UNIVERSITY OF CALIFORNIA SAN DIEGO

The Circadian Transcription Factor, *Bmal1*, is Required for GH Axis Function and Hepatic Gene Expression

A thesis submitted in partial satisfaction of the
requirements for the degree Master of Science

in

Biology

by

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The Thesis of McKenna Ruth Sinkovich is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

Co-chair

Chair

University of California San Diego

2018

Dedication

I'd like to dedicate my thesis to:

Mom, Dad, and Tyler, for their unconditional love and support.

Jessica, Laura, and Austin, for the coffee, the food, and the laughs in lab.

Samantha, Jackie, and Maddy, for the years of friendship.

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LIST OF ABBREVIATIONS

BMAL1	Brain and muscle Arnt-like protein 1
CRY1/2	Cryptochrome 1/2
Cyps	Cytochrome p450s
GH	Growth hormone
GHRH	Growth hormone-releasing hormone
IGF-1	Insulin-like growth factor 1
KO	Knock-out
MUPs	Major Urinary Proteins
qPCR	Quantitative polymerase chain reaction
SCN	Suprachiasmatic nucleus
SST	Somatostatin
VNO	Vomeronasal organ
WT	Wild-type

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ABSTRACT OF THE THESIS

The Circadian Transcription Factor, *Bmal1*, is Required for GH Axis Function and Hepatic Gene Expression

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Master of Science in Biology

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Professor Pamela L. Mellon, Chair

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Circadian rhythms are the endogenous 24-hour cycle that coordinates physiology with time of day. The molecular feedback loop that regulates circadian rhythms also regulates the secretion of many hormones, including growth hormone (GH). GH is secreted in a sexually dimorphic pattern, which drives sexually dimorphic expression of many genes in the liver, including the Major Urinary Protein (MUP) pheromone family in mice, and the drug-metabolizing Cytochrome p450 (Cyp) family. Interestingly, some MUPs and Cyps are expressed in a circadian pattern, indicating that their expression is regulated not only by the GH axis, but also by circadian clock genes. Thus, we sought to establish the role of a core circadian clock gene, *Bmal1*, in regulating the GH axis and

sexually dimorphic hepatic genes. *Bmal1* knockout (KO) male mice have decreased MUP expression and feminized GH pulse patterns. Surprisingly, *Bmal1* KO males have dysregulated, but not feminized, Cyp expression, indicating that feminization of the GH pulses is not solely responsible for the disruption. To determine if *Bmal1* is acting in the liver to regulate hepatic gene expression, we deleted *Bmal1* from hepatocytes with an Albumin Cre; *Bmal1*$^{flox/flox}$ (*Bmal1* liver KO) mouse. The males have decreased MUP expression, and similar Cyp expression to the full-body *Bmal1* KO, indicating that *Bmal1* acts in the liver to regulate MUPs and Cyps. Together, our study indicates that the circadian transcription factor *Bmal1* plays a vital role in regulating GH pulsatility and masculine hepatic gene expression.

INTRODUCTION

Circadian rhythms are the molecular 24-hour cycles that coordinate many physiologic processes to the time of day. The mechanisms involved in the regulation of these rhythms play a role in the regulation of the many physiological processes, such as metabolism, stress, and fertility, and notably influence the synthesis and release of hormones. Circadian rhythms have been shown to impact the function of hormonal axes, including the reproductive axis and the stress axis, though the full extent and mechanisms of circadian regulation of different axes is currently unknown. However, the effects of disrupted circadian rhythms on health and disease are well studied and can result in pathologies such as cancer, cardiac disease, and metabolic syndrome. The growth hormone axis, responsible for the proliferation of tissues during development and maintenance of growth hormone secretion in adulthood, is one such axis regulated by circadian rhythm proteins. The GH axis has profound effects on many tissue types, such as bone and fat tissue, although its effects on the liver are especially potent. In the liver, GH regulates the expression of a class of sexually dimorphic genes important in drug metabolism called the Cytochrome p450s, and a class of sexually dimorphic pheromones in mice involved in behavioral regulation called the Major Urinary Proteins (MUPs). Due to the wide effects of GH, the dysregulation of the axis leads to a variety of consequences, including obesity and deterioration of bone, and an alteration in the expression of Cytochrome p450 drug-metabolizing enzymes. Many Cytochrome p450 genes are also expressed in a circadian pattern, indicating that disruptions in circadian rhythms could alter their expression at multiple levels. As increasing numbers of the human population are exposed to disrupted circadian rhythms through shift work and

artificial lighting, understanding the effects of dysregulated circadian rhythms on the body, and in the liver, is becoming more important.

Circadian rhythms and the Bmal1 KO mouse model

Circadian rhythms are regulated by a transcriptional feedback loop comprised of the four core clock proteins: BMAL, CLOCK, PERIOD, and CRYPTOCHROME. Light input from the optic nerve synchronizes the suprachiasmatic nucleus (SCN), the central clock in the hypothalamus, to the day-night cycle. The SCN then synchronizes the molecular clock in tissues throughout the whole body (**Figure 1**). The four core proteins act as transcription factors by binding to enhancer regions (E-boxes) on DNA to modulate the expression of circadian genes.

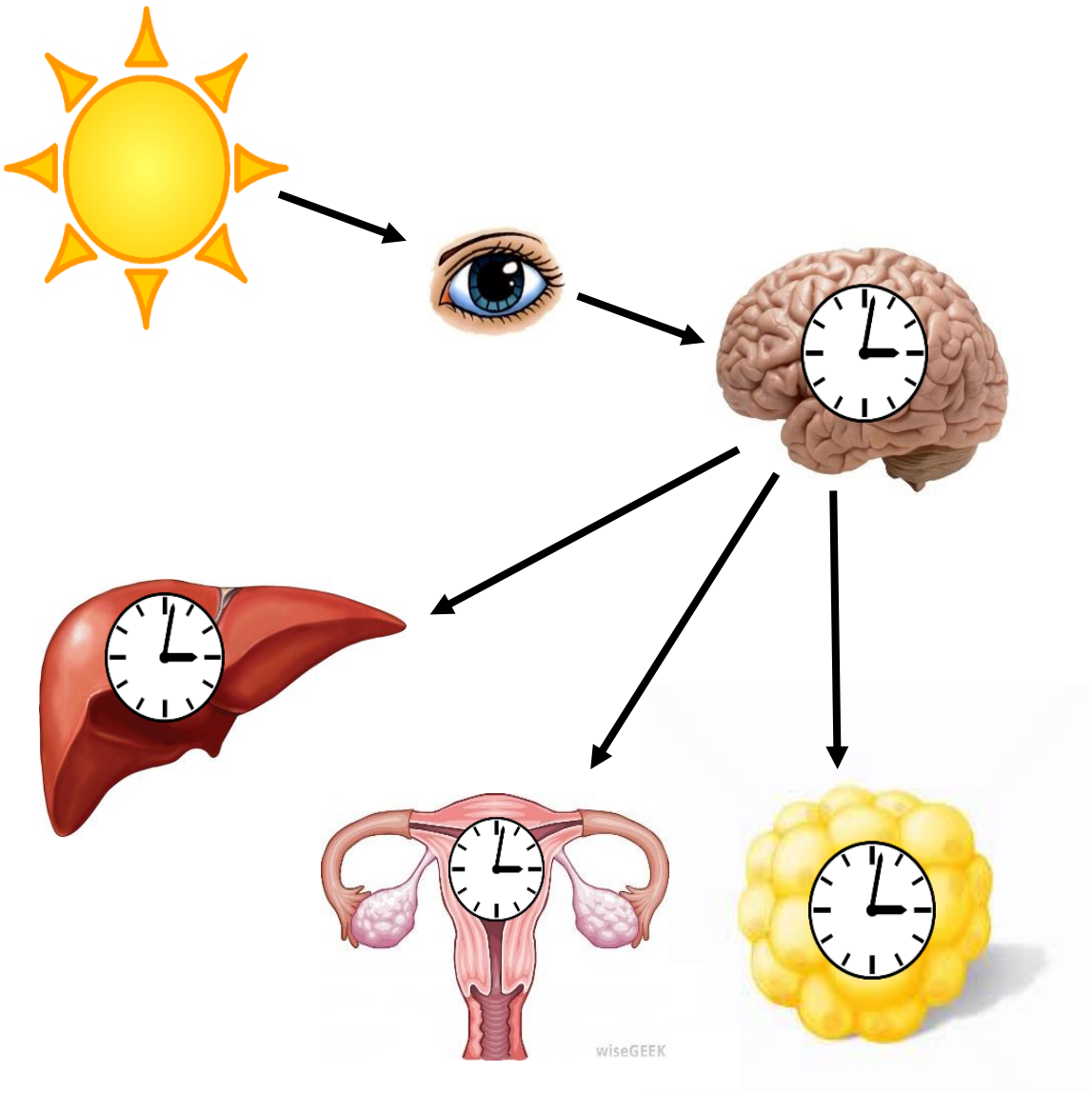

Figure 1. The SCN coordinates circadian rhythms to time of day. Light input enters through the eyes, and is integrated in the hypothalamus. The molecular feedback loop in the SCN, considered the Master Clock, is coordinated with the light cues. Circadian molecules clocks in other tissues are then synchronized to the loop in the SCN.

Disruptions in circadian rhythms have been implicated in endocrine and metabolic disorders, highlighting the regulatory effects of circadian rhythms in multiple systems. Dysregulations in circadian rhythms are associated with subfertility and metabolic disorders, such as obesity and alterations in glucose homeostasis [1]. As anywhere between 17-60% of the US population are subject to disruptions in circadian rhythms due to shift work, understanding the underlying mechanisms of circadian regulation of gene expression in various tissues will have a huge impact on human health [2, 3].

Mouse models with genetic disruptions or deletions of circadian clock genes are useful for understanding the role of circadian rhythms in disease. The *Bmal1* KO mouse exhibits several physiological defects, including increased risk for cancer, heart defects, diabetes, and infertility [4-6]. Similar disease states are observed in human populations subject to circadian disturbances, who exhibit an increased risk for breast cancer [7] and increased risk for metabolic syndrome [1].

Circadian rhythms mediate some of their downstream effects by regulating the secretion of hormones. Growth hormone is one hormone that has been linked to circadian regulation, though the extent and the mechanism remain to be determined. Growth hormone is responsible for the proliferation of many tissue types during development, including bone, adipose tissue, and cardiac tissue, and the maintenance of those tissues in adulthood.

The GH axis

GH in adulthood plays a role in maintaining homeostasis of many tissue types. Adults with GH deficiency show increased body fat [8], decreased bone density [9], and

alterations in cardiac function [10]. Growth hormone is secreted in a pulsatile manner from the pituitary gland and the pattern of growth hormone release is maintained by the growth hormone (GH) axis (**Figure 2**). Growth-hormone releasing hormone (GHRH) neurons in the hypothalamus release GHRH into the pituitary portal system. Once in the anterior pituitary, GHRH binds to its receptors on the somatotrope cells to induce the release of GH to the body. The GH from the pituitary binds to growth hormone receptors in many tissues, including the liver. GH receptor activation in the liver drives the expression of many hepatic genes, including Major Urinary Proteins (MUPs), a class of pheromones in mice, and the cytochrome p450s (Cyps), which regulate lipid and steroid metabolism. GH also induces the expression of insulin-like growth factor-1 (IGF-1) in the liver [11]. IGF-1 is released into the bloodstream, then acts upon various tissues, including bone, cardiac muscle, skeletal muscle, and adipose tissue to regulate cell growth and turnover.

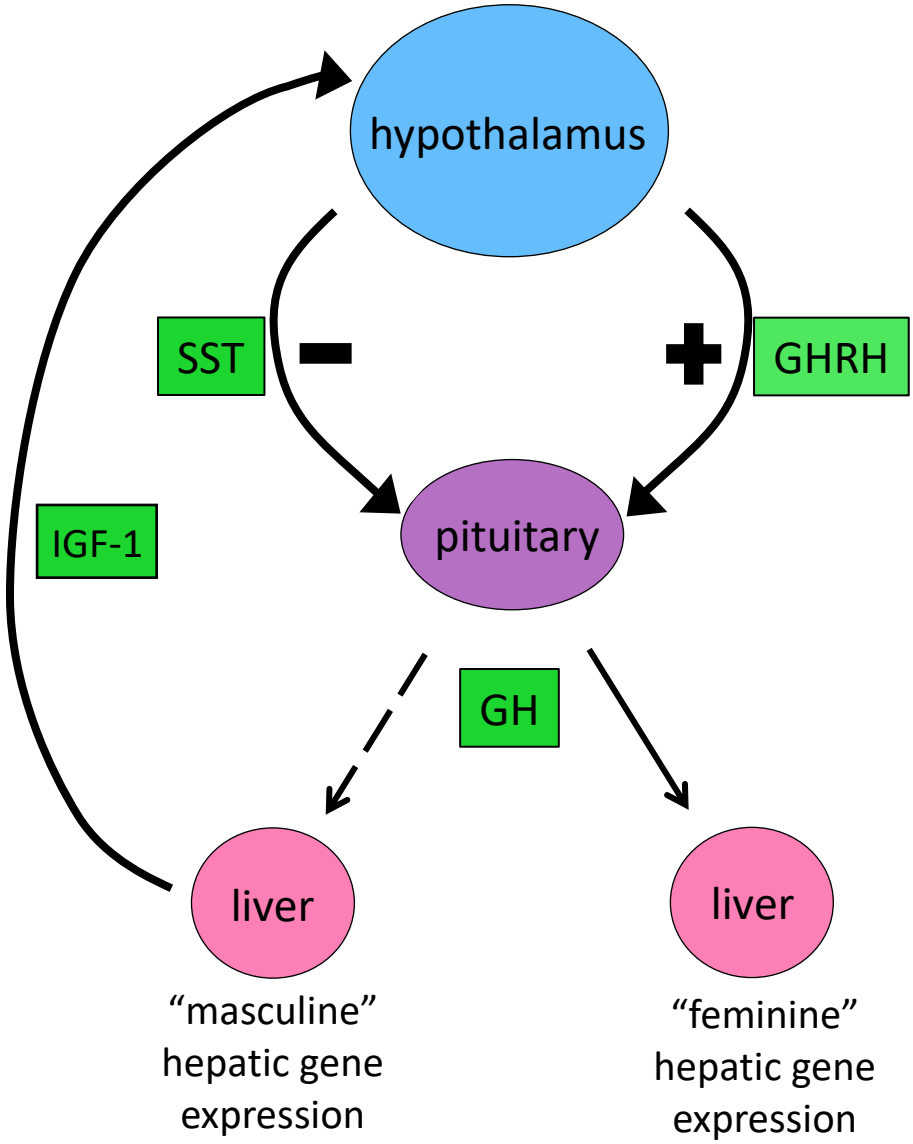

Figure 2. The mammalian growth hormone (GH) axis. The hypothalamus releases growth hormone-releasing hormone (GHRH) to the anterior pituitary via the portal system. The pituitary then releases GH to the body. GH binds to receptors in the liver to activate hepatic gene expression. IGF-1 is produced in response to GH, and acts to increase somatostatin (SST) expression in the hypothalamus.

Up to 90% of sexual dimorphism in the liver is due to the differential pattern of growth hormone (GH) release from the pituitary [12]. Male mice release GH in high-amplitude pulses, with an "off-period", or trough, of about 90 minutes between peaks. Female mice release GH more continuously; female GH pulses are more frequent and have higher baseline serum concentrations [13, 14]. The sexually dimorphic pattern of GH release is, in part, responsible for the different hepatic gene expression profiles between the sexes (**Figure 3**, adapted from [15]). Pulsatile GH release in males drives male-like hepatic gene expression through the "off-period" of low serum GH concentrations, which allows GH receptors in the liver to re-sensitize between GH pulses [16]. In male rodents, the pulsatile GH drives the expression of the Major Urinary Proteins (MUPs), which are highly expressed in males. In females, the lack of an "off-period" causes GH receptors to become de-sensitized to GH stimulation, therefore GH binding to the GH receptor does not activate gene expression of the male-specific hepatic genes. The different GH patterns between the sexes cause differential gene expression in many sexually dimorphic gene families, including the cytochrome p450s and MUPs.

The mechanism of this sexual dimorphism is not fully elucidated, but there are notable sexual dimorphisms in the hypothalamus and pituitary, which may contribute to the differential pulse patterns of GH between males and females. Both the stimulatory peptide (GHRH) and the inhibitory peptide somatostatin (SST), are expressed at higher levels in the male versus the female rat hypothalamus [17, 18]. The elevated levels of GHRH drive the high-amplitude GH pulses seen in males [19], whereas the elevated SST drives the trough [20]. At the level of the pituitary, males have an increased number of somatotrope (GH-expressing) cells, and these cells respond differently to GHRH [21-23].

IGF-1 is thought to be partially responsible for the negative feedback that regulates GH secretion. There is evidence that IGF-1 decreases GHRH mRNA and increases SST mRNA in the hypothalamus [24, 25], and IGF-1 deficiencies are correlated with high circulating levels of GH [26]; however, the mechanism of action is still an active area of study.

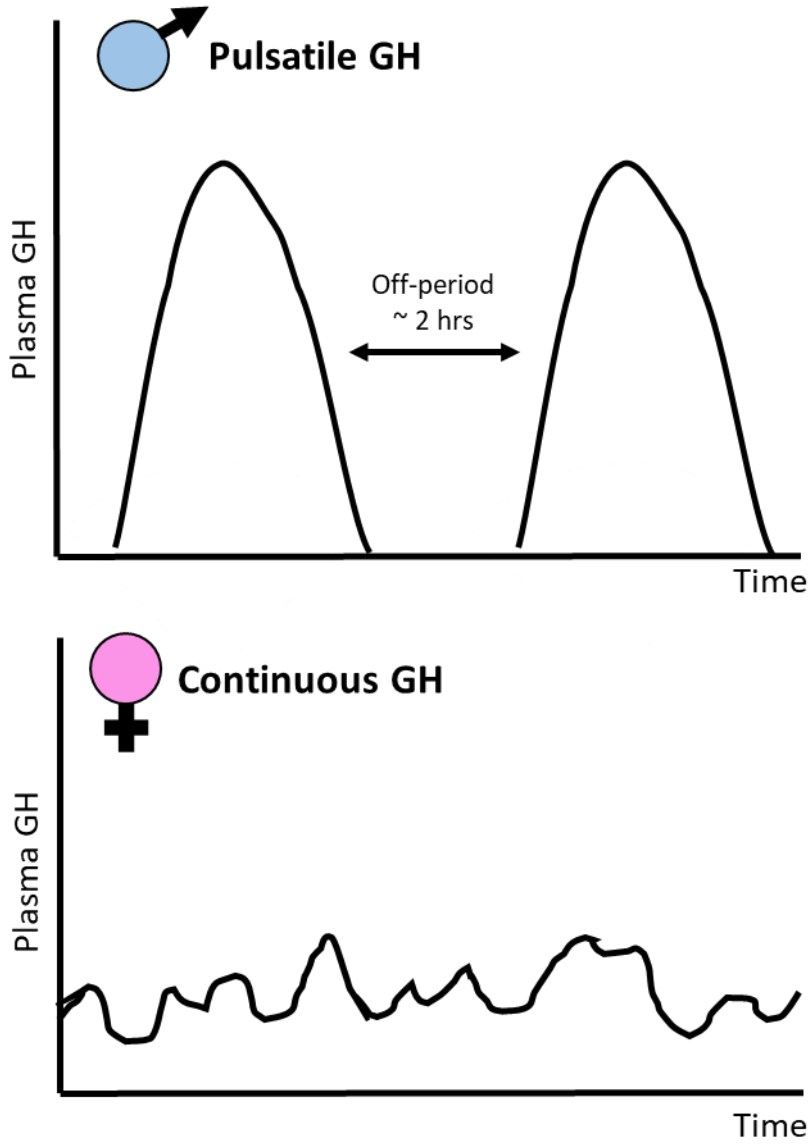

Figure 3. GH release is sexually dimorphic. In males, GH is released in high-amplitude pulses, separated by off-periods driven by SST. In females, SST is produced at much lower levels, thus GH release is more continuous.

Cytochrome p450s

The cytochrome p450 genes (Cyps) are transcribed in the liver and play a critical role in metabolizing steroids and toxicants. Absence of Cyps, or abnormalities in their expression, leads to altered rates of drug metabolism [27]. Several of the Cyp genes are expressed in a sexually dimorphic fashion, resulting in different rates of drug metabolism between sexes. This sex-specific expression is largely due to differential growth hormone (GH) release patterns between males and females [28]. However, until recently, the role of circadian rhythms in the maintenance of sex-specific hepatic gene expression was not evaluated in depth.

Many Cyps are also expressed in a circadian pattern, causing the metabolism of some compounds to differ depending on time of day [29, 30]. Some Cyps have been shown to interact with circadian proteins, implicating their regulation by the circadian genes [31]. Thus, it is possible that the clock genes regulate the expression of Cyps at the level of the GH axis as well as at the level of the liver itself.

Major Urinary Proteins (MUPs)

MUPs, a class of pheromones in mice, are a sexually dimorphic gene family regulated by GH pulse patterns, and are often used as a marker of liver sexual dimorphism [16, 32]. Pheromones are chemical cues used as a form of communication in many animal species. Rodents, like most other mammals, have two olfactory systems, including the main olfactory epithelium (MOE) and the vomeronasal organ (VNO). The MOE projects to the olfactory bulb in the brain, and is primarily responsible for detecting volatile odorants [33]. The VNO projects to the accessory olfactory bulb, and is

predominantly responsible for detecting pheromones in the environment [33]. Pheromone cues are then processed in the hypothalamus and can result in specific innate behavioral responses, such as mating, aggression, and fear [33].

Both reproductive and aggressive behaviors in mice are regulated in large part by the Major Urinary Protein (MUP) pheromone family [34]. These large molecular weight proteins are transcribed in the liver and excreted in the urine. MUPs are sexually dimorphic, and are expressed at very high levels in males, but at low levels in females. It is not simply the degree of total MUP production, however, that cues behavior in mice. There are at least 21 identified MUPs, most of which have highly similar sequence homology [35]. The different quantities and combinations of MUPs in urine send different signals to recipient mice [34, 36]. For example, MUP3 and MUP20 are both implicated in male-male aggression, yet only MUP20 plays a role in female attraction to males [34, 37].

MUP levels are often used as a non-invasive marker of hepatic masculinization, which occurs as a result of differential growth hormone signaling [16, 32]. The male-like GH pulse pattern drives the expression of MUPs, whereas the continuous GH pattern in females results in lower MUP expression. Certain MUPs, such as MUP2, are expressed in a circadian pattern, implicating the molecular clock as a regulatory mechanism of MUP gene expression [38]. Thus, the expression of MUPs in the liver is driven by both masculine GH pulses and by circadian genes; however, the interaction between the GH axis and circadian rhythms on hepatic gene expression has not yet been determined.

Circadian regulation of the GH axis

There is evidence that the SCN is important for regulating the GH axis, and therefore the molecular clock may influence sex-specific gene expression in the liver by modulating GH release. Lesions of the SCN in male rats results in female-like hepatic gene expression, including Cyps [39], indicating a central role for circadian rhythms in regulating gene expression. In fact, the GHRH neurons of the hypothalamus show a circadian pattern of *Ghrh* mRNA expression [40]. Somatostatin is also released in a circadian pattern [41], indicating that the clock genes play a role in regulating the GH axis, at least at the level of the hypothalamus. Although the role of *Bmal1* in somatotrope cells within the pituitary has not been established, it is an important regulator of the gonadotrope cells, another pituitary cell-type, indicating a role for the core clock genes in pituitary function [42]. *Cry1/Cry2* double knock-out mice, which lack molecular clock function throughout the whole body, also lack sex-specific Cyp and MUP expression. Interestingly, when these mice were injected with GH in a male-like pattern, hepatic expression of several genes was rescued to male-like levels, indicating that female-like GH expression in the male mice was the underlying cause of the abnormality [43]. Together, these data indicate a role for the molecular clock in regulating the GH axis, and therefore the sexually dimorphic gene expression in the liver.

Circadian Regulation of MUP and Cyp Gene Expression

Many hepatic genes are expressed in a circadian pattern, thus suggesting that *Bmal1* and the other circadian transcription factors may play a role in gene regulation at the level of the liver. For example, *Mup2*, one of the male-specific MUP proteins [35], is

regulated by both the CLOCK/BMAL1 heterodimer binding to two E-boxes and the glucocorticoid receptor binding to a glucocorticoid response element within the promoter region of the *Mup2* gene [38]. Many of the sexually dimorphic Cyp genes are also expressed in a circadian pattern. *Cyp2b10*, a female-predominant gene, has higher expression around midnight, whereas *Cyp4a10* and *Cyp4a14*, also female-predominant, show highest expression in the evening and early night. *Cyp7b1*, a male-predominant gene involved in the synthesis of many steroids, also shows a circadian expression pattern, with highest expression during the day [44]. However, the precise mechanisms through which circadian rhythms regulate hepatic gene expression is still being investigated.

Hypothesis

Together, these data indicate that sex-specific hepatic gene expression is regulated by multiple factors, including growth hormone expression patterns and circadian clock genes. We hypothesize that the circadian gene, *Bmal1*, influences the expression of sexually dimorphic hepatic gene expression through multiple cooperative mechanisms, by modulating growth hormone secretion patterns and by transcriptional regulation in the liver.

MATERIALS AND METHODS

Mice

Bmal1 floxed mice were obtained from Jackson Laboratories. Full-body *Bmal1* KO mice were created by crossing a *Bmal1*$^{flox/flox}$ mouse with a ZP3-Cre mouse [45], resulting in a germline deletion of Exon 8 of the *Bmal1* gene, producing a non-functional version of the BMAL1 protein. *Bmal1* KO males were obtained through heterozygous matings, and WT littermates were used as controls for all experiments. Males at 8-16 weeks of age were used for all experiments, and were group housed unless otherwise stated. Mice were housed in 12-hour light and 12-hour dark conditions, with ad libitum access to food and water. All procedures were approved by the University of California, San Diego, Animal Care and Use Committee.

Bmal1$^{flox/flox}$ mice were crossed with Albumin-Cre mice obtained from the Jackson Laboratories to create a *Bmal1* liver-specific KO. This results in a deletion of Exon 8 of the *Bmal1* gene in hepatocytes, producing a non-functional BMAL1 protein in the liver. *Bmal1* liver KO males were obtained through crossing an Albumin-Cre +; *Bmal1*$^{flox/flox}$ female with a *Bmal1*$^{flox/flox}$ male. Cre– littermates were used as controls for all experiments. Males at 8-16 weeks of age were used for all experiments, and were group housed. Mice were housed in 12-hour light and 12-hour dark, with ad libitum access to food and water. All procedures were approved by the University of California, San Diego, Animal Care and Use Committee.

Gonadectomy and testosterone replacement

Male mice at 8-12 weeks of age were anesthetized with isoflurane, and bilaterally gonadectomized (Gdx). A testosterone pellet from SILASTIC brand (Dow Corning Corp; internal diameter 1.02 mm; external diameter 2.16 mm) capsule-packed testosterone (Sigma; T1500; 6 mm) was implanted sc. Implants have been shown to produce elevated physiological levels of testosterone (11.1 +/- 0.8 ng/mL) [46]. Mice were allowed to recover for 2 weeks before experiments, as this time frame of T replacement is known to restore mating behaviors in gonadectomized males [47].

Urine analysis

Urine was collected from male mice at 4-hour intervals throughout a 24-hour period. Samples taken at night were obtained under red light settings. Urine was stored at -20˚C until analysis. Samples were prepared as 3 parts 2x Laemmeli buffer and 1 part urine, then heated for 5 minutes at 95˚C. 4 µl of sample was run through a 12% SDS-PAGE gel. Gels were washed 3x with MilliQ water, and then incubated at RT overnight with Thermofisher PAGE Blue Dye (catalog # 24620). Protein content was quantified using ImageJ software. Samples were normalized to the WT male at ZT0.

Behavioral assays

Male mice were singly housed to 2 weeks prior to experimentation. A resident-intruder paradigm was used, which allows for the determination of the test mouse (the intruder) to invoke an aggressive response from the resident. Each trial was recorded, and the seconds of aggression were counted over the 15 minutes of total assay time.

Aggressive behaviors included biting, scratching, and wrestling. Trials were ended early if the total time of aggression exceeded 2 minutes and 30 seconds, or if one of the mice was bleeding excessively.

Hormone assay

For growth hormone measurement, male mice were handled daily for at least 28 days prior to experiment. On the day of the experiment, mouse cages were moved to hood early in the morning, and the test began at 10:00 am. Every ten minutes for 6 hours, 4 μl of tail blood was collected in a capillary tube. Blood was allowed to clot for 20 minutes at room temperature, then spun at 2000 *x g* for 15 minutes to separate serum. 1.5 μl serum was added to 23.5 μl matrix buffer from Milliplex MAP mouse pituitary magnetic bead panel (MPTMAG-49k). Serum was kept at -20˚C until the assay was run. Hormone levels were measured using the Luminex Magpix. Pulses were determined using MatLab Peakfinder, and interpulse intervals were measured using MatLab software.

For testosterone assay, 150 μl of tail blood was collected in a capillary tube. Blood was allowed to clot for 90 minutes at room temperature, then spun at 2000 *x g* to separate serum. Serum was kept at -80˚C until assay. Testosterone was measured by the University of Virginia Hormone and Ligand Core (RRID:SCR_004318).

Quantitative RT-PCR

Quantitative RT-PCR was performed on Bio-Rad CFX Connect (Bio-Rad Laboratories). Tissues were removed from animals after euthanasia, and kept at -80˚C

until assay. Tissue was homogenized in 1 mL Trizol (Invitrogen) using a glass tissue grinder. RNA was extracted with 200 μL of chloroform and precipitated with 500 μL of isopropanol and 1.5 μL of linear acrylamide (Ambion) as a coprecipitate. The pellet was then resuspended in ribonuclease- and deoxyribonuclease-free water, and genomic DNA was removed with Turbo DNA-free (Ambion) following the manufacturer's instructions. One microgram of total mRNA was converted to cDNA using iScript cDNA synthesis kit (Bio-Rad Laboratories). 20 ng of cDNA was loaded in each qPCR reaction. All primers were tested for specificity and efficiency (**Table 1**). Data were collected from threshold values from the Bio-Rad CFX Manager 3.0 software (Bio-Rad Laboratories).

Table 1. Primer list. All primers were obtained through Integrated DNA Technologies. Primers were diluted and tested for specificity and efficiency.

Gene	Forward Primer (5-')	Reverse Primer (3-')
Actin	ACCTTCTACAATGAGCTGCG	CTGGATGGCTACGTACATGG
Bmal1	ACTGGAAGTAACTTTATCAAACTG CTGACCAACTTGCTAACAATTA	CTCCTAACTTGGTTTTTGTCTGT
Cre	GCATTACCGGTCGTAGCAACGAGTG	GAACGCTAGAGCCTGTTTTGCACGTTC
Cyp2a4	TGTGCTGAAAGACCCCAAG	TCCTTCTCCGAAACAATACCG
Cyp2b9	TTTTCTGCCCTTCTCCACAG	TCTTTGGGAGTGAGGTCAATG
Cyp4a12a	GTTCCTACAGATTTCTAGCTCCC	AGAGTCTGCCATGATTTCCG
Cyp7b1	CCTCTTTCCTCCACTCATACAC	GTCCAAAAGGCATAACGTAAGTC
Elovl3	ATGCAACCCTATGACTTCGAG	ACGATGAGCAACAGATAGACG
Fmo3	CACCATCCCCATAACTGACC	CCATACCATTTGAACTTTTCCCC

VNO neuron activity

Transient increases in free Ca^{2+} concentration in dissociated VNO neurons were determined by ratiometric Fura-2 fluorescence [36, 48]. WT male mice were killed, and VNO tissue was collected and dissociated and loaded with Fura-2 to detect increases in CA^{2+}, indicative of neuron firing. Whole urine was separated into high and low molecular

weight fractions using Amicon Ultra centrifugal filters (Millipore). VNO neurons were perfused with freshly collected WT or *Bmal1* KO male low molecular weight urine followed by the high molecular weight fraction of the respective urine.

IGF-1 assay

Protocol from Chaudhari et al. was followed [49]. Briefly, serum IGF-1 levels were measured via RayBio Mouse IGF-1 ELISA 96-Well Plate Kit. 10 µl of whole blood was collected every 4 hours for a 24-hour period starting at ZT0 and stored at -80˚C until use. 2 µl of serum was diluted 50-fold in buffer provided in RayBio kit. The sandwich ELISA was performed with two antibodies – the secondary antibody was linked to biotin. Horseradish peroxidase and TMB substrate from kit were used for colorimetric detection, and optical density was measured at 450 nm. A standard curve was created with manufacturer-provided mouse IGF-1. All samples and standards were run in duplicate.

Statistical Analysis

Statistical analyses were performed using either Unpaired t-test, One-Way ANOVA or Two-Way ANOVA, followed by *post hoc* analysis by Tukey, Sidak, or Bonferroni as indicated in figure legends, with $p < 0.05$ to indicate significance.

RESULTS

CHAPTER I: *Bmal1* is required for GH axis function and normal hepatic gene expression

As circadian rhythms have been implicated in other hypothalamus-pituitary axes [42, 50, 51], we hypothesized that the core circadian gene, *Bmal1*, would play a role in the regulation of the GH axis. Previous studies have shown that other core circadian genes, the *Cry1/Cry2* complex, are required for proper GH signaling in males [43]. Thus, we hypothesized that *Bmal1* is involved in the regulation of the GH axis, and, by extension, the regulation of hepatic gene expression.

Bmal1 KO males do not elicit aggressive responses from WT males

Previous studies have shown that *Bmal1* knockout (KO) male mice do not respond to pheromone cues in their environment, resulting in a loss of reproductive, fear, and aggressive behavior [42]. An intruder assay was performed to determine whether *Bmal1* KO male mice would elicit an aggressive response from a WT male. We tested this by introducing either a wild-type (WT) male or a *Bmal1* KO male intruder into the cage of a WT male resident. When the intruder was a WT male, the WT resident demonstrated aggressive behavior towards the intruder, while a KO intruder elicited fewer aggressive responses from the WT resident. The assay was quantified by counting the number of seconds of aggression per the 15 minutes of the assay, and we found that the WT resident showed significantly more aggression towards the WT intruder than the *Bmal1* KO intruder (53.4 ± 19.22 vs 4.33 ± 4.33, p=0.0411) (**Figure 4**).

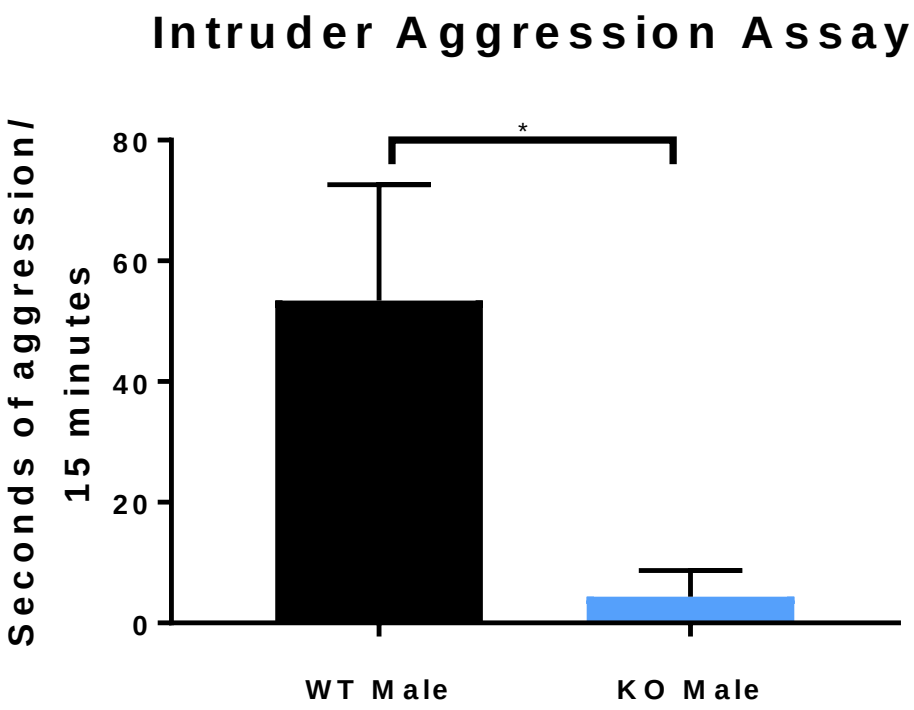

Figure 4. *Bmal1* KO males do not elicit aggression. A WT male resident mouse was introduced to either a WT male intruder (n=5) or a *Bmal1* KO male intruder (n=6). Seconds of aggression (biting, scratching, wrestling) were counted over the 15 minute assay. Statistical analysis by Mann-Whitney test, *$p<0.05$.

There is some evidence that testosterone levels in *Bmal1* KO males are lower than WT males [50], and because testosterone is a known regulator of aggression in mice [52], we measured levels of serum testosterone in WT and KO males. Testosterone is released in a pulsatile fashion, making it difficult to interpret testosterone levels with a singular measurement. However, due to assay sensitivities, measuring pulsatile testosterone through serial sampling is technically challenging. We measured serum levels of testosterone, and we found no significant difference between the WT male and *Bmal1* KO male testosterone levels (332.9 ± 94.62 vs 332.8 ± 113.4) (**Figure 5**).

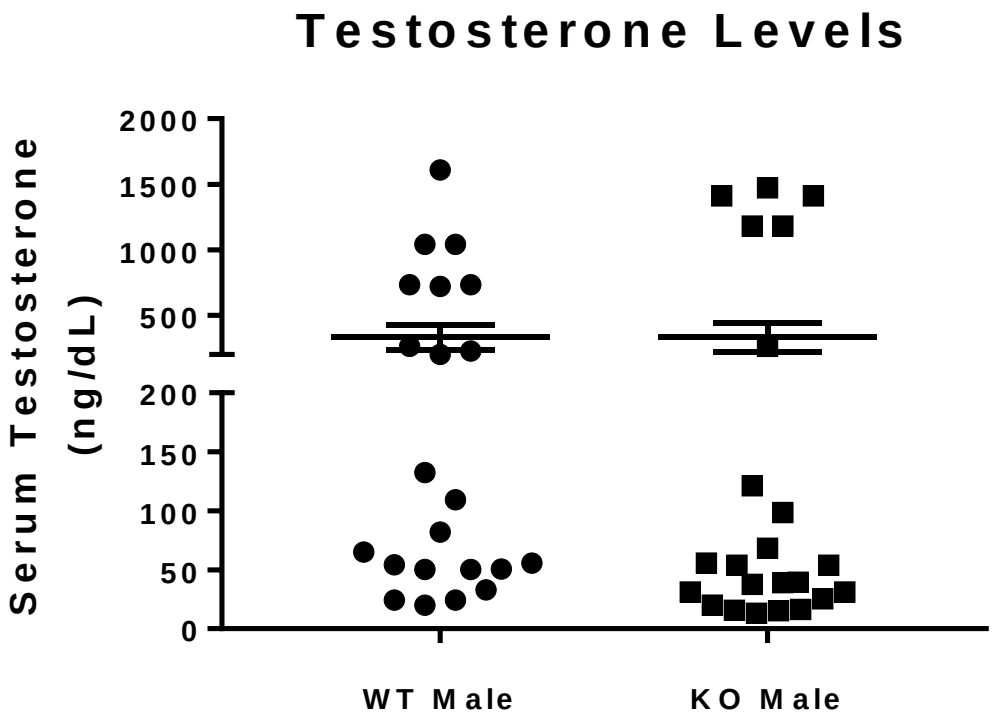

Figure 5. *Bmal1* KO males have testosterone levels comparable to WT males. Whole blood was taken from WT males (n=22) and *Bmal1* KO males (n=23). Serum was separated and testosterone was measured via the University of Virginia Hormone and Ligand Core. Statistical analysis by Unpaired t-test, *p<0,05.

Despite the comparable testosterone levels, we repeated the intruder assay with mice that had been gonadectomized and given a testosterone replacement, which has been shown to raise testosterone levels to 11.1 ± 0.8 ng/mL (gdxT) [46]. Even when controlling for testosterone levels, the *Bmal1* KO male mice do not elicit the same aggressive response as WT males (59.0 ± 29.1 vs 0 ± 0) (**Figure 6**).

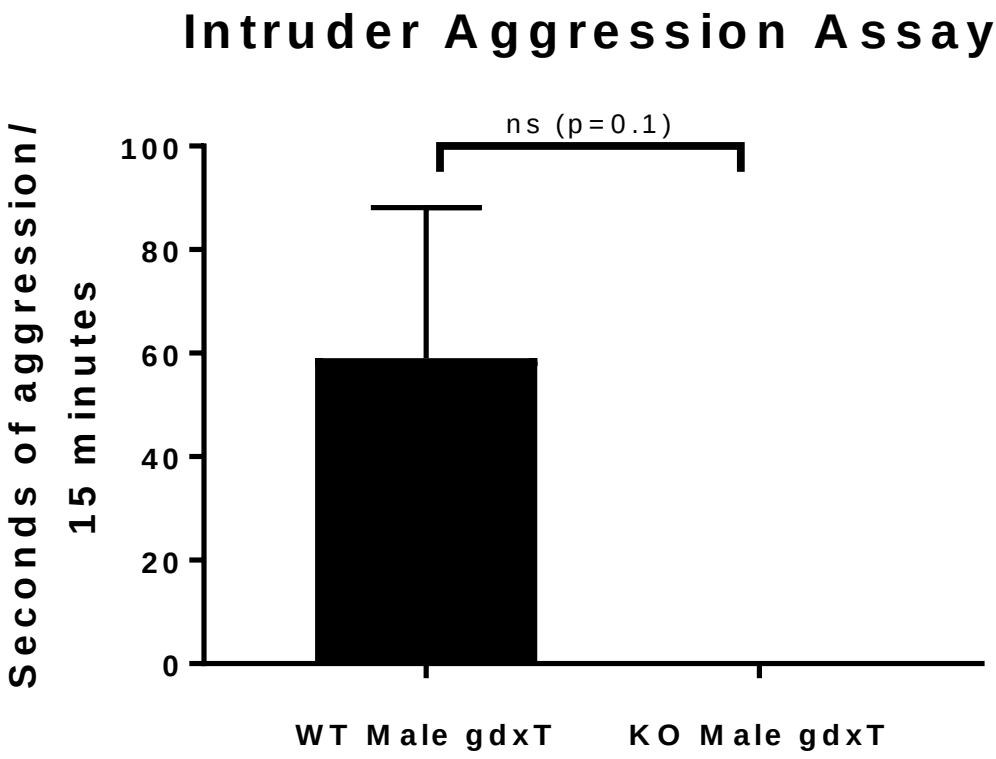

Figure 6. Testosterone replacement does not rescue aggression. WT males (n=3) and *Bmal1* KO males (n=3) were gonadectomized (gdx) and given a testosterone replacement (T) from SILASTIC (Dow Corning Corp). Mice were given 2 weeks to recover, then the intruder assay was run. Statistical analysis by Mann-Whitney test.

Bmal1 KO males do not produce WT levels of MUPs

Major Urinary Proteins (MUPs) are a class of high molecular weight pheromones made in the liver and secreted in the urine. They play an important role in both reproduction and aggression. Female urine has low levels of MUPs, while male urine contains high levels of MUPs. We hypothesized that one reason the *Bmal1* KO males do not elicit aggression from a WT male could be due to decreased urine MUP concentration. Urine was collected over 24 hours, and protein content was quantified. *Bmal1* KO males exhibited significantly lower MUP protein expression than the WT males at ZT 4 (1.298 ± 0.0722 vs. 1.078 ± 0.0335, p=0.0496) and ZT 8 (1.373 ± 0.0528 vs. 1.118 ± 0.0593, p=0.0158) (**Figure 7**).

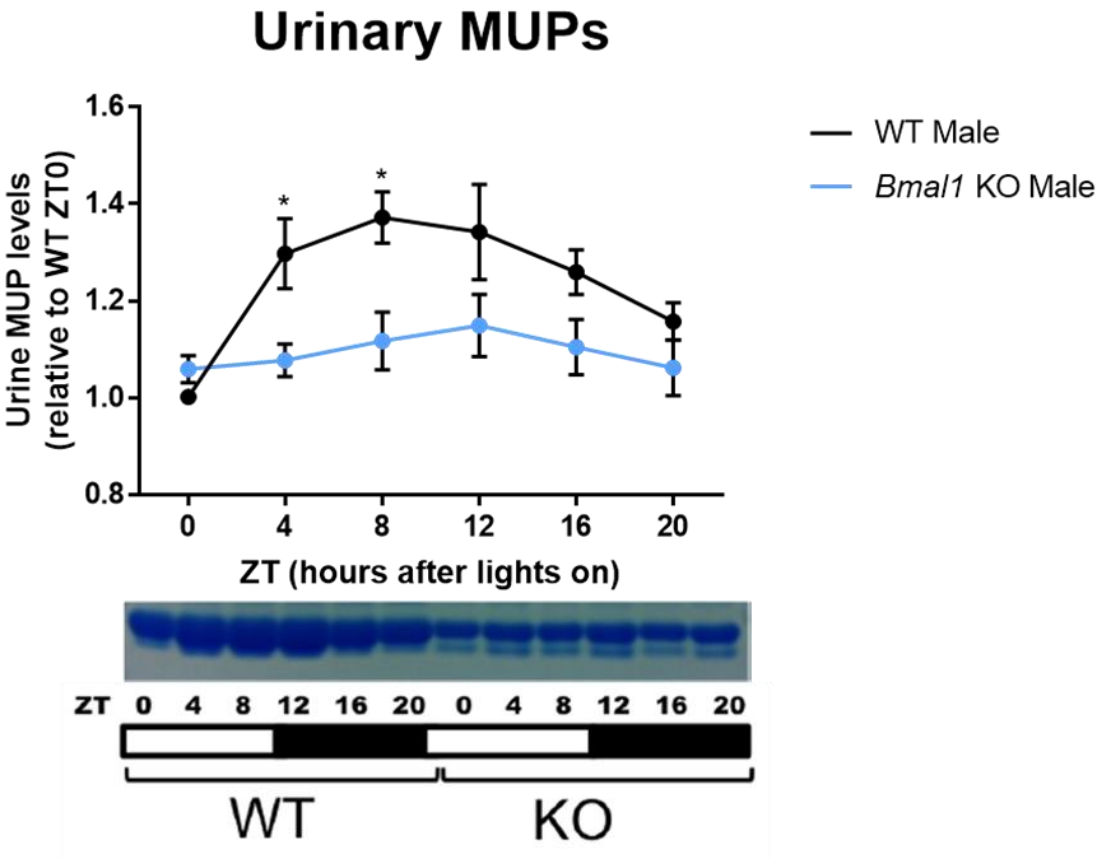

Figure 7. *Bmal1* KO males have decreased MUPs. Urine was collected from WT males (n=4) and *Bmal1* KO males (n=4) every 4 hours for 24 hours. Urine was run through a 12% SDS-PAGE, and total protein was stained with Thermofisher Page Blue Dye overnight. Statistical analysis by Two-way ANOVA, with Sidak's multiple comparison, *$p<0.05$.

Testosterone plays an important role in regulating the sexually dimorphic MUP expression in the liver [32]. In order to control for testosterone, the mice were gonadectomized and given a testosterone replacement pellet, and then 24-hour urine collection was performed (**Figure 8**). The *Bmal1* KO mice still showed significantly decreased MUPs when compared to WT mice at ZT 4 (1.221 ± 0.0959 vs. 0.767 ± 0.168, p=0.0241) and ZT 8 (1.304 ± 0.0911 vs. 0.849 ± 0.0983, p=0.0235) indicating that testosterone defects were not responsible for the decreased MUP concentration in *Bmal1* KO males.

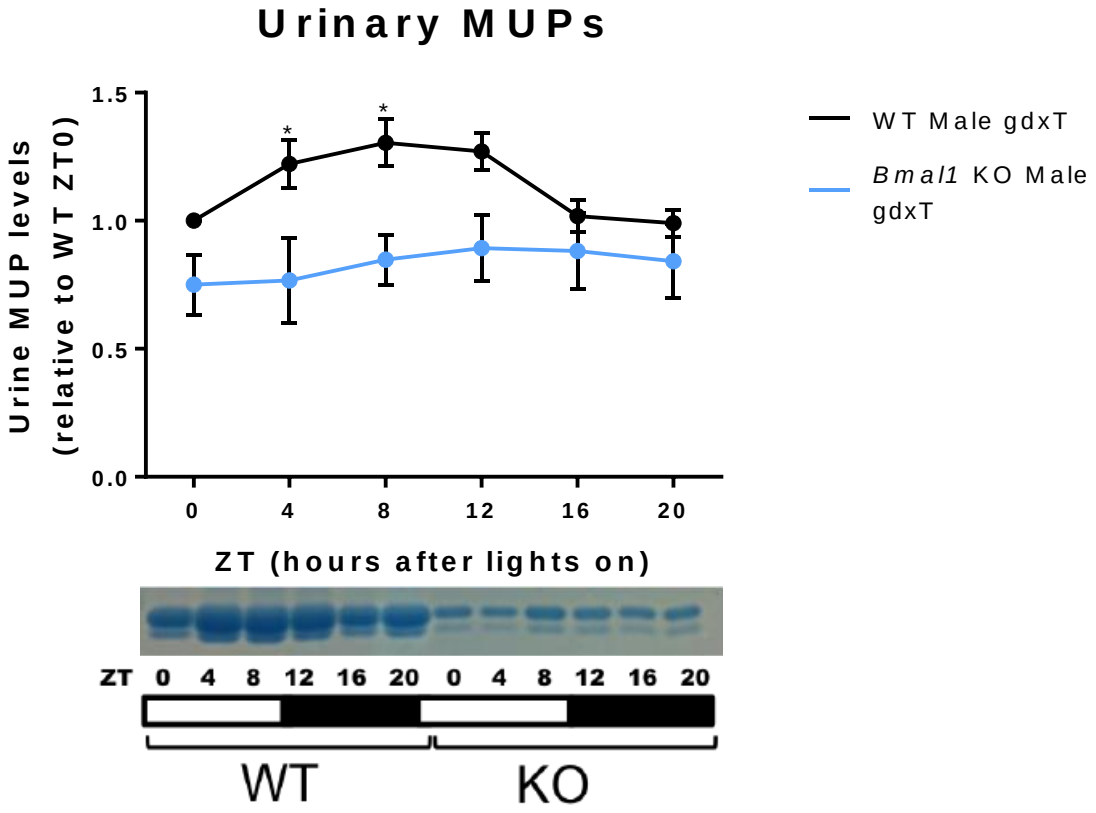

Figure 8. Testosterone replacement does not rescue MUP expression. Mice were gonadectomized and given a testosterone replacement pellet (gdxT), then allowed to recover for 2 weeks. Urine was collected from WT gdxT males (n=7) and *Bmal1* KO gdxT males (n=7) every 4 hours for 24 hours. Urine was run through a 12% SDS-PAGE, and total protein was stained with Thermofisher Page Blue Dye overnight. Statistical analysis by Two-way ANOVA, with Sidak's multiple comparison, *p<0.05.

Bmal1 KO male MUPs activate a different subset of WT VNO neurons

Pheromones bind to V2R receptors in the vomeronasal organ (VNO), and then the signals are transduced to the accessory olfactory bulb, where they are processed and result in a behavioral output [36]. We hypothesized that the *Bmal1* KO males may have a different combination of MUPs in their urine, thus sending a different signal to the animals who come into contact with them. Because MUP gene sequences are highly homologous, designing effective primers for qPCR analysis is difficult. Therefore, we utilized calcium imaging techniques to measure neural activation in response to urine pheromone signals. We found that the high molecular weight fraction of *Bmal1* KO urine containing MUPs activated smaller percentage of WT VNO neurons than WT urine (2.896 ± 0.7497 vs. 0.7186 ± 0.1755, $p=0.0475$), and a different subset of WT VNO neurons than WT urine (**Figure 9**). This suggests that *Bmal1* KO males are perceived differently than their WT male littermates.

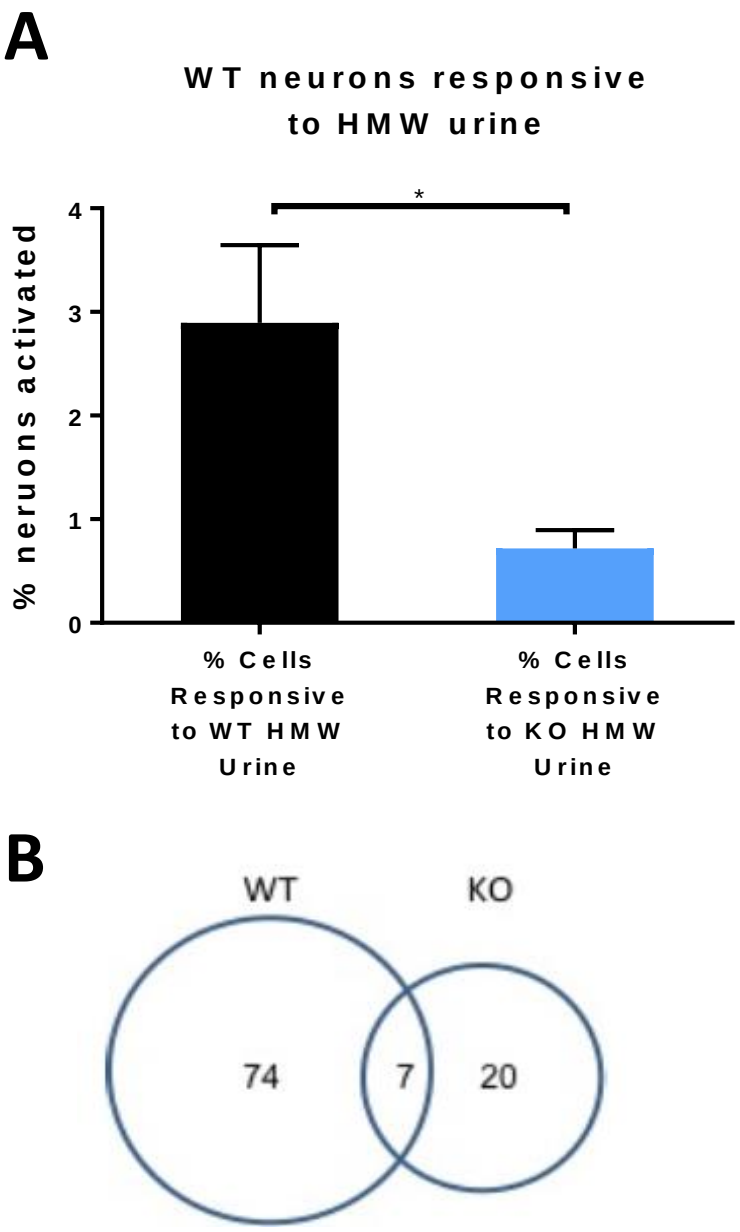

Figure 9. *Bmal1* KO urine activates different subset of WT male VNO neurons. VNO tissue was removed from WT males and loaded with Fura-2. Urine was collected from WT males (n=3) and *Bmal1* KO males (n=3) and fractionated. The high molecular weight (HMW) fraction was perfused over neurons, and increases in Ca^{2+} were measured **(9A)**. Neurons activated by the WT and/or *Bmal1* KO urine were counted, and subsets compared **(9B)**. Statistical analysis by Unpaired t-test, *p<0.05.

Bmal1 KO males have abnormal GH pulse patterns

As shown in Figure 8, controlling for testosterone levels in the *Bmal1*KO males does not rescue MUP expression, indicating that a different regulatory mechanism of MUP production is causing the deficiency. Growth hormone (GH) is known to regulate liver gene expression via sexually dimorphic pulse patterns. The highly pulsatile release of GH drives "male-like" gene expression in the liver, and is partially responsible for driving MUP expression [16, 43]. We hypothesized that the *Bmal1* KO males expressed a "female" GH pattern, with more continuous release that would result in a decrease in MUP expression.

The pulse patterns for *Bmal1* KO males, WT males, and WT females were determined via a serial blood sampling (**Figure 10A-C**, representative GH profiles shown). The WT males had highly pulsatile GH release, with low-frequency and high-amplitude pulses separated by an intermittent interval where GH was below the level of detection. WT females had more continuous release, with more pulses and a higher basal serum concentration. Interestingly, the *Bmal1* KO males had a pulse profile more similar to the WT females than the WT males. The *Bmal1* KO males had pulses with higher baseline GH values, similar to those of the WT female mice. They also had more pulses over the 6-hour time course than the WT males (3.667 ± 0.33 vs 6.5 ± 1.19, p=0.18), and a significantly shorter interpulse time than the WT males (60.65 ± 7.8 vs 41.0 ± 3.9, p=0.045) (**Figure 11A-B**).

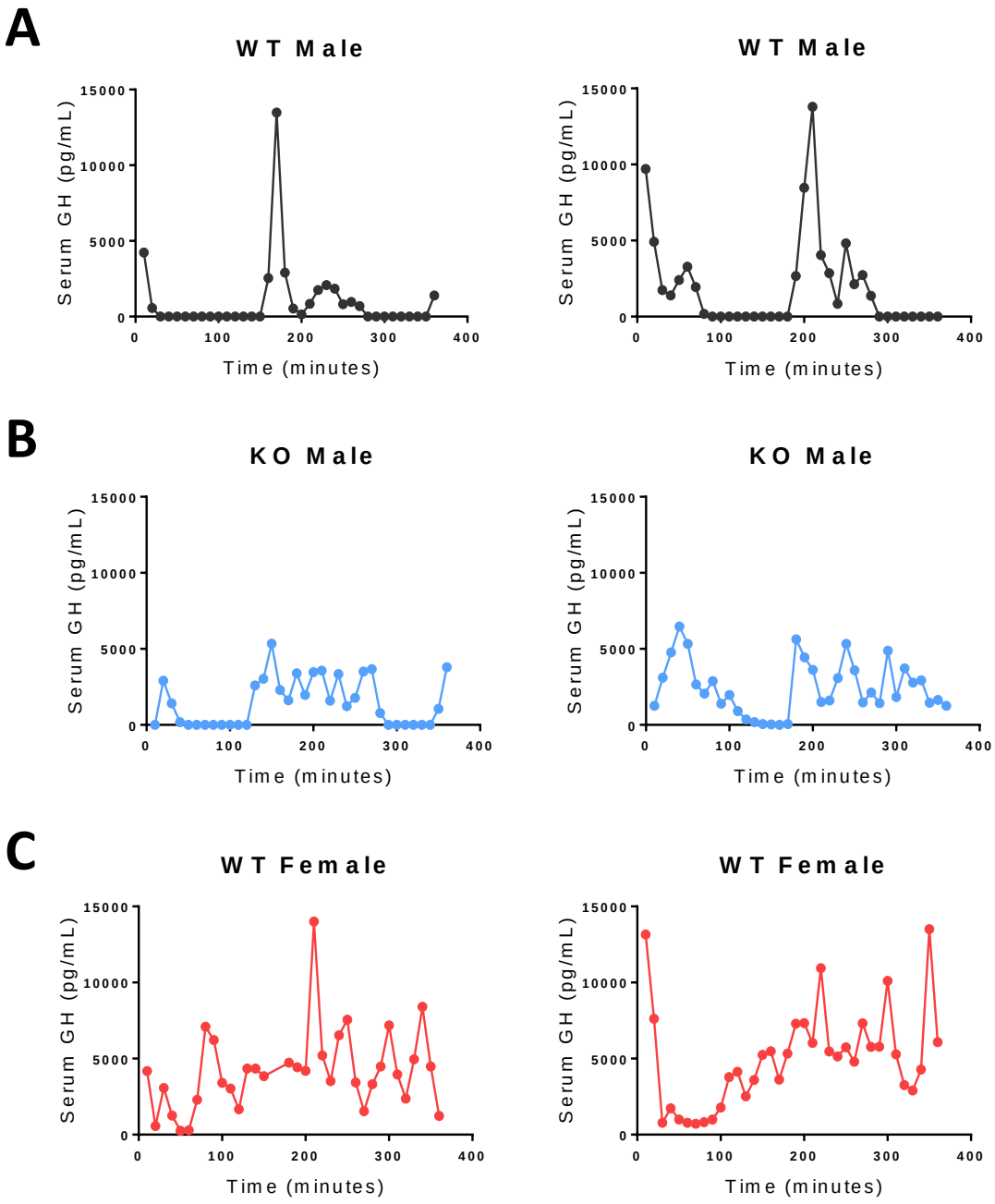

Figure 10. *Bmal1* KO males have abnormal GH pulses. Mice were handled daily for 1 month prior to testing. Tail blood was collected every 10 minutes for 6 hours from WT males (n=3) **(10A)**, *Bmal1* KO males (n=4) **(10B)**, and WT females (n=5) **(10C)**. Serum was separated and GH was measured via a Luminex assay. Representative profiles shown.

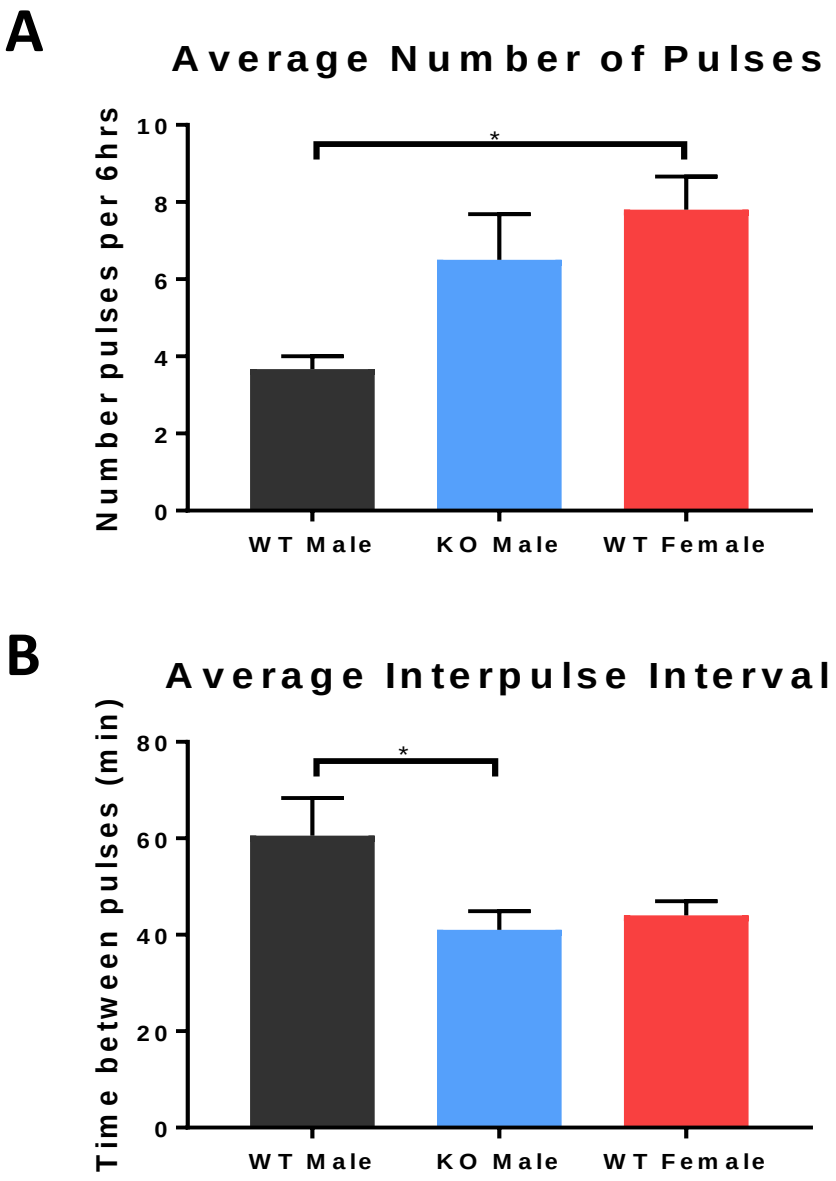

Figure 11. *Bmal1* KO males have feminized GH pulses. The number of pulses from mice in **Figure 10** were quantified using MatLab Peakfinder software (**11A**), and the time between pulses, or interpulse interval, was found using MatLab software (**11B**). Statistical analysis by One-way ANOVA with Tukey's multiple comparison, *p<0.05.

Bmal1 KO males have abnormal hepatic gene expression

As GH pulses regulate a majority of sexually dimorphic hepatic gene expression [28], we hypothesized that other liver genes besides MUPs in the *Bmal1* KO males would be feminized as the MUPs were. *Cytochrome p450s,* or *Cyps*, are a family of sexually dimorphic hepatic genes that play a role in drug and steroid metabolism [28]. To test their expression in the *Bmal1* KO males, we performed qRT-PCR analysis of liver cDNA. We first examined three male-predominant hepatic genes: *Cyp7b1*, responsible for the hydroxylation of steroids [53], *Cyp4a12a*, involved in the hydroxylation of ω-fatty acids [54] and *Elovl3*, responsible for the elongation of fatty acids [55]. The genes *Cyp7b1* and *Elovl3* were expressed at significantly higher levels in the *Bmal1* KO males than in the WT males (1.82 ± 0.65 vs. 10.8 ± 2.71, $p = 0.0078$; 1.14 ± 0.15 vs. 12.13 ± 0.67, $p<0.0001$), whereas *Cyp4a12a* was downregulated in the *Bmal1* KO males (1.578 ± 0.497 vs. 0.45 ± 0.157, $p>0.05$), showing a more feminine expression pattern (**Figure 12A**). We then investigated three female-predominant genes: *Fmo3*, responsible for the metabolism of certain drugs, such as codeine [56] , *Cyp2b9,* induced by phenobarbital and sex steroids [57, 58], and *Cyp2a4*, responsible for the hydroxylation of sex steroids [59]. *Fmo3* and *Cyp2a4* were both further downregulated in the *Bmal1* KO males (0.37 ± 0.21 vs 0.01 ± 0.007, $p>0.05$; 0.690 ± 0.167 vs. 0.069 ± 0.0115, $p>0.05$). *Cyp2b9*, however, showed an upward trend, indicating feminization (0.42 ± 0.17 vs. 1.27 ± 0.14, $p>0.05$) (**Figure 12B**).

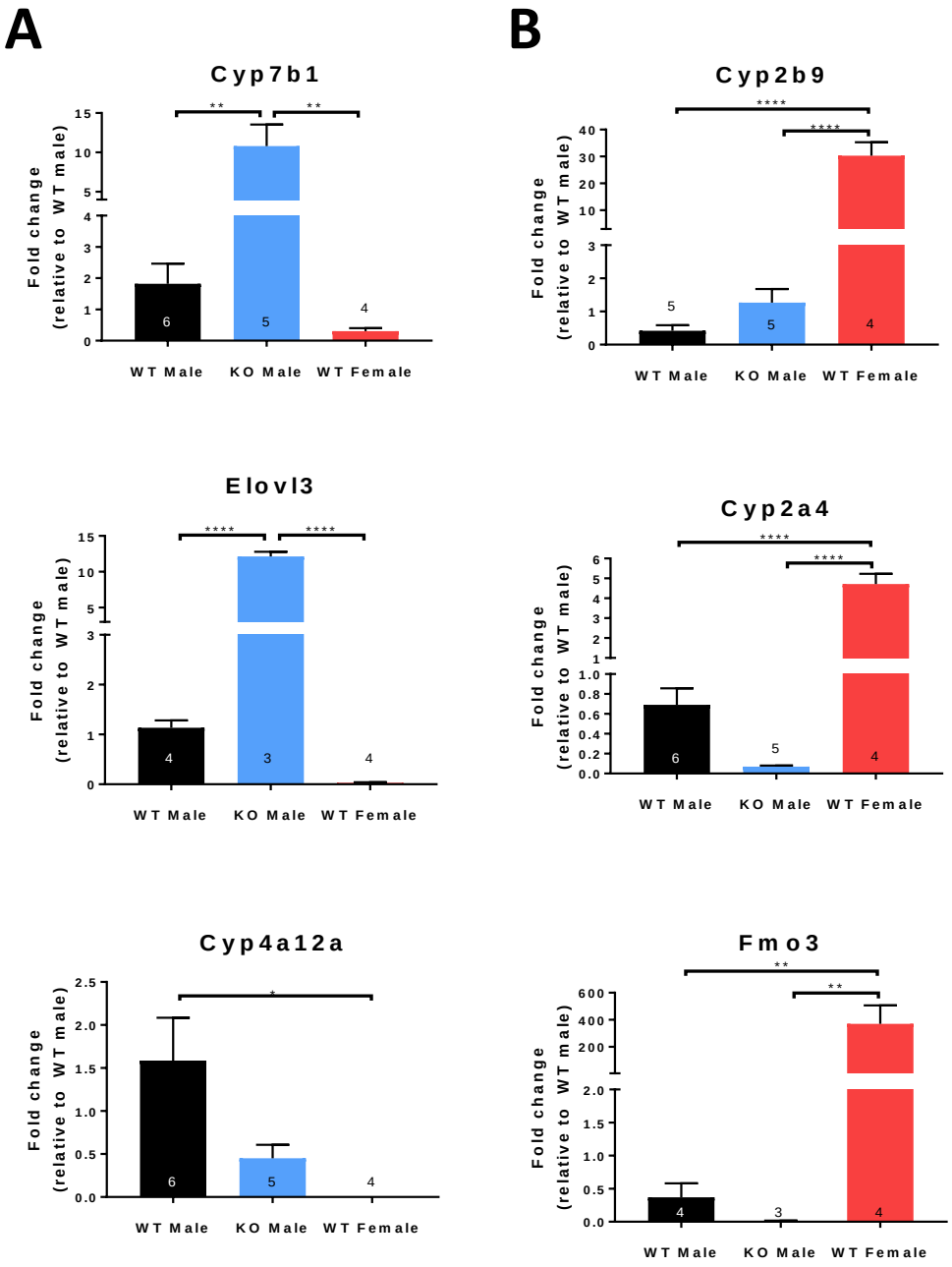

Figure 12. *Bmal1* KO males have disrupted, but not feminized, liver Cyp expression. Male-predominant genes (**12A**) and female-predominant genes (**12B**) were analyzed in liver samples via qRT-PCR. Statistical analysis by One-way ANOVA with Tukey's multiple comparison, *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

As testosterone plays a role in regulating sexually dimorphic gene expression [60], we performed the qPCR analyses on mice that had been gonadectomized and given a testosterone replacement (**Figure 13A-B**). We observed similar trends, with *Bmal1* KO males having dysregulated expression of *Cyp7b1* (2.1 ± 0.52 vs 12.74 ± 2.68, p=0.0041), *Elovl3* (1.44 ± 0.13 vs 16.66 ± 2.15, p<0.0001), *Fmo3* (0.06 ± 0.01 vs 0.003 ± 0.001, p>0.05) and *Cyp2a4* (0.4153 ± 0.0792 vs. 0.0396 ± 0.007, p>0.05). However, we observed feminization of *Cyp2b9* (0.06 ± 0.007 vs 0.41 ± 0.19, p>0.05) and *Cyp4a12a* (1.858 ± 0.630 vs. 0.596 ± 0.15, p>0.05).

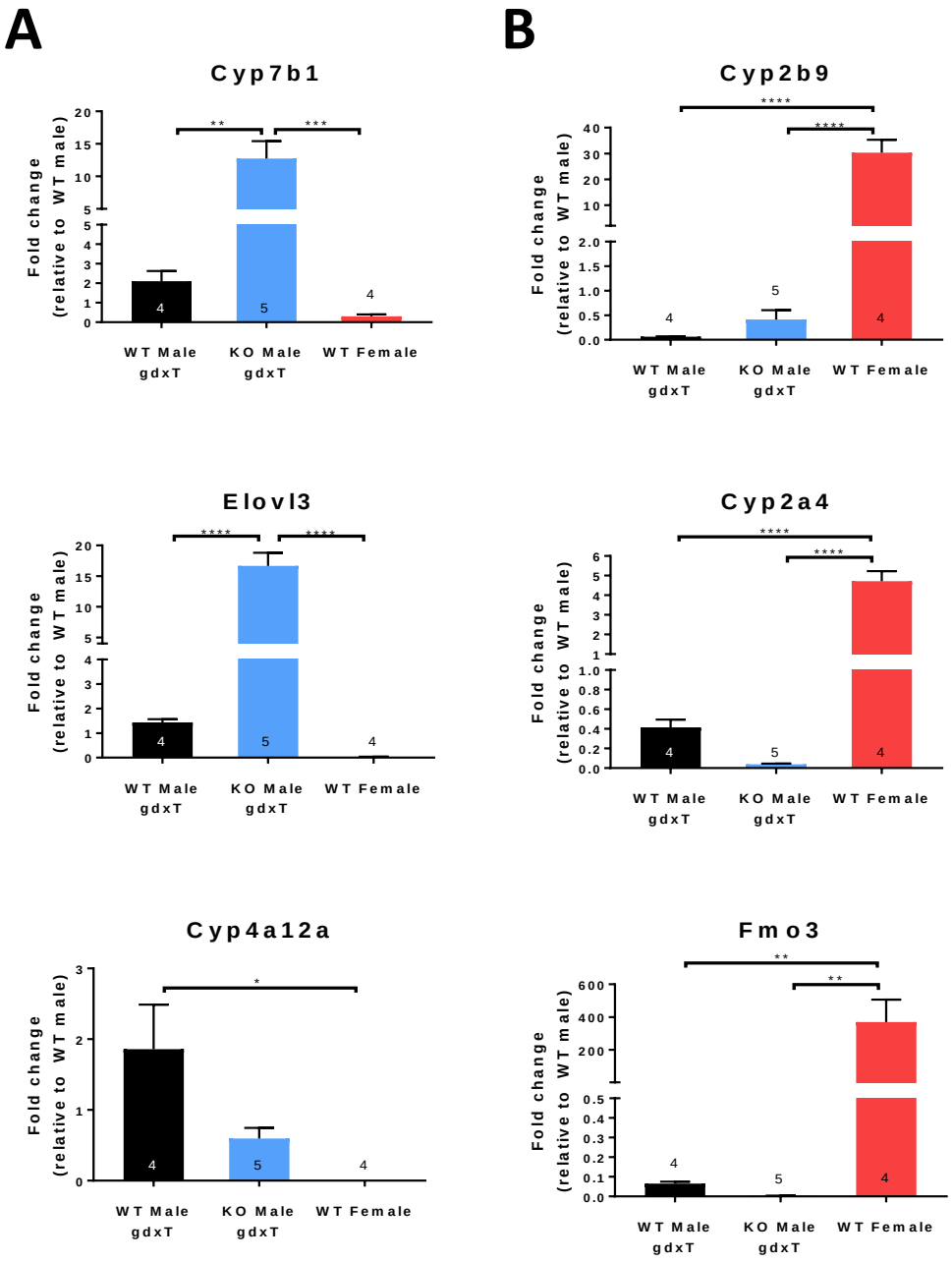

Figure 13. gdxT does not rescue Cyp expression in *Bmal1* KO male liver. Male-predominant genes (**13A**) and female-predominant genes (**13B**) were analyzed in livers from gdxT WT and KO males and WT females with qRT-PCR. Statistical analysis by One-way ANOVA with Tukey's multiple comparison, *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

IGF-1 is decreased in Bmal1 KO males

In response to GH binding to the GH receptor, the liver increases expression of IGF-1, which is then secreted to the body. IGF-1 acts upon various tissues to promote glucose uptake, cell proliferation, and cell growth. It is also thought to inhibit GH secretion by upregulating SST mRNA and downregulating GHRH mRNA in the hypothalamus [24, 25], although the specific mechanisms of action regulating this negative feedback are still under study. IGF-1 production shows circadian rhythmicity, with higher serum levels during the early morning, thus regulation of IGF-1 by *Bmal1* is possible [49].

Because GH concentrations were higher in the *Bmal1* KO males, we hypothesized that there might be a deficiency in IGF-1 feedback. *Bmal1* KO male mice had reduced IGF-1 throughout the 24 period compared to their WT male littermates (ZT 0: 1085 ± 220.1 vs. 511.3 ± 48.28, p=0.068; ZT 4: 1612 ± 259.2 vs. 787.7 ± 29.25, p=0.073; ZT 8 1130 ± 184.4 vs. 786 ± 78.18, p=0.188; ZT 12: 873.9 ± 78.49 vs. 642.9 ± 56.26, p=0.057; ZT 16: 1108 ± 196.4 vs. 795.1 ± 34.96, p=0.22; ZT 20: 1293 ± 170.6 vs. 734.6 ± 20.74, p=0.026), indicating that *Bmal1* may play a role in regulating IGF-1 production (**Figure 14**).

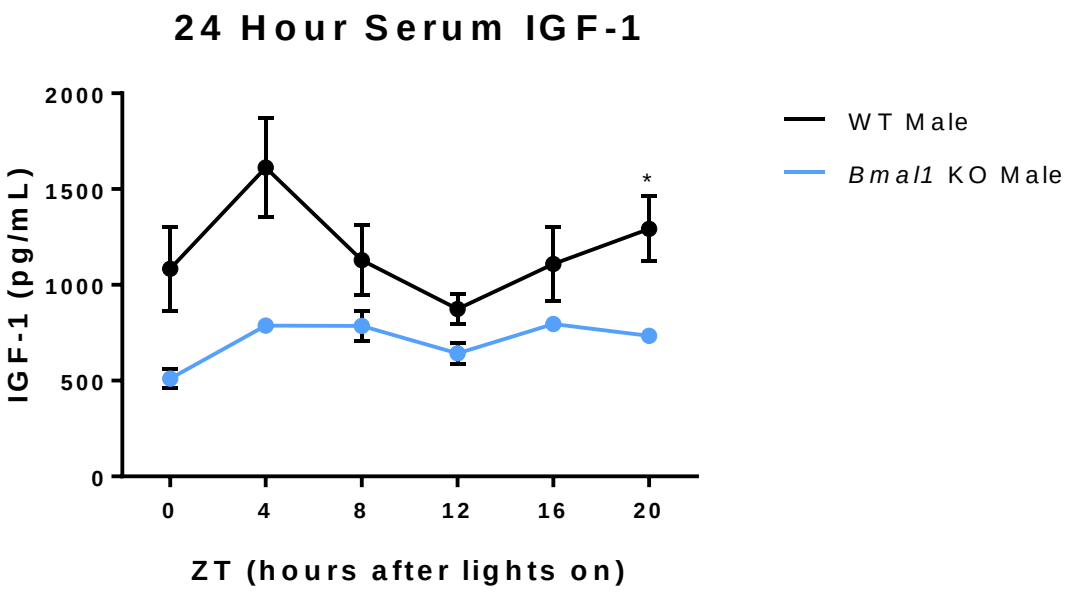

Figure 14. *Bmal1* KO males have decreased serum IGF-1. Whole blood was taken every 4 hours for 24 hours from WT males (n=10) and *Bmal1* KO males (n=6). Serum was separated and serum IGF-1 was measured via RayBio Mouse IGF-1 ELISA Kit. Statistical analysis by Unpaired t-test at each time point, *p<0.05.

Summary of Chapter I

Together, our data indicate that *Bmal1* plays a role in the regulation of the GH axis, either at the level of the brain or at the level of the liver through IGF-1 feedback. The female-like GH pulse pattern in the male *Bmal1* KO mice is associated with a downregulation in the expression of MUPs in the urine, a phenotype that could drive the loss of aggression from WT mice. However, our qPCR data suggests that *Bmal1* also plays a role in directly regulating the expression of sexually dimorphic genes, as the Cyp profiles measured were not entirely feminized, indicating that the genes measured were regulated by *Bmal1* and not the female GH secretion profile.

Chapter 1, in part, is currently being prepared for submission for the publication of material. I would like to thank the following co-authors for allowing me to share our data: Erica Schoeller, Sandeepa Dey, Rujing Shi, Lisa Stowers, and Pamela Mellon.

CHAPTER II: *Bmal1* in the liver is required for MUP and Cyp expression, but not IGF-1

The Albumin Cre; Bmal1$^{flox/flox}$ mouse model

To separate the effects of *Bmal1* in the liver from *Bmal1* throughout the whole body, we used the Albumin Cre; *Bmal1*$^{flox/flox}$ mouse (*Bmal1* liver KO) (**Figure 15**). By crossing a *Bmal1*$^{flox/flox}$ mouse with an Albumin Cre^{+} mouse, we deleted *Bmal1* expression specifically from the liver, which allowed us to analyze the direct effects of *Bmal1* on hepatic gene expression (**Figure 15**).

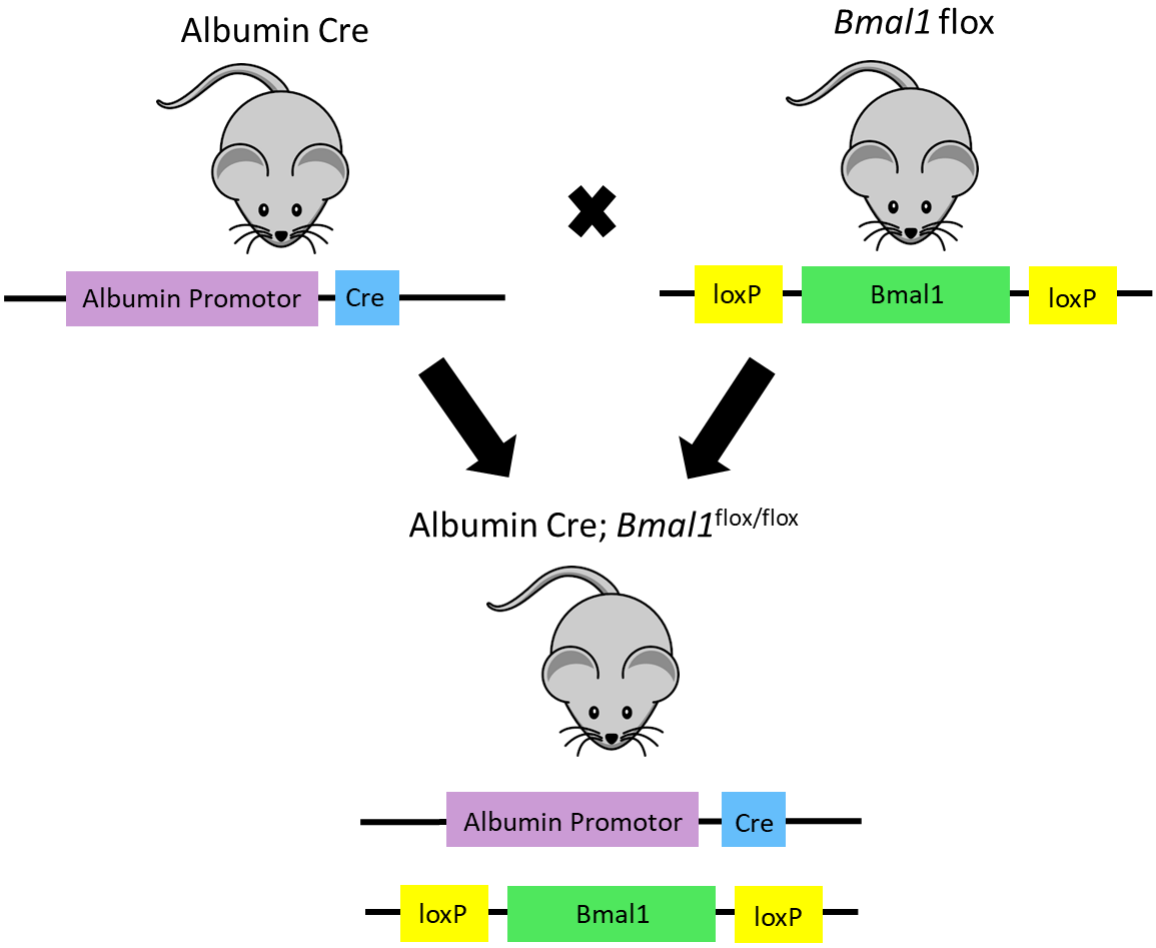

Figure 15. Albumin Cre; *Bmal1*$^{flox/flox}$ mouse model. The Cre gene was linked to the promoter for Albumin, a protein made exclusively in the liver. The Albumin Cre mouse was crossed with a *Bmal1* floxed mouse, with loxP sites were placed on either side of Exon 8 of the Bmal1 gene. When Cre is expressed in the liver, *Bmal1* is removed from the DNA, thus removing *Bmal1* from hepatocytes.

Bmal1 liver KO males do not produce WT levels of MUPs

As GH pulses regulate MUP expression, and because *Bmal1* was removed only from the liver, we hypothesized that MUP levels in the *Bmal1* liver KO males would not be different from WT male MUP levels. To determine if the liver KO males produced MUPs similarly to WT males, a 24-hour urine collection was performed. Interestingly, the *Bmal1* liver KO male mice had lower levels of MUPs in their urine than the WT males at ZT4 (1.064 ± 0.061 vs. 0.559 ± 0.143, p=0.015), ZT8 (1.146 ± 0.034 vs. 0.611 ± 0.131, p=0.0094), ZT12 (1.187 ± 0.069 vs. 0.627 ± 0.141, p=0.0062), and ZT16 (0.967 ± 0.063 vs. 0.524 ± 0.120, p=0.036) similar to the whole-body *Bmal1* KO males (**Figure 16**), suggesting that *Bmal1* mediates MUP expression in the liver, and not through modulation of GH pulses.

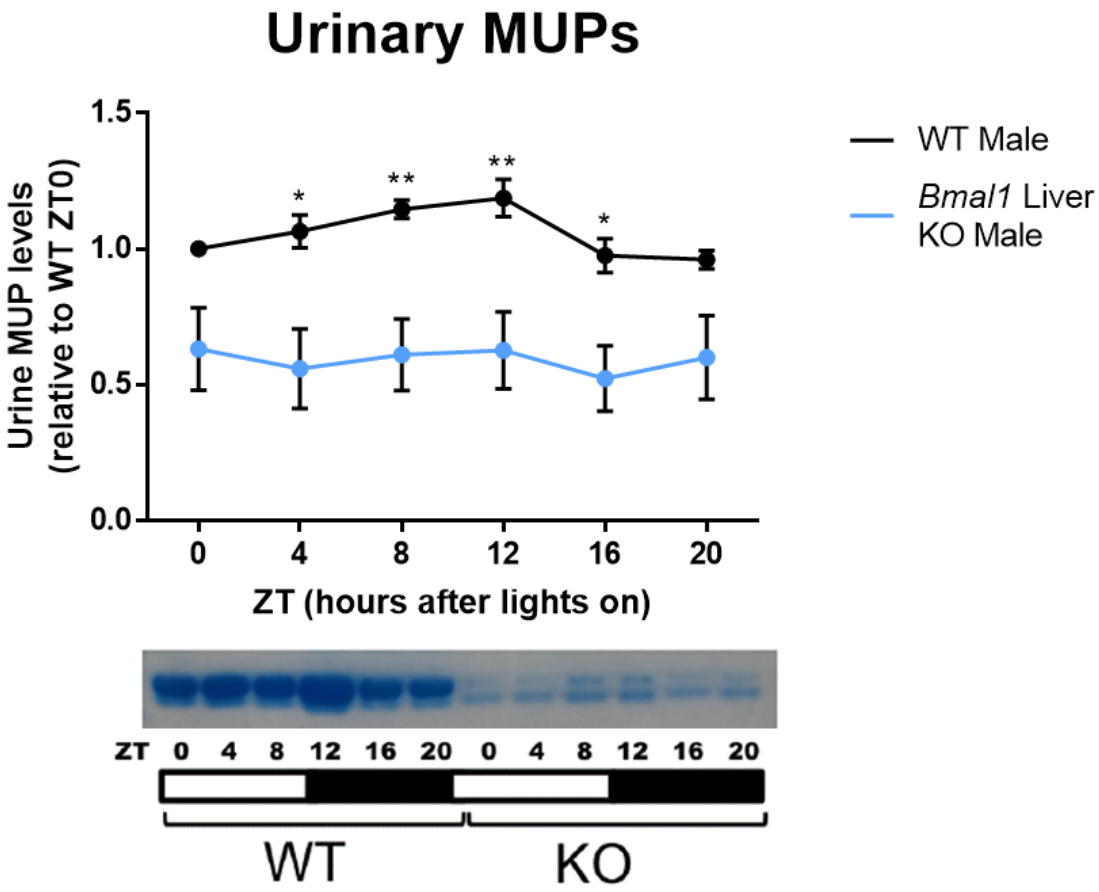

Figure 16. *Bmal1* liver KO males have decreased MUPs. Urine was collected from WT males (n=3) and *Bmal1* liver KO males (n=3) every 4 hours for 24 hours. Urine was run through a 12% SDS-PAGE, and total protein was stained with Thermofisher Page Blue Dye overnight. Statistical analysis by Two-way ANOVA, with Sidak's multiple comparison, *$p<0.05$.

Bmal1 liver KO males have disrupted hepatic gene expression

We had expected that the feminized GH pulses in the full-body *Bmal1* KO males would result in feminine hepatic gene expression, as GH pulse patterns are largely responsible for sexually dimorphism in the liver. Because the *Bmal1* KO males had dysregulated hepatic gene expression, rather than feminized gene expression, however, we hypothesized that *Bmal1* was acting in the liver to directly regulate expression of sexually dimorphic genes instead of through GH pulses. Using qRT-PCR, we analyzed the liver profile of the *Bmal1* liver KO males. The same pattern was observed in the *Bmal1* liver KO as in the whole-body *Bmal1* KO in *Cyp71b* (1.54 ± 0.56 vs. 4.19 ± 2.28, $p>0.05$), *Cyp2b9* (1.74 ± 0.41 vs. 7.03 ± 2.5, $p>0.05$), *Elovl3* (1.71 ± 0.78 vs. 5.65 ± 2.05. $p = 0.041$) *Fmo3* (0.92 ± 0.43 vs. 0.29 ± 0.20, $p>0.05$), and *Cyp2a4* (0.7652 ± 0.187 vs. 0.2637 ± 0.160, $p>0.05$. The genes were not feminized, but differentially expressed, indicating a regulatory role for *Bmal1* at the level of the liver. Interestingly, the *Bmal1* liver KO males showed levels of *Cyp4a12a* similar to the WT males (0.9414 ± 0.141 vs. 0.934 ± 0.090, $p>0.05$), indicating that liver-specific *Bmal1* expression does not regulate *Cyp4a12a* (**Figure 17A-B**).

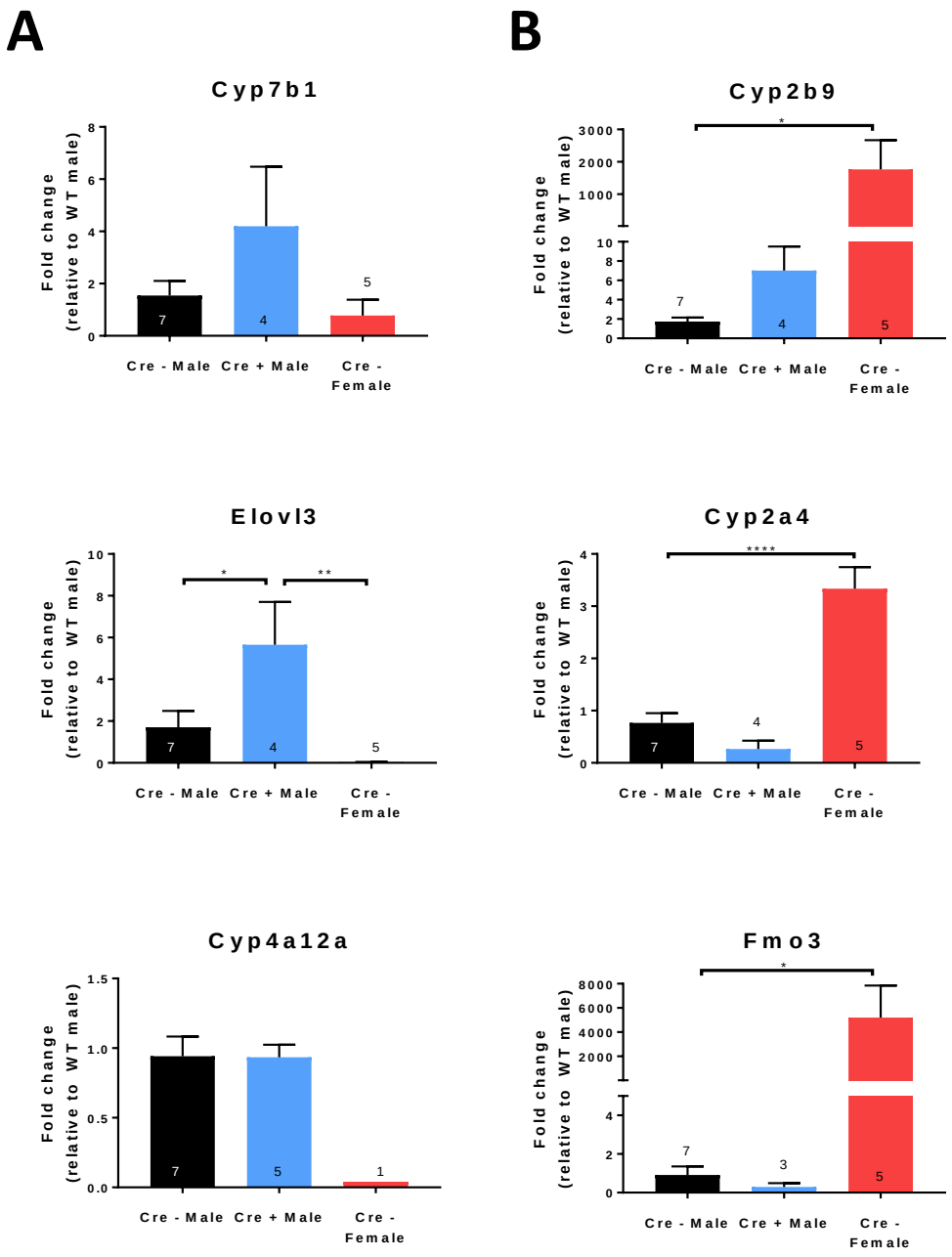

Figure 17. *Bmal1* liver KO males have disrupted Cyp expression. Liver gene expression of male-predominant genes (**17A**) and female-predominant genes (**17B**) was analyzed with RT-qPCR. Statistical analysis by One-way ANOVA with Tukey's multiple comparison, *p<0.05, ** p<0.01, ***p<0.001, ****p<0.0001.

IGF-1 is produced at normal levels in Bmal1 liver KO males

As MUP expression and various other sexually dimorphic genes were found to be regulated by *Bmal1* in the liver, we hypothesized that IGF-1 would be regulated by *Bmal1* as well, thus causing abnormal secretion in the *Bmal1* liver KO males. To determine if the liver KO mice had decreased levels of IGF-1, a 24-hour serial tail bleed was performed, as in **Figure 14**. Interestingly, the *Bmal1* liver KO males had IGF-1 levels comparable to WT males at every time point (ZT 0: 1085 ± 220.1 vs. 662.2 ± 120.9, $p>0.05$; ZT 4: 1612 ± 259.2 vs. 1464 ± 173.6, $p>0.05$; ZT 8 1130 ± 184.4 vs. 748.8 ± 161.4, $p>0.05$; ZT 12: 873.9 ± 78.49 vs. 719.9 ± 169.2, $p>0.05$; ZT 16: 1108 ± 196.4 vs. 977 ± 267.7, $p>0.05$; ZT 20: 1293 ± 170.6 vs. 1060 ± 266.8, $p>0.05$) (**Figure 18**). This indicates that serum IGF-1 levels are not modulated by *Bmal1* within the liver, and that an external factor contributes to serum IGF-1 rhythmicity.

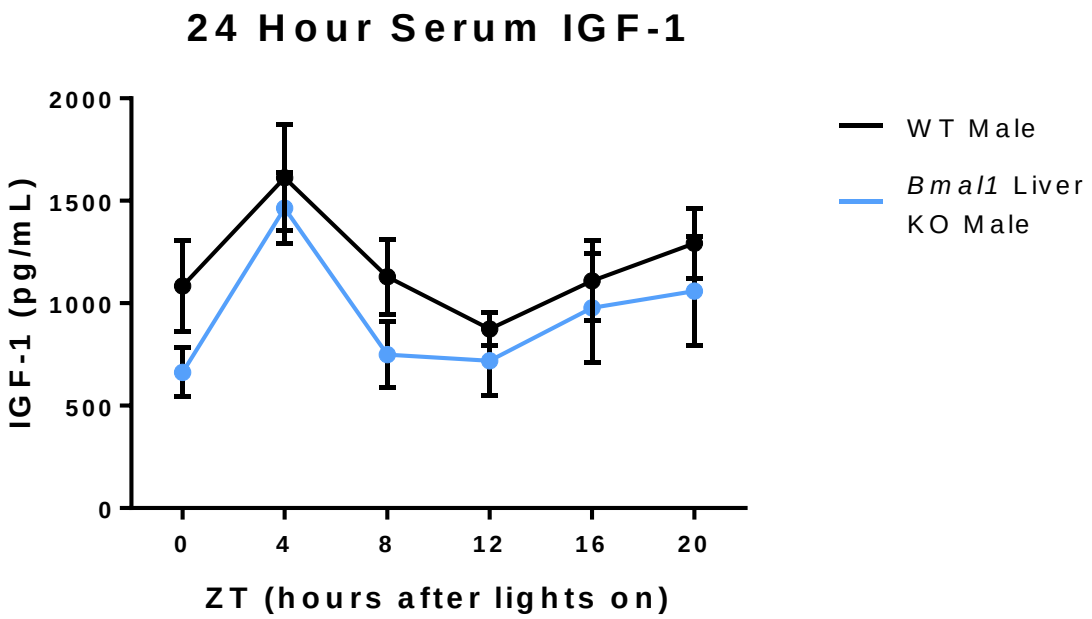

Figure 18. *Bmal1* liver KO males have normal serum IGF-1. Whole blood was taken every 4 hours for 24 hours from WT males (n=10) and *Bmal1* liver KO males (n=4). Serum was separated and serum IGF-1 was measured via RayBio Mouse IGF-1 ELISA Kit. Statistical analysis by Unpaired t-test at every time point.

Summary of Chapter II

Together, our data indicate that *Bmal1* regulates the expression of some sexually dimorphic genes in the liver, such as MUPs and members of the Cyp family. However, other genes appear to be regulated through another mechanism, and are unaffected by the loss of liver *Bmal1*. Our project reveals a novel regulatory mechanism through which *Bmal1* directly mediates sexually dimorphic gene expression, independent of the effects of the GH axis.

Chapter 2, in part, is currently being prepared for submission for publication. I would like to thank the following co-authors for allowing me to share our data: Erica Schoeller, Sandeepa Dey, Rujing Shi, Lisa Stowers, and Pamela Mellon.

DISCUSSION

Bmal1 is required for masculine GH pulses and normal hepatic gene expression

The molecular clock is comprised of 4 core clock genes, *Bmal1*, *Clock*, *Per*, and *Cry*. The molecular clock regulates the body's endogenous circadian rhythms, which coordinates physiological processes with time-of-day. The suprachiasmatic nucleus in the hypothalamus, or the SCN, is considered the "Master Clock". The SCN synchronizes to the 24-hour day-night cycle by integrating light signals from the optic nerve with the molecular clock, and then sends hormonal and chemical signals to align the molecular clock in peripheral tissues with the master clock.

The removal of *Bmal1* from the mouse genome disrupts circadian rhythms, leading to numerous detrimental phenotypes. Although *Bmal1* KO mice entrain to light cues, wheel-running patterns in 24-hour darkness show a complete ablation of circadian running rhythms [31]. Both the male and female *Bmal1* KO mice are infertile [42, 51], and they display metabolic defects, early aging, and an increased rate of cancer, indicating that *Bmal1* plays an important regulatory role in many systems within the body [5, 6, 61]. These effects are also observed in humans with disrupted circadian rhythms via shiftwork; shiftworkers are subfertile and are at higher risk for cancer and cardiac disease [1, 7].

Circadian rhythms can also impact behavioral responses in mice, including reproductive, aggressive, and fear behavior [42]. While studying the behavior of the *Bmal1* KO male mice, we observed that they did not elicit aggressive responses from WT males, as other WT males typically would. In an intruder assay, a WT resident male will

defend the home cage territory from other males, who would be seen as a competitor for resources, such as food and females. However, the WT males did not attack the *Bmal1* KO males. The WT resident investigated the pheromone scent of the *Bmal1* KO male intruder, but did not proceed to initiate attack behavior (**Figure 4**). This was in stark contrast to the WT male behavior towards other WT males. WT intruders provoked an aggressive response from the WT resident, including wrestling, biting, and scratching behaviors. This suggests that the pheromone cues from the *Bmal1* KO males are not perceived as a threat to the WT resident male.

Testosterone, which plays a role in aggression, is thought to be suppressed in *Bmal1* KO males [50], although the evidence for that phenotype is inconsistent. Testosterone is released from the gonads in pulses, and thus measuring serum concentrations at only a single time point will result in data with large variation, as some measurements will catch a peak while others catch a trough. However, measuring testosterone at multiple times points is technically challenging and requires large amounts of blood, so a single time point measurement was performed. Though the levels of serum testosterone in the *Bmal1* KO males were similar to those of WT males (**Figure 5**), we still normalized the testosterone levels the animals were gonadectomized and given a testosterone pellet at a level to restore the endogenous levels of sex steroids, thus normalizing testosterone levels between the *Bmal1* KO males and WT males. Even when controlling for testosterone levels, the *Bmal1* KO males did not elicit an aggressive response from the WT resident males, indicating that defects in testosterone levels were not the cause of the behavioral difference (**Figure 6**).

Most mammals communicate through chemicals signals, or pheromones, which are detected by the vomeronasal organ and the main olfactory epithelium. The chemical cues are then integrated in the hypothalamus, where they are processed to induce behavioral changes. In mice, the pheromone family MUPs, expressed at very high levels in males, are involved in behaviors such as reproduction, fear, and aggression. MUPs are made in the liver, secreted in urine, and then detected by other mice to communicate information about the social, reproductive and nutritional status of the animal. We hypothesized that the *Bmal1* KO males were failing to synthesize or secrete these pheromones, which could alter other animals' response to the *Bmal1* KO mice. After measuring MUP levels in urine over a 24 hour period, we concluded that the *Bmal1* KO males did secrete lower levels of MUPs than their WT littermates (**Figure 7**). These data suggest that the *Bmal1* KO males were not being perceived in the same way as WT males, resulting in a less aggressive response from the resident WT mice.

Decreased levels of MUPs, however, do not necessarily translate to differences in eliciting behavioral responses such as aggression. There are at least 21 known MUPs [62], many of which have high sequence homololgy, and different combinations of these proteins in urine send distinct signals to the recipient animal [34]. To test whether the *Bmal1* KO male urine communicated similar signals as WT urine, we perfused WT and *Bmal1* KO male urine over WT male VNO neurons, and recorded the response of the VNO neurons via calcium imaging. The data show that the urine from the *Bmal1* KO males do not activate as many VNO neurons as the WT urine, and the neurons that are activated by the *Bmal1* KO urine are not the same population activated by the WT urine (**Figure 9**). This suggests that the *Bmal1* KO males are perceived differently than WT

males, which provides a plausible explanation for the lack of aggressive responses from WT males. It is possible that the makeup of MUPs expressed in the *Bmal1* KO male mice is markedly different than the WT males, which would send a different signal to the mouse responding to the pheromones. To further test this hypothesis, the MUP composition of *Bmal1* KO males and the WT males could be compared via 2D gel separation follow by analysis by mass spectrometry [63]. The alteration in overall MUP expression, however, is not caused by testosterone differences (**Figure 8**), suggesting that there is a disruption in another driving factor of hepatic gene expression in the *Bmal1* KO males.

As GH is known to play a role in liver masculinization [28], and *Bmal1* is involved in the regulation of other aspects of hypothalamic and pituitary function [42, 50, 51], we hypothesized that the secretion of GH was disrupted, potentially causing a dysregulation of sexually dimorphic genes in the liver. In females, GH is constantly secreted, which desensitizes the GH receptors in the liver, and results in female-like hepatic gene expression. GH binding to its receptor in the liver results in a rapid downregulation of the GH receptor protein, and a desensitization of the JAK2/STAT5b signaling pathway, and although the desensitization is not yet wholly understood, it is thought to drive the expression of feminine hepatic genes [64]. In WT males, GH is secreted in high amplitude pulses, which drives male-like gene expression in the liver, separated by "off-periods", which allows the GH receptors in the liver to be replaced on the cell surface, and the JAK2/STAT5b pathway to regain its ability to respond [64]. We found that the *Bmal1* KO males have feminized GH pulses, with shorter "off-periods" and an increased number of peaks (**Figures 10 & 11**), suggesting that the decrease in

MUP expression could be a result of liver feminization. This finding was similar to the data found in the Bur et al. study (2009), which found that circadian mutant *Cry1/Cry2* double KO male mice had increased levels of GH, consistent with female GH pulse patterns [43]. These data indicate that the circadian clock genes are involved in regulating the growth hormone axis, although the level at which this regulation occurs has not yet been established. Continuous GH secretion in the *Bmal1* KO males and in the *Cry1/Cry2* double KO males may drive feminized MUP expression. We hypothesized that the feminized GH pulse pattern seen in the *Bmal1* KO males could be feminizing other sexually dimorphic hepatic genes as well.

Another class of sexually dimorphic genes in the liver is the Cytochrome p450s, or Cyp genes, which are involved in the metabolism of toxicants, lipids, and steroids. Male-like GH pulses play an important role in regulating the expression of sexually dimorphic genes in the liver, thus a disruption in the GH axis causes a change in the expression of the sexually dimorphic Cyps. The *Bmal1* KO male mice, thus far, had been very similar to the *Cry1/Cry2* KO circadian mutants, in that both mouse lines showed feminized MUP expression and a feminized GH profile. Increased release of GH in the *Cry1/Cry2* double KO males may have desensitized the liver receptors, and resulted in a Cyp expression pattern more similar to WT females than WT males. A twice daily injection of GH into the *Cry1/Cry2* KO males had rescued the masculine expression of some, but not all, feminized Cyps, indicating that the pulsatile GH pattern had been responsible for the feminization of those Cyps, including *Cyp7b1* and *Cyp4a12* [43]. As the *Bmal1* KO males' phenotype was consistent with the *Cry1/Cry2* KO, we hypothesized that the *Bmal1* KO males' feminized GH pulse pattern would result in the

same Cyp feminization seen in the Bur et al study. We examined three male-predominant hepatic genes: *Cyp7b1*, responsible for the hydroxylation of steroids [53], *Cyp4a12a*, involved in the hydroxylation of ω-fatty acids [54] and *Elovl3*, responsible for the elongation of fatty acids [55] (**Figure 12A**). All three genes are expressed at higher levels in WT males than in WT females. Interestingly, rather than being feminized, *Cyp7b1* and *Elovl3* were further upregulated in the *Bmal1* KO males. *Cyp4a12a* was slightly downregulated, indicating possible feminization, although the decrease was non-significant. We also examined three female-predominant genes: *Fmo3*, responsible for the metabolism of certain drugs, such as codeine [56] , *Cyp2b9*, induced by phenobarbital and sex steroids [57, 58], and *Cyp2a4*, responsible for the hydroxylation of sex steroids [59] (**Figure 12B**). All are expressed at higher levels in WT females than in WT males. *Fmo3* and *Cyp2a4* are further downregulated in the *Bmal1* KO males. However, *Cyp2b9* is upregulated in the *Bmal1* KO males, indicating a feminization. Interestingly, although many of the Cyp genes were differentially expressed, most were not feminized, unlike the pattern seen in the circadian mutant mice in Bur et al. (2009). *Cyp7b1* and *Elovl3* are dysregulated in the *Bmal1* KO males, rather than feminized as in the *Cry1/Cry2* double KO males. As many sexually dimorphic hepatic genes are regulated by testosterone, we repeated the analysis with males who had been gonadectomized and given a testosterone replacement (gdxT). The gdxT males showed the same expression pattern (**Figure 13A-B**), indicating that possible testosterone differences in the *Bmal1* KO males are not driving the phenotype. Since most of the Cyp genes were not feminized as we had expected, given the feminized GH pulses, we hypothesized that *Bmal1* regulates the expression of these genes independently of the GH axis, possibly at the level of the liver.

BMAL1 acts as a transcription factor in many tissues, and directly regulates many circadian genes [38], thus the removal of *Bmal1* from the liver could result in the loss of an important regulator of the expression of Cyps and other hepatic genes.

A key feedback regulator of the GH axis, IGF-1, is produced in the liver in response to GH receptor activation. IGF-1 then inhibits the GH axis at the level of the hypothalamus and the pituitary, and is thought to upregulate SST in the hypothalamus [25]. We hypothesized that the *Bmal1* KO males would secrete less IGF-1, as a result of increased GH secretion. If loss of *Bmal1* results in decreased IGF-1 secretion, this would feed back onto the hypothalamus and pituitary to increase GH secretion. We found that the *Bmal1* KO males had decreased serum IGF-1 levels over a 24-hour period (**Figure 14**). This indicates that *Bmal1* in the liver may play a key role in the regulation of IGF-1.

Together, these data indicate that *Bmal1* is a key regulator of the GH axis, and that the disruption of circadian rhythms ablates normal pulsatility in males. As *Bmal1* is known to play a role in other hypothalamic-pituitary axes at the level of the hypothalamus and the pituitary [65, 66], it's plausible that *Bmal1* regulates the GH axis at one or both levels. *Ghrh* and *Sst* mRNA levels in the hypothalamus are not significantly different between WT males and *Bmal1* KO males. The levels of *Gh* mRNA in the pituitary does not significantly differ between the WT males and the *Bmal1* KO males, although the *Bmal1* KO males are trending towards a downregulation (unpublished data, not shown). The removal of *Bmal1* could result in a decrease of GH production in the males, thus a decrease in IGF-1 production. Together, these phenotypes would cause a more feminine GH pulse profile, as seen in the *Bmal1* KO male mice. In order to test this

hypothesis, a pituitary-specific *Bmal1* KO mouse model could be used, which would allow us to explore the role of *Bmal1* specifically in somatotrope cells.

In the liver, *Bmal1* regulates the expression of the many of the Cytochrome p450 genes, though its action overrides the potential feminizing effects of the feminized GH pulse patterns, causing the Cyps to be differentially expressed rather than feminized. Had the GH disruption been solely responsible for the change in hepatic gene expression, we would have observed a feminization in the Cyp expression, as in the Bur et al. study (2009). However, the Cyps are dysregulated rather than feminized, thus suggesting that the role that *Bmal1* plays in the liver contributes to their expression more than the male-like GH pulse pattern does.

IGF-1 production is also disrupted in the *Bmal1* KO males, either due to the continuous GH pulses, or by *Bmal1* regulation. A decrease in IGF-1 could then cause the *Bmal1* KO male mice to have feminized GH pulses, as IGF-1 levels might not be high enough to result in an increase in SST to drive the “off-periods”.

Bmal1 is required in the liver for normal hepatic gene expression

To isolate the effects of *Bmal1* action in the liver, Cre-lox technology was used to generate a liver-specific *Bmal1* KO. A *Bmal1*$^{flox/flox}$ mouse was crossed with an Albumin Cre mouse, generating the Albumin Cre; *Bmal1*$^{flox/flox}$ mouse model, or the *Bmal1* liver KO (**Figure 15**). These mice lack the *Bmal1* gene only in hepatic cells, which allows us to determine the regulatory effects of *Bmal1* in the liver, rather than in the whole body. Because the full body *Bmal1* KO males showed altered, but not feminized, hepatic gene expression, we hypothesized that *Bmal1* in the liver was directly regulating the gene expression. Thus, we expected the *Bmal1* liver KO males to show similar patterns in their hepatic gene expression profile.

We first analyzed MUP concentration in the urine. As masculine GH pulses are partially responsible for driving MUP expression, and because the *Bmal1* KO males had feminized MUPs, we had hypothesized that the *Bmal1* KO males' GH pulse patterns were causing the downregulation of MUPs. However, MUP expression is circadian, with high expression at the onset of the night, and lower expression during the day. This circadian expression could be achieved by direct regulation via *Bmal1* in the liver. Thus, we hypothesized that the *Bmal1* liver KO males would have disrupted MUP production. After observing MUP levels in urine for 24 hours, we found that the *Bmal1* liver KO males had decreased MUPs compared to their Cre- littermates throughout most of the day (**Figure 16**), indicating that *Bmal1* regulates MUP expression at the level of the liver.

The expression of the Cytochrome p450 genes was then analyzed via qPCR. In the *Bmal1* KO males, we had expected the feminized GH pulse pattern to feminize the expression of Cyps; however, we found that the Cyp genes were differentially expressed,

rather than feminized. As many Cyp genes are expressed in a circadian pattern, we hypothesized that the differential regulation was caused by *Bmal1* acting directly in the liver. The actions of *Bmal1* were more potent than the feminization effects of the GH pulse patterns, thus leading to a dysregulation of Cyp expression. We hypothesized that removing *Bmal1* specifically from the liver would cause a dysregulation similar to the full-body *Bmal1* KO. For all the genes we examined except *Cyp4a12a*, the expression pattern in the *Bmal1* liver KO is the same as in the full-body KO (**Figure 17A & 17B**). The Cyp genes were dysregulated rather than feminized. These data indicate that *Bmal1* regulates the expression of Cyp genes in the liver, and that the regulation by *Bmal1* is more potent than the effects of a feminized GH pulse pattern. This finding was in contrast to the Bur et al. study, which indicated that the feminization of GH pulse patterns was a more potent regulator of Cyp expression than *Cry1/Cry2*, as injecting the mice with male-like GH had rescued the phenotype, despite the loss of *Cry1/Cry2*. Interestingly, *Cyp4a12a* in the *Bmal1* liver KO males looks to be expressed at the same level as in the WT males, indicating that *Bmal1* in the liver does not influence its expression. This finding is in agreement with the Bur et al. study, as the feminization of the GH pulse pattern by the loss of *Bmal1* was more potent than *Bmal1* at the level of the liver. However, to verify this hypothesis, male-like GH must be introduced to the full-body *Bmal1* KO males to rescue the expression of *Cyp4a12a*. Together, the hepatic gene expression profile in the *Bmal1* liver KO males indicates that *Bmal1* acts in the liver to regulate sexually dimorphic circadian genes.

Since *Bmal1* influenced the expression of other hepatic genes with circadian expression, we hypothesized that IGF-1 might by directly regulated as well. Like MUPs,

IGF-1 is produced at higher levels during the day. We found that IGF-1 levels are reduced in the *Bmal1* KO males. This could be caused by the increased GH secretion in the *Bmal1* KO males down-regulating IGF-1 production. Alternatively, *Bmal1* could be directly regulating the expression of *Igf-1*. We examined the *Bmal1* liver KO males' IGF-1 levels over 24 hours, and found that they were comparable to their Cre- littermates (**Figure 18**). This suggests that *Igf-1* expression is not regulated by *Bmal1* in the liver, thus the decrease in the full-body *Bmal1* KO males could be because *Bmal1* regulates the expression in all tissue types that produce IGF-1, including the skeletal muscle, reproductive tissues, and adipose tissue. The full-body *Bmal1* KO could affect IGF-1 production in all tissues, thus reducing the total serum levels of IGF-1. However, in the *Bmal1* liver KO males, IGF-1 production would only be affected at the level of the liver, while the other tissues' production would remain intact, or possibly upregulate their IGF-1 production in response to the loss of liver IGF-1. This could result in a non-significant change in serum levels of IGF-1, despite a loss of production from the liver.

This study shows that the circadian transcription factor, BMAL1, is an important regulator of masculine GH pulses and hepatic gene expression. The loss of *Bmal1* leads to decreased MUP levels, and differential expression of the MUP genes. Upon examining the GH pulse pattern in these mice, we discovered that GH was released in a feminine pattern, with more continuous secretion, leading to the hypothesis that the feminized GH was driving female-like hepatic gene expression. However, we found that the Cytochrome p450 genes were differentially expressed rather than feminized, suggesting that *Bmal1* was directly regulating their expression in the liver. Using the *Bmal1* liver KO

mouse model, we found that MUPs and Cyps were both directly regulated at the level of the liver. However, the decreased levels of IGF-1 in the full-body *Bmal1* KO males are likely due to the feminized GH pulse pattern, as the *Bmal1* liver KO males did not show the same disruption in IGF-1 production.

Future Directions

In future experiments, the functional result of altered Cyp expression should be explored. As *Bmal1* KO males produce more of some Cyps, we would expect to see a faster metabolism of the associated substrates; the opposite would be true for the downregulated Cyps. This could play an important role in determining drug doses for humans with disrupted circadian rhythms, as some drugs may be metabolized at different rates.

To verify that *Bmal1* is a more potent regulator of Cyp expression, the full-body *Bmal1* KO and the *Bmal1* liver KO should be subjected to the GH injections used in the Bur et al. study. In the *Cry1/Cry2* KO males, this rescued both Cyp and MUP expression. If *Bmal1* does in fact regulate the Cyp genes directly, we should see that the male-like GH injections do not rescue the Cyp expression in either animal model.

Conclusions

As increasing numbers of the human population are subject to circadian disruptions via shift work, jet lag, and artificial light at night, understanding the role in *Bmal1* in various tissues is becoming more important in improving health outcomes of these individuals.

Our project indicates that *Bmal1* acts directly in the liver to influence the expression of the Cytochrome p450s, a family of genes involved in the metabolism of steroids and drugs. *Bmal1* also regulates the GH axis, and the loss of *Bmal1* causes feminized GH pulses in male mice, likely resulting in a decrease of IGF-1, an important growth factor expressed in the liver in response to male-like GH pulses. Our research suggests that circadian disruptions result in an alteration of the GH axis and a dysregulated hepatic gene profile, possibly leading to altered growth and drug metabolism.

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