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Acute and Chronic Effects of Rotating Shift Work on Running Wheel Activity, Energy Balance, and Molecular Alterations

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by

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Dissertation

Presented to the Faculty of the Graduate School of

The University of Texas at Austin

in Partial Fulfillment

of the Requirements

for the Degree of

Doctor of Philosophy

The University of Texas at Austin

May 2022

Acknowledgements

I owe my gratitude to the generous support and help of many individuals, without whom I would not have been able to complete my research or my doctoral degree!

I'd want to convey my gratitude to my family for their support and encouragement in helping me finish this dissertation. My beloved and supportive husband, Shabrish, who has always been by my side and has motivated me greatly in completing this research work and my adoring daughter, Sameeksha, who has inspired me to complete this project. My heartfelt gratitude to my parents and in-laws who have cared for Sameeksha during my absence for these past 7+ years.

I'd want to express my gratitude to the Bray Lab team at the University of Texas at Austin, especially my supervisor, Dr. Molly Bray, for her wisdom and experience, which guided and enabled me to go above and beyond in my research work. Thanks to Dr. Laura Lashinger for her constant support and opportunities to expand my knowledge in the metabolic niche. I am grateful to Dr. Ryan Gray for allowing me use Gray lab equipment, which made bench work more productive. Thank you, Dr. Diana Gutierrez, Johanna Wright, Ashley Neely, Jesse Balderrama, and Montavius Coleman, for the stimulating scientific discussions, long days of working together, putting up with my stresses and moans, and for all the fun we have had in the last few years. I thank Graham Salmun, Dr. Geoff Solares, Dr. Jaehyun Joo, Dr. Fei Dong, Meredith First, Masumi Yoshimoto, Lawrence Chu, Jessica Tan, Tanvi Ingle, Sarah Koo, Layan Al-Sukhni, Zahver Momin, Ana Csitkovits, and Eliza Plummer for their help in the implementation and completion of this huge research study.

My sincere thanks to committee members, Drs. deGraffenried, Stefano Tiziani, Mary Steinhardt and Audrey Stone for the approval and recognition of my work.

Abstract

Acute and Chronic Effects of Rotating Shift Work on Running Wheel Activity, Energy Balance, and Molecular Alterations

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The University of Texas at Austin, 2022

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Endogenous molecular clocks influence physiology, metabolism, and behavior. Shift workers are exposed to repeated changes in the timing of light, feeding, and activity and, consequently, are at increased risk for development of chronic disease associated with disruptions of circadian rhythms. The objectives of this research were to determine the behavioral, physiological, and molecular effects of acute and chronic exposure to rotating shift work (RSW) using murine models and evaluate the effects of timed exercise and timed feeding independently and in combination, within the context of high and low-fat diets, in a model of chronic rotating shift work (CRSW). To simulate human RSW, mice were housed in either normal or RSW conditions, consisting of 12-hour shifts in the light/dark cycle every 3 or 4 days for either 3- or 14-day in the acute experiment (RSW) or 10 weeks in the chronic experiment (CRSW). Under CRSW, six groups of mice were examined for metabolic and behavioral outcomes, including control (C), shift work control (SC), shift work chronic exercise (S-RW), shift work timed exercise (S-TE), shift work timed feeding (S-TF), and shift work timed exercise and feeding (S-TFEX). We

report disrupted wheel running activity patterns in acute and chronic exposure to RSW. Differential effects of low- and high-fat diet were observed in wheel running activity in CRSW. Substantial alterations in the diurnal expression of core clock and metabolic genes were observed in a tissue-specific manner. After 14 days of RSW exposure, most tissues lost complete clock rhythmicity and resulted in impaired fasting glucose. CRSW resulted in significantly greater weight gain and lower glucose tolerance in S-TF and S-TFEX mice. The S-TE mice weighed lower and displayed higher glucose tolerance on low-fat diet compared to S-TF condition. Acute exposure to RSW disrupts molecular functions in a tissue-specific manner, which may precede the metabolic consequences of chronic exposure to RSW. The present study provides evidence that the variable food intake patterns associated with shift work may result in deleterious metabolic outcomes. A short intense voluntary bout of exercise every 24 hours may serve as a behavioral cue for eating behavior.

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Chapter 1: Introduction and literature review

A BRIEF HISTORY OF CHRONOBIOLOGY

Humans have evolved as a diurnal species in a natural light/dark cycle, with physical activity and feeding carried out primarily during the light phase and sleep taking place in the dark phase in a day. The periods of sleep and activity follow an approximately 24-hr rhythm that coincides with the earth's rotation on its axis [4, 5]. The hypothesis that a biological timekeeping system existed that regulated physiological rhythms was first proposed by the French astronomer and botanist, Jean Jacques d'Ortous de Mairan, in the 1700s, who observed the rhythmic opening and closing of plant leaves (*mimosa pudica*), even in extended darkness [6]. Augustin de Candolle, a Swiss botanist in the 1800s, later hypothesized that an endogenous clock was responsible for controlling the rhythmic movement of leaves in *Mimosa pudica*. The idea that organisms have an intrinsic circadian clock only came to be accepted in the 1900s [5]. In 1926, Maynard Johnson provided evidence that an internal clock was responsible for the locomotor activity rhythms in deer mice housed under constant darkness. He observed that, in the absence of an external time cue, the activity rhythms of the mice had a delayed start of about 6 minutes each day. By the end of one month, the activity rhythms had drifted by around 4-hr [7]. Since that time, many researchers have contributed to the discovery of the molecular mechanisms that regulate the circadian clock. These discoveries have helped to inform and advance research in the field of chronobiology [6].

Molecular architecture of circadian clock in mammals. Endogenous circadian clocks regulate many physiological, metabolic, and behavioral processes. Biological clocks allow adaptation in anticipation of environmental changes, such that environmental cues and physiology are optimally synchronized each day [8]. Each cell contains its own clock mechanism, requiring a pacemaker to keep the clocks in synchrony with each other [9]. The mammalian circadian system is hierarchically

organized to coordinate behavior and physiology to alterations in the environment. At the top of this hierarchy is the suprachiasmatic nucleus (SCN), which is located above the optic chiasm of the hypothalamus and stimulated by light [9]. The SCN synchronizes the organism to the daily environmental light/dark cycle and communicates these time cues to the peripheral clocks present in tissues external to the SCN via neuro-humoral factors. In turn, the peripheral clocks regulate specific physiological processes in a tissue-specific manner [4].

The molecular clock is comprised of an intracellular auto-regulatory process governed by interacting positive and negative transcriptional-translational feedback loops (TTFL; see Figure 1.1). The rhythmic expression of core clock genes is followed by their translation as transcription factor and regulatory proteins that drive and maintain rhythmicity of the overall clock mechanism. The genes driving the core clock mechanism include *circadian locomotor output cycles kaput* (*clock*), *brain and muscle-Arnt-like 1* (*bmal1*), *period1* (*per1*), *period2* (*per2*), *period3* (*per3*), *cryptochrome1* (*cry1*), *cryptochrome2* (*cry2*), *retinoic acid receptor-related orphan receptor-alpha/beta* (*ror-α/β*), and *nuclear receptor rev-erb-alpha/beta* (*rev-erbα/β*) [10]. At the core of the molecular clock mechanism are two basic helix-loop-helix (bHLH)-Per-Arnt-Sim (PAS) transcription factor proteins, CLOCK and BMAL1, which form heterodimers and bind to the transcriptional regulatory (E-box) sequences of themselves and repressor genes *per1/2/3*, *cry1/2*, and *rev-erbα/β* [10] to activate transcription and form a positive loop of the TTFL. The PER and CRY proteins interact to form heterodimers and translocate to the nucleus to repress CLOCK:BMAL1-mediated transcription of genes, forming the primary negative loop of the TTFL and ultimately decreasing the abundance of the repressor genes [11, 12]. As CLOCK:BMAL1 inhibition is relieved, a new cycle of circadian clock transcription begins. A second interlocking accessory loop driven by *ror-α/β* and *rev-erbα/β* provides additional regulation of the rhythmic expression of *bmal1*. REV-ERBA/B competes with RORA/B to bind the retinoic acid-related orphan receptor elements (ROREs) on the promoter regions of *bmal1*, *clock*, and *npas2*. Binding of RORA/B activates transcription, while binding of REV-ERBA/B inhibits transcription of

these core clock components [10], in part through interaction with PER2 [13]. A third transcriptional loop is formed via CLOCK:BMAL1-driven transcription of *D-box binding protein* (*dbp*), combined with *nuclear factor interleukin-3-regulated protein* (*nfil3*) transcriptional activation and repression mediated by the action of ROR/REV-ERB. The resulting protein dimer, NFIL3/DBP, activates transcription of *ror-α/β*. The three loops of the circadian clock mechanism additionally regulate the transcriptional activity of clock controlled genes (Ccg's) by binding the E-box, RRE, and DBP elements at the regulatory regions of target genes [11].

Post-translational modifications fine-tune the rhythms of day and night. Post-translational regulation determines the length of the biological "day and night". Without regulation, if the repressor proteins PER and CRY translocate to the nucleus and immediately repress the CLOCK:BMAL1 transcription of target genes, then the rhythmic cycle would take a few hours to complete rather than a 24-hr cycle. To maintain the periodicity of a circadian cycle to 24-hr, a significant delay in the repression of transcriptional activation is required. This delay in transcriptional repression occurs via regulation of post-translational modifications of core repressor proteins. Phosphorylation of repressor proteins regulates many important processes such as nuclear entry, formation of heterodimeric complexes, and proteasomal degradation [14].

Post-translational modifications provide stability to the core repressor proteins. The period length depends on the phosphorylation state of the PER and CRY proteins. Casein kinase (CK) phosphorylates PERs leading to the recruitment of β-transducin repeat-containing protein (βTrCP) mediated proteasomal ubiquitination and degradation of PERs [14]. Phosphorylation of PER by CK1ε/δ masks the nuclear localization signal (NLS) and marks the protein for proteasomal degradation by E3 ligases; however, the formation of the PER-CRY-CK1ε/δ complex also protects PER from proteasomal degradation promoting nuclear accumulation [15, 16]. A mutation (*tau*) in casein kinase 1ε (CK1ε) results in phosphorylation of a regulatory site on the PER protein, targeting it for ubiquitination and proteasomal degradation, which speeds up the clock and shortens

the period [14, 17]. Phosphorylation by CKs regulates the nuclear entry of PER1/3 through regulation of the nuclear localization signals (NLS), and phosphorylation of PER2 by glycogen synthase kinase-3β (GSK3β) further regulates nuclear entry in mammals [14]. Phosphorylation events can also affect CRY protein stability. CRY phosphorylation by adenosine monophosphate (AMP)-activated protein kinase (AMPK) promotes the binding of F-box and leucine-rich repeat protein 3 (FBXL3) to CRY1 and marks the protein for E3 ligase mediated ubiquitination and proteasomal degradation in the nucleus [18]. F-box and leucine-rich repeat protein 21 (FBXL21) is the primary E3 ligase of CRY in the cytoplasm. FBXL21 has strong interaction with CRY but with reduced efficiency in CRY ubiquitination compared to FBXL3. The involvement of AMPK-mediated phosphorylation of CRY provides a link to metabolism via sensitivity of AMPK to cellular energy status [18]. Both CRY and PER protein post-translational phosphorylation events independently affect the period length in mammals, providing an additional level of clock regulation.

Peripheral tissue clocks. Virtually all tissue types and cells in the body outside of the SCN, referred to as peripheral clocks, exhibit 24-h circadian rhythmicity, similar to the mechanism observed in the master regulator (SCN). Peripheral clocks are required for the normal functioning of an organ system. The cyclic expression of *clock-controlled genes* (*ccg)* downstream of the core circadian oscillator includes some significant number of genes that are key enzymes in nutrient metabolic pathways. The nature, number, and phase of rhythmic expression of genes are highly tissue-specific and dependent on the organ-specific needs. Studies have provided evidence that endogenous clocks regulate ~10% of the mammalian transcriptome, which varies by tissues [19]. The cyclically expressed core clock genes are in a consistent period (24-hr) across all tissues, given the unique profiles of the tissue-specific output of circadian transcriptome. This congruent phase relationship suggests a shared circadian oscillator among organs and the functional integration of molecular and cell-type-specific transcriptional regulation [20]. The timing

of metabolism in peripheral clocks is driven by systemic cues from the SCN or the core clocks in peripheral tissues [9].

CIRCADIAN CLOCK ENTRAINMENT

The rhythms of endogenous biological clocks occur with a period of ~24-h in the absence of external time cues. Daily exposure to environmental zeitgebers (from the German word for 'time-givers'), for example solar cycles, maintains rhythmicity of approximately 24-hr. Zeitgebers provide signals of time that induce molecular changes in the endogenous clock, facilitating adaptation of physiology and behavior [21]. Light is the dominant zeitgeber of the master circadian oscillator (SCN) [22, 23]. The SCN consists of approximately 20,000 neurons and is divided into two main sub-divisions based on the differential expression of neuropeptides. The core (ventrolateral) of the SCN lies adjacent to the optic chiasm, and the shell (dorsomedial) encloses the core [24]. Each neuron can maintain *in vitro* endogenous rhythmicity, indicating the presence of an active autonomous oscillator driven by core clock gene TTFL. Environmental light information that varies in intensity throughout the day signals the SCN through complex mechanistic pathways, as briefly described below. The resetting of the master circadian oscillator by light is referred to as photic entrainment. Additionally, non-photic zeitgebers, including neurohumoral factors released upon food intake [25, 26] and physical activity [27, 28], have been demonstrated to entrain the circadian rhythms of the peripheral oscillators.

Photic zeitgeber. The SCN is the key area of the brain that links external photic signals to internal output of time communication via neuro-humoral signals to the rest of the body. The setting of the SCN clock is achieved by specialized retinal cells called the intrinsically photosensitive retinal ganglion cells (ipRGCs) that transfer light information from the environment to the SCN as they express the pigment melanopsin. These photic signals are transmitted via the retinohypothalamic tract (RHT) [9]. Upon light stimulation of the retina, the terminal synapses of the RHT release neurotransmitters, glutamate (Glu) and pituitary adenylate cyclase-activating polypeptide (PACAP). Glutamate binds to the

N-methyl-d-aspartate receptors (NMDA), increasing calcium influx and activation of calcium-/calmodulin-dependent protein kinase (CaMK) [29]. The co-release of PACAP with Glu has a dual functional role. At the presynapse, PACAP increases the release of glutamate and activates the adenylyl cyclase (AC)- protein kinase A pathway (PKA) at the presynapse and postsynapse, respectively [30]. The downstream stimulation of the ERK/MAPK pathway phosphorylates cAMP response element-binding protein (CREB). The binding of p-CREB to cAMP response elements (CRE) in the promoter regions activates transcription of immediate early genes (IEG) and CLOCK/BMAL1 mediated transcription of *Per1* and *Per2* [31-33] (Figure.1.2). The core TTFL expresses vasoactive intestinal polypeptide (VIP) and gastrin-releasing peptide (GRP), while the shell expresses vasopressin [24]. These neurotransmitters play a critical role in synchronizing neurons to generate coherent rhythmic activity. Peak electrical activity in the SCN occurs during the subjective day and is lowest during the subjective night under constant conditions, where subjective day and subjective night refer to the day or night portion of a light/dark cycle, respectively, under constant conditions. The peak in SCN activity coincides with the sleep phase of nocturnal mice, while in diurnal beings, the peak SCN activity corresponds with the active behavioral phase [24, 30, 34]. Changes in electrical activity were measured in the rat SCN exposed to external light and electrical activity was recorded. Upon light stimulation only a section (32%) of rat SCN neurons showed electrical activity spikes [35], subsequently inducing the expression of clock genes *Per1* and *Per2*. The duration of transcriptional activation of *Per* depends on the intensity of light [31, 36]. Exposure to light stimuli provided during the early subjective night phase delays the physical activity rhythm. In contrast, the same light stimuli provided during late subjective night advances the phase of the activity rhythm in diurnal rodents [37]. Entrainment to a new light cycle relies on these phase dependent responses to light [24] and takes several cycles to re-synchronize [38, 39].

Integration of photic information. The SCN innervates within different areas of the brain to prepare the body and individual organ systems in anticipation of light/dark

cycle via hormonal secretion associated with these changes [40]. The dense SCN innervations within the medial hypothalamus coordinate the release of hormones and regulate the autonomic nervous system [40]. Tracing techniques have revealed SCN innervations in the medial preoptic area (MPO), sub paraventricular area (sPVN), dorsomedial hypothalamus (DMH), arcuate nucleus (ARC), and the lateral hypothalamus.

The circadian regulation of glucocorticoid (GC) release has been a well-explored subject in influencing molecular clocks in peripheral cells. A peak in corticosterone levels occurs about the transition from sleep to wake cycle in nocturnal rodents, which is comparable to a peak at the beginning of the wake cycle in humans [41]. The rhythmic release of GC involves neuro-humoral communication between the SCN and adrenal cortex via the hypothalamic-pituitary-adrenal (HPA) axis and sympathetic nervous system. Photic stimulation of the SCN activates the rhythmic release of corticotrophin-releasing hormone (CRH) from the PVN, which induces the release of adrenocorticotropin hormone (ACTH) from the anterior pituitary. Rhythmic ACTH and parallel stimulation of the autonomic nervous system regulate the diurnal variation in glucocorticoid release from the adrenal cortex [42, 43]. The intrinsic circadian oscillator of the adrenal cortex determines the phase at which response to ACTH is most effective. The responses to ACTH stimulation in cultured adrenal slices of wild-type animals are dependent on the time-of-day stimulus application, and adrenal slices with a non-functional clock lack this capacity of time-dependent response to ACTH [42]. GCs exert their effects by binding to glucocorticoid receptors (GR) expressed ubiquitously in all tissues except the SCN. GRs function as ligand-activated transcription factors that translocate to the nucleus upon binding of GC. GRs in the nucleus binds to the glucocorticoid responsive element (GRE) on promoter sequences of target genes, thereby regulating transcriptional activity [40]. A bidirectional interaction between GRs and core clock proteins exists. GCs activate transcriptional expression of core clock genes in peripheral clocks [44, 45]. Reciprocally, clock proteins control the nuclear localization of GR and GR transcriptional activity. CLOCK, via its histone acetyltransferase (HAT) activity, acetylates GR, decreasing its transcriptional activation, and CRYs rhythmically

repress GR activation by associating with GREs [46, 47]. Collectively, these processes help to define a temporal window of the sensitivity of GC response to gene regulation, and an optimal chronophysiological response occurs when the central rhythm in GC release and peripheral rhythms in GR expression are temporally aligned [41].

Another key synchronizer of circadian signals between central and peripheral clocks is melatonin. Plasma melatonin rhythm peaks during the night in both nocturnal and diurnal animals and is also termed as the 'hormone of darkness'. Peak in melatonin and GC occurs during dark phase in nocturnal animals. In contrast, GC peaks during the day and melatonin peaks at night in diurnal animals. Circadian release of melatonin is sympathetically regulated via the SCN's neural innervations of the pineal gland and is tightly aligned with the light/dark cycle. Because of its nocturnal release, melatonin provides rhythmic endocrine signals during the nighttime [40]. The nightly release of the sympathetic neurotransmitter norepinephrine (NE) stimulates adenylyl cyclase via the β_1-adrenergic receptors of the pineal membrane, resulting in a rise in intracellular Ca^{2+} and PKC activity. A subsequent increase in cAMP levels phosphorylates CREB, which binds to CRE in the promoter region of *arylalkylamine N-acetyltransferase* (*Aanat*) [48, 49]. *Aanat*, a key enzyme in the pathway of melatonin synthesis, exhibits diurnal rhythmicity in its expression. Melatonin exerts its effects by melatonin receptors1/2 (MT1 and MT2) widely distributed throughout the body and brain structures, including the SCN [50]. Melatonin readily passes through the cellular membrane because of its lipophilic nature and may interact with other cellular proteins to exert its effects. Melatonin has various functional roles as a free radical scavenger, an antioxidant, and as a regulator of the entrainment of circadian rhythms of peripheral clock gene expression, sleep, blood pressure, and metabolism [51, 52]. *In vitro* analysis of melatonin exposure during the simulated night in adipocytes increased expression of core clock genes, increased leptin and lipid synthesis, and reduced lipid breakdown [52]. Pinealectomy studies in rats resulted in attenuation of glycogen synthesis in both liver and muscle, while administration of melatonin resulted in lowered blood glucose, increased glucose tolerance, and increased glycogenesis following glucose infusion in the liver and muscle

[53-55]. Recent studies using pinealectomized animal models demonstrated diabetic phenotypes characterized by glucose intolerance and insulin resistance in peripheral and central tissues [56, 57]. Deficient insulin signaling and reduction in GLUT4 expression and translation have been observed in the absence of melatonin in pinealectomized models [56, 58]. Furthermore, melatonin is known to activate tyrosine kinase β-subunit of insulin receptor as well as downstream signaling cascades involving IRS-1/PI(3)-kinase, serine AKT, MAP-kinase, and phosphorylation of STAT3 [59-61]. Additionally, melatonin influences the synthesis and secretion of glucagon from pancreatic islets [62] and phosphorylation of the IGF-1 receptor [63]. In summary, internal synchronization of environmental time involves the coordinated activity of chemical neurotransmitters and electrical coupling in the SCN and other brain areas. The relay of this coordinated information to the peripheral organs occurs via neuro-humoral signals that synchronize to align physiology in anticipation of behavior changes in a day.

Non-photic zeitgebers. Although the SCN is a master regulator of circadian rhythms in the body, other zeitgebers exist that, along with local clocks, play a key role in integrating environmental information to align physiological responses independent of SCN control. Food intake is one such behavior that occurs in a cyclic manner, alternating between feeding and fasting cycles. Accordingly, physiological responses to the environment are designed to either drive energy storage during nutrient abundance or mobilize nutrient stores in fasting [64]. In addition to feeding, physical activity can also entrain peripheral clocks, especially in the skeletal muscle [65].

Feeding. Food intake is a strong zeitgeber for peripheral tissue clocks. Mice fed during the light phase of light/dark cycle, uncouples the phase of SCN and peripheral clocks. As a result, the phase of the SCN is fixed to the timing of external light/dark cycle while that of peripheral clocks is fixed to the timing of food intake [66]. Animal models in which food availability has been manipulated provided notable insights in understanding the mechanisms of energy homeostasis and the consequences of nutrient

dyssynchrony. Neurohumoral signals associated with feeding serve as both clock output and clock input factors in peripheral tissues, as these tissues relay signals to the brain via metabolites (e.g., glucose) and humoral factors (e.g., ghrelin, leptin, insulin) [67]. Free access to a high-fat diet in mice results in obesity, hyperinsulinemia, hyperglycemia, hypercholesterolemia and dyslipidemia compared to mice on low-fat chow [68]. Feeding restricted to the sleep phase in animal models caused disruptions of circadian rhythms [66] that resulted in increased body weight, reduced glucose tolerance, dampened rhythms of glucose and corticosterone, and altered gene expression in liver clock and metabolic genes [69, 70]. A study in rats with food restricted to 4-hr during the rest phase entrained the liver rhythms to the new feeding schedule within two days, with a large phase shift of 10-hr in *per1* luciferase rhythmicity, while the phase of the SCN rhythm remained locked to the light cycle, providing evidence that restricted feeding uncouples peripheral oscillators from the SCN [71]. Timing of macronutrient intake can also affect energy homeostasis, as seen in studies that restricted high fat diets to either the dark phase or light phase. High fat food intake during the light phase resulted in increased body weight when compared to a feeding schedule restricted to the dark phase [72]. Bray et al. [73] studied four different feeding schedules in mice and demonstrated that a high fat diet at the end of the active phase combined with a low fat diet at the beginning of the active phase resulted in increased weight gain, adiposity, decreased glucose tolerance, and increased insulin, leptin and triglyceride levels, compared to animals eating a waking meal high in fat, combined with a late meal low in fat. In contrast, Hatori et al. reported that a high-fat diet provided during the normal active phase improved glucose tolerance, insulin sensitivity, adiposity, serum cholesterol and leptin levels, compared to animals fed ad libitum [74]. In a rodent model of night shift work, feeding restricted to the rest phase resulted in a 6-hr phase shift in triacylglycerol (TAG) rhythms and a 9-hr phase shift in temperature rhythms. TAG and temperature rhythms peaked during the sleep phase in the rest phase feeding group compared to rhythmic peaks that occurred in the active phase in the control groups [75]. Rest phase restricted feeding also promoted weight gain and accumulation of fat. Deleterious outcomes in metabolism were prevented when food was

restricted to normal activity phase (dark phase) [75]. Together these studies indicate a crucial role for timing of food and macronutrient intake in maintaining metabolic rhythms.

Integration of feeding information. The crosstalk between the circadian clock and nutrient metabolism is not confined to the peripheral cellular level, but it also occurs at the systemic level. For example, mammals predict mealtime when food is restricted by increasing their locomotor activity prior to receiving food, and this increase in activity is termed as food anticipatory activity (FAA) [9]. Peripheral energy status of the body is communicated to the brain to regulate feeding homeostasis. The circulating peripheral hormones ghrelin and leptin are implicated in this crosstalk [9]. Ghrelin is released from the oxyntic cells of the stomach in anticipation of a habitual mealtime. The binding of ghrelin to the growth hormone secretagogue receptor (GHSR) in regions of the brain promotes feeding by increasing orexigenic peptides and reduction of anorexigenic peptides [76]. The binding of ghrelin to GHSR encourages feeding and increases the FAA [9]. Ghrelin and the core clock repressor proteins, PER1/2, are expressed together rhythmically in the oxyntic cells. *Per1/2* double KO mice do not express ghrelin, suggesting a role for molecular clock regulation in ghrelin expression and/or release [77]. In *Per2* KO mice, the FAA is absent, and the FAA is decreased in mice lacking ghrelin receptors [77, 78]. These findings indicate that ghrelin elicits its effects via GHSRs regulating feeding in anticipation of mealtime and the integration of cellular clock mechanism regulating temporal expression of regulatory factors at both tissue and systemic level.

One other neuroendocrine factor responsive to feeding in the peripheral tissues exhibiting diurnal variation is leptin. Leptin is released from white adipose tissue proportional to fat depot and is expressed under direct regulation of CLOCK/BMAL1 transcription [79]. Rest phase feeding in mammals reverses serum leptin rhythms to peak during the light phase of the light/dark cycle compared to peak in dark phase when rats are fed *ad libitum* [80], suggesting a role for feeding regulating the 24 h rhythmicity in

serum leptin levels. In contrast, SCN lesions in rodents completely abrogated the rhythmic pattern of plasma leptin levels [81], suggesting that a functional master clock is essential for diurnal variation in plasma leptin levels. Circadian misalignment of behavior (fed/fast and sleep/wake cycles) as seen in shift work and jet lag, resulted in constant baseline levels of leptin throughout a 24 h period when participants food intake and rest times occurred 12 h out-of-phase from their normal routine [82] suggesting differential effects of circadian disruption in humans and rodents.

Several cellular regulators including nicotinamide adenine dinucleotide (NAD^+/NADH) [83], peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α) [84], *Rev-erb* isoforms [19], and adenosine monophosphate-activated protein kinase (AMPK) [18] are all controlled by CLOCK/BMAL1 transcriptional regulation. These regulators control rhythmic metabolic processes in cell function through downstream pathways. The core clock proteins CLOCK/BMAL1 and NPAS2/BMAL1 exhibit sensitivity to intracellular redox changes based on the ratio of $NAD(P)^+$/NAD(P)H when binding to E-box sequences. A higher ratio of reduced forms of redox cofactors stimulates binding to E-box sequences while a higher ratio of oxidized forms inhibits binding [85]. Additionally, the CLOCK/BMAL1 heterodimers regulate the expression of a key enzyme in the NAD^+ salvage pathway, nicotinamide phosphoribosyltransferase (NAMPT) [83], suggesting cellular redox status influencing a reciprocal regulation between circadian oscillator and cellular redox status. A second energy sensing cellular factor corresponds to AMPK. When cellular energy levels are low characterized by elevated levels of AMP (adenosine monophosphate), then AMPK, as a food sensor upregulates intracellular energy generation pathways. AMPK regulates CRY1 and PER2 protein stability by phosphorylation and marking them for degradation [18, 86]. In a in vivo model or PER2:Luciferase mice, Koruda et al. investigated the influence of meal frequency of 2, 3, 4 or 6 meals on the phase shift of circadian clocks in the liver, kidney, and submandibular gland. The phase of clock gene rhythms in these organs was not affected by the different frequency of meals when provided at equally spaced time intervals. Three meals at unequally spaced intervals during a 24-hr period, on

the other hand, resulted in significant phase alterations in peripheral tissue clocks. Length of fasting interval between feeding time points in the tested meal frequency is a critical aspect in resetting peripheral clocks, and a longer fasting interval has a significant impact on the phase resetting process [87]. Cellular energy sensors (i.e., AMPK) that engage in energy-producing pathways during fasting could be a potential mechanism for the resetting effects of peripheral clocks.

Rev-erb isoforms, like AMPK and NAD, are nuclear receptors that are expressed in a rhythmic manner [88] and can bind heme as their ligand [89]. The association of REVERBs with the nuclear receptor corepressor complex (NCoR) is regulated by binding of heme. Furthermore, heme binding to REVERBA inhibits the expression of *g6pase* and *pepck*, two key enzymes in the gluconeogenic pathway, tying the circadian gene to regulating metabolism. Transcriptional coactivator proliferator-activated receptor gamma coactivator 1 (PGC-1α and PGC1- β) is another factor that integrates metabolism and circadian clock. *Pgc-1α* is induced during fasting regulating hepatic gluconeogenesis [90] while intake of saturated fats regulates *Pgc1- β* expression [91]. Both *Pgc-1α* and *Pgc1- β* display rhythmic expression in liver and skeletal muscle [84]. PGC-1α directly upregulates the nuclear factors, receptors, and transcription factors that mediate the regulation of gluconeogenesis [90, 92]. Additionally, PGC-1α coactivates *rorα* thereby inducing core clock gene expression (*bmal1*, *clock*, *per2*, *rev-erbα*) [93]. These findings indicate an important role for nuclear receptors and transcriptional coactivators in response to feeding behavior and nutrients to integrate clock mechanism and metabolism.

Exercise. Exercise is the most commonly prescribed therapeutic in both health and disease conditions. Owing to the large mass of skeletal muscle and high metabolic capacity, it has the potential to influence other tissue functions throughout the body. Apart from exercise promoting quality of life, emerging data have suggested a potent role for exercise as non-photic zeitgeber for circadian clocks. Exercise has been shown to induce a phase shift in the rhythms of melatonin [28] and peripheral skeletal muscle clocks [65, 94]. Scheduled voluntary wheel running under constant darkness entrains

sleep-wake rhythms in C57BL/6 mice to the timing of wheel availability [95]. Scheduled late-night access to running wheels in a circadian disrupted model (VIP deficient mice) helped recover the entrainment deficits in the SCN and peripheral clocks under standard light/dark condition in mice [96]. Wolff et al. tested the effects of 2-h scheduled exercise in advancing the PER2::LUC rhythms in lung and three different skeletal muscles (soleus, extensor digitorum longus, and flexor digitorum brevis) in mice housed under 12h light/12h dark cycle. The study reported that scheduled voluntary or involuntary exercise significantly phase advanced PER2::LUC bioluminescence rhythms in three different skeletal muscles and lungs [65]. Scheduled voluntary wheel running activity presented at the beginning of a light phase facilitated re-entrainment to a new light/dark cycle in skeletal muscle and lung, while the SCN re-entrained to the new light phase conditions in both exercise and control groups [94]. A more recent study by the same group tested the re-entrainment effects of wheel running to advanced and delayed light/dark protocol with running wheel presented at the end of the phase delayed protocol and provided evidence that the phase advances were unaffected by wheel running while re-entrainment by phase delay was hindered in lung and skeletal muscle [97]. *In vivo* experiments investigating the effects of forced (treadmill or wheel running) vs voluntary (wheel running) exercise during scheduled times showed that larger phase shifts in circadian clock gene expression in peripheral tissues occurred in forced exercise compared to voluntary exercise group [98]. Scheduled 1-hr of forced treadmill or voluntary wheel running activity during the inactive (zt4 to zt5) period phase advanced the clock while the same activity scheduled during the active (zt20 -zt21) period phase delayed the clock [98], suggesting differential effects of the same stimulus provided at different times which correspond to the phase response curve for exercise [99]. (PRC; PRC describes the relationship between a stimulus (exercise) and response (direction of phase shift) in relation to the time of stimulus (exercise) presented). Larger phase advances/delays in clock gene expression (*Per2*, *Bmal1*, and *Rev-erbα*) in liver, kidney and the submandibular gland were reported in the forced treadmill exercise vs voluntary wheel running groups. Phase advances/delays in clock gene expression observed in

skeletal muscle and lung, did not differ by exercise [98]. In a recent study, 1-hr muscle contractions, induced by either treadmill running or *in vitro* electrical pulse stimulation of myotubes in culture, regulated expression of clock genes in skeletal muscle tissue and myotubes in a time-of-day dependent manner. Treadmill exercise at the middle (zt5) or the end of the inactive phase (zt11) phase advanced and phase delayed the PER2::LUC rhythms in skeletal muscle explants, respectively [100]. Similarly, time-of-day effects were observed in *in vitro* C2C12 myotube cultures in response to a single bout of electrical contractions. A single bout of 60-min electrical contractions applied at either 22-hr or 28-hr post synchronization of culture, significantly, phase advanced or phase delayed Bmal1:Luc expression, respectively, and were maintained for many days after treatment [100]. The exercise-induced phase shifting effects of circadian rhythms have also been reported in human studies. Yamanaka et al. reported that timed physical activity involving activity occurring at two 2-hr time points facilitated re-entrainment of the sleep-wake cycle to an 8-hr advance in sleep schedule [101]. Altered clock gene expression in human skeletal muscle also has been reported in response to resistance training [102, 103] and endurance exercise [103]. These findings indicate an essential role for exercise as a zeitgeber in regulating the rhythmicity of peripheral circadian clocks.

Although the mechanisms involved in exercise-induced phase shifts of circadian clocks are elusive, the pathways activated during stress have also been implicated in this phenomenon. Two potential pathways in exercise-induced entrainment of peripheral clocks are the HPA and sympathetic-adreno-medullar (SAM) axes. HPA and SAM secrete GCs or catecholamines, respectively. These molecules have been implicated in influencing the phase of peripheral circadian clocks via their respective receptors, GRs and β-adrenoreceptors. Several studies in animals have examined the stimulation of these axes in response to exercise [104-109]. GCs elicit transcriptional regulation directly via the GREs on promoter sequences of clock genes, *Per1*, *Per2*, and *Nfil3* [44, 45] and have also shown decreased transcription of *Rev-erbα* in hepatocytes [110]. Adrenergic receptors belong to the family of G protein-coupled receptors (GPCRs) that have been

functionally well characterized [111]. Noradrenaline induces expression of *Per1*, *Per2*, and *Nfil3* in mouse hepatocytes by activating the α_1-adrenergic receptor and downstream involvement of the MAPK signaling pathway [109], suggesting a role for autonomic nervous system in synchronizing peripheral tissue clocks. A recent study reported the role of corticosterone and norepinephrine in the entrainment of peripheral clocks by forced treadmill and forced wheel running exercise. The levels of both serum corticosterone and tissue norepinephrine were increased using the forced activity model, and the corticosterone rhythm was phase advanced, suggesting that both corticosterone and norepinephrine may be involved in exercise-mediated phase adjustments in peripheral clocks. The mechanism for these responses to forced exercise is yet to be elucidated. These responses to forced activity suggest a similarity between exercise and stress response [98]. Apart from the activity-related effects on skeletal muscle clock, clock gene expression in the absence of motor nerve activity has also been determined via denervation techniques by removal of small segment of sciatic nerve in the upper thigh under anesthesia. Although denervation moderately altered the phase, amplitude, and expression of core clock genes, several rhythmically expressed non-core clock genes displayed altered phase, amplitude, and expression [112]. The calcineurin-NFAT pathway played a key role in activity-dependent rhythmic gene expression via nuclear localization of NFAT and, thereby, regulation of target genes [112]. Nerve activity-dependent phase advances in muscle clock have also been reported by Nakao et al. The group reported decreased expression of core clock genes, *Bmal1*, *Per1*, *Per2*, *Rorα*, *Nr1d1*, and *Dbp* but an increased expression of *Clock* as a result of sciatic denervation [113]. These findings suggest potential roles of implicated pathways that need further investigation for a greater degree of understanding of the exercise-induced peripheral clock phase adjustments.

CIRCADIAN REGULATION OF NUTRIENT METABOLISM

Core clock proteins have pleiotropic functions outside of the molecular clock mechanism. The functional relevance of the molecular circadian clock in metabolism has been illustrated using knockout (KO) and dominant-negative models.

Clock regulation of glucose homeostasis. Recent studies have provided support for the role of pancreatic and liver clock genes in the development of type 2 diabetes [114, 115] and the control of glucose tolerance [116] respectively. The liver is an important regulator of glucose homeostasis. Liver stores glycogen during an abundance of glucose availability. During times of physiological demand, circulating glucose levels are maintained by liver gluconeogenesis or glycogenolysis. The adaptation of metabolism to glucose availability throughout the circadian period is directly dictated by the levels of glucose. The circadian clock helps to provide baseline rhythmic regulation to the occurrence of periodic daily events, such as food intake, after an overnight fast [117]. The SCN controls rest/activity and feeding/fasting rhythms resulting in rhythmic nutrient uptake and periodic fluctuations in blood glucose, while the liver clock functions to buffer these fluctuations to maintain homeostasis in blood glucose levels [117]. Evidence of this buffering action in glucose homeostasis comes from liver-specific *Bmal1* deletion in mice, which results in the loss of rhythmic expression of *Glut2*. *Glut2* mRNA and protein in wild type mice show peak expression during the resting/fasting phase in mice and a trough expression during the active/feeding phase in mice. The peak expression of the *Glut2* glucose transporter during the fasting phase facilitates the export of glucose into the circulation when food intake is low. In contrast, low expression of *Glut2* during the feeding phase favors glucose import and storage when food intake is maximum. Liver-specific deletion of *Bmal1* in mice results in fasting phase hypoglycemia and exaggerated glucose clearance in the presence of normal insulin production [116, 118], suggesting a defect in hepatic glucose export into the circulation. These mice also exhibit reduced liver glycogen storage and loss of circadian expression of clock-controlled hepatic glucose metabolism genes (*G6pt1- glucose 6 phosphatase 1*, *Gck - glucokinase*,

Lpl – liver pyruvate kinase) [116]. Conversely, whole-body *Bmal1* KO mice exhibit decreased plasma insulin levels and increased adiposity, along with fasting hypoglycemia [119-121]. The core CLOCK protein regulates rhythms in hepatic glycogen synthesis, and *Clock* mutants displays diminished rhythms in hepatic glycogen content and mRNA and protein levels of the rate limiting enzyme, hepatic glycogen synthase 2 (*Gys2*) [122]. Similar metabolic alterations in glucose homeostasis are observed in mutant models of the core clock repressor proteins, PER and CRY. *Per2* mutant mice exhibit fasting hypoglycemia, loss of circadian fluctuation in hepatic glycogen storage, elevated plasma insulin levels due to increased glucose-mediated pancreatic insulin response, and impaired gluconeogenesis [123, 124]. Overexpression of *Cry1* specifically in the liver of streptozotocin-induced diabetic mice increased responsiveness to insulin and lowered blood glucose. In the same study, the cryptochrome genes, *Cry1* and *Cry2*, were shown to regulate transcriptional activation of gluconeogenic genes via CREB by interacting with G-protein coupled receptors, blocking cAMP accumulation [125]. Cry1 glucocorticoid receptor interaction in hepatocytes results in downstream transcriptional repression of the rate-limiting gluconeogenic enzyme, phosphoenolpyruvate kinase (PEPCK) [46]. In summary, the liver circadian clock and its interaction with the time-of-day dependent availability of nutrients together play a vital role in maintaining glucose homeostasis.

Skeletal muscle is a major tissue for postprandial insulin-responsive glucose uptake under the regulation of the muscle circadian clock [126, 127]. Skeletal muscle-specific *Bmal1* KO results in impaired glucose uptake due to a reduction in protein levels of glucose transporter 4 (GLUT4) and TBC1 domain family member 1 (TBC1D1), an oscillating GTPase involved in the membrane translocation of GLUT4 [127, 128]. Inactivation of *Bmal1* in skeletal muscle also significantly reduced hexokinase (*Hk*), pyruvate dehydrogenase kinase (*Pdk*) and pyruvate dehydrogenase phosphatase 1 (*Pdp1*), all enzymes involved in the catabolism of glucose [127]. Skeletal-muscle specific *Bmal1* KO mice favor glucose storage, as indicated by reduced expression and activity of phosphofructokinase 1 (*Pfk1*), increased glycogen content, decreased levels of glycolytic

intermediates (glyceraldehyde-3-phosphate, 2-/3-bisphosphoglycerate, and phosphoenolpyruvate), and elevated lactate, suggesting preferential glucose storage versus glucose oxidation in the absence of *Bmal1* in skeletal muscle [129]. Reduced activity of pyruvate dehydrogenase complex (PDC) [127] in this KO model may divert glycolytic intermediates into alternative pathways, which may explain the increase in lactate production. Although influx of glucose is lower in the *Bmal1* KO model, levels of TCA cycle intermediates were unaffected. Additionally, several amino acids that serve as precursors for TCA cycle intermediates were increased, suggesting protein mobilization for energy generation. Low levels of citrate and isocitrate further support the shift in nutrient substrate utilization [129].

The α- and β-cells of the endocrine pancreas secrete glucagon and insulin, respectively. These two hormones play a significant role in regulating glucose homeostasis and also exhibit day and night fluctuations in their secretion that occurs independently of feeding. The rapid secretion of these hormones in response to a nutritional challenge (i.e., bolus of glucose) occurs as repeated pulses [130, 131]. Similar to other tissues, global and tissue-specific KO models of clock genes in the pancreas have provided insights on clock-regulated insulin secretion and the development of diabetes. *Clock* mutants, along with pancreas-specific *Bmal1* KO in mice, display increased circulating glucose, decreased pancreatic insulin secretion, and glucose intolerance [120]. Reduced pancreatic islet size and impaired insulin release were also observed in *Clock* mutants, along with reduced or altered gene expression patterns for genes involved in glucose uptake, glucose metabolism, β-cell growth and proliferation, and insulin signaling [120]. Isolated islet cells from *Clock* mutants revealed defective insulin exocytosis in response to a glucose challenge, lack of glucose-induced Ca^{2+} increase prior to insulin secretion, and reduced translation of proteins involved in insulin vesicle transport [120]. Conditional ablation of *Bmal1* in the pancreas resulted in defective insulin secretion by β-cells, increased glucose levels, and impaired glucose tolerance in mice [114, 115], whereas *Per2* knockouts displayed increased glucose-responsive insulin

secretion and reduced insulin clearance [123]. Additional to the core clock genes, *Clock* and *Bmal1*, *Rev-erbα* also plays a vital role regulating glucose homeostasis in the pancreas. Downregulation of *Rev-erbα* in the pancreas via RNA silencing resulted in impaired glucose responsive insulin secretion and a decrease in exocytotic genes [132], which is in agreement with the study findings in *Clock* mutant mice described above [120].

Clock regulation of lipid metabolism. Hepatic lipid metabolism involves the production of lipoproteins, lipid uptake, lipid synthesis, fatty acid β-oxidation, and cholesterol and bile acid synthesis. The majority of these processes involve circadian clock regulation in the liver. *Clock* mutant C57Bl/6 mice on a normal diet experience a significant decrease in gene expression of *hypocretin neuropeptide precursor* (*Hcrt1*), *ghrelin* (*Ghrl*), and *cocaine-amphetamine regulated transcript* (*Cart*) in the brain, resulting in an obesity phenotype [133]. Homozygous *Clock* mutants develop steatosis in the liver and hepatosteatosis with age when fed a regular chow diet. These mice were characterized by obesity, increased accumulation of triglycerides in the liver, and increased plasma alanine aminotransferase (ALT). Genes involved in lipid uptake (*Cd36*), synthesis (*Dgat2*, *Fas*, *Srebp1c*), and lipoprotein assembly (*Mttp*) were upregulated in the *Clock* mutant mice, while those involved in fatty acid oxidation (*Cpt1*, *Pparα*) were downregulated in *Clock* mutant animals. These changes preceded the endoplasmic reticulum stress related alterations in gene expression. Hepatosteatosis was accelerated when these mutant mice were fed high fat and Western diets [134]. A recent study in which the mutant mice received wild-type *Clock* DNA vectors injected through the tail vein partially rescued the metabolic impairments observed in *Clock* mutant mice fed a high-fat diet via hepatic gene therapy [135]. The gene therapy was associated with normal body weight gain, recovered diurnal rhythmicity in feeding behavior, and energy expenditure comparable to wild type mice. The application of intact CLOCK protein ameliorated fatty liver in mutant mice, suggesting a role for circadian clock regulation of hepatic lipid metabolism [135].

Like *Clock*, *Bmal1* has also been implicated in roles outside of the core clock mechanism regulating metabolism. Liver-specific *Bmal1* deletion results in dyslipidemia with high circulating free fatty acids and increased hepatic triglycerides [118]. Deleting liver-specific *Bmal1* and *ApoE* and inhibition of liver lipoprotein lipase (*Lpl*) activity in mice displayed hyperlipidemia and atherosclerosis. Microsomal triglyceride transfer protein (MTP) is involved in hepatic lipoprotein synthesis. In the *Bmal1* and *ApoE* KO model, higher MTP activity was reported, which explains the increased lipoprotein production; these effects were lessened when *Bmal1* was overexpressed in the liver, providing evidence for the role of *Bmal1* as a regulator of lipid levels in both tissue and circulation [118].

Another member of the core clock mechanism involved in regulation of lipid metabolism in various tissues is *Rev-erbα*. *Rev-erbα*, plays a vital role in epigenetic modulation of lipid metabolism by colocalizing with histone deacetylase 3 (HDAC3) to chromatin structures and repressing transcriptional activation of target genes. REVERBA and HDAC3 together have been reported to colocalize at 130 gene sites in hepatocytes, repressing transcription of genes involved in the biosynthesis of lipids including *accα* and *fasn* (*fatty acid synthase*) [136]. The absence of HDAC3 or *Rev-erbα* in mice results in hepatic steatosis due to a lack of repression of lipid synthetic genes suggesting a crucial role in lipid homeostasis [136]. *Rev-erbα* plays an influential role in regulating lipid metabolism through the diurnal expression of Srebp-1c controlled lipid metabolic pathways in the liver. Lipid oxidation is favored during the daytime, while lipid production is enhanced during the nighttime, as substantiated by upregulation of *Lpl* (in muscle) and *Fas* (in muscle and white adipose tissue), respectively, in nocturnal mice. Lpl is constitutively expressed in *Rev-erbα* KO model due to lack of regulation by *Rev-erbα* in muscle and increased expression of *Cd36* levels. Together these changes reflect increased fatty acid uptake and metabolism in muscle [19]. Similarly, HFD fed *Rev-erbα* KO mice displayed increased expression of *Lpl* and *Cd36* along with the increased expression of *Srebp1c*, *Fas*, *acetyl-CoA carboxylase* (*Acc*), and *ELOVL fatty acid elongase 6* (*Elovl6*) in hepatocytes, consistent with the metabolic phenotype in this model

[19]. These findings describe the wide-ranging roles of *Rev-erbα* at the genomic level regulating chromatin structures to influencing key genes involved in lipid metabolism.

White adipose tissue primarily functions to store energy as triglycerides (TGLs). Rhythmic clock gene expression has been described in different murine and human depots of adipose tissue. Several adipose tissue transcripts and adipose-derived hormones display rhythmic variation in expression. Many of these are important regulators of appetite and lipid metabolism in adipose and influence metabolic processes in other tissues as well [137]. *In vivo* deletion of *Bmal1* either globally or in an adipose-tissue specific manner results in adiposity in mice younger than 20 weeks of age. By contrast, identical deletion of *Bmal1* in mice older than 30 weeks of age revealed a significant reduction in adiposity that may be a result of the premature aging phenotype observed in this model [138]. *Bmal1* has been reported to function in adipogenesis as observed in adipocyte-specific *Bmal1* KO models with decreased expression of enzymes promoting fatty acid biosynthesis. These enzymes include, *Elovl6* and *stearoyl CoA-desaturase* (*Scd1*) [139]. Exposure of *Cry* double KO mice to high-fat diets resulted in hyperinsulinemia and weight gain, despite reduced food intake. The increased secretion of insulin, coupled with increased insulin sensitivity and lipid uptake in adipose tissue in *Cry* double KO mice, explains the weight gain in this model despite lower calorie intake than littermate controls. Weight gain in these mice is further confirmed by increased expression of *Fas*, *Lpl*, *Acc*, *Acsl4*, *Dgat1/2*, and *lep* [140]. *Rev-erbα* KO mice display increased expression of *Lpl*-mediated lipid uptake in adipocytes on both chow as well as HFD [19]. A double KO of both isoforms of *Rev-erb* increases preferential mobilization of free fatty acids for energy [141], and similar findings were observed in the *Rev-erbα* only KO models [19]. Along with driving the core clock mechanism, the above findings provide evidence for non-clock functions of core clock genes in white adipose tissue.

White adipose tissue mobilizes stored triglycerides during energy deficient states such as during fasting with a role for clock genes in this process. The *staggerer* mice with functionally deficient *Rorα* display resistance to DIO, with the phenotype attributable to increased expression of *medium-chain acyl-CoA dehydrogenase* (*Mcad*) resulting in an

increased influx of fatty acids through β-oxidation in white adipose tissue, coupled with the decreased fatty acid synthesis in the liver (corresponding to decreased *Srebp1c* expression) [142], demonstrating a role for *Rorα* in lipid metabolism.

Together these findings convey a better understanding of the intimate interplay of the cellular circadian clock and metabolism in mammals via the non-canonical clock functions of core clock genes. These findings also support the observation that many types of disruption of the clock can result in a significant alteration in macronutrient metabolism and risk for development of chronic diseases.

SHIFT WORK – HISTORY, CHARACTERISTICS, AND CONSEQUENCES

Shift work is an example where the normal synchrony is disrupted between the light/dark cycle, physical activity, feeding and sleep. Repeated out-of-phase instances of daily activities induce a multitude of changes in the physiological state of an individual [143]. This section will briefly discuss the epidemiology and consequences of shift work influencing lifestyle factors such as sleep, food intake, and activity and eventually discuss the mechanisms and pathways that link shift work, circadian rhythms, and impact on health.

A brief history. Evidence of shift work traces back to ancient Romans when shift schedules included evening, midnight, and early morning shifts [144]. Shift work schedules disappeared during medieval times and later got revitalized during the industrial revolution, which paralleled the breakthrough discovery of electricity by Michael Faraday [145] and the invention of the first electric bulb patented by Thomas Edison [146] during the 1800s. These scientific advances set the initial stage for an effortless extension of daytime work into the night. As labor laws developed, policy makers prohibited children (1833 Factory Act) from working night shifts to prevent abuse of children working very long night shifts in the United Kingdom. In 1906, the International Association for Labor Protection (Switzerland) prohibited night work for women to prevent abuse of women and protect their health [144]. The past century reinstated shift work due to growing demands in production and availability of services

round the clock associated with the beginning of World War I, advances in industrial development, expansion of global economy, travel/communication, and growth of urban areas [4, 144]. These transitions have made working outside of the regular 7:00 am to 6:00 pm job environment not only commonplace but necessary, which has created more jobs for new shift workers [147]. Shift work in our 24-hour society is not limited to critical services such as public safety by police and fire personnel, healthcare, transportation, and utilities such as power and water. Shift work has expanded into round-the-clock availability of groceries, gas, food in restaurants, and many other essential conveniences and services [147].

Shift work characteristics and prevalence. Shift work schedules can vary between individuals and may be different, depending upon the work environment. Shift work schedules can be fixed, where individuals live and work on only one shift throughout the week, or discontinuous, with work interrupted during weekends or interspersed with work that does not involve a night shift [148]. At the organizational level, shift work systems vary by several factors related to the occupational demand. These factors include the length of shift cycle, the duration of shifts, the number of individuals working shifts, the timing of the shift cycle, the speed and direction of shift rotation, the number of rest days, the position of rest days between work shifts, and the regularity of shift schedules [148]. Shift workers periodically alternate on different shift work schedules by combining the factors mentioned above at the organizational level [148].

According to a report from the Bureau of Labor Statistics (BLS), nearly 16 percent of the American workforce over the age of 15 y work in non-daytime schedules or shift work schedules (including evening, night, rotating, split shift, or an irregular work schedule) in the year 2017-2018. Of the total workforce, the non-daytime workforce consisted of 18% men, compared to 15% women [149]. Approximately 9% of shift workers held a bachelor's degree or higher. Approximately 33% of individuals worked in service-related occupations (such as health care, protective service, food preparation and

serving, building maintenance, personal care and service), 29.2% in transportation and material moving, 25.4% in production, and 25.1% in sales and related professions [149]. The proportion of shift workers was highest in the leisure and hospitality industry (36.8%), followed by transportation and utilities (26%), and wholesale and retail trade (25.4%) and the lowest in construction (3.2%) industries [149]. Although these changes have enabled an efficient and convenient society, they have also introduced challenges related to social life, physiological adaptation, work schedule tolerance, and deleterious impacts on health.

Consequences of shift work. Shift work involves working outside of the regular daytime hours of work, as described previously. Altered or irregular shift timing forces individuals to eat and be active at a time of day that is out of sync with natural rhythms of light and dark and is associated with increased risk for several disease conditions. The risk for health impairment may be attributed to a combination of factors including sleep deprivation and alterations in the timing and frequency of food intake and physical activity, among several other factors affected by a shift work schedule.

Sleep. Sleep is an essential aspect of daily living necessary for survival. Humans have evolved to sleep/rest at sunset and be awake and active at sunrise. Optimal health occurs when the sleep/wake cycle and physiology are synchronized to the external daily light/dark cycle. Healthy sleep involves a sufficient amount of sleep occurring at an appropriate time every day in the absence of interfering factors or disorders affecting sleep [150, 151]. The classical “two-process” model of sleep regulation has two components, the homeostatic pressure to sleep (homeostatic sleep drive) and circadian rhythms. The homeostatic sleep drive and wakefulness promoted by circadian rhythms increases during daytime. However, the rise in wakefulness overrides homeostatic sleep drive resulting in awakening. Under normal conditions, sleep occurs when pressure for sleep is high and wakefulness is low synchronizing homeostatic and circadian components of the two-process model of sleep [152, 153]. Shift work interferes with the normal sleep/wake cycle, often resulting in short daytime sleep and accumulation of sleep

debt due to lack of recovery of sleep [152, 153]. For instance, when a night shift worker is not accustomed to their shift schedule, both homeostatic sleep drive and circadian rhythm for wakefulness increase during the day, forcing the individual to be awake. When wakefulness drops at night, it signals the individual to sleep, increasing sleep pressure furthermore when awake at night. As a result, night shift workers may fall asleep immediately after their work shift but may also have difficulty remaining asleep long enough to satisfy the need for rest, as circadian rhythms for wakefulness are increasing during their daytime sleep. Due to this sleep debt, the homeostatic pressure for sleep does not decrease entirely [153] (Figure. 1.3).

Multiple hormones are involved in the regulation of sleep. Melatonin, a sleep hormone, is secreted from the pineal gland, and the levels vary in response to the time of day. Melatonin secretion begins to increase near the onset of darkness, peaks between 3:00 am - 4:00 am and remains low throughout the day until the next evening. Cortisol, which is associated with wakefulness and is part of the stress-response system, peaks upon awakening and fall close to midnight in individuals on a normal daytime schedule [154]. Weibel et al. reported that the peak in cortisol rhythm coincides with night workers' daytime sleep schedule, with low cortisol during the active work time at night, negatively impacting sleep and wakefulness, respectively (Figure. 1.4). As a result of opposing hormonal signals with night workers' sleep/wake behavior, the daytime sleep schedule is short and fragmented, giving rise to homeostatic sleep pressure deficits and dampened arousal during night work time [154]. The combination of opposing hormonal signals and sleep debt may explain the common complaint of excessive sleepiness among early morning and night shift workers or in shift workers with reduced time for recovery between shifts as seen in rapidly rotating shift schedules [155]. However, when peak melatonin secretion is aligned with daytime sleep schedules or when a greater proportion of melatonin secretion occurs during the daytime sleep of fixed night workers, sleep bouts tended to be longer [156, 157] . A harmonious coordination between the homeostatic and circadian sleep processes promotes continual bouts of sleep and

wakefulness in a 24-hr day, as observed in daytime work schedules [158]. When the peak melatonin rhythms are aligned with shift workers' shifted sleep schedules, sleep bouts tend to be longer [156]. Behaberou-Brun et al. examined the relationship of melatonin secretion and daytime sleep quality in night shift nurses using 6-sulphatoxy-melatonin (UaMT6s) excretion in 24-hr urine samples as a marker of circadian time [157]. The quantity of UaMT6s excreted during the day (aligning with the nurses' sleep daytime sleep schedules) was associated with improved sleep quality and shorter naps during night shift work [157].

The Centers for Disease Control and Prevention (CDC) recommends ≥ 7 hours of quality sleep per day for adults between 18-60 years. In 2014, a CDC report indicated that 35% of adults in the United States sleep less than 7-hr per day [159]. The Sleep Health Index (SHI) is a valid research tool developed by National Sleep Foundation (NSF). This tool measures three distinct yet related elements of sleep – duration, quality, and disorders. According to NSF report for the last quarter of 2018, the average SHI score was 77 out of a possible 100 among American adults (18 – 65+ yr and multi ethnic participants), and the average sleep duration sub-index score was approximately 79 out of a likely 100 [160]. In a metanalysis study examining sleep length in fixed evening (shifts that start between 2 and 5 pm), fixed night (shifts that start between 10 pm and midnight) and rotating (morning, evening, and night) shifts compared to fixed day shift control group [161], the shortest sleep duration occurred after night shift among fixed night workers and after morning shift (shifts that started between 6 and 9 am) and night shift in rotating shift schedules (~ avg. 5-6 hr). The most prolonged sleep duration was observed after an evening shift in fixed evening shift group (~ avg. 8-hr) [161]. In summary reduced sleep duration is a common problem prevalent among the general population as well as amongst shift work individuals working in shifts not aligned with day/night and/or hormonal rhythms.

According to the American Academy of Sleep Medicine (AASM), shift work disorder (SWD) is classified as a circadian rhythm sleep-wake disorder. The diagnostic criteria for SWD include reduced sleep time and sleep efficiency, increased insomnia and

daytime sleepiness [155], and the persistence of symptoms for at least three months, according to the international classification of sleep disorders [162]. The prevalence of SWD among night workers is around 32%, as reported in both community-based [155] and random population cohort studies [163]. The prevalence of SWD is specific to the occupation. The prevalence of SWD was lower among oil rig workers [164] compared to nursing staff, which consists primarily of females [165]. The environment of oil rig workers is more conducive for adaptation to night work with the absence of interfering factors and social demands [164]. In contrast, female nurse night workers may carry significant responsibility for domestic duties and child care after work [165]. Evidence suggests that a fixed night work schedule is associated with circadian adaptation to shift work, with additional challenges resulting from rotating night work [166, 167].

Sleep deprivation (sleep debt) is associated with negative physiological effects on health, work performance deficits, and work-related accidents. Perkins (2001) reports that individuals on a night shift schedule may experience periods of altered state of awareness that may last up to 30 seconds due to cumulative sleep deprivation [168]. During this brief duration, the individual may lack focus and responsiveness to the surroundings, leading to work-related accidents [168]. A quantitative experiment from Reid *et al*. compared psychomotor performance after 28 hours sleep deprivation to measured blood alcohol concentration in individuals who were asked to consume 10-15g of alcohol every 30 minutes until the average blood alcohol concentration was 0.10% in two different experiments. After being sleep deprived for more than 24 hours, the cognitive psychomotor performance level of the participants was comparable to the performance level of participants with a blood alcohol of 0.10% [169]. High rates of sick leave have also been associated with rotating daytime (work schedules rotated between morning and afternoon shifts) workers [170]. Reports of frequent sick leave are typical among night and evening shift nurses [170], as well as rotating daytime workers [171] as mentioned earlier. However, a systematic review was inconclusive when examining the link between sick leave and rotating shift work [172]. Needless to say, insufficient sleep and

low sleep quality have significant negative influence on the health and lifestyle of shift workers and their families.

Food intake. The unconventional work hours observed in shift work schedules can induce changes in several aspects of eating behavior [173]. Shift work may alter macronutrient intake, calories, quality of food, and the timing of food intake. A systematic review of total caloric intake did not differ significantly between daytime and shift workers [174]. However, another cross-sectional study reported higher caloric intake among high-frequency night shift workers compared to the day workers, although diet quality was similar between both groups [175]. A similar cross-sectional study compared macro- and micro-nutrient intake in fixed-day workers, shift work without night work, and shift work with night work conditions [176]. Energy intake was highest in the shift work with night work condition across all age groups compared [176]. Micronutrient intake among young (20-29 yr) shift workers with night work was the lowest of all groups, as this was not observed in older shift workers [176]. These findings suggest negative influence of shift work on both macro- and micro-nutrient intake.

Several factors influence eating patterns among shift workers. A recent systematic review analyzed the factors influencing food intake under four themes corresponding to when shift workers eat, what type of food shift workers eat, the source of food during shift work, and the stimuli to eat during shift work [177]. The meal timings of shift workers tends to be defined by work schedules [178-180], availability of down time during work [180-182], the interplay between shift work and family life [182, 183], and local cultural influences, which vary across industries and around the globe. The type of work shift influences the nutrient profiles of meals, snacks, and meal size. For instance, vegetable intake among night shift bus drivers is less than their day working colleagues [184]. Resident physician interns consumed less fruit in proportion to the increased number of work hours [185]. Overweight and obese nurses consumed fewer servings of fruit and vegetables than nurses who were ideal weight or underweight [186]. Additionally, in nurses, the length of the work shift was positively associated with consumption of deep-fried foods [187]. Night shift and rotating shift workers consumed

significantly higher saturated fats and less carbohydrate compared to day-working individuals, respectively [176]. These findings demonstrate that shift workers in general consumed lesser fruit and vegetables in their diets compared to day workers. These choices in food intake are mostly influenced by several factor as described above.

The availability of food plays a crucial role in shaping eating habits [180]. The sources where shift workers can obtain food typically include cafeterias, on-the-job meals, vending machines, take-away foods, break rooms, and home-cooked food [177]. Although cafes in some facilities are open at night, nurses report a preference for vending machines to save the time required to making a trip to the canteen [182]. The lack of cafes during nighttime work hours in some facilities limits the choice of available foods [188, 189]. Industries related to airlines, food services, and others may provide meal(s) during shift work time. This provision helps to standardize meals for all workers but may also prove a barrier to healthy eating with continuous access to food [190, 191]. An intervention study to promote fruit intake among shift workers, resulted in increased fruit consumption and dietary fiber [192]. A similar intervention of pre-packed meals provided for lunch significantly decreased total fat intake and increased fiber and water intake among the entire working population (including day and shift workers) during the intervention period compared to the control period. In contrast, shift workers significantly consumed a higher amount of water during the intervention period compared to control period, while all other dietary elements did not reach significance during this period, suggesting differential efficacy of the intervention among day and night shift workers [193]. Finally, Gupta *et al*. explored the motivation behind food consumption during shift time in a systematic review [177]. The findings suggest that availability of down time, social pressure at work, work-related barriers to implementing healthy behaviors (e.g., stress eating), and years of experience as shift worker may negatively influence quality of food intake [177].

Several studies in humans have identified altered food intake patterns, such as frequent nibbling of energy dense snacks, altered timing of intake, and frequency of

intake, as independent risk factors for weight gain, impaired glucose metabolism, insulin resistance, and dyslipidemia [194-197]. Nutritional surveys of shift workers via 24-hour dietary recall, food diary, and questionnaires have demonstrated that total daily energy intake between different shift work schedules did not differ [176, 198-200]; however, frequent nibbling of energy dense snacks has been reported to be high during the night shift [199, 201]. Nocturnal animals, which typically consume a higher proportion of food during active phase (dark phase), fed only during their sleep phase (light phase) experience disruptions in metabolic rhythms, along with increased body weight, reduced glucose tolerance, dampened rhythms of glucose and corticosterone, and phase shifts in gene expression in the liver and other tissues [66, 69, 72]. A disruption in circadian rhythms of neurohumoral factors can explain the mechanism that links metabolic alterations to a disturbed temporal eating pattern. Several metabolic hormones and nutrient metabolic pathways are under circadian regulation. Studies in humans have indicated increased levels of ghrelin, increased insulin resistance, and reduced leptin levels in the presence of single nucleotide polymorphism (SNP) variants in circadian genes [202] or in response to altered timing of sleep/wake cycle [82]. Altogether these studies indicate the importance of timing in food intake and their influence in regulating numerous metabolic processes, ultimately influencing health in individuals. A more detailed discussion on the crosstalk between circadian rhythms and metabolism is provided in the section titled ‘Circadian regulation of nutrient metabolism.’

Physical activity. Many studies provide evidence that physical activity protects against the risk of chronic disease and all-cause mortality [203, 204]. The World Health Organization (WHO) recommends at least 150 minutes of moderate-intensity physical activity per week to benefit health [205]. Despite these health benefits, it is often challenging for shift workers to meet the recommended physical activity guidelines. Participation in team sports, group activities, or other scheduled leisure activities may be difficult for shift workers due to their incompatible work schedules [206]. A survey of professional social care workers investigated the quality of life, life satisfaction, and

happiness among individuals in different work schedules. Shift workers with night shifts reported reduced opportunities to change behaviors related to physical well-being, leisure activities, and personal growth compared to day workers or shift workers without night work [207]. Occupational factors such as job title, work hours, work status, and high work demand are associated with physical inactivity [208]. Approximately 49% of nurses did not meet the recommendations for physical activity of 30 minutes on most days of the week according to a cross-sectional study that examined the health behaviors of nursing staff [209]. Physical inactivity among shift workers is a global concern, as reported in several studies [208, 210, 211]. In contrast to the above findings, leisure time and occupational physical activity measured objectively in hospital shift workers using accelerometers was similar to physical activity in non-shift workers [212]. Another prospective cohort study of nurses evaluated physical activity under different work schedules. No significant differences were observed at baseline and 6-year follow-up in physical activity under different work schedules [213]. For shift workers who prefer individual activities, shift work-associated disruption was not a concern [206]. These individuals could participate in activities at convenient times, while also benefitting from discounted rates for using health club facilities during non-peak hours [206]. As several studies reported similar physical activity levels among different work schedules and between shift workers and non-shift workers, one may hypothesize that the impaired metabolic consequences observed among shift workers may result from sleep deprivation [214] or temporal alterations in eating behaviors [215] or that physical inactivity contributes equally to shift and non-shift workers.

Physical activity interventions at the workplace have reported beneficial effects on workers' health [216, 217], along with increased productivity and reduced health care costs [218]. A recent systematic review of randomized controlled trials (RCTs) evaluated worksite physical activity interventions in shift workers. All studies reported that physical activity improved anthropometric and biological risk factors [219]. Nevertheless, none of the seven studies included in the review measured changes in physical activity behaviors and activity timing or described the setting where the activity occurred [219]; therefore, it

is still unclear how and whether these interventions influenced physical activity behaviors among shift workers in the long term. Despite the positive outcomes associated with a physical activity intervention, a mere 6.4% of individuals showed interest in being recruited, suggesting substantial barriers to physical activity participation in the shift work population [220].

MECHANISM AND PATHWAYS RELATED TO HEALTH CONSEQUENCES AMONG SHIFT WORKERS

Shift work is characterized by alterations in several aspects of daily living. Kecklund *et al*. (2016) proposed a theoretical model of mechanisms and pathways of shift work-related alterations in behaviors in the development of chronic diseases. The first of these alterations is exposure to light at night and darkness during day, which is inverse to the light/dark cycles in which living beings have evolved. Second, food intake patterns are altered, with food consumed late at night when humans have normally evolved to sleep, increased frequency in eating, irregular eating patterns, and limited availability/access to healthy food choices, all commonly observed in shift work-related food intake behaviors. Third, shift work results in shorter sleep duration and early termination of sleep, causing excessive sleepiness and fatigue during work hours. Lastly, shift work also increases adverse health behaviors, including increased smoking and alcohol consumption and reduced physical activity. These alterations in lifestyle result in circadian disruption, disturbed sleep, overweight/obesity, sedentary lifestyle, and psychosocial stress. In this model, a cluster of all these factors ultimately increases physiological stress, with the net effect of increased risk for chronic disease development and accidents [221].

SHIFTWORK, CIRCADIAN MISALIGNMENT, AND HEALTH

Increasing exposure to light at night, as evidenced in shift work, can significantly alter the temporal organization of biological rhythms in humans, increasing the risk for the development of obesity [222], metabolic dysfunction [223, 224], cancer [225], sleep

disturbances [226], and cognitive impairments [227, 228]. The connection between shift work-related circadian disruptions and disease risk has gained increased attention. Metabolic syndrome is characterized by elevated triglycerides, central obesity, high blood pressure, and impaired fasting glucose and is a condition that is increasing in epidemic proportions globally [229]. Several combinations of factors are involved in the development of this disorder, including circadian misalignment, sleep deprivation, alterations in timing and quality of meals, conflicts between personal and work schedules, and the type of shift rotation [230]. Insulin resistance and type 2 diabetes are commonly prevalent among shift workers [231, 232] with a higher percentage observed in rotating shift workers [233, 234]. These findings are in agreement with a meta-analysis of observational studies that reported significantly increased risk for type 2 diabetes mellitus among rotating shift workers [235]. Additionally, a higher number of night shifts per month [234] and higher work hours (>40 hours/week) was positively associated with increased risk for type 2 diabetes [236]. Cardiovascular events (myocardial infarction, stroke, and arrhythmias) frequently occur during the early hours of the morning [237]. The likely mechanisms of this temporal occurrence of events may be explained by increased blood pressure, loss of endothelium-dependent diurnal variation in vasodilation, changes in heartbeat dynamics, the peak level of steroid hormones (testosterone, adrenaline, and cortisol), and increased sympathetic activity during the early morning [238]. The first scientific article that described the associations of cardiovascular disease (CVD) in shift workers was published in 1984 [239]. About 40% of shift workers have an increased risk of cardiovascular disease. Although the mechanism of shift work-related increased risk for CVD is not fully known, the possible contributing factors may relate to circadian disruption, lifestyle factors including smoking, alterations in food intake, and stress underlying psychosocial issues [240]. Circadian dyssynchrony of clocks, as evidenced in shift work, is associated with high blood pressure, insulin resistance, dyslipidemia, and obesity [237]. In addition to a high prevalence of metabolic syndrome and CVD, shift work is also associated with multiple health issues related to psychological, reproductive, gastrointestinal, metabolic, and sleep

disorders, along with cancer. The international Agency for Research on Cancer (IARC) has classified shift work associated disruption in circadian rhythms as a probable human carcinogen [148].

Animal models using dim light (~5 lux) exposure at night closely mimic light exposure in human shift workers. In a study by Fonken et al., dim light at night (dLAN) in a 14h:10h light/dim light cycle in mice resulted in increased body mass with increases in epididymal fat mass also resulting in time-, tissue- and gene-specific alterations in core clock genes. Overall, core clock gene expression levels in the hypothalamus (*per1*, *per2*, and *cry1*), liver (*bmal1*, *per1*, *per2*, *cry1*, *cry2*, and *rev-erbα*), and white adipose (*rev-erbα*) tissues were reduced with dLAN exposure compared to mice housed under 14h:10h light/dark cycle. In the SCN, both gene and protein expression of *Clock* were suppressed in dLAN conditions [241]. In another study, mice exposed to the light/dLAN (14h:10h) developed metabolic disturbances (increased body weight, fat mass and glucose intolerance) caused by a shift in substantial amount of food consumed in the light phase vs nocturnal mice who consume most food in the dark phase [242]. When the normal light/dark cycle was reinstated, the metabolic disturbances in weight gain, epididymal fat mass, and glucose metabolism were reversed [243].

Feeding behavior in animal models that closely mimic shift work involves food availability being shifted to the light/rest phase of the day. Bray et al. studied the effects of light phase restricted feeding in mice using the CLAMS (Comprehensive Lab Animal Monitoring System) that allows for simultaneous measurement of numerous metabolic parameters (oxygen consumption, carbon dioxide production, respiratory exchange ratio, food consumption, locomotor activity levels, core body temperature, and heart rate) and avoids direct human contact, which has also been shown to induce stress-related weight gain in animals. Light phase-restricted feeding altered energy homeostasis and increased body weight, caloric intake, RER, and decreased energy expenditure [69]. Significant phase differences in liver clock and metabolic gene expression were observed compared to other peripheral tissues (epididymal fat, gastrocnemius muscle, and heart) [69]. In

summary shift work causes desynchronization between the environment and the organism at multiple levels that may occur: 1) between central and peripheral clocks [71], 2) among peripheral clocks in different organs [69], 3) between the molecular clock mechanism and clock-controlled genes [70], and 4) within the molecular clock itself [244]. Altogether, dyssychrony results in suboptimal functioning of many physiological processes affecting health.

INTERVENTIONS TO MITIGATE HEALTH EFFECTS OF SHIFT WORKERS

Alleviating the disruptive effects of shift work on health is a complex process. Several efforts have been made to lessen the risk of development of chronic diseases among shift workers, including implementing ergonomic work schedules [245-248], controlled exposure to light/dark cycle [249, 250], physical activity and lifestyle changes [251], and pharmacotherapy to promote adaptation [252, 253]. Ergonomic work schedules allow for optimal adaptation to shift work and consider the personal preferences of shift workers in addition to organizational requirements. Accordingly, the strategies investigated involve changing from backward (3xEvening/1x day off/ 3xMorning/ 3xNight/ 5xday off shift) to forward shifts (2xMorning/ 2xEvening/ 2xNight shift/ 4x day off) and increasing rest time between shifts [245-247], working 12-hr shifts instead of 8-hr [248, 254, 255], flexibility in work schedules [256, 257], and or self-scheduling work time [258, 259]. Studies involving a transition from backward shift to forward shift or changing the speed at which shift rotation occurs (3xEvening/1x day off/ 3xMorning/ 3xNight/ 5xday off shift versus 2xMorning/ 2xEvening/ 2xNight shift/ 4x day off) have shown recovery in the form of increased sleep quantity and sleep quality, lowered serum glucose and triglyceride levels and decreased systolic blood pressure [245-247]. Interventions involving 12-hr versus 8-hr shifts were associated with improved sleep quality and duration, with a fewer number of broken sleep pattern episodes in the 12-hr worktime groups [248]. In contrast to these findings, Axelsson et al. did not find any significant differences in sleep quality or duration between 8- and 12-

hour week schedules, with sleepiness corresponding to sleep duration and physical work rather than shift duration [255]. Ergonomic shift scheduling interventions significantly improved work-life conditions and sleep hygiene, reduced mental stress, and reduced the need for recovery when shift workers were given limited worktime control [259]. Boggild et al. reported improvement in lipoprotein profiles when shift workers agreed upon selected predefined ergonomic scheduling criteria, represented by decreased low-density lipoproteins and total cholesterol-to-high-density lipoprotein (HDL) ratio, with an increase in HDL levels [256]. Systematic reviews evaluating organizational level interventions affecting health and work-life balance report beneficial effects specific to the intervention. Most benefits associated with sleep were observed for speed of rotation, with a shift from slow to fast rotation (switch from slow to fast rotation included changing from 6 to 7 consecutive shifts of the same shift type to 3 to 4 consecutive shifts), shift direction involving backward (night/ afternoon/ morning) to forward (morning/ afternoon/ night) rotation, and lastly self-scheduling worktimes improved sleep and work life balance [258].

Exposure to bright light and the use of goggles that shield light during commute time after a night shift or before sleep time have been investigated independently and in combination as an intervention strategy to improve sleep health among shift workers. The use of light-shielding goggles allows for release of melatonin while lowering cortisol and body temperature [249, 250, 260]. A combination of brief exposure to bright light (7,000 to 10,000 lux for ≥ 30 min) during mid-way of a night shift and use of dark goggles after shift work was investigated in a randomized controlled study of rotating shift nurses. The findings reported significant improvements in daytime sleep and nocturnal alertness upon exposure to bright light, while morning light attenuation was not effective in improving clinical insomnia in the recruited nurses [261]. A recent cross-over design study exposed sleep-deprived individuals to bright light near the end of a conventional night shift. The sources of light therapy were either a light therapy box (10,000 lux) or light therapy glasses (2,000 lux LED blue light) that significantly improved alertness, cognition, and

mood, suggesting an important practical application in night shift workers. However, the sample size in this study was very small, and the participants recruited were young and not true shift workers (may have a history of shift work but not doing any shift work during study recruitment) and may have differential effects than actual shift workers [262]. The effects of using blue light blocker glasses after a night shift work increased sleep duration and decreased fragmented sleep, suggesting an application that may help to improve daytime sleep among perpetual night shift workers [263].

The use of pharmacotherapy or stimulants among shift workers to promote sleep, vigilance, or adaptation to shift work includes sleep promoting agents such as melatonin or stimulants like caffeine. In a randomized, double-blinded, placebo-controlled crossover trial of night shift nurses, administration of 5 mg of melatonin prior to usual sleep time significantly improved sleep quality and decreased sleep onset latency, providing an effective treatment to overcome difficulty in falling asleep among night workers [252]. In contrast to these findings, melatonin was ineffective in improving sleep quality in a randomized, double-blind, placebo-controlled crossover trial involving pediatric residents [253]. It should be noted that the dose of melatonin and the time of melatonin administration in these two trials varied. Caffeine is a common stimulant used among shift workers to counteract sleepiness. Caffeine intake at 2 mg/kg of body weight during a stimulated night shift reduced sleepiness as measured by multiple sleep latency (multiple sleep latency test, which tests physiological daytime sleepiness by measuring the time taken to fall asleep during a day) onset and increased performance measured using a computerized simulated assembly line task (SALT). Consumption of caffeine did not interfere with the sleep after the simulated night shift work [264]. A systematic review of caffeine intake and work injuries and errors among shift workers provided evidence that caffeine is an effective performance enhancer, as measured by improved reasoning, memory, attention, and perception parameters. Due to very limited number of trials available, the effects on injuries and errors were not assessed [265]. The effect of

the use of caffeine or other stimulants is not clear due to limited research on the long-term effects of caffeine supplementation on sleep.

Interventions related to behavioral changes for improving health among shift workers are limited. Most behavioral interventions have targeted physical activity and/or dietary changes. Interventions occurring at the workplace have more potential for positive lifestyle changes. A physical training intervention (running, jogging, skiing, swimming, walking, gymnastics for 4-months) study involved 2-6 exercise sessions at 60% to 70% of maximal heart rate values customized in relation to age and other health parameters. The participants were female nurses and were evaluated via questionnaires, laboratory tests and field study pre- and post- intervention [251]. Post intervention, the nurses showed a significant increase in sleep length after an evening shift and decrease in fatigue during worktime and increase in physical fitness in the physical training group [251]. A systematic review of randomized controlled trials (RCTs) of physical activity interventions in shift workers indicated that physical activity mitigated risk factors associated with the development of chronic diseases, including a reduction in body weight, body mass index (BMI), waist circumference, and fat mass post physical activity intervention. The duration of physical activity interventions in the primary studies included in the review varied between 2 weeks to 6 months. The physical activity sessions occurred 2 to 6 sessions per week at moderate to vigorous intensity. The modes of physical activity included walking, jogging, rowing, or a combination of aerobic and resistance training. Some primary studies in this review also included evaluation of behavioral aspects such as sleep quality and duration, general health, and low back pain [219]. Additional to improvements in sleep quality and sleep duration, improvements in lipid profiles and a cardiovascular marker, cathepsin, were reported [219]. The function of cathepsins is to degrade nonessential intracellular proteins; however, studies have discovered other roles of cathepsins in the development and progression of atherogenesis [266]. A 10-week intermittent walking (10 min of brisk walk 3 times/day during night work) program for 3-days a week in night shift workers significantly reduced levels of

serum cathepsin isoforms suggesting intermittent exercise as an effective strategy in preventing atherosclerosis in night shift workers [266].

Knutsson et al. (2002) evaluated the efficacy of meal timing during night work on physiological parameters of blood glucose, insulin and triglycerides. The intervention involved intake of identical meals at three different time points (7:30 pm, 11:30 pm, and 3:30 am) during night work for all nurses. Peak levels in glucose and insulin response were observed after the 11:30 pm meal, while triglycerides peaked after 7:30 pm meal. The results indicate that food intake at an inappropriate time of day may have health implications, increasing the risk for chronic diseases [267]. A prospective trial among fire fighters included team and individual centered programs for one year. Outcome measures included dietary, physical activity, body weight and general health pre- and post-interventions. Post intervention assessments included significantly lower weight gain and increased fruit and vegetable consumption among participants promoting changes in behavior towards better health [268]. Currently there are very few studies evaluating the effects of dietary interventions among shift workers. Future studies assessing intervention in larger sample sizes and examining the timing of macronutrient intake, combined with group or individual oriented interventions, may be helpful to apply findings to shift workers.

CONCLUSION

Shift work plays an integral role in our modern-day society. Research to promote long term health and well-being in this population has been somewhat ignored and very limited. Although several interventions to promote health in this population have been investigated, the application of these findings to a broader population are limited by sample size and varied shift characteristics. The approach for future studies should focus on the use of advanced technologies and a holistic approach to provide personalized guidance that may help to increase adherence to behavioral changes and long-tern improvements in health. Evaluating and translating the findings from non-shift work

research and animal models to human shift workers may help to provide some meaningful insights. Evaluating the effects of time-restricted feeding, intermittent fasting, ketogenic diets, or timed activity in shift workers could prove valuable. Although, interventions in this specific group are challenging, the long-term benefits on health, safety, and costs likely outweigh the difficulties.

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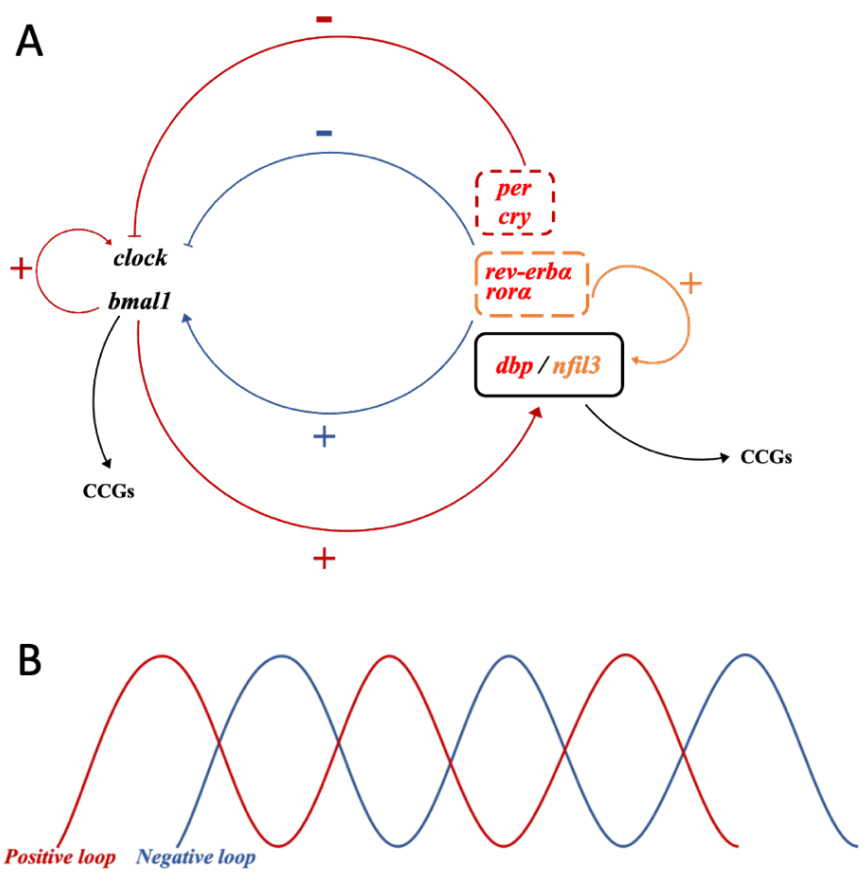
A
-
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bmal1
rev-erbα
rorα
+
+
dbp / nfil3
CCGs
CCGs
+
+
B
Positive loop
Negative loop

Figure 1.1.

Figure 1.1: Molecular clock mechanism

The molecular clock mechanism is driven by the interacting positive and negative transcriptional translational feedback loops (TTFLs). At the core of the clock mechanism CLOCK/BMAL1 activate transcription of themselves and repressor genes *per*, *cry*, *rev-erbα* forming the positive loop of the TTFL. The PER and CRY proteins form a complex and inhibit the CLOCK/BMAL1-mediated transactivation of genes which forms the negative feedback loop of the TTFL. As CLOCK/BMAL1 inhibition is relieved from PER/CRY complex and the levels of these proteins begin to decline, this begins a new cycle of circadian clock transcription. A second accessory loop driven by *rorα/ rev-erbα* regulates the diurnal expression of *bmal1* stabilizing the clock mechanism. Binding of the RORA to the promoter regions of *clock* and *bmal1* activates transcription, while binding of REV-ERBA to the same promoter sequences inhibits transcription of these core genes. A third transcriptional loop of the clock mechanism is driven by CLOCK/BMAL1 transcription of *dbp* and ROR/REV-ERB driven transcription of *nfil3*. The resultant DBP/NFIL3 proteins regulate the transcription of *rorα*. Additionally, the three loops of the circadian clock mechanism regulate the transcriptional activity of clock-controlled genes (CCGs) by the transcription factors (CLOCK/BMAL1, DBP, and REV-ERBA/RORA) binding the promoter regions of target genes (A). Diurnal rhythmicity in expression occurs when the genes that form the positive loop begin to rise causing the genes of the negative loop to rise inhibiting the transactivation of genes in positive loop, thereby reducing their level of expression and relieving inhibition of transactivation, beginning a new cycle (B). Abbreviations: CLOCK, circadian locomotor output cycles kaput; BMAL1, brain and muscle-Arnt-like 1; PER, period; CRY, cryptochrome; ROR, retinoic acid receptor-related orphan receptor; REV-ERB, nuclear receptor rev-erb; DBP, D-box binding protein; NFIL3, nuclear factor interleukin-3-regulated protein; CCGs, clock-controlled genes.

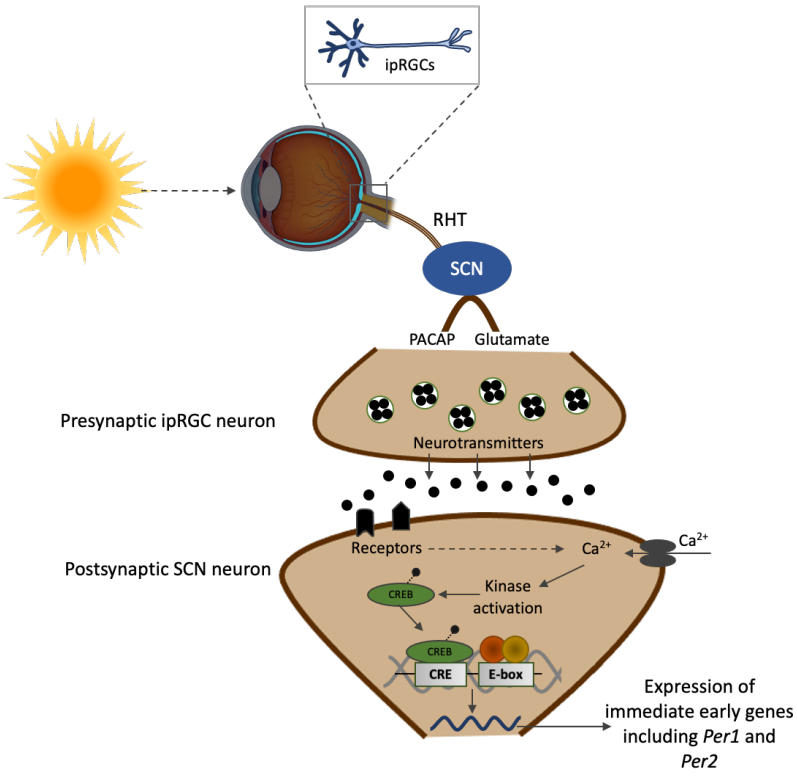

Figure 1.2: Mechanism of light induced expression of immediate early genes in the suprachiasmatic nucleus (SCN) of the hypothalamus.

External light activates the ipRGCs expressing melanopsin. The light signals are transmitted via the RHT which release neurotransmitters, glutamate and PACAP. Glutamate binds to specific receptors which increases calcium influx and activates CaMK. Additionally, PACAP increases the release of glutamate and activates PKA pathway. The resultant downstream stimulation of ERK/MAPK pathway phosphorylates CREB. Phosphorylated CREB binds to CRE in promoter regions activating transcription of IEG and CLOCK/BMAL1-mediated transcription of *per1* and *per2*. Image adapted from Sultan A (2018) [3]. Abbreviations: ipRGCs, intrinsically photosensitive retinal ganglion cells; RHT, retinohypothalamic tract; PACAP, pituitary adenylate cyclase activating polypeptide; CaMK, calcium-/calmodulin-dependent protein kinase; PKA, protein kinase A; ERK/MAPK, extracellular signal-regulated kinase/ mitogen activated protein kinase; CREB, cAMP response element-binding protein; CLOCK, circadian locomotor output cycles kaput; BMAL1, brain and muscle-Arnt-like 1; PER1/2, period1/2.

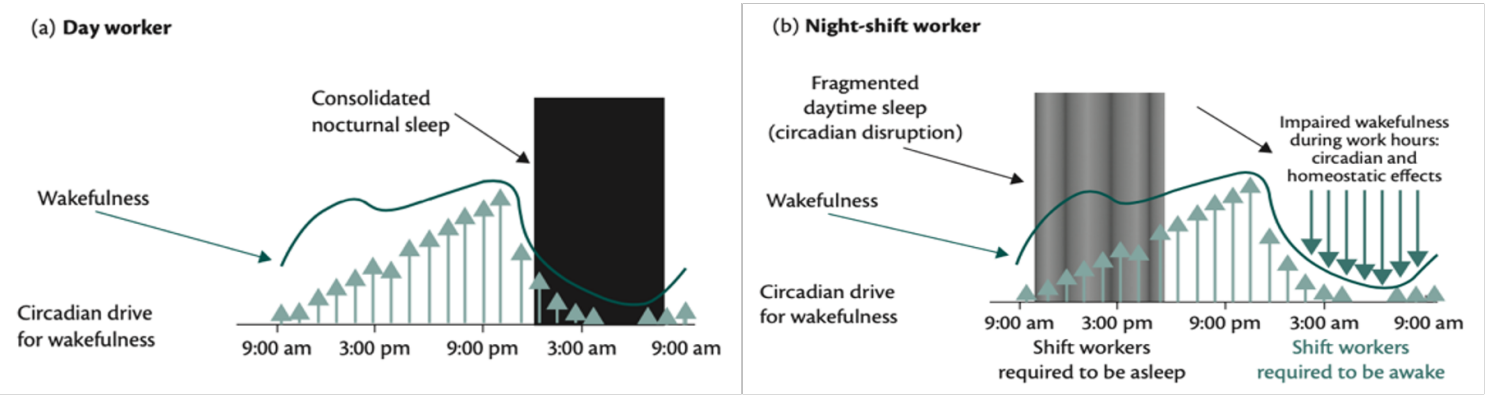

Figure 1.3: Two process model of sleep.

The components of the two-process model of sleep includes homeostatic sleep drive and circadian rhythms. In day workers, homeostatic sleep drive and circadian rhythms work together to promote wakefulness during the daytime and sleep during the night (A). Among shift workers, the circadian alerting signal is strong during the day, which leads to short and fragmented sleep resulting in sleep debt. During the night, when the worker is required to remain awake, sleep debt coupled with blunted nighttime circadian arousal can result in excessive sleepiness. The image was taken from Pillai et al. (2017) [2].

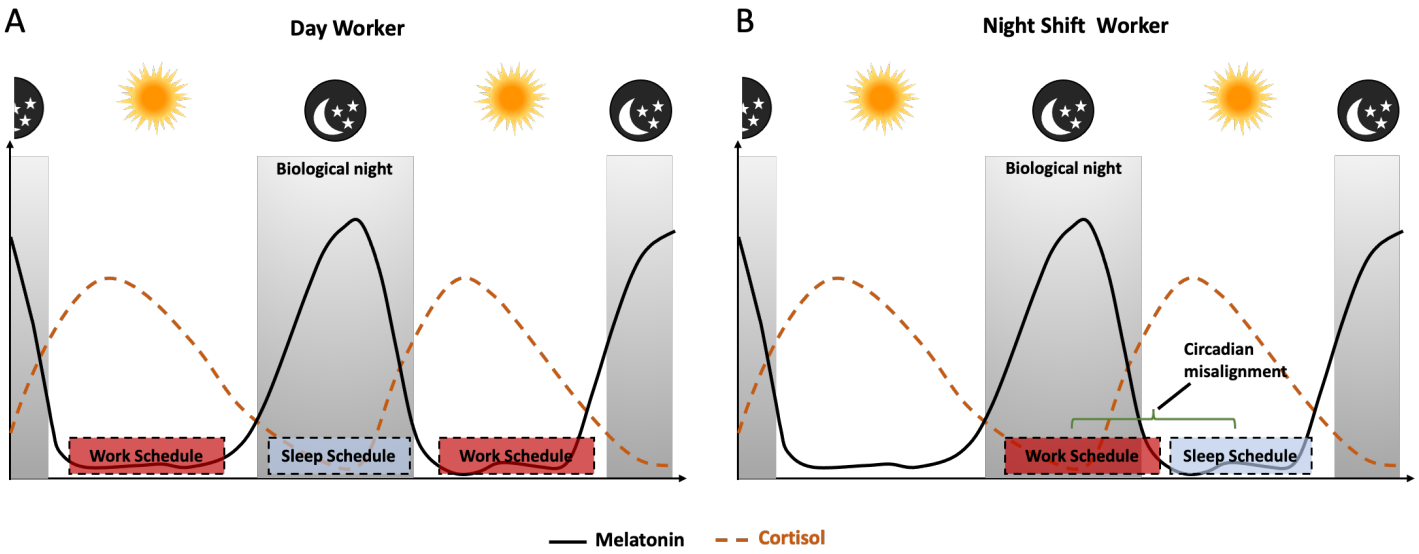

Figure 1.4: Melatonin and cortisol rhythms in day and night shift workers.

(A) Among day workers, cortisol peaks during the day stimulating wakefulness aligned with day work schedule while melatonin peaks during the night in line with sleep schedules of day worker. (B) In night-shift workers, cortisol rhythm is misaligned with daytime sleep schedule while melatonin is misaligned with night work schedule. The image was adapted from Meijer, K (2017) [1].

Chapter 2: Acute exposure to rotating shift work alters activity patterns, core clock, and molecular gene expression in mice

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Running Title: Implications of acute exposure to rotating shift work.

Number of figures: 7

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ABSTRACT

Background: Endogenous molecular clocks influence physiology, metabolism, and behavior. Shift workers are exposed to repeated changes in the timing of light, feeding, and activity and, consequently, are at increased risk for development of chronic disease associated with disruptions of circadian rhythms. The objective of this study was to determine the physiological, behavioral, and molecular alterations associated with acute exposure to rotating shift work (RSW).

Methods: Mice (n=160) were housed in either normal or RSW conditions, consisting of 12-hour shifts in the light/dark cycle every 3 or 4 days, and sacrificed every 4 h for 24 h after either 3 or 14 days exposure to RSW.

Results: Wheel running activity patterns were disturbed within 3 days of exposure to RSW. Substantial alterations in the diurnal expression of core clock and metabolic genes were observed in a tissue-specific manner. After 14 days of RSW exposure, most tissues lost complete clock rhythmicity. Acute exposure to RSW resulted in impaired fasting glucose by the end of 2-week protocol.

Conclusion: Acute exposure to RSW disrupts molecular functions in a tissue-specific manner, which may precede the metabolic consequences of chronic exposure to RSW.

BACKGROUND

Many of our body's biological processes follow approximately 24-hour rhythms that regulate and maintain physiological functions in mammals. Endogenous timekeeping is achieved by the molecular circadian clock system, with the central circadian oscillator in the suprachiasmatic nuclei (SCN) within the hypothalamus of the brain and interdependent cellular oscillators in peripheral tissues. The molecular clock is comprised of an intracellular auto-regulatory process governed by interacting positive and negative transcriptional-translational feedback loops (TTFL). At the core of the molecular clock mechanism, CLOCK (Circadian Locomotor Output Cycles Kaput protein) and BMAL1 (Brain and Muscle-Arnt-Like 1) proteins form heterodimers and bind to the transcriptional regulatory (E-box) sequences of themselves and repressor genes *per1/2/3* (*period1/2/3*), *cry1/2* (*cryptochrome1/2/3*), and *rev-erbα/β* (*nuclear receptor rev-erb-alpha/beta*) [1] to activate transcription, forming the positive loop of the TTFL. The PER and CRY proteins bind to form heterodimers that repress CLOCK:BMAL1-mediated transcription, forming the negative loop of the TTFL [2, 3]. A second interlocking accessory loop driven by *ror-α/β* (*retinoic acid receptor-related orphan receptor-alpha/beta*) and *rev-erbα/β* provides additional regulation to the molecular clock. REV-ERBA/B competes with RORA/B to bind the retinoic acid-related orphan receptor elements (ROREs) on the promoter regions of *bmal1* and *clock*. Binding of RORA/B activates transcription, while binding of REV-ERBA/B inhibits transcription of these core clock components [1]. The TTFL additionally regulates the transcriptional activity of clock controlled genes (CCGs), which include key enzymes in metabolic pathways [2]. The molecular circadian clock synchronizes internal clock timing with external cues (e.g.,

light, food, exercise) [4-7]. Light is the dominant zeitgeber (timekeeper) of the master circadian oscillator, which resets endogenous physiological and behavioral rhythms to match the 24 h day [8]. Additionally, non-photic zeitgebers, including neurohumoral factors associated with food intake [9, 10] and exercise [11, 12], have been demonstrated to entrain the circadian rhythms of the peripheral oscillators.

In the past century, transitions in industrialization, globalization, travel/communication, and urbanization have created a "24-hour society" designed to cater to the modern lifestyle's increasing social and economic demands [13]. These transitions have made working outside of the regular 8:00 am to 5:00 pm job environment not only commonplace but necessary [14]. Although these changes have enabled an efficient society, they have also introduced challenges related to physiological adaptation to this lifestyle. Humans have evolved as a diurnal species in a natural light/dark (LD) cycle, with activity and feeding primarily constrained to the light phase and sleep to the dark phase of the day. The cycle of sleep and activity follows an approximately 24 h rhythm, which coincides with the rotation of the earth on its axis [13, 15].

Evidence suggests that the ever-increasing exposure to light at night can significantly alter the temporal organization of biological rhythms in humans, increasing the risk for the development of obesity [16], metabolic dysfunction [17, 18], cancer [19], sleep disturbances [20], and cognitive impairments [21, 22]. Shift work is an example of the new "24-hour society," leading to disruptions in the normal synchrony between the LD cycle, physical activity, feeding, and sleep. In addition, these out-of-phase daily activities induce a multitude of changes in the physiological state of an individual [14], including decreased levels of leptin and increased levels of blood glucose, insulin, and blood pressure, among many other metabolic alterations [23].

Shift work has up until now been investigated using repeated phase shifts of the LD cycle, forced activity during sleep phase, sleep restriction models, and altered timing of food intake. We used an innovative mouse model that closely mimics human rotating shift work through repeated 12-hr alterations in LD cycles every 3 to 4 days. Our goal was to determine whether acute exposure to a model of simulated human rotating shift work alters metabolism, energy balance, adiposity, and/or molecular circadian rhythms in clock and metabolic gene expression compared to a normal LD cycle.

MATERIALS AND METHODS

Animals. Eleven-week-old FVB/N male mice (Jackson Laboratories, Farmington, CT, USA) (n=160) were individually housed in microisolator cages at the Animal Resource Center facility at the University of Texas at Austin (Austin, TX, USA). Before undergoing experimental conditions, the animals acclimated to a 12 h light/12 h dark schedule for one week, with *ad libitum* access to standard chow (LabDiet, St.Louis, MO, USA; Kilocalories from protein 24.5%, fat 13.1%, and carbohydrates 62.4%) and water. Approval for all experimental procedures was granted from the Institutional Animal Care and Use Committee (IACUC) at the University of Texas at Austin.

Experimental design. Mice were randomized to two experimental conditions, control or shift work. The control conditions consisted of an unvarying daily 12 h light/12 h dark cycle, with the lights on at ZT0 (6:00 am) and light off at ZT12 (6:00 pm). In the shift work condition, mice were exposed to alternating light/dark cycle inversions to simulate the rotating shift work paradigm, as described below and illustrated in Figure 2.1. All mice received *ad libitum* access to standard chow and water during the experimental period.

Rotating shift work experimental design. A novel murine model of rotating human shift work was implemented, as shown in Figure 2.1. LD cycle inversions occurred each week to simulate a rotating shift work paradigm with alternating patterns of 3 days "on shift"/ 4 days "off shift" during week 1, followed by 4 days "on shift"/ 3 days "off shift" during week 2. "Off shift" is defined as normal light/dark cycle with lights on at ZT0 and lights off at ZT12, and "on shift" is defined as an inversed LD cycle with lights on at ZT12 and lights off at ZT0. Each transition from the on shift to off shift schedule and vice versa was punctuated by extended (24 h) periods of light or dark, respectively, as illustrated in Figure 2.1.

Mice underwent terminal circadian dissection at the end of 3 days for control (3DC) and shift work (3DS) and at the end of 14 days control (14DC) and shift work (14DS). Six mice from each treatment group were dissected every four hours (i.e., at ZT0, ZT4, ZT8, ZT12, ZT16, and ZT20) over 24 hours. Harvested tissues were snap-frozen in liquid nitrogen and stored at -80^0C for molecular analysis.

A subset of mice (n=8/group) was used to characterize daily voluntary wheel activity, weekly body weight, body composition, and glucose response after exposure to control (C) and rotating shift work (S) conditions for 2 weeks. Voluntary daily activity in mice was measured using a low-profile wireless running wheel (Med Associates Inc., St. Albans, VT, USA) placed inside each cage. These mice were not included in the terminal dissection or gene expression assays.

Body weight and body composition. Body weight was measured weekly using a digital scale (Ohaus, Parsippany, NJ, USA), with both groups of mice measured when lights were on. In addition, we measured body weight in all mice prior to dissection. Mice were placed into a tared container, and weight was recorded when the movement of the

animal stabilized. Body composition in live animals was measured at the end of the study using a non-invasive MRI (Echo MRI LCC, Houston, TX, USA).

Glucose tolerance test (GTT). Before GTT, mice fasted for 12 h during their sleep phase (i.e., regular lights on). All measurements of glucose were made using tail blood. Fasting glucose levels were measured in duplicate using a Freestyle Lite glucometer (Abbott Laboratories, Abbott Park, IL, USA). Glucose levels were recorded at 15, 30, 60, 90, and 120 minutes after intraperitoneal administration of 10% D-glucose at 1g/kg body weight. The tests were performed two hours prior (i.e., at ZT10) to the normal light-to-dark transition.

RNA extraction and real-time RT-qPCR. Total RNA was extracted using standard extraction procedures from the liver, gastrocnemius muscle (GC), brown adipose tissue (BAT), visceral adipose tissue (VAT), and subcutaneous adipose tissue (SAT). RNA was quantified using spectrophotometry (Cytation 3, BioTek Instruments. Inc, Winooski, VT, USA), and concentrations were normalized across all samples and tissues. RNA was reverse transcribed to cDNA using RNase H+ MMLV reverse transcriptase enzyme (Bio-Rad, Hercules, CA, USA) according to the manufacturer's protocol. Quantitative RT-PCR was performed to measure the expression of the core clock and metabolic genes on the CFX platform and analyzed using the Maestro software (BioRad Laboratories Inc, Hercules, CA, USA). All samples were run in duplicate, and relative gene expression was calculated using a standard curve. Gene expression data are represented as mRNA molecules in femtograms (fg). cDNA ranging from 12.5 to 60 ng which is within the detection range for the gene expression assay from BioRad.

Statistical Analysis. Statistical analysis was performed using one-way ANOVA for body composition, fasting glucose, total physical activity, and AUC (area under the curve) for glucose. Weekly body weight was analyzed using repeated measures ANOVA,

with time as the within-subjects factor and group as the between-subjects factor. Mixed modeling was used for analyzing daily wheel activity data. All graphical results are expressed as mean ± SEM (standard error of the mean). All analyses were performed using Stata 17 SE (Stata Corp., San Antonio, TX).

The circadian period for wheel-running activity was determined by a chi-square periodogram using the Circadian Physiology software package (Circadian Rhythm Laboratory, New Orleans, LA). Rhythmicity of the 24 h gene expression data was examined using nonlinear cosinor analysis. Data were visualized using box plots by group and zeitgeber time. Gene expression data points marked above 1.5 IQR of the upper quartile and below 1.5 IQR of the lower quartile [24] were identified as outliers and excluded from cosinor analysis for all gene expression data. Given the predetermined light/dark cycles, the period was fixed at 24-hr in the following cosinor equation:

$$f(\mathrm{t}) = A * Cos\,[(2\pi t/T) + \phi] + C$$

Where A is the amplitude of oscillation (distance between peak and trough of the rhythm/2); t is zeitgeber time (0, 4, 8, 12, 16, or 20); T is the period of oscillation (24 h); ϕ a measure of time of peak expression; and C is the intercept (rhythm adjusted mean). The percent variance accounted for by the rhythmic model is given by the R^2 value. F-statistic and corresponding p-values were calculated using the mean square model (MS-model) and mean square residual (MS-residual) from cosinor output. For data with a significant cosinor fit, rhythmic parameter comparisons for phase and amplitude of core clock gene expression were tested for differences between groups. Groups were significantly different if the 95% confidence intervals for each parameter did not overlap.

For metabolic gene expression data, mean expression levels were analyzed between treatment conditions using ANOVA. Cosinor analysis was performed for genes whose mean expression levels were significantly different by experimental condition. A p-value of <0.05 was considered significant for all tests. All cosinor analyses for gene expression data were performed using zt time because of altered LD cycles every 3 to 4 days. The sample size for cosinor analysis varied between 3-6 at each time point for both core clock and metabolic gene expression.

RESULTS

Acute exposure to a rotating shift work (RSW) paradigm did not alter body weight, body composition, and glucose tolerance in mice. Animals with *ad libitum* access to running wheels displayed similar body weights, fat mass, and lean mass in both control and rotating shift work light conditions (Figure 2.2A) at the end of 2-week experimental protocol. The body weights of the mice that underwent circadian dissection showed a significant effect of time for both 3- and 14-day exposure to experimental protocols (Figure 2.2B and C); however, no effects of treatment condition on body weight independently or as a function of time were observed. Shift work animals without a running wheel were heavier on average than control animals, with high variability within the groups. The small but non-significant difference in body weights between the shift work mice with and without running wheels at the end of 2-weeks (Running wheel: 29.16 g ± 2.23 vs. No running wheel: 30.0 g ± 2.09) suggests that the presence of the running wheel may have attenuated the effect of the shift work light exposure.

Acute exposure to RSW resulted in statistical differences in fasting blood glucose, which was significantly higher for shift work mice compared to controls in animals with

ad libitum access to running wheels (C: 79.06 ± 15.1 mg/dL vs S: 129.71 ± 38.7 mg/dL; $p<0.05$; Figure 2.3A). Area under the curve for glucose was not statistically different between the experimental light conditions (Figure 2.3B). Normalizing AUC glucose (AUC glucose divided by total activity) for wheel activity measured as distance did not result in significant differences by experimental condition (Figure 2.3C).

Acute exposure to RSW altered rhythmic pattern in physical activity. Examination of wheel running activity patterns under control conditions revealed distinct patterns of high and low activity during the dark and light phases, respectively (Figure 2.4A). In contrast, mice in the shift work condition lost this rhythmic pattern of physical activity (Figure 2.4B). Although total activity measured as distance was not statistically different between control and shift work animals (113.8 km ± 36.2 vs 86.6 ± 35.2 km, $p=0.1789$) (Figure 2.4C), the activity levels differed significantly by light and dark phase. The control mice displayed significantly higher dark phase activity compared to shift work mice (105.6 ± 40.1 km vs 50.6 ± 27.9 km; $p=0.0114$) (Figure 2.4D). In contrast, shift work mice displayed significantly higher light phase activity compared to control mice (36.0 km ± 17.5 vs 8.19 ± 6.0 km; $p=0.0018$) (Figure 2.4D). Within treatment conditions, the control mice displayed significantly higher dark phase activity compared to light phase (105.6 ± 40.1 km vs 8.19 ± 6.0 km; $p<0.0001$). While, within shift work mice, activity was similar by dark and light phase (50.6 ± 27.9 km vs 36.0 ± 17.5 km; $p>0.05$; Figure 2.4D). Additionally, both groups of mice displayed a progressive increase in wheel running activity for 2 weeks of the study. Linear mixed modelling was used to test the effect of time and treatment on wheel running activity distance. The wheel running distance increased significantly over time ($p<0.0001$) in both control and shift work mice. However, this trend of time was not significantly different between treatment

conditions controlling for the effect of zeitgeber time and light status (whether lights were on or off). The wheel running activity period (i.e., estimated length of the activity cycle) was determined individually for mice from both groups for 14 days of study duration. The average period in wheel running activity was not significantly different between groups (C: 24.14 ± 0.4 vs S: 24.25 ± 2.9; $p>0.05$); however, mice in the control group displayed a period range between 24 to 25 h, while the wheel running activity period in the shift work mice was highly variable with a range between ~19 to 27 h.

Acute exposure to a RSW paradigm significantly altered molecular peripheral rhythms in circadian clock genes in a tissue-specific manner. In the present study we evaluated the hypothesis that exposure to a 3- or 14-day rotating shift work protocol alters rhythmic expression of core clock genes. We investigated the rhythmic expression of *bmal1*, *per2*, *rev-erbα*, and the clock-controlled gene *dbp* (*D-box binding protein*) in liver, gastrocnemius muscle, brown adipose, visceral adipose, and subcutaneous adipose tissues. Figures 2.5 and 2.6 represent clock gene expression after three and 14 days, respectively, and each row represents a different tissue. Cosinor parameter data for the core clock genes are presented in Tables 2.1 and 2.2. The *dbp gene is an immediate CCG that* provides a readout of clock function. *Dbp* exhibited a decreased amplitude across all tissues after 3-day exposure to the RSW protocol, and the rhythmicity in expression of *dbp* was completely lost after 14-d exposure to rotating shift protocol across all tissues except for liver, where the amplitude was significantly lower and the phase delayed by ~10 h in shift work animals compared to controls (Figure 2.6A and Table 2.1). Consistent with *dbp* expression patterns, rhythmicity in expression of *bmal1* and *per2* displayed reduced amplitude after 3-day exposure and loss of rhythmicity in expression after 14-day exposure to RSW conditions in GC, VAT, and SAT.

However, liver and BAT continued to display significant rhythmicity in expression of *bmal1* after 14-day exposure to protocol, with the phase advanced in both liver and BAT (Figure 2.6B) compared to the control group. *Per2* expression after 14-day exposure to the shift work protocol was rhythmic only in SAT, with *per2* amplitude significantly lower and peak phase advanced compared to the control group (Figure 2.6C; Table.2.2). Rhythmic expression in *rev-erbα,* an important regulator of adipocyte differentiation as well as circadian clock function, was lost with 3-day exposure to rotating shift work in liver and GC, while all three adipose tissues maintained significant rhythmicity in expression of *rev-erbα* (Figure 2.5D). After 14-d exposure to the shift work protocol, BAT, VAT and GC tissues displayed rhythmic expression of *rev-erbα* while liver and SAT were non-rhythmic (Figure 2.6D). Expression of *rev-erba* was very low in GC in both conditions after 14 days.

Acute exposure to RSW paradigm altered rhythmic expression of genes involved in fatty acid, lipid, and glucose metabolism in peripheral tissues. Key genes in metabolic pathways related to lipid, fatty acid, glucose, and energy metabolism were examined for potential alterations in metabolism associated with circadian clock disruption (Figure 2.7). We measured the rhythmic expression of fatty acid and lipid metabolism genes, including regulatory transcription factors (*peroxisome proliferator activated receptor α* [*pparα*] and *peroxisome proliferator activated receptor γ* [*pparγ*]), triglyceride hydrolysis of lipids in chylomicrons (*lipoprotein lipase* [*lpl*]), triglyceride synthesis (*diacylglycerol O-acyltransferase 2* [*dgat2*]), and *de novo* fatty acid synthesis (*acetyl-CoA carboxylase α* [*accα*]). Genes directly related to glucose metabolism included *glucose transporter 2* (*glut2,* liver) and *glut4* (GC, adipose) and *pyruvate dehydrogenase kinase 4* (pdk4). We also examined the adipocyte-specific genes

uncoupling protein 1 (*ucp1,* BAT only), *atgl* (*adipose triglyceride lipase*), *hormone sensitive lipase* (lipe), and *leptin* (*lep*). We first analyzed differences in mean expression by treatment conditions. For the genes that showed significant differences in mean expression, we determined whether the genes displayed 24 h rhythmicity in expression.

Two weeks of RSW exposure in the liver resulted in significant difference in mean *dgat2* and *glut2* expression levels, with shift work mice having higher expression levels than controls. *Dgat2* was the only gene that displayed significant diurnal rhythmicity in expression with no differences in amplitude and peak phase of expression between treatment conditions (Figure 2.7 A-B and Table.2.3).

In brown adipose tissue, *pparα*, *dgat2*, and *ucp1* showed significant differences in mean expression in response to RSW; however, these differences were variable by duration of exposure to protocol. Expression of *pparα* was significantly higher compared to control mice under both durations of exposure, while only control mice displayed significant rhythmicity in *pparα* expression (Figure 2.7C). *Dgat2* expression was higher in RSW after 2-week of exposure to protocol compared to control mice, although significant 24 h rhythmicity in expression was observed only in shift work mice (Figure 2.7D). Similar to *pparα* and *dgat2*, *ucp1* expression was significantly higher in shift work mice after 3-day exposure to RSW, and none of the treatment conditions displayed significant rhythmicity in expression (Figure 2.7E).

Accα expression was upregulated under 3- and 14-day exposure to RSW compared to controls in SAT. Only controls displayed significant rhythmicity in expression of *accα* for both durations of exposure, while rhythmicity in *accα was observed in RSW mice* only after 14-day exposure to protocol (Figure 2.7F). None of the cosinor parameters were different by treatment conditions for *accα* after 14-day exposure to RSW. Expression of *lipe* was higher in shift work mice after 3-day protocol compared

to controls while significant diurnal rhythmicity was observed only in the control condition (Figure 2.7G).

In contrast to liver and brown adipose tissue, the mean expression level for *dgat2* in visceral adipose was significantly lower after three days of exposure to RSW conditions compared to controls, and diurnal rhythmicity in expression was not observed for either control or shift work mice (Figure 2.7H).

No significant differences were observed in mean expression levels for *pparα* (liver and GC), *pparγ* (liver, VAT, SAT), *lpl* (liver, SAT), *pdk4* (liver, GC), *accα* (liver, VAT), *dgat2* and *glut4* (GC, SAT), *atgl* (VAT, SAT) and *lep* (SAT) and several of these genes displayed variable responses in rhythmic expression (data not shown).

DISCUSSION

The present study investigated the behavioral, physiological, and molecular effects of acute exposure to a rotating shift work model. Acute exposure to RSW resulted in disrupted wheel-running activity patterns, which was noticeable as early as three days of exposure to RSW (Figure 2.4). We observed substantial alterations in the diurnal expression of core clock genes (Figure 2.5 and 2.6), as well as metabolic genes in liver, gastrocnemius muscle, and subcutaneous, visceral, and brown adipose tissues (Figures 2.7). The effects of RSW on molecular alterations were more striking after 14-day exposure as most tissues experienced a complete loss in core clock rhythmicity (Figure 2.6). The physiological consequences of these acute alterations were not observed in body weight or body composition (Figure 2.2A-D); however, glucose homeostasis (Figure 2.3A) was affected by the end of two weeks of exposure to RSW, resulting in impaired fasting glucose. These results suggest that molecular alterations occur early on

in a tissue-specific manner, which may precede the metabolic consequences of chronic exposure to RSW.

The short duration of exposure to altered LD cycle did not result in immediate alterations in gross physiology. Body weight and body composition were similar between control and shift work mice independent of access to *ad libitum* running wheel. Our results contrast with previous findings using dim light at night (DLAN) to model shift work. Two-week exposure to DLAN conditions significantly increased body weight in Swiss-Webster mice [25]. Similarly, two other studies using DLAN to simulate shift work reported increased body weight [26, 27]. The increase in body weight in these studies were attributed to altered nutrient utilization [25], reduced energy expenditure [25], or modified timing of food intake with food (~55%), mostly consumed during the sleep phase in the DLAN exposure group [26]. Increases in body weight also corresponded to increased fat mass in these studies [26, 27]. Similarly, differential effects of diet on body weight gain under a 12 h shift in light schedules were observed in rats initially fed with a chow diet and later switched to either low- or high-fat diets [28].

In contrast to the above studies, our work and a rat study of dark phase light contamination did not observe significant increases in body weight with acute exposure to RSW [29]. These contrasting findings may be due to differences in food intake, 24 h access to a running-wheel, short duration of exposure to treatment, or strain-specific responses to shift work. The current study did not measure daily food intake, and the 24 h access to a running wheel may have influenced body weight results in that subgroup of animals. Voluntary wheel running activity reduces weight gain and body fat mass by decreasing food intake in mice [30], and our findings are consistent with animals receiving *ad libitum* access to a running wheel [31] under regular LD cycle conditions. However, we did not observe significant differences in body weight in the larger subset

of mice without a running wheel, possibly pointing to the short duration of the protocol to observe such gross differences. Similar body weight in control and shift work mice suggests strain-specific effects on physiology, as FVB/N mice used in our study are considered a diet-induced obesity-resistant model [32].

Exposure to altered light/dark cycles also influences glucose homeostasis in mammals. Contamination of the dark phase with a light intensity of 0.20 lux for two weeks altered the phase of the diurnal rhythm in glucose while maintaining normoglycemia [29]. In contrast with these findings, DLAN resulted in impaired glucose tolerance following exposure to DLAN conditions [26] or rotating shift work conditions [33] with a light intensity in these studies being 5 lux and 4μ W/cm^2, respectively. Our data indicate impaired fasting glucose (Figure 2.3A) in mice exposed to rotating shift work while the area under the curve for glucose was not different in shift work mice compared to the controls (Figure 2.3C) despite controlling for wheel-running activity. However, voluntary wheel-running activity in type 2 diabetic mice exposed to 6 h advance in LD cycle improved glucose tolerance [34], suggesting that the presence of a running wheel may have mediated the effects of the treatment conditions in the short term.

The present study demonstrated significant influences of repeated alteration in LD cycles on running-wheel activity patterns as early as three days of exposure. Mice typically display high wheel-running activity during the dark phase that gradually declines to baseline levels in the late dark phase [35, 36], and our data are consistent with these wheel-running activity patterns in control mice. In contrast, shift work mice in the present study displayed lower and higher wheel running activity during dark and light phases, respectively (Figure 2.4D). We report no significant differences in total wheel-

running activity between control versus shift work mice, and this finding agrees with previous studies that measured locomotor activity in either DLAN or LD cycle alterations [25-27] (Figure 2.4C). However, the temporal pattern of activity shifted with high activity occurring in the light phase compared to the dark phase in our study. Previous research using timed sleep restriction or slow rotating drums to model shift work reported a gradual decline in dark phase locomotor activity [37] and reduction in mean locomotor activity [38] at the end of 2-week experimental protocol. In contrast, control and shift work mice in our study displayed an increasing trend in wheel-running activity during the 2-week exposure to a novel environment containing a running wheel (Figure 2.4A-B), and such increase in activity was also reported in a study evaluating the effects of physical activity on emotional behaviors [39]. Light schedules can strongly influence wheel-running behavior. Mice exposed to a 6 h phase shift in LD cycle require 5-7 days for complete re-entrainment of wheel-running activity to the new LD cycle [40]. In the present study, the mice failed to re-entrain to the new LD cycle before LD cycles shift again. These findings suggest that the acute effects of RSW are similar to previous reports related to activity behavior. Frequent transitions between cycles of light and dark do not provide sufficient time (only 3-4 days) to re-entrain wheel-running activity within 2-weeks of exposure to protocol. Catecholamines released during exercise and physical activity play a significant role as zeitgebers for peripheral clocks [41]; thus, alterations in physical activity patterns have the potential to significantly disturb circadian clock function, particularly in skeletal muscle.

The effects of RSW on the rhythmic expression of core clock genes in gastrocnemius muscle were more disruptive than liver and the three adipose tissues (Figure 2.5 and 2.6) in the present study. Interestingly, while *dbp* (a readout of core clock

function) preserved rhythmic expression across all tissues with reduced amplitudes after 3-day exposure to the shift work conditions, rhythmic expression of *dbp* was lost after 14-day exposure to protocol across all tissues except the liver. While many core clock genes maintained rhythmic expression after a 3-day light shift (Table 2.1), a 14-day exposure to RSW profoundly altered core clock gene expression in a tissue- and gene-specific manner, with most core clock genes losing rhythmicity in expression. Some genes preserved rhythmic expression, for example, *rev-erbα*, consistent with the known non-clock regulatory functions for *rev-erbα* as transcriptional repressors controlling adipocyte differentiation [42] and lipid metabolism in adipose tissues [43]. Our results of dampened amplitude are in agreement with previous studies of LD cycle disruption by DLAN [44, 45], constant light conditions [46], or repeated phase shifts in the LD cycle [47]. Szantoova et al. reported robust rhythmic expression of core clock genes (i.e., *bmal1*, *per2*, and *rev-erbα*) in rat livers and hearts exposed to simulating rotating shift work (8 h phase delays in LD cycle every two days) and delayed peak expression of core clock genes [47]. In contrast, Wu et al. reported no alterations in hepatic core clock gene expression at the end of a 7-day reversal of the LD cycle [48]. However, feeding in this study was restricted to the dark/active phase of the LD cycle, which was maintained even under an altered LD cycle, suggesting feeding as a dominant driver of peripheral core clock gene expression, which may have reset at the end of 7-day period [48]. Our studies provide evidence that alterations in the LD cycle influence the peripheral core clock gene expression in a tissue-specific manner with almost complete suppression of circadian clock function after two weeks of exposure to RSW.

Studies have suggested that entrainment of peripheral oscillators to an inverted LD cycle takes about 2 to 7 days under a time-restricted feeding paradigm in a tissue-

dependent manner, with liver clocks entraining more rapidly compared to heart tissue [48]. A plausible explanation for the differential effects of LD cycle alterations across the different tissues in our study suggests independent routes of entrainment and the tissue-specific response to signals in the peripheral environment, such as alterations in patterns of food intake [10, 49-51]. These tissue-specific differences in response to physiological and behavioral signals may have resulted in the differential effects in peripheral core clock gene expression observed in our data under continually alternating LD cycles.

Multiple studies have investigated the circadian clock gene rhythmicity under different models, including DLAN [44], forced activity during the rest phase [45], constant light conditions [46], restricted feeding [10, 52-54], and 8 h phase delay in LD every two days [47]. In general, these studies reported attenuated amplitude and phase shifts in clock and metabolic gene expression specific to tissue with liver being most sensitive to temporal changes in zeitgeber exposure. While much is known about the chronic effects of shift work on metabolic outcomes, our study examined the acute effects of simulated rotating shift work. To our knowledge, there are no studies that investigated the consequences of repeated, short-term alterations in LD cycles on crucial genes in the circadian clock and metabolic pathways in multiple peripheral tissues.

In the current study, expression of *dgat2*, a rate-limiting enzyme in triglyceride production [46], was increased in liver and brown adipose in response to 14-day RSW. In contrast to our findings, an acute exposure to constant light conditions upregulated *dgat2* expression in white adipose tissue but was suppressed in liver, and restricted feeding restored *dgat2* expression levels in liver [46], emphasizing the importance of nutrient cues. Upregulation of *dgat2* has been implicated in the development of fatty liver disease [55] indicating its importance in metabolic health. Furthermore, *dgat2* expression is

rhythmically expressed in both liver (peaks during mid dark phase) and adipose tissues [46]. We observed rhythmic expression of *dgat2* in liver and BAT in response to 2-week RSW, suggesting diurnal variation driven by factors other than alteration in LD cycle in these tissues. Additional to *dgat2*, RSW was associated with higher expression of *pparα* compared to controls at both 3-day and 14-day time points, although rhythmicity of expression was not observed in shift work mice. Under normal physiological conditions, *pparα* expression in the BAT peaks during the light phase of the LD cycle [56] to facilitate lipid catabolism during fasting, and our data are consistent with these findings in control mice. *Pparα* induces *ucp1* expression by binding PPRE (PPAR- response element) on its promoter [57]. UCP1 is involved in non-shivering thermogenesis that uncouples fatty acid oxidation from ATP synthesis to produce heat [58], suggesting an important role in energy expenditure and reducing lipid accumulation (in BAT) as observed in our data

Accα, a key enzyme in *de novo* fatty acid synthesis, was higher in SAT after 3- and 14-day exposure to RSW, compared to control animals. Control mice in our study displayed peak expression of *accα* in the dark phase consistent with previous findings [51]. Although shift work mice displayed diurnal variation in the present study after 14-day exposure to RSW, peak expression in *accα* occurred during the light phase, suggesting a shifted phase in diurnal variation. Temporal expression of *accα* occurs via AMPK (a cellular energy sensor), suggesting a role for feeding in the regulation of its expression [59, 60]. A previous study using DLAN to simulate shift work reported a shift in the timing of feeding to the light phase of the LD cycle with increases in fat mass [26], which may explain the phase shift observed for *accα* in our study due to sensitivity to cellular energy status that is directly tied to nutrient status.

Lipe, also commonly known as *hormone sensitive lipase* (*hsl*), is a key enzyme hydrolyzing triglycerides, and CLOCK/BMAL1 heterodimers regulate its transcriptional rhythmicity in expression [61]. In the present study, the peak phase of *lipe* expression in control mice was in contrast to previous findings [46]. RSW eliminated 24 h diurnal oscillation in *lipe* expression, resulting in consistent high levels of expression regardless of timing. Catecholamines enhances and insulin inhibits LIPE activity, implying that LIPE activity is elevated during fasting to mobilize fat stores for energy or in diabetes mellitus as a result of impaired insulin sensitivity in adipose tissue [62]. Since temporal expression of *lipe* was not in agreement with previous studies [46], this variability in expression may be related to signals of nutrient response as the mice were fed at *ad libitum* prior to tissue harvest.

Glut2 was the only gene involved in carbohydrate metabolism that was considerably higher in shift work mice compared to control mice in the present study. Previous findings have reported robust rhythmic expression of *glut2* [37], with a peak expression occurring during the early dark phase [63]. However, we found no diurnal variation in *glut2* expression in the livers of control and shift work mice, with shift work mice consistently expressing greater amounts of *glut2* independent of timing. Our data indicated impaired fasting glucose in shift work mice and elevated *glut2* expression. GLUT2 is indispensable for hepatic glucose uptake while [64]. These findings may indicate that acute exposure to RSW resulted in normal hepatic uptake of glucose, as evidenced by increased *glut2* expression.

The experimental protocol in the present study involved *ad libitum* access to food for both control and shift work mice. Such natural (*ad libitum*) feeding patterns are normally represented by ~65% of food intake during the dark phase and the remainder during the light phase of a regular LD cycle [65]. These patterns in food intake suggest

that, under a regular LD cycle and *ad libitum* feeding conditions, the central pacemaker may be strongly involved in the phase resetting process of peripheral clocks [65] while food intake may weakly influence the resetting process. In contrast, time-restricted feeding (TRF) is a potent zeitgeber in the phase resetting process of peripheral tissue clocks independent of central clock regulation [10, 52] because of long fasting intervals not observed under *ad libitum* feeding [65, 66] or the uncoupling of peripheral oscillators from the master oscillator in the hypothalamus [52]. Mice in our study were in different metabolic/fed states at each time point, which may be one of the reasons we see variability in metabolic gene expression.

Although we report altered core clock mechanism and metabolic gene expression in multiple tissues exposed to two different durations of RSW, several questions remain to be answered. First, fasting/feeding cues and LD cycles influence diurnal variation of core clock and metabolic genes in the periphery [13, 67]. Our experimental protocol involved *ad libitum* access to rodent chow. Although the present experiment was focused on examining the acute changes in adiposity, behavior, and gene expression following exposure to repeated alterations in the light/dark cycles, ascertaining how food intake and feeding patterns are altered in a rotating shift work model will facilitate our understanding of the influence of feeding on physiology and molecular alterations in peripheral tissues that closely regulate nutrient metabolism. Second, our data reported insignificant influences on body weight and body composition, in contrast to previous reports [25-27]. Nevertheless, our results are important in demonstrating that, even in a putative animal model (i.e., FVB/N) of resistance to diet-induced obesity, acute alterations in the circadian clock and in physical activity behavior occur that may have long term effects on metabolic health. Rotating shift work can be replicated in animal models more susceptible to diet-induced obesity (e.g., C57BL/6) to examine similar

outcomes (i.e., body weight, body composition etc.) with and without access to a running wheel. Third, measuring protein expression for the genes involved in critical metabolic pathways may shed light on the functional significance of our findings, as gene expression does not always equal protein translation. Additionally, posttranslational regulation of mRNA transcripts is yet another level of regulation in circadian metabolism [68]. Fourth, the mice were not fasted before circadian dissection, which may have influenced gene expression results as the metabolic profiles may have been different between mice because feeding can influence gene expression patterns of both core clock and metabolic genes [69, 70]. Finally, understanding the differential effects of high fat versus low fat diets in this protocol is important, as shift workers have easy access to such foods [71-73], and the type [74] and timing of macronutrient intake [9, 75] can influence diurnal expression patterns of core clock and metabolic gene expression.

In summary, we report that acute exposure to RSW disrupts behavior, physiology, and molecular functions in the present study, and these effects are evident as early as 3 days after exposure to a shift in the LD cycle. Although gross changes in body weight and body composition are not evident with an acute exposure to shift work in our model, rapid molecular alterations are evident in our study. RSW did not provide an opportunity to adapt to the repeated exposure to disrupted light dark cycle every 3 to 4 days, because of brief period of time available between shifts. As a result, shift workers may be at a disadvantage and may not adapt to repeated change in shift schedules with a consequence of increased risk for chronic diseases. Future studies should focus on developing interventional strategies, such as timed exercise and timed feeding, as they are potential zeitgebers that may facilitate adaptation in peripheral tissues and thereby reduce the risk for metabolic diseases.

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Tissue	Cosinnor Parameter	Mesor		Amplitude		Phase	
	Group	3DC	3DS	3DC	3DS	3DC	3DS
Liver	*dbp*	5.54 (4.52, 6.56)	5.07 (3.55, 6.59)	7.46 (8.89, 6.03)	5.02 (2.71, 7.33)	6.64 (5.92, 7.39)	7.46 (5.93, 8.99)
	bmal1	0.24 (0.21. 0.27)	0.21 (0.17, 0.24)	0.26 (0.22, 0.30])	0.18 (0.13, 0.23)	19.56 (18.94, 20.17)	20.31 (19.22, 21.40)
	per2	0.79 (0.67, 0.91)	0.90 (0.78, 1.02)	0.59 (0.41, 0.76) †	0.21 (0.04, 0.37)	12.65 (11.54, 13.77)	14.10 (10.92, 17.28)
	rev-erbα	0.58 (0.41, 0.74)	NR	0.72 (0.48, 0.95)	NR	4.24 (3.02, 5.46)	NR
Gasctrocnemius	*dbp*	20.06 (15.82, 24.31)	19.34 (13.79, 24.90)	18.81 (12.65, 24.97)	12.31 (4.62, 20.00)	7.61 (6.42, 8.80)	9.60 (7.13, 12.08)
	bmal1	0.21 (0.13, 0.30)	NR	0.23 (0.11, 0.34)	NR	20.40 (18.34, 22.46)	NR
	per2	0.33 (0.25, 0.41)	NR	0.35 (0.23, 0.46)	NR	11.03 (9.76, 12.30)	NR
	rev-erbα	0.43 (0.17, 0.69)	NR	0.50 (0.14, 0.86)	NR	1.38 (-1.42, 4.19)	NR
Brown adipose	*dbp*	1.55 (0.92, 2.18)	1.23 (0.92, 1.55)	1.98 (1.09, 2.88)	0.93 (0.48, 1.38)	6.92 (5.20, 8.64)	8.97 (7.14, 10.80)
	bmal1	2.31 (2.00, 2.61)	1.87 (1.53, 2.21)	2.46 (2.03, 2.89) †	1.39 (0.91, 1.87)	20.20 (19.53, 20.87)	20.35 (19.04, 21.67)
	per2	0.42 (0.39, 0.45)	NR	0.33 (0.29, 0.37)	NR	10.56 (10.04, 11.07)	NR
	rev-erbα	1.13 (1.00, 1.26)	1.16 (0.98, 1.34)	1.10 (0.91, 1.29) †	0.33 (0.07, 0.59)	4.35 (3.72, 4.99)	7.66 (4.69, 10.64)
Visceral adipose	*dbp*	4.59 (3.47, 5.71)	3.05 (2.47, 3.63)	4.57 (2.97, 6.17) †	2.00 (1.17, 2.83)	8.14 (6.83, 9.45)	9.50 (7.95, 11.07)
	bmal1	2.03 (1.53, 2.54)	1.22 (0.85, 1.59)	1.86 (1.17, 2.60)	0.81 (0.18, 1.23)	0.18 (18.73, 21.64) †	0.19 (-2.68, 3.06)
	per2	0.96 (0.82, 1.10)	NR	0.44 (0.24, 0.64)	NR	10.76 (9.02, 12.50)	NR
	rev-erbα	13.12 (10.62, 15.62)	13.07 (10.28, 15.85)	12.37 (8.78, 15.95)	8.78 (4.83, 12.72)	5.02 (3.94, 6.10) †	8.29 (6.58, 10.01)
Subcutaneous adipose	*dbp*	3.21 (2.54, 3.89)	2.74 (2.26, 3.22)	3.26 (2.32, 4.21)	1.71 (1.03, 2.39)	8.05 (6.93, 9.17)	9.67 (8.16, 11.17)
	bmal1	0.04 (0.03, 0.06)	0.06 (0.04, 0.08)	0.06 (0.04, 0.08)	0.05 (0.02, 0.07)	8.32 (16.94, 19.70) †	22.12 (19.88, 24.36)
	per2	0.38 (0.29, 0.46)	NR	0.26 (0.14, 0.38)	NR	9.68 (7.96, 11.40)	NR
	rev-erbα	0.23 (0.15, 0.31) †	1.31 (1.06, 1.56)	0.15 (0.03, 0.26) †	0.89 (0.53, 1.25)	7.05 (4.14, 9.95)	7.72 (6.22, 9.23)

† Significant differences in the corresponding cosinor parameter between experimental light conditions (3DC vs 3DS). Data are reported as parameter estimate and 95% confidence intervals in parentheses. Abbreviations: 3DC, 3-day control; 3DS, 3-day shift work; NR, not rhythmic. The data are represented as mean ± sem.

Table 2.1: Influence of 3-day exposure to rotating shift work on the mesor, amplitude and phase of core clock genes in peripheral tissues.

Tissue	Cosinor Parameter	Mesor		Amplitude		Phase	
	Group	14DC	14DS	14DC	14DS	14DC	14DS
Liver	*dbp*	6.32 (4.68, 7.97)	4.17 (2.70, 5.64)	9.82 (7.43, 12.20) †	3.66 (1.56, 5.77)	7.52 (6.64, 8.39) †	17.33 (15.17, 19.49)
	bmal1	0.26 (0.23, 0.29)	0.26 (0.22, 0.31)	0.28 (0.25, 0.32) †	0.17 (0.10, 0.24)	19.88 (19.38, 20.39) †	6.70 (2.18, 8.18)
	per2	1.20 (1.06, 1.34)	NR	1.12 (0.91, 1.33)	NR	12.60 (11.92, 13.28)	NR
	rev-erbα	0.47 (0.34, 0.61)	NR	0.69 (0.50, 0.88)	NR	4.11 (3.09, 5.13)	NR
Gasctrocnemius	*dbp*	14.44 (11.36, 17.53)	NR	10.86 (6.46, 15.25)	NR	7.02 (5.49, 8.55)	NR
	bmal1	0.13 (0.10, 0.16)	NR	0.14 (0.10, 0.19)	NR	21.76 (20.59, 22.94)	NR
	per2	0.15 (0.12, 0.17)	NR	0.08 (0.05, 0.12)	NR	11.38 (9.68, 13.08)	NR
	rev-erbα	0.20 (0.13, 0.27)	0.12 (0.09, 0.15)	0.19 (0.10, 0.28) †	0.05 (0.01, 0.09)	1.72 (-0.20, 3.63) †	21.80 (18.86, 24.75)
Brown adipose	*dbp*	2.68 (2.28, 3.09)	NR	3.36 (2.80, 3.92)	NR	7.61 (6.95, 8.27)	NR
	bmal1	1.90 (1.57, 2.24) †	2.72 (2.31, 3.12)	2.09 (1.62, 2.56) †	1.07 (0.50, 1.65)	20.76 (19.9, 21.63) †	9.34 (7.31, 11.38)
	per2	0.38 (0.31, 0.45)	NR	0.26 (0.16, 0.36)	NR	11.74 (10.30, 13.17)	NR
	rev-erbα	0.89 (0.78, 1.00) †	1.40 (1.17, 1.63)	0.91 (0.75, 1.06) †	0.40 (0.09, 0.72)	4.44 (3.78, 5.09) †	17.80 (14.73, 20.83)
Visceral adipose	*dbp*	3.27 (2.58, 3.97)	NR	3.40 (2.40, 4.39)	NR	8.54 (7.44, 9.64)	NR
	bmal1	1.98 (1.68, 2.29)	NR	1.33 (0.91, 1.75)	NR	19.74 (18.46, 21.02)	NR
	per2	0.81 (0.65, 0.97)	NR	0.53 (0.31, 0.76)	NR	13.69 (12.03, 15.34)	NR
	rev-erbα	12.21 (9.51, 14.91)	10.85 (8.61, 13.09)	9.87 (5.99, 13.73)	5.32 (2.10, 8.53)	5.85 (4.39, 7.30) †	18.82 (16.58, 21.06)
Subcutaneous adipose	*dbp*	2.20 (1.72, 2.69)	NR	2.38 (1.70, 3.07)	NR	8.95 (7.86, 10.05)	NR
	bmal1	0.04 (0.02, 0.05)	NR	0.04 (0.02, 0.06)	NR	19.49 (17.78, 21.20)	NR
	per2	0.26 (0.17, 0.34)	0.30 (0.23, 0.37)	0.20 (0.08, 0.33)	0.14 (0.04, 0.24)	13.39 (11.12, 15.65) †	6.43 (3.69, 9.14)
	rev-erbα	0.69 (0.46, 0.91)	NR	0.81 (0.49, 1.12)	NR	6.92 (5.40, 8.44)	NR

† Significant differences in the corresponding cosinor parameter between experimental light conditions (14DC vs 14DS). Data are reported as parameter estimate and 95% confidence intervals in parentheses. Abbreviations: 14DC, 14-day control; 14DS, 14-day shift work; NR, not rhythmic. The data are represented as mean ± sem.

Table 2.2: Influence of 14-day exposure to rotating shift work on the mesor, amplitude and phase of core clock genes in peripheral tissues.

Tissue	Parameter	Mean		Amplitude		Phase	
	Group	3DC	3DS	3DC	3DS	3DC	3DS
Brown adipose	*ppara*	3.71 ± 0.38 †	5.51 ± 0.40	2.04 (1.13, 2.95)	NR	3.58 (1.96, 5.19)	NR
	ucp1	265.1 ± 11.9 †	319.11 ± 20.5	NR	NR	NR	NR
Subcutaneous adipose	*accα*	1.68 ± 0.29 †	3.01 ± 0.31	1.33 (0.64, 2.03)	NR	17.75 (15.70, 19.70)	NR
	lipe	0.33 ± 0.05 †	0.92 ± 0.08	0.20 (0.05, 0.35)	NR	16.54 (13.90, 19.18)	NR
Visceral adipose	*dgat2*	33.62 ± 3.31 †	23.72 ± 2.69	NR	NR	NR	NR
Tissue	14-day Exposure to Rotating Shift work						
	Group	14DC	14DS	14DC	14DS	14DC	14DS
Liver	*dgat2*	13.32 ± 0.76 †	16.29 ± 0.48	4.04 (2.27, 5.81)	1.87 (0.66, 3.11)	13.08 (11.45, 14.72)	16.16 (13.63, 18.69)
	glut2	14.68 ± 0.50 †	16.30 ± 0.62	NR	NR	NR	NR
Brown adipose	*pparα*	4.22 ± 0.40 †	7.79 ± 0.51	2.26 (1.39, 3.13)	NR	2.08 (0.66, 3.50)	NR
	dgat2	30.71 ± 1.80 †	54.05 ± 2.12	NR	10.65 (6.13, 15.19)	NR	13.10 (11.33, 14.88)
Subcutaneous adipose	*accα*	0.96 ± 0.14 †	1.65 ± 0.28	0.55 (0.20, 0.91)	1.19 (0.47, 1.91)	26.53 (13.97, 19.08)	6.68 (4.38, 9.01)

† Significant differences in the corresponding mean expression level between experimental light conditions (3DC vs 3DS or 14DC vs 14DS). The data are represented as mean ± sem and parameter estimate and 95% confidence intervals for amplitude and phase.
Abbreviations: 3DC, 3-day control; 3DS, 3-day shift work; 14DC, 14-day control; 14DS, 14-day shift work; NR, not rhythmic.

Table 2.3: Influence of 3- and 14-day exposure to rotating shift work on mean expression, amplitude and phase of key metabolic genes involved in fatty acid/lipid and glucose metabolism across multiple tissues.

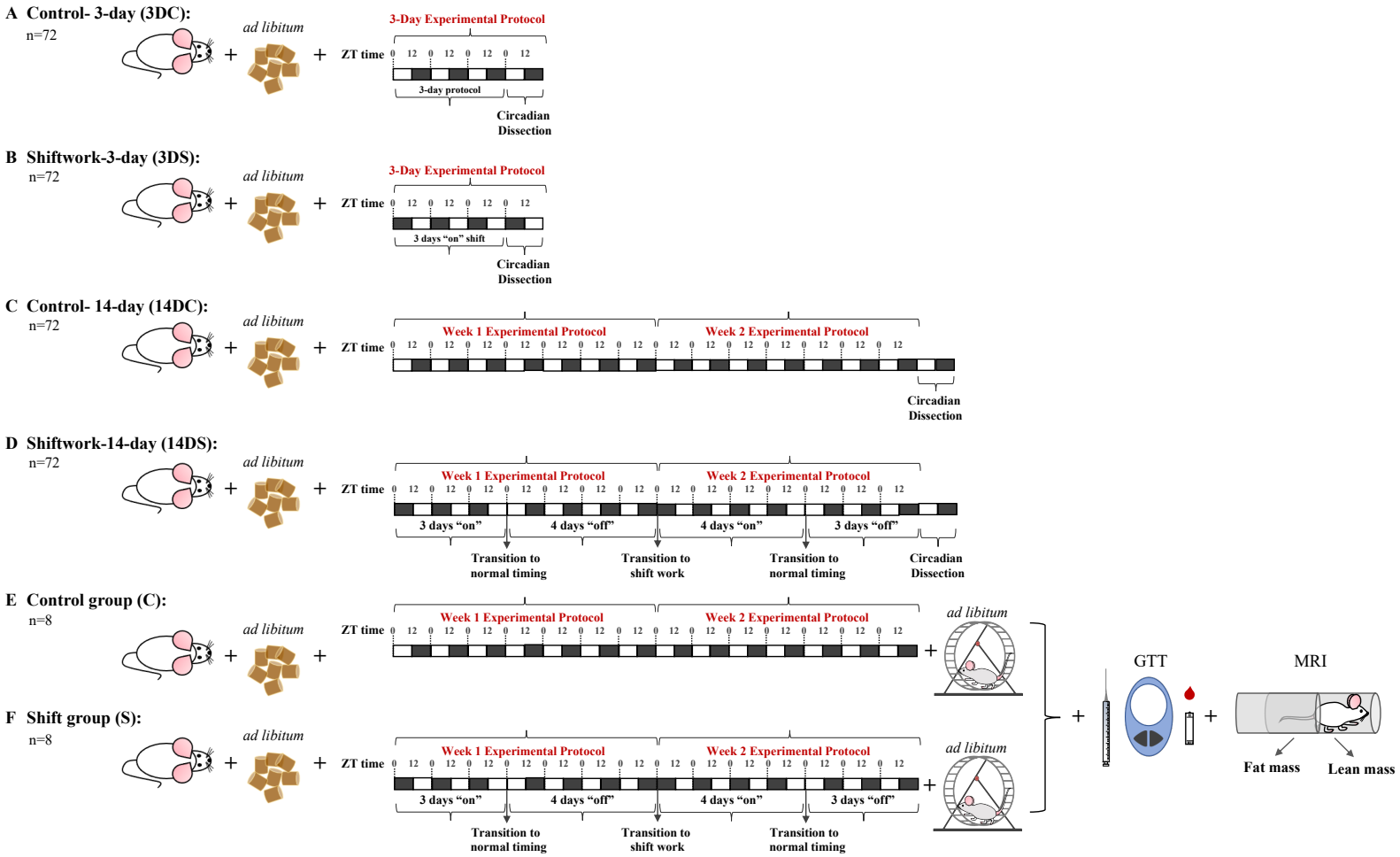
A Control- 3-day (3DC):
n=72
ad libitum
ZT time
3-Day Experimental Protocol
3-day protocol
Circadian Dissection
B Shiftwork-3-day (3DS):
n=72
ad libitum
ZT time
3-Day Experimental Protocol
3 days "on" shift
Circadian Dissection
C Control- 14-day (14DC):
n=72
ad libitum
ZT time
Week 1 Experimental Protocol
Week 2 Experimental Protocol
Circadian Dissection
D Shiftwork-14-day (14DS):
n=72
ad libitum
ZT time
Week 1 Experimental Protocol
Week 2 Experimental Protocol
3 days "on"
4 days "off"
4 days "on"
3 days "off"
Transition to normal timing
Transition to shift work
Transition to normal timing
Circadian Dissection
E Control group (C):
n=8
ad libitum
ZT time
Week 1 Experimental Protocol
Week 2 Experimental Protocol
ad libitum
F Shift group (S):
n=8
ad libitum
ZT time
Week 1 Experimental Protocol
Week 2 Experimental Protocol
3 days "on"
4 days "off"
4 days "on"
3 days "off"
Transition to normal timing
Transition to shift work
Transition to normal timing
ad libitum
GTT
MRI
Fat mass
Lean mass

Figure 2.1.

Figure 2.1: Rotating shift work (RSW) experimental design.

Mice were randomly assigned to either control 3-day: 3DC (A), shift work 3-day: 3DS (B), control 14-day: 14DC (C), shift work 14-day: 14DS (D), control: C (E), or shift work S: (F) experimental groups. All groups of control mice (3DC, 3DS, and C) were housed under regular 12 h light/ 12 h dark cycles and shift work mice were housed under repeated shifts in 12 h light/ 12 h dark cycle every 3 to 4 days for the corresponding durations of exposure to experimental protocols. Open squares represent 12 h light and filled squares represent 12 h dark. For shift work experimental groups, the transition from 'on shift' to 'off shift' and vice versa is marked by 24 h light or 24 h dark cycle, respectively. Brown pellets represent food provided at *ad libitum*. Only groups C and S received 24 h access to running wheel and underwent glucose tolerance test and body composition measures (MRI) as indicated in the study design figure. Sample size is indicated by n for each group. Abbreviations: ZT, zeitgeber; GTT, glucose tolerance test; MRI, magnetic resonance imaging.

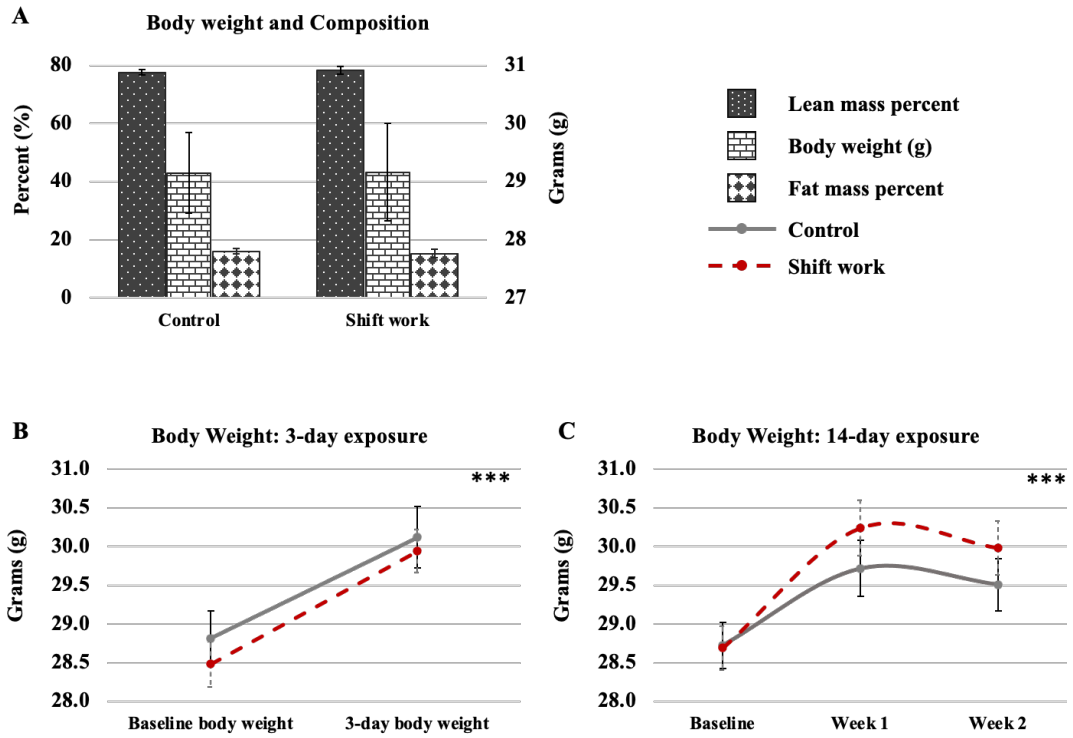

Figure 2.2: Acute exposure to rotating shift work did not alter body weight and body composition.

End of study body weight and body composition between C (n=8) and S (n=7) mice (A). Body weight in mice exposed to 3-day (3DC vs 3DS; n=36/group) (B) and weekly body weight in mice exposed to 14-day (14DCvs 14DS; n=36/group) experimental protocol (C). Repeated measures ANOVA was used to analyze body weight in B and C. *** $p<0.0001$ represents significant main effects of time.

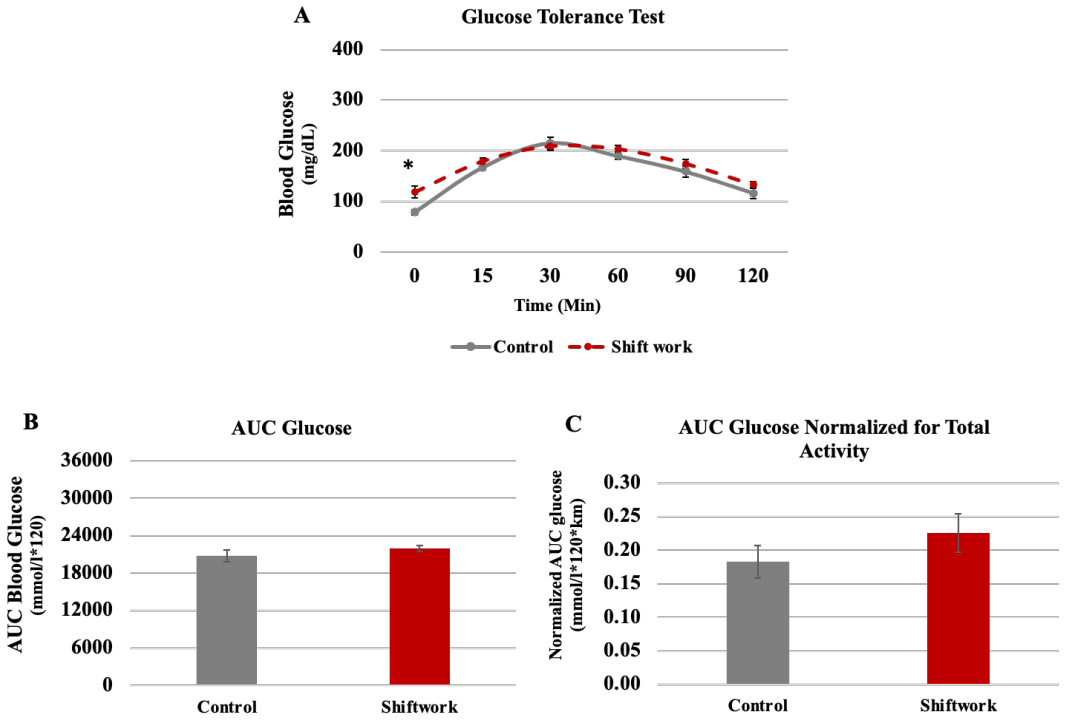

Figure 2.3: Acute exposure to rotating shift work resulted in impaired fasting glucose in mice with ad libitum access to running wheels.

Glucose tolerance test was conducted for C (n=8) and S (n=7) mice at the end of 14-day exposure to experimental conditions (A). Area under the curve (AUC) for glucose between C (n=8) vs S conditions (n=7) (B). AUC glucose controlled for total activity between C (n=7) vs S (n=6) (C). * $p<0.05$

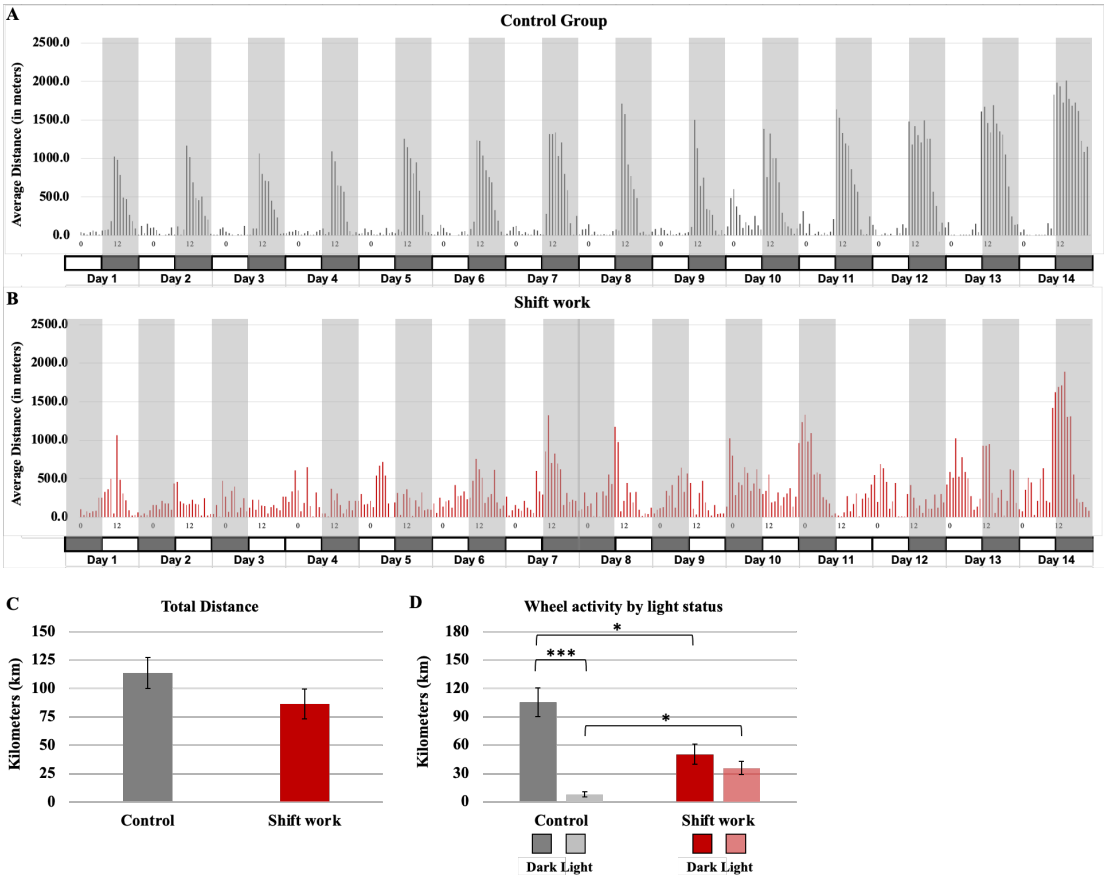

Figure 2.4: Acute exposure to rotating shift work disrupts wheel running activity.

Average hourly wheel running activity pattern in mice exposed to 2-week of control (A) and shift work (B) conditions. Open and filled squares at the bottom of x-axis in (A) and (B) represent light and dark phases of light/dark cycle, respectively. Gray shaded areas represent dark phase of light/dark cycle. The numbers 0 and 12 on the x-axis represent zeitgeber time. Day 10 running wheel activity between zt0 and zt12 is an artefact due to cage change. Average total distance by group (C), and average total distance run by light status within and between groups (D). n=7/group; * $p < 0.05$ and *** $p<0.0001$

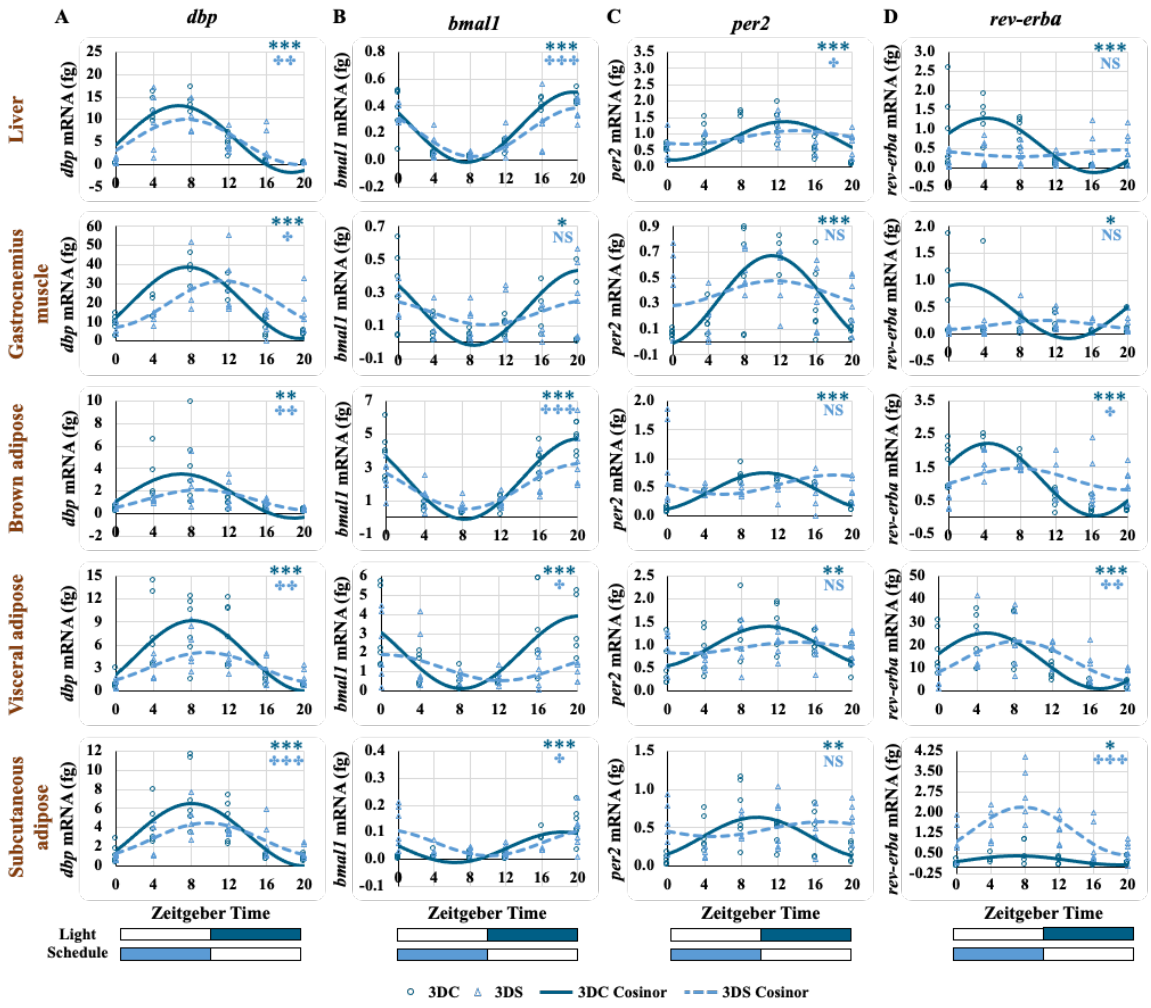

Figure 2.5: Acute exposure to rotating shift work dysregulates core clock gene expression after 3-day exposure to protocol in a tissue specific manner.

Dbp (A), *bmal1* (B), *per2* (C), and *rev-erbα* (D). Dark and light blue filled bars represent the dark phase of light/dark cycle in control and shift work groups, respectively. Units on y-axis are represented as femtograms (fg) mRNA per 12.5 to 60 ng of total RNA. A significant cosinor fit is represented by * $p < 0.05$, ** $p<0.001$ and *** $p<0.0001$for control data and ✣ $p<0.05$, ✣✣ $p<0.001$ and ✣✣✣ $p<0.0001$ for shift work data. Significance or non-significance indicated on the top right of individual graphs refers to cosine fit of data for corresponding treatment conditions. Abbreviations: *bmal1*, *brain and muscle-Arnt-like 1*; *per2*, *period 2*; *nr1d1*, *nuclear receptor family1, group D, member 1*; *dbp*, *D-box binding protein*; NS, non-significant; n=3-6/time point/group.

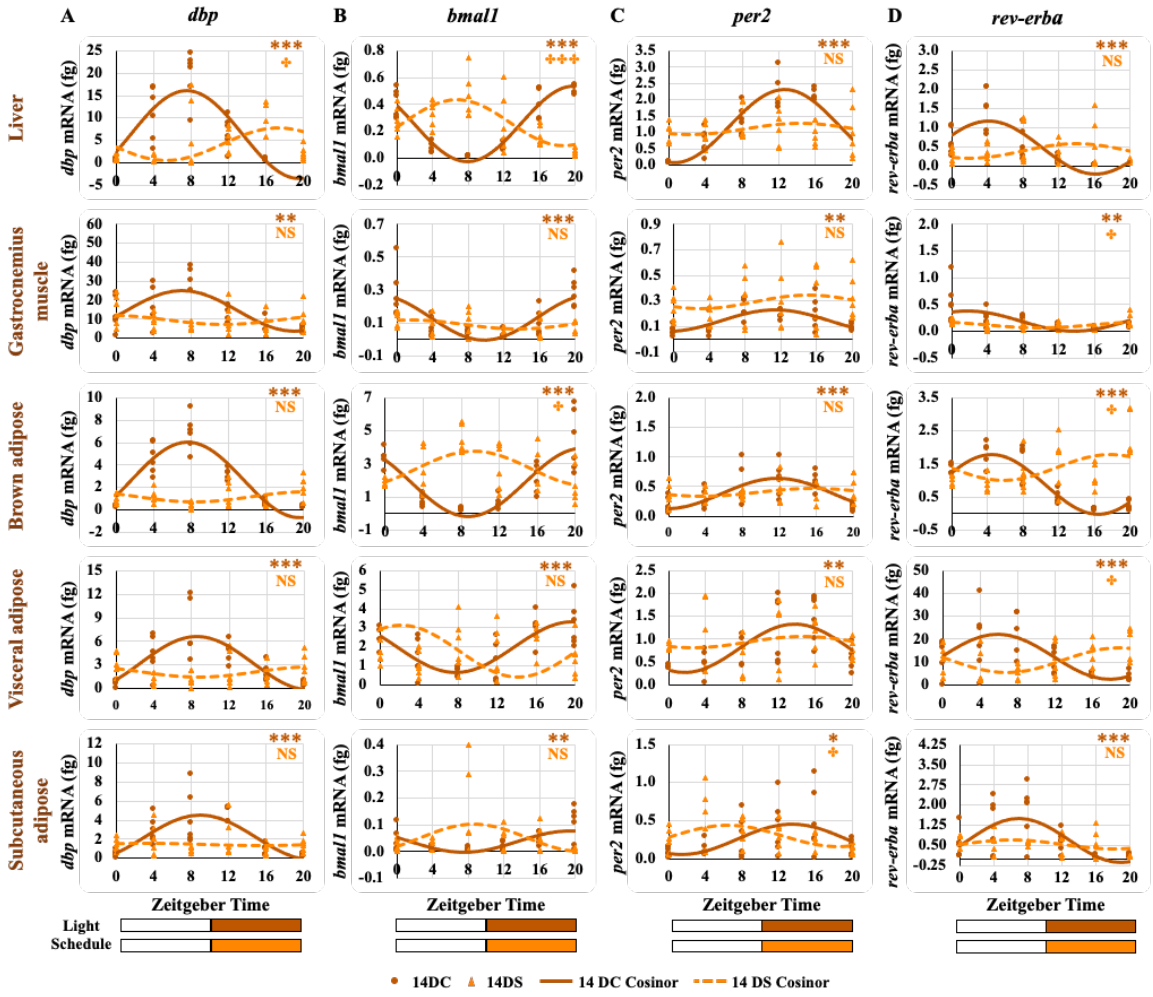

Figure 2.6: Acute exposure to rotating shift work dysregulates core clock gene expression after 14-day exposure to protocol in a tissue specific manner.

Dbp (A), *bmal1* (B), *per2* (C), and *rev-erbα* (D). Dark and light orange filled bars represent the dark phase of light/dark cycle in control and shift work groups, respectively. Units on y-axis are represented as femtograms (fg) mRNA per 12.5 to 60 ng of total RNA. A significant cosinor fit is represented by * $p < 0.05$, ** $p<0.001$ and *** $p<0.0001$for control data and ✣ $p<0.05$, ✣✣ $p<0.001$ and ✣✣✣ $p<0.0001$ for shift work data. Significance or non-significance indicated on the top right of individual graphs refers to cosine fit of data for corresponding treatment conditions. Abbreviations: *bmal1*, *brain and muscle-Arnt-like 1*; *per2*, *period 2*; *nr1d1*, *nuclear receptor family1, group D, member 1*; *dbp*, *D-box binding protein*; NS, non-significant; n=3-6/time point/group

1

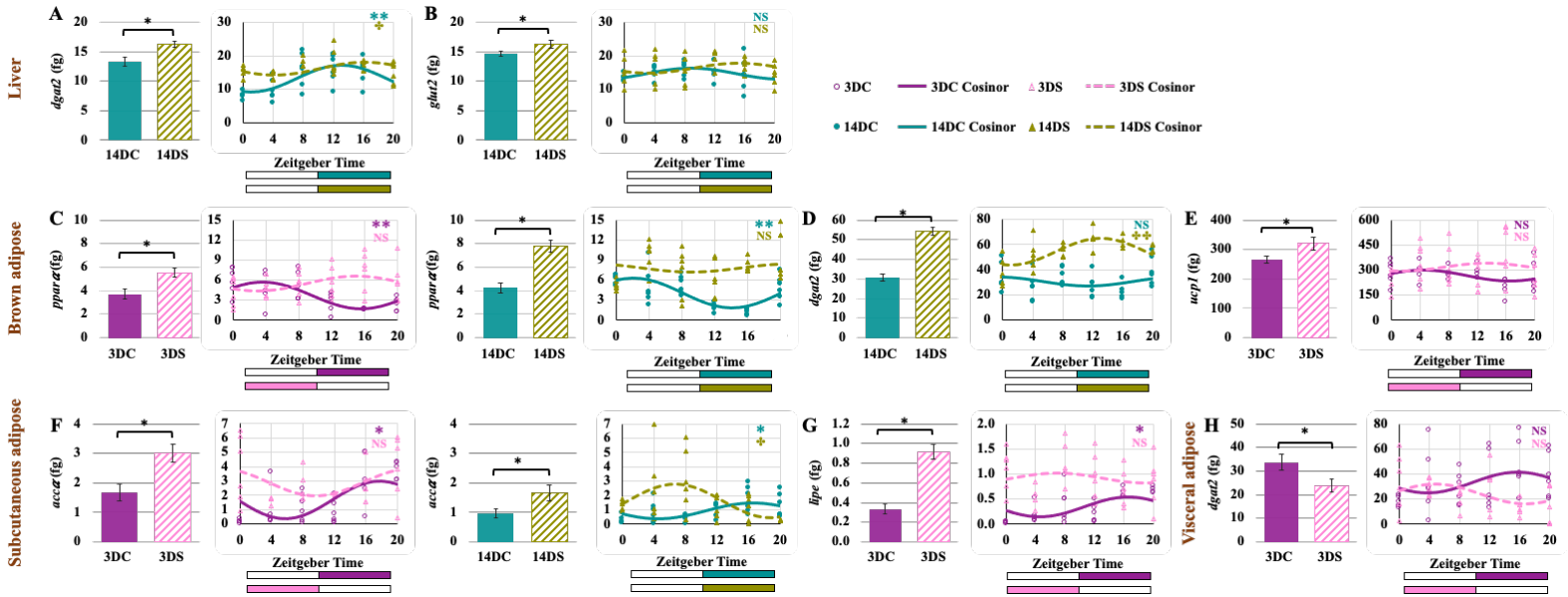
Liver
Brown adipose
Subcutaneous adipose
Visceral adipose
A
B
C
D
E
F
G
H
dgat2 (fg)
ghrl2 (fg)
pparα (fg)
acca (fg)
lipe (fg)
ucp1 (fg)
14DC
14DS
3DC
3DS
Zeitgeber Time
NS
3DC
3DC Cosinor
3DS
3DS Cosinor
14DC
14DC Cosinor
14DS
14DS Cosinor

Figure 2.7

1

Figure 2.7: Acute exposure to rotating shift work dysregulates metabolic gene expression after 3- and 14-day exposure to protocol in a tissue specific manner.

A set of two graphs represent mean expression level (bar graphs) and cosine fit of data for the corresponding gene. Liver expression of *dgat2* (A) and *glut2* (B); brown adipose tissue expression of *pparα* (C), *dgat2* (D), and *ucp1* (E); subcutaneous adipose tissue expression of *accα* (F) and *lipe* (G); and visceral adipose tissue expression of *dgat2* (H). Dark purple and dark green filled bars represent the dark phase of light/dark cycle for control groups exposed to 3- and 14-day experimental conditions, respectively. Light pink and light green filled bars represent the dark phase of light/dark cycle for shift work groups exposed to 3- and 14-day experimental conditions, respectively. Open bars represent the light phase of light/dark cycle in both control and shift work groups. A significant cosinor fit is represented by * $p < 0.05$ and ** $p<0.001$ for control data and ✣ $p<0.05$ and ✣✣ $p<0.001$ for shift work data; NS, non-significant. Significance or non-significance indicated on the top right of individual graphs refers to cosine fit of data for the corresponding treatment groups and duration of exposure. Abbreviations: *dgat2*, *diacylglyerol O-acyltransferase 2*; *pparα*, *peroxisome proliferator activated receptor α*; *ucp1*, *uncoupling protein 1*; *accα*, *acetyl-CoA carboxylase α*, *glut2*, *glucose transporter 2*.

Chapter 3: Effects of timed feeding and timed exercise on behavioral and physiological changes associated with chronic exposure to rotating shift work

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Running Title: Chronic exposure to rotating shift work and the differential effects of diet and interventions.

Number of figures: 8

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ABSTRACT

Background. Cellular circadian clocks are found in virtually every cell in the body and serve to regulate the transcriptional rhythmicity of many metabolic genes in anticipation of daily fluctuations in the environment (e.g., light/dark, availability of food, etc.). Shift workers experience repeated alterations in the timing of feeding, sleep, and physical activity, predisposing them to increased risk for obesity and metabolic disorders. Timed feeding and timed exercise may serve as non-photic zeitgebers (timekeepers) in preventing the metabolic disruptions associated with shift work. Our objective for this study was to evaluate the effects of timed exercise, timed feeding, and combination of timed feeding and exercise, within the context of high and low-fat diets, in a model of chronic rotating shift work (CRSW).

Methods: Mice (n=96) were housed in either normal or CRSW conditions, consisting of 12-hour shifts in the light/dark cycle every 3 or 4 days. Six groups of mice were examined for metabolic and behavioral outcomes following 10 weeks in variable conditions, including control (C), shift work control (SC), shift work chronic exercise (S-RW), shift work timed exercise (S-TE), shift work timed feeding (S-TF), and shift work timed exercise and feeding (S-TFEX).

Results: CRSW resulted in significantly greater weight gain in S-TF (LF: 3.94 ± 1.6 g; HF: 3.55 ± 1.4 g) and lower glucose tolerance ($p<0.05$) in the S-TF and S-TFEX groups on both low- and high-fat diets. Mice receiving 1-hr of scheduled exercise at the same time every day weighed significantly lower ($p<0.05$) and displayed lower glucose intolerance ($p<0.05$) on low-fat diet compared to S-TF condition. Exposure to CRSW

resulted in disrupted activity patterns and differential effects of low- and high-fat diets on these activity patterns during early study vs late study.

Conclusions: The present study provides evidence that the variable food intake patterns associated with shift work result in deleterious metabolic outcomes. Exercise may serve as a behavioral cue for eating behavior.

BACKGROUND

Virtually every cell in the body contains a molecular circadian clock, which is comprised of a coordinated set of genes that regulate the transcriptional rhythmicity of themselves and many downstream metabolic genes in anticipation of daily fluctuations in the environment. Daily exposure to environmental zeitgebers (from the German word for 'time-givers') such as solar cycles, provide signals of time that enable anticipation of environmental changes, such that environmental cues and physiology are optimally timed each day [1]. Light is a potent zeitgeber of the master transcriptional oscillator in the suprachiasmatic nuclei (SCN) of the hypothalamus via the light-sensitive retinal ganglion cells [2]. The SCN is the key area of the brain that processes external photic signals into an internal output of time communication via neurohumoral signals to the rest of the body [3]. Additionally, non-photic zeitgebers, including neurohumoral factors associated with food intake [4] and physical activity [5], have been demonstrated to entrain the circadian rhythms of the peripheral oscillators.

The connection between shift work-related circadian disruptions and disease risk has gained increasing attention. Metabolic syndrome, which is characterized by elevated triglycerides, central obesity, high blood pressure, and impaired fasting glucose [6], is associated with circadian misalignment, sleep deprivation, alterations in timing and quality of meals, conflicts between personal and work schedules, and the type of shift rotation [7]. Insulin resistance and type 2 diabetes are commonly prevalent among shift workers [8, 9], with a higher percentage observed in rotating shift workers (counter clockwise rotation of 5 shifts each for day, night and evening; or 3 or 4 shifts each of day, night and evening shifts) [10, 11]. About 40% of shift workers have an increased risk for CVD, due at least in part to circadian disruption, lifestyle factors including smoking,

alterations in food intake, and stress underlying psychosocial issues [12]. Circadian dyssynchrony of clocks, as evidenced in shift work, is associated with high blood pressure, insulin resistance, dyslipidemia, and obesity [13]. In addition to a high prevalence of metabolic syndrome and CVD, shift work is also associated with multiple health issues related to psychological, reproductive, gastrointestinal, metabolic, and sleep disorders, along with cancer. The international Agency for Research on Cancer (IARC) has classified shift work associated disruption in circadian rhythms as a probable human carcinogen [14].

Alleviating the disruptive effects of shift work on health is a complex process. Several efforts have been made to lessen the risk of chronic diseases among shift workers, including implementing ergonomic work schedules [15-18], controlled exposure to light/dark cycle [19, 20], physical activity and lifestyle changes [21], and pharmacotherapy to promote adaptation [22, 23]. Behavioral interventions targeting physical activity and/or dietary changes in shift workers [21, 24-27] are associated with decreased body weight, BMI, waist circumference, adiposity, and hyperlipidemia and increased health behaviors such as fruit and vegetable intake. However, the application of these findings to other types of shift work and are limited by sample size and varied shift characteristics.

Research in animal models has shown that the timing of feeding can have positive or negative metabolic consequences in rodent models under standard light/dark conditions [28-30], as well as under constant light conditions [31] and in mice lacking circadian clock function [32]. We and others have shown that mice fed only during normal periods of sleeping/rest have disrupted metabolic rhythms and increased energy intake and adiposity [29, 30]. Emerging data have provided evidence for exercise as a non-photic zeitgeber for circadian clocks, with the potential to entrain peripheral

circadian clocks in rodents [5, 33-35] and humans [36-38] under both regular and circadian disrupted conditions. These findings suggest a key role for timed feeding and exercise as potential intervention strategies for rotating shift workers who are exposed to frequent alterations in feeding, activity, and sleep.

The goal of the present study was to, 1) investigate the effects of chronic exposure to rotating shift work (CRSW) in comparison to regular light/dark schedule on food intake, body weight, and glucose homeostasis and 2) evaluate the effects of behavioral interventions, including time-restricted feeding and timed exercise, independently and in combination on physical activity patterns, food intake, body weight, and glucose homeostasis. Additionally, we investigated the differential effects of low and high fat diets under chronic exposure to CRSW and timed feeding and timed exercise conditions.

MATERIALS AND METHODS

Animals. Eleven-week-old male mice on FVB/N background (n=96) (Jackson Laboratories, Farmington, CT, USA) were individually housed in microisolator cages with alpha-dry bedding (Shepherd Specialty Papers, Inc. Richland, MI, USA) at the Dell Pediatric Research Institute (Austin, TX, USA) under controlled conditions. The animals were acclimated to a 12-hr light/ 12-hr dark cycle for one week with *ad libitum* access to regular chow (LabDiet, St. Louis, MO, USA) and water, prior to undergoing the experimental conditions. All experimental protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Texas at Austin.

Experimental model. A light-based model of human rotating shift work (CRSW) was used, as depicted in Figure.3.1. Each week light/dark cycle inversions occurred to simulate a rotating shift work paradigm, with alternating patterns of 3 days “on shift”/ 4

days “off shift” during odd weeks, followed by 4 days “on shift”/ 3 days “off shift” during even weeks. Off shift was defined as normal light/dark cycle with lights on at ZT0 and lights off at ZT12, and on shift is defined as an inversed light dark cycle with lights on at ZT12 and lights off at ZT0. Each transition from the on shift to off shift schedule and vice versa was punctuated by extended (24-hour) periods of light and dark respectively, as illustrated in Figure 3.1.

Experimental design. Mice were randomized to six experimental groups (Figure 3.1) to test the effects of chronic exposure to CRSW schedules and 2) to test timed feeding and timed exercise interventions independently and in combination under the altered light/dark schedules for 10-week duration. All experimental conditions were repeated in two independent sets of mice. The groups included: 1] control (C), 2] shiftwork control (SC), 3] shiftwork chronic exercise (S-RW), 4] shiftwork timed exercise (S-TE), 5] shiftwork timed feeding (S-TF), and 6] shiftwork timed exercise and feeding (S-TFEX). The experimental group light schedules and access to food and running wheels are visually illustrated in Figure 3.1. Control group mice were housed in regular 12-hr light/ 12-hr dark schedules for the entire duration of the study with 24-hr access to food. The 5 shift groups were housed under altered light/dark schedules as described above to mimic human shift work. A 2-week light/dark schedule mimicking rotating shift work pattern under different experimental conditions is illustrated in Figure.3.1. This 2-week pattern of light schedules was repeated for the 10-week duration of study.

Diet and food intake. All experimental groups received either high-fat (20% kcal protein, 45% kcal fat, and 35% kcal carbohydrates) or low-fat (20% kcal protein, 10% kcal fat, and 70% kcal carbohydrates) diets (Research Diets. Inc, New Brunswick, NJ, USA). Food intake was measured weekly in all groups. Food access was provided *ad*

libitum for C, SC, S-TE, and S-RW groups. Food was restricted to the dark phase of the altered light/dark cycle for S-TF and S-TFEX groups, simulating eating only during "work" hours. Access to food and water was restricted for S-TE and S-TFEX groups during the 1 h access to voluntary running wheels (described below). All groups received *ad libitum* access to water unless specified.

Food intake behavior in real time was monitored in representative animals in the SC, S-TE, S-TF, and S-TFEX groups. Mice from these groups were housed in a BioDAQ automated food intake monitoring system (Research Diets. Inc, New Brunswick, NJ, USA). The cages were equipped with gated food hoppers and an Automated Gate Controller that programmed the gates to open and close at specified times, limiting researcher interactions. Representative gate controllers are mounted to live scales that quantitatively measure and record episodic food intake in individual animals. The system is also equipped to capture food spillage.

Physical activity. Low-profile wireless running wheels (ENV-047, Med Associates. Inc, St. Albans, VT, USA) were used to quantitatively measure physical activity in mice. Each wheel was equipped with sensors that wirelessly transmitted wheel revolution counts every 30 seconds to a receiver hub. Wheel manager data acquisition software (SOF-860) was used to record the revolution counts from the hub. S-RW groups received *ad libitum* access to running wheels in home cages. S-TE and S-TFEX groups received daily 1-hr access to wireless running wheels at the same time each day, independent of altered light/dark cycles. The running wheels were placed into the home cages for 1-hr and promptly removed when the exercise bout was completed. During 1-h access to running wheels, access to water and food were removed for both S-TE and -TFEX groups.

Body weight. Body weights for all animals were measured using a digital scale (Ohaus, Parsippany, NJ, USA) once every week. Mice were placed into a tared tall cup and weight recorded when movement was stabilized.

Glucose tolerance test (GTT). Animals were fasted for 12-hours during their sleep phase prior to GTT. All glucose measurements were taken from tail blood. Fasting glucose levels were measured in duplicate using a Freestyle Lite glucometer (Abbott Laboratories, Abbott Park, IL, USA). Fasting glucose levels were measured after administration of 10% D-glucose at 1g/kg body weight intraperitoneally, and glucose levels were measured and recorded at 15, 30, 60, 90, and 120 minutes. The tests were performed two hours prior to the normal light-to-dark transition (ZT10).

Statistical Analysis. Statistical analysis was performed using ANOVA for end of study body mass, body mass change, fasting glucose, glucose AUC (area under the curve), and wheel running activity to evaluate the differential effects of the experimental conditions by diet. *Post hoc* analysis was carried out for significant global ANOVA test by one-way ANOVA using Scheffe correction. End-of-study body mass was residualized for food intake and residuals were analyzed with ANOVA. Linear mixed models were used to analyze wheel activity across time. All results are expressed as mean ± SD (standard deviations). All analyses were performed using Stata 17 SE (Stata Corp., San Antonio, TX). Significance level was set at $p<0.05$.

RESULTS

Timed feeding altered end of study body mass and body mass change similarly on both diets. After controlling for food intake, exposure to CRSW altered body mass compared to control mice in a diet-specific manner. Significant differences in body mass were observed for C vs SC mice on high fat diet ($F_{1,13} = 7.67$, $p<0.05$; Figure

3.2E) controlling for total kilocalorie intake with SC mice weighing less than C mice. However, no differences in body mass were observed for mice on low fat diets (Figure 3.2A), and body mass gain did not differ between C vs SC mice on either diet, controlling for food intake (Figures 3.2B and 3.2F). For mice in the shift work conditions, timed feeding (S-TF: 31.6 ± 2.2 g) mice gained significantly higher body mass compared to SC (29.3 ± 1.6 g; $F_{1,12} = 8.81$, $p<0.05$) and S-TE (28.8 ± 1.6 g; $F_{1,12} = 14.90$, $p<0.01$) mice on low-fat diet (Figure 3.2C), controlling for total kilocalorie intake. On the high fat diet (Figure 3.2G), S-TF (30.9 ± 1.6 g) mice had significantly higher body mass, controlling for total kilocalorie consumption, compared to both groups with access to running wheels (S-RW: 28.6 ± 2.8 g; $F_{1,12} = 10.75$, $p<0.01$ and S-TE: 29.4 ± 3.8 g; $F_{1,12} = 5.82$, $p<0.05$). There were significant group differences in body weight change from baseline to the end of the study on both low (Figure 3.2D) and high fat (Figure 3.2H) diets controlling for food intake. The S-TF group gained the most weight on both diets, after controlling for total food intake. S-TF mice on the low-fat diet (Figure 3.2D) gained 3.94 ± 1.6 g, which was significantly higher than SC (1.26 ± 0.9 g, $F_{1,12} = 20.23$, $p<0.001$), S-RW (0.30 ± 1.7 g, $F_{1,12} = 12.34$, $p<0.01$), and S-TE (0.62 ± 1.25 g, $F_{1,12} = 22.84$, $p<0.001$) mice. On the high fat diet (Figure 3.2H), body weight for S-TF (30.9 ± 1.6 g) was significantly higher than S-RW mice (28.6 ± 2.8 g, $F_{1,10} = 13.10$, $p<0.01$).

Although body weight and body weight gain differed significantly specific to diet and treatment conditions, total kilocalorie intake did not differ between treatment conditions (Figure 3.3A-D), with an exception for SC mice on low fat diet. SC (832.8 ± 55.8 kcal) mice consumed significantly more kilocalories compared to C (774.9 ± 31.3 kcal) mice on low-fat diet ($F_{1,14} = 6.55$, $p<0.05$). In general, S-TFEX consumed the least number of calories on both diets but gained the similar amount of body mass as S-TF groups and S-TE consumed the highest number of kilocalories on high-fat diet.

Timed feeding on CRSW disrupted glucose homeostasis on both low- and high-fat diets. There were no differences in fasting glucose (Figures 3.4A and 3.4E) or glucose tolerance (Figures 3.4C and 3.4G) in SC mice compared to C mice on either diet. Under shift work conditions, there were no differences in fasting plasma glucose for mice on the low-fat diet (Figure 3.4B); however, S-TF and S-TFEX mice displayed significantly higher fasting glucose (153.8 ± 18.6 mg/dL and 148.6 ± 12.6 mg/dL, respectively) compared to S-RW mice (102.9 ± 29.9 mg/dL) on the high-fat diet ($F_{4,32}$ = 3.96, $p<0.05$, Figure 3.4F). In terms of glucose tolerance, S-TF mice had significantly higher glucose AUC (mean ± SD) compared to SC (27606.5 ± 4787.2, $p<0.05$), S-RW (23893.3 ± 4931.2, $p<0.05$), and S-TE (20141.5 ± 2879.6, $p<0.05$) receiving low-fat diet (Figure 3.4D). On the high fat diet, S-TF (153.8 ± 18.6 mg/dL) and S-TFEX (148.6 ± 12.6 mg/dL) mice on high fat diet displayed significantly higher fasting glucose compared to S-RW (102.9 ± 29.9 mg/dL, $p<0.01$) mice (Figure 3.4H). There were no differences in fasting blood glucose or glucose tolerance between either timed feeding group.

Chronic exposure to rotating shift work disrupted 24-hr physical activity rhythms on both low- and high-fat diets. Chronic exposure to shift work conditions disrupted 24-hr wheel-running activity rhythms under both low- and high-fat diets for S-RW mice. We have previously shown [39] that mice receiving standard chow display regular patterns of physical activity during normal LD conditions, with high physical activity during the dark phase and low physical activity during the light phase. In contrast, chronic exposure to rotating shift work disrupted this pattern of high dark and low light phase physical activity, with mice displaying equal amounts of wheel-running

activity in both light and dark conditions in S-RW mice under both diets (Figure 3.5A and D and 3.6). In a two-way ANOVA of total distance run by light status (on vs. off), there was significant main effects of diet (Figure 3.5A, low- vs high-fat diet; $F_{1,22} = 26.5$, $p<0.0001$) but not for light (Figure 3.5D). S-RW mice on the high-fat diet (average total distance: 353.9 km; lights on: 147.6 ± 45.7 km; lights off: 206.4 ± 43.3 km) ran significantly greater distance than mice on the low-fat diet (average total distance: 159.7 km; lights on: 77.0 ± 50.0 km; lights off: 82.7 ± 51.2 km) diets. We analyzed the trend in physical activity for the S-RW group across the 10 weeks of the study using linear mixed models, which revealed a significant trend in wheel-running activity measured as distance on both low and high fat diets (Figure 3.6A and B). Mice on the low-fat diet ran ~333 meters less than mice on high fat diets ($p<0.001$). A significant interaction between study duration and diet showed that mice on low fat diet increased distance run by 0.22 meters for every additional hour in study duration ($p<0.001$). The trend in wheel-running activity significantly differed by early weeks to late weeks of study protocol. Main effects of early vs late study activity ($p<0.0001$), diet ($p<0.0001$) and interaction ($p<0.0001$) were significant. On average, mice on low fat diet ran significantly more distances during late study (coefficient: +201.3 m) compared to early study (coefficient: - 247.1 m) while the opposite trend was true for mice on high fat diet with decreased activity during late weeks (coefficient: -108.9).

1-hr of daily timed voluntary physical activity was similar by diet and treatment condition in S-TE and S-TFEX. Given the pre-determined exposure to running wheels in the S-TE and S-TFEX groups (i.e., one hour of running wheel time every 24 hours), there were no differences between the S-TE and S-TFEX groups for either diet, and the total distance run was significantly higher in the S-RW group (HF:

353.9 ± 31.6 km; LF: 159.7 ± 97.0 km) compared to the S-TE (HF: 40.5 ± 17.4 km; LF: 35.1 ± 7.1 km) and S-TFEX (HF: 44.5 ± 11.4 km; LF: 39.5 ± 11.0 km) groups on both low- ($F_{2,18}$ = 11.0, $p<0.001$) and high-fat ($F_{2,19}$ = 500.8, $p<0.0001$) diets and (Figure 3.5B). However, 1 h of wheel-running activity at zt0 in the S-TE and S-TFEX mice resulted in significantly longer distances run compared to the same 1 h time period in the S-RW group ($F_{2,18}$ = 7.61, $p<0.01$, Figure 3.5D) on the low-fat diet.

1-h of scheduled voluntary bout of exercise altered feeding behavior in mice on high fat diet. Real-time feeding behavior monitored in representative mice exposed to S-TE condition on low- and high-fat diet are shown in Figure 3.8. 1-h bout of exercise appeared to serve as a behavioral zeitgeber for feeding, with majority of food consumed immediately following an exercise bout and low or no feeding until after a bout of exercise the following day. This change in feeding behavior was observed after two weeks of exposure to protocol. Although this feeding behavior was observed in low-fat mice, consistency in feeding pattern after bout of exercise was more prominent in high-fat fed mice. Interestingly, such change in feeding pattern was evident in SC mice not receiving a bout of exercise on low-fat diet after 1-week of exposure to protocol (Figure 3.7A). These findings are only observational as no formal statistical tests were performed on a sample size of two mice per condition and diet.

DISCUSSION

In the current study we investigated the long-term consequences of exposure to altered LD cycles (SC) in chronic rotating shift work (CRSW). We also tested the physiological outcomes of CRSW combined with physical activity via continual access to

running wheels (S-RW), 1-h scheduled voluntary timed exercise (S-TE), timed feeding (S-TF), or a combination of timed exercise and timed feeding (S-TFEX) interventions on low- and high-fat diets. Exposure to CRSW displayed differential effects of diet on body mass compared to control conditions (Figure 3.2E). Mice in the timed feeding condition, with or without 1-h exercise, displayed the greatest body mass gain (Figure 3.2C, D, G and H), the highest fasting glucose (Figure 3.4F), and the lowest glucose intolerance (Figure 3.4F and H) in a diet-specific manner. Exposure to CRSW resulted in disrupted activity patterns and differential effects of low- and high-fat diets on these activity patterns (Figure.3.5 and 3.6) during early study vs late study. These results suggest that timing food intake to coincide with wakefulness or "work" resulted in the poorest metabolic outcomes, which were not attenuated by a daily bout of exercise. This observation is important because many individuals working on shift-work schedules may use this strategy, either by necessity or to limit food intake. A recent study quantifying energy intake of rotating shift work nurses who worked at least 2-3 consecutive nights shifts reported increased proportion of total daily energy intake during night shifts, even though total energy intake was similar between night- and day-shift nurses. The proportion of energy intake during night shifts also increased for each consecutive day of night shift [40]. Nurses on night shift distributed their food intake to coincide with work at nights while not increasing their total energy intake.

Exposure to CRSW did not alter body mass in mice on the low-fat diet but SC mice weighed much lower on high-fat diet compared to control mice despite the fact that they consumed more kilocalories than control mice. Our finding is in contrast to increased body mass reported by previous studies using dim light at night (DLAN) [41-43], constant light [31], and repeated shifts in light/dark (LD) cycle [44]. The control mice in this study were meant to characterize normal growth in a regular LD cycle,

however these mice differed by location, caging, and feeding systems in order to be able to maintain them under LD cycles different from shift work mice, suggesting that these factors may have influenced the results. Additionally, the control mice started on protocol heavier than SC mice and maintained this difference in linear growth till the end. Due to these reasons our findings are not in agreement with reports from previous studies [42, 45].

The interventions used to facilitate adaptation to CRSW showed differential effects of treatments on body mass. Although no treatment conditions were significantly different for kilocalories consumed, S-TF and S-TFEX groups displayed significant body mass gains. We hypothesized that timing feeding to coincide with waking/active periods (i.e., lights off in nocturnal animals) might be a good strategy for facilitating metabolic adaptation to CRSW. In fact, our experimental model of CRSW combined with timed feeding resulted in the most deleterious effects on metabolic outcomes. In addition to body mass gains, we also report dysregulation of glucose homeostasis in response to timed feeding on both low- and high-fat diets. Feeding is a strong zeitgeber for peripheral tissue clocks, and the timing of food intake can influence positive or negative metabolic outcomes. Our hypotheses regarding the timing of food intake were based on observations made by our lab and others who have reported that restricting food access to the normal sleep phase (lights on) in mice was associated with disruptions of circadian rhythms that resulted in increased body weight, reduced glucose tolerance, dampened rhythms of glucose and corticosterone, and altered gene expression in liver clock and metabolic genes [30, 46]. On the contrary, timed restricted feeding during the normal dark phase (zt12 to zt0) under constant light conditions has been reported to restore diurnal rhythmicity in plasma glucose levels [31]. Similarly, time restricted feeding

(TRF) of a high-fat diet fed only during the regular dark phase when mice normally eat most of their food also prevents obesity and metabolic perturbations associated with *ad libitum* access to high-fat diet [28]. A recent study compared the effects of 10-hr early TRF (start of dark phase) versus late TRF (initiated 4-hr into dark phase) in mice and reported attenuation of weight gain under both TRF conditions but to a lesser extent in the late TRF. The study also reported improved glucose tolerance under both TRF timings [47]. TRF is generally associated with regular fasting intervals each day [48]. In our model, timed feeding/fasting intervals lasted for 24 hr during the transition periods between on-shift and off-shift days every 3-4 days. This irregular feeding schedule and lack of consistency in long fasting intervals may have negatively influenced the resetting of the peripheral clocks to repeated changes in feeding timing, resulting in altered metabolic outcomes which were not attenuated by a 1-h bout of exercise. It is important to note that altering the timing of food intake when transitioning from on-shift to off-shift schedules is a common practice among shift workers.

Constant access to a running wheel in our study, showed lower body mass and body mass gain for mice fed either a low or high fat diet. The gain in body mass in the S-RW group was considerably lower than for either timed feeding group. This finding is consistent with a recent study of chronic access to voluntary running wheel (VWR) in a diet-induced obesity weight loss model that reported substantial weight loss associated with VWR corresponding to a reduction in fat pad mass [49]. Additionally, mice compensated for decreased energy stores [49, 50] and increased daily energy expenditure [51] as a result of VWR by increasing dietary food intake [49, 50]. We did not observe alterations in food intake in response to chronic access to running wheel and weight change. Foright et al. (2020) evaluated the compensatory eating behaviors in rats in

response to four weeks of forced treadmill training scheduled during the last 2-hr of light cycle. The study reported differential effects of sex where female rats increased food intake in compensation to exercise while male rats decreased intake [52], supporting our results. Average wheel activity in S-RW mice on the high fat diet was considerably higher compared to mice on the low-fat diet, which may explain the body weight change on high fat diet. However previous studies have reported the opposite of increased physical activity observed in our study with high-fat diet, with decline in locomotor activity in mice feeding on a western diet high in fat [53]. The effect of study duration on physical activity in mice receiving a high fat diet did demonstrate significant decline in activity at a later time in our study, consistent with previous findings [53] when controlled for time. *Ad libitum* high-fat diet alters feeding behavior in mice by increasing food intake during the rest phase with a resultant increase in body mass and glucose intolerance [54]. Similarly, exposure to shift work conditions show decreased tolerance for glucose in rodents and increased body mass in response to timing of feeding shifted to mostly occur in rest phase, which may be linked to increased insulin resistance, although this was not measured [55]. S-RW mice on both diets in the present study showed considerable tolerance to glucose as demonstrated by lower AUC levels compared to timed feeding groups with or without 1-hr exercise. One of the reasons for higher tolerance to glucose is that these mice had chronic access to a running wheel. Nascimento et al. (2016) reported higher tolerance to glucose in a diabetic mouse model to altered LD cycle in the presence of voluntary running wheel [56]. Exercise is known to increase glucose uptake by skeletal muscle in an insulin-independent mechanism of GLUT4 translocation to the plasma membrane [57], while also sensitizing the muscle to insulin action in response to 30 min of aerobic exercise in rats fed a high fat diet [58]. These

findings support our reported results of higher glucose tolerance with chronic access to a voluntary running wheel.

One hour of scheduled voluntary exercise given daily at the same time in our CRSW model resulted in considerably lower body mass compared to the timed feeding groups on both diets, despite the fact that these mice consumed the highest number of calories from high fat diet, although not significant. Several studies involving scheduled exercise have reported the potential for scheduled exercise to either recover the deficits of circadian disruption [59, 60] or entrainment of molecular circadian rhythms under regular [33] and altered LD cycle [34, 60]. One study in rats evaluated the effects of 12-hr forced scheduled wheel running activity at a scheduled timing under 12-hr repeated shifts in LD cycle which reported lower mean body weight compared to rats without forced running wheel under a shifted LD cycle [61]. In contrast to forced activity, wheel running in our study was voluntary, resulting in considerable variability within treatment condition while also simulating the natural activity behavior and avoiding influence of stress in mice [62]. Studies comparing the benefits of forced vs voluntary activity demonstrated equally beneficial effects in suppression of metabolic risk factors, including reduced body weight and fat mass, reduction in adipocyte size, and increased mitochondrial biogenesis [63] yet also demonstrate distinct characteristics in molecular adaptation in different skeletal muscles by shift in the type of muscle fiber and altering training-induced gene expression of markers in muscle [64]. These findings may suggest that 1 h of physical activity in mice may be too little time to observe significant effects. It is possible that changing the timing of the 1-h exercise bout may produce more pronounced effects. Sasaki et al. evaluated three 4-h voluntary running wheel exercise schedules (morning, noon, and evening) in combination with 4-h restricted feeding (morning, noon, and

evening) in mice. The results of a series of experiments reported lower body weight and fat mass when scheduled exercise occurred after a period of eating with a corresponding decrease in resting energy ratio and increased energy expenditure compared to food intake after exercise [65], indicating the importance of timing of exercise to maximize metabolic benefits.

A recent study investigated the effects of voluntary wheel running in a circadian disruption model of short photoperiod (5hr light/ 19hr dark) and reported higher tolerance to glucose compared to rats with no wheel access [60], suggesting the importance of physical activity in positively improving glucose homeostasis under circadian disruption. To date, most studies have modeled timed or scheduled exercise to include several hours of scheduled activity [61, 65]. Our study is the first one to test the effects of 1-hr scheduled voluntary exercise in CRSW model, which was designed to act as a zeitgeber to set the daily timing of molecular clocks in peripheral tissues. Despite the fact that scheduled 1-hr voluntary exercise had no statistically significant effects on quantitative measures, mice receiving exercise for 1-hr gained less body mass than mice without exercise, indicating that 1-h physical activity could be beneficial in shift work models. Also, mice actively used the running wheel during 1-h access more than the mice with 24-hr access during the same hour (Figure 3.5). Interestingly, one hour of wheel activity appeared to serve as a behavioral zeitgeber, resulting in food intake immediately following 1-hr activity, however this was only an observation based on data from two mice (Figure 3.8B). A recent study reported increased meal frequency and decreased meal size in male rats forced to exercise at a scheduled time compared to sedentary rats [52]. Although rats in this study were fed low and high fat diets, the differential effects of exercise and diet on meal pattern were not reported [52]. Future studies should

investigate the role of physical activity in driving feeding behavior in low and high fat diets under *ad libitum* and TRF conditions.

The present study contributes some noteworthy findings to the literature, yet has some limitations. First, to accommodate differing LD schedules between control and shift work conditions, the control mice were housed in a different room and differed in caging and feeding system, which may have influenced the comparison of the normal LD and shifted LD controls. Future studies should minimize differences in cage size and location [66]. Second, the interventions of timed feeding and timed exercise were compared to a shift work control group only to examine their role as potential zeitgebers in a shift work paradigm. To gain a better understanding of effects of interventions, future studies could examine whether these strategies are effective under normal LD conditions. Third, real time feeding was only measured in representative animals under each treatment condition, resulting in insufficient statistical power for effect analysis. Fourth, we tested the effects of scheduled activity at only one timing and future studies should include multiple timings for scheduled activity, as timing of activity can influence outcomes [65].

In conclusion, we report that chronic exposure to rotating shift work altered physiological parameters of body mass and gain on both diets. Physical activity patterns were disrupted, and differential effects of diet were observed in these patterns. Timed feeding and combination of timed feeding and exercise as modeled in this study were not beneficial strategies in preventing metabolic disruptions. Rather than facilitating adaptation, the outcomes were detrimental as demonstrated by increase mean body mass and dysregulation of glucose homeostasis. Chronic access to voluntary running wheel and 1-h bout of exercise showed lower mean body mass and higher glucose tolerance

compared to timed feeding similarly on both diets. Future studies must look at addressing the limitations of this study to gain better insights and increase the potential of these interventions to lessen the health burden of shift workers.

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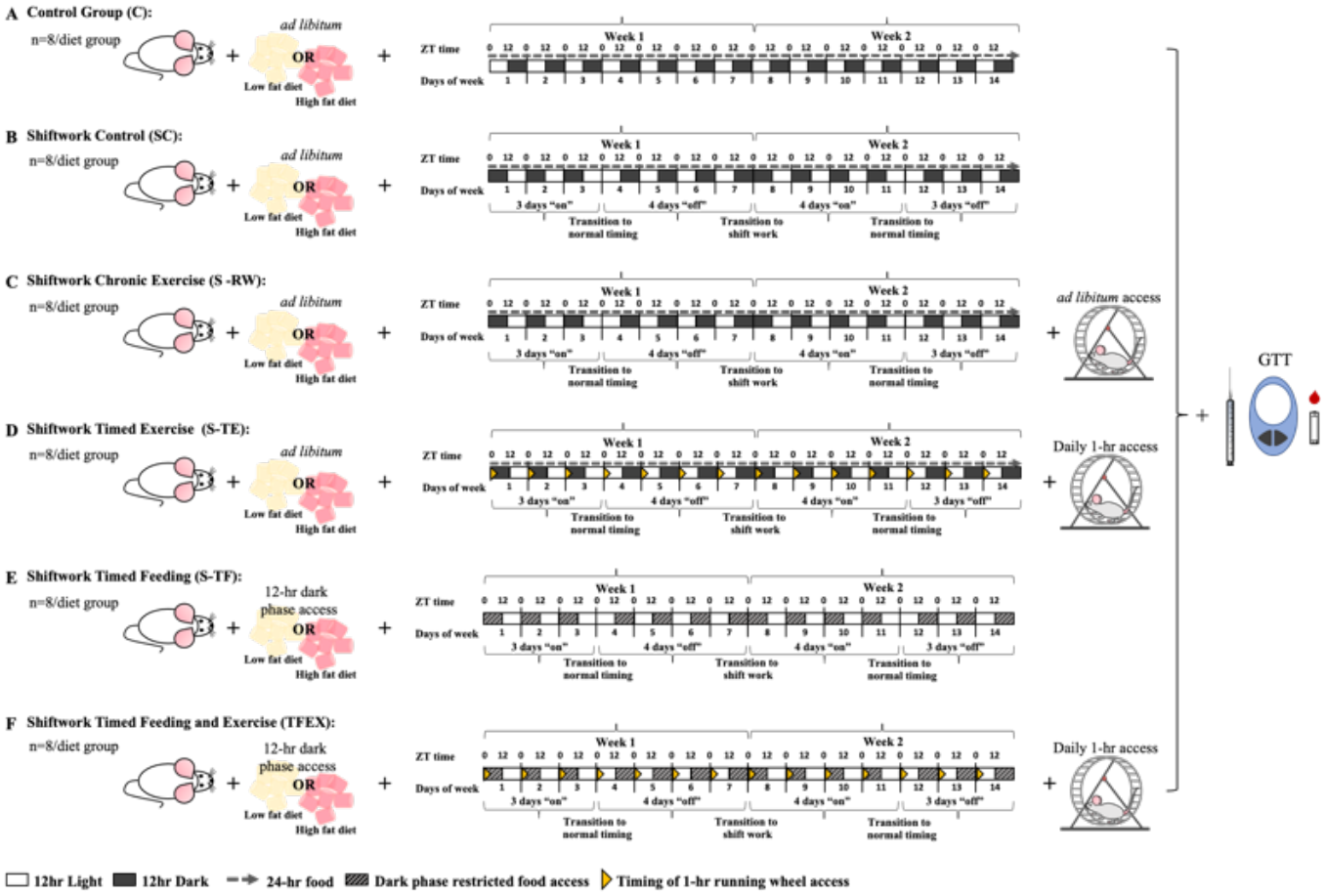
A Control Group (C):
n=8/diet group
ad libitum
OR
Low fat diet
High fat diet
ZT time
Week 1
Week 2
Days of week
B Shiftwork Control (SC):
3 days "on"
4 days "off"
4 days "on"
3 days "off"
Transition to normal timing
Transition to shift work
C Shiftwork Chronic Exercise (S -RW):
ad libitum access
GTT
D Shiftwork Timed Exercise (S-TE):
Daily 1-hr access
E Shiftwork Timed Feeding (S-TF):
12-hr dark phase access
F Shiftwork Timed Feeding and Exercise (TFEX):
12hr Light
12hr Dark
24-hr food
Dark phase restricted food access
Timing of 1-hr running wheel access

Figure 3.1.

Figure 3.1: Experimental study design.

Mice were randomly assigned to either control: C (A), shiftwork control: SC (B), shiftwork chronic exercise: S-RW (C), shiftwork timed exercise: S-TE (D), shift work timed feeding: S-TF (E), or shiftwork timed exercise and timed feeding: S-TFEX (F) experimental groups. Control group mice (C) were housed under regular 12 h light/ 12 h dark cycles, and all other groups (SC, S-RW, S-TE, S-TF, S-TFEX) were housed under repeated shifts in the 12 h light/ 12 h dark cycle every 3 to 4 days and exposed to the corresponding experimental protocols for 10 weeks. Open squares represent 12 h light and filled squares represent 12 h dark. For shift work experimental groups, the transition from 'on shift' to 'off shift' and vice versa is denoted by 24 h light or 24 h dark cycle, respectively. Yellow and pink pellets of food represent low- and high-fat diet, respectively. The sample size is indicated by n for each experimental condition and diet. Abbreviations: ZT, zeitgeber; GTT, glucose tolerance test.

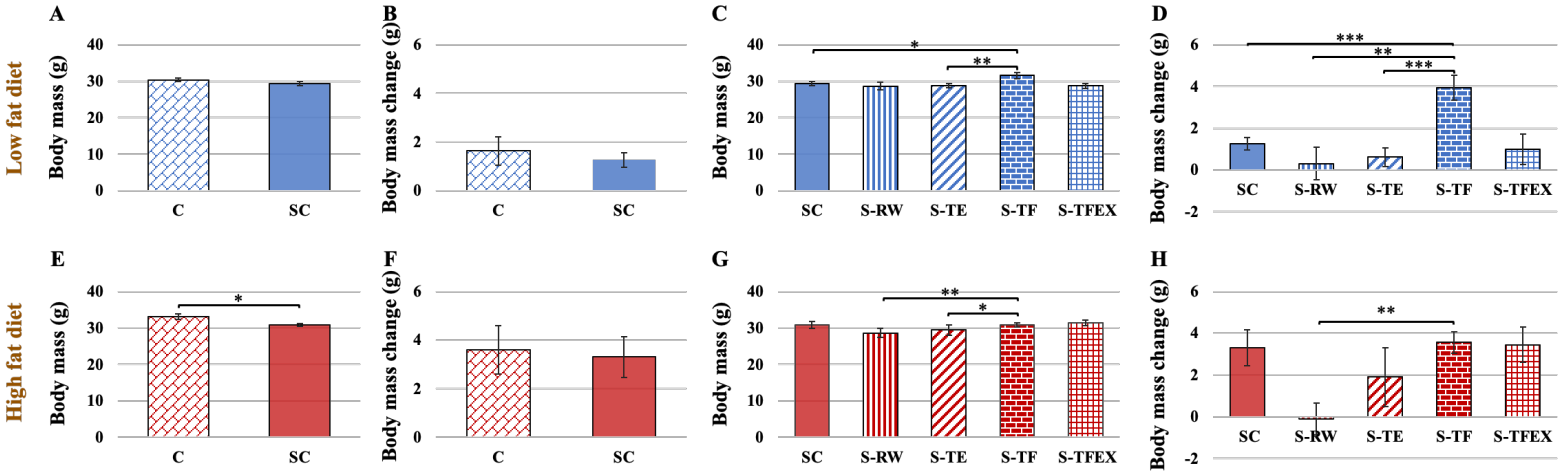

Figure 3.2: Effect of chronic exposure to rotating shift work and timed exercise and time feeding on body mass and body mass gain.

End of study body mass and body mass gain – C vs SC on low- (A and B) and high-fat (E and F) diets; S-TF vs all other shift work experimental conditions on low-fat (C and D) and high-fat (G and H) diet. n=5 to 8/group and diet. Two-way ANOVA was used to analyze data. * $p<0.05$; ** $p<0.01$; *** $p<0.001$. Abbreviations: C, control; SC, shift work control; S-RW, shift work chronic exercise; S-TE, shift work timed exercise; S-TF, shift work timed feeding; S-TFEX, shift work timed feeding and timed exercise.

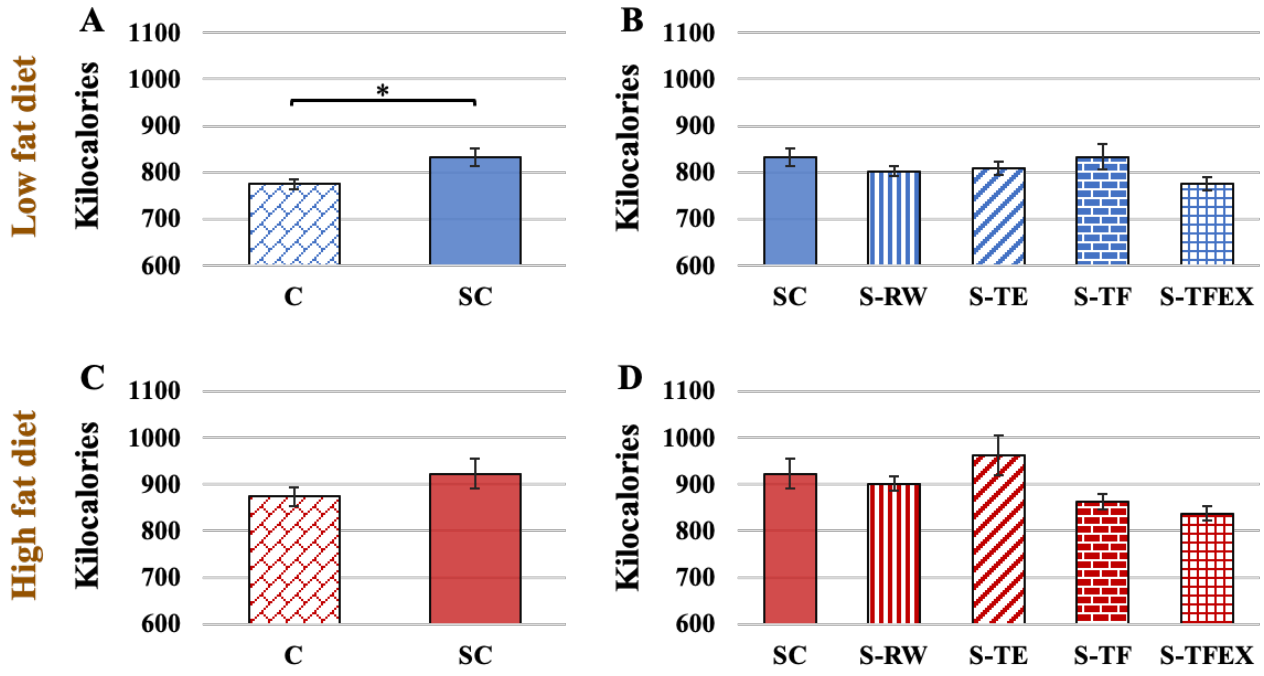

Figure 3.3: Effect of chronic exposure to rotating shift work on average kilocalorie consumption on low- and high-fat diets.

Average kilocalorie intake between C and SC on low- (A) and high-fat (C) diets; average kilocalorie intake between all shift work experimental conditions on low- (B) and high-fat (D) diets. n=5 - 8/diet group. One-way ANOVA was used to analyze data A to D. * $p<0.05$. Abbreviations: C, control; SC, shift work control; S-RW, shift work chronic exercise; S-TE, shift work timed exercise; S-TF, shift work timed feeding; S-TFEX, shift work timed feeding and timed exercise.

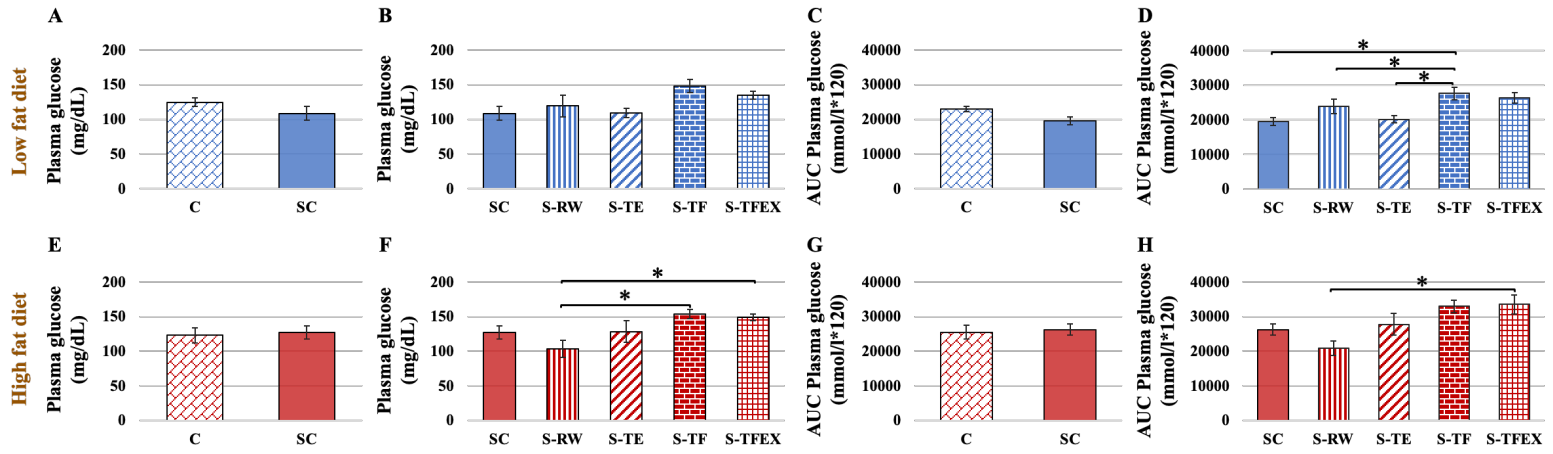

Figure 3.4: Effect of chronic exposure to rotating shift work protocol on plasma fasting glucose and glucose tolerance.

Average fasting plasma glucose – C vs SC on low- (A) and high-fat (E) diets; average fasting plasma glucose between all shift work experimental conditions on low- (B) and high-fat (F) diets; AUC for glucose – C vs SC on low- (C) and high-fat (G) diets; AUC for glucose between all shift work experimental conditions on low- (D) and high-fat (H) diets; n=5 to 8/group and diet. One-way ANOVA was used to analyze data in A – H. * $p < 0.05$. Abbreviations: C, control; SC, shift work control; S-RW, shift work chronic exercise; S-TE, shift work timed exercise; S-TF, shift work timed feeding; S-TFEX, shift work timed feeding and timed exercise.

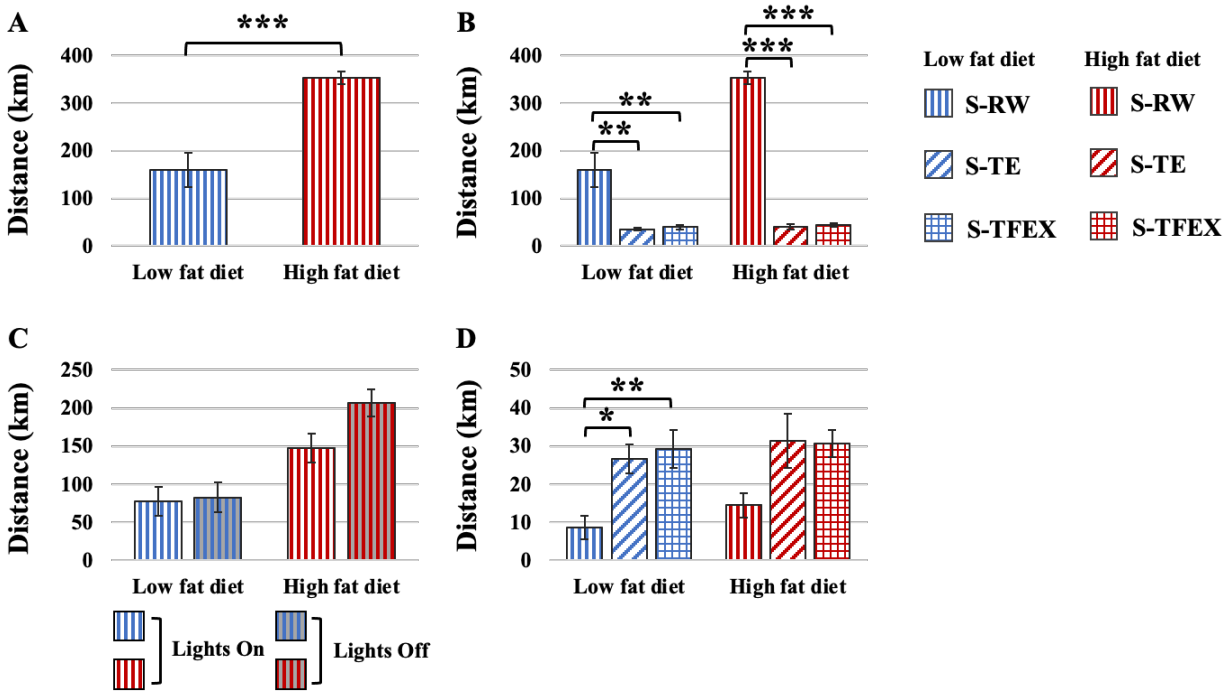

Figure 3.5: Average physical activity for S-RW, S-TE and S-TFEX.

Average total distance run by diet in S-RW (A); S-RW vs S-TE and S-TFEX (B). Average total distance run by light status and diet in S-RW mice (C). Average total distance run at zt0 between S-RW vs S-TE and S-TFEX (D). S-RW: n=6-7/diet group, S-TE: n=7-8/diet group, and S-TFEX: n=7-8/diet group. One-way ANOVA was used for A, B, and D and Two-way ANOVA by light status and diet in B. * $p<0.05$ ** $p<0.01$ *** $p<0.0001$. Significant main effects observed for diet in C. Abbreviations: S-RW, shift work chronic exercise; S-TE, shift work timed exercise; S-TFEX, shift work timed feeding and timed exercise.

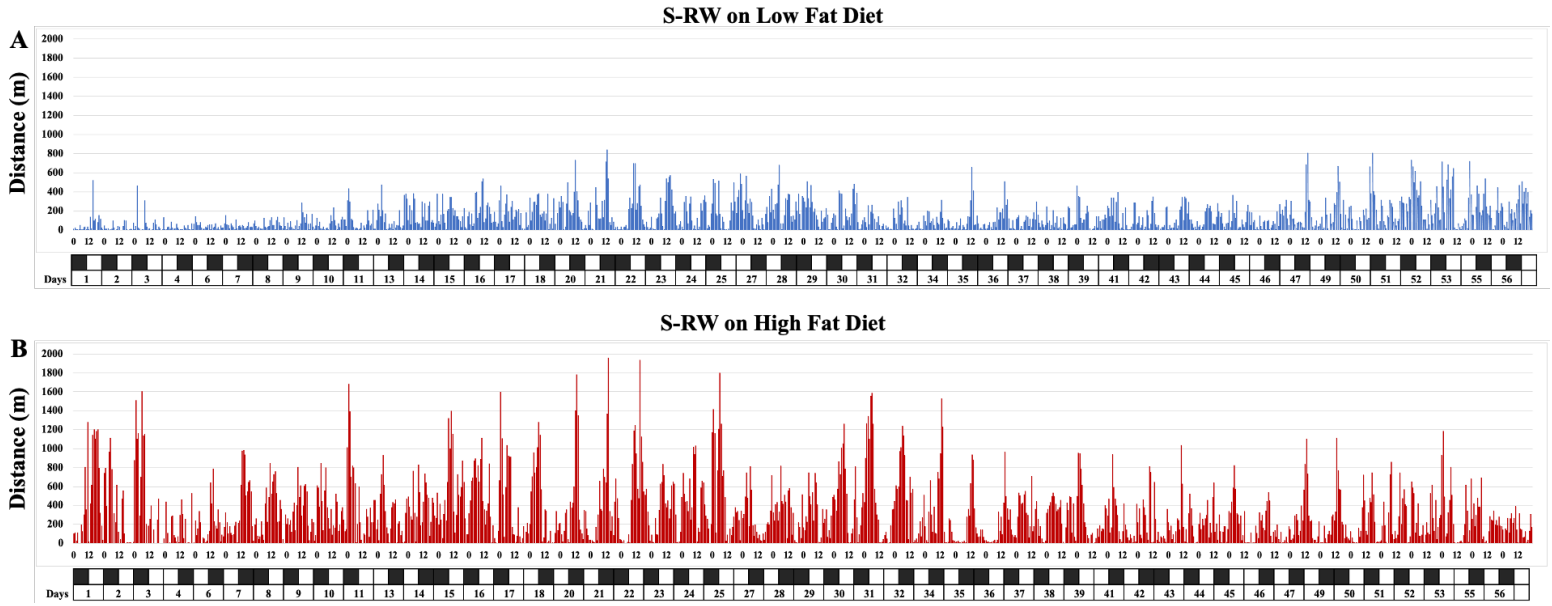

Figure 3.6: Chronic exposure to CRSW disrupts physical activity patterns in low- and high-fat diet groups.

8-week hourly physical activity patterns of S-RW mice on low- (A) and high-fat (B) diets. LF (n=7) and HF (n=6) mice. Linear mixed modeling was used to analyze the trends in physical activity. Abbreviations: S-RW, shift work chronic exercise; LF, low-fat diet; HF, high-fat diet.

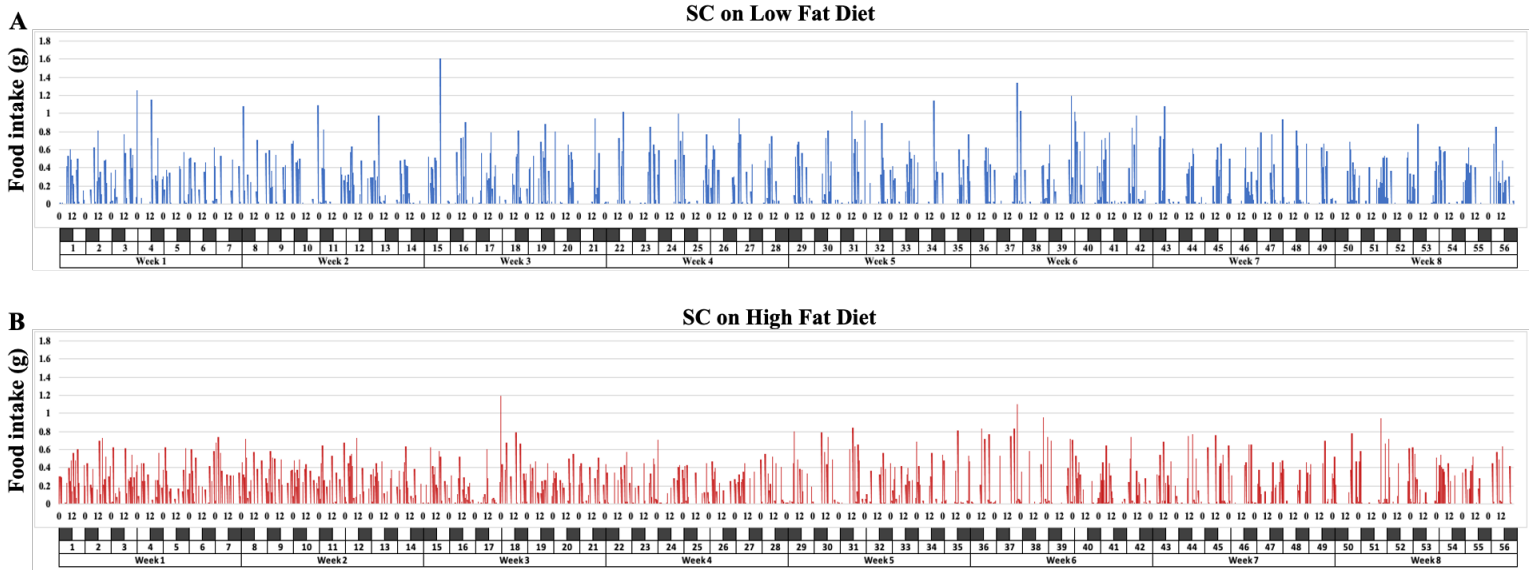

Figure 3.7: Feeding pattern in shift work control (SC) mice on low- and high-fat diet.

8-week real time feeding pattern in SC mice on low- (A) and high-fat (B). The data are representative for one individual animal on each diet. Abbreviations: SC, shift work control.

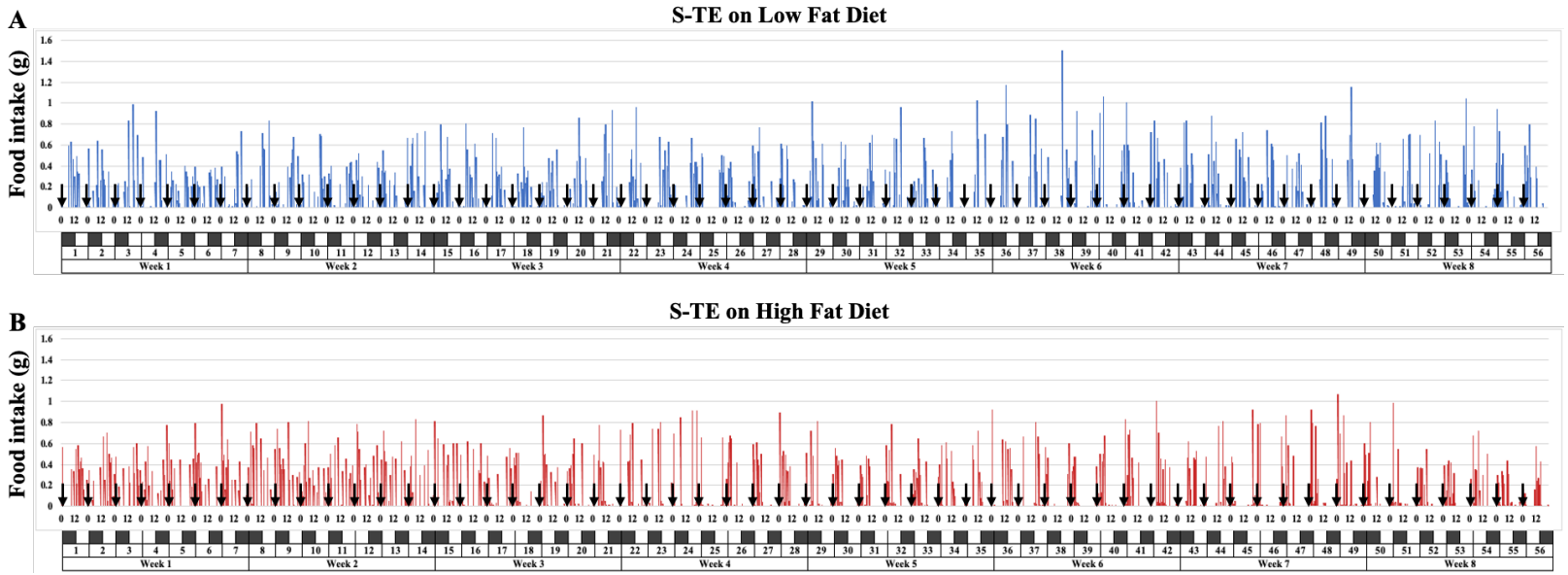

Figure 3.8: Feeding pattern in shift work timed exercise (S-TE) mice on low- and high-fat diet.

8-week real time feeding pattern in S-TE mice on low- (A) and high-fat (B). The data are representative for one individual animal on each diet. Abbreviations: SC, shift work control.

Chapter 4: Conclusion and Future Directions

CONCLUSION

The objective of this research was to investigate the acute and chronic effects of exposure to a simulated human rotating shift work (RSW) in mice and evaluate the impact of chronic exercise, timed exercise, timed feeding, and a combination of timed feeding and exercise in facilitating adaptation to rotating shift work under and the differential effects of low- and high-fat diets in these experimental conditions. Acute exposure to RSW disrupted behavioral activity rhythms and molecular rhythmic expression of core clock and metabolic genes as early as 3-day exposure to protocol. However, gross changes in body mass and body composition were not observed. Acute exposure also altered hepatic glucose metabolism, as evidenced by impaired fasting glucose. These findings may indicate that cellular alterations occur early on and precede the future development of metabolic disorders.

Chronic exposure to shift work, like acute exposure, did not demonstrate gross changes in anthropometric measures on both low- and high-fat feeding. However, the interventions of timed feeding and a combination of timed feeding and exercise displayed impaired fasting glucose, glucose intolerance while also gaining the most weight for food consumed. Combining 1-hr exercise with timed feeding displayed similar adverse effects as timed feeding alone. Timed exercise mice weighed considerably lower and were more tolerant to glucose than timed feeding mice. Interestingly, timed exercise appeared to behave as a behavioral zeitgeber, driving food intake immediately after 1-hr activity, however this observation is only based on data from two mice. Chronic exposure to RSW also resulted in disrupted activity patterns with differential effects by diet. These findings suggest that timed feeding has more disruptive outcomes rather than facilitating

adaptation to chronic RSW, and chronic exercise or 1-hr exercise may have potential as an intervention to minimize metabolic disturbances in shift workers with further research in this area.

LIMITATIONS

The empirical findings presented here should be viewed in the context of some limitations. A commonly used mouse model in diet induced obesity studies are the C57Bl/6J. C57Bl/6J mice when fed a high-fat diet gain considerable weight corresponding to increased adipose stores, display impaired fasting glucose, increased circulating triglycerides and increased total cholesterol [1, 2]. In contrast the animal model we used in our present work is FVB/N which is thought to be resistant to diet induced obesity (DIO) when fed a high-fat diet [3]. FVB/N are highly preferred for their high reproductive performance [4]. This model although does not display significant alterations in body weight gain on high-fat diet, the mice do display glucose intolerance, liver steatosis, and white adipose tissue inflammation [1]. However, this model may not be right choice for studying the consequences of shift work because they did not exhibit adverse physiological and metabolic outcomes after acute or chronic exposure to RSW conditions when fed a high-fat diet, contrasting to findings from previous reports of shift work models [5, 6], which may suggest species specific effects of coping with shift work related stress. FVB/Ns are prone to rapid macular degeneration as early as within a few postnatal weeks [7]. As a result, these mice are highly active with constant running in circles which is highly variable that may have influenced our results.

In our acute study of RSW we measured body mass, body composition, and glucose tolerance in mice after acute exposure to RSW in the presence of ad libitum running wheel, which may have influenced the outcomes we observed. We also did not

evaluate the differential effects of high fat diet under acute RSW conditions to understand the outcomes form a real world perspective, as shift workers have easy access to such foods [8-10], and the type [11] and timing of macronutrient intake [12, 13] can influence diurnal expression patterns of core clock and metabolic gene expression. Fasting the mice before performing circadian dissection is critical to control for differences in metabolic profiles of the animals in our study, as feeding can influence core clock and metabolic gene expression [14, 15].

In our chronic RSW study, we did not have comparable groups of control mice evaluating the effects of intervention to help distinguish if the outcomes we observed were genuinely due to shift work. We did not measure gene expression in chronic study to understand the molecular consequences of chronic RSW. This would have revealed if acute alterations observed in gene expression were transient or persistent after 10 weeks of exposure to RSW. Real time feeding was monitored only a representative sample of mice. Measuring daily food intake in a larger sample will help decipher important insights into the outcomes we reported under various interventions examined, as well as give sufficient statistical power to detect effects. Lastly, only one scheduled time of physical activity was evaluated. Because timing of activity can influence metabolic parameters [16], its critical to examine multiple times of exercise bout to understand the resulting influential effects to maximize benefits of exercise.

The next section will address some of the limitations more deeply recommending future directions to improve the quality, applicability, and understanding of shift work research.

FUTURE DIRECTIONS

Our research has contributed noteworthy findings in simulated shift work modeled by frequent alterations in light/dark cycles, from changes in behavioral factors to modifications in the rhythmicity and quantity of gene expression and the role of timing of feeding and exercise in energy balance and metabolism. These findings carry important implications for translation to human trials. Nevertheless, several follow-up questions related to the current work can help improve our understanding of the physiological and molecular alterations associated with shift work and deepen our knowledge about the interventions currently used. Future research work in the area of rotating shift work should consider the limitations addressed here to gain better insights and increase the potential of the interventions evaluated to lessen the burden of health in shift workers.

Our findings have reported molecular alterations in the rhythmic gene expression of core clock and metabolic gene expression in response to acute exposure to rotating shift work within 3 days. However, no whole body changes (body mass and composition) were evident by the end of the 2-week protocol. Most of our current knowledge about rhythmic regulation is focused on transcriptional regulation, with recent studies that have described translational regulation of mRNA transcripts, to proteins adding a layer of complexity to rhythmic gene expression and translation into functional proteins [17, 18]. Understanding the temporal regulation of gene expression to protein translation and, eventually, the functional significance of these alterations will help to improve our understanding of shift work-related circadian disruption at various levels of molecular biology. Measuring protein expression of genes in future studies will highlight the functional relevance of these alterations to outcomes of rotating shift work. Furthermore, investigating these transcriptional and translational modifications under shift work and

variable diets will further advance our knowledge of real-world implications of scientific findings, as many shift workers consume high fat and highly processed foods while on shift [8-10]. We and others have shown that the timing of feeding and the timing of macronutrients [12, 13] can influence molecular adaptation to feeding time.

Another area of focus related to rotating shift work involves the development of non-pharmacological interventional strategies to facilitate adaptation to shift work. Most recently, timed feeding and timed exercise in combination have demonstrated positive outcomes in preventing obesity and improving metabolic disturbances associated with a high-fat diet under a regular light/dark cycle [19], but these interventions have not been explored in a circadian disruption model. Our present work evaluated timed feeding and timed exercise independently and combined on both low-and high-fat diets in chronic RSW conditions. Our findings show that timed feeding resulted in the most adverse physiological effects, while the addition of 1-hr activity with timed feeding could partially restore these adverse effects. One note to focus on here is that our experimental design modeled timed feeding by shifting feeding time to match with the shift in dark phases of RSW every 3-4 days, mimicking the manner in which many shift workers eat. Future studies should examine the effect of normalizing food intake to a regular pattern of feeding and fasting while accommodating the shift work schedule on human health. Previous timed feeding studies have primarily modeled consistent patterns in restricted feeding schedules [16, 19]. Future studies of RSW could also explore timed feeding by consistently restricting feeding at the same time, independent of the shift in light/dark cycles. Such studies will provide considerable insights about the timing of food intake in circadian disrupted models such as RSW and address the consequences of restricting feeding in frequently altered light schedules.

Exercise has long been used as a therapeutic in health and disease, which also has the potential to influence peripheral tissue clocks [20, 21]. In our present work, chronic access to a voluntary running wheel was associated with the least weight gain for food intake. The results of the 1-hr of the timed exercise were promising, suggest a potential entrainment of food intake to physical activity. A daily 1 h bout of exercise is a promising intervention for shift workers because 1-hr of exercise, combined with an altered pattern of feeding was able to mitigate some of the adverse effects in our model. Future iterations of this work should consider examining different times of running wheel access, as the timing of exercise can elicit distinct metabolic outcomes [16] and molecular signatures [22]. In humans, the best coordination, fastest reaction times, and the greatest cardiovascular efficiency and muscular strength all occur in a time of dependent manner [23-25], suggesting timing the daily exercise bout in sync with peak performance and cardiovascular efficiency for optimal effects. Investigating the mechanisms governing the timing of exercise-induced metabolic outcomes could help us better understand the relationship between exercise and circadian rhythms in RSW. Exercise could be a beneficial therapeutic for mitigating the effects of circadian disturbances in several populations such as shift workers.

Finally, our current model of rotating shift work did not reveal substantial deviations in physiological measures from normal control conditions in response to chronic exposure to RSW. It will be necessary to determine if the effects observed in acute exposure to RSW are transient, eventually resulting in adaptation to RSW with chronic exposure. Further, investigating the underlying molecular mechanisms in chronic RSW will help better understand the results observed in this current work. Researching RSW in diet-induced obesity models may be beneficial to understanding species-specific

responses, and the results may be more translatable to humans as we currently live in an obesogenic environment.

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