INVOLVEMENT OF TISSUE-TYPE PLASMINOGEN ACTIVATOR IN THE REGULATION OF CIRCADIAN RHYTHMS

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by

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TABLE OF CONTENTS

LIST OF FIGURES……iv

ACKNOWLEDGEMENTS……....vi

Introduction.....……..…….……..1

Suprachiasmatic Nucleus Background…….....……...1

Entrainment…….…….……1

Tissue-type Plasminogen Activator in the Brain…….……4

Restricted Feeding Influences Circadian Physiology and Behavior…....…7

Restricted Feeding Influences Peripheral Tissues….……8

The Food-Entrainable Oscillator….…….……...……10

Restricted Feeding and the SCN…..…….…….……...……..12

Goals.…….……13

Methods…..……..…16

Animals……..16

Experiment 1……..16

Experiment 2……..17

Experiment 3……..20

Data Analysis…….22

Results……24

Discussion……..46

References……..57

LIST OF FIGURES

Introduction

Fig 1: tPA and its Associated Signal Cascade of Events……...……...…6

Fig 2: Relay of Information between Central and Peripheral Tissues.……...…..9

Methods

Fig 3: Diagram of Restricted Feeding Protocol……19

Results

Fig 4: Experiment 1 Light-Induced Phase Shift Response……...24

Fig 5: Experiment 1 Representative Actograms……...25

Fig 6: Experiment 2 48hr Fast Activity and Sex Differences…...……28

Fig 7: Experiment 2 1st 4hr RF: FAA and Sex Differences…….29

Fig 8: Experiment 2 1st 4hr RF: Activity Profile…...……..……30

Fig 9: Experiment 2 Actograms of Male Activity During RF…..…….31

Fig 10: Experiment 2 Actograms of Female Activity During RF…...……..…32

Fig 11: Experiment 2 2nd 4hr RF: FAA and Sex Differences……...……...33

Fig 12: Experiment 2 2nd 4hr RF: Activity Profile…..…….34

Fig 13: Experiment 2 Percent Weight Change Across RF……...35

Fig 14: Experiment 3A Liver *Clock* and *Per1* Expression……...37

Fig 15: Experiment 3A Liver *Per2* Expression……38

Fig 16: Experiment 3A Liver *uPA* and *PAI-1* Expression……..……..39

Fig 17: Experiment 3A Liver *tPA* Expression……..……...40

Fig 18: Experiment 3B SCN Pre- and Post-Capture..……..42

Fig 19: Experiment 3B SCN *Clock* and *PAI-1* Expression……...43

Fig 20: Experiment 3B SCN *Per1* and *Per2* Expression……..44

Fig 21: Experiment 3B SCN *VIP* Expression……...45

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Introduction:

The suprachiasmatic nucleus (SCN) of the hypothalamus is the master circadian pacemaker in mammals. It coordinates the sleep-wake cycle as well as endocrine and metabolic functions that allow an organism to adapt to and function in its environment. The SCN is comprised of approximately 20,000 neurons (Riley and Moore, 1977; van den Pol, 1980; Güldner, 1983) and is located in the anterior hypothalamus superior to the optic chiasm and bilateral to the third ventricle (Cassone et al, 1988). Circadian rhythms are biological cycles having a period of approximately 24 hours. These rhythms are entrained to environmental cues referred to as zeitgebers. Lesions of the SCN abolish circadian behavioral and physiological rhythms (Moore and Eichler, 1972; Stephan and Zucker, 1972). This arrhythmicity can be rescued via transplantation of donor SCN tissue, with the recipient exhibiting the period length of the SCN donor (Ralph et al., 1990; LeSauter et al., 1996). Light serves as the predominant zeitgeber to which circadian rhythms entrain. However, nonphotic environmental cues such as scheduled feeding, cage changes or introduction of a novel wheel to rodents, among many others, can also serve as cues to entrain the master circadian clock. In the absence of an external cue, circadian rhythms persist and are said to "free-run" with intrinsic periods of approximately 24 hours (Aschoff, 1967).

Entrainment

Photic cues entrain SCN activity via both monosynaptic and polysynaptic pathways between retinal ganglion cells and SCN neurons. Retinal ganglion cells directly innervate the SCN via the retinohypothalamic tract (RHT), which carries a signal down the optic nerves and through the optic chiasm to the SCN. This projection uses glutamate as its primary neurotransmitter (Meijer et al., 1988). Severing the RHT prevents photic entrainment of locomotor activity to the light/dark cycle (Johnson et al., 1988), and application of glutamate receptor antagonists prevents activation of the SCN via RHT both *in vivo* and *in vitro* (Cahill & Menaker, 1989). The intergeniculate leaflet serves as an indirect input to the SCN relaying information about photic and nonphotic cues via Neuropeptide Y (NPY) and GABA-ergic innervations (Rusak and Boulos, 1981; Harrington et al., 1987; Moore and Speh, 1993), and the raphe nuclei located in the midbrain also relay nonphotic cues and modulate photic responses: The median raphe directly innervates the SCN via serotonergic projections, while the dorsal raphe innervates the IGL (Morin & Meyer-Bernstein, 1999).

In turn, the SCN relays rhythmic output, via neural and humoral signals, to other secondary pacemakers located in many peripheral cells in turn influencing physiology and behavior, such as body temperature, endocrinology, sleep-wake cycles, and gastrointestinal, heart, kidney, and liver function (as reviewed by Gachon, 2004). In the absence of the SCN, the cycles of peripheral clocks dampen and virtually disappear after just a few oscillations unless entrained by an external cue. Therefore, the clocks in peripheral tissues are referred to as “slave oscillators” since their rhythmicity is dependent on and entrained to signaling from the master circadian pacemaker. SCN-

dependent cues synchronize and sustain circadian organization in the periphery which ensures behavioral, physiological, and metabolic events occur at the appropriate time.

The entrainment of peripheral functions is dependent upon the circadian oscillations of core clock gene expression within the SCN. In simplified terms, this oscillator consists of autoregulatory transcriptional feedback loops of clock genes and their protein products and has both a positive and negative element. The positive arm depends on the transcription and translation of Bmal1 and Clock genes into their protein products. The Clock gene is expressed constitutively in the SCN throughout the circadian period (Vitaterna et al., 1994) while Bmal1 expression oscillates with a circadian rhythm, peaking in the middle of the dark phase (Ikeda & Nomura, 1997). Bmal1 and Clock proteins form heterodimers and acting as transcription factors bind to a specific regulatory DNA sequence, referred to as an E-box, located within the promoter region of many genes. If a gene has an E-box in its promoter region it is likely to be expressed in a circadian rhythm, and therefore is referred to as a clock controlled gene. Bmal1/Clock heterodimers activate the negative limb of the feedback loop by binding to the E-box of Period (Per) and Cryptochrome (Cry) genes and initiate the production of their protein products. Per and Cry proteins form homo- and heterodimers and suppress the E-box dependent gene activation across the next 12 hours. Per and Cry proteins are gradually degraded by proteasomes, thereby lessening the suppression of clock controlled gene activation, allowing Clock/Bmal1 heterodimers to reinitiate the cycle (as reviewed by Hastings et al., 2008). An accessory loop consisting of proteins RORA and Rev-erbα

enhances the activity of the core oscillatory cycle via activation and suppression, respectively, of Bmal1 expression through acting on RORE regulatory sequences (as reviewed by Hastings et al., 2008).

Exposure to light can have profound effects on the behavior of an animal, depending on when the light exposure occurs during the circadian period. Such effects can be defined as a phase shift, during which the animal deviates its active phase to begin or end earlier or later than expected, depending on whether the light pulse is given during the subjective day or night of the animal. A light pulse given during the subjective day has no effect on activity rhythms. However, a light pulse given early in subjective night causes a phase delay, and a light pulse given late in subjective night causes a phase advance (Daan & Pittendrigh, 1976). Glutamate acts as a neurotransmitter and binds to NMDA receptors in the SCN, triggering a signal cascade in events that ultimately results in the phase-shifting of activity (Ding et al., 1994; Mintz & Albers, 1997; Mintz et al., 1999).

Tissue-type Plasminogen Activator in the Brain

The extracellular protein, tissue-type plasminogen activator (tPA), is a serine protease traditionally known for its fibrinolytic functions in the blood, but which also has been shown to modulate normal CNS physiology including processes such as synaptic plasticity and learning and memory. Sappino et al. (1993) first detected tPA in neural tissues and observed that tPA-dependent proteolysis occurs in discrete brain regions. In the CNS, much like in the bloodstream, tPA is responsible for converting plasminogen

into its active form, plasmin, which in turn targets several other substrates for cleavage, including the neurotrophin brain-derived neurotrophic factor (BDNF) (Plow et al., 1995). Plasmin is responsible for cleaving proBDNF into its active form, mature BDNF, which in turn modulates neuronal survival and synaptic activity and plasticity through activation of its tyrosine kinase receptor TrkB (Lee et al., 2001) (Note: See Figure 1 for diagram illustrating tPA and its associated signal cascade). Both BDNF and the TrkB receptor have been localized in the SCN (Liang et al., 1998), and BDNF is expressed rhythmically in the SCN, peaking during the early subjective night (Liang et al., 1998). The localization of BDNF and TrkB expression in the SCN-optic chiasm region raises the possibility of BDNF mediation of signaling interactions between the RHT and SCN cells (Liang et al., 1998). Further supporting this, BDNF +/- mice exhibit reduced light-induced phase shifts, and when given a TrkB antagonist both light and glutamate-induced phase shifts in the SCN are blocked *in vivo* and *in vitro* (Liang et al., 2000; Michel et al., 2006; Mou et al., 2009). tPA and all proteins associated with its activation cascade have been identified in the SCN and are critical to its normal physiology, specifically mediating glutamate-induced phase shifts via BDNF activation of TrkB receptor (Mou et al., 2009). In the presence of pharmacological inhibitors of tPA and its associated proteins, the shifting capability of the SCN is markedly reduced, indicating that BDNF is acting as a "gatekeeper", and its presence is critical to normal SCN activity (Mou et al., 2009).

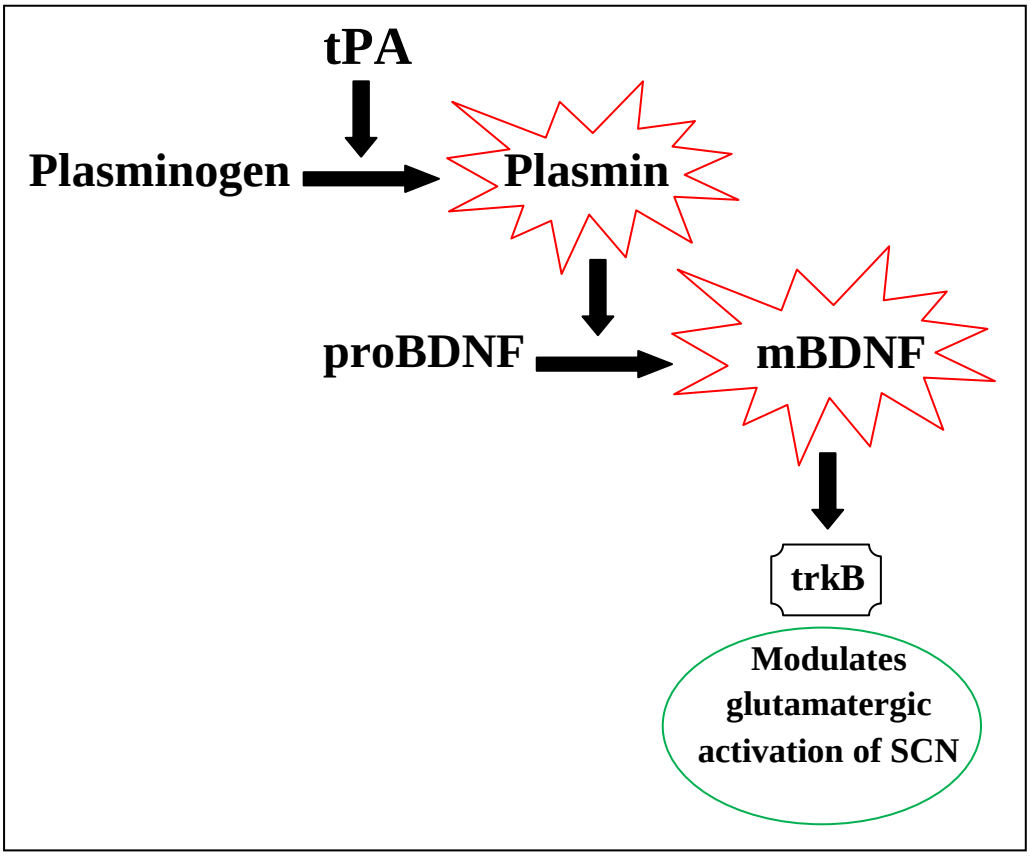


Figure 1: Diagram illustrating tPA and its associated signal cascade of events.

Restricted Feeding Influences Circadian Physiology and Behavior

The SCN controls the phase of peripheral tissues by orchestrating a rest/active cycle, which in turn determines a daily feeding cycle (Mendoza, 2006). When food availability is limited to a short time interval at a particular time each day, mammals quickly develop a new component of daily behavioral activity referred to as food anticipatory activity (FAA). During FAA the animal displays increased blood glucose, locomotor activity, blood plasma corticosterone and body temperature shortly before the time of daily food presentation (as reviewed by Feillet, 2006). However, upon lesioning of the SCN, FAA still persists, indicating the presence of a separate oscillator referred to as the food-entrainable oscillator (FEO) (Stephan et al., 1979a,b). Therefore, the SCN entrains circadian rhythm expression to the light/dark cycle, while the food-entrainable oscillator entrains to food availability. Since rodents have limited energy stores and can only survive a mere few days without food, FAA is believed to be an adaptive mechanism that induces the animal to forage and actively seek out food. FAA is robust and stable over many daily cycles, and is still exhibited when food availability is limited to the inactive phase of an animal during the light/dark cycle (Storch et al., 2009). When feeding is shifted, the expression of FAA shifts with it.

During *ad libitum* feeding the activity of the FEO and SCN are in sync, and the activity component controlled by each cannot be distinguished. Evidence of coupling between the FEO and SCN is indicated by the finding that arginine vasopressin (AVP) release from the SCN has a delayed onset and an earlier decline in rats fed during the

light phase (Kalsbeek et al., 1998). The uncoupling of activity between the FEO and SCN during restricted feeding (RF) has also been observed, and phase advances of circadian rhythms of gene expression in the liver, kidney, heart, pancreas, but not the SCN are observed during FAA (Damiola et al., 2000; Stokkan et al., 2001; Hara et al., 2001).

Restricted Feeding Influences Peripheral Tissues

The liver is directly involved with food processing and energy homeostasis, and most rhythmically expressed hepatic genes code for enzymes or regulatory proteins involved in these functions (Damiola et al., 2000). During restricted feeding, while under a 12:12 light/dark cycle, uncoupling of SCN activity and the periphery occurs, during which the circadian clock in the liver shifts and entrains to the restricted feeding schedule while SCN activity remains entrained to the light/dark cycle (Damiola et al., 2000; Stokkan et al., 2001; Hara et al., 2001). This uncoupling may be facilitated by behavioral and physiological changes associated with the altered feeding schedule and sustained by the FEO. The FEO and SCN may directly or indirectly communicate with peripheral oscillators through sympathetic and parasympathetic neurons of the autonomic nervous system (as reviewed by Mendoza, 2006) (Note: See Figure 2 for possible relay between FEO, SCN, and peripheral tissues). Although the FEO can function in the absence of the SCN, the SCN may be required to maintain the amplitude of meal-entrained rhythms and may control the period and phase of the FEO as long as food availability is unrestricted (Stephan, 2002).

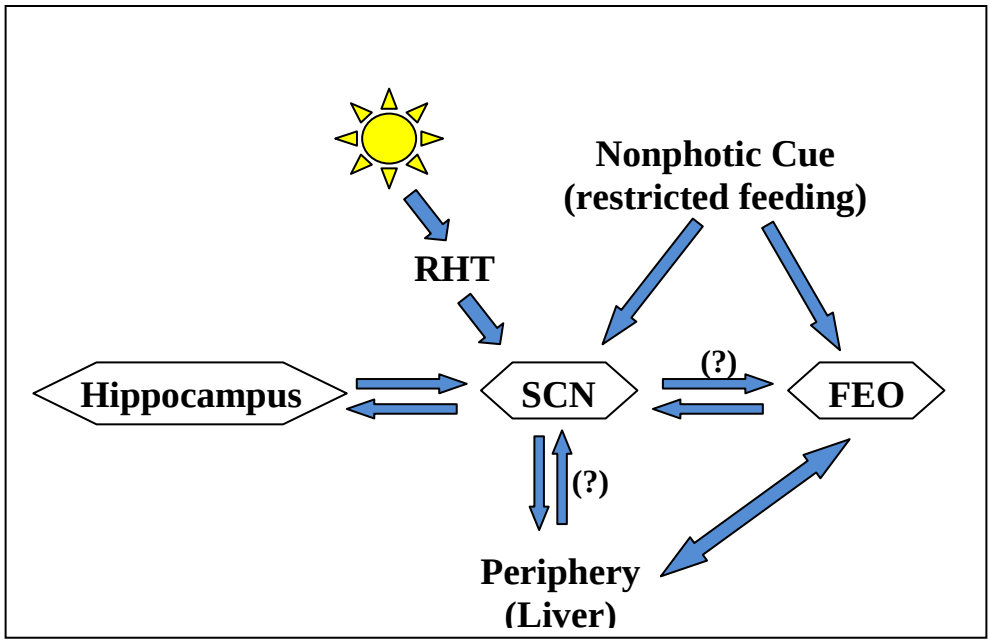


Figure 2: Schematic of possible relay of photic and nonphotic information between central and peripheral tissues.

The Food-Entrainable Oscillator

There has been much controversy over the exact anatomical location of the FEO—whether it arises from a single central or peripheral structure or multiple central and peripheral regions. Scientists have examined the gastrointestinal system, since organs such as the intestine, kidney, or liver can be entrained by RF, indicating that the FEO could possibly be located there. However, rats made cirrhotic with CCl_4 injections still exhibited FAA during RF (Escobar et al., 2002). If the FEO were localized in a particular organ of the gastrointestinal system, Davidson et al. (2003) expected to observe a correlation between the phase of the organ's rhythms and the phase of FAA which would peak diurnally and persist after the termination of restricted feeding. Using Per1-luciferase transgenic rats, it was observed that FAA did not arise as an output of rhythms from the liver, esophagus, stomach, or colon as *Per1* expression shifted and peaked nocturnally when rats were switched back to ad libitum feeding (Davidson et al., 2003). Therefore, this suggests that these organs may receive efferent signals from the FEO, and the FEO may arise as a central region or network of regions that receive and integrate information from the periphery, which in turn generates a new signal that is delivered to the peripheral organs (Feillet et al., 2006). The brainstem nuclei which receive information from the gastrointestinal system exhibit increased *c-fos* expression after food presentation in rats on a restricted feeding schedule, supporting this possible relay of information between central and peripheral structures (Angeles-Castellanos et al., 2005). Lesions of central structures such as the hippocampus, neocortex, ventral medial nucleus

of the hypothalamus, arcuate nucleus of the hypothalamus, area postrema, and olfactory bulbs do not abolish FAA (as reviewed by Feillet et al., 2006). However, specific damage to the core but not the shell of the nucleus accumbens reduces FAA (Mendoza et al., 2005a). Lesions of the parabrachial nucleus altered FAA and the rise in body temperature; but it is believed to relay information to or from the FEO and is not the FEO itself (Davidson et al., 2000). In support of the hypothesis that the FEO consists of a network of structures, c-fos immunoreactivity was observed before and after meal time in food entrained rats in regions such as the nucleus accumbens, basolateral and central amygdala, bed nucleus of the stria terminalis, lateral septum, prefrontal cortex, and paraventricular nucleus of the thalamus (Angeles-Castellanos et al., 2007).

A particular structure of interest is the dorsomedial hypothalamus (DMH) because it projects to brain regions critical for the regulation of sleep and body temperature, and receives efferent signals from the SCN. It integrates circadian and energy information to modulate physiological and behavioral processes, including the sleep/wake cycle, body temperature, and locomotor activity (as reviewed by Mendoza, 2006). Chemical lesions of the DMH impair FAA expression, but electrolytic lesions of the DMH do not affect the expression of FAA (Gooley et al., 2006; Landry et al., 2006). Also, clock gene expression in DMH neurons is induced by a restricted feeding schedule, and these neurons exhibit a daily rhythm of clock gene expression that is set by mealtime. However, upon DMH ablation rhythmic clock gene expression in the arcuate nucleus, nucleus of the solitary tract, and area postrema, FAA, and the accompanying rise in body

temperature are unaffected, indicating that the DMH is sensitive to the daily restricted feeding but is not the primary oscillator driving FAA (Moriya et al., 2009).

Restricted Feeding and the SCN

Food restriction can synchronize peripheral clocks with or without an effect on clock gene expression in the SCN, depending on the amount of caloric intake. Conflicting results have been reported for SCN entrainment in response to restricted feeding of mice housed in constant dark (DD). Caloric content (i.e., hypocaloric vs. normocaloric) of food during restricted feeding as well as timing of food availability in relation to the start of the free running rhythm appear to influence SCN entrainment to RF during DD in mice. Thus, depending on experimental design, restricted feeding may or may not be an effective zeitgeber to SCN neurons, but is certainly a dominant zeitgeber to peripheral tissues. When implementing hypocaloric RF during which food is limited to approximately 60-70% of normal caloric intake, SCN circadian rhythmicity is affected in both rats and mice. In rats under 12:12 light/dark schedule with hypocaloric RF, there is a phase advance of circadian rhythms of locomotor activity, body temperature, and melatonin in comparison to *ad libitum* animals (Challet et al., 1997). These results suggest that a hypocaloric RF has an effect on SCN since all the rhythms influenced are controlled by the SCN. Mice exposed to hypocaloric RF exhibit phases advances in behavioral running activity and in *Per1*, *Cry2*, and *AVP* expression in the SCN (Mendoza et al., 2005b). The alterations of mRNA and protein levels in the SCN may result from competition between food and light synchronizing signals on the

circadian molecular loops (Mendoza, 2006). Therefore, the question arises: Why does hypocaloric RF strongly affect SCN activity while RF alone may or may not? This may be due to hypometabolic state of the animals in response to chronic calorie restriction, which could lead to a decreased sensitivity of the SCN clock to light-resetting properties.

Changes in metabolic status affect the circadian feedback loop: NADPH (i.e., the metabolic indicator of high energy) increases affinity of clock proteins for their target DNA's promoter E-box, while NADP (i.e., the metabolic indicator of low energy) inhibits clock proteins from binding to target DNA (Rutter et al., 2001). Hypocaloric RF may also modify circadian responses to nonphotic cues. Since nonphotic entrainment of SCN activity works via serotonergic and NPY-ergic projections from raphe nuclei and intergeniculate leaflets to the SCN, respectively, these brain regions may be responsible for conveying information directly or indirectly from the FEO to the SCN about the metabolic state of the body during hypocaloric RF (as reviewed by Mendoza, 2006).

Goals

The experiments described in this thesis utilized mice deficient in tPA (tPAKO) in order to determine the significance of this extracellular protease in circadian physiology, and the mechanisms through which it modulates normal brain physiology. Since BDNF activity is a necessary component of the photic entrainment pathway, we sought to determine the impact of the loss of tPA on entrainment of circadian activity rhythms. Michel et al. (2006) observed that in the presence of trkB receptor inhibitor K252a that BDNF-induced phase advances and delays were significantly reduced in the SCN,

indicating BDNF activation of trkB is critical to normal phase-shifting behavior. We will examine the light-induced phase response of tPAKO mice in comparison to wildtype (WT) mice and hypothesize that since tPAKO mice are going to have little mBDNF, due to deficiency of the key protease responsible for its generation in the CNS, tPAKO mice will exhibit a reduced light-induced phase shift response in comparison to wildtype (WT) mice.

Huang et al. (1996) demonstrated that tPAKO mice exhibit deficiencies in the late phase of long-term potentiation in the hippocampus, and tPAKO mice display impaired performance in context conditioning tests (a hippocampus-related task) and two-way avoidance tests (a striatum-dependent task) (Calabresi et al., 2000). Upon exposure to a nonphotic cue such as restricted feeding, we hypothesized that tPAKO mice will have difficulty learning the daily restricted feeding schedule and thus will exhibit reduced FAA in comparison to WT mice.

Damiola et al. (2000) as well as Hara et al. (2001) observed a shift in the expression of *Per1* and *Per2* in the liver of mice on a restricted feeding schedule with virtually no effect on clock gene expression in the SCN. On the other hand, Castillo et al. (2004) reported a shift in *Per2* expression in the SCN of mice exposed to restricted feeding. We will examine clock gene expression in the SCN and liver during the phase of FAA in tPAKO mice in order to determine the central and peripheral mechanisms through which tPA may modulate the circadian behavioral and physiological response to restricted feeding. We hypothesize that tPAKO mice will exhibit a differential circadian

response to a restricted feeding schedule and will have altered period gene expression in comparison to WT mice.

Methods:

Animals

Male and female tPAKO mice were generated at and purchased from the Jackson Laboratory (Bar Harbor, Maine, USA). The tPAKO mice were fully backcrossed into the same background C57BL/6J strain as wildtype (WT). WT and tPAKO mice were bred and group-housed in the Kent State University animal facility in a 12h-light/12h-dark cycle (12L:12D) and fed *ad libitum*. Male and female mice aged 6-12 weeks of age at the beginning of each experiment were used. Animals used for each study were age-matched as closely as possible. Experiments were conducted in accordance with Kent State University Institutional Animal Care and Use Committee.

Experiment #1: Light-Induced Phase Shift Response of tPAKO vs. WT mice

Animals were individually housed in constant dark for at least 10 days with free access to running wheels and activity monitored using ClockLab software. tPAKO and WT mice were given a light pulse of either 300, 50, or 5 lux for 15 min at CT16 or 300 lux at CT22, determined by activity onset defined as CT12. Light intensities were measured at the cage bottom using a lux meter, and each animal was exposed to only one light pulse. Phase shifts were calculated using ClockLab software. This software utilizes a linear regression method proposed by Daan and Prittendrigh (1976). A line was fitted to activity onsets 10 days prior to the light pulse. A second line was fitted to activity

onsets 4-10 days after light pulse. Days 1-3 after the light pulse were not included in the data analysis due to transitory effects. The phase shift was equal to the difference between the two regression lines. All data shown in the results represents mean ± standard error of the mean.

Experiment #2: Behavioral Response of tPAKO vs. WT mice to Restricted Feeding

Male and female tPAKO and WT mice were individually housed in 12L:12D with free access to running wheels and food and water *ad libitum* for at least 10 days and activity monitored using ClockLab software. The experiment was run in three series and all data grouped and analyzed collectively. In the first RF series, 4 tPAKO male and 4 WT male were used, 3 female and 4 male from each genotype were used in the second series, and 3 female and 3 male from each genotype were used in the third series (Note: See Fig. 3 for diagram of feeding protocol). When put on restricted feeding schedule, animals were fed *ad libitum* for at least 3 days then food deprived for 48 hours, starting at ZT12. After the 48hr food deprivation, animals were fed *ad libitum* for 3 consecutive days starting at ZT12 followed by 2 consecutive days of 8hr RF (ZT4-ZT12), 2 consecutive days of 6hr RF (ZT4-ZT10), then 4 consecutive days of 4hr RF (ZT6-ZT10). Animals were food deprived the following day totaling a 44 hour food deprivation, and 4hr RF was resumed for the next 6 consecutive days (ZT6-ZT10). Animals were switched to 6 days of *ad libitum* feeding, then food deprived for 48hrs, starting at ZT12. Animals were weighed during beginning *ad libitum* feeding, after the first 48hr fast, at the end of the 3 consecutive days *ad libitum*, the last day of 8hr RF, the last day of 6hr

RF, the fourth day of 4hr RF, the last day of 4hr RF, and at the end of the last *ad libitum* feeding to monitor for differential weight gain/loss between genotypes. Food was put on the bottom of the cage during RF in order to optimize access to food and for easier retrieval of food in case animals became lethargic as mentioned by Pendergast et al. (2009).

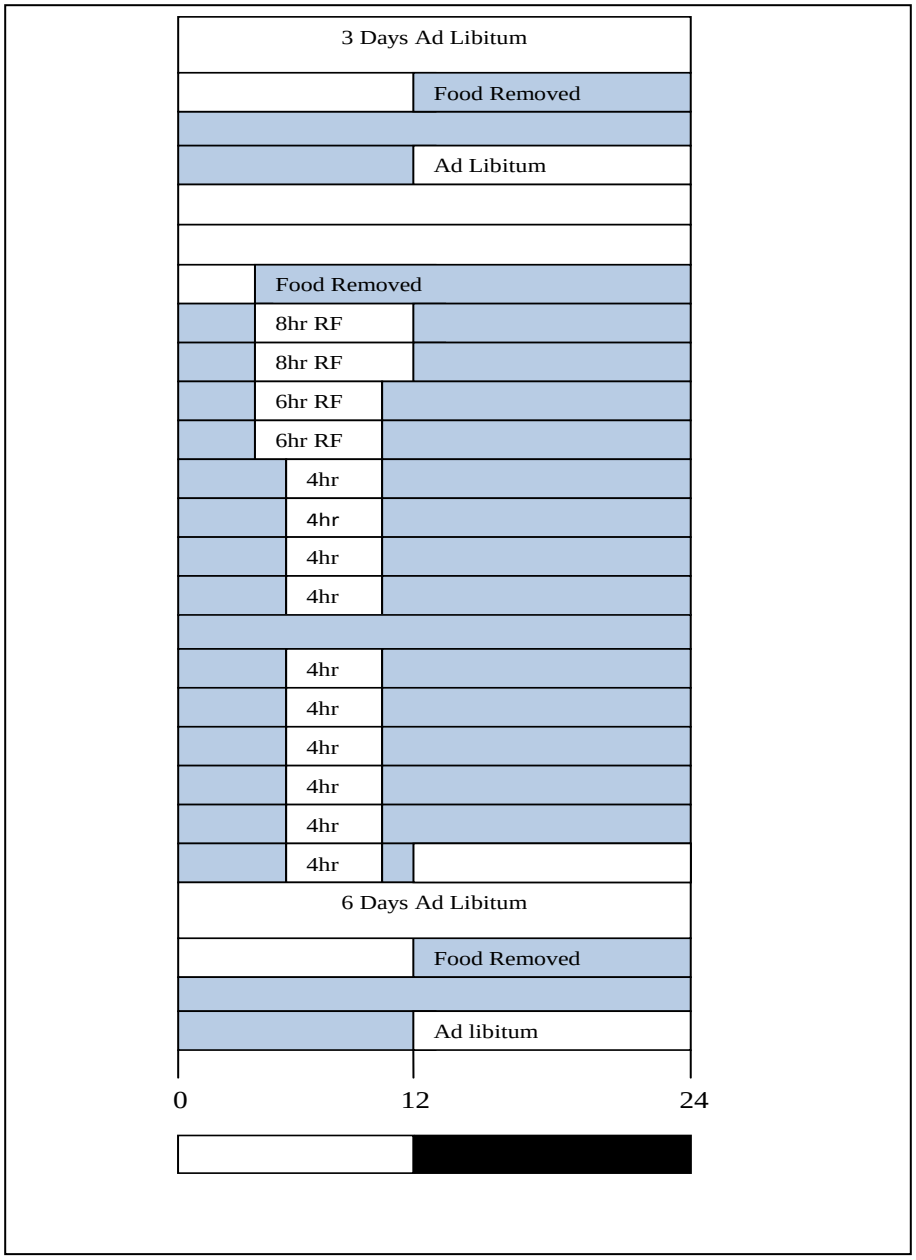


Figure 3: Diagram depicting restricted feeding protocol adapted from Pendergast et al. (2009). Animals were housed 12L:12D LD with lights off at ZT12. Clear regions indicate time of food availability, while blue shaded regions indicate time of food withdrawal.

Experiment #3: Effects of Food Restriction and Genotype on Gene Expression in the Liver and SCN

Male tPAKO and WT mice were individually housed in 12L:12D with free access to running wheels and food and water *ad libitum* for at least 10 days. Only males were used in this experiment since females of both genotypes failed to exhibit significant FAA during RF. 7 tPAKO and 7 WT mice were put on a restricted feeding schedule, while 7 tPAKO and 7 WT mice were fed *ad libitum* as the control. When put on restricted feeding schedule, animals were fed *ad libitum* for at least 3 days then switched to 2 consecutive days of 8hr RF (ZT4-ZT12), 2 consecutive days of 6hr RF (ZT4-ZT10), followed by 4-5 consecutive days of 4hr RF (ZT6-ZT10). Animals were sacrificed at ZT5, in the midst of FAA expression and one hour prior to feeding time, via cervical dislocation. Brains and livers were rapidly removed and immediately frozen in isopentane or on dry ice, respectively, and stored at -80°C.

SCN Gene Expression

Brains were sliced into 12 µm sections on the cryostat and immediately mounted on positively charged glass slides. Sections were then processed for hemotoxylin staining in order to visualize the SCN. First sections were submerged in 75% ethanol solution for 30 seconds, rinsed in water to remove excess ethanol, then submerged in hemotoxylin (Vector Laboratories) for 90 seconds. Then excess hemotoxylin was rinsed off in molecular biology grade water. Sections were then submerged in a dehydration series: 30 seconds in 75% ethanol solution, 30 seconds in 95% ethanol solution, and 30

seconds in 100% ethanol. Sections were then immersed in xylenes for at least 5 minutes before capture. Sections were removed from xylenes and the xylenes allowed to evaporate before being loaded into a laser capture microscope (Arcturus Autopix, MDS Analytical Technologies). The SCN was identified and captured using Capsure HS LCM Caps (Molecular Devices, MDS Analytical Technologies). Six consecutive SCN sections were captured from each animal, and pre- and post-capture images were taken in order to confirm the specificity of the SCN. The samples were purified using an RNA purification kit (Picopure, MDS Analytical Technologies), including a DNase treatment. Purity of the samples was established using a 1 µl aliquot from each using Nanodrop Spectrophotometer ND-1000 (Thermo Scientific). Samples having a 260/280 ratio higher than 2.0 and and 260/230 ratio between 2.0-2.2 were used in the assay. RNA was stored at -80°C until assayed.

Quantitative real-time PCR (qPCR) was carried out using Applied Biosystems Prism 7000 sequence detection system. Purified RNA from each sample was reverse transcribed in a 100 µl reaction using Taqman Reverse Transcription reagents kits. For quantification of each gene, a total of 2 µl of cDNA was used per triplicate reaction. *Clock*, *Per1*, *Per2*, *uPA*, *tPA*, and *PAI-1* were the genes assayed. *GAPDH* served as the constant expression control gene, and *VIP* was used to confirm SCN specificity of the samples as *VIP* is expressed throughout the SCN but not in the surrounding tissue. To insure that *GAPDH* could serve as a control gene, a 2-way anova was performed on the CT values of *GAPDH*, having genotype and feeding treatment as the two factors. No

significant effects were detected, suggesting that *GAPDH* expression did not differ between genotypes and feeding treatments.

Liver Gene Expression

Approximately 20 mg of each liver sample was homogenized using a sonicator and purified using an RNA purification kit (Qiagen RNeasy Mini Kit), including a DNase treatment. Purity of the samples was established using a 1 µl aliquot for each using Nanodrop Spectrophotometer ND-1000 (Thermo Scientific). Samples having a 260/280 ratio higher than 2.0 and and 260/230 ratio between 2.0-2.2 were used in the assay. Quantitative real-time PCR (qPCR) was carried out using an Applied Biosystems Prism 7000 sequence detection system. Purified RNA from each sample was reverse transcribed in a 100 µl reaction using Taqman Reverse Transcription reagents kits. For quantification of each gene, a total of 6 µl of cDNA was used per 60 µl triplicate reaction, having 20 µl per well. *Clock, Per1, Per2, uPA, tPA,* and *PAI-1* were the genes assayed. *GAPDH* served as the constant expression control gene for qPCR analysis.

Data Analysis

T-tests were performed on data from Experiment #1 to evaluate significant differences between genotypes using Microsoft Excel software. Data from Experiment #2 were analyzed using repeated measures two-way ANOVA (sex and genotype as the factors, and the day of RF as the repeated factor). The percent change in body weight across the RF protocol was analyzed using two-way ANOVA (sex and genotype as the factors). Data from Experiment #3 were also analyzed using a two-way ANOVA

(genotype and feeding treatment as the factors). Tukey-Kramer post-hoc tests were performed to determine differences between individual factor combinations. NCSS Statistical software was used to perform all ANOVA and post-hoc analyses.

Results:

Experiment 1: Light-Induced Phase Shift Response of tPAKO vs. WT mice

In this experiment, the light-induced phase response of tPAKO mice was evaluated in comparison to WT mice. tPAKO mice did not exhibit a significantly different light-induced phase response in comparison to WT mice when given a light pulse at either ZT16 at 300 (tPAKO = -2.1 ± 0.39 and WT = -2.5 ± 0.59, p=0.59), 50 (tPAKO = -1.9 ± 0.08 and WT = -1.8 ± 0.27, p=0.84), or 5 lux (tPAKO = -1.4 ± 0.19 and WT = -1.2 ± 0.27, p=0.55) or ZT22 at 300 lux (tPAKO = 0.4 ± 0.02 and WT = 0.5 ± 0.16, p=0.67) (Figure 4 & 5).

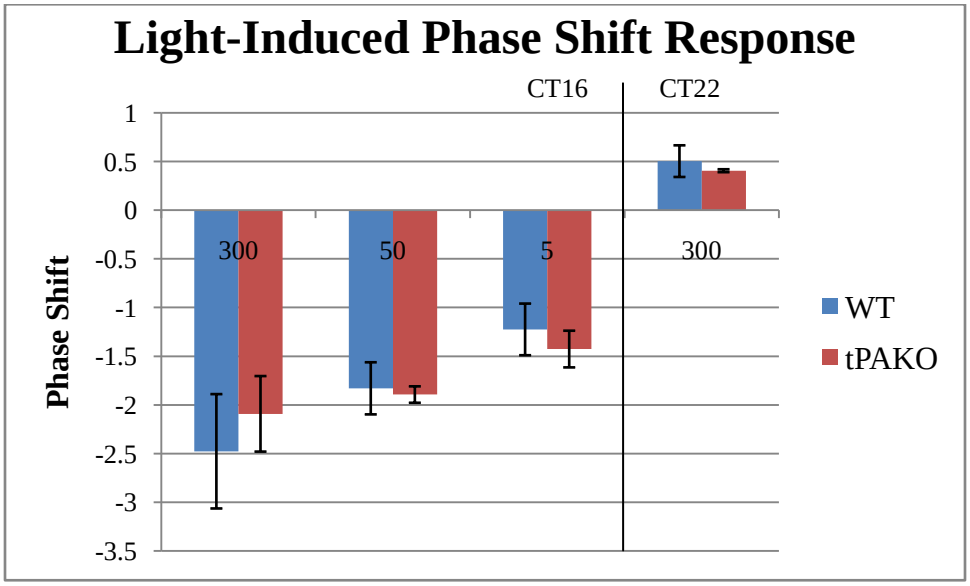


Figure 4: There was no significant difference in light-induced phase shift response in tPAKO vs. WT mice at 300 (p=0.59), 50 (p=0.84), or 5 lux (p=0.55) at CT16 or 300 lux (p=0.67) at CT22. Error bars represent the standard error of the mean.

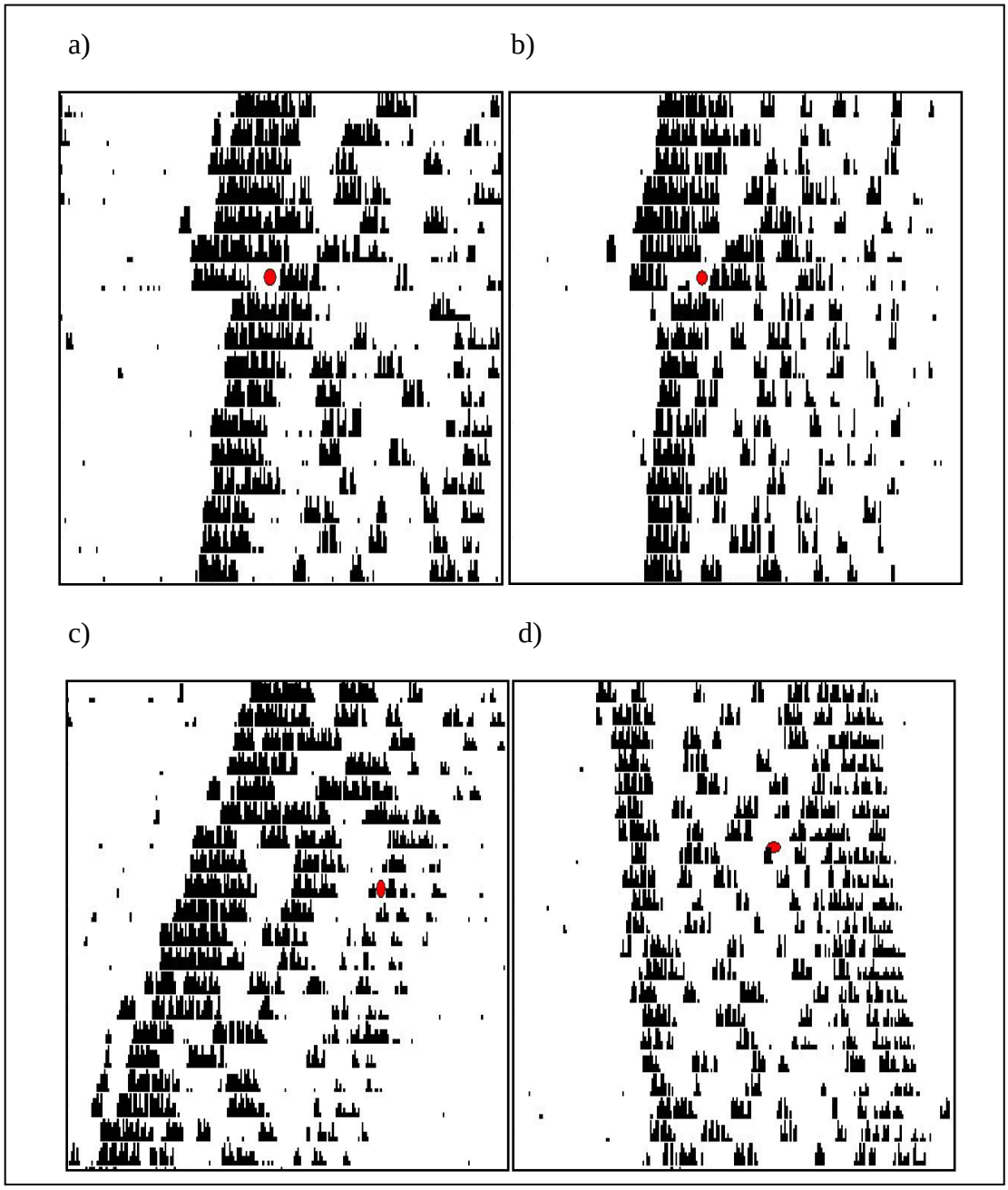


Figure 5: Representative actograms showing light-induced phase responses of WT and tPAKO mice. Phase delay of (a) WT and (b) tPAKO mice receiving a light pulse of 300 lux at CT16. Phase advance of (c) WT and (d) tPAKO mice receiving a light pulse of 300 lux at CT22. Red circles indicate time of light pulse.

Experiment 2: Behavioral Response to Restricted Feeding Schedule

In this experiment, wheel-running activity in general and food anticipatory activity specifically of tPAKO in comparison to WT mice during *ad libitum* feeding or a restricted feeding schedule were evaluated. There was a significant difference in the expression of FAA (ZT3 to ZT6) between genotypes during the first four days of 4hr restricted feeding ($p=0.0075$) as well as between sexes ($p=0.0019$) (Figure 7 & 8). Also, a significant interaction between genotype and sex was observed ($p=0.03$). tPAKO mice exhibited significantly enhanced FAA compared to WT mice (Figure 8). Indicative of a behavioral sex difference, the increase in FAA was restricted to males since no significant increase in FAA was observed in female tPAKO mice (Figures 9 & 10).

Following the interim 44 hour fast, FAA expression was boosted on the immediate subsequent days (Figure 12). As the restricted feeding schedule continued across the next six days, the significant difference in FAA expression between genotypes gradually disappears (Figure 9). However, there is still a significantly dampened FAA expression in females of both genotypes ($p=0.0017$) as compared to males, and no significant interaction observed ($p>0.05$).

Animals were exposed to a 48hr fast to monitor fast-induced activity (Figure 6). Interestingly, the baseline activity of females of both genotypes was higher in comparison to males, but they exhibited significantly lower fast-induced activity in comparison to males ($p=0.02$). There was no significant difference detected between genotypes

($p>0.05$). Therefore, this suggests that the enhanced FAA expression of tPAKO mice is specific to the restricted feeding schedule.

Animals were weighed throughout the RF protocol in order to monitor for differential weight gain/loss between genotypes (Figure 10). There is no significant difference between genotypes ($p>0.05$) for percent weight change between initial *ad libitum* and after the 1st 48hr fast, indicating that each genotype loses weight at the same rate during fasting. Also, there is no significant difference between genotypes ($p>0.05$) for percent weight change between weight measured before the start of RF and the last day of 4hr RF, exhibiting that the RF paradigm does not result in differential weight loss between genotypes.

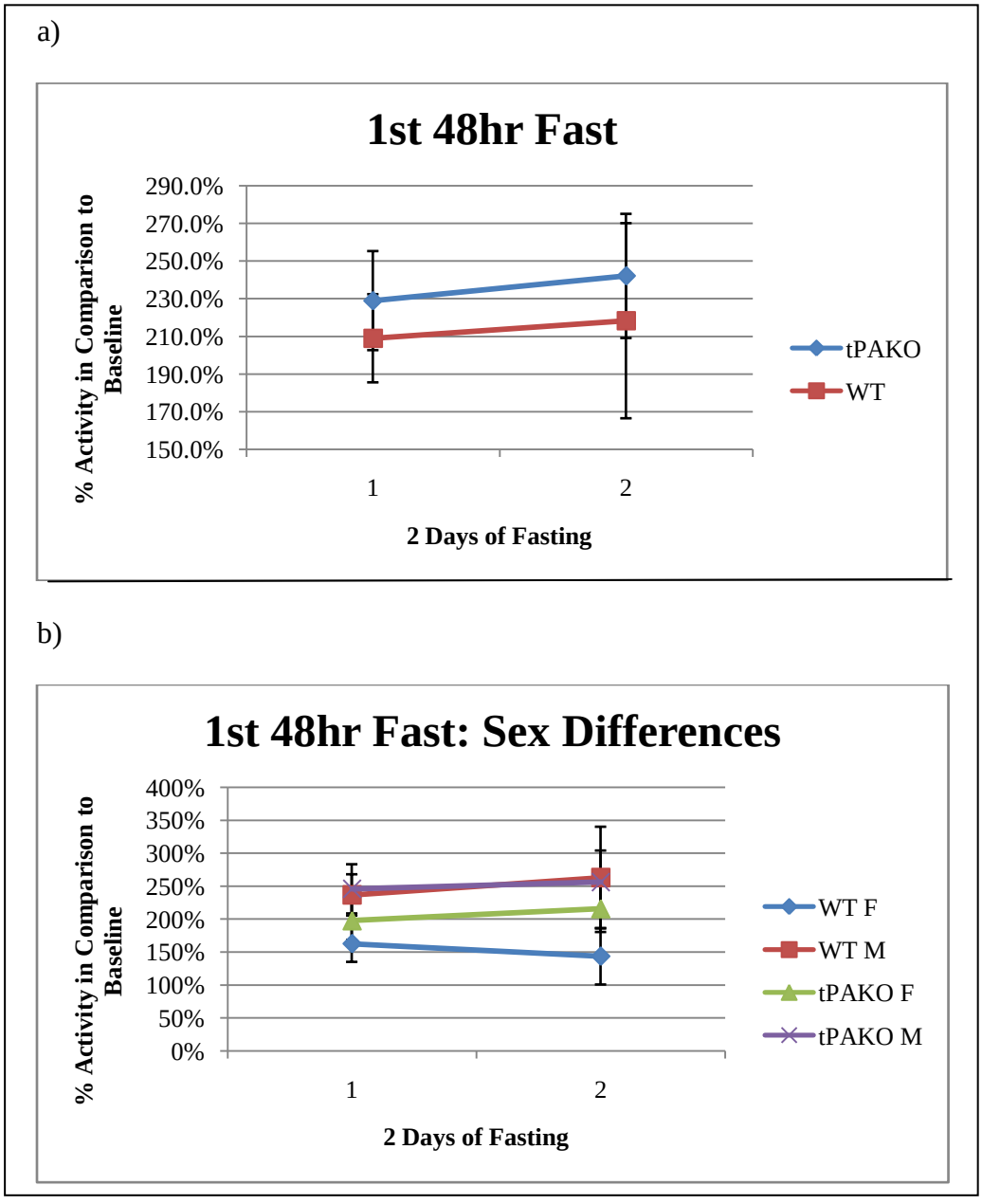
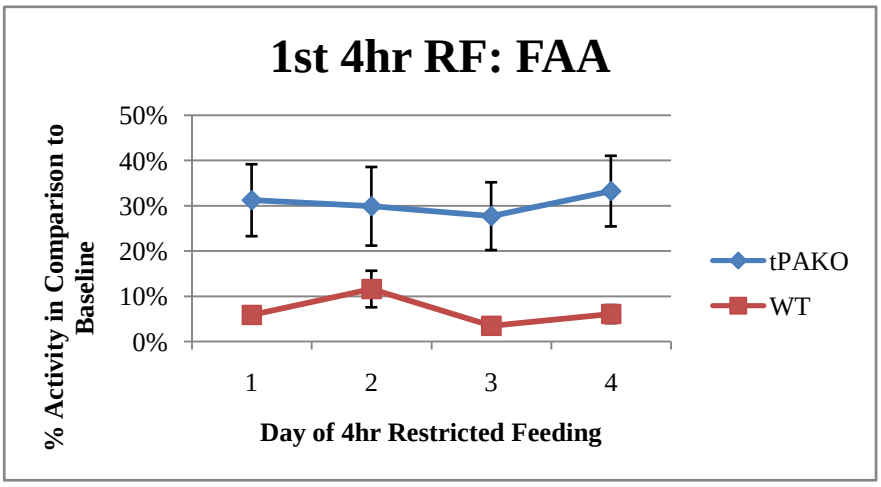


Figure 6: (a) Both tPAKO and WT mice show an increase in activity in comparison to baseline, but no significant difference in fast-induced activity between genotypes was detected ($p>0.05$). (b) Females of both genotypes exhibit significantly lower fast-induced activity in comparison to males ($p=0.02$) (b). Data have been normalized and is expressed as a percentage of overall baseline activity to account for variability in the amount each animal runs on a daily basis. Error bars represent the standard error of the mean.

a)



b)

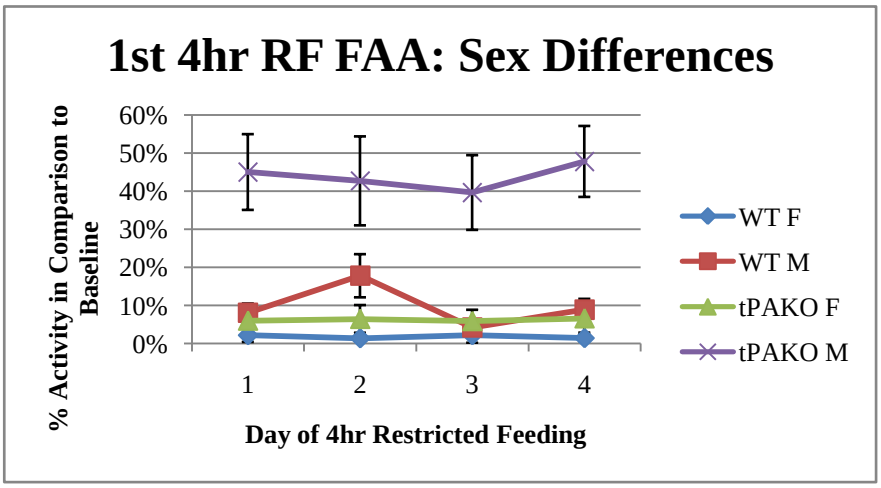


Figure 7: There is a significant difference in the expression of FAA between genotype (a) and sex (b). tPAKO mice show significantly enhanced FAA (p=0.0075) in comparison to WT mice during the first 4 days of restricted feeding. Females of both genotypes exhibit significantly reduced FAA in comparison to males (p=0.0019). Data have been normalized and is expressed as a percentage of overall baseline activity to account for variability in the amount each animal runs on a daily basis. Error bars represent the standard error of the mean.

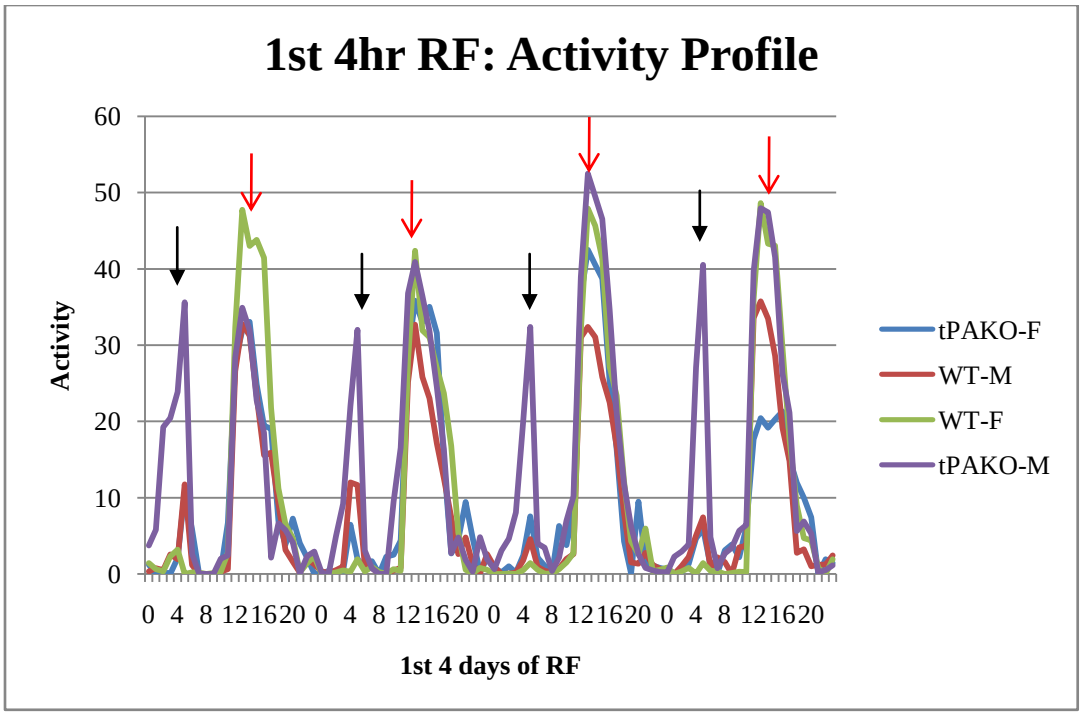


Figure 8: Activity profile of the 1st 4 days of restricted feeding. Black arrows indicate food anticipatory activity, while red arrows indicate nocturnal activity.

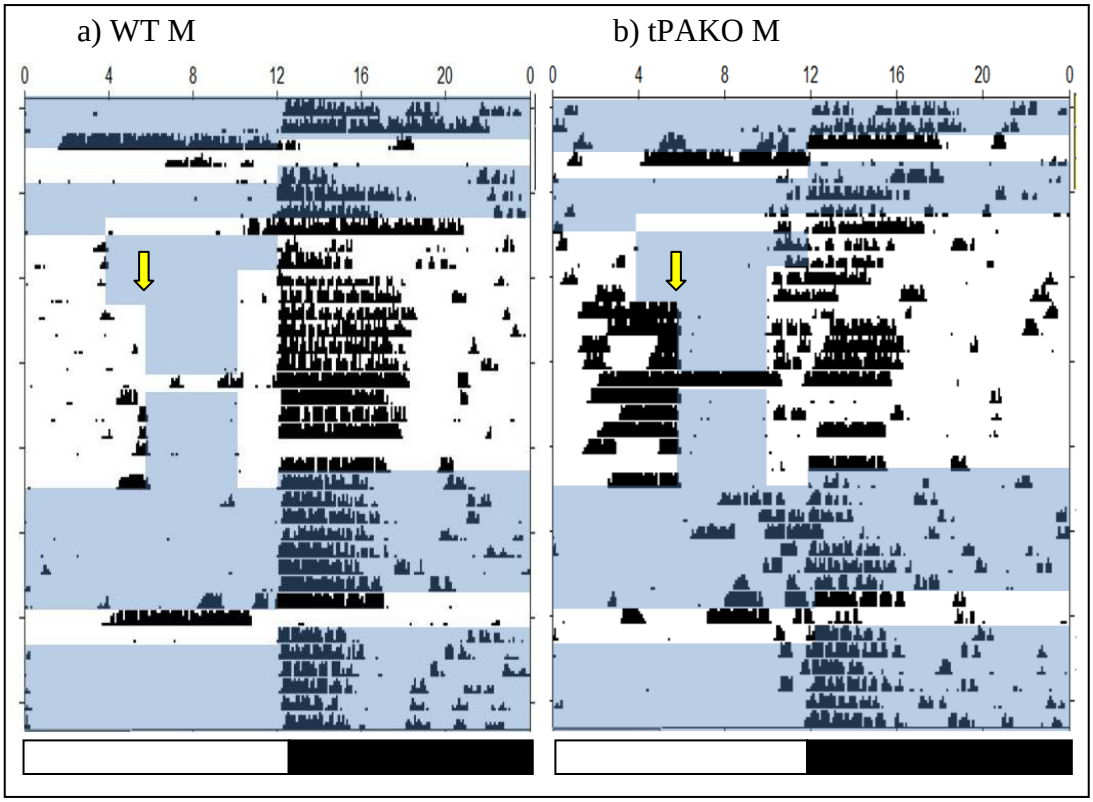


Figure 9: Actogram of WT male mice (a) and tPAKO male mice (b) on restricted feeding schedule. Yellow arrows indicate time of food presentation (ZT6) during 4hr restricted feeding. FAA is activity occurring 2-3 hours prior to food presentation, while SCN-controlled activity is characterized by activity onset during the dark phase, starting at ZT12. tPAKO mice exhibit significantly enhanced FAA in comparison to WT mice ($p=0.0075$). White bar represents time of lights on, and black bar represents time of lights off (12L:12D). The shaded areas indicate the hours of food availability.

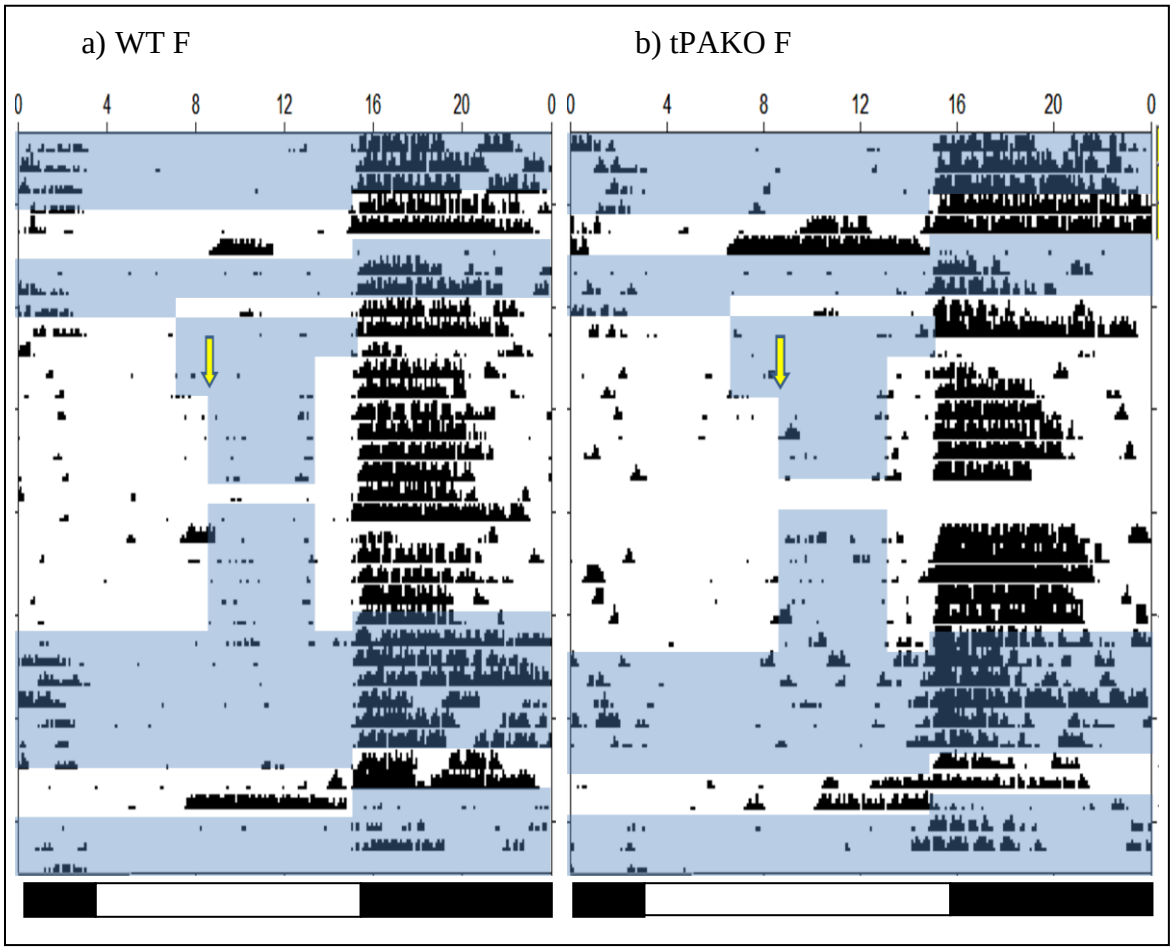
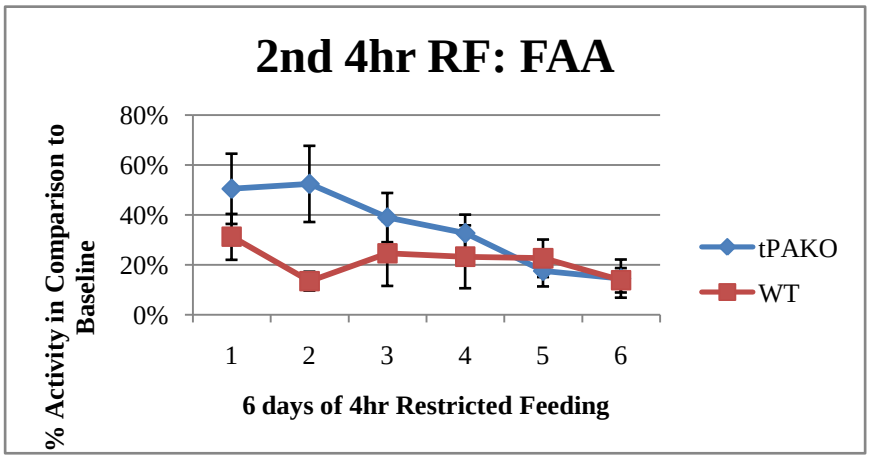


Figure 10: Actogram of WT female mice (a) and tPAKO female mice (b) on restricted feeding schedule. Yellow arrows indicate time of food presentation during 4hr restricted feeding. FAA is activity occurring 2-3 hours prior to food presentation, while SCN-controlled activity is characterized by activity onset during the dark phase, starting at ZT16. White bar represents time of lights on, and black bar represents time of lights off (12L:12D). The shaded areas indicate the hours of food availability.

a)



b)

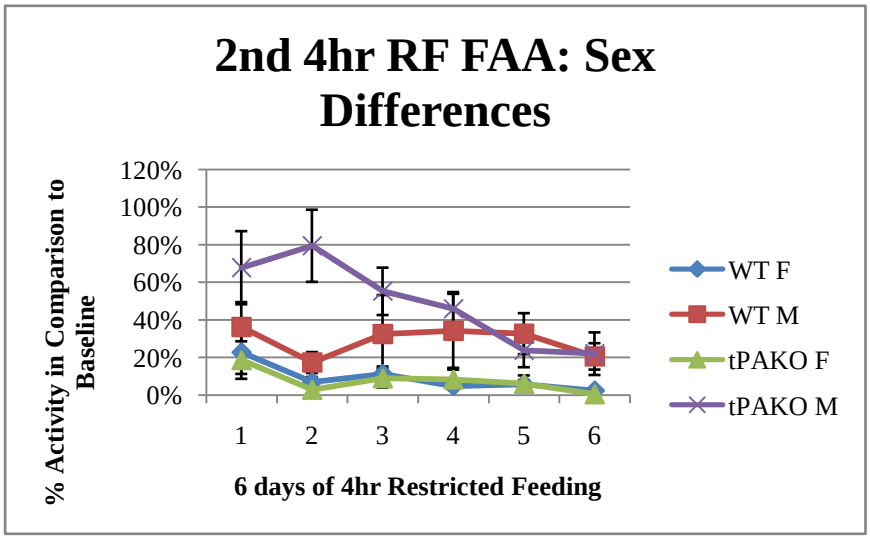


Figure 11: The enhanced FAA expression in tPAKO mice gradually decreases across the last 6 days of restricted feeding, and the significant difference between genotypes disappears ($p>0.05$) (a). Females of both genotypes continue to show markedly reduced FAA in comparison to males ($p=0.0017$) (b). Data have been normalized and is expressed as a percentage of overall baseline activity to account for variability in the amount each animal runs on a daily basis. Error bars represent the standard error of the mean.

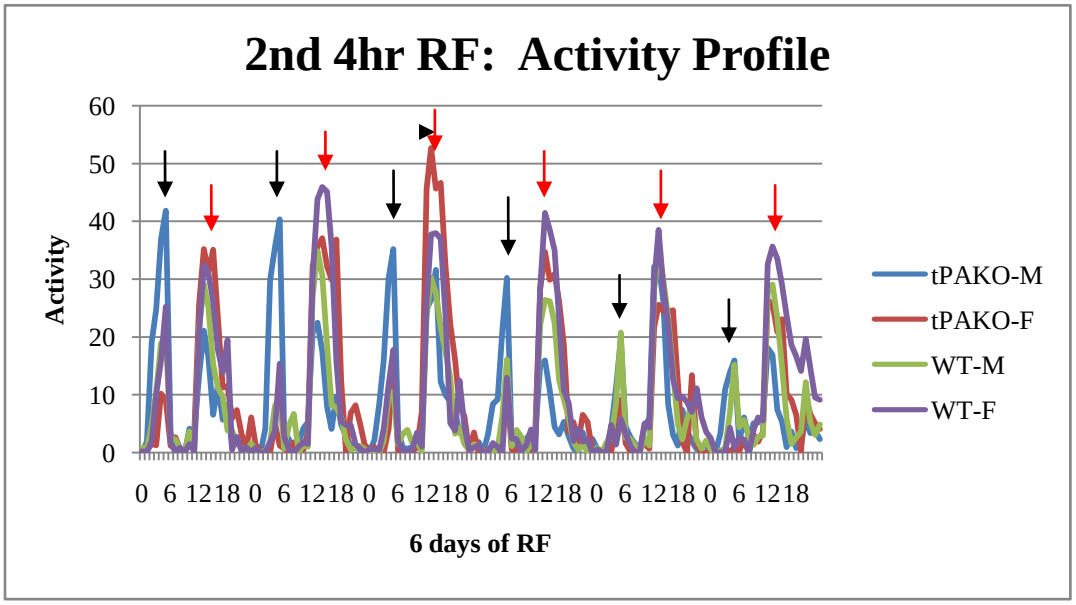


Figure 12: Activity profile of the 6 days of the 2nd 4hr RF. Black arrows indicate food anticipatory activity, while red arrows indicate nocturnal activity.

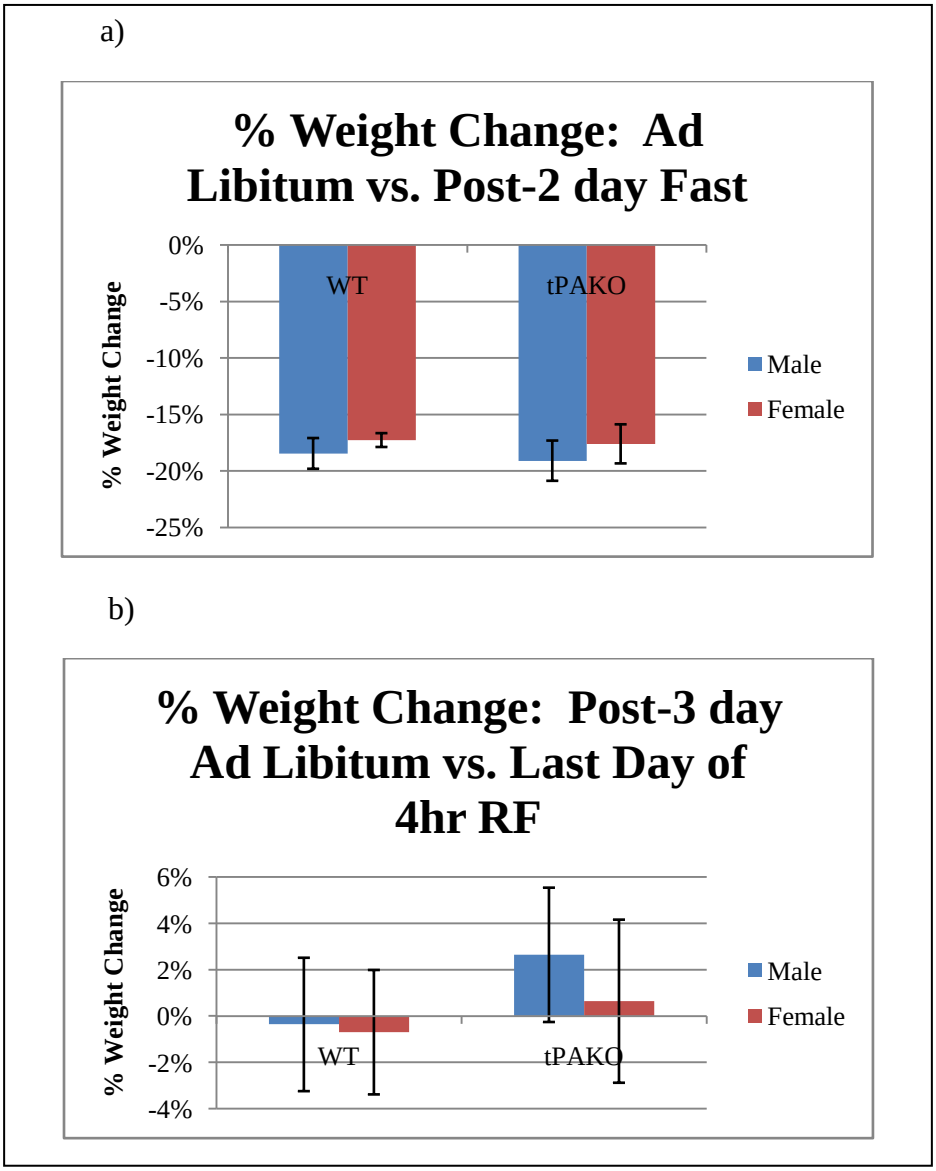


Figure 13: Percent change of weight loss/gain between tPAKO and WT mice across the RF protocol. (a) There is no significant difference between genotypes ($p>0.05$) for percent weight change between initial *ad libitum* and after the 1st 48hr fast, indicating that each genotype loses weight at the same rate during fasting. (b) There is no significant difference between genotypes ($p>0.05$) for percent weight change between weight measured after 3-day *ad libitum* and the last day of 4hr RF, exhibiting that the RF paradigm does not result in differential weight loss between genotypes. Error bars represent the standard error of the mean.

Experiment 3A: Differential Gene Expression of the Liver During Phase of FAA

In this experiment, differential expression of genes *Clock*, *Per1*, *Per2*, *uPA*, *tPA*, and *PAI-1* in the liver during *ad libitum* (AL) and restricted feeding (RF) were evaluated during the time of FAA (ZT5) in WT and tPAKO mice in order to examine peripheral clock function as well as genes related to tPA that could explain the significantly enhanced FAA expression in tPAKO mice. There was a significant difference in the expression of the genes *Clock* ($p=0.005$) and *Per1* ($p=0.00002$) between RF and AL but not between genotypes ($p>0.05$). *Clock* expression was significantly reduced and *Per1* was increased during RF in both genotypes (Figure 11). *Per2* gene expression exhibited a significant increase between genotypes ($p=0.047$) as well as between RF and AL ($p=0.00005$). Both tPAKO and WT mice exhibited a significant increase in *Per2* expression during RF in comparison to AL, but *Per2* expression was significantly higher in tPAKO mice than WT mice during RF (Figure 12). There was no significant difference detected in *PAI-1* and *uPA* gene expression between genotypes or feeding protocol ($p>0.05$) (Figure 13). Lastly, WT mice exhibited no significant difference in *tPA* gene expression between RF and AL ($p>0.05$) (Figure 14).

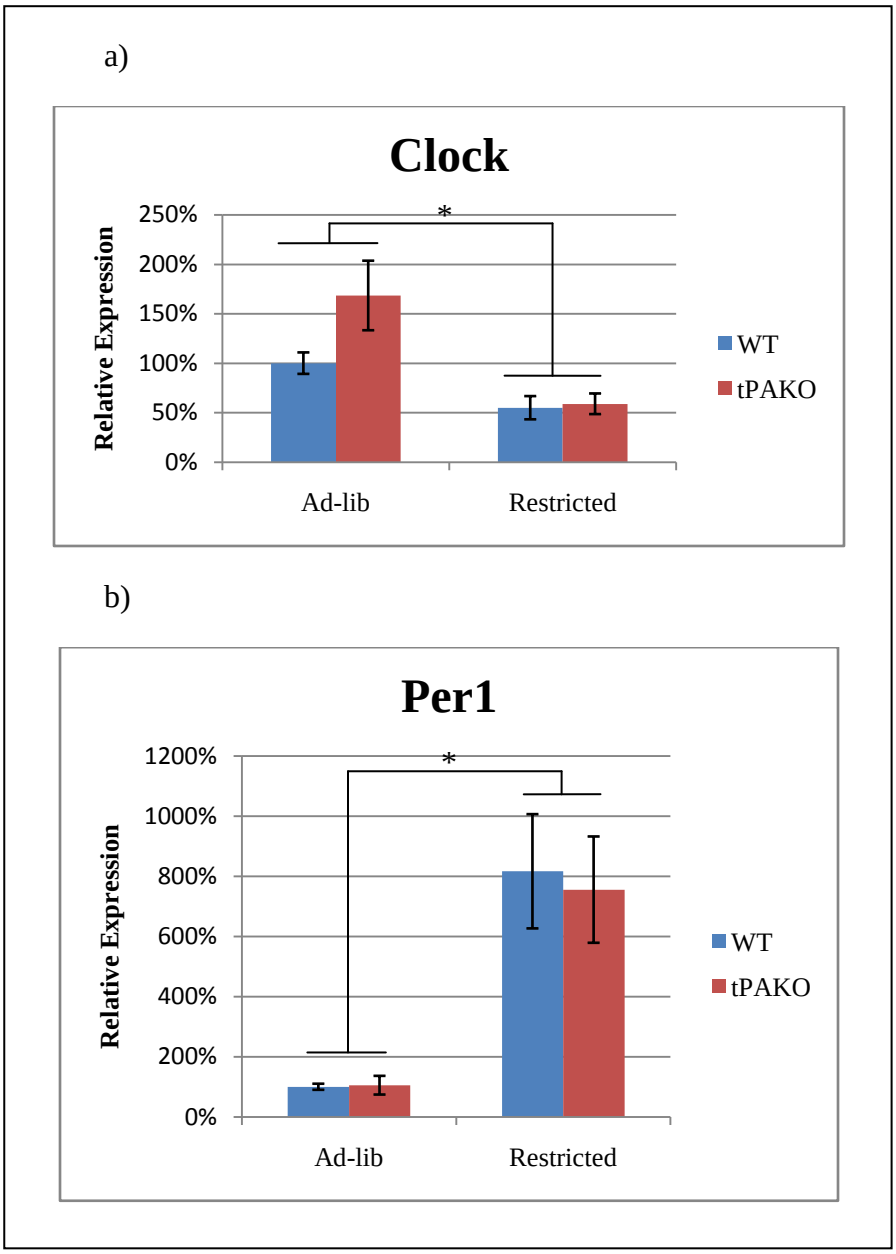


Figure 14: There was a significant difference in the expression of the genes *Clock* (p=0.005) and *Per1* (p=0.00002) between RF and AL but not between genotypes (p>0.05). Error bars represent the standard error of the mean.

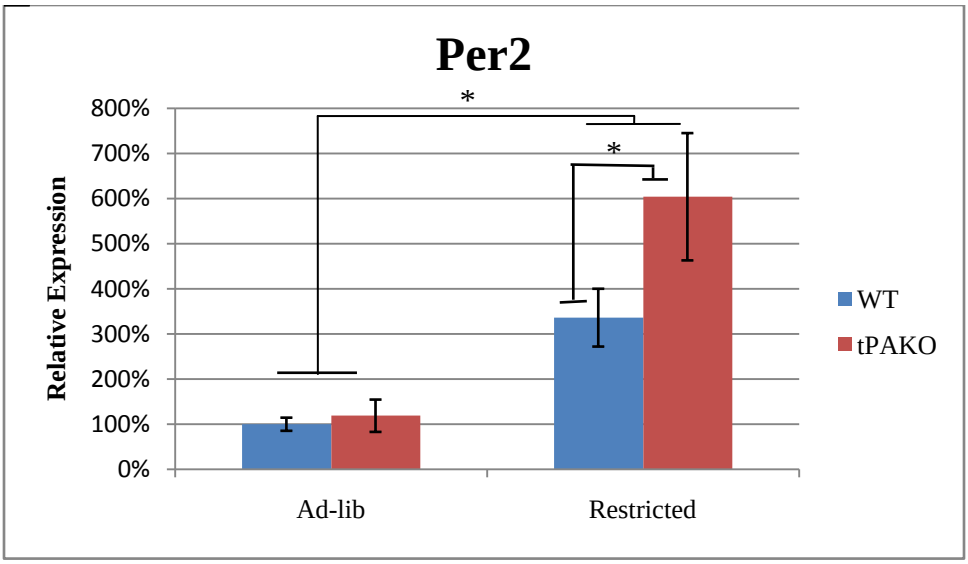


Figure 15: There is a significant difference in *Per2* gene expression between tPAKO and WT mice (p=0.047) as well as between feeding protocol (p=0.00005). Error bars represent the standard error of the mean.

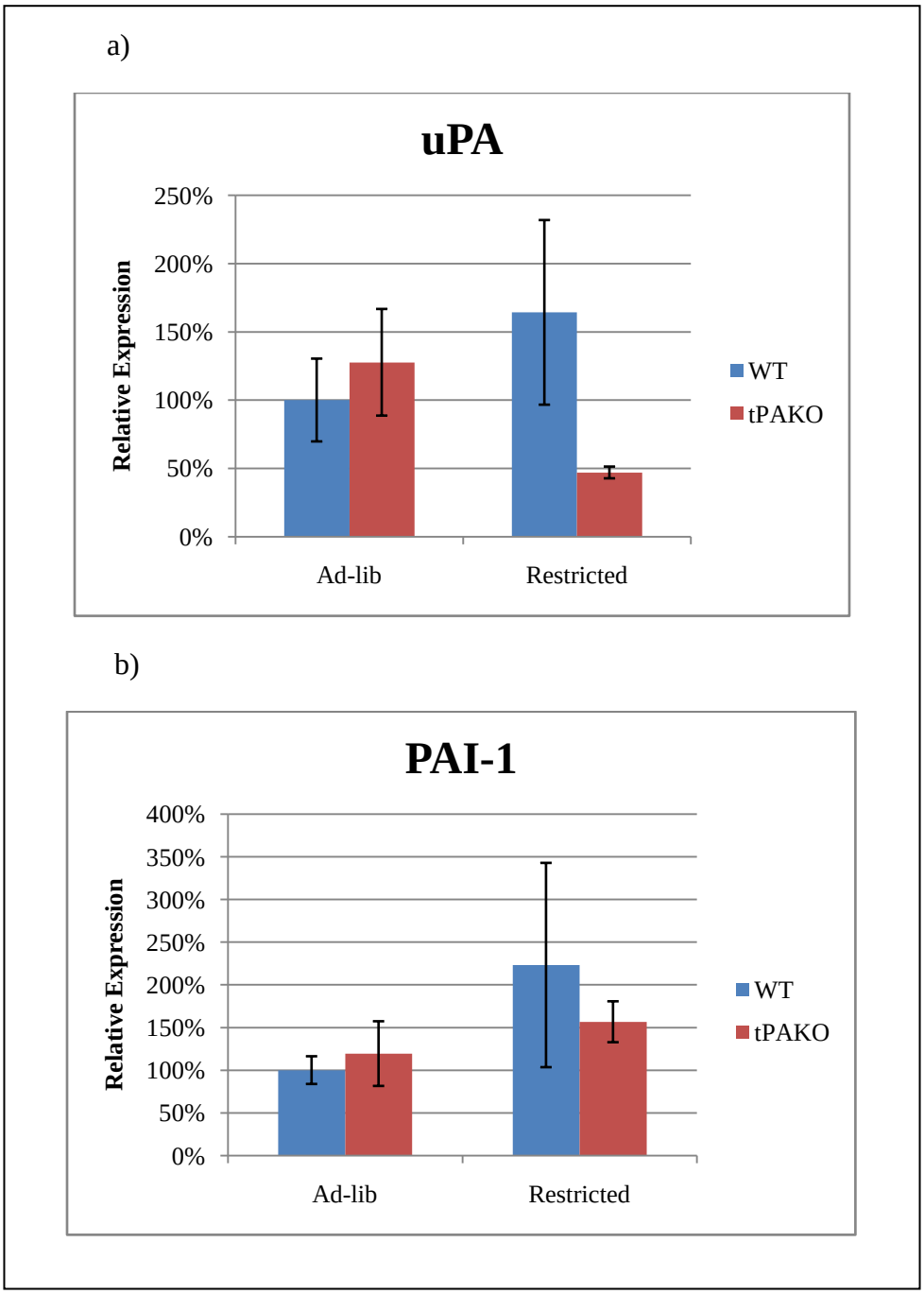


Figure 16: There is no significant difference detected in *PAI-1* and *uPA* gene expression between genotypes or feeding protocol ($p>0.05$). Error bars represent the standard error of the mean.

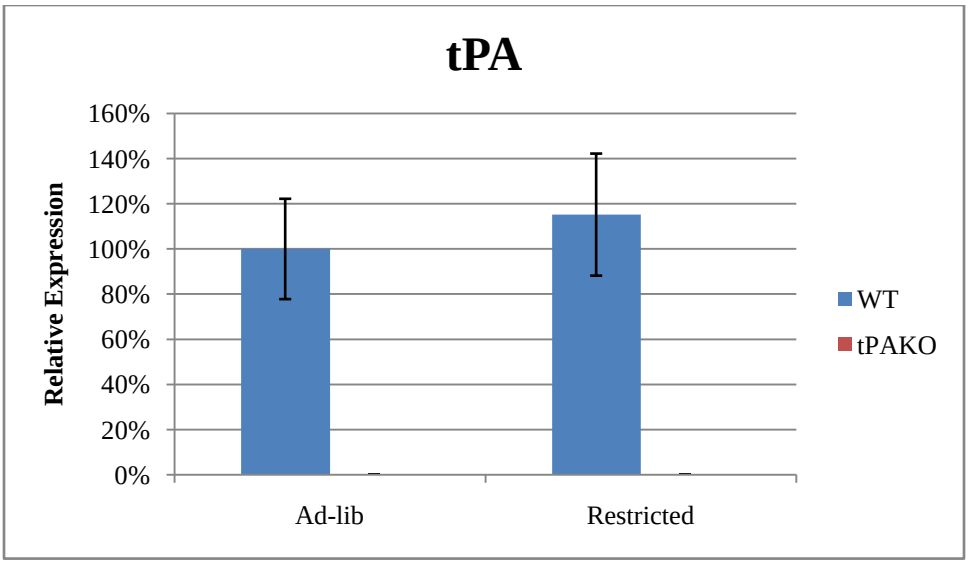


Figure 17: WT mice exhibited no significant difference in *tPA* gene expression between feeding protocols ($p>0.05$). Error bars represent the standard error of the mean.

Experiment 3B: Differential Gene Expression of the SCN During Phase of FAA

In this experiment, differential expression of genes *Clock*, *Per1*, *Per2*, and *PAI-1* in the SCN during restricted feeding and *ad libitum* were evaluated during the time of FAA (ZT5) in WT and tPAKO mice in order to examine central clock function as well as genes related to tPA that could explain the significantly enhanced FAA expression in tPAKO mice. The expression of *VIP*, a gene expressed ubiquitously throughout the SCN but not in surrounding tissues, was used as a marker and evaluated as well to verify that the arrays were SCN-specific. There was no significant difference in the expression of the gene *Clock* and *PAI-1* in the SCN between genotypes or feeding RF vs. AL ($p>0.05$). However, a significant interaction in *Clock* and *PAI-1* expression between genotype and feeding regimen is observed: Expression of *Clock* as well as *PAI-1* is significantly higher in WT mice than tPAKO mice during AL ($p=0.002$, $p=0.02$, respectively) (Figure 16). There was no significant difference in *Per1* and *Per2* expression between genotype or feeding regimen ($p>0.05$) (Figure 17). *VIP* expression exhibited no significant difference between genotypes or feeding regimen ($p>0.05$), indicating that all samples used were specific to the SCN (Figure 18). *uPA* and *tPA* gene expression were also evaluated; however, the results were inconclusive due to failure of their detection in the majority of samples.

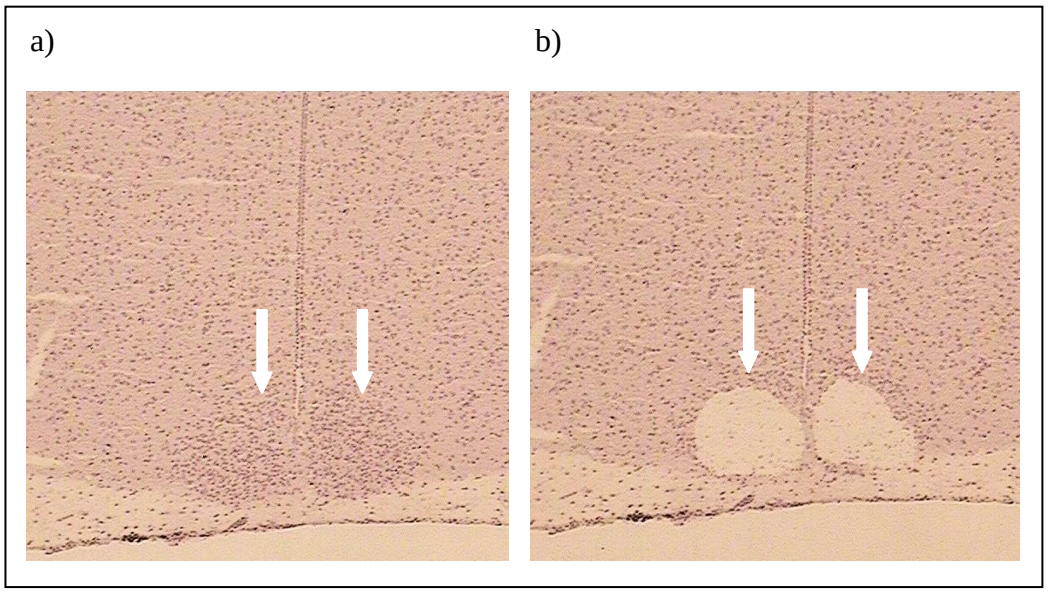


Figure 18: (a) 12 µm thick coronal section of the SCN (indicated by arrows) post-hemotoxylin staining before LCM. (b) Section after laser capture of the SCN.

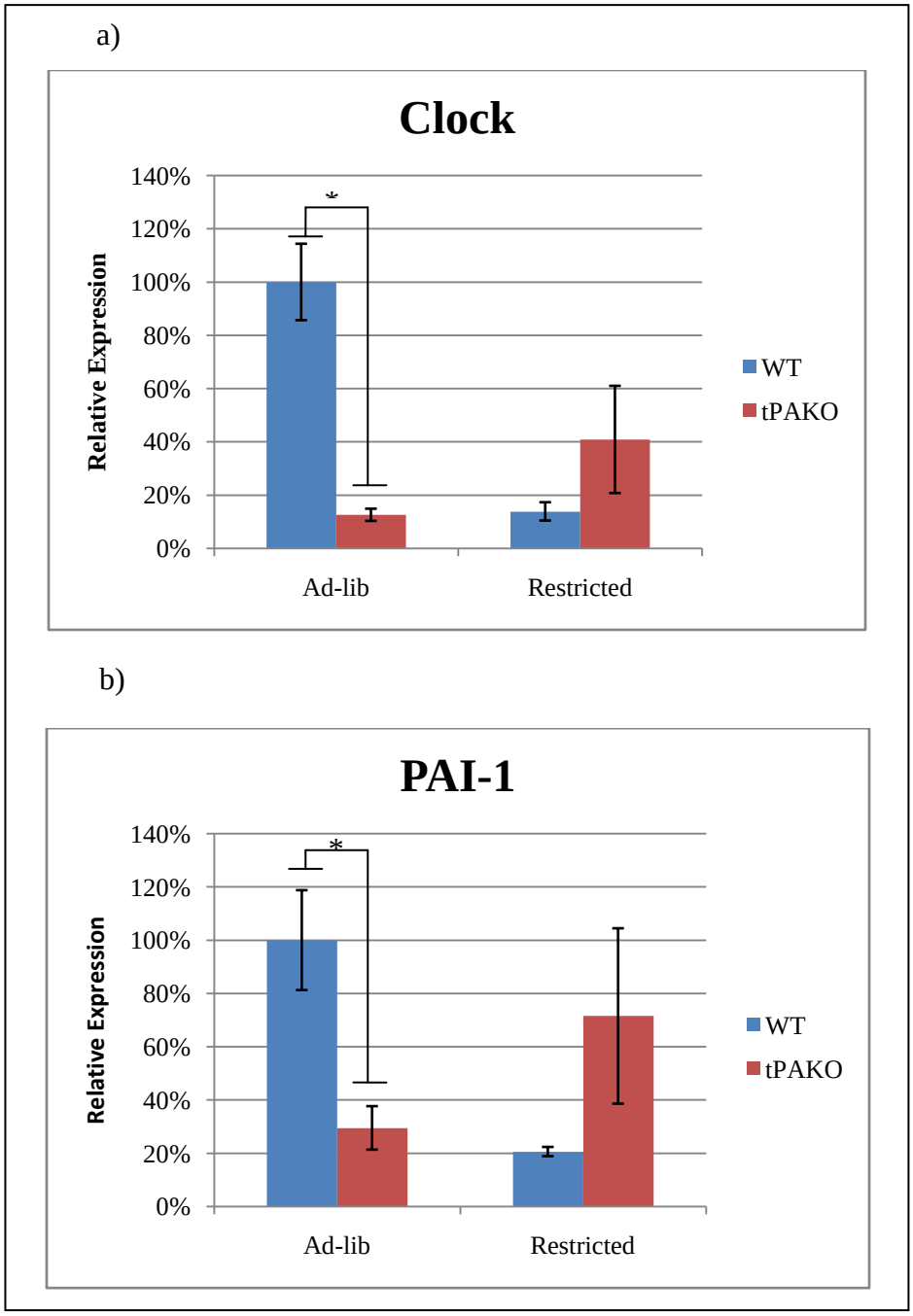


Figure 19: There is no significant difference in *Clock* (a) and *PAI-1* (b) gene expression in the SCN between genotypes or feeding regimen ($p>0.05$). However, a significant interaction is observed in that WT mice have significantly higher *Clock* and *PAI-1* in comparison to tPAKO mice during AL ($p=0.002$, $p=0.02$, respectively). Error bars represent the standard error of the mean.

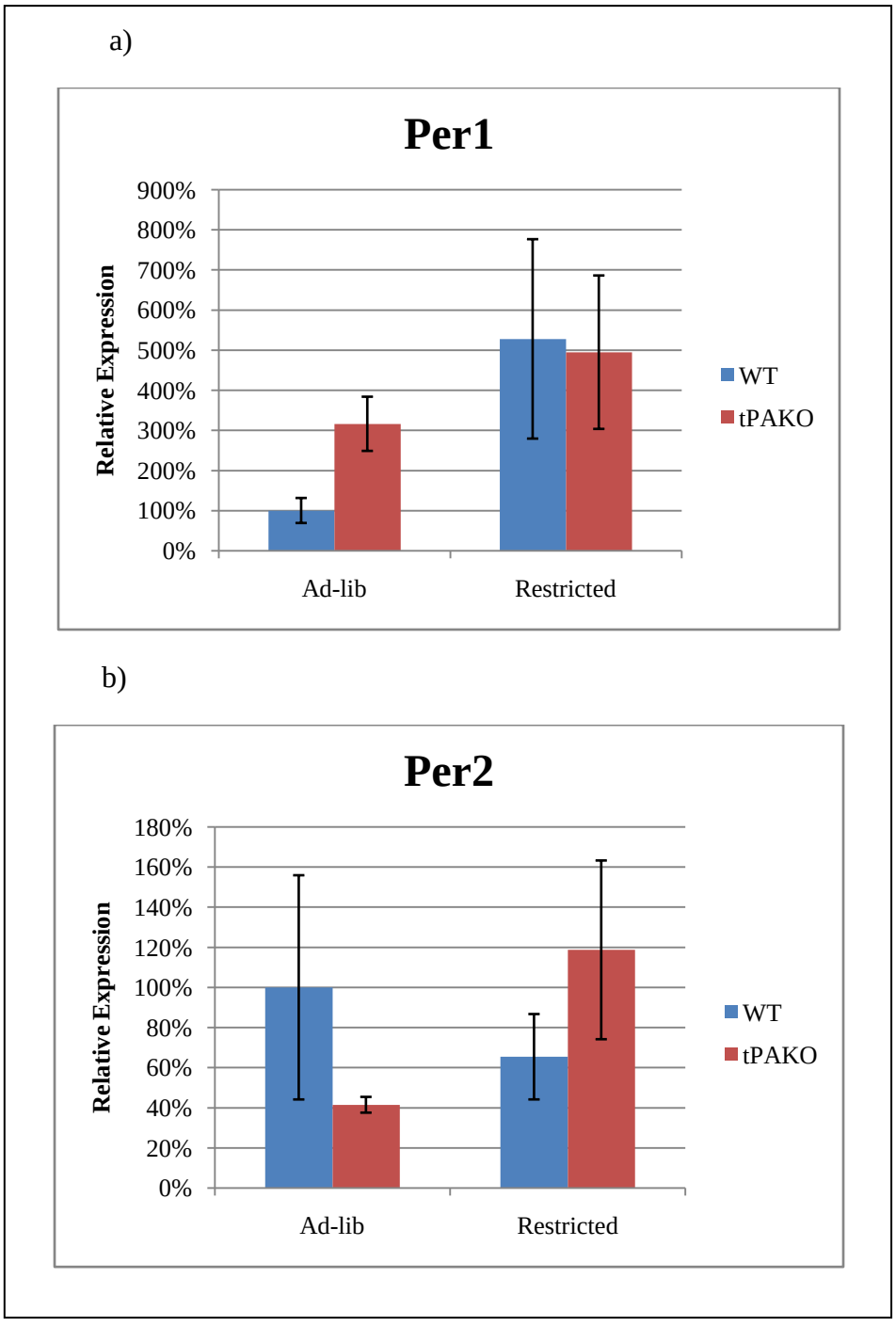


Figure 20: There is no significant difference in *Per1* or *Per2* gene expression in the SCN between genotypes and feeding regimen ($p>0.05$). Error bars represent the standard error of the mean.

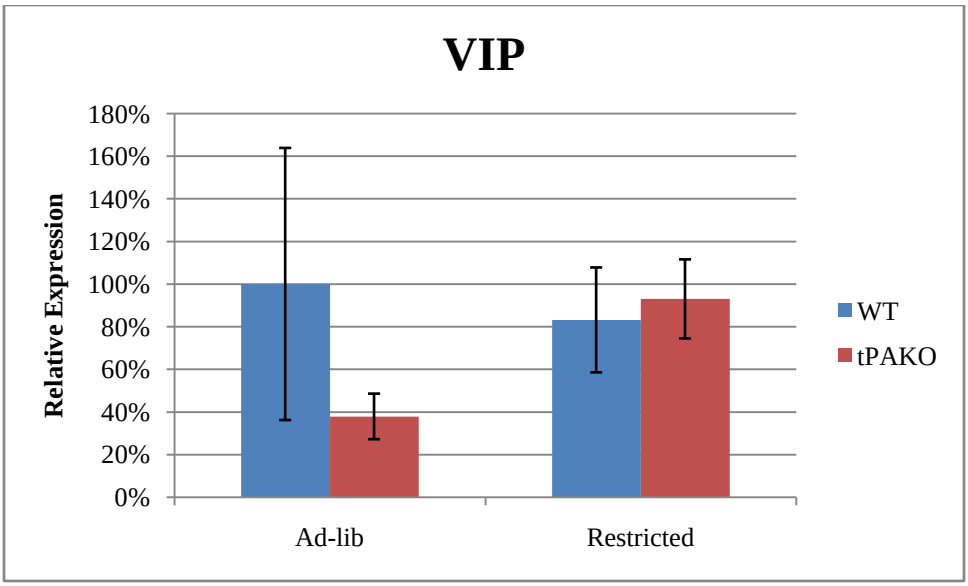


Figure 21: There is no significant difference in *VIP* gene expression between genotype and feeding regimen ($p>0.05$). Error bars represent the standard error of the mean.

Discussion:

BDNF and its tyrosine kinase receptor trkB have been localized to the SCN and in the presence of a trkB antagonist both light and glutamate-induced phase shifts in the SCN are blocked *in vivo* and *in vitro* (Liang et al., 2000; Michel et al., 2006; Mou et al., 2009). Mou et al. (2009) demonstrated that BDNF generation in the SCN is critical to its phase-shifting behavior, and is dependent on the actions of tPA and its associated signal cascade. The inhibition of tPA or one of its associated downstream proteins results in insufficient phase-shifting behavior, therefore designating BDNF as the "gatekeeper" of SCN phase-shifting response via modulation of glutamatergic synaptic transmission. We were interested to determine if mice deficient in the tPA, the key protease responsible for BDNF generation, would exhibit markedly reduced phase-shifting behavior in response to light. tPAKO mice express decreased mBDNF levels as well as decreased mBDNF: proBDNF in the SCN in comparison to WT mice (Mou et al., unpublished data). Based on the fact that tPAKO mice exhibit deficient BDNF levels, we hypothesized that tPAKO would exhibit a reduced light-induced phase-shifting response in comparison to WT mice.

Interestingly, we found that there was no significant difference in light-induced phase-shifts between tPAKO and WT mice at various light intensities during either early or late subjective night. Upregulation of trkB in tPAKO mice in order to compensate for

the BDNF deficiency has been suspected but not confirmed. Future experiments will address this. trkB mRNA has been shown to peak in the frontal cortex and hippocampus during the beginning of the inactive phase in rats (Bova et al., 1998), but it is unknown whether trkB expression in the SCN is rhythmic. Neurotrophin-4 (NT-4) also activates the trkB receptor in the CNS; however, its functional significance in the SCN is unknown at this time. Conover et al. (1995) reported that mice deficient in NT-4 exhibit no obvious neurological defects and live a normal life span. BDNF homozygous mutant mice die shortly after birth and display severe neurological deficits, indicating that the expression of BDNF is much more critical to normal brain development and function than NT-4 (Ernfors et al., 1994). The presence of urokinase plasminogen activator (uPA) has been detected in the brain as well, but to a much lesser extent than tPA (Verrall & Seeds, 1988). uPA is also capable of cleaving plasminogen into plasmin, though, whether it does so in the SCN is unknown. The possibility arises that uPA may be overexpressed in tPAKO mice, exerting a developmental compensatory effect. However, our data shows that *uPA* expression in the SCN of tPAKO mice was too low to be detected using RT-PCR.

Since tPAKO mice did not exhibit a significant differential behavioral response to light in Experiment #1, the next objective was to monitor the behavioral response to a nonphotic cue in tPAKO mice. LTP-inducing tetanic stimulation of the CA1 (Patterson et al., 1992) and dentate gyrus (Castren et al., 1993; Dragunow et al., 1993) exhibit enhanced *BDNF* mRNA expression, indicating BDNF involvement in late phase long-

term potentiation. Also, a rapid increase in *BDNF* levels in the dentate gyrus and hippocampus of rats during radial arm maze training (Mizuno et al., 2000), inhibitory avoidance training (Ma et al., 1998; Alonso et al., 2002), and hippocampus-dependent contextual learning (Hall et al., 2000) has been reported. *tPA* mRNA rapidly increases after onset of L-LTP induction (Qian et al., 1993), and tPAKO mice exhibit selective deficits in L-LTP (Huang et al., 1996; Calabresi et al., 2000). Pang et al. (2004) linked the interconnection of BDNF to tPA/Plasmin by exhibiting that the actual cleavage of proBDNF by tPA/Plasmin is critical to long-term hippocampal plasticity. Upon exposure to a nonphotic cue of a restricted feeding schedule, we hypothesized that tPAKO mice would have difficulty learning the daily restricted feeding schedule and thus would exhibit reduced FAA in comparison to WT mice.

Contrary to our hypothesis, tPAKO mice exhibited significantly enhanced FAA in comparison to WT mice during the first four days of restricted feeding. The enhanced FAA gradually diminished over a period of 10 days during which the significant effect disappeared, and no interaction was observed. A plausible explanation for this divergence from our hypothesis could be that since tPAKO mice have deficits in LTP and memory but are not completely impaired, it simply takes them longer to learn the restricted feeding paradigm than WT mice. The enhanced FAA could serve as a mechanism through which tPAKO mice start their activity earlier and continue more robustly since they have difficulty remembering the time of food presentation. This also serves to explain why the significantly enhanced FAA of tPAKO mice disappears over

time—they gradually learn and adjust their activity to the restricted feeding schedule. Alternatively, tPA might be acting at an extra-SCN brain site in such a way that increases the locomotor response to timed food restriction.

To explain the enhanced FAA of tPAKO mice, we were interested to see if the amount of FAA could be explained by differences in weight loss during the restricted feeding regimen, with the idea that tPAKO mice might be losing additional weight and therefore showing greater FAA. To verify this, animals were weighed throughout the restricted feeding paradigm in order to monitor for differential weight gain/loss between genotypes, and there were no significant differences in the absolute or percent weight change observed between tPAKO and WT mice. Therefore, tPAKO mice do not appear to have a differential metabolic rate that could explain why they exhibit such enhanced FAA. If tPAKO mice did have a metabolic deficit that was the cause of the enhanced activity, one would expect that tPAKO mice would exhibit significantly greater fast-induced activity in comparison to WT mice. However, this is not the case, since there was no significant difference in fast-induced activity monitored during the 48hr fast between genotypes, indicating that enhanced activity observed in tPAKO mice is specific to the restricted feeding schedule and not simply caused by the fasting condition.

Maintenance of the metabolic balance of an organism is influenced by circadian rhythms in hypothalamic-pituitary-adrenal (HPA) axis activity and glucocorticoid secretion controlled by the SCN and FEO. The SCN drives the rhythm of corticotrophin-releasing hormone (CRH) secretion and subsequent adrenocorticotropic hormone

(ACTH) release (Dallman et al., 1978 & 1993; Damiola et al., 2000) and autonomically innervates the adrenal gland (Buijs et al., 1999 & 2003; Ishida et al., 2005; Jasper & Engeland, 1994). Cortisol/corticosterone levels peak during the early subjective day of mammals during *ad libitum* feeding. However, upon introduction of restricted feeding regimen, time of meal presentation serves as a potent zeitgeber to the glucocorticoid rhythm during which cortisol/corticosterone levels peak 1-2 hours before food presentation. This response is believed to be controlled by the FEO, since it still occurs in the absence of a functional SCN (Stephan, 2002) as well as in adrenalectomized mice (Segall et al., 2008).

A role for tPA in elicitation of the stress response is supported by the report that tPA activity increased in the amygdala of WT mice 30 minutes after they were subjected to a restraint test; the amygdala is a region of the brain critical to the interpretation of noxious vs. harmless stimuli and the fight or flight response (Pawlak et al., 2003). Corticosterone levels of mice subjected to restraint stress were 30% higher in tPAKO mice than WT mice while baseline levels were similar in both genotypes, indicative of a possible impairment of the HPA-axis in tPAKO mice (Matys et al., 2004). Damiola et al. (2000) and Stephan (2002) reported a peak in corticosterone 1-2 hours prior to food presentation in food restricted mice. It would be of interest to measure corticosterone levels of tPAKO mice and WT mice across the restricted feeding regimen in order to determine if there is a significant correlation between glucocorticoid secretion and FAA expression.

Dopamine (DA) serves as the chief neurotransmitter in regions of the brain involved in reward-related behavior such as the ventral tegmental area (VTA), nucleus accumbens (NAcc), amygdala, hippocampus, prefrontal cortex, among other areas. DA synthesis appears to be regulated by a circadian clock as daily rhythms of tyrosine hydroxylase (the rate-limiting enzyme in DA synthesis) expression in the NAcc and VTA have been reported (Webb et al., 2009). *C-fos* expression in reward-related areas of the brain have been observed in rats exposed to a restricted palatable food regimen, indicating that rewarding stimuli may be necessary for the development and maintenance of FAA, and the possible FEO influence over the mesolimbic system (Mendoza et al., 2005c). Nagai et al. (2004) reported that the tPA-plasmin system directly regulates DA release in response to the rewarding effects of morphine as tPAKO mice exhibited reduced morphine-induced DA release in the NAcc that could be rescued by injection of exogenous tPA or plasmin. tPAKO mice also have been shown to exhibit increased sensitivity to cocaine and over-respond to cocaine-self administration, indicating tPA modulation of the behavioral response to cocaine (Maiya et al., 2009; Ripley et al., 1999). Therefore, the possibility arises that the significantly enhanced FAA expressed by tPAKO mice may be a product of altered dopaminergic activity in reward-related regions of the brain.

Interestingly, FAA in tPAKO mice appears to be a male-associated behavior, as female mice of both genotypes failed to exhibit virtually any FAA. No pattern could be distinguished upon examination of female daily activity throughout the restricted feeding

protocol that corresponded to the estrous cycle. Sex differences in response to stress and in learning and memory could be a possible explanation for the lack of FAA expression in females. Genn et al. (2003) observed that food-deprived female rats exposed to a social interaction stress test exhibited decreased locomotor activity believed to be a result of energy conservation. Decreased locomotor activity as a means to conserve energy could be a plausible explanation since female mice of both genotypes exhibited significantly lower fast-induced activity in comparison to males. However, to determine if this is the case with the current experiment, future studies using ovariectomized females could further clarify this phenomenon.

As a result of our surprising finding of enhanced FAA expression in tPAKO mice, we decided to examine molecular clock gene expression as well as tPA and associated genes in the SCN and peripherally in the liver to determine whether central and peripheral circadian clocks were differentially responding in the two genotypes. Damiola et al. (2000) as well as Hara et al. (2001) observed a shift in the expression of *Per1* and *Per2* in the liver of mice on a restricted feeding schedule with virtually no effect on clock gene expression in the SCN. In support of this evidence Girotti et al. (2009) also reported that restricted feeding had no effect on clock gene expression in the SCN in rats. On the other hand, Castillo et al. (2004) reported a shift in *Per2* expression in the SCN of mice exposed to restricted feeding. Since tPAKO mice exhibit a greater circadian response to restricted feeding, we hypothesized that tPAKO mice would exhibit differential period gene expression in comparison to WT mice.

We observed a significant difference in the expression of genes *Per1* and *Clock* in the liver between restricted feeding and *ad libitum* but not between genotypes. *Per1* expression was significantly increased during restricted feeding, while *Clock* expression was significantly decreased during restricted feeding. Stokkan et al. (2001) reported a shift in *Per1* gene expression in the liver of Per1-luciferase transgenic mice exposed to restricted feeding. *Per2* also exhibited a significant increase in expression during restricted feeding as well as a significant effect between genotypes ($p=0.047$). Hara et al. (2001) and Damiola et al. (2000) also observed a shift in *Per1* and *Per2* gene expression in mice under a restricted feeding regimen, indirectly supporting our finding of the increase in *Per1* and *Per2* gene expression in the liver. Conflicting results have been reported on expression of the gene *Clock* in the liver: Takata et al. (2002) reported constitutive circadian expression of *Clock* in the liver of mice under *ad libitum* feeding while Froy et al. (2009) observed oscillations in the expression of *Clock* with mRNA levels peaking around ZT18. Based on our results, restricted feeding may suppress expression of *Clock* in the liver; or it may impose a rhythm, and the time of sacrifice happened to fall at the time of lowest *Clock* expression. In order to determine which scenario is occurring and if *Per1* expression has been shifted, an experiment during which animals are sacrificed across 24 hours during restricted feeding for measurement of clock gene expression should be done.

There was no significant difference observed in *uPA*, *PAI-1*, or *tPA* (exclusively to WT mice) gene expression in the liver between genotypes or feeding treatment. To

our knowledge, no studies examining the expression of genes *uPA*, *PAI-1*, and *tPA* in the liver during restricted feeding have been reported. However, it does strengthen the argument that *uPA* and *PAI-1* are not involved in developmental compensatory effects influencing peripheral physiology of tPAKO mice.

Studies examining the effects of normocaloric restricted feeding on clock gene expression of the SCN have reported conflicting data such that SCN gene expression was virtually unaffected or a shift in Per2 expression was observed. A significant interaction was observed in that *PAI-1* and *Clock* expression in the SCN were significantly higher in WT mice than tPAKO mice during *ad libitum* feeding. The Clock gene is expressed constitutively in the SCN during *ad libitum* feeding across the circadian period (Vitaterna et al., 1994). To our knowledge no studies examining molecular clock function in the SCN of tPAKO mice have been reported. Mendoza et al. (2007) reported that *Clock* expression in the SCN was constitutively expressed across *hypocaloric* RF except for a significant decrease 2 hrs following feeding. However, to our knowledge no studies examining *Clock* or *PAI-1* expression in the SCN during normocaloric RF have been reported, but it would be of interest to examine the circadian expression of clock genes, especially of *Clock*, during RF since our data indicates its constitutive expression has been disrupted. The reduced expression and the functional significance of *PAI-1* in the SCN during RF remain unclear.

Girotti et al. (2009), Damiola et al. (2000), and Wakamatsu et al. (2001) all reported that *Per1* and *Per2* expression in the SCN during normocaloric RF were

unaffected. Our data is consistent with this as there was no significant difference in *Per1* and *Per2* expression between genotypes or feeding regimen. Castillo et al. (2004) observed that *Per2* expression entrained to RF without caloric restriction in mice under constant dark. This effect is said to depend on how close the free-running period is to the period of food availability, and if free-running animals are kept on RF long enough, the SCN would entrain and an interaction between the two activity components could be observed. However, our data is consistent with other studies reporting that restricted feeding cannot outcompete and reset SCN clock gene activity when exposed to the principle influence of light as well.

VIP gene expression was also examined in order to confirm SCN specificity as it is expressed throughout the SCN but not in the surrounding tissue. There was no significant difference detected in *VIP* gene expression between genotype or feeding regimen, indicating uniformity of all samples collected. However, *VIP* expression appears to be sensitive to restricted feeding as Morin et al. (1993) reported a phase advance of *VIP* expression in the SCN when rats were restricted to daytime feeding, and Andrade et al. (2004) observed a significant reduction in *VIP* mRNA levels in rats under hypocaloric food restriction. This could be due to the catabolic state of the animal during which clock and clock-controlled gene expression are disrupted; however, future research examining the sensitivity of *VIP* expression to RF will be necessary as well as a larger sample size to further strengthen our results.

Overall Conclusions

In our attempts to further examine tPA modulation of the suprachiasmatic nucleus, we surpisingly discovered tPAKO mice do not exhibit a differential behavioral response to photic cues but are very sensitive to restricted feeding. The present data clearly indicate a role for tPA modulation of FAA, as its absence clearly leads to the disinhibition and enhancement of its behavioral expression. This could be due to the tPA-plasmin system's influence over SCN phase-shifting capabilities, learning and memory retrieval in the hippocampus, and/or dopaminergic signaling in reward-related areas of the brain. Lesion studies trying to localize the FEO have proved inconclusive and as a result strengthen the argument that the FEO appears to be a network of interconnected central structures that influence foraging and feeding behaviors. Our data are consistent with the uncoupling of peripheral tissues from the suprachiasmatic nuclei in response to RF, during which SCN clock gene expression is virtually unaffected and liver clock gene expression is altered. However, a future experiment examining circadian gene expression in the liver and SCN across restricted feeding needs to be done in order to fully verify the uncoupling effect and shift in liver gene expression. Feedback occurring between the SCN, FEO, peripheral organs, and the current metabolic status of the animal influence the expression of FAA, and further research will be necessary to identify the mechanisms through which tPA modulates central structures and influences peripheral feedback during restricted feeding.

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