrachial Nucleus

MnPO – Median Preoptic Nucleus

mPBN – Medial Parabrachial Nucleus

mpk – Milligram Per Kilogram

MUP3 – Major Urinary Protein 3

NAV1.8 – Sodium Channel V1.8

NEFA – Non-Esterified Fatty Acids

Nes – Nestin

NG – Nodose Ganglion Of The Vagus Nerve (Tenth Cranial Nerve)

NTS – Nucleus Of The Solitary Tract; Nucleus Tractus Solitarii

OVLT – organum vasculosum of lamina terminalis

PCR – Polymerase Chain Reaction

PEPCK – Phosphoenolpyruvate Kinase

Per1 – Period 1

Per2 – Period 2

PGC1 – Peroxisome Proliferative Activated Receptor, Gamma, Coactivator 1 Alpha

PH – posterior hypothalamic area

Phox2b – Paired-Like Homoeobox 2b

PMV – Ventral Premammillary nucleus

PNMT – Phosphatidylethanolamine N-Methyltransferase

PNS – Peripheral Nervous System

PPAR – Peroxisome Proliferator Activated Receptor

PVH – Paraventricular Nucleus

QPCR – Quantitative Real Time PCR

RCN – Retrochiasmatic Nucleus

Reverba –

RF– restricted feeding

Rgs16 – Regulator of G-Protein Signaling 16

RXR – Retinoid X Receptor

Sac – Sacrifice

SCD1 – Stearoyl-Coenzyme A Desaturase 1

SCN – Suprachiasmatic Nucleus

SEM – Standard Error of the Mean

SlcO1a1 – Solute Carrier Organic Anion Transporter Family, Member 1a1

SNS – Sympathetic Nervous System

SOCS2 – Suppressor Of Cytokine Signaling 2

StaAR– Steroid Acute Regulatory Protein

Sult1d1 – Sulfotransferase 1d1

Sult1e1 – Sulfotransferase 1e1

Tg – Transgenic

TH – Tyrosine Hydroxylase

Thal AD – Anterodorsal Thalamus

tKBs– Total Ketone Bodies

Trigs – Triglycerides

Veh – Vehicle

Vgx – Vagotomy

VIP – Vasoactive Intestinal Peptide

vlVMH– Ventrolateral Ventromedial Nucleus

VTA – Ventral Tegmental Area

WAT – White Adipose Tissue

CHAPTER ONE

INTRODUCTION

Mammals possess multiple pathways that partition nutrients, one of which is through members of the peroxisome proliferator (PPAR) class of nuclear receptors, which regulate the expression of a key endocrine molecule fibroblast growth factor 21 (FGF21). PPARα regulates its expression in the liver in response to prolonged fasting, resulting in coordination of fatty acid oxidation and gluconeogenesis (Badman et al., 2007; Inagaki et al., 2007; Potthoff et al., 2009). Conversely, PPARγ regulates FGF21 expression in the adipose and induces a lipogenic program in response to feeding (Dutchak et al., 2012). Overall, FGF21 acts to coordinate whole-body fat utilization and energy expenditure, resulting in lower serum glucose, insulin, and triglycerides in both a lean and pathophysiological setting of diabetes and obesity (Potthoff et al., 2012). Thus, these PPAR-FGF21 relationships comprise a complex feedback loop between tissues resulting in metabolic integration throughout the body. But the primary target tissue upon which circulating FGF21 acts is unclear. Identification of the anatomical substrates of what is posited as a promising anti-diabetic will offer insight into its pharmacodynamics as well as potential side effects. Moreover, it will certainly reveal previously unrecognized ways in which organisms achieve physiologic integration in the face of metabolic challenges.

As discussed in this chapter, several observations indicate that FGF21 secreted from the liver can act on the brain to govern aspects of FGF21 biology and pharmacology. In Chapter 2, I provide neuroanatomical evidence of FGF21 action.

Chapter 3 describes the involvement of a sympathetic efferent response as an underlying mechanism of FGF21's pharmacological properties, while in Chapter 4, I demonstrate the importance of the body's master clock in mediating the behavioral and many physiological outcomes of chronically elevated FGF21. In summation, my studies provide direct evidence for the importance of the nervous system as a major contributor to FGF21-elicited biology.

The Autonomic Nervous System Mediates Communication between Body and Brain

Metabolic homeostasis requires the coordinate regulation of molecular programs between a variety of tissues including the liver, adipose, adrenals, pancreas, intestine, and brain. The principal sites in the brain for sensing and integrating information regarding an animal's metabolic status are the hypothalamus and hindbrain. The autonomic nervous system acts as the conduit between the body and the brain, and has two main branches. The sympathetic branch uses norepinephrine as its main neurotransmitter and is thought of as catabolic, mediating "fight or flight" responses. The sensory component of the sympathetic system resides in the dorsal root ganglia located throughout the spinal column. On the other hand, the parasympathetic, "rest and digest" division is regarded as anabolic, and uses acetylcholine. Its sensory neurons comprise the vagus nerve, also known as the Xth cranial nerve, whose cell body is the nodose ganglion. Information regarding nutrient status can be conveyed by means of sensing metabolites or hormones locally by direct innervation of target tissues or via direct action on nervous system sites in the brain after transit in the blood stream. In turn, effector responses may be delivered as post-synaptically released neurotransmitters or neuropeptides. Conversely, hormonal

systems such as ACTH and anti diuretic hormone exist. Together, the autonomic nervous system controls all homeostatic processes throughout an organism (Lowey, 1990).

We have shown that the primary source of circulating FGF21 is from the liver secreted into the blood in animals treated with PPARα agonists (Dutchak et al., 2012), which is representative of a fasted state. Therefore, my studies regarding FGF21 were done within the context of the fasting response.

The Fasting Response Has Several Stages

The brain is the most metabolically active tissue in the body, consuming about 25% of the body's glucose as its primary source of fuel during normal conditions. Maintenance of the electrochemical gradients necessary for neurotransmission accounts for the majority of the brain's energy budget (Byrne, 2004). Although the brain does store and use a small amount of glycogen, it cannot process fatty acids for fuel. Therefore, the brain is highly dependent on the storage and mobilization of energy sources from peripheral tissues. Given the importance of maintaining adequate energy to sustain life, it is reasonable that higher organisms would evolve both reflexive and adaptive systems to respond to privation, and that many of these would share overlapping features, while having their own unique properties.

The Reactive Phase

A decline in the level of glucose in the blood is sensed both at the cellular level, and on an organismal level. The brain is exquisitely sensitive to changes in blood glucose concentrations, utilizing cellular level mechanisms much like that of the pancreatic islet

(Levin et al., 2001; Marty et al., 2007). Upon sensing lower glucose levels, brainstem and hypothalamic nuclei elicit a reflexive sympatho-adrenal response that targets multiple peripheral tissues [recently reviewed in (Watts and Donovan, 2010)]. Sympathetic motor efferents directly target the endocrine pancreas to repress insulin secretion, while inducing glucagon release. Additionally, the adrenal medullae are stimulated to release epinephrine, which then acts on the liver to stimulate glycogenolysis, further potentiated by glucagon released from the pancreas, and on the adipose to elicit lipolysis. As fasting proceeds and liver glycogen is depleted, gluconeognesis begins from amino acids and glycerol, while fatty acids released by lipolysis are converted to acetyl-CoA for ketogenesis. Neither process is possible until insulin concentration drops. Eventually, muscle amino acids are spared with the rise in ketone bodies, which feedback to repress proteolysis and prevent muscle wasting. Some studies indicate that lipolysis may also be inhibited by elevated ketone bodies acting through the GPCR, GPR109 (Taggart et al., 2005).

The Adaptive Phase

Adaptive mechanisms eventually come into play. Ghrelin secretion from the stomach is likely elicited upon sympathetic outflow (Zhao et al., 2010b), which can lead to growth hormone release (Goldstein et al., 2011; Zhao et al., 2010a). Furthermore, glucocorticoid synthesis and secretion are increased. Eventually, FGF21 is induced, an effect aided by the cooperation of glucagon and fatty acids activating PPARα (Badman et al., 2007; Berglund et al., 2010; Inagaki et al., 2007), and possibly through a feed-forward mechanism involving corticosterone activation of the glucocorticoid receptor

(GR) in the liver (Bookout and Cummins, manuscript in preparation). In small animals, the brown adipose initiates a program of thermogenesis to buffer against a declining body temperature [recently reviewed in (Richard and Picard, 2011; Whittle et al., 2011). Additionally, animals will increase food-seeking behavior, mediated both by ghrelin and orexin pathways (Yamanaka et al., 2003).

The Starvation Phase

During prolonged food deprivation, ketone bodies satisfy the energetic requirements of the brain, while other tissues continue to use fatty acids (Cahill, 2006). Chronic nutrient deficits lead to down-regulation of catabolic processes aided by sympathetic tone [lipolysis, proteolysis;(Guyton, 2000)]. Moreover, energetically costly activities are inhibited such as immune function, locomotion, growth, and reproduction (Ahima et al., 1996; Bronson and Marsteller, 1985; Connors et al., 1985; Guyton, 2000).

Liver-Derived Endocrine FGF21 Acts During Adaptive and Chronic Fasting States

FGF21 belongs to an exceptional subclass of the FGF superfamily, along with FGF15/19 and FGF23. They are unique in that they lack the heparin binding domain characteristic of other FGFs, a feature allowing diffusion away from tissues in which they are produced. Thus, they act hormonally as a means to coordinate responses between organs. Four FGF receptors exist (FGFR1-4), the first 3 of which undergo alternative splicing to produce numerous isoforms. Endocrine FGFs are also unique in their requirement for a co-receptor to initiate signaling through FGFR IIIc isoforms. FGF23 needs Klotho, while FGF15/19 and 21 utilize βKlotho to elicit biological responses

(Kharitonenkov et al., 2008; Kurosu et al., 2007; Ogawa et al., 2007). Additionally, FGF15/19 can signal through a 3rd Klotho family member, Lctl (Fon Tacer et al., 2010). FGFRs are receptor tyrosine kinases, and as such elicit a downstream signaling cascade through phosphorylation of FRS2a and ERK1/2, among others. While the FGFRs are expressed in a multitude of tissues, βKlotho is restricted in its distribution (Fon Tacer et al., 2010), thus conferring specificity of either FGF15/19 or FGF21 actions.

Late in the transition from a fasted state to starvation, FGF21 contributes to ketogenesis (Badman et al., 2007; Inagaki et al., 2007; Potthoff et al., 2009), reduces glucagon secretion (Berglund et al., 2009; Kharitonenkov et al., 2005), and observations from my studies suggest that it inhibits ghrelin release (see Chapter 4), while leading to growth hormone resistance (Inagaki et al., 2008). Moreover, FGF21tg animals are very small, females are infertile, and if fasted, these animals undertake a unique hibernation-like state called torpor, in which core body temperature and locomotor activities drop as a means of energy conservation (Inagaki et al., 2007; Inagaki et al., 2008).

Evidence for a Central Mechanism of FGF21 Action

Injection of recombinant FGF21 lowers plasma insulin within minutes following a single administration (Xu et al., 2009). Coordinately, in the liver, a gluconeogenic gene expression signature [glucose-6-phosphatase (G6Pase), phosphoenolpyruvate carboxykinase (PEPCK), receptor-gamma coactivator 1 α (Pgc1α)] is induced during the same time frame [(Potthoff et al., 2009); Fig.1]. Moreover, observations in our lab and others indicate that a thermogenic program is induced in the brown adipose tissue [PGC1α, BMP8, Deiodinase 2; (Coskun et al., 2008)]. The reduction in insulin levels is

not due to direct actions on the beta cells of the islets, nor an increase in glucagon secretion (Potthoff et al., 2009; Xu et al., 2009). Moreover, upregulation of G6Pase, PEPCK, and PGC1α requires an intact animal as shown by a lack of effect on these genes by FGF21 in isolated perfused livers, or primary hepatocyte cultures (Potthoff et al., 2009).

βKlotho is expressed in whole hypothalamus (Coskun et al., 2008) and direct infusion of FGF21 into brains of diabetic rats leads to improvements in insulin sensitivity and energy expenditure (Sarruf et al., 2010). FGF21 has been shown to cross the blood brain barrier (Hsuchou et al., 2007) and has been detected in human cerebrospinal fluid (Tan et al., 2011). More recently, intraperitoneal injection of FGF21 was shown to increase phosphorylation of ERK western blots of whole hypothalamus (Yang et al., 2012).

Taken together, the acute injection studies and the phenotypes of the FGF21tg animals suggest that FGF21 has properties overlapping all phases of the fasting response and may require nervous system sites to elicit these effects.

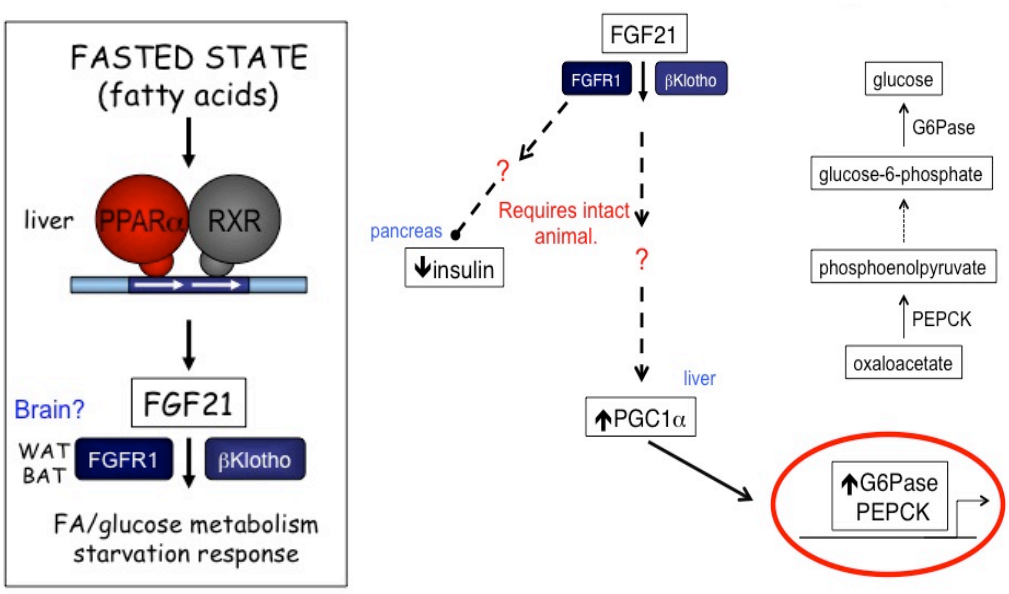


Fig 1) FGF21 is elevated by and mediates aspects of the fasting response.

CHAPTER TWO
Results

NEUROANATOMIC BASIS FOR FGF21 ACTION

Introduction

As outlined in Chapter 1, there are several lines of evidence strongly implicating the nervous system as a key player in FGF21 biology, but the first step was to ascertain the possibility by mapping the receptors and co-receptors in key neuronal sites. Furthermore, it was essential to establish that FGF21 applied directly to the brain could elicit an effect in the periphery.

The FGFR1-IIIc/βKlotho Complex is Expressed in Key Neuronal Sites

Traditionally, gene expression mapping in brain sections is performed by in situ hybridization in conjunction with immunohistochemistry when an appropriately validated antibody is available [see (Saper, 2009) for discussion of antibody usage in histology]. Several commercially available βKlotho antibodies failed to produce signal in any tissue tested by immunohistochemistry. In situ hybridization of the mRNA proved particularly unreliable as well. Several attempts were made using ^{33}P instead of ^{35}S-labeled riboprobes as a means to enhance the sensitivity of the assay at the cost of the fine resolution afforded by the less energetic isotope. Although we were able to get a working probe, replication of the assay was inconsistent. Fortunately, in the Elmquist lab I was able to contribute to the development of a highly sensitive quantitative, real-time PCR (QPCR) assay while working with post-doctoral fellows Syann Lee and Laurent Gautron. We used anatomically-guided laser capture microdissection (LCM) to isolate specific

hypothalamic and brainstem nuclei from the mouse brain, in addition to sensory ganglia from the parasympathetic (nodose) and sympathetic (dorsal root ganglia; DRG) divisions of the peripheral nervous system. The sites were chosen based on known roles of each nucleus in metabolic homeostasis, expression of receptors for key hormones such as leptin and insulin, and general interest to those in the lab. QPCR revealed that FGFRs and the Klotho family co-receptors for the endocrine FGFs are expressed in several hypothalamic sites (Fig 2.1). Most surprisingly, the FGF21 receptors FGFR1-IIIc and βKlotho are found in the suprachiasmatic nucleus (SCN), the area postrema (AP) and the nucleus tractus solitarii (NTS), which are the sensory nuclei of the dorsal vagal complex (DVX), and nodose. FGF15, FGF21, and FGF23, ligands for these receptors, were not detected in these samples.

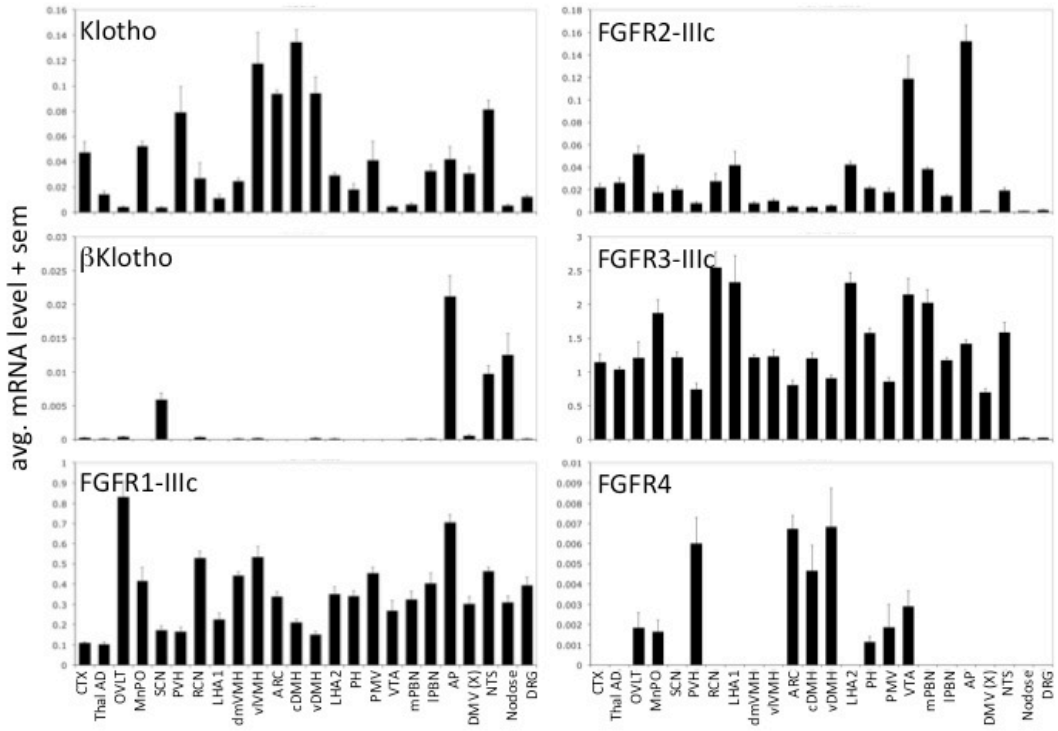


Fig 2.1) FGFR and Klotho family members are expressed in hypothalamic, brainstem, and peripheral nervous system sites. QPCR data are shown as average mRNA levels + sem from n=4-5 male C57 BL/6J mice harvested at the start of the light cycle. Key sites were chosen to represent non-hypothalamic (CTX, Thal AD), hypothalamic (OVLT through PMV), midbrain (VTA through lPBN), hindbrain (AP through NTS), and peripheral (nodose, DRG) neuronal populations.

The Nodose Ganglion

The nodose is the cell body of the vagus nerve, the 10th (X) and longest cranial nerve. It is a major player in conveying chemical, thermal, and hormonal information from visceral tissues it directly innervates, especially from the gut (Berthoud, 2008). Being a mixed nerve, the vagus has motor efferents as well as sensory afferents that transmit information from the body to the brain by way of the NTS. It is composed of multiple cell types including neurons and axonal fibers. Again, we used LCM to enrich

neuronal and axonal RNA specimens independently. Nav1.8 is a sodium channel expressed specifically in the sensory neurons of the dorsal root and nodose ganglia (Stirling et al., 2005), while myelin basic protein is in axon fibers; both are shown as controls for the fidelity of the dissections. While FGFR1 is in both cell types, βKlotho is found only in the neuronal population (Fig 2.2), implicating the vagus as a potential sensory site for FGF21. Recent data suggests that vagal sensory neurons may be key integrators of changing levels of circulating metabolites and hormones, and are directly involved in insulin sensitivity (German et al., 2009; Latour and Lautt, 2002; Uno et al., 2006). Since FGF21 dramatically enhances insulin sensitivity, I hypothesized that the vagus may be mediating the insulin-lowering response to FGF21 administration. One way of testing the involvement of the vagus is by surgical resection, or vagotomy, though there are many caveats to interpretation of results from such studies (Berthoud, 2004; Norgren and Smith, 1994; Powley et al., 1983). Another way is to genetically delete the receptor of interest using cre-loxP based conditionally deletion. In the Elmquist lab, 2 cre driver lines were available: the Nav1.8-cre knock-in generated by the laboratory of John Wood (Stirling et al., 2005) and the Phox2b-cre transgenic, developed by Mike Scott in the Elmquist group (Rossi et al., 2011; Scott et al., 2011). The Nav1.8-cre model targets sensory neurons of the vagus (Gautron et al., 2011; Stirling et al., 2005), while the Phox2b-cre (line 4) shows cre recombinase activity in vagal motor and sensory neurons, in addition to AP, NTS, DMV, and some hypothalamic neurons (Rossi et al., 2011; Scott et al., 2011). I crossed these cre driver lines to the floxed βKlotho mouse made by Xunshan Ding in the Mangelsdorf/Kliewer lab with the rationale that through comparison of the 2 models, I could identify which effects of FGF21 are mediated either by the vagus

or the hindbrain.

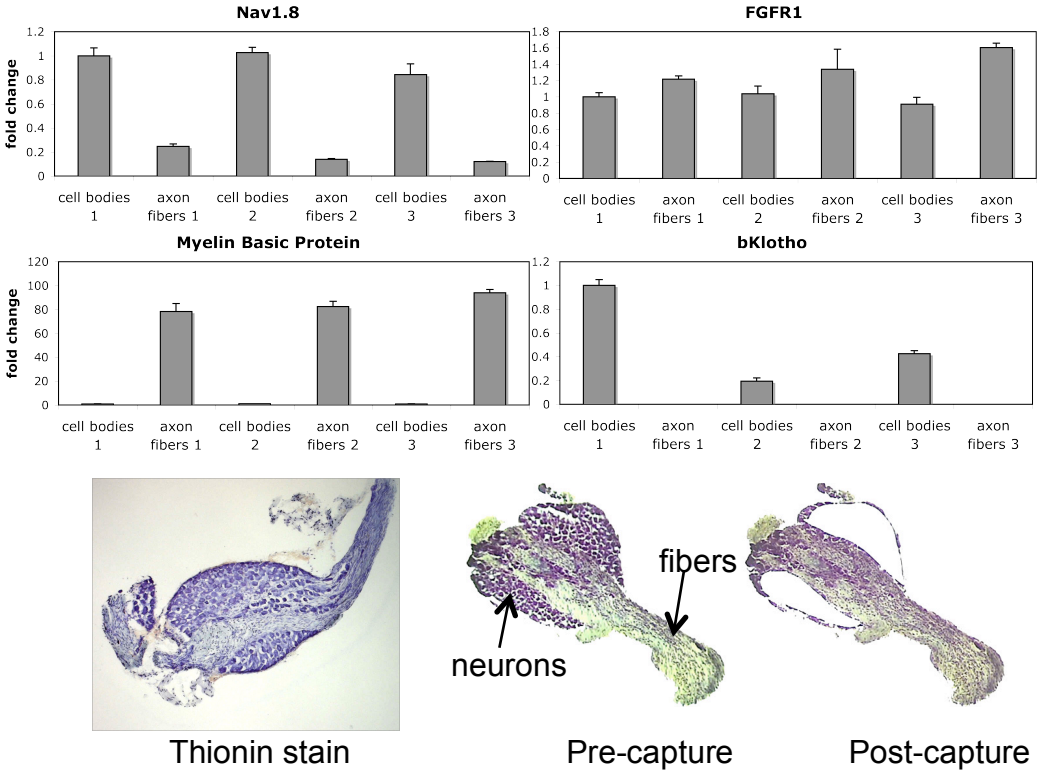


Fig 2.2) βKlotho is expressed in neurons of the nodose ganglion. QPCR data are shown for individual male C57 BL/6J mice harvested at the start of the light cycle. Lower left, thionin staining illustrates the heterogeneity of cell types in the ganglion. Lower right, images of a ganglion pre- and post-laser capture.

The Dorsal Vagal Complex

The dorsal vagal complex (DVX) is composed of the sensory components, AP and NTS, and the motor component, the dorsal motor nucleus [DMV;(Lowey, 1990; Travagli et al., 2006)]. The AP is a circumventricular nucleus that is considered to be outside of the blood-brain-barrier and has intimate connection with the NTS (Lowey, 1990). Due to this, the AP and NTS will be considered as a functional unit. The NTS is a

major site of sensory integration from the periphery, including spinal sensory neurons as well as vagal parasympathetic neurons. It sends projections to diverse brain regions, especially in the hypothalamus, some of which send reciprocal connections (e.g., arcuate and paraventricular nuclei). It is regarded as sort of a hub of communication between the sensory innervations of peripheral organs and higher forebrain structures. The DMV is the preganglionic parasympathetic that exerts efferent motor control over gastrointestinal and pancreatic secretions in response to information relayed by the NTS. Little is known about the chemical identity of the AP and NTS neurons except that distinct populations are noradrenergic and are important for hypoglycemic counter-regulatory responses [recently reviewed in (Ritter et al., 2011)]. The interesting expression of βKlotho in the AP and NTS, but not the DMV, suggests a role for this sensory complex in mediating the gluconeogenic responses to FGF21. To test this I planned to use the Phox2b-cre model described above.

The Suprachiasmatic Nucleus

The SCN is known as the master clock and as such orchestrates entrainment of organisms to their external environment. It is a symmetrical bilateral structure that sits on top of the optic tracts and receives photic information from the eyes. It contains several cell types, but mainly those expressing VIP or AVP. It gates the timing of many daily rhythms such as wakefulness, glucocorticoid secretion, and the LH surge in rodents [reviewed in (Dibner et al., 2010; Huang et al., 2011; Kalsbeek et al., 2006; Maywood et al., 2007)]. Neuronal sites influenced by the SCN are diverse, but just a few, such as the PVH, are directly innervated by SCN neurons. Projections from the SCN mainly are

found on interneurons that in turn target major nuclei under the control of the SCN. Therefore the SCN is viewed as sort of a relay center (Saper et al., 2005a; Saper et al., 2005b). The SCN is not typically regarded as being influenced by dietary derived factors, but it does show expression of leptin, ghrelin, and other metabolically related receptors (Hakansson et al., 1998; Unger et al., 1989; Zigman et al., 2006). The expression of βKlotho in the SCN implicated a role for liver-derived FGF21 in relaying nutritional status to the SCN. I hypothesized that this may be the nucleus responsible for longer-term effects of FGF21 on glucose homeostasis, and perhaps torpor. In the absence of an SCN-specific cre driver, I planned to use the supposedly pan-neuronal cre under the control of the nestin promoter available from Jackson. Through our collaboration with Joseph Takahashi, I later learned that the nestin-cre driver was inefficient for SCN deletions since this nucleus wires postnatally (Beltramo et al., 1994), and nestin is expressed embryonically. Fortunately, a driver was available that begins expression in the late post-natal period, just prior to weaning age. Several lines of the CamKIIα-cre exist, and each has its own pattern and timing of cre activity. Mariko Izumo in the Takahashi lab provided us with the appropriate line for conditional deletion from the SCN (Casanova et al., 2001).

FGF21 Delivery into the Brain Alters Liver Gene Expression

The presence of βKlotho in neuronal sites is strong evidence that FGF21 may act there, but could circulating FGF21 get into the brain, and if it did, what was the appropriate readout to test this? In order to test the action of any compound in the brain, intracerebroventricular (icv) experiments can be used where cannulae are implanted into

brain ventricles and the compound is administered either by acute injection directly into the cannula or by constant infusion via a subcutaneous mini-pump. Previous work from our lab indicated that gluconeogenic liver genes such as G6Pase, PGC1α, and RGS16 would potentially serve as good markers of FGF21 action (Potthoff et al., 2009). Given the expression of βKlotho in the SCN and the link between clock output and hepatic function [reviewed in (Bass and Takahashi, 2010; Dibner et al., 2010)]. I also measured known clock genes. As shown in Fig 2.3, 14 days of icv administration of FGF21 (1ug/day/brain) into the 3rd ventricle of C57 BL/6J males resulted in elevation of liver gene expression, confirming that indeed, FGF21 delivered into the brain could elicit a response in peripheral tissues. An important control in this type of experiment is to ensure that the protein did not leak from the brain. Since the recombinant protein is made from the human coding sequence, I was able to differentiate it from the endogenous mouse protein using an ELISA specific to human FGF21 and to rule out possible leakage into the periphery. A previous report indicated that icv delivery of FGF21 into diet-induced obese rats results in changes in food intake and insulin sensitivity (Sarruf et al., 2010). However, I did not observe any changes in food intake or plasma insulin levels (data not shown).

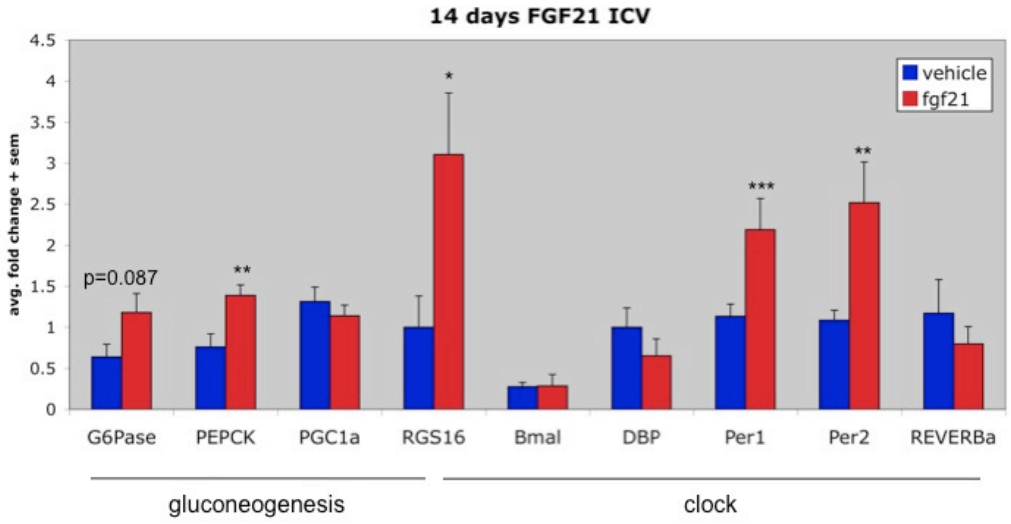


Fig 2.3) FGF21 administered into the brain results in changes in liver gene expression. QPCR data are shown as average mRNA levels + sem from livers of n=4-5 male C57 BL/6J mice following 2 weeks of continuous 3rd ventricle icv FGF21. *p≤0.05, **p≤0.01, ***p≤0.001

Validation of and Technical Considerations for Cre Models

A summary of the neuronal sites and the cre drivers used to conditionally delete βKlotho is shown in Fig 2.4. I measured the degree of βKlotho deletion from each of these models by QPCR of blunt dissections of the SCN, DVX, and whole nodose. The Nav1.8 line showed no deletion from the SCN or DVX, and highly variable deletion from the nodose; the Phox2b line showed no SCN effect, 50-70% DVX deletion, but no nodose deletion; the CamK line gave greater than 90% deletion from SCN, ~50% from DVX, and no nodose deletion; the nestin line showed about 50% deletion from SCN and DVX, and greater than 90% deletion from nodose, though this was effect was variable. In order to assess the contribution of each of these neuronal sites to the chronic effects of FGF21, in lieu of an ample supply of recombinant protein for chronic injections, I

crossed the Phox2b, CamK, and nestin-cre; floxed βKlotho lines onto the FGF21tg model.

Cre conditional deletion strategies have proven very useful for assigning tissue-specific function to many physiological pathways. This is especially true in neuroscience and the Elmquist lab, in conjunction with the Lowell lab, has contributed to the creation of numerous cre driver lines. However, caution is warranted in interpreting studies using these types of approaches particularly due to potential "off target" effects of the cre driver itself, in addition to unexpected expression of the cre in tissues previously unrecognized to express the driver gene. Crossing the cre driver to a reporter line such as lacZ or a fluorescent reporter is useful to assess this. Additionally, it is important to measure the effects of the cre model itself. In this regard, we have validated the Nav1.8-cre line, and have seen intestinal expression of the Phox2b-cre. Importantly, as I describe in Chapter 3, the nestin-cre has several reported problems.

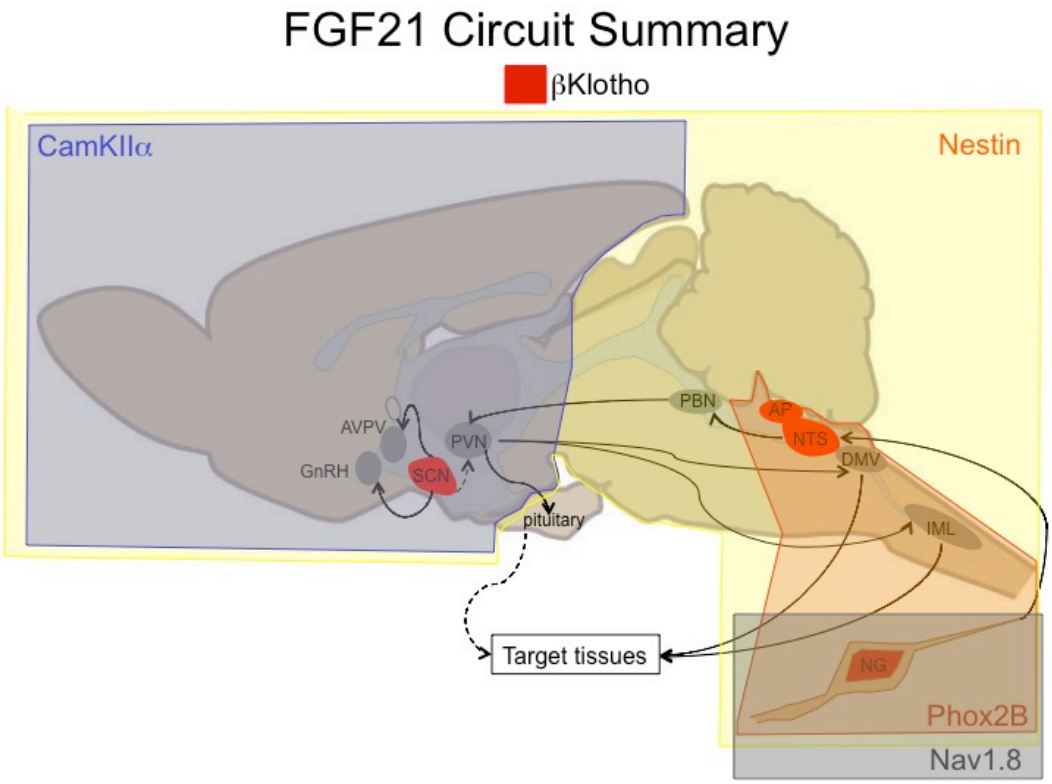


Fig 2.4) Summary of neuronal sites expressing βKlotho overlayed with cre drivers employed for conditional deletion of βKlotho. βKlotho-expressing brain regions are shown in red. Other nuclei with potential importance for responses to FGF21 are shown in gray. Arrows show afferent/efferent projections hypothesized to be involved in responses to FGF21. Shaded areas indicate expected coverage of each cre driver. CamKIIα (blue), nestin (yellow), Phox2b (red), Nav1.8 (gray). See text or list of abbreviations for definitions.

CHAPTER THREE
Results

FGF21 ACTION ON GLUCONEOGENESIS IS MEDIATED NEURONALLY

Introduction

The vagus regulates insulin secretion, while the hindbrain is involved in counter-regulatory responses to hypoglycemia. The presence of the FGF21 receptor/co-receptor complex in vagal neurons and in the sensory sites of the dorsal vagal complex (AP and NTS) suggests that these nuclei may be key in regulating the acute insulin-lowering and gluconeogenic gene expression profile of exogenously administered FGF21. In other words, these sites may be the neuroanatomic substrates of FGF21 that underlie its acute pharmacological properties. My hypotheses, as outlined in Chapter 2, regarding the role of these parts of the nervous system in the action of FGF21 is supported by the studies in this chapter.

FGF21 Reduces Insulin Secretion

Lowering of insulin can be achieved either by suppression of secretion or by enhancing clearance. In order to differentiate the two, c-peptide, a cleavage product of the insulin prepro-hormone that is not subject to the same clearance mechanism as inulin can be used as a surrogate marker of insulin secretion. As shown in Fig 3.1, FGF21 lowers plasma insulin by blocking secretion as indicated by concurrently reduced plasma c-peptide, but has no effect on glucose concentration in these lean animals.

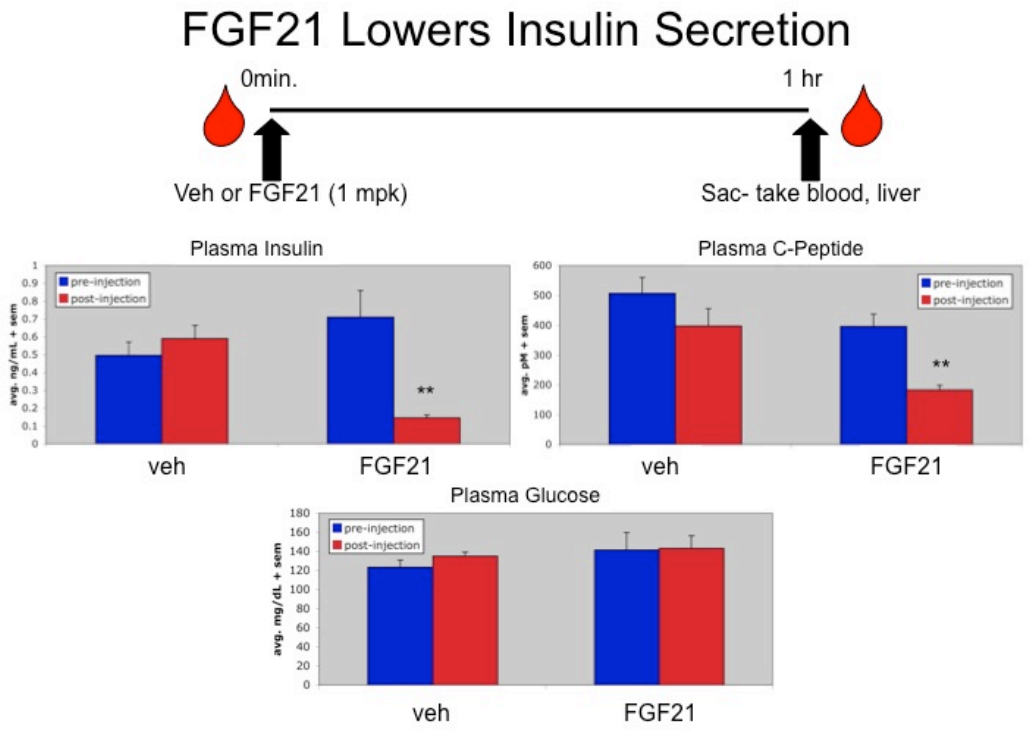


Fig 3.1) Acutely administered FGF21 reduces insulin secretion. Top, experimental paradigm. Tail blood was sampled from C57 BL/6 male mice (n=5) prior to i.p. injection with vehicle or 1mpk recombinant FGF21. After 1 hour, animals were sacrificed and blood and liver were collected. Plasma insulin, c-peptide, and glucose levels are shown plotted as avg. + sem. **p≤0.01

The Vagus Nerve is Required for Insulin-Lowering by FGF21

Insulin levels were not affected by central administration of FGF21 (see Chapter 2), suggesting that this effect was mediated in the periphery. The rapidity with which insulin levels drop following FGF21 administration (Xu et al., 2009) suggested a neuronal mechanism. Given the expression of βKlotho in neurons of the vagus and the known role of the vagus in insulin secretion [reviewed in (Miller, 1981)], we tested this using a surgical model, vagotomy (vgx). C57 BL/6 adult males underwent bilateral, sub-

diaphragmatic vgx at Jackson Labs. After recovery and shipment, a CCK test was administered to test the fidelity of the surgical procedure based on the method of (Fan et al., 2004). CCK is a hormone that acts on the vagus nerve to reduce feeding, so successfully vagotomized animals will not respond to this hormone and continue to eat. This behavior, combined with an overall slower intestinal motility is apparent in the increase in stomach weight (Fig 3.2). Animals were challenged with an intraperitoneal dose of FGF21 and sacrificed an hour later. Plasma insulin measurements indicate that the insulin-lowering effect of FGF21 requires an intact vagus nerve (Fig 3.2).

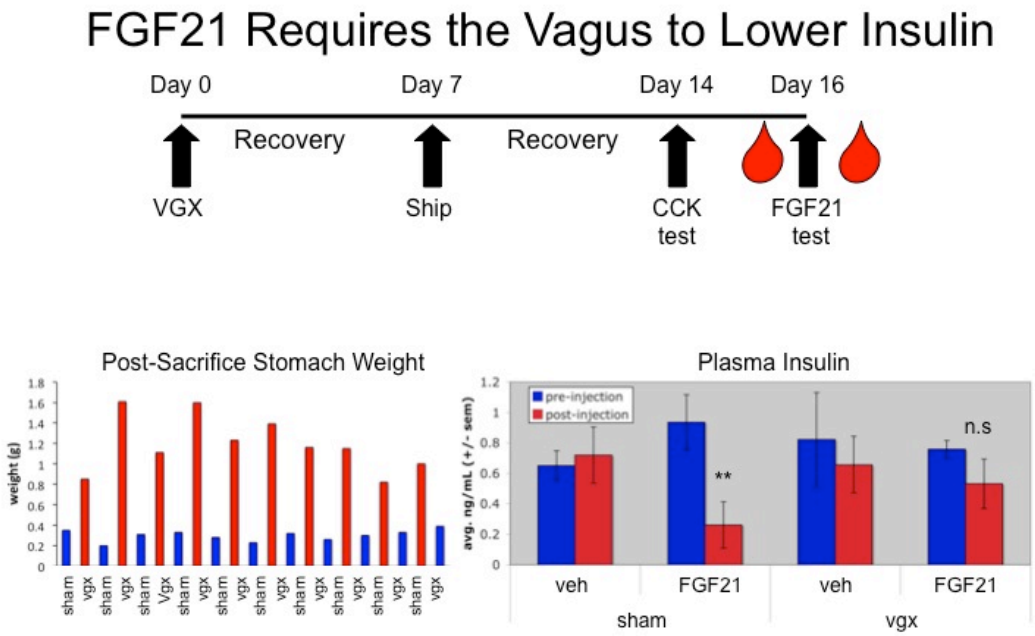


Fig 3.2) An intact vagus nerve is required for FGF21 to lower insulin. Top, experimental paradigm. Bottom, individual stomach weights (grams) following sacrifice are shown as proof of successful surgery (blue sham, red vgx). Plasma insulin level is shown plotted as avg. + sem, n=5. **p≤0.01

However, QPCR data of liver and brown adipose (bat) gene expression show that gluconeogenic and thermogenic genes are still induced (Fig 3.3). Egr1 is an immediate-early gene previously identified to be upregulated after FGF21 treatment and is shown as a positive control. Liver G6Pase expression is likely lost in the vgx animals due to the lack of reduction in insulin. Since this surgical vgx procedure ablates parasympathetic afferent and efferent signals, while leaving sympathetic efferent signals intact, it is possible that FGF21 is acting at another site in the brain to indirectly affect liver and bat gene expression.

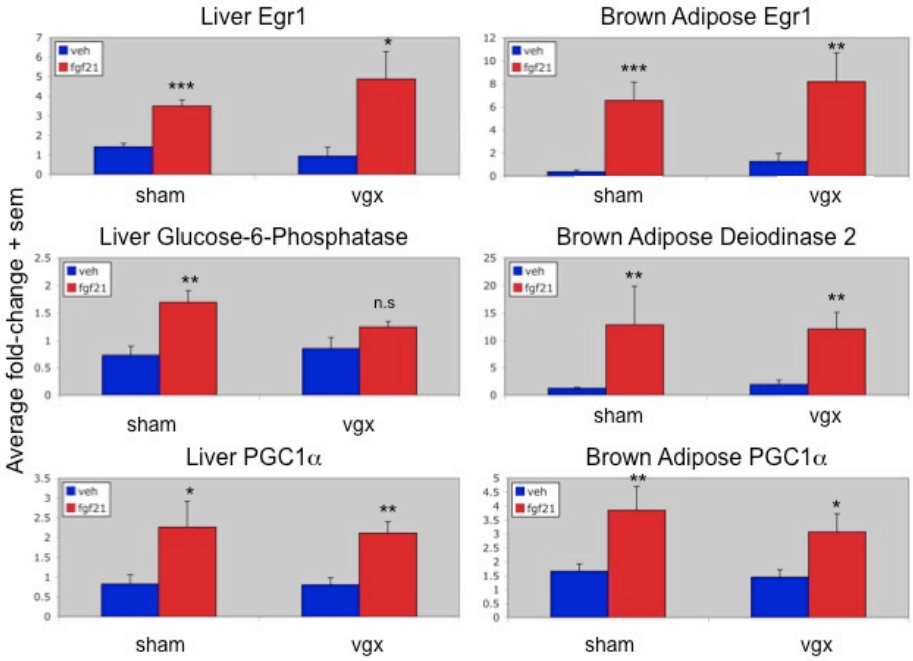


Fig 3.3) The vagus nerve does not mediate FGF21-induced gene expression. QPCR of liver and bat genes from animals in Fig 3.3. Data are shown plotted as avg. + sem, n=5. *p≤0.05, **p≤0.01, ***p≤0.001

In order to differentiate between afferent and efferent parasympathetic involvement in mediating the insulin-reducing response to FGF21, surgical resection is not sufficient since it is not possible to physically separate the nerves. Therefore, I undertook a genetic approach to delete βKlotho from afferent sensory vagus neurons using cre recombinase driven by either the Nav1.8 or Phox2b promoters. These genetic vgx animals were then subjected to the same FGF21 treatment paradigm as the surgical vgx animals. In both cases, the genetic vgx animals still responded to FGF21 by lowering insulin equally as well as in control animals lacking cre (data not shown). However, the degree to which βKlotho is deleted in these models, if at all, has been difficult to establish given the labile nature of the in situ probe and the possible presence of contaminating adipose, which expresses βKlotho, in whole nodose isolations (see Chapter 2). Further validation of these animal models is needed before drawing any firm conclusions about the role of vagal sensory neurons in insulin-lowering by FGF21.

Insulin-lowering and Gene Expression are Mediated by a Sympathetic Response

The results described above show that hepatic gluconeogenic gene expression is not due to a direct effect of FGF21 on the liver. That this response is intact after surgical vgx implies that FGF21 may be acting at other sites for this action. One possibility is that FGF21 acts centrally to mediate a sympathetic efferent response that could indirectly affect target tissue gene expression by increasing catecholamine or glucocorticoid release from the adrenal glands. Indeed, plasma glucocorticoids are increased in FGF21tg animals (see Chapter 4), but I reasoned that a catecholamine response was more plausible given the short time in which both liver and bat genes are induced. As a surrogate

measure of autonomic tone, I measured genes related to adrenergic (sympathetic) and muscarinic (parasympathetic) pathways in livers and adrenals from wildtype and FGF21tg mice. As shown in Fig 3.4, the mRNAs for catecholamine biosynthetic enzymes (TH, DBH, PNMT) are decreased in the adrenals, in addition to an upregulation of the degrading enzyme MAO-A in the liver, perhaps leading to reduced synthesis and increased clearance of epinephrine. A compensatory elevation of the adrenergic receptors α2ba and β2 in the liver may indicate sensitization of these GPCRs due to low ligand. Conversely, the muscarinic acetylcholine receptor 3 (Chrm3), known to be expressed in liver (Vatamaniuk et al., 2003), is reduced, perhaps as a means to desensitize this GPCR, while the enzymes that degrade acetylcholine are increased, likely to clear excess substrate. Taken together, this profile implies elevated parasympathetic tone at the liver. This is further supported by the report that FGF21tg mice have elevated liver glycogen (Potthoff et al., 2009), as the vagus regulates glycogen content in the liver (Ritter, 1992; Uyama et al., 2004). With respect to glucose homeostasis, the importance of the liver as a target of parasympathetics is unclear given the recent report that Chrm3 ko mice have no apparent metabolic phenotype (Li et al., 2009). Regardless, on the surface, these results were opposite to the prediction that FGF21 works by initiating adrenergic responses. But given that the FGF21tg is a model of chronic excess FGF21, it stood to reason that such a catabolic process would be down regulated in a longer-term setting. Indeed, chronic fasting eventually results in decreased sympathetic tone (Hayashi and Nagasaka, 1983; Sakaguchi et al., 1988; Young and Landsberg, 1977), perhaps favoring fuel conservation. With this notion in mind, I undertook adrenergic blockade experiments to determine if FGF21 engages a sympathetic response acutely.

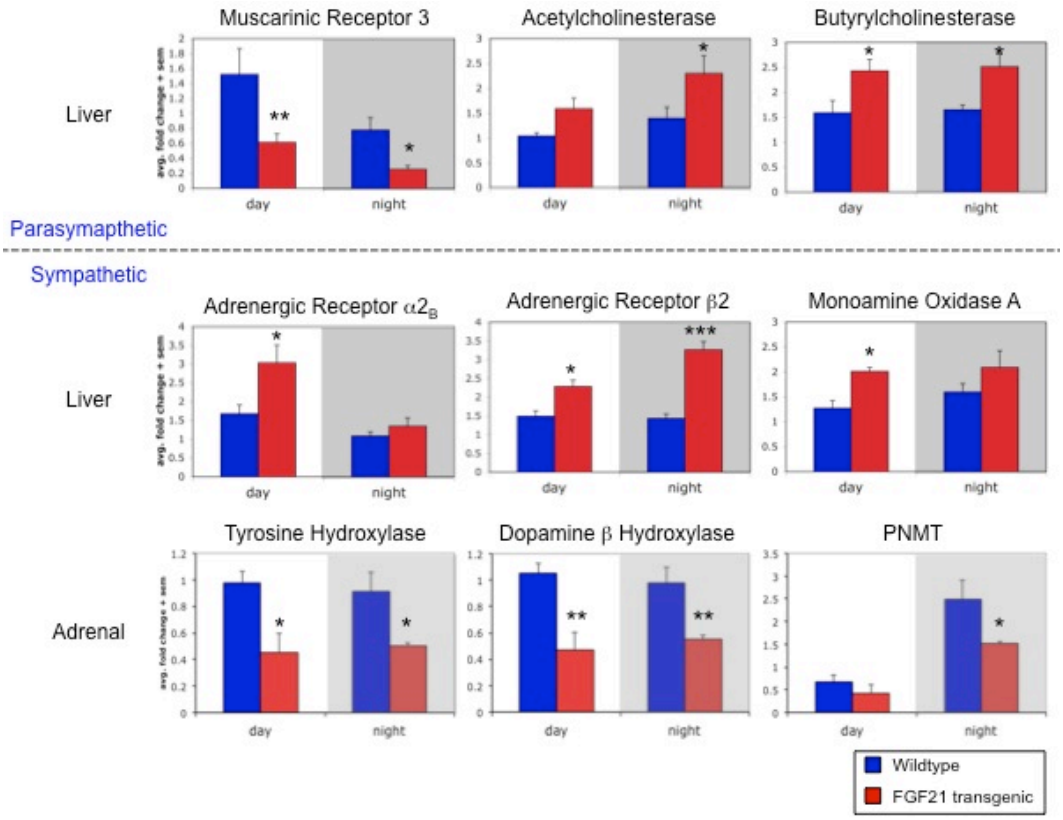


Fig 3.4) Autonomic tone is altered by FGF21. QPCR of liver and adrenal genes. Data are shown plotted as avg. + sem, n=5. *p≤0.05, **p≤0.01

It has been reported that the α2-adrenergic receptor mediates insulin lowering by sympathetic nervous innervation of pancreatic islets (Peterhoff et al., 2003). In order to test the possibility that FGF21 lowers insulin by this mechanism, C57 BL/6 males were administered vehicle or the α2-adrenergic antagonist, yohimbine, 30 minutes prior to administration of recombinant FGF21. Blood was sampled before the start of the experiment and 1 hour after FGF21 injection. Plasma glucose and insulin were measured.

The known glucose-lowering effect serves as a positive control for the drug. As shown in Fig 3.5, the insulin-lowering effect of FGF21 is mediated by the α2-adrenergic receptor. This implies that FGF21 can act on the central nervous system to increase sympathetic outflow to the pancreas.

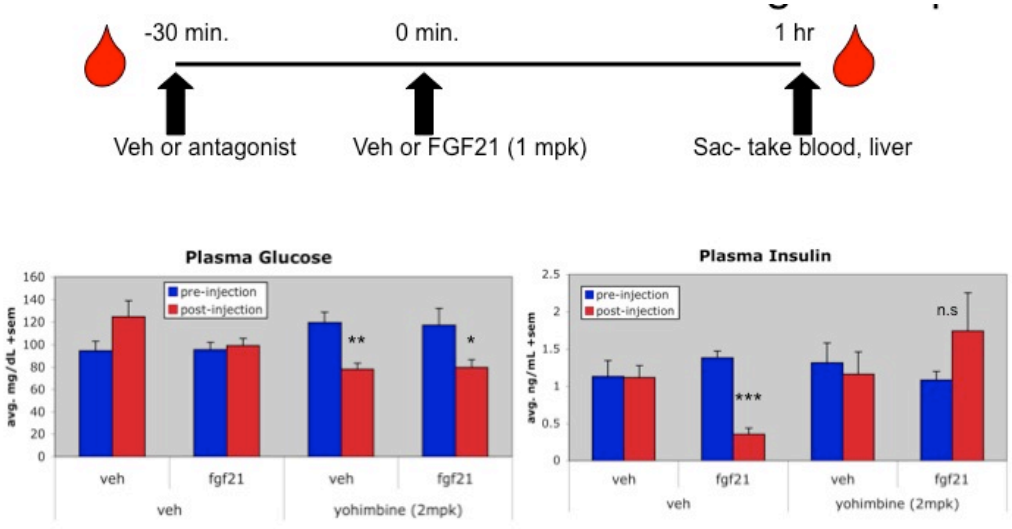


Fig 3.5) FGF21 lowers insulin through α2-adrenergic receptor activation. Experimental outline is shown at top. C57 BL/6 males, n=5 were pre-treated with yohimbine 30 min. prior to ip FGF21. Glucose and insulin levels are shown plotted as avg. + sem, n=5. *p≤0.05, **p≤0.01, ***p≤0.001

In order to test the possibility that FGF21 also induces gluconeogenic and thermogenic genes by inducing catecholamine release, beta-adrenergic blockade was undertaken. Using the same experimental paradigm as just described, the combination of β1, 2- (propranolol) and β3-adrenergic (SR59230A) antagonists abolished the effect of FGF21 on liver and brown adipose gene expression (Fig 3.6). Therefore,

pharmacologically administered FGF21 likely engages the sympathetic nervous system to induce a whole-body gluconeogenic response. In order to address whether these effects of FGF21 are mediated by the hindbrain directly, better genetic tools are needed, as none of our models sufficiently deletes βKlotho from the DVX (see Chapter 2).

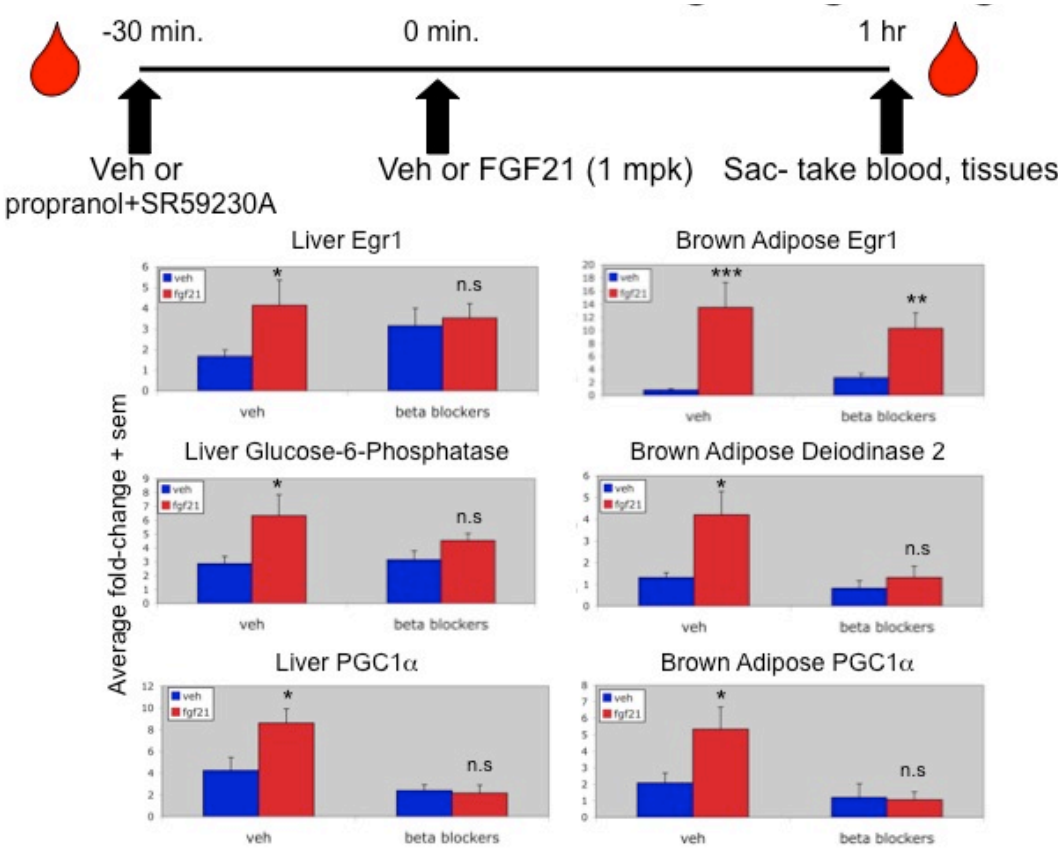


Fig 3.6) FGF21 induces liver and bat gene expression through β-adrenergic receptor activation. Experimental outline is shown at top. C57 BL/6 males, n=5 were pre-treated with a cocktail of β-blockers 30 min. prior to ip FGF21. QPCR data are shown plotted as avg. + sem, n=5. *p≤0.05, **p≤0.01, ***p≤0.001

Is the Vagus Involved in the Chronic Effects of FGF21 on Adipose?

In order to determine the contribution of the nervous system to the phenotypes reported for the FG21tg, I crossed the Phox2b-, CamKIIα–, and nestin-cre βKlotho$^{fl/fl}$ conditional deletion models onto the FGF21tg background. The Phox2b line showed no apparent differences with respect to cre expression in plasma or liver and adipose gene expression parameters examined (data not shown). The CamKIIα line is discussed in Chapter 4. Nestin-cre was expected to result in lack of any neuronal βKlotho expression. However, as stated in Chapter 2, the SCN and DVX deletion was highly variable, and about 50% effective at best. However, nodose deletion was complete. Examination of the βKlotho$^{fl/fl}$;FGF21tg;nestin-cre line revealed striking effects on plasma lipid parameters (Fig 3.7) in addition to lipogenic gene profiles in the white adipose tissue (Fig 3.9), while the liver seems unaffected by the absence of βKlotho from vagal sensory neurons (Fig 3.8). If true, this is suggestive of a potential role for the vagus in the fed-state FGF21 effects given that this white adipose tissue phenotype mirrors that recently reported by our group in terms of the FGF21 ko model (Dutchak et al., 2012). Although it is tempting to be excited about the possibility that the vagus regulates adipose tissue in response to FGF21, much caution is warranted in interpreting these results. Namely, the nestin-cre driver itself has been reported to have a phenotype regarding insulin sensitivity and body size (Briancon et al., 2010). Indeed, liver gene expression in these animals shows a distinct effect on the GH axis irrespective of the presence of the floxed βKlotho allele (Fig 3.8). While βKlotho expression seems unaltered in bat or wat (compare cre neg to cre + without the FGF21tg), nestin is noted to be expressed both in adipocyte and

pancreatic progenitors (Hunziker and Stein, 2000; Mendez-Ferrer et al., 2010). Therefore, more rigorous control studies are warranted.

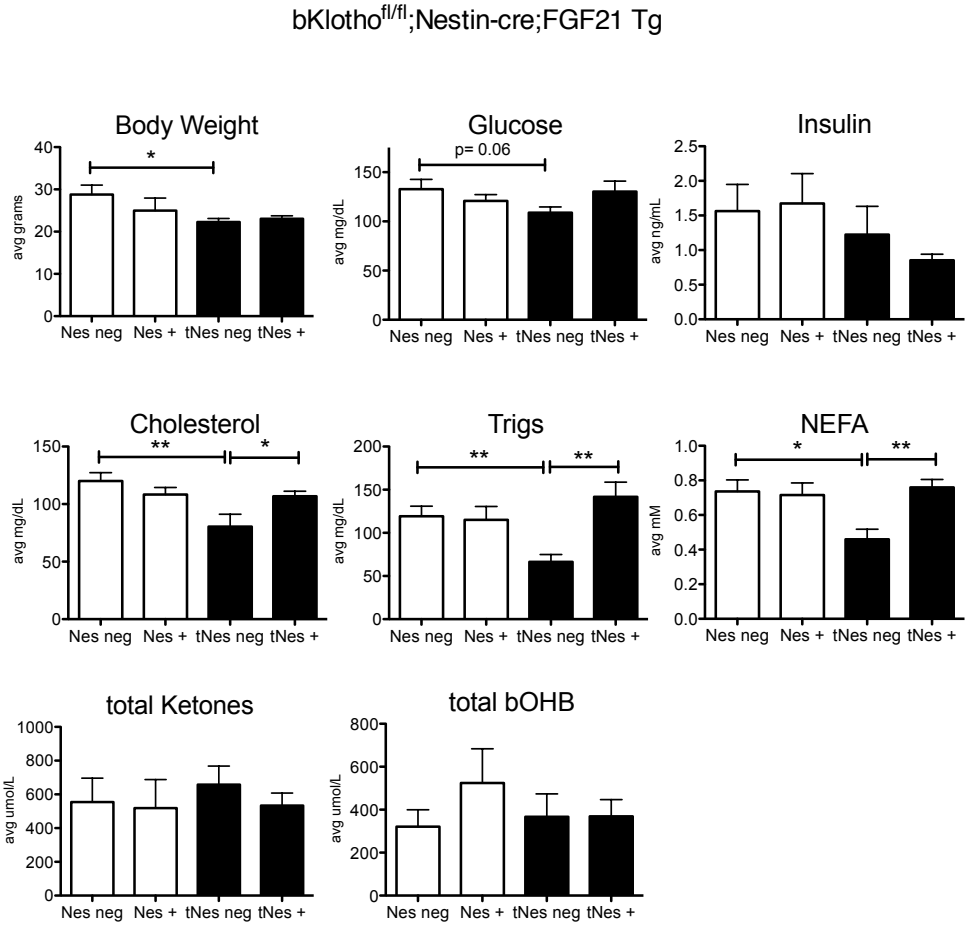


Fig 3.7) Body weight and plasma parameters for βKlotho$^{fl/fl}$;FGF21tg;nestin-cre mice. Males were sacrificed at the beginning of the light cycle. White bars are wildtype, while black bars indicate FGF21tg. Data are shown plotted as avg. + sem, n=5-6. *p≤0.05, **p≤0.01, ***p≤0.001

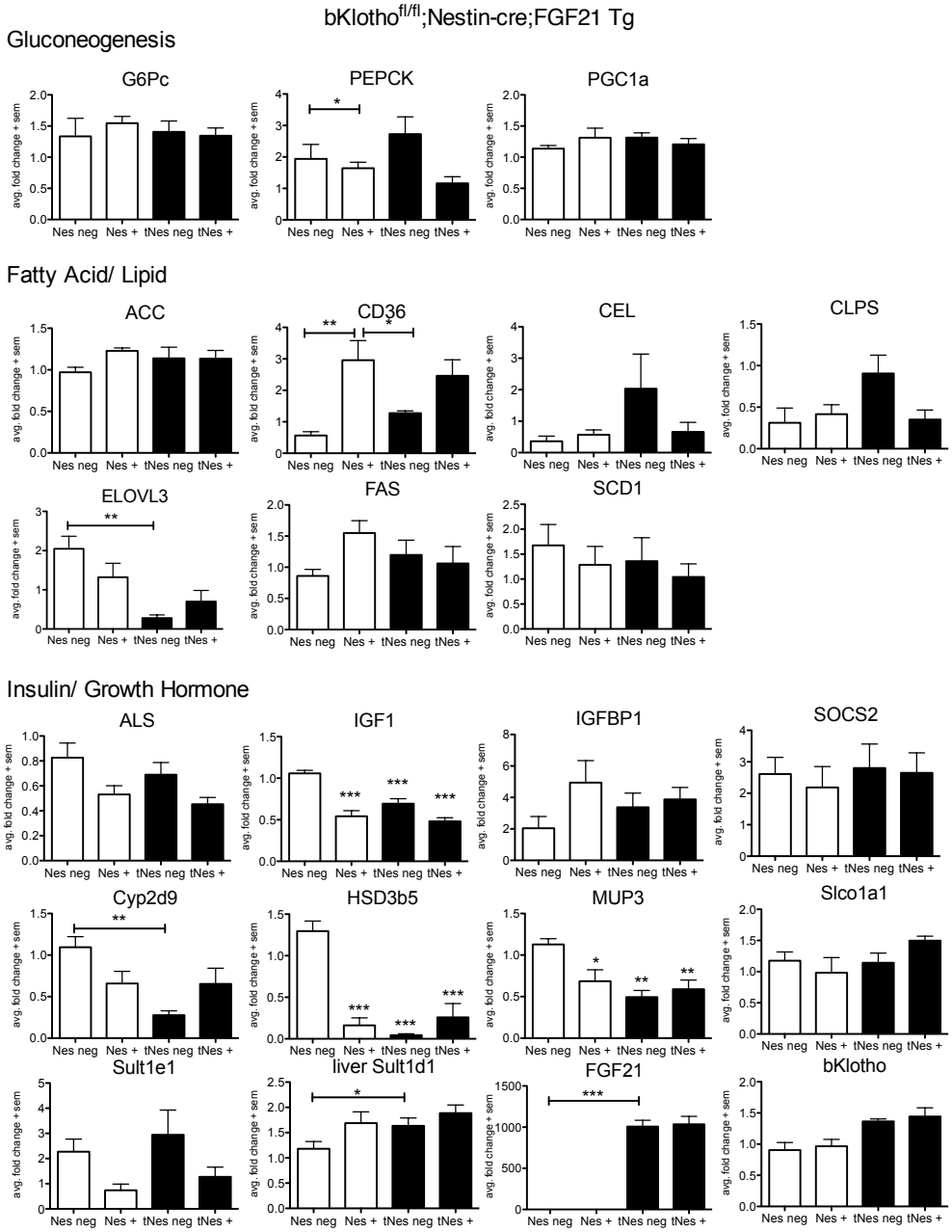


Fig 3.8) Liver gene expression for βKlotho$^{fl/fl}$;FGF21tg;nestin-cre mice. Males were sacrificed at the beginning of the light cycle. White bars are wildtype, while black bars indicate FGF21tg. Data are shown plotted as avg. + sem, n=5-6. *p≤0.05, **p≤0.01, ***p≤0.001

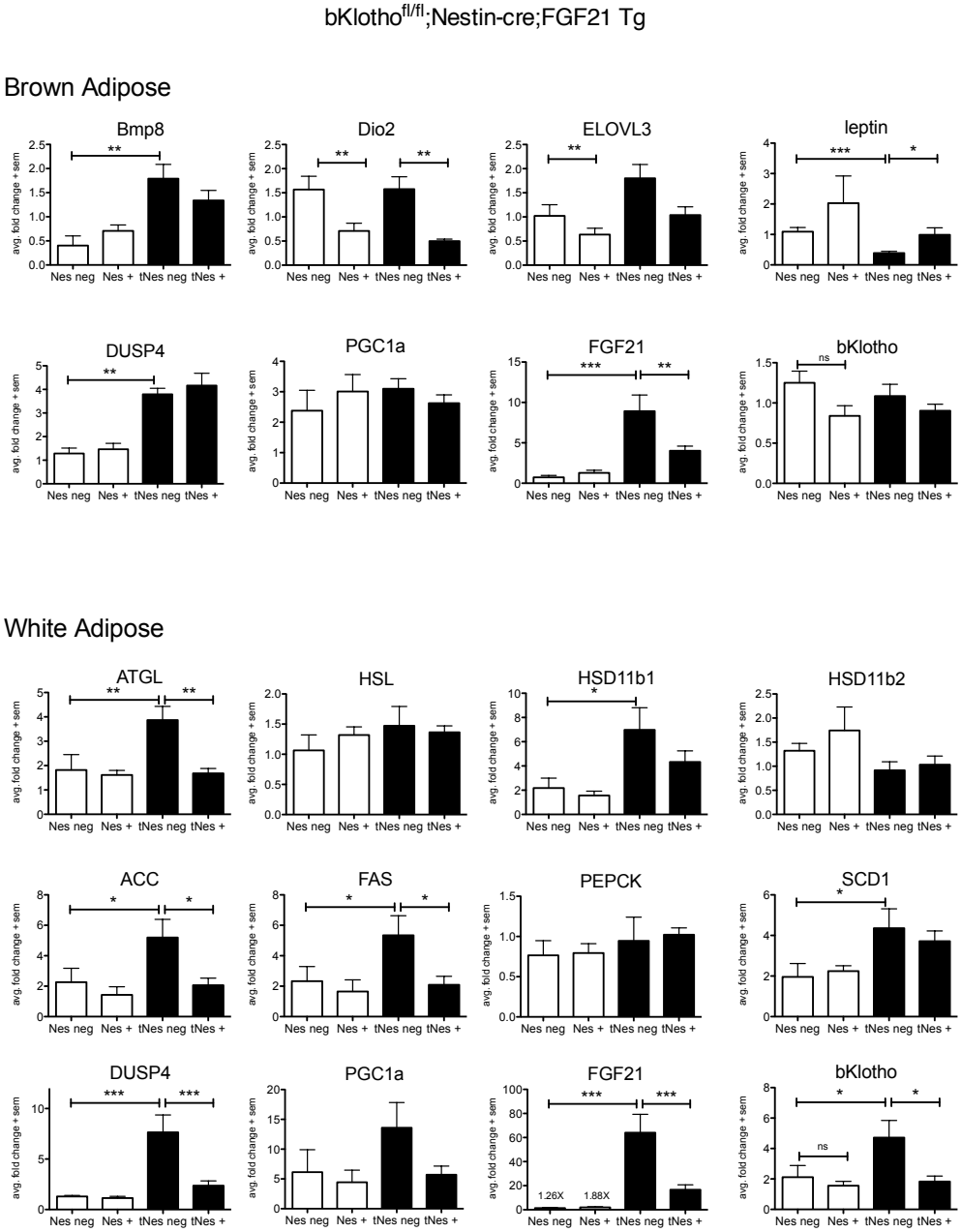


Fig 3.9) Adipose gene expression for βKlotho$^{fl/fl}$;FGF21tg;nestin-cre mice. Males were sacrificed at the beginning of the light cycle. White bars are wildtype, while black bars indicate FGF21tg. Data are shown plotted as avg. + sem, n=5-6. *p≤0.05, **p≤0.01, ***p≤0.001

CHAPTER FOUR
Results

SUPRACHIASMATIC NUCLEUS OUTPUT IS INHIBITED BY FGF21

Introduction

The SCN is regarded as the body's master clock, but its role is certainly under-represented. The discrete expression of βKlotho here suggested a role for FGF21 in circadian biology. As described in the following studies, I reveal a dramatic role for the SCN in responding to FGF21 as a global starvation signal.

FGF21 Expression is Rhythmic

Due to the role of the SCN in the regulation of timing, I determined the profile of FGF21 expression in key metabolic tissues, liver, brown and white fat, and muscle under ad libitum and food restricted conditions over the course of a day (Fig 4.1). Food entrainment is performed by limiting food availability to a 4 hour period during the animals' regularly inactive phase, namely during the day for mice. It is a means by which to segregate the effects of the intrinsic clock, controlled by the SCN, from those of the so-called food entrainable oscillator (FEO), which is distinct from and does not require the SCN, but can override the inhibition of activity imposed by the SCN during the light phase (Blum et al., 2012; Damiola et al., 2000; Mistlberger and Antle, 2011; Saper and Fuller, 2007). The rationale for this was that FGF21 might be a signal from the liver that, since it is a fasted-state hormone, could somehow overcome the restrictions enacted by the SCN. Interestingly, while the liver shows an anticipatory rise in FGF21 expression just prior to meal time, paradoxically, bat and wat express the highest levels of FGF21

following the 4 hours of re-feeding. The role of FGF21 in the white adipose following refeeding is the subject of a recent report by our group (Dutchak et al., 2012), and it may have to do with lipogenesis as a means to store ingested fuel following a period of fasting (Potthoff et al., 2012). The pattern of liver FGF21 expression in the food-entrained group is reminiscent of that seen for glucocorticoids (Le Minh et al., 2001), and led to the assessment of adrenal function in the FGF21 animal models (see Fig4.11). That hepatic FGF21 is rhythmic and entrainable supports the notion that it could relay nutrient timing information to the brain from the periphery.

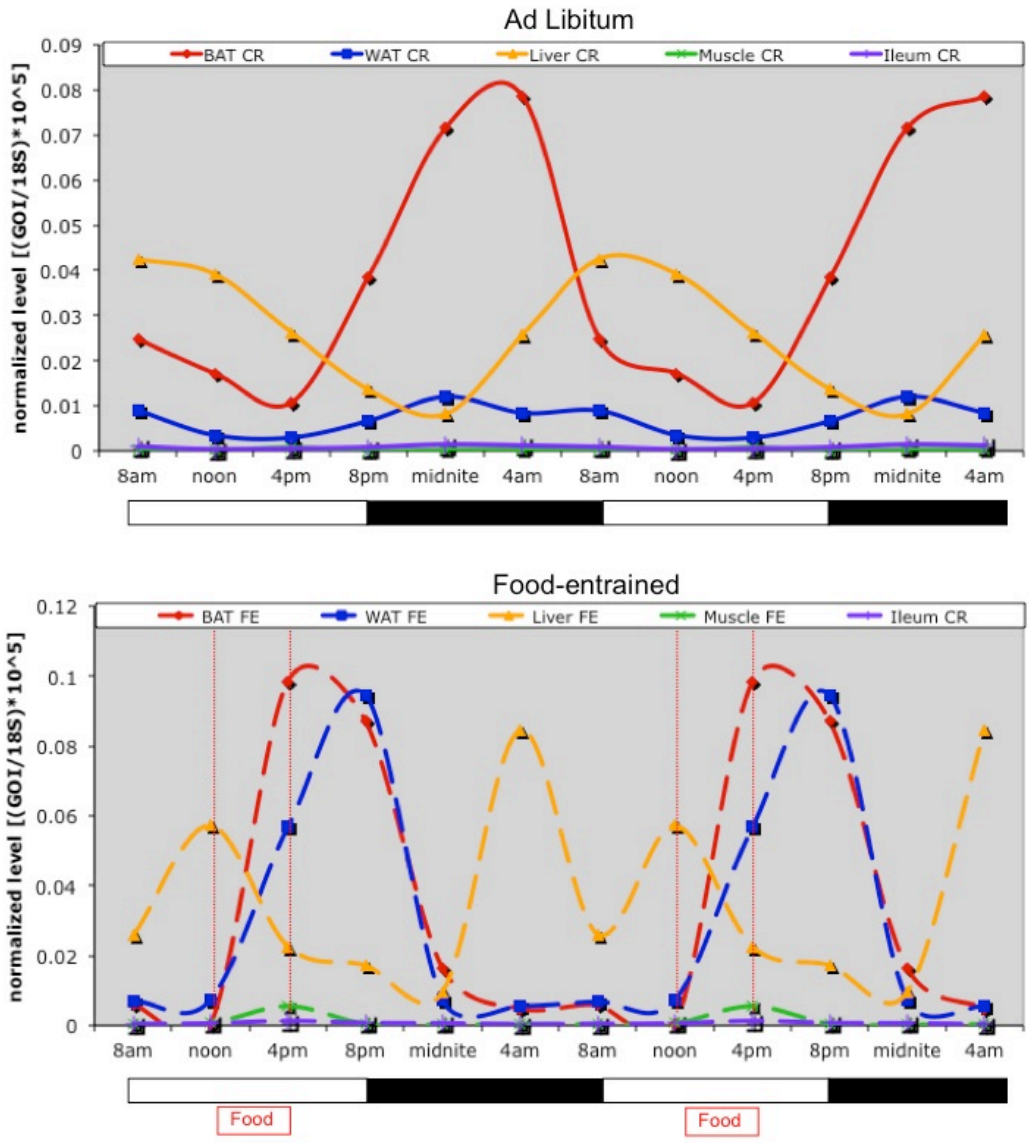


Fig 4.1) FGF21 expression is rhythmic and responds to feeding time differently between tissues. C57/BL6 males, 8-10 weeks old, were divided into circadian/ad libitum fed and food entrained, n=6 for each time point. The entrained group underwent restricted feeding for 4 hours during the day, noon to 4pm, for 14 days QPCR data are of pooled RNA for each treatment condition at each time point.

FGF21 Alters Circadian Behavior but Not Canonical Clock

Because the SCN controls the timing of daily activity cycles, one very robust way of testing the influence of FGF21 on the SCN is to measure daily wheel running behavior. In collaboration with Marleen de Groot in the laboratory of Joseph Takahashi, I was able to undertake these experiments. Wheel running behavior was recorded for FGF21tg males and their wildtype counterparts under various lighting conditions. Representative actograms are shown in Fig 4.2 (see Appendices for all individual actograms). Quantitative assessment of the activity shows that overall, elevated FGF21 reduces wheel running, but that these transgenic animals do not consolidate their running to the dark phase (Fig 4.3). One possibility for this response is that the animals may not sense or respond appropriately to light. However, the further overall reduction in activity during constant lighting (called masking) is intact in FGF21tg mice (Fig 4.2), thus ruling out defective photic responses as an explanation. Since the SCN is active during light to inhibit activity, the increased nocturnality implies that elevated FGF21 is sufficient to inhibit the function of the SCN. Analysis of data collected during constant darkness was used to calculate tau, or the free-running period, as a measure of the fidelity of the intrinsic molecular clock. Since the tau of all animals observed was essentially identical (23.5 ± 0.1 hours), this indicates that the behavioral alteration seen in the FGF21tg is not an effect on the molecular clock per se, but rather more likely a result of inhibiting the output of the clock.

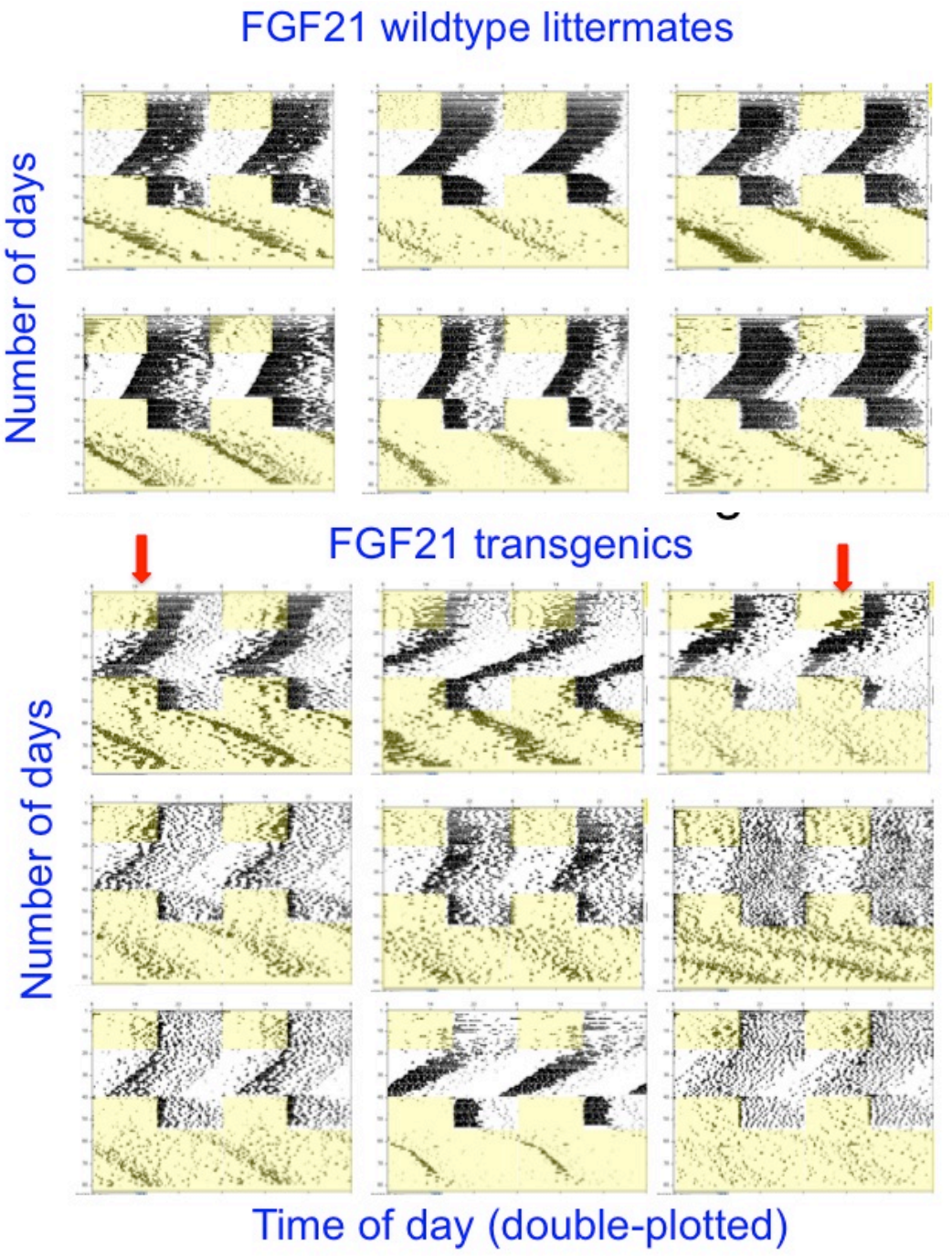


Fig 4.2) FGF21tg mice display altered wheel running behaviors. Individual actograms are shown double-plotted. Yellow indicates light. Red arrows indicate examples of daytime running behaviors.

In order to test the requirement for FGF21 in mediating the altered circadian behaviors as we observed in the FGF21tg mice, it was necessary to induce a similar metabolic context, i.e., a starvation-like physiological profile, in wildtype and FGF21 ko animals. Obviously it was not possible to completely remove access to food in our long-term wheel-running paradigms, and caloric restriction imposed a technical complication not easily overcome. Namely, a pre-measured food pellet would have to be given to animals each day, which if done at the same time would superimpose an alternate entrainment method (food entrainment), but if given at random times would disturb normal behavioral patterns, thus putting "noise" into our analyses. Therefore, a different dietary route was taken. Ketogenic diet is very high fat, low protein, and low carbohydrate, mimics the physiological state seen in FGF21tg mice (Badman et al., 2007; Inagaki et al., 2007; Kennedy et al., 2007). Chow-fed FGF21 ko mice behaved essentially as wildtypes. When challenged with the ketogenic diet, wildtype mice began to behave as the FGF21tg in terms of reduced overall activity and a propensity to run during the light phase (Fig 4.3). While reduced activity previously was previously reported for FGF21 ko animals on ketogenic diet in wheel running assays (Oishi et al., 2010), the effect was reduced for this genotype in our experiments. Therefore, not only is FGF21 sufficient to alter the amount and timing of daily activity bouts, it is also a major contributor.

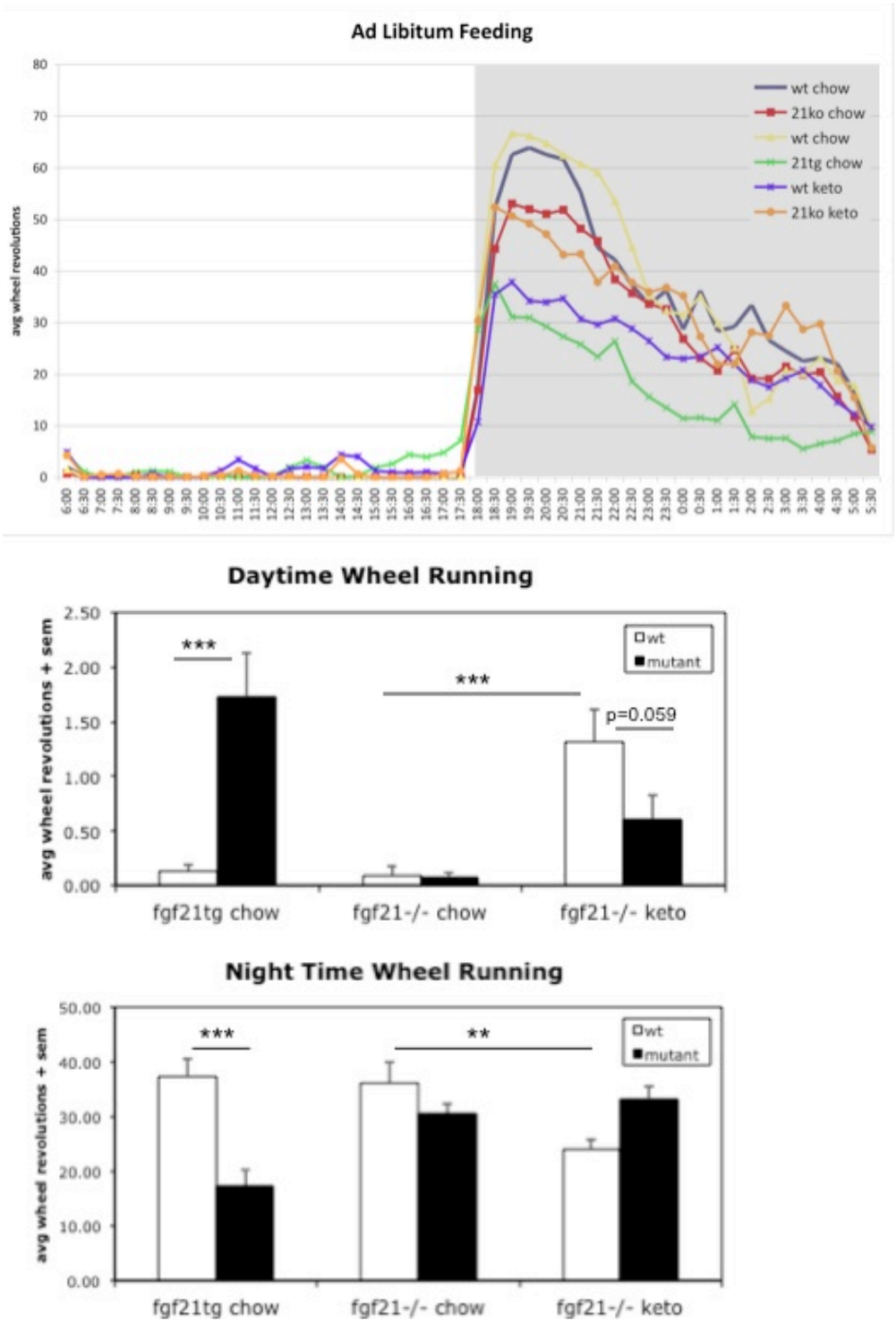


Fig 4.3) Elevated FGF21 alters nocturnality. Top, average waves summarize the daily wheel running activity of n=6-12 animals over 6 days. Gray indicates night. Bottom, quantification of daytime and night time wheel activities for the indicated models. Black bars represent the FGF21tg or ko. *p≤0.05, **p≤0.01, ***p≤0.001

FGF21 Does Not Enhance Food Anticipatory Behavior

Given that FGF21 is a fasting-related hormone and that the FGF21 transgenic is a model of chronic starvation, this may represent the need for the animal to be active in order to find food at any time of day. Therefore, food entrainment studies were undertaken as a normal portion of the overall wheel running paradigms with the expectation that the FGF21tg mice would show elevated anticipation of meal time. Surprisingly, the response observed was exactly opposite to that; the FGF21tg did not anticipate feeding time (Figs 4.4 and 4.6). One explanation is that the animals were going into torpor, as we have previously seen in this model (Inagaki et al., 2007). However, telemetry experiments, in which a radio telemeter is implanted into the abdominal cavity as a means to measure core body temperature, revealed that the FGF21tg animals actually resisted a torpid state early in the entrainment paradigm, while wildtype animals easily experienced declines in body temperature (Fig 4.5). While superficially this is in opposition to our own report (Inagaki et al., 2007), it is important to consider the different experimental approach. Our original study describes the torpor response following an immediate full 24-hour food deprivation, while the studies described herein employ a gradual decrement in the availability of food over the course of 10 days, with a full 24-hour deprivation of the final day. The resistance of the FGF21tg to torpor during a slow decrease in food availability is line with a role for FGF21 in bat-mediated thermogenesis. Indeed, this somewhat counter-intuitive physiological response is part of torpor biology (Geiser, 1998; Heldmaier et al., 1989). However, it is also in line with the observation that FGF21tg animals have decreased sympathetic tone (see Chapter 3), and that expression of torpor requires sympathetic activity (Swoap and Weinshenker, 2008). More

careful examination of the data in Fig 4.5 indicates that when animals undergo a full 24-hour fast on the final day of the paradigm, indeed the FGF21tg animals achieve torpor more deeply than their wildtype controls.

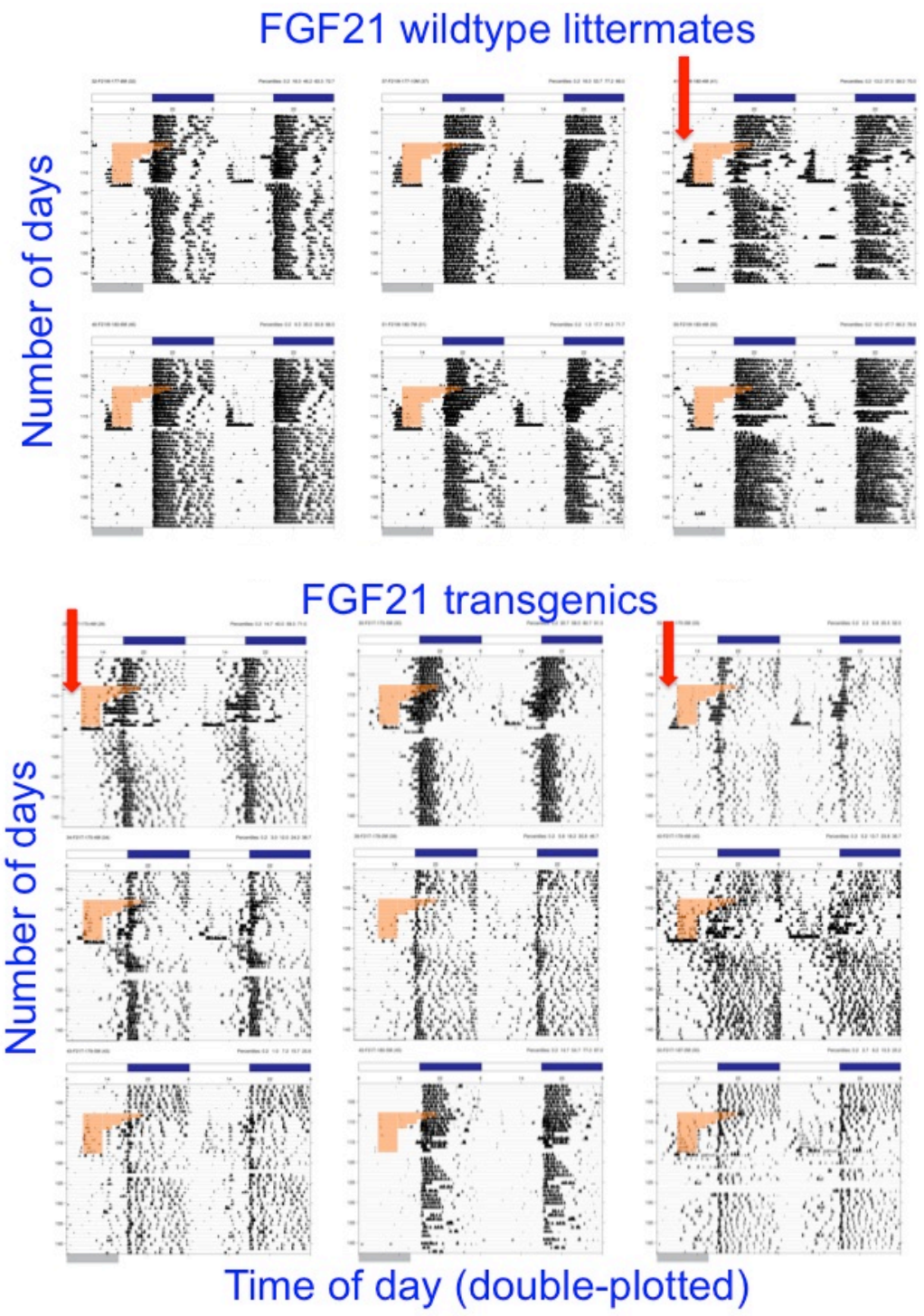


Fig 4.4) FGF21tg mice do not anticipate feeding time during a restricted feeding paradigm. Individual actograms are shown double-plotted. Orange indicates feeding time. Red arrows indicate examples of anticipatory behavior (wildtype, top) or lack of anticipation (transgenic, bottom).

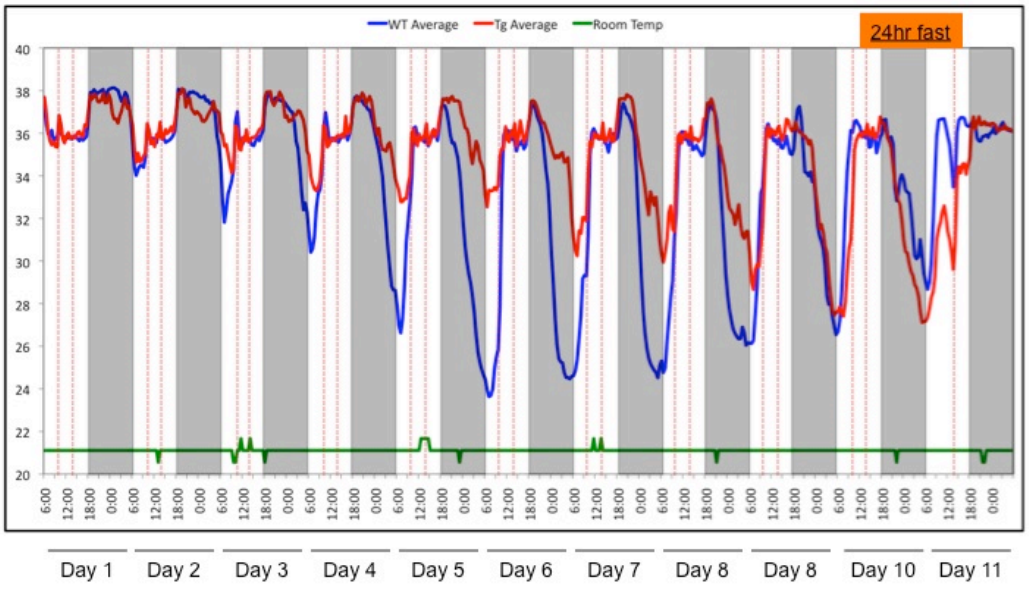


Fig 4.5) Lack of food anticipation in FGF21tg is not due to torpor. Average body temperature data recorded by telemetry are shown over the 10-day course of food entrainment. On the 11th day, animals were fasted for 24 hours. Gray indicates night; red vertical line denotes feeding time

Examination of the responses of the FG21 ko mice fed ketogenic diet revealed that this lack of food anticipatory activity was independent of FGF21, and likely related to the starvation-like state seen with FGF21 overexpression due to genetic (FGF21tg) or dietary (ketogenic) means (Fig 4.6). A factor that may contribute to the apparent lack of anticipatory motivation is the reduced ability for FGF21tg animals to increase ghrelin in response to fasting (Fig 4.6). Indeed, recent studies in the ghrelin receptor ko model support a role for ghrelin in food anticipatory activity (LeSauter et al., 2009; Yannielli et al., 2007).

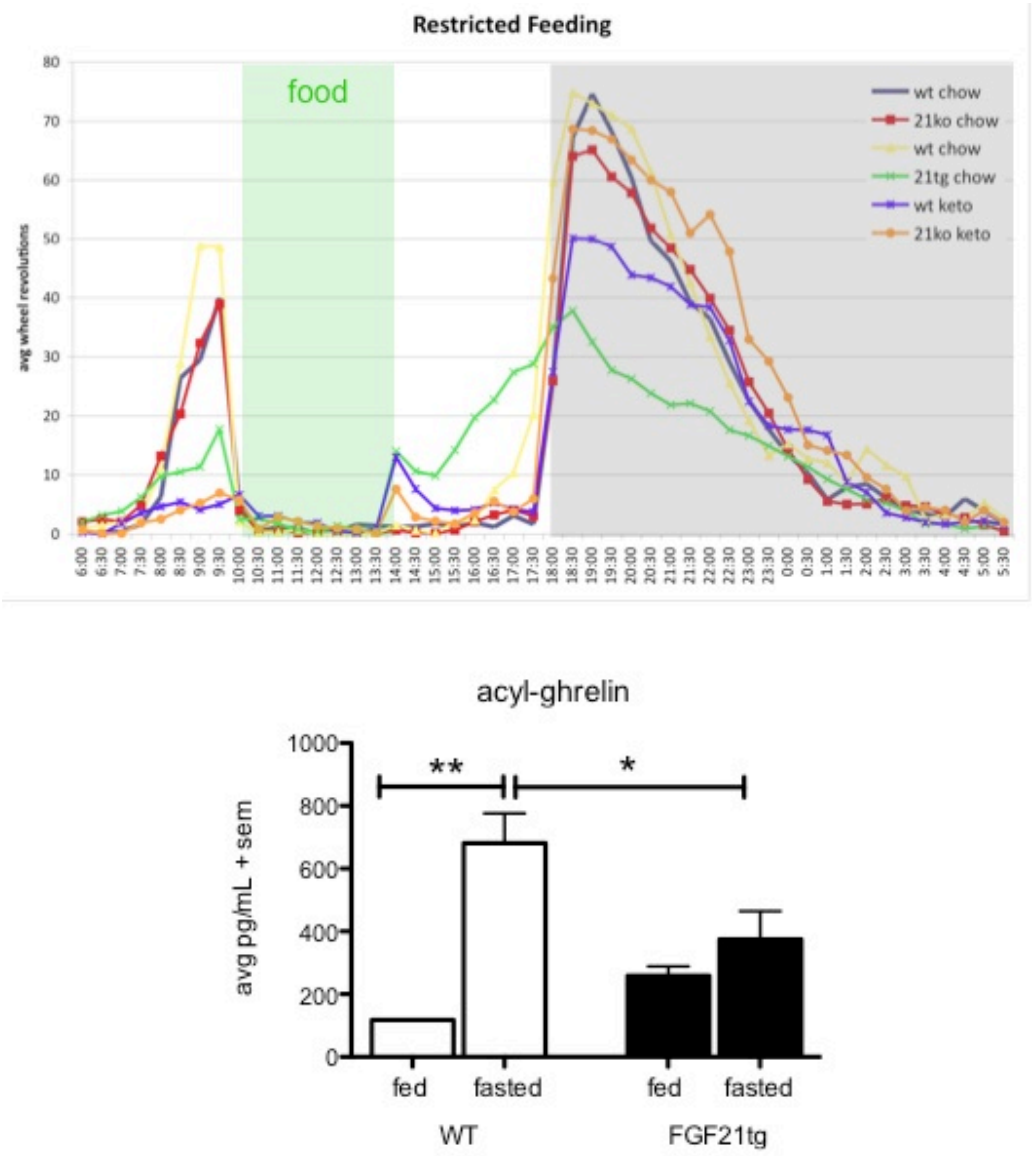


Fig 4.6) Lack of food anticipation is due to starvation. Top, average waves summarize the daily wheel running activity of n=6-12 animals over 6 days of food entrainment. Gray indicates night; green denotes feeding time. Bottom, Plasma ghrelin in fed or fasted animals. Black bars represent the FGF21tg *p≤0.05, **p≤0.01

The SCN is the Target of FGF21 Action on Behavior

The expression of βKlotho in the SCN raised the exciting prospect that FGF21 is a liver-derived hormone that could alter SCN function. In order to test the possibility that the behavioral alterations observed for the FGF21tg mice were mediated by the SCN, I subjected βKlotho$^{fl/fl}$; FGF21tg, CamKIIα-cre animals to our wheel-running protocols. As seen in Fig 4.7 and Appendix G, deletion of βKlotho from the SCN rescues the daytime running behavior as well as the overall reduction in activity. These animals do not anticipate feeding time as described in the previous section, however, reinforcing the notion that the SCN is not required for food entrainment, and that this apparent lack of effect is also independent of FGF21 (Appendix H, Fig 4.6). While wheel running studies per se are esoteric, they have revealed that the SCN can be inhibited by a peripheral signal, as manifested by relaxed inhibition of activity during the light phase. The overall implication that a hormone secreted from the liver can relay information regarding metabolic status to the brain via the SCN is an important finding and exposes an unrecognized role for this nucleus in starvation-related physiology as outlined by the studies that follow.

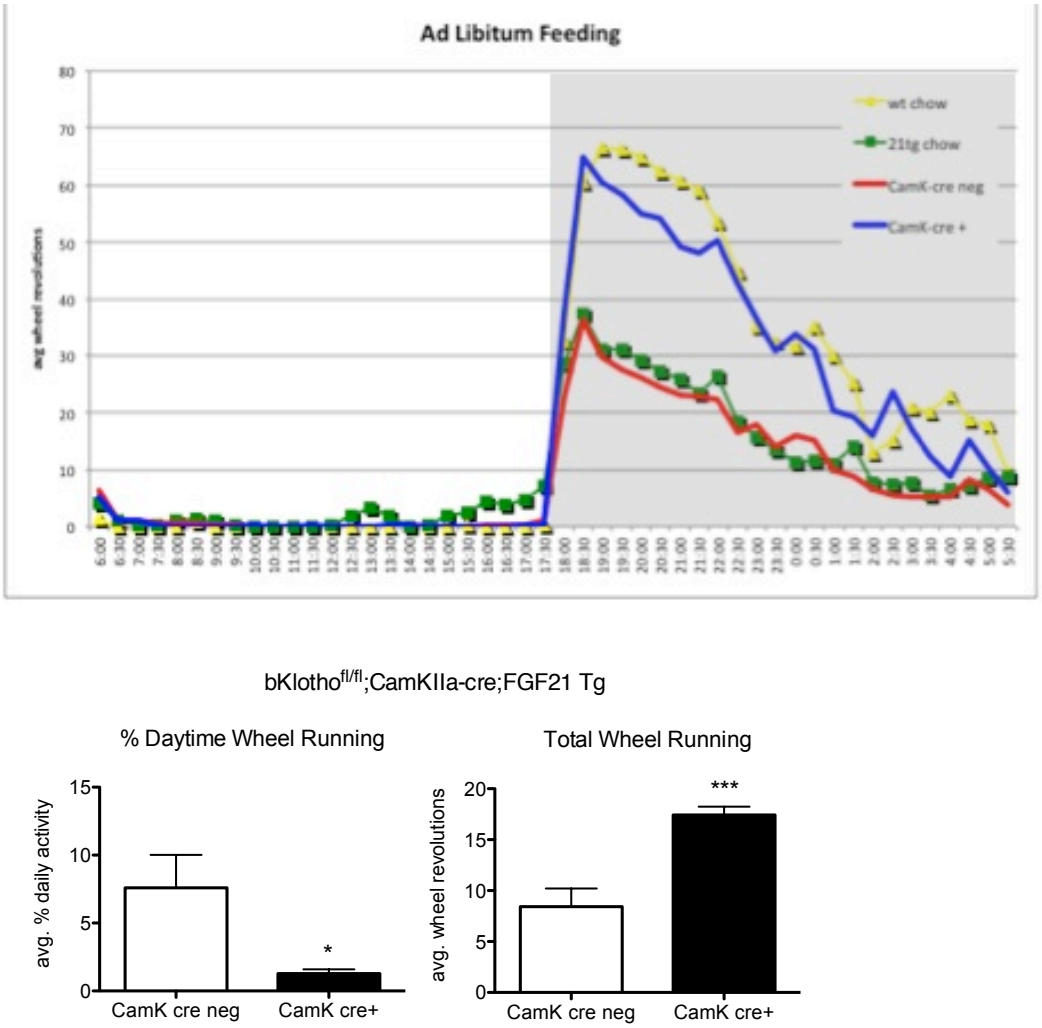


Fig 4.7) Deletion of βKlotho from SCN rescues wheel running phenotype of FGF21tg mice. Top, average waves summarize the daily wheel running activity of n=12 animals over 6 days. CamK neg and + animals are also FGF21tg. WT and FGF21tg from Fig 4.3 are re-plotted here for comparison. Gray indicates night. Bottom, quantification of % daytime and total wheel activities. Black bars represent the FGF21tg. *p≤0.05, **p≤0.01, ***p≤0.001

The SCN is the Target of FGF21 Effects on the Growth Hormone Axis

Growth hormone (GH) secretion is pulsatile throughout the day, but shows a sharp rise during early deep sleep (Czeisler and Klerman, 1999) . Neuroendocrine control of GH occurs via stimulation by GHRH and inhibition by somatostatin. The underlying neural circuit is complex and poorly understood. Gating of this rise may occur through the SCN, although other nuclei such as the arcuate and ventromedial nuclei, which are reactive to fluctuating glucose concentrations, elicit GH secretion, such as during hypoglycemia or chronic protein deficiency (Guyton, 2000; Muller et al., 1999). We have previously shown that FGF21tg mice have elevated GH, but are smaller in size due to blockade of the GH pathway (Inagaki et al., 2008). Interestingly, deletion of βKlotho from the forebrain using CamKIIα-cre rescues this defect, both in body weight and liver gene expression (Figs 4.8 & 4.9). While not statistically significant, there was a trend toward reversal of the low insulin levels characteristic of FGF21tg mice, likely due to restored body weight. Similarly, reduced expression of G6Pase is probably a consequence of restored insulin concentration.

Interestingly, adipose gene expression was largely unaffected by deletion of βKlotho from the forebrain (Fig 4.10), except in the case of BMP8 and ELOVL3 in the bat. The SCN is reported to have a role in thermoregulation by way of its projections to the DMH and MnPO (Morrison and Nakamura, 2011). While this may hint at a role for the SCN in regulating bat thermogenesis by FGF21, further studies are needed.

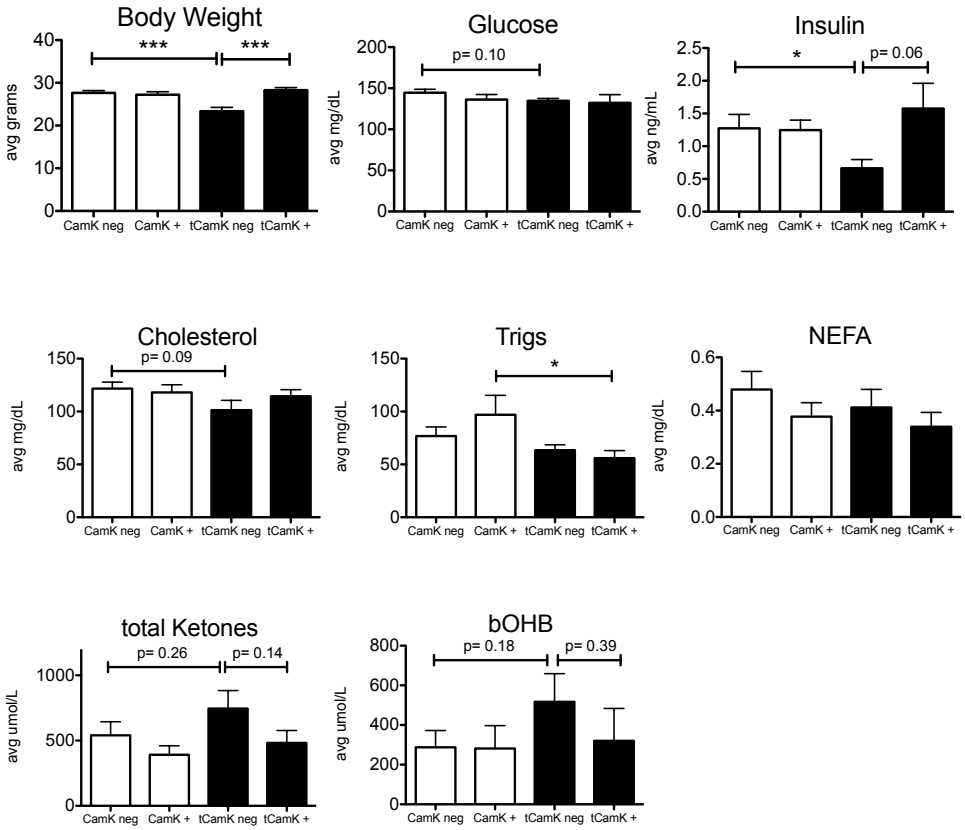


Fig 4.8) SCN deletion of βKlotho rescues the body weight phenotype of FGF21tg mice. Males were sacrificed at the beginning of the light cycle. White bars are wildtype, while black bars indicate FGF21tg. Data are shown plotted as avg. + sem, n=5-6. *p≤0.05, **p≤0.01, ***p≤0.001

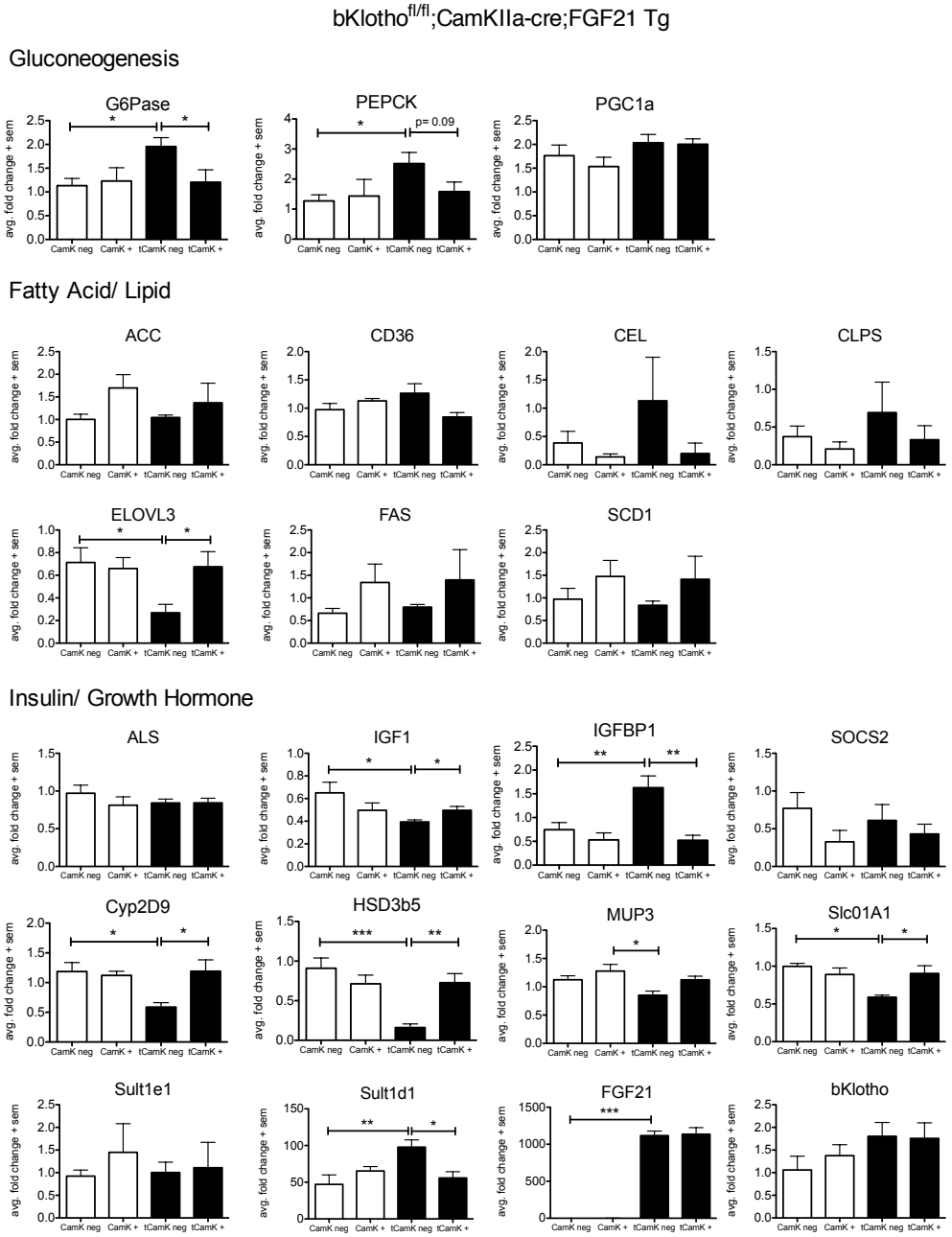


Fig 4.9) SCN deletion of βKlotho reverses the defects of the GH axis in livers of FGF21tg mice. Males were sacrificed at the beginning of the light cycle. White bars are wildtype, while black bars indicate FGF21tg. Data are shown plotted as avg. + sem, n=5-6. *p≤0.05, **p≤0.01, ***p≤0.001

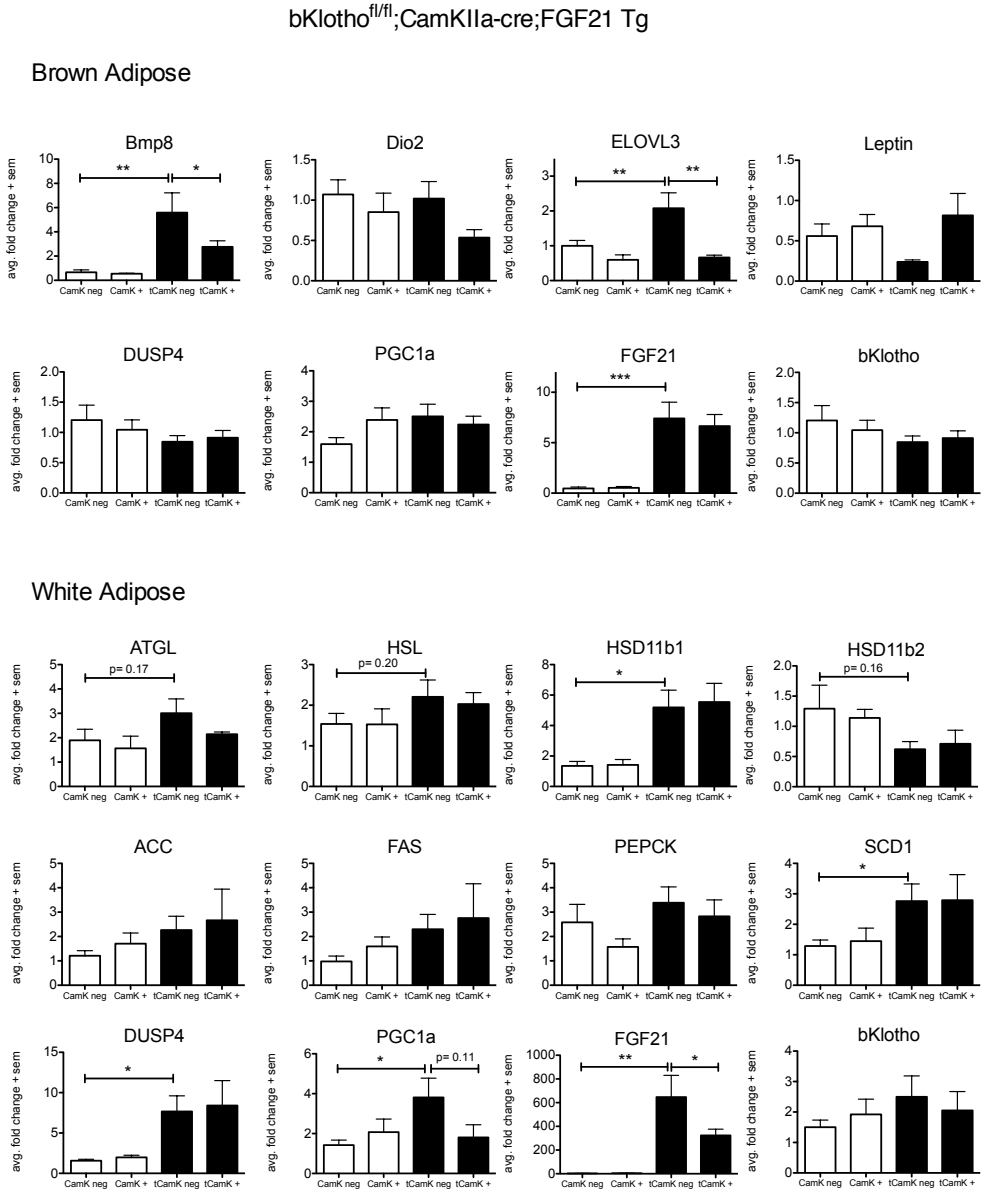


Fig 4.10) SCN deletion of βKlotho has modest effects on adipose of FGF21tg mice. Males were sacrificed at the beginning of the light cycle. White bars are wildtype, while black bars indicate FGF21tg. Data are shown plotted as avg. + sem, n=5-6. *p≤0.05, **p≤0.01, ***p≤0.001

The SCN is Likely the Target of FGF21 on Glucocorticoid Secretion

The SCN projects to the PVH and controls the timing of glucocorticoid secretion to allow its rise in anticipation of daily awakening. Additionally, the SCN plays a role in the timing of adrenal sensitivity to ACTH (Oster et al., 2006; Ulrich-Lai et al., 2006). I observed elevated corticosterone and a concomitant decrease in ACTH in plasma from FGF21tg mice (Fig 4.11), a few hours prior to the onset of the dark phase. White adipose of FGF21tg may be a source of extra-adrenal glucocorticoids due to elevated expression of HSD11b1 and decreased HSD11b2 (Fig 4.10). However, data from primary adrenal cultures of FGF21tg mice implicate the adrenals as the source of extra circulating glucocorticoids. Indeed, adrenal explants from FGF21tg mice harvested in the afternoon secrete more corticosterone and are more sensitive to ACTH, while those of FGF21-/- animals show the opposite phenotype (Fig 4.11). Recently, the catecholamine sulfotransferase, Sult1d1 was shown to be a transcriptional target of GR (Wong et al., 2010). Gene expression data from liver of FGF21tg mice indicate that Sult1d1 is elevated, and that this is increase is lost with the CamKIIα-cre deletion of βKlotho (Fig 4.9). Given the observed elevation in corticosterone and the reversal of an elevated glucocorticoid-responsive gene in livers of mice that lack βKlotho expression in the SCN, it is likely this nucleus is responsible for the elevated corticosterone with FGF21 overexpression. Further assays are planned to address this prediction.

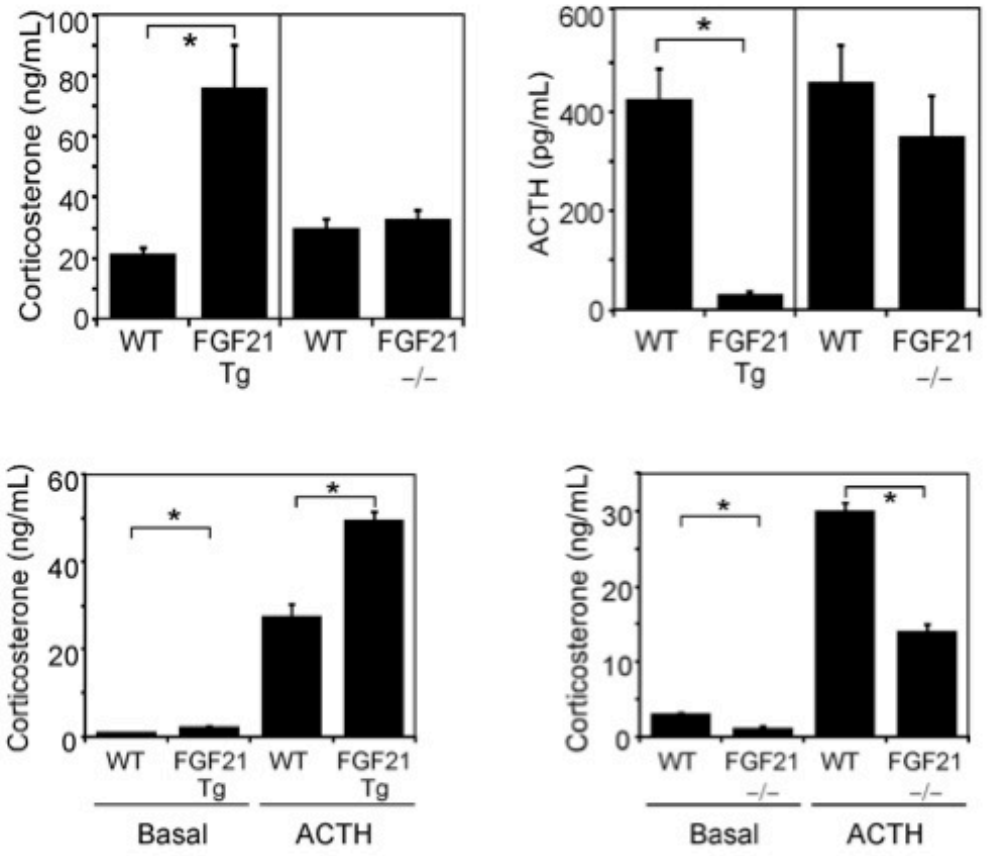


Fig 4.11) FGF21tg mice have elevated glucocorticoid. Top, Males were sacrificed 4 hours prior to the beginning of the dark cycle. Plasma corticoterone and ACTH levels are shown. Bottom, primary adrenal cultures from FGF21tg and FGF21 ko animals. Supernatants were collected and measured for corticosterone. Data are plotted as avg. + sem, n=5-6. *$p \leq 0.05$

Is the SCN the Target of FGF21 for Torpor?

The SCN is linked to torpor (Ruby, 1989), an energy conserving process in small animals characterized by inhibited locomotor activity, blunted metabolic processes, and a rapid drop in body temperature to at least below 33°C. Microarray analyses of livers from FGF21tg mice revealed seemingly ectopic elevation of the pancreatic lipases CEL and CLPS (Inagaki), which hinted at a role for FGF21 in torpor given that this liver gene expression response is seen in hibernation (Zhang, 2006, Andrews, 1998). While FGF21 is not required (Oishi, 2010), it is sufficient for deepened bouts of torpor (Inagaki). Given the connection between the SCN and the torpor response, I predict that this FGF21tg phenotype will be reversed with CamKIIα-cre deletion of βKlotho. Experiments are ongoing in order to test the requirement of the SCN for this effect.

CHAPTER FIVE

PERSPECTIVES & FUTURE DIRECTIONS

FGF21 Action on the Brain is Both Reactive and Adaptive

The summation of my work demonstrates roles for the nervous system in mediating both pharmacological and physiological effects of FGF21 (summarized in Fig 5). Namely, sympathetic responses to acute injection of FGF21 indicate a sort of reactive reflex that acts to drive metabolic status towards ketogenesis by dropping insulin levels in order to promote a permissive setting for gluconeogenesis and ketone body formation by the liver. Further, FGF21-elecited sympathetic activation may aid in initiating or sensitizing brown adipose to a thermogenic program, ultimately resulting in mobilization of stored fat from white adipose, thus leading to the weight loss and insulin-sensitizing effects seen in rodent and primate diabetic models. Chronic elevations of FGF21 initiate an SCN-mediated adaptive response resulting in down-regulation of energetically costly activities such as locomotion and growth, while increasing glucocorticoid levels. The importance of these responses warrants consideration in terms of side effects of extended pharmacotherapeutic use of FGF21.

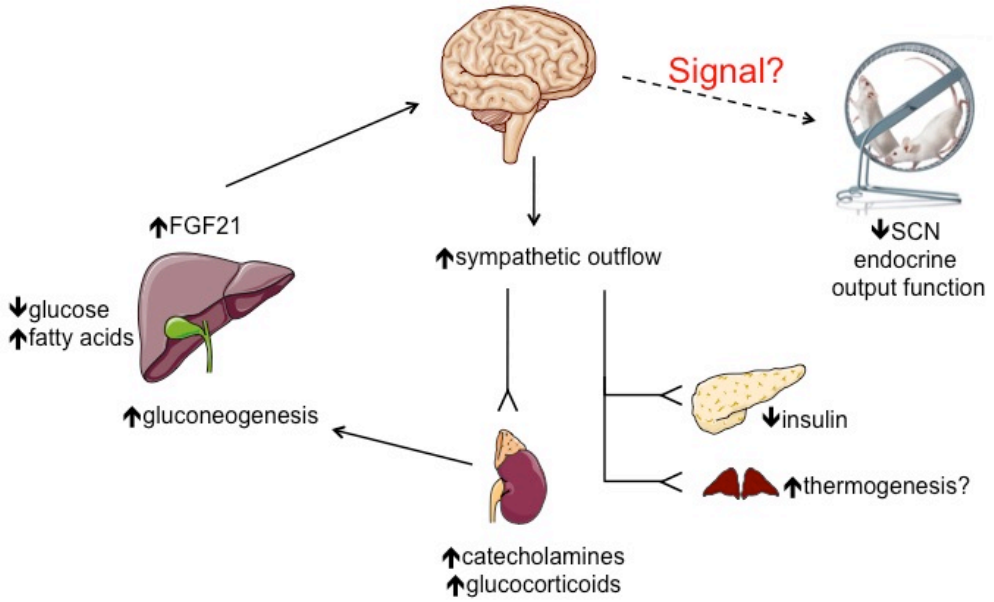


Fig 5) Model of FGF21 Actions Mediated by the Nervous System.

Open Questions

While my work has uncovered key ways in which FGF21 alters physiology through the brain, they are but the beginning and many questions remain.

What are the Chemical Identities of βKlotho Expressing Neurons?

The chemical makeup of neurons is related to how they communicate information and is comprised of both chemical and peptidegic neurotransmitters. Nuclei are typically composed of mixtures of different neuronal cell types. A classical example is the arcuate POMC, NPY, and kisspeptin cell populations. In the NTS, TH-positive noradrenergic cells are well known, while POMC, GLP1 and other neuropeptides have

been identified. The SCN is typically regarded as having two main populations that demarcate the architecture of this nucleus: the VIP cells of the core and the AVP cells of the shell, each with assigned output functions. Suitable in situ probes and/ or antibodies against βKlotho are necessary. Identification of the cell types that express βKlotho will aid in understanding mechanisms underlying the effects of FGF21 on these neuronal sites.

Are the Responses Electrophysiological or Transcriptional?

That the gluconeogenic effect induced by acute injections of FGF21 is so fast suggests that it is mediated by an electrophysiological response. While bFGF2 has been reported to change the biophysical properties of neurons as evidenced by enhanced long-term potentiation (Zhao et al., 2007), electrophysiological studies for the FGF family are lacking. FGF21 has been shown to elicit Ca^{+2} signaling in 3T3-L1 adipocytes (Moyers et al., 2007), so it will be interesting to see if it can initiate neurotransmission in acute brain slice preparations. These studies are hampered by the lack of knowledge of the cellular identity of $\beta Klotho^{+}$ neurons since electrophysioligical experiments require the use of cell type-specific fluorescent reporter mice. However, a candidate approach is currently underway.

The chronic effects of FGF21 mediated by the SCN seem to indicate transcriptional reprogramming of the nucleus. Indeed, I have observed reduced AVP mRNA in hypothalami of FGF21tg animals. Whether this is a direct effect of FGF21 on SCN cells, or rather an effect due to the metabolic environment within these animals as a result of elevated FGF21 remains to be determined. However, a direct effect downstream

of ERK signaling is supported by a recent report showing increased pERK in whole hypothalamus in response to acute FGF21 injection (Yang et al., 2012).

What is the Nature of the Signal from Brain to Periphery?

While the adrenergic blockade studies of Chapter 3 are indicative of a sympathetic signal from the brain to the pancreas, liver, and bat, other signals from the brain may mediate some of the longer-term adaptations to elevated FGF21. Again, identification of the makeup of βKlotho^{+} neurons will offer clues.

Do Other Endocrine FGFs Act in the Brain?

Klotho expression is much more widespread than βKlotho in the nuclei examined (Fig 2.1). This raises the potential for FGF23 action on these nuclei. However, Klotho differs from βKlotho in that it can shed its extracellular domain, thus conferring FGF-independent properties such as regulation of ion channels [reviewed in (Huang, 2012)]. Data showing that FGF23 can cross the blood-brain-barrier are lacking, but the potential for CNS effects warrants further study.

FGF15/19 also binds βKlotho and an early study in which the recombinant protein was delivered icv showed an effect on energy homeostasis (Fu et al., 2004). Care should be taken in interpreting these results since the dose administered was later shown to be the same at which FGF21 was determined to leak out into peripheral circulation (Sarruf et al., 2010). Although not the same protein, FGF15/19 and 21 have similar molecular weights, thus raising the possibility of entry and exit from the brain. While my

profiling studies do not show overlap of FGFR4 with βKlotho in the neuronal sites examined, FGF15/19 is able to signal as efficiently through FGFR1, thus allowing it to act at the same sites as FGF21. Its reported effects on carbohydrate metabolism may not be cell-autonomous (Kir et al., 2011; Potthoff et al., 2011). Therefore, it will be interesting to test the contribution of the brainstem or vagus nerve to the glycogen-storing effect of FGF15/19.

Behavioral studies in FGF15/19 and FGFR4 animal models will be difficult owing to limitations of background strain. These knockout models are lethal in the C57 BL/6 strain, and thus must be maintained as mixed or other pure strain, such as 129. In terms of circadian studies, this renders these models less than ideal (Siepka and Takahashi, 2005). Nonetheless, since they were available in the Mangelsdorf/Kliewer lab, I tested both models in the wheel running assay. As expected, the effect of background strain on the behavior was apparent and limited interpretation of the results (Appendix K, L). Conditional deletion and transgenic strategies are greatly needed here.

What is the Relative Importance of the Brain versus Other Tissues in FGF21 Biology?

FGF21 as an injectable drug encompasses both PPARα and PPARγ effects. βKlotho is expressed at its highest levels in brown and white fat (Fon Tacer et al., 2010) and our group has recently demonstrated the importance of FGF21 action in white adipose (Dutchak et al., 2012). FGF21 likely owes its weight loss properties to thermogenesis by the bat (Coskun et al., 2008). Therefore both types of adipose are probably major players in the biology of FGF21. Additionally, the endocrine pancreas of diabetic rodent models seems to benefit directly from FGF21 treatment (Hart et al.,

2000), thus contributing to improvements in glycemic control. Since each of these tissues contribute to overall homeostasis orchestrated by the nervous system, it is difficult to consider them in isolation. Nonetheless, based on my own studies, it seems that FGF21 can elicit systemic responses largely mediated by the brain, in addition to the cell-autonomous responses we reported for wat (Dutchak et al., 2012). The relative contribution of each tissue is an active area of work in our group and remains to be determined.

CHAPTER SIX

MATERIALS & METHODS

Animal Models

All procedures and use of animals were approved by the Institutional Animal Care and Use Committee of UT Southwestern Medical Center Dallas. Unless otherwise stated in specific dietary studies, all animals were maintained on 2916 Global Diet (Harlan Teklad). Male mice were used in all studies. C57 BL/6J mice used for LCM and recombinant FGF21 injections were obtained from Jackson, or from the UT Southwestern mouse breeding core, which also sources its colony breeders from Jackson.
FGF21 ko and tg mice were made in the Mangelsdorf/Kliewer laboratory (Inagaki et al., 2007; Potthoff et al., 2009). The floxed βKlotho mice were also made in the Mangelsdorf/Kliewer laboratory by post-doctoral fellow Xunshan Ding. Nav1.8 mice were generated by and used in collaboration wit the Elmquist with John Wood. The Phox2b-cre (line 4) mice were generated by Michael Scott in the Elmquist laboratory (Rossi et al., 2011; Scott et al., 2011). Nestin-cre mice were purchased from Jackson. CamKIIa-Cre mice from the laboratory of Gunther Schutz were characterized by Marioko Izumo in the Takahashi lab to efficiently target the SCN (Casanova et al., 2001).

Brain and Ganglia Collection; Laser Capture Microdissection

At the start if the light cycle, C57 BL/6 males were deeply anesthetized with chloral hydrate and decapitated. Left nodose ganglion and mid-thoracic dorsal root ganglia were rapidly collected and frozen in liquid nitrogen. Brains were collected, slow-

frozen on dry ice, and stored at -80C until sectioning. Brains were cryosectioned at 30um and thaw-mounted onto silane-coated PEN membrane glass slides (Molecular Devices, Sunnyvale, CA) and stored at -80˚C. Slides were lightly fixed in 75% ethanol immediately prior to thionin staining. Slides were then dehydrated in a graded ethanol series followed by 5 minutes in xylenes. The Arcturus Veritas Microdissection System (Molecular Devices) was used to isolate each nucleus based on neuroanatomical boundaries defined by Paxinos and Franklin (2001) mouse brain atlas.

Peripheral ganglia RNA was isolated using Ambion's Ribopure kit, while LCM nuclei RNA was extracted using the PicoPure RNA Isolation Kit (Molecular Devices) with on-column DNAseI treatment (Qiagen, Valencia, CA), and stored at -80˚C. RNA quality was assessed using the Experion Automated Electrophoresis system (Bio-Rad, Hercules, CA).

cDNA was synthesized using the High Capacity cDNA Kit (Applied Biosystems), followed by cDNA pre-amplification of specific gene targets using TaqMan Preamplification Buffer (Applied Biosystems), according to the manufacturer's directions. Due to sufficiently high expression, it was not necessary to pre-amplify 18S rRNA, which was used as the normalizer gene. All gene expression levels were measured with an Applied Biosystems 7900HT Sequence Detection System using the efficiency-corrected ΔCt method as previously described (Bookout et al., 2006; Cravo et al., 2011). Samples with cycle times (Ct) ≥ 30 were considered to be below detection. The anatomic specificity of the LCM dissections was confirmed by qPCR for the expression of known marker genes in each nucleus.

Quantitative Real Time PCR

Gene expression studies of liver, wat, bat, and adrenal were performed using the ΔΔCt assay essentially as described (Bookout and Mangelsdorf, 2003).

Recombinant FGF21

Human recombinant FGF21 was a generous gift from M. Mohamedi and R. Goetz at NYU. Original preps were unstable, so 50% glycerol was included in later preps

ICV

C57 BL/6 males received 3rd ventricular cannula implants tethered to subcutaneously implanted 2-week osmotic minipumpt (Alzet) containing either FGF21 or vehicle in saline adjusted to deliver a total of 1 μgram of FGF21 per animal per day.

Vagotomy

Surgically manipulated C57 BL/6 males were purchased from Jackson Labs. Briefly, animals underwent bilateral sub-diaphragmatic vagotomy and were allowed to recover for 1 week prior to shipment.

Primary Adrenal Cultures

Primary adrenal explants were performed as described (Cummins et al., 2006).

Blood Analytes

Trunk blood was collected into K-EDTA tubes following rapid decapitation to avoid stress responses. Glucose, ketones, cholesterol, nefa, and triglycerides were measured by colorimetric assays (Wako); corticosterone and acth by radioimmuneassay (MP Biomedical); insulin, c-peptide, and ghrelin by ELISA (Crystal Chem, Alpco, Cayman).

Telemetry

Radio telemetry experiments were performed as described (Inagaki et al., 2007).

Wheel Running

Wheel running experiments were performed in collaboration with the Takahashi lab using the guidelines in (Siepka and Takahashi, 2005), following the paradigms:

Protocol 1- FGF21 Models

Lighting Condition	Feeding Condition	# of days
12:12 LD	*Ad Libitum*	18
DD	*Ad Libitum*	21
12:12 LD	*Ad Libitum*	15
LL	*Ad Libitum*	33
12:12 LD	*Ad Libitum*	20
12:12 LD	RF - 12 h	1
12:12 LD	RF - 10 h	1
12:12 LD	RF - 8 h	1
12:12 LD	RF - 6h	1
12:12 LD	RF - 4h	6
12:12 LD	24 h food deprivation	1
12:12 LD	*Ad Libitum*	6
12:12 LD	24 h food deprivation	1
12:12 LD	*Ad Libitum*	6
12:12 LD	24 h food deprivation	1
12:12 LD	*Ad Libitum*	6
12:12 LD	24 h food deprivation	1
12:12 LD	*Ad Libitum*	4
		143

Protocol 2- Telemetry

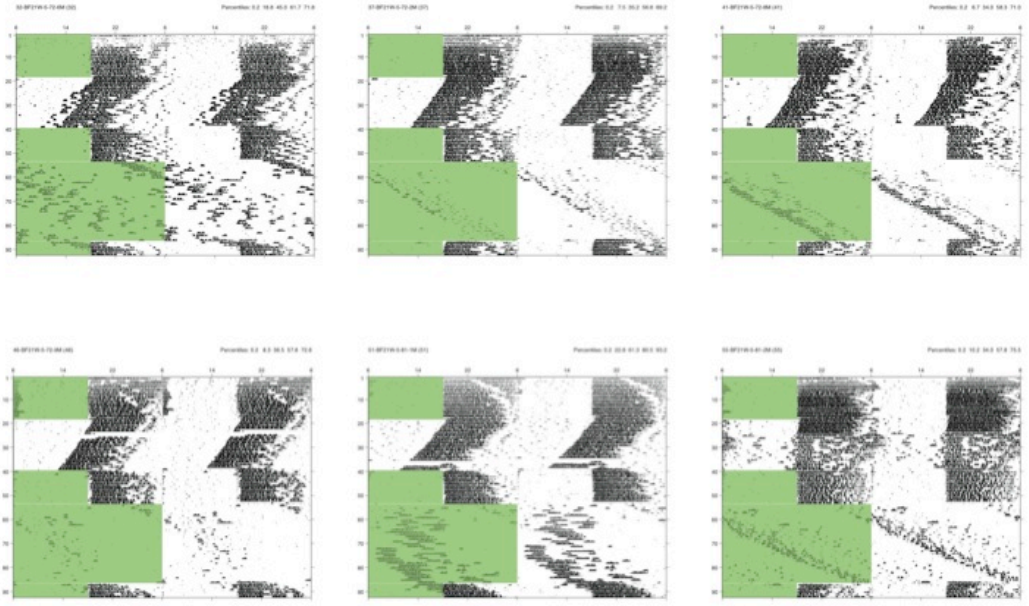
Lighting Condition	Feeding Condition	# of days
12:12 LD	*Ad Libitum*	16
12:12 LD	RF - 12 h	1
12:12 LD	RF - 10 h	1
12:12 LD	RF - 8 h	1
12:12 LD	RF - 6h	1
12:12 LD	RF - 4h	6
12:12 LD	24 h food deprivation	1
12:12 LD	*Ad Libitum*	6
12:12 LD	24 h food deprivation	1
12:12 LD	*Ad Libitum*	6
12:12 LD	24 h food deprivation	1
12:12 LD	*Ad Libitum*	6
12:12 LD	24 h food deprivation	1
12:12 LD	*Ad Libitum*	4
		52

Protocol 3- βKlotho models

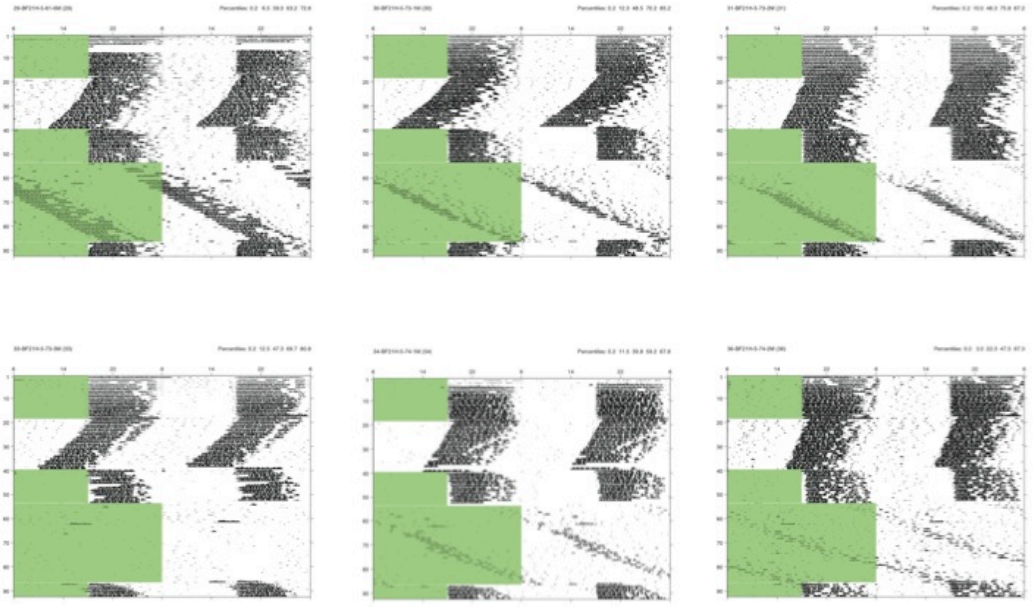
Lighting Condition	Feeding Condition	# of days
12:12 LD	*Ad Libitum*	18
DD	*Ad Libitum*	21
12:12 LD	*Ad Libitum*	26
12:12 LD	RF - 12 h	1
12:12 LD	RF - 10 h	1
12:12 LD	RF - 8 h	1
12:12 LD	RF - 6h	1
12:12 LD	RF - 4h	6
12:12 LD	24 h food deprivation	1
12:12 LD	*Ad Libitum*	6
12:12 LD	24 h food deprivation	1
12:12 LD	*Ad Libitum*	6
12:12 LD	24 h food deprivation	1
12:12 LD	*Ad Libitum*	6
12:12 LD	24 h food deprivation	1
12:12 LD	*Ad Libitum*	4
		101

APPENDIX A

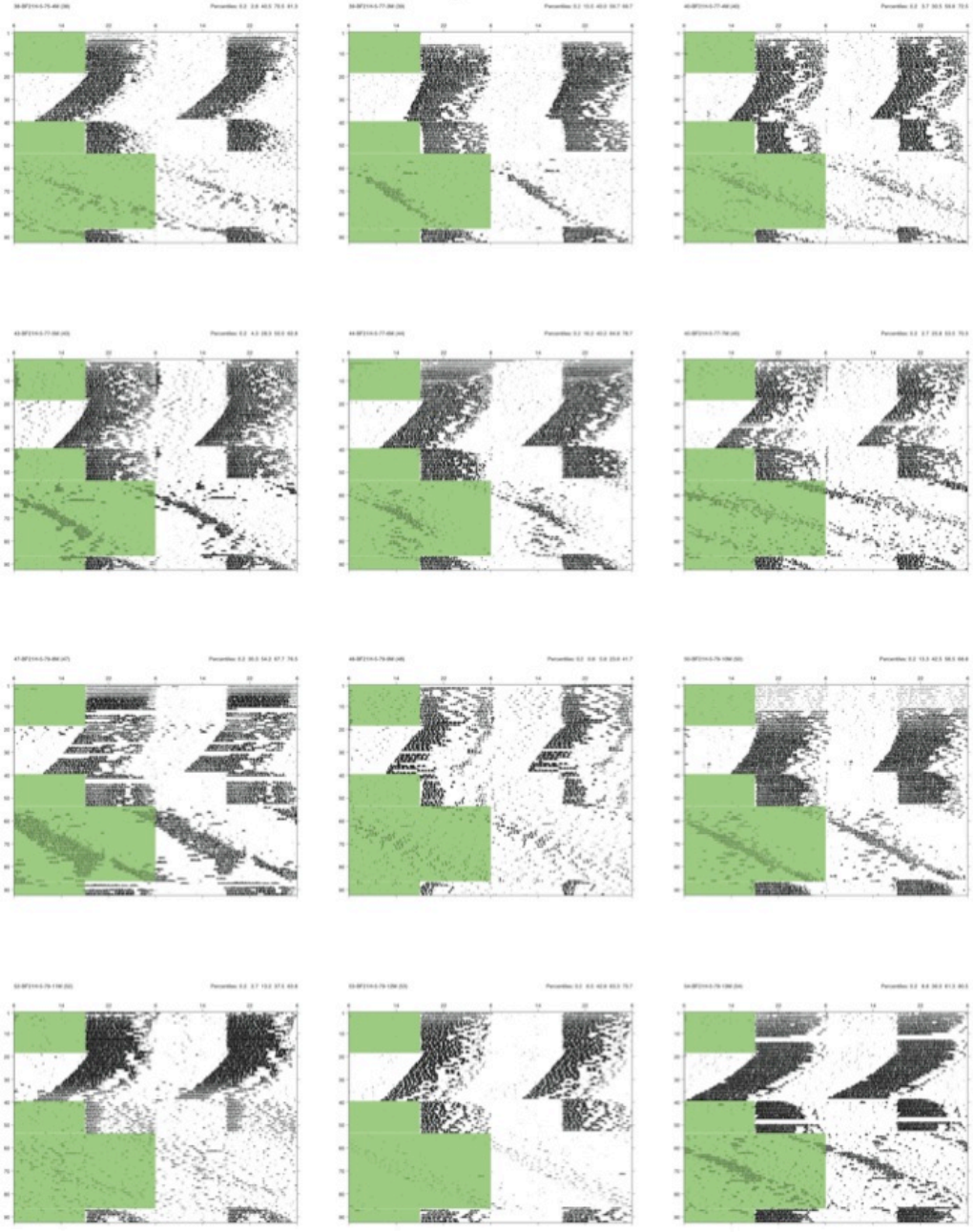
FGF21 wt chow



FGF21 ko chow

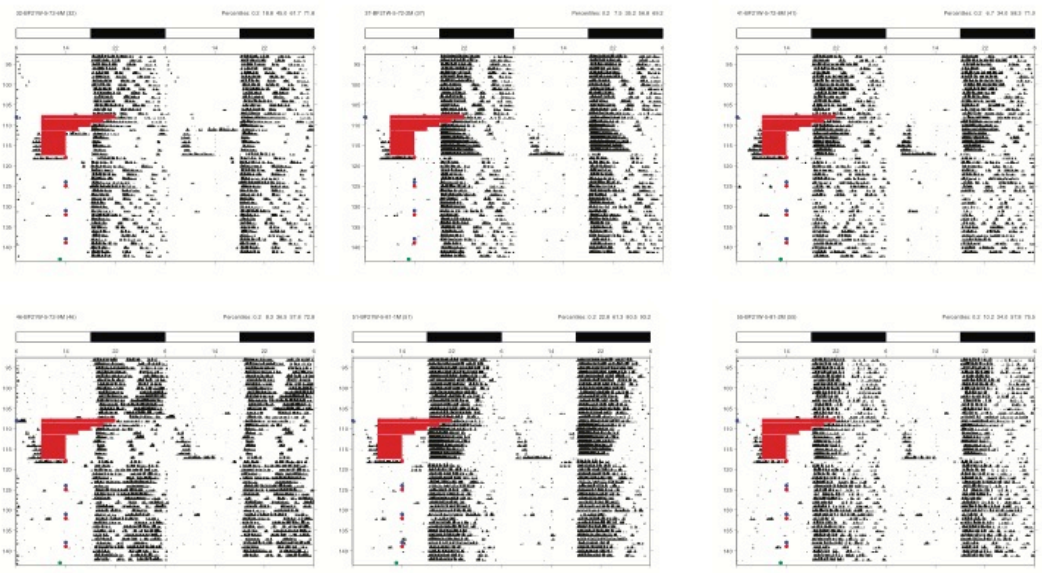


FGF21 ko chow (cont)

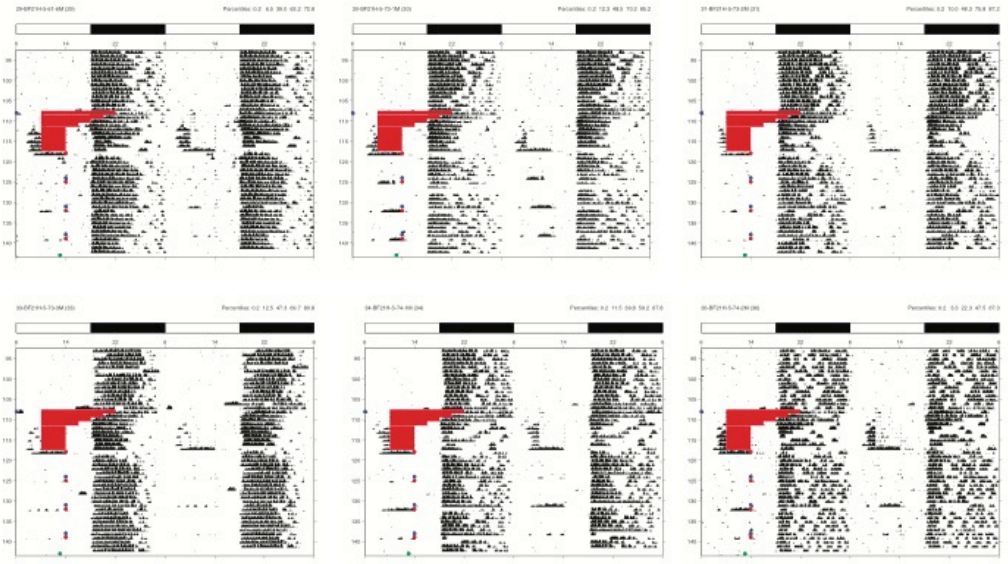


APPENDIX B

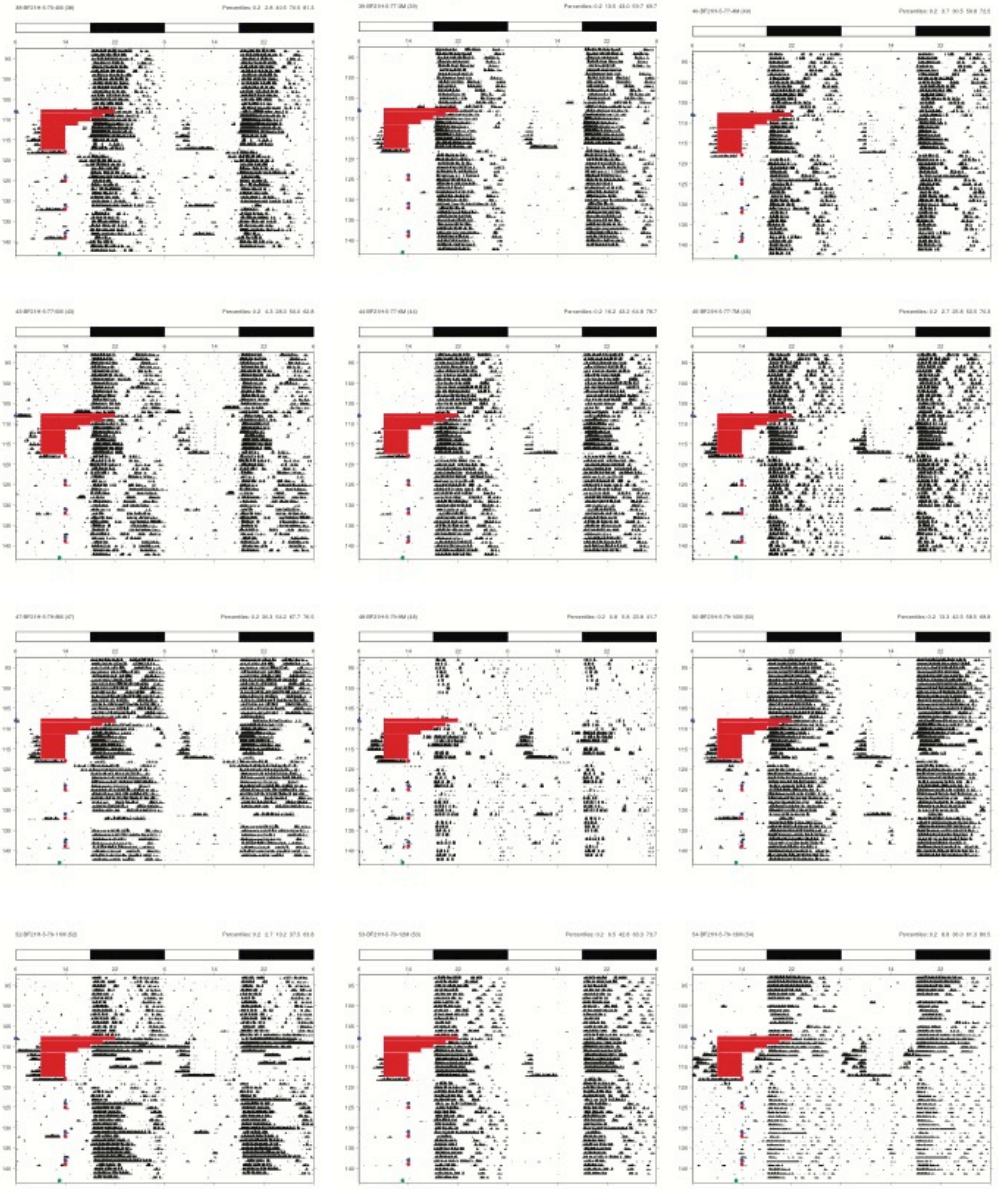
FGF21 wt chow



FGF21 ko chow

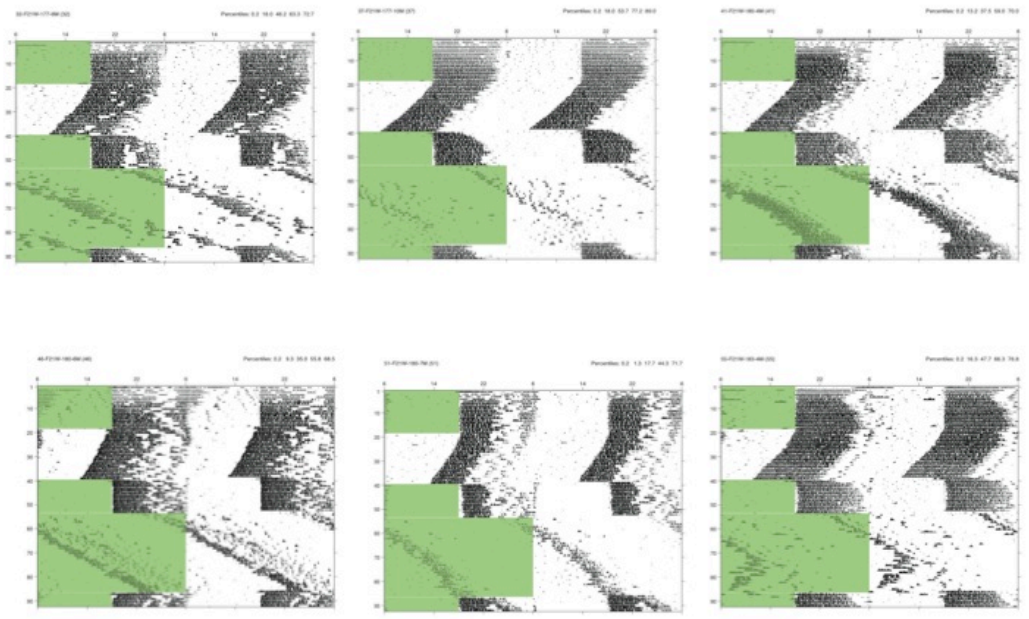


FGF21 ko chow (cont)



APPENDIX C

FGF21 wt chow



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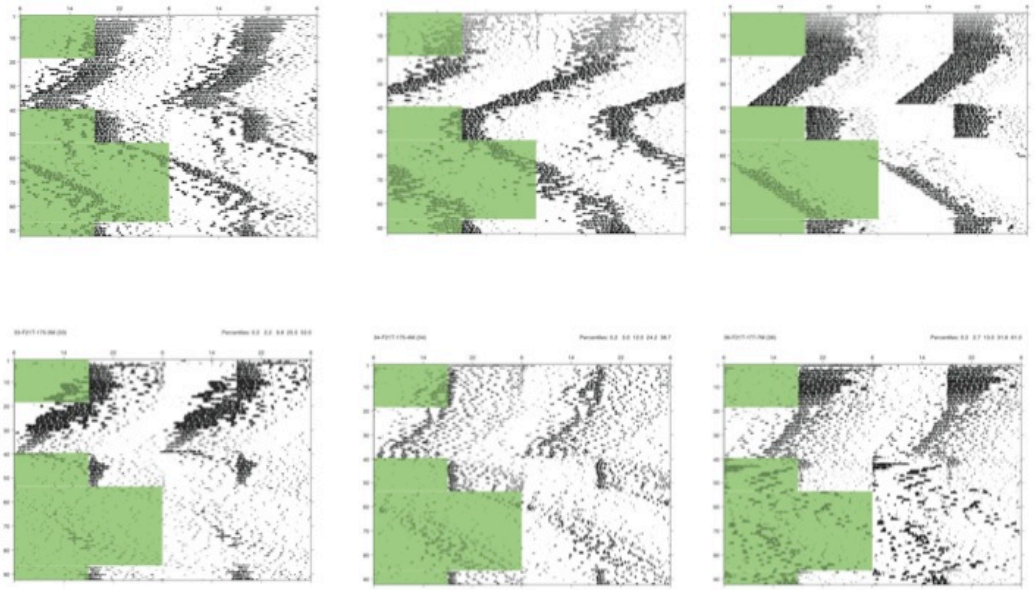

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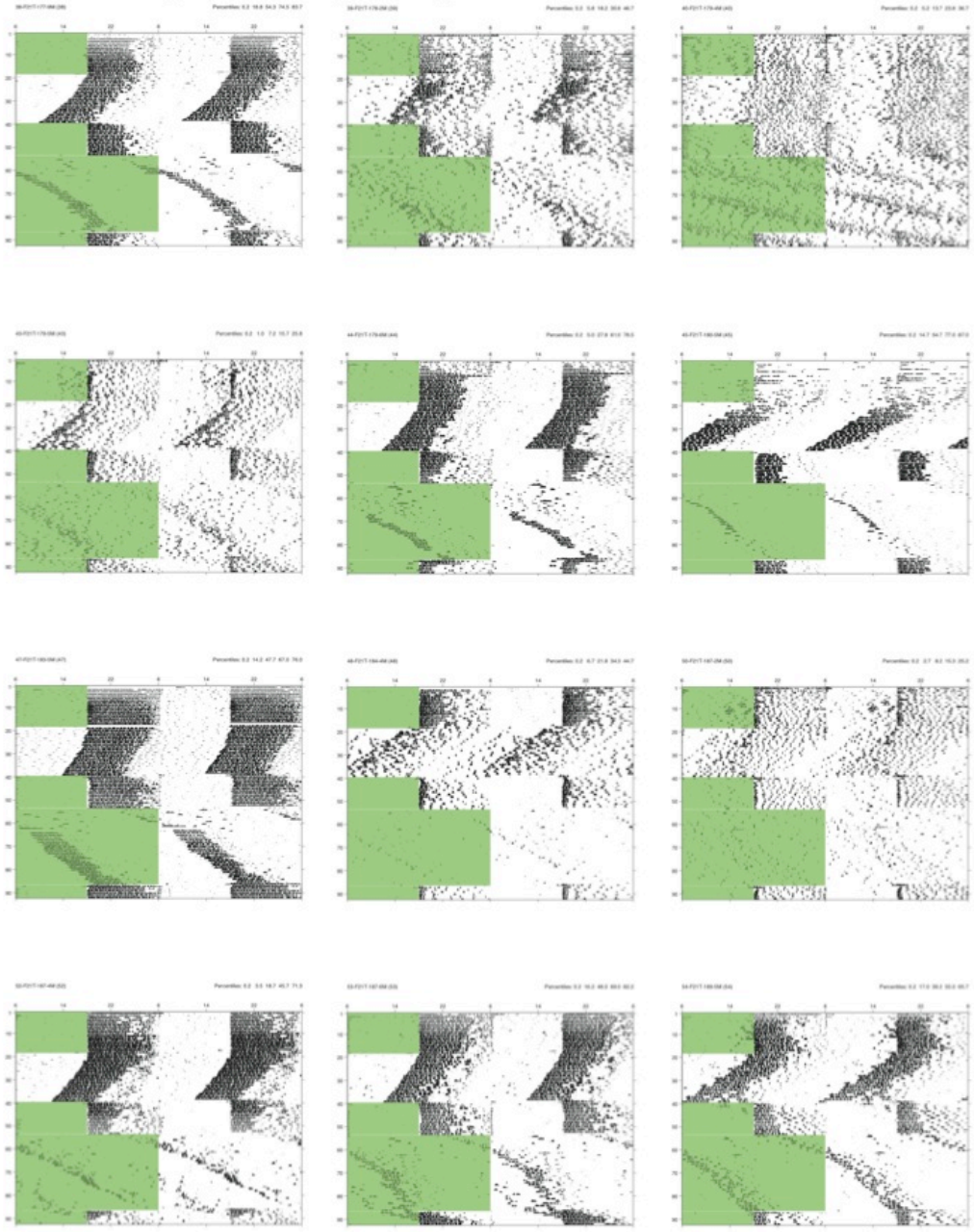
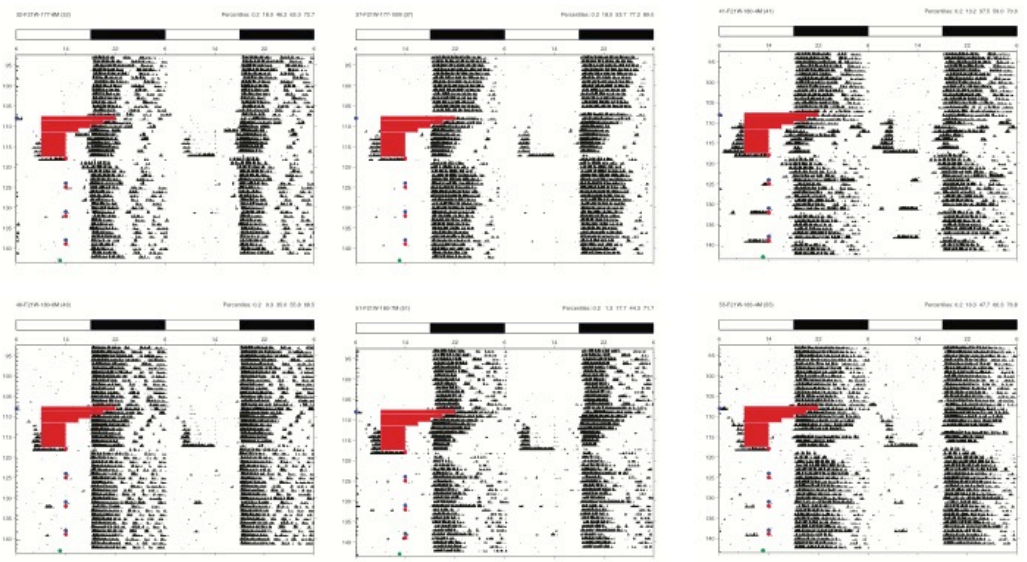


APPENDIX D

FGF21 wt chow



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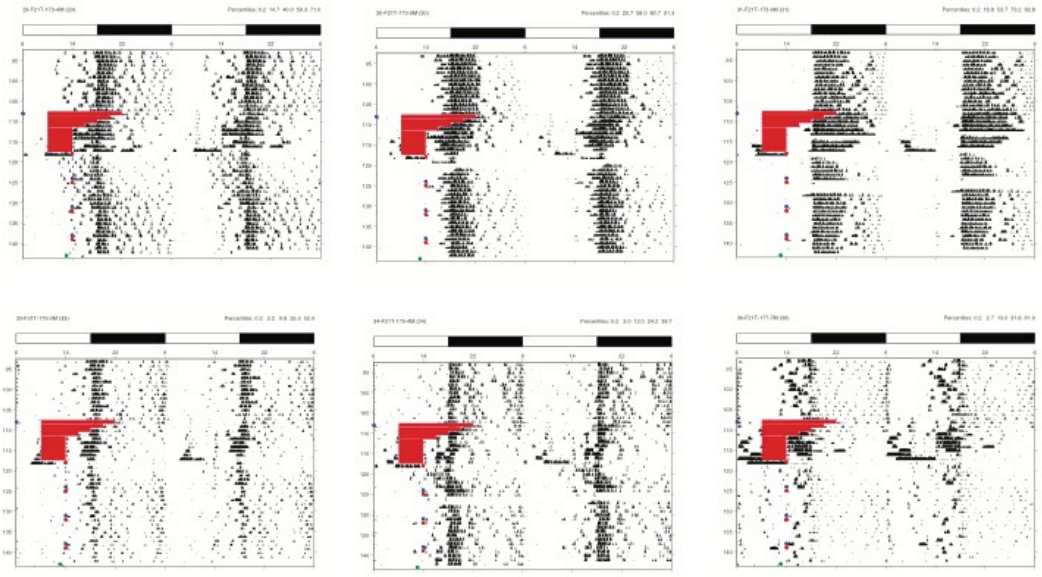

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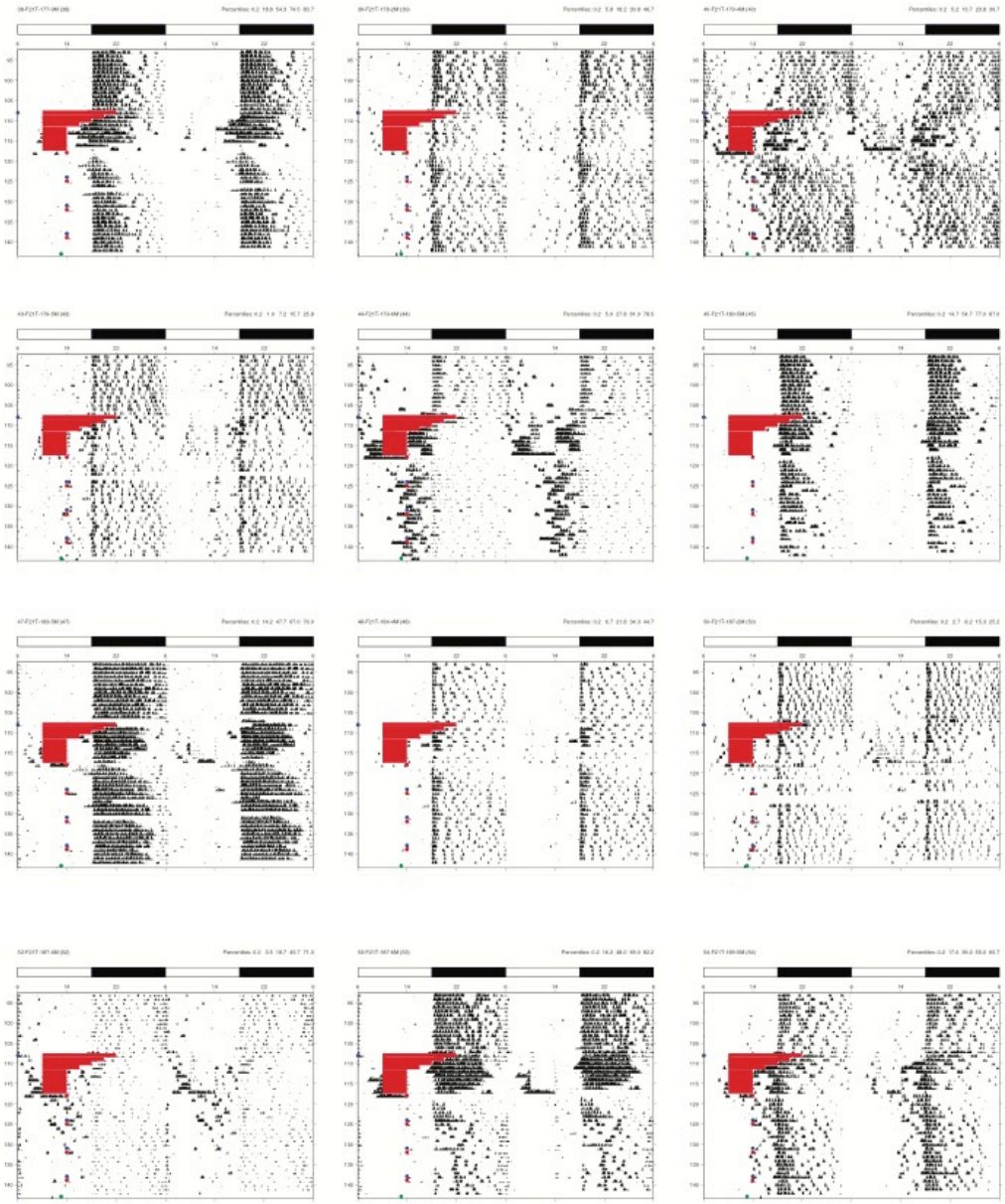
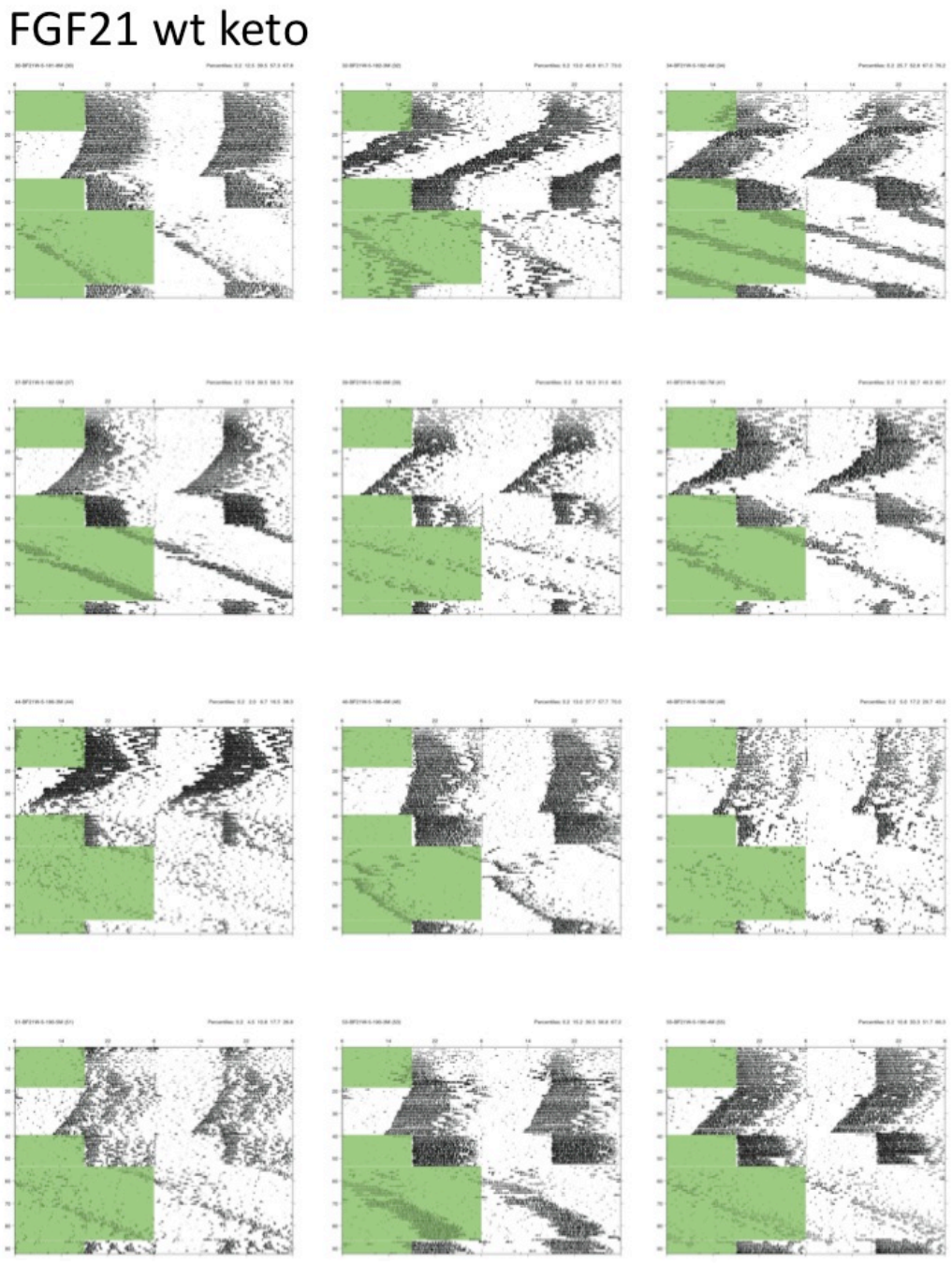
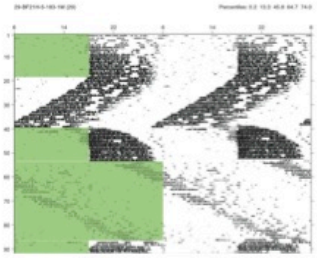
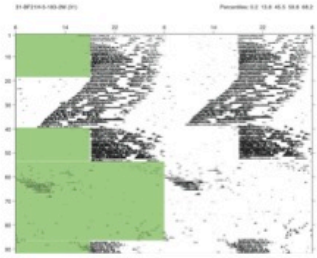
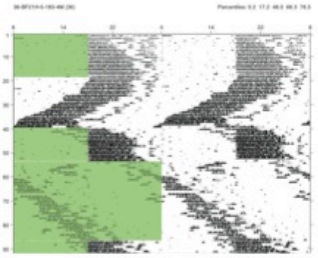
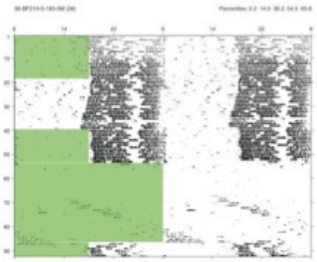
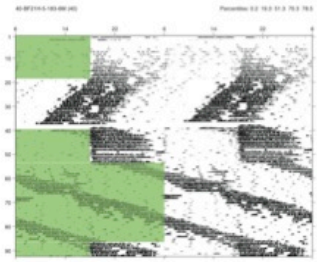
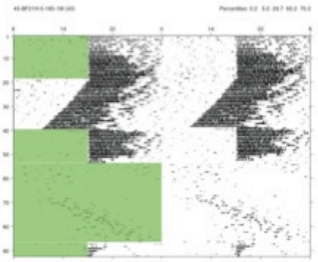
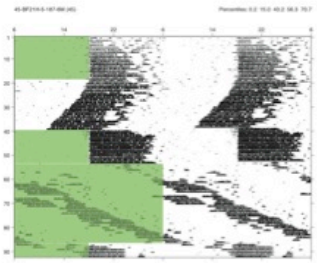
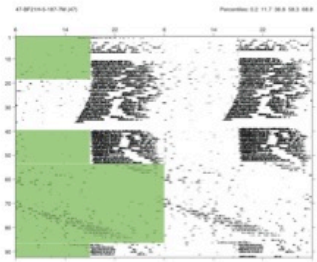
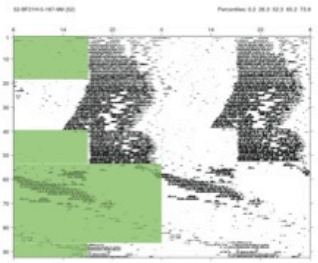
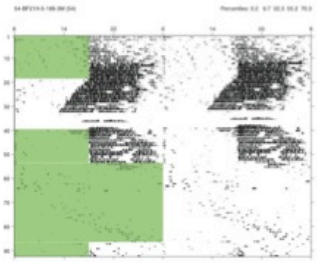


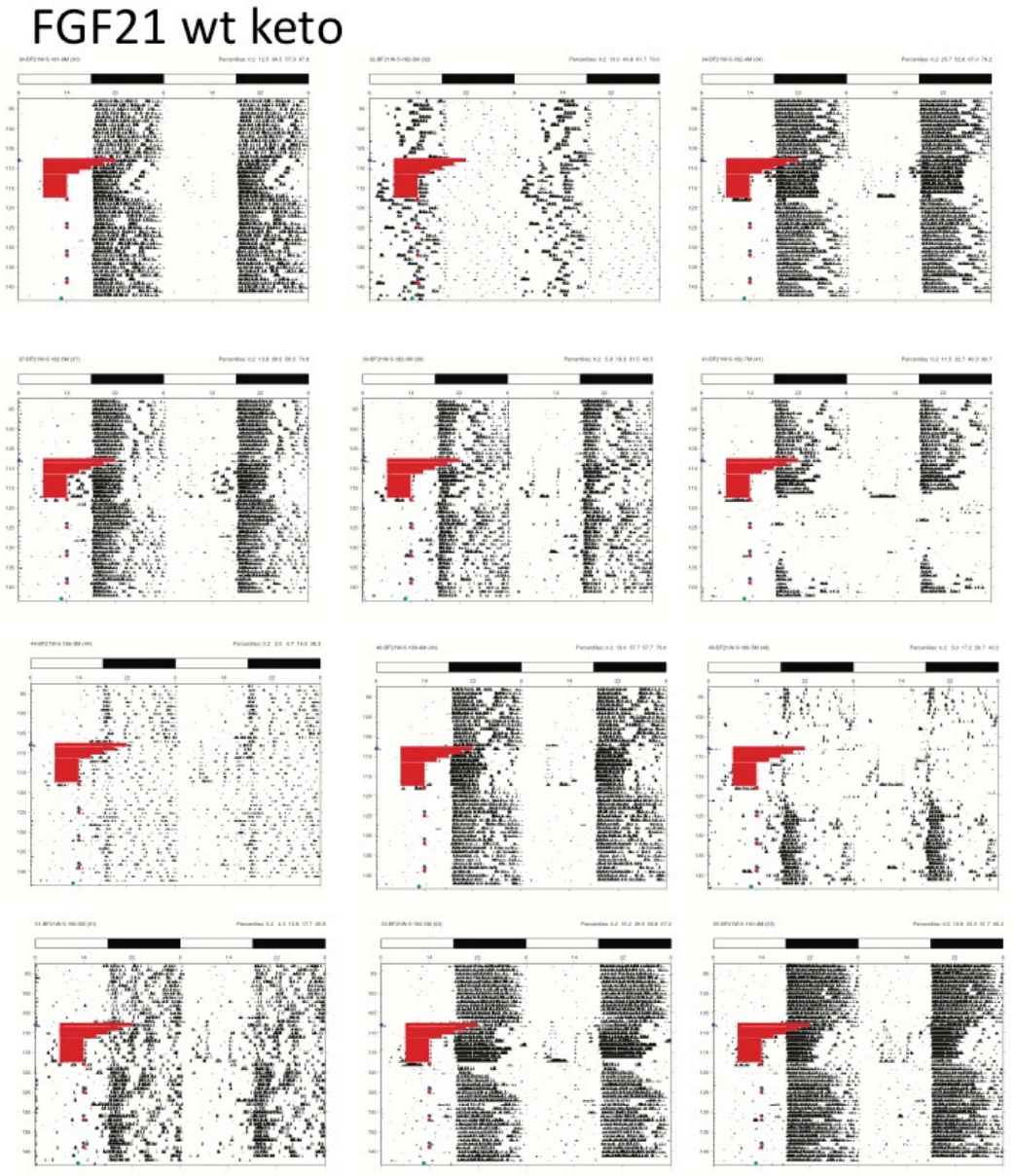
APPENDIX E



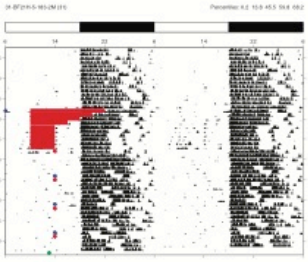
FGF21 ko keto

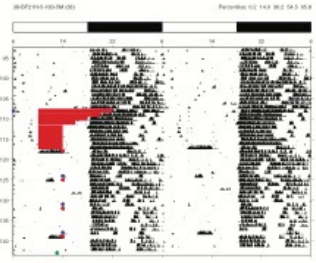
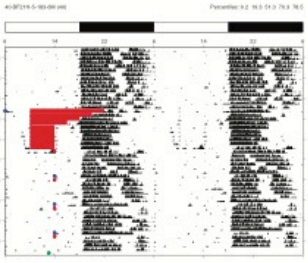
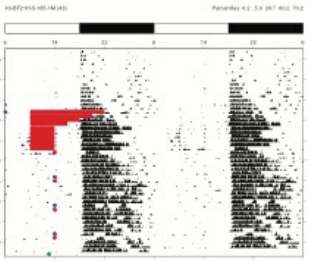
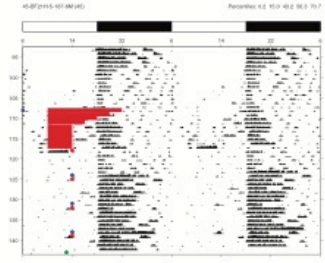











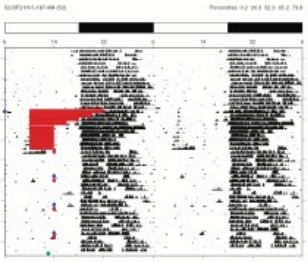
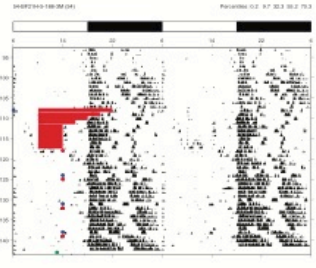
APPENDIX F



FGF21 ko keto

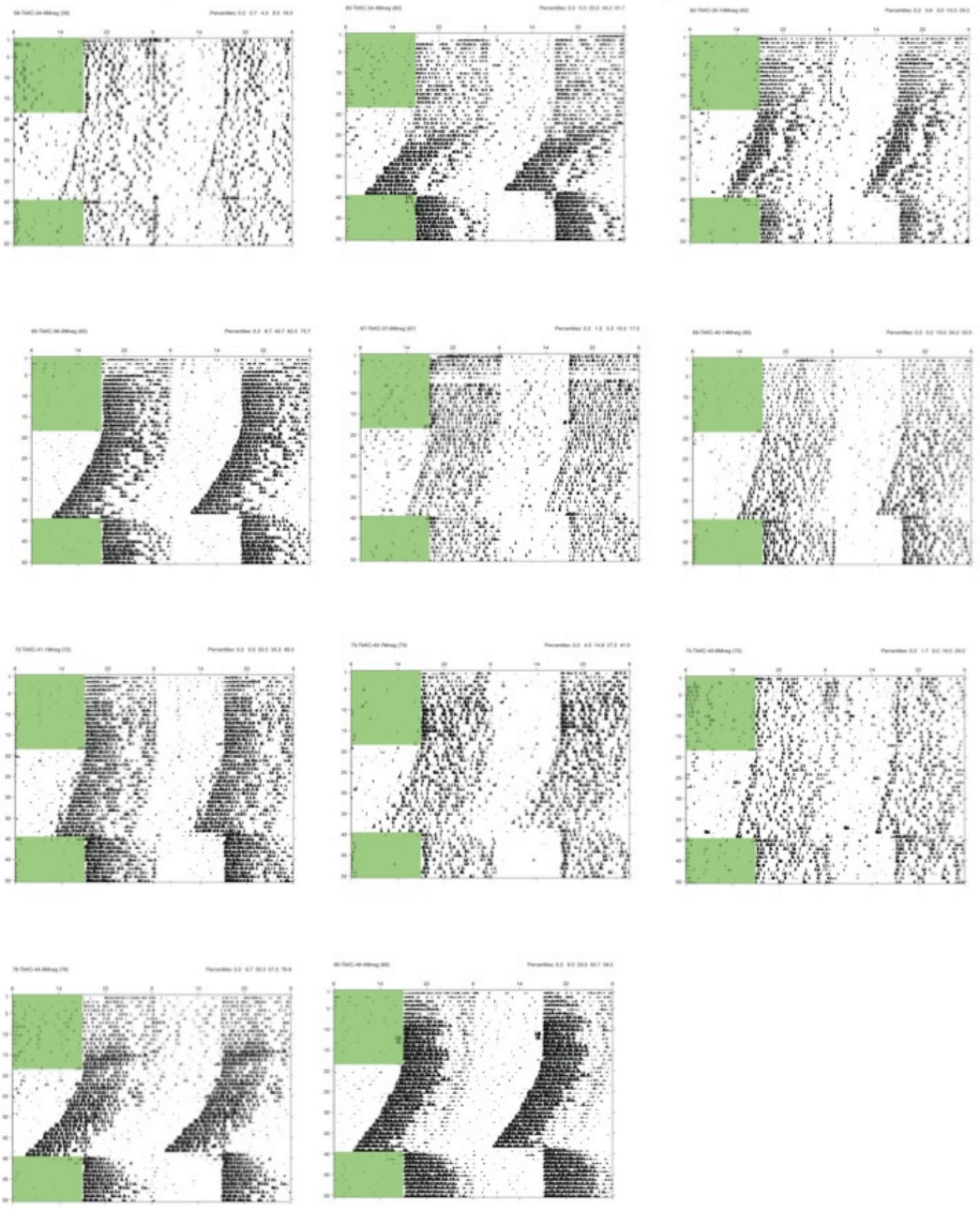



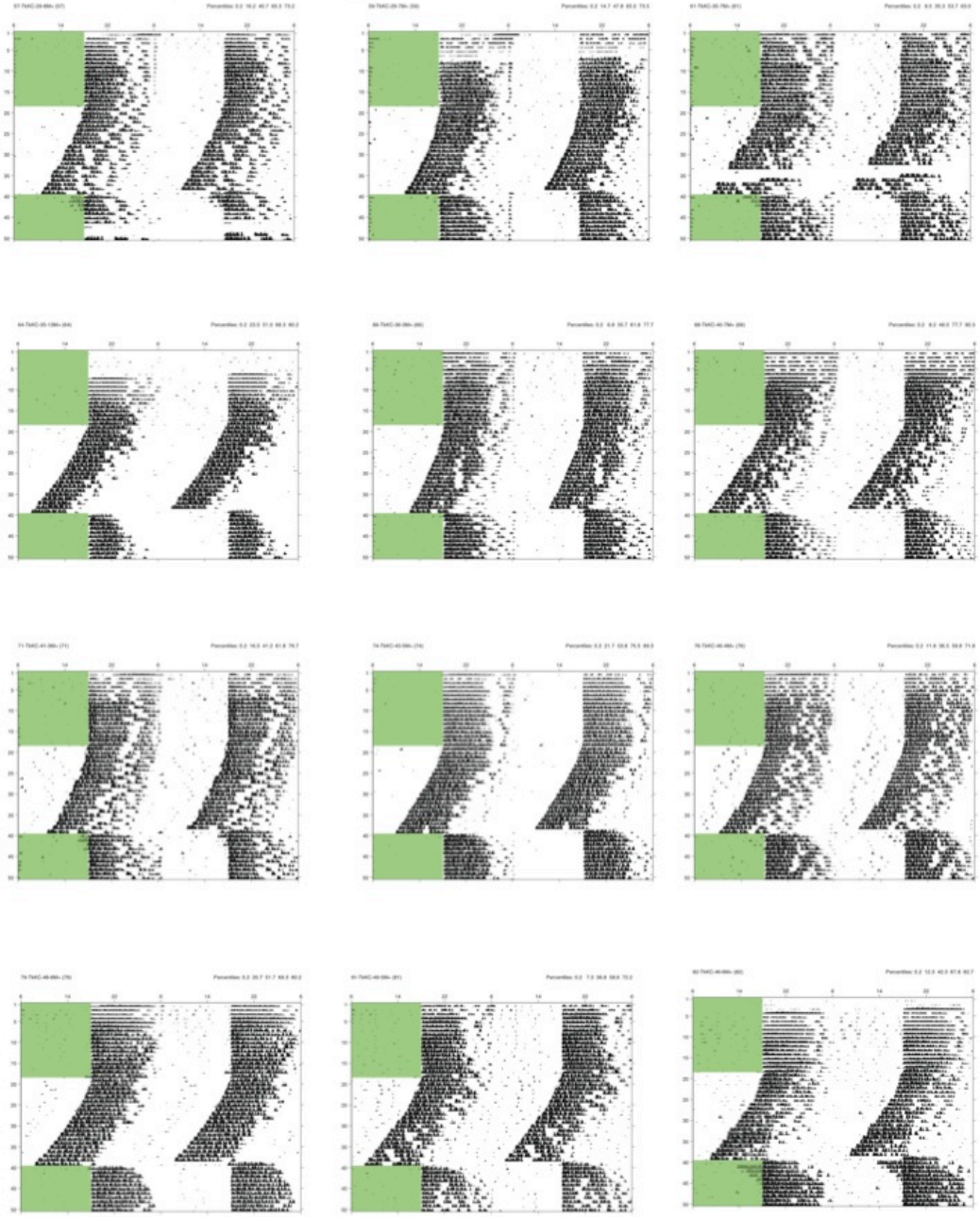



APPENDIX G

fl/fl KLB; FGF21tg; CamK-cre neg

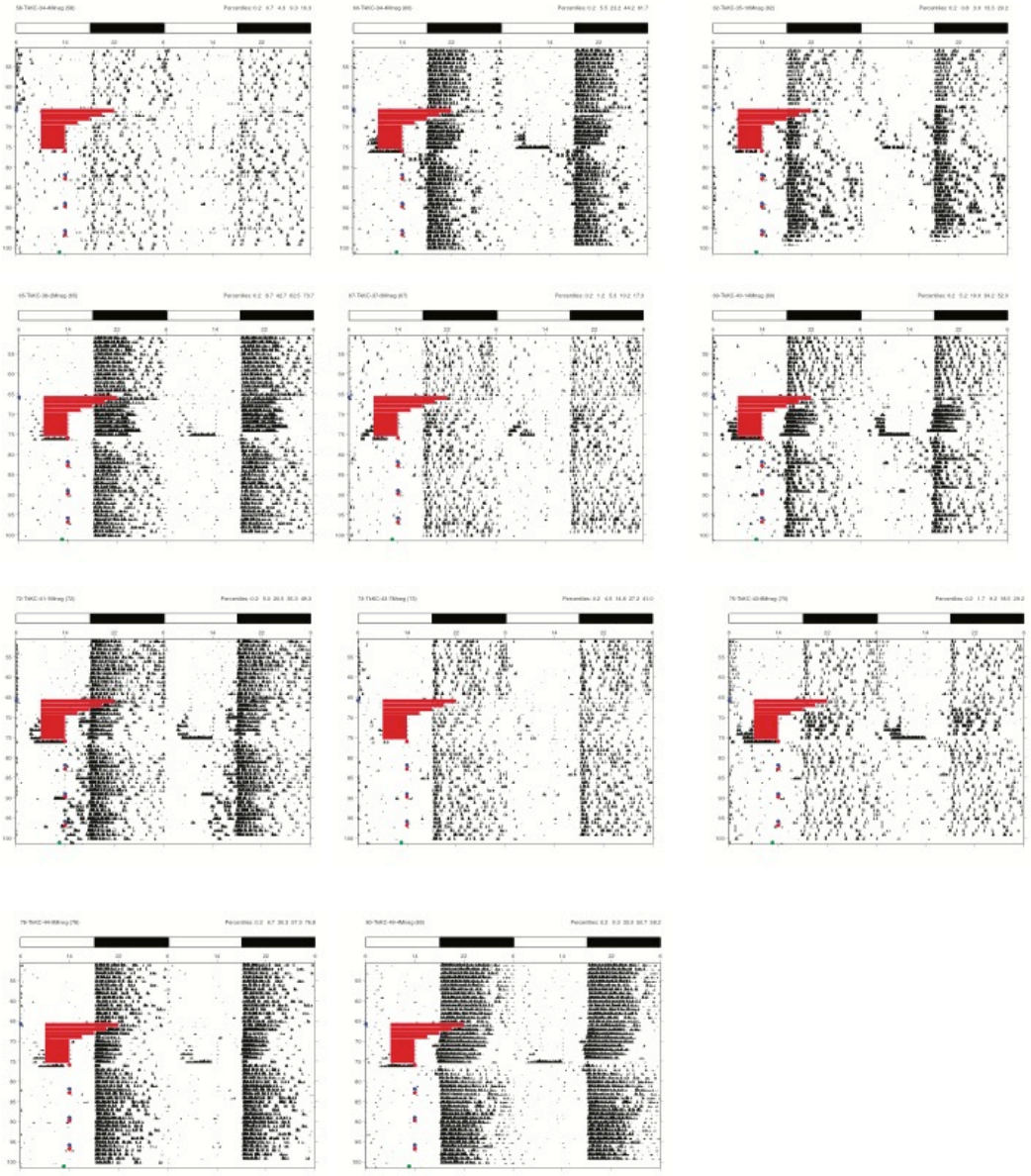


fl/fl KLB; FGF21tg; CamK-cre +

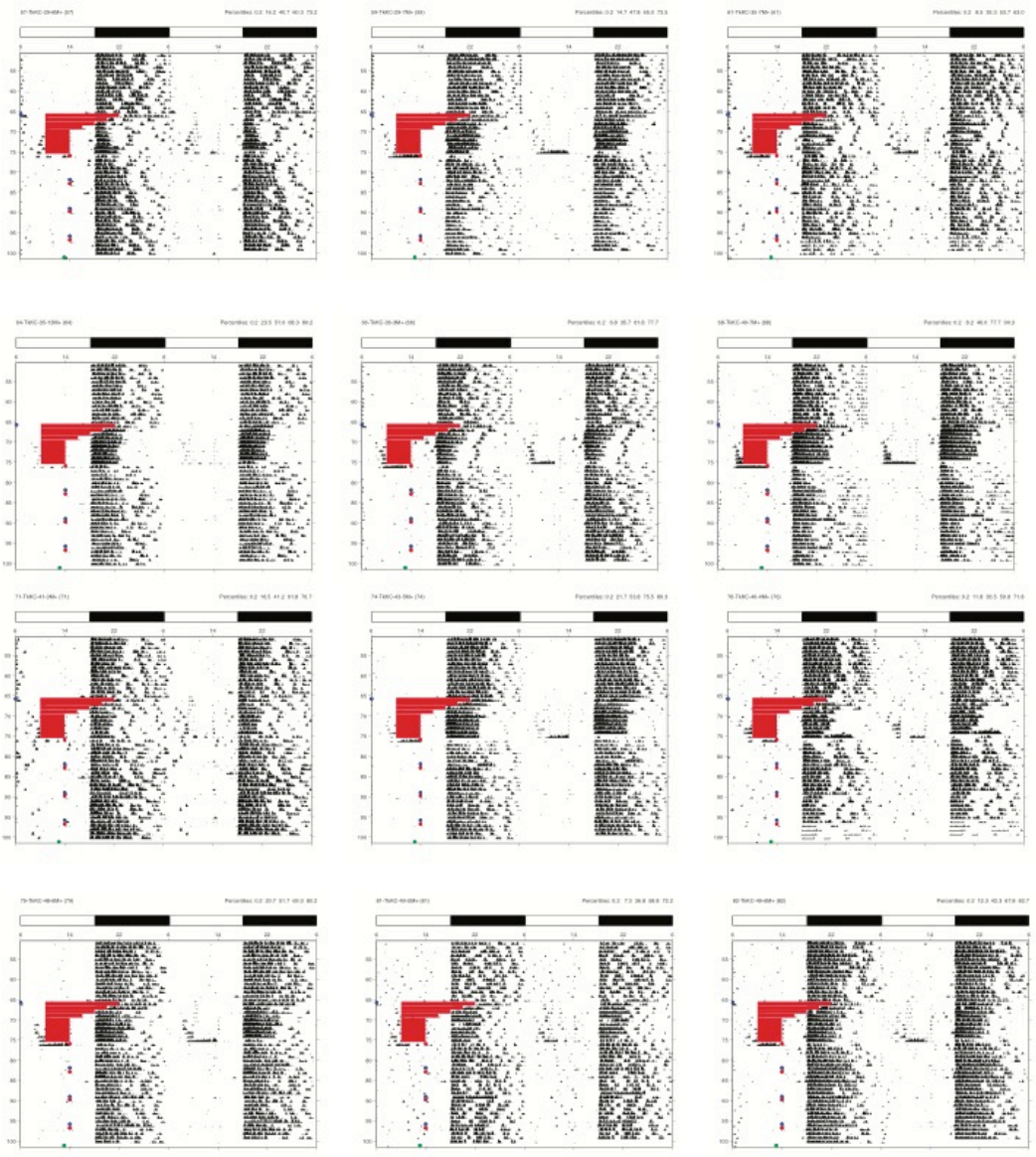


APPENDIX H

fl/fl KLB; FGF21tg; CamK-cre neg

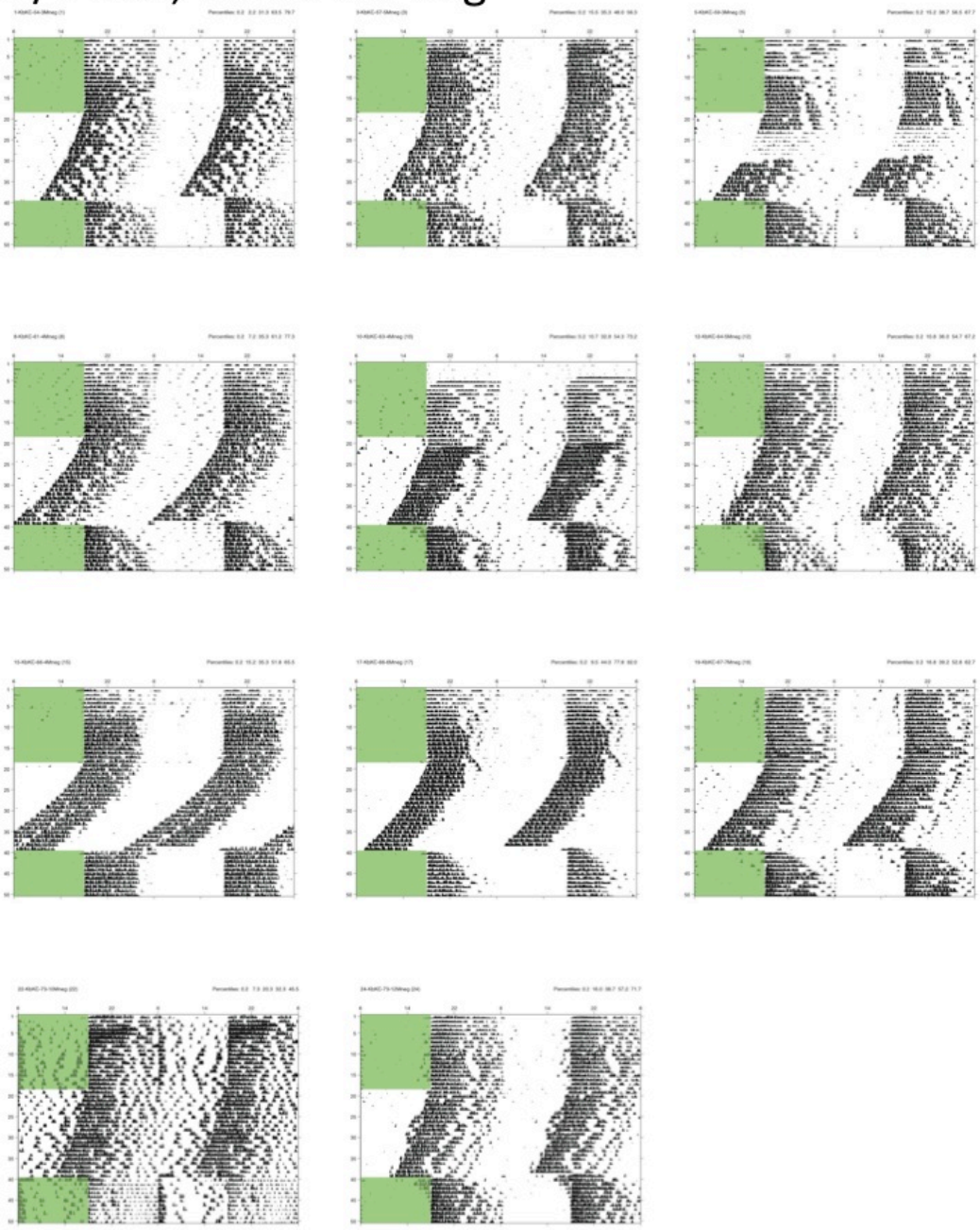


fl/fl KLB; FGF21tg; CamK-cre +

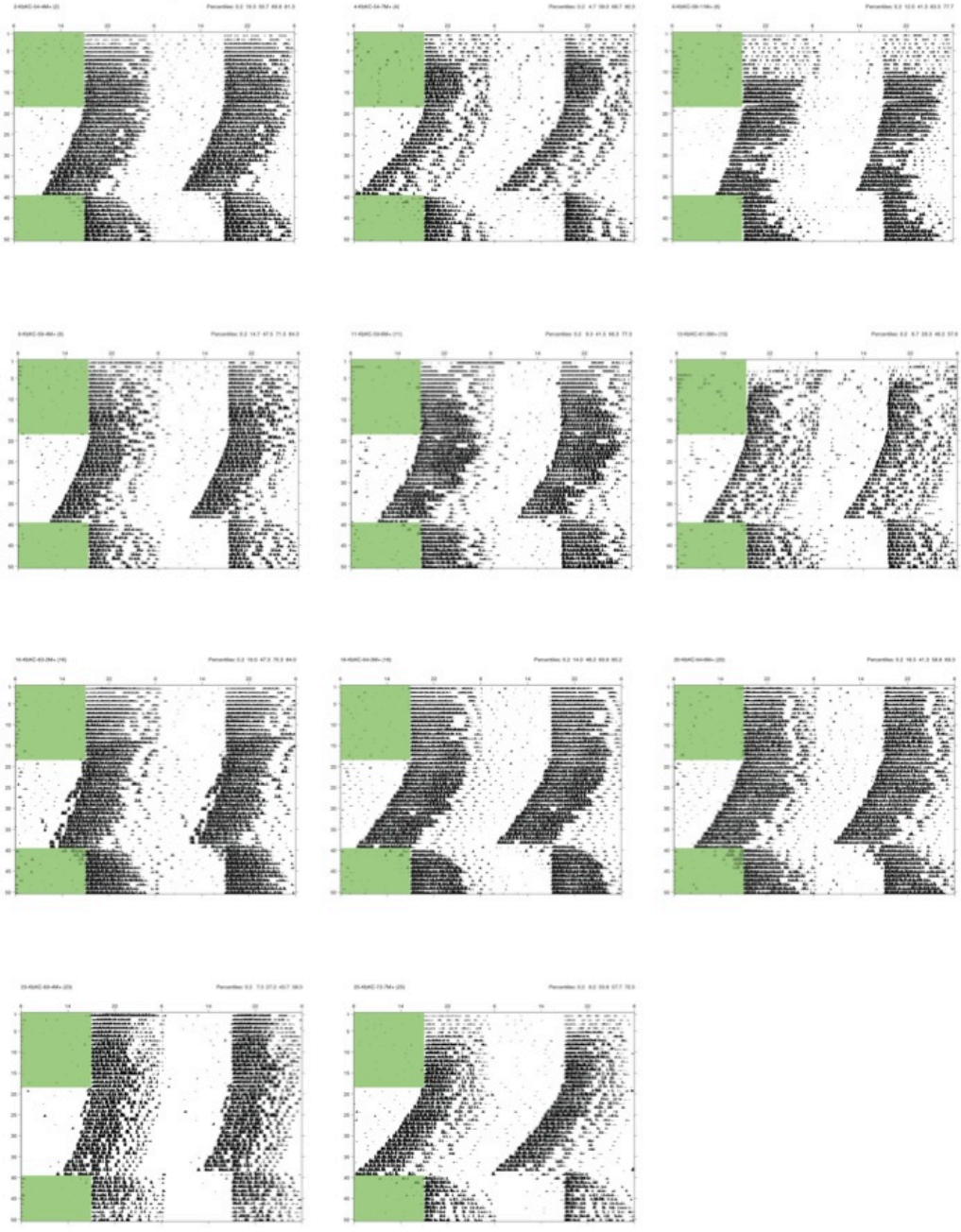


APPENDIX I

fl/fl KLB; CamK-cre neg

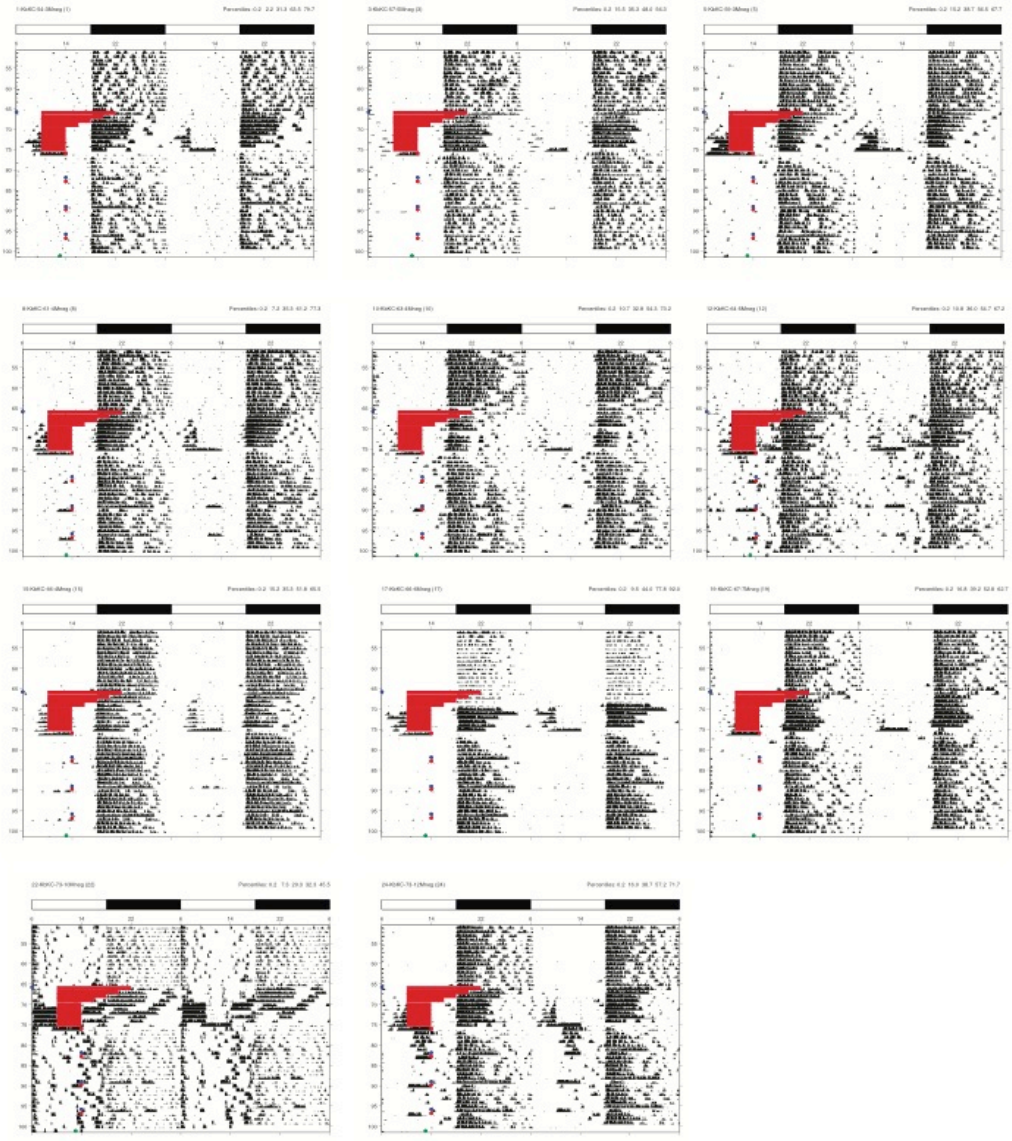


fl/fl KLB; CamK-cre +

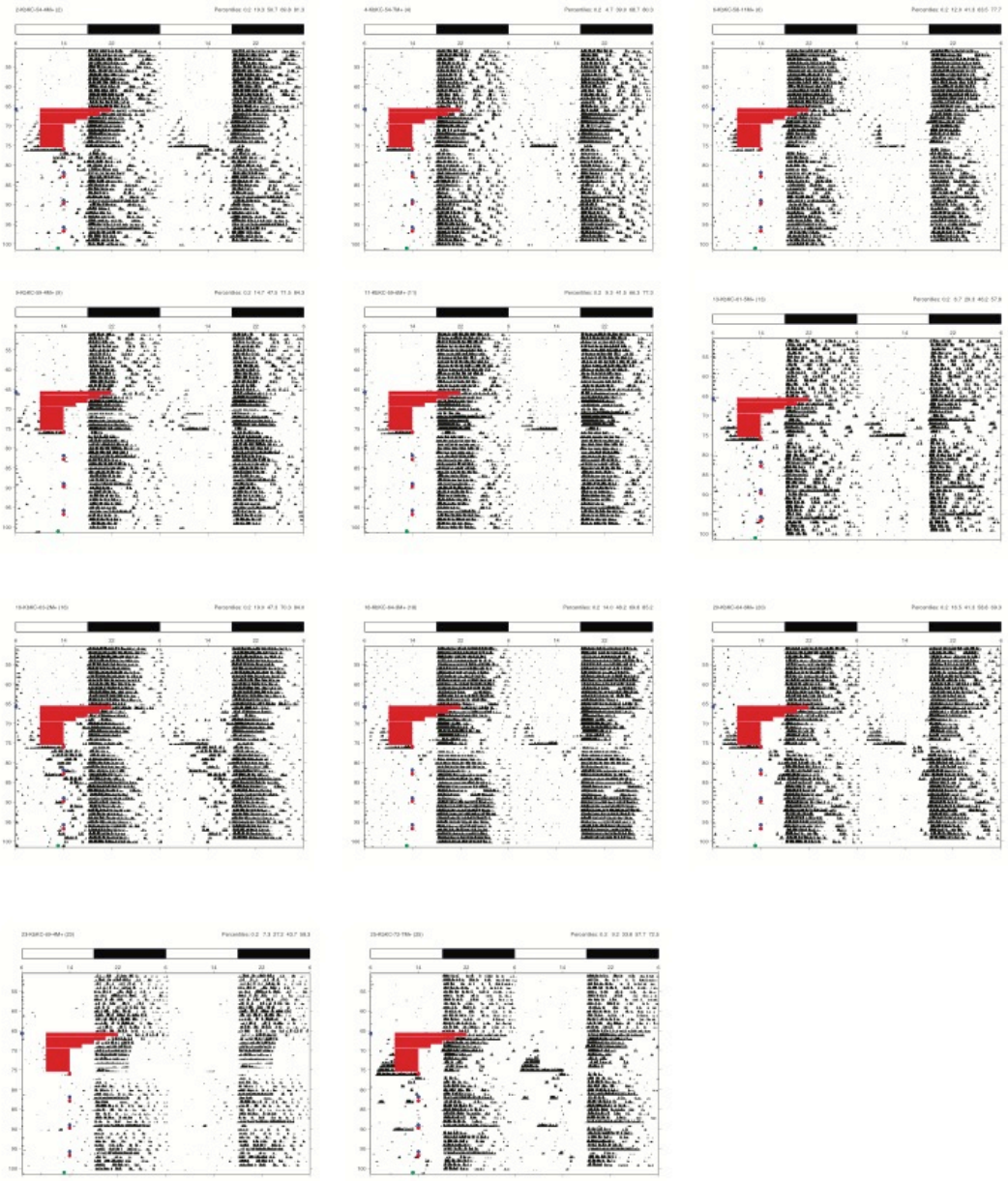


APPENDIX J

fl/fl KLB; CamK-cre neg

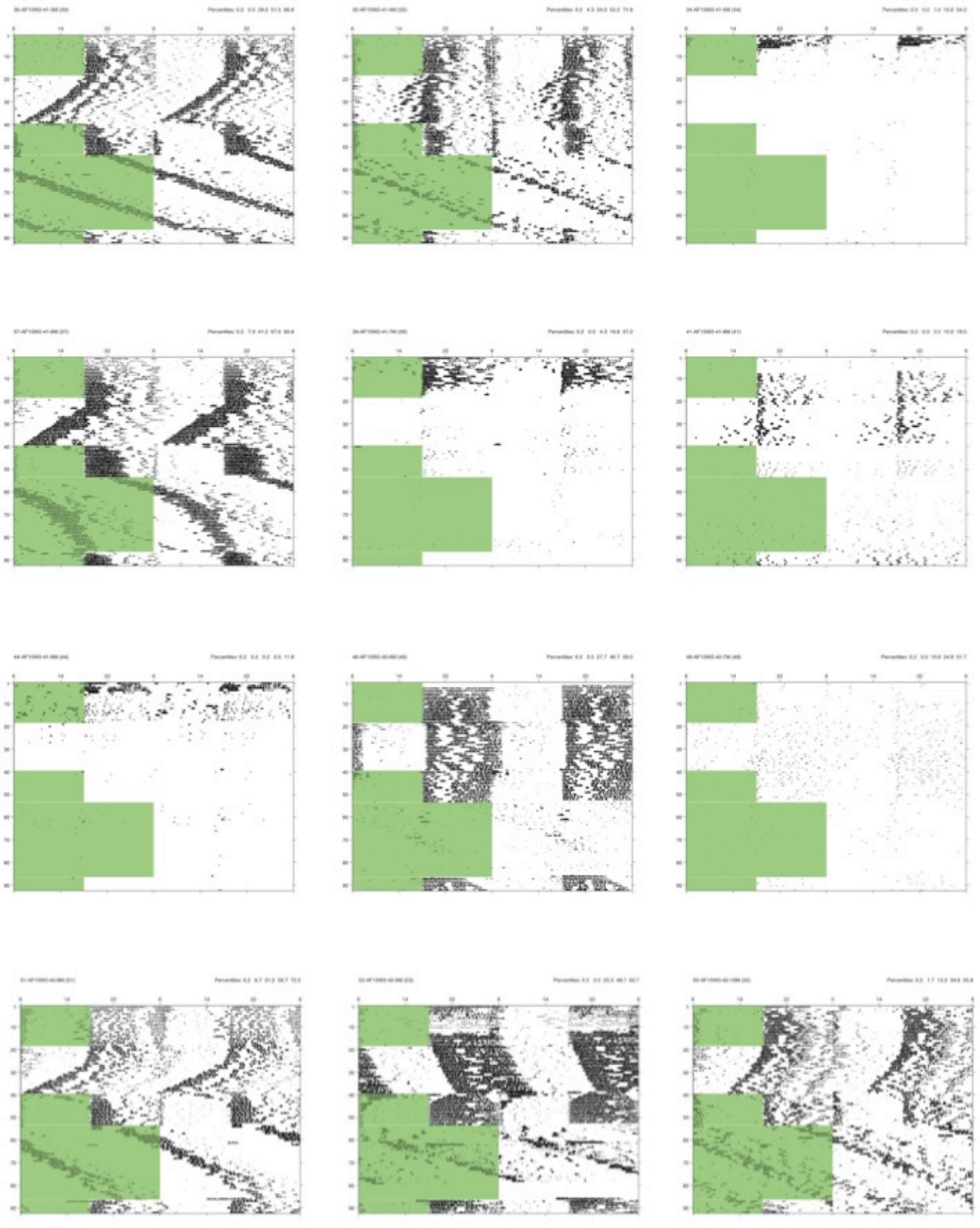


fl/fl KLB; CamK-cre +

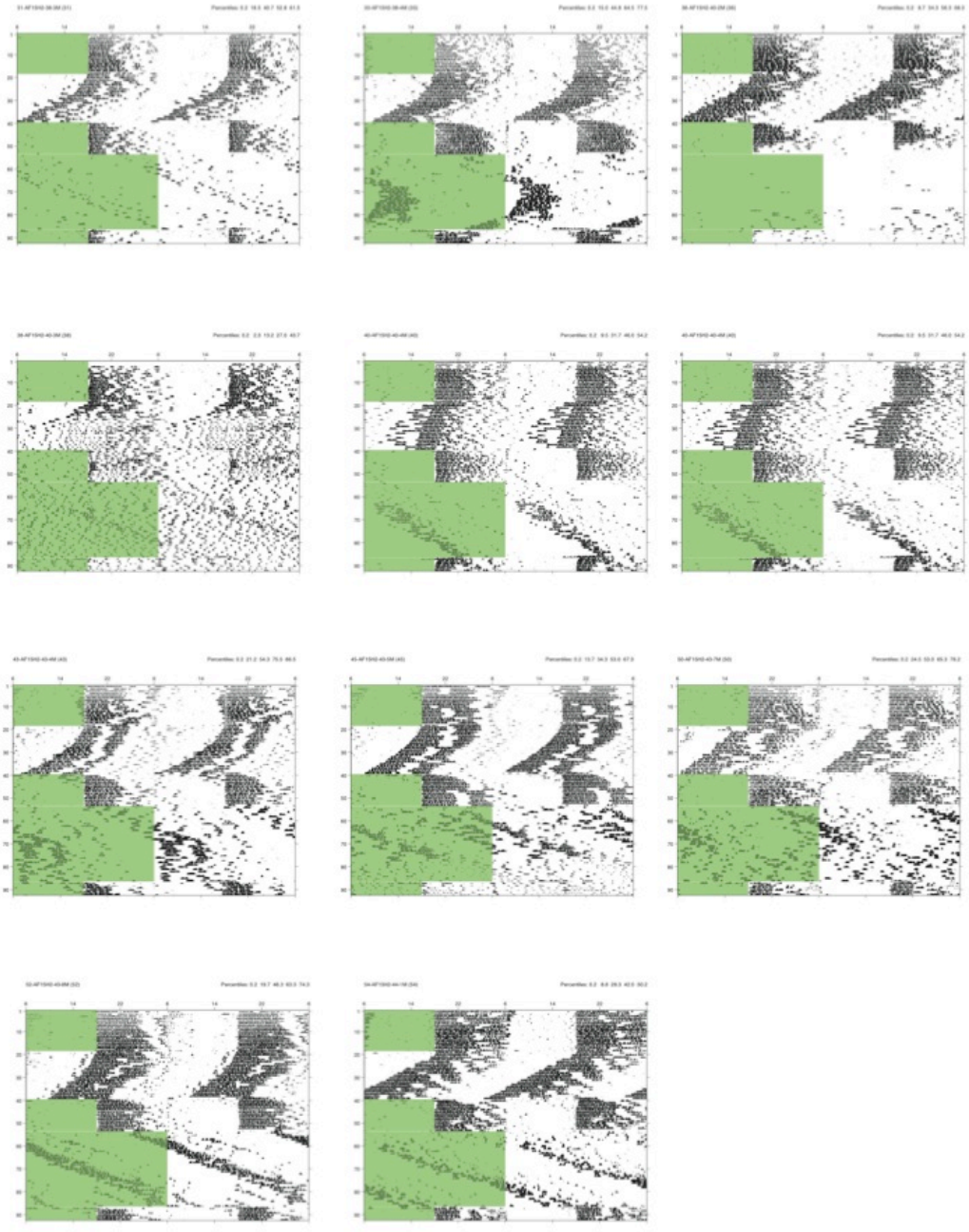


APPENDIX K

FGF15 wt chow

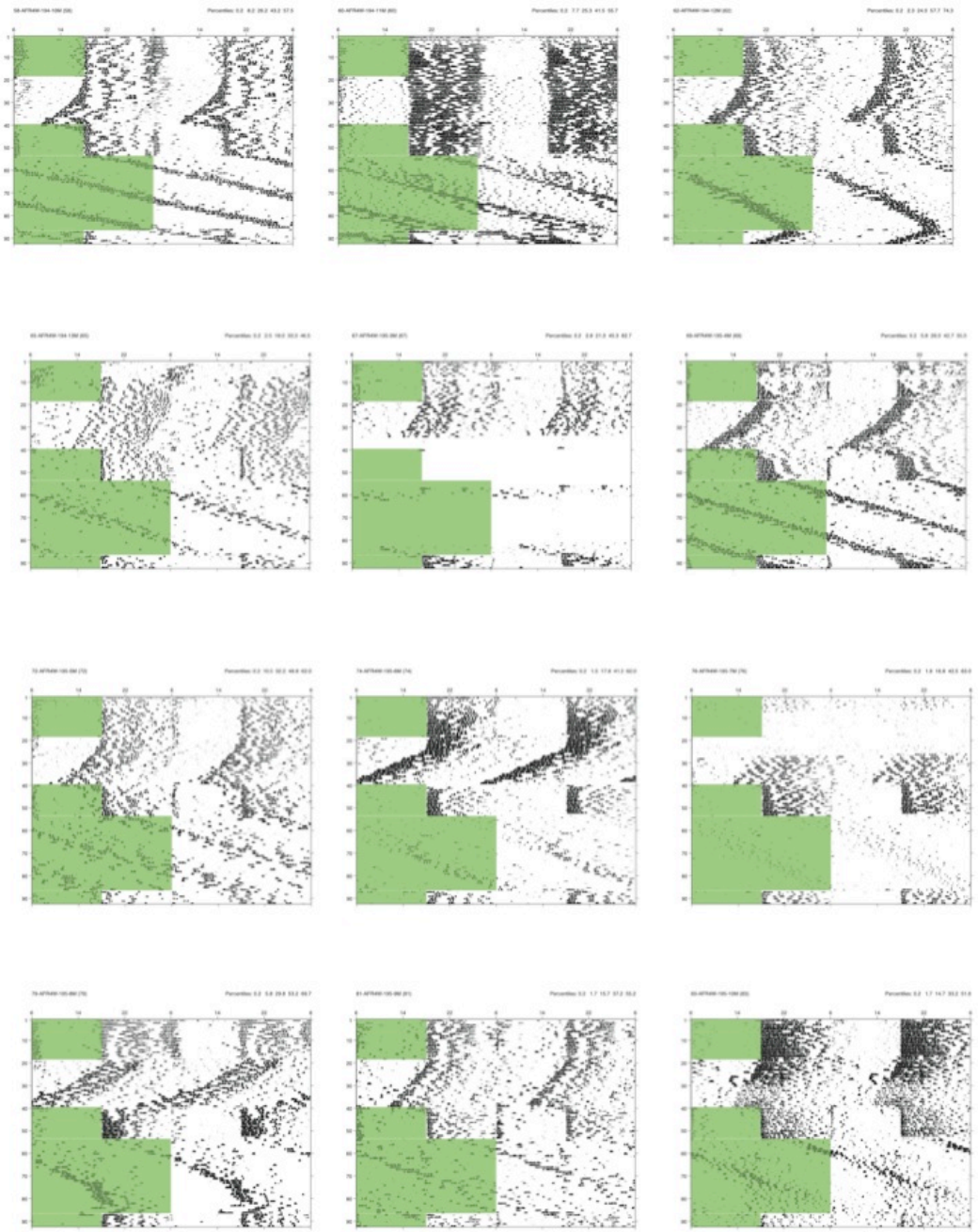


FGF15 ko chow

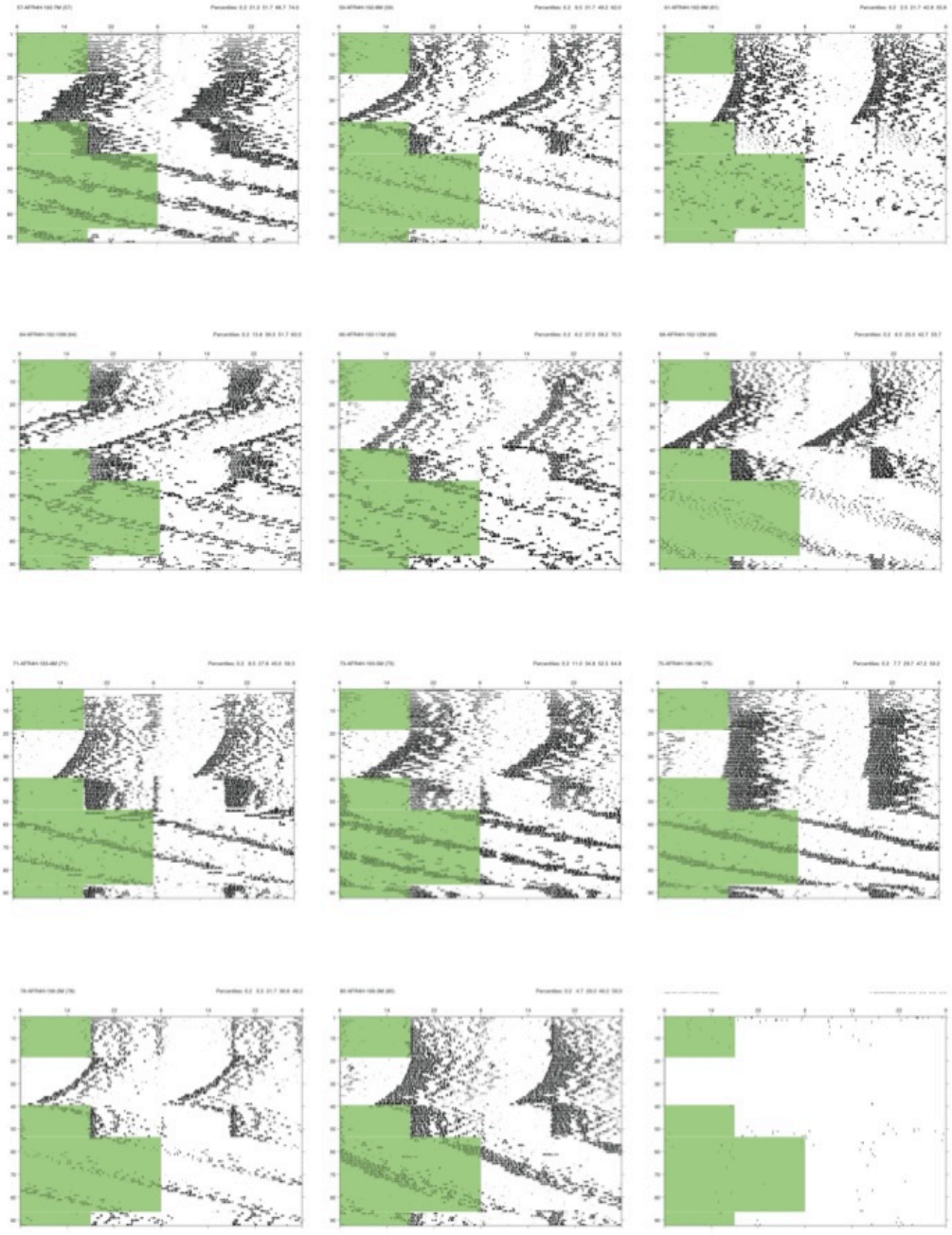


APPENDIX L

FGFR4 wt chow



FGFR4 ko chow



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