UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO

DOCTORADO EN CIENCIAS BIOMÉDICAS

INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

LA RELACIÓN ENTRE LOS RITMOS CIRCADIANOS Y EL METABOLISMO: IMPLICACIONES PARA EL SÍNDROME METABÓLICO

TESIS
QUE PARA OPTAR POR EL GRADO DE:
DOCTORA EN CIENCIAS

PRESENTA:
REBECA MÉNDEZ HERNÁNDEZ

DIRECTOR DE TESIS:
DR. RUDOLF M. BUIJS
INSTITUTO DE INVESTIGACIONES BIOMÉDICAS

COMITÉ TUTOR
DRA. GLORIA ACACIA BENÍTEZ KING
INSTITUTO NACIONAL DE PSIQUIATRÍA RAMÓN DE LA FUENTE

DR. ARMANDO ROBERTO TOVAR PALACIO
INSTITUTO NACIONAL DE CIENCIAS MÉDICAS Y NUTRICIÓN SALVADOR ZUBIRÁN

CIUDAD DE MEXICO, SEPTIEMBRE 2022

LA RELACIÓN ENTRE LOS RITMOS CIRCADIANOS Y EL METABOLISMO: IMPLICACIONES PARA EL SÍNDROME METABÓLICO

Programa de Doctorado en Ciencias Biomédicas

Esta tesis fue realizada con el apoyo de CONACYT (beca 329239 asignada a

Rebeca Méndez Hernández) y UNAM-DGAPA-PAPIIT (IG201321).

Agradecimientos

A la Universidad, por todas las oportunidades que me ha brindado, no solo una excelente educación, sino también un ambiente enriquecedor que me permitió convertirme en la persona que soy.

Al programa de doctorado en Ciencias Biomédicas, por brindarme la infraestructura para mi desarrollo académico.

A PAEP, por el apoyo para asistir a los congresos “International Congress of Neuroendocrinology 2018” y “XVI European Biological Rhythm Society Congress 2019”

A mi comité tutoral, la Dra. Gloria Benítez y el Dr. Armando Tovar, siempre amables y dispuestos a ayudar en mi proyecto. Les agradezco por su tiempo y enseñanzas.

A la Dra. Carolina, por su tiempo, enseñanzas y buenos momentos compartidos.

To Dr. Kalsbeek, for your substantial input and support throughout our joint work.

To Valentina and Ewout, for your hard and fruitful work.

A la Dra. Limei, por su apoyo durante mi tiempo en el posgrado.

A Azucena Ayala, por su apoyo y disposición amable.

To Ruud, thank you for always pushing me to be a better scientist, for your commitment to my training and the good times inside and outside the lab.

A Maricarmen, que fue un pilar muy grande de mi trabajo en el laboratorio y, personalmente, alguien con quien siempre contar.

A Mara, porque valoro mucho tu apoyo y admiro la forma en que piensas. Gracias por hacer del trabajo algo divertido y fructífero.

A Gaby, porque siempre tienes un meme, un *paper,* o alguna idea para compartir conmigo.

A Eva, por acompañarme con tu amistad durante todo este proceso.

A Esteban, porque haces la vida del lab (y fuera de ella) más interesante y divertida.

A Rodrigo, porque sé que cuento contigo para apreciar un chiste simple o hacer análisis complicados.

A Betty, por todos los momentos de diversión y especulación.

A Yeni y Richi, por las risas y buenas discusiones.

A Natalí, por tu apoyo y los buenos momentos fuera del lab.

A Beto, por todas las veces que has sido mi apoyo emocional, mental e incluso físico (haciendo muestreos a deshoras). Gracias por ser el mejor compañero de vida.

A mi mamá, no sé ni por dónde empezar a agradecerte, siendo que me has dado tanto. Te quiero.

A Gisela, por ser mi hermana favorita, aunque yo arruine todo con mi ñoñez.

A mi papá que, aunque ya no está conmigo, lo encuentro en cada instante.

A Xesus, por tu apoyo y cariño.

A Javier y Pau, por su amistad y apoyo incondicional.

A León, Mario y Jonathan, por hacer de las clases de posgrado algo divertido.

To Anneloes, for your overseas love and support.

A Salvador, por ser un buen amigo en toda la extensión de la palabra.

La relación entre los ritmos circadianos y el metabolismo:

implicaciones para el síndrome metabólico

RESUMEN

Todos los seres vivos han desarrollado mecanismos que les permiten adquirir energía y optimizar su uso. Estos mecanismos permiten que se adapten a un entorno cambiante donde la disponibilidad de recursos fluctúa dependiendo de la hora del día. De este modo, diversas conductas y funciones, como la alimentación y la utilización de glucosa por los tejidos, se encuentran reguladas de manera circadiana. El sistema circadiano funciona gracias a que el cerebro, específicamente, el hipotálamo, coordina las funciones de diversos órganos a través de múltiples vías de comunicación. En contraste, el estilo de vida actual implica que las personas llevan a cabo conductas opuestas a lo que dicta su sistema circadiano, por ejemplo, el trabajo nocturno, la alimentación nocturna y la exposición constante a la luz artificial. Estas condiciones generan un conflicto en el sistema circadiano y predisponen a los individuos a las enfermedades metabólicas.

En esta tesis, exploramos la relación entre el sistema circadiano y el metabolismo utilizando a la rata como modelo experimental. El capítulo 1 introduce la relación entre el sistema circadiano y el metabolismo con detalle y aborda las condiciones en las cuales esta relación se ve comprometida. Se detalla cómo es que el hipotálamo funciona como integrador del sistema circadiano, cómo las funciones metabólicas fluctúan de acuerdo con la hora del día y cómo las condiciones como

la alimentación en el horario de descanso y la luz por la noche pueden afectar al sistema circadiano y al metabolismo.

El capítulo 2 explora el conflicto entre la luz y la alimentación durante el día utilizando el paradigma experimental del fotoperiodo esqueleto, que consiste en eliminar la luz la mayor parte del día. Se expuso a distintos grupos de animales al fotoperiodo esqueleto o a un fotoperiodo normal, restringiendo la alimentación al día, la noche, o manteniendo libre acceso al alimento. Se observó que el conflicto entre luz y alimentación en el día solo contribuye parcialmente a la aparición de problemas metabólicos como la intolerancia a la glucosa y la acumulación de grasa. Sin embargo, la exposición al fotoperiodo esqueleto indujo intolerancia a la glucosa independientemente del horario de alimentación. Este fotoperiodo también incrementó la adiposidad de los animales y modificó sus ritmos circadianos de temperatura, actividad locomotora y gasto energético, generando una falta de anticipación al periodo de actividad. En este capítulo se describe por primera vez al fotoperiodo esqueleto como una nueva condición que genera problemas metabólicos como consecuencia de pequeñas pero consistentes modificaciones en los ritmos metabólicos, lo que podría proporcionar una base para entender cómo los trabajadores nocturnos que están expuestos a horarios irregulares de luz pueden desarrollar enfermedades metabólicas.

El capítulo 3 describe la influencia de la leptina, una hormona considerada como un inhibidor de la ingesta de alimento, en la regulación de los ritmos circadianos de temperatura en ratas. Se observó que administrar esta hormona en dosis bajas a ratas modifica la temperatura corporal de los animales dependiendo de la hora del

día y que el hipotálamo responde diferente a la misma dosis de leptina dependiendo del horario. Además, se proporciona evidencia de que la leptina podría ser importante para mantener los ciclos circadianos de temperatura en condiciones de saciedad. Estos datos proporcionan una base para entender mejor qué moléculas y regiones cerebrales están implicadas en la regulación de los ritmos circadianos de temperatura en condiciones de ayuno y obesidad.

Por último, el capítulo 4 recapitula lo anterior para proponer los mecanismos que generan intolerancia a la glucosa y acumulación de grasa en las diversas condiciones de disrupción circadiana como luz constante, alimentación en el horario de descanso y el fotoperiodo esqueleto. También se discuten algunas propuestas para restituir la función metabólica en estas condiciones. La información generada en este trabajo proporciona las bases para poder entender mejor cómo es que se desarrollan las enfermedades metabólicas a consecuencia de estilo de vida nocturno que prevalece en una gran parte de la población.

The relationship between circadian rhythms and metabolism:

Implications for metabolic syndrome

SUMMARY

All living beings have developed different mechanisms to acquire energy and optimize its use. In this way, organisms can adapt to a changing environment where resource availability depends on the time of the day. Several behaviors and physiological processes, such as feeding and glucose utilization, are regulated in a circadian manner. These circadian fluctuations occur thanks to the coordination of organ function by the brain, specifically the hypothalamus, through multiple communication pathways. In contrast, many people currently engage in behaviors that oppose what their circadian system dictates; for example, they work at night, eat at night, and are constantly exposed to artificial light. These conditions generate a conflict in the circadian system and predispose individuals to metabolic diseases.

This thesis explores the relationship between the circadian system and metabolism using the rat as an experimental model. Chapter 1 introduces the relationship between the circadian system and metabolism in more detail and addresses the conditions that compromise this relationship. We discuss the hypothalamus as the integrator of the circadian system, the circadian oscillation of metabolic function, and the influence of eating during the resting phase and light at night on the circadian system and metabolism.

Chapter 2 explores the conflict between light and food during the day using the experimental paradigm of a skeleton photoperiod, in which light is eliminated for most of the day. Several groups of animals were exposed to the skeleton or a normal photoperiod, and food access was restricted to the day, night, or left *ad libitum*. We observed that the conflict between light and food only partially contributes to the appearance of metabolic issues like glucose intolerance and fat accumulation. However, exposure to the skeleton photoperiod induced glucose intolerance regardless of the feeding schedule. This photoperiod also increased adiposity and modified the circadian rhythms of temperature, locomotor activity, and energy expenditure, generating a lack of anticipation to the activity period. This chapter describes, for the first time, the skeleton photoperiod as a new condition that generates metabolic problems as a consequence of small but consistent changes in metabolic rhythms. These data provide the basis to understand how people that are exposed to irregular light schedules might develop metabolic diseases.

Chapter 3 describes the influence of leptin, a hormone that inhibits food intake, in regulating circadian temperature rhythms in rats. We observed that administering small doses of this hormone modifies the body temperature of the animals depending on the time of administration. In addition, the hypothalamus responds differently to the hormone depending on the time of the day. We also provide evidence that leptin might be important to maintain the circadian temperature rhythm in satiety conditions. These data provide knowledge regarding the molecules and brain regions implicated in regulating circadian temperature rhythms in fasting conditions and obesity.

Finally, Chapter 4 reprises the above to propose the mechanisms behind glucose intolerance and fat accumulation in several conditions of circadian disruption, such as constant light, feeding during the resting phase, and the skeleton photoperiod. We also propose potential ways to restore metabolic functions under these conditions. This thesis gathers information that aids in understanding the development of metabolic diseases as a consequence of the nocturnal lifestyle that prevails among a large part of the population.

Content

Chapter 1. General Introduction

1.1 Energy regulation in mammals

1.1.1 Overview of energy metabolism

One of the most important functions of living beings is their capacity to store and transform energy, i.e., energy metabolism. In mammals, energy is mainly produced by the oxidation of glucose or fatty acids acquired through the ingestion of complex molecules, which are then digested and distributed throughout the body. In addition, mammals have a wide array of mechanisms that balance the consumption and storage of energy depending on the changing environment. In this sense, organs such as the liver and adipose tissue are essential for storing energetic molecules. Among its many functions, the liver provides the rest of the organism with a constant glucose supply; it produces glucose (gluconeogenesis) and stores it after feeding in the form of glycogen. The liver can also produce ketone bodies, which serve as a fuel source for the brain in conditions of low glucose supply. Moreover, the white adipose tissue stores fatty acids and glycerol in the form of triglycerides, which can be hydrolyzed and released into the circulation to supply other organs with energy substrates. Many other organs, such as the gut, the pancreas, the adrenal gland, etc., also play a role in regulating energy metabolism. Coordination of these organs' functions is essential to maintaining metabolic homeostasis. This coordination is mainly carried out by hormonal and neural systems, orchestrated by the brain and, specifically, the hypothalamus.

1.1.2 The role of the hypothalamus in metabolism.

The role of the hypothalamus in metabolism is clearly exemplified in early lesion studies. Lesioning rats' lateral nucleus of the hypothalamus profoundly inhibited food intake, making rats extremely lean and eventually leading to death (Broberger, 1998). In contrast, lesioning the ventromedial nucleus of the hypothalamus produced overfeeding in animals, leading to obesity (King, 2006). More recent studies have provided a more integrated view and implicated many different hypothalamic and brainstem nuclei in regulating food intake. Notably, the hypothalamus regulates food intake, and the same hypothalamic feeding circuits also regulate energy expenditure, core body temperature, and glucose uptake, as will be discussed below.

1.1.3 Hypothalamic feeding circuits

The hypothalamic circuit regulating feeding behavior mainly comprises the lateral hypothalamus (LH), the ventromedial nucleus (VMH), the arcuate nucleus (ARC), the paraventricular nucleus (PVN), and the suprachiasmatic nucleus (SCN).

The arcuate nucleus is at the center of this circuit for several reasons. First, it has direct access to blood-borne molecules (Rodríguez, Blázquez, & Guerra, 2010). The ARC can detect different circulating metabolites that signal the organism's metabolic state, such as glucose, fatty acids, postprandial hormones like insulin or leptin, and hunger-related hormones like ghrelin. Second, it has two antagonistic populations of neurons, which, once stimulated, can promote or inhibit feeding behavior. The former, orexigenic neurons, express neuropeptide Y (NPY) and agouti-related

peptide (AgRP). In contrast, anorexigenic neurons express proopiomelanocortin (POMC), a peptide that can be split into smaller anorexigenic peptides like α-melanocyte-stimulating hormone (α-MSH) (Belgardt, Okamura, & Brüning, 2009).

Both POMC and AgRP neurons in the ARC communicate with the PVN, a nucleus that is considered the output of the hypothalamus (R. M. Buijs, Escobar, & Swaab, 2013). This nucleus projects to other brainstem regions, such as the parabrachial nucleus (PBN), to regulate feeding behavior (Li et al., 2019; Shah et al., 2014). In addition, the PVN has pre-autonomic neurons that influence the metabolic function of peripheral organs. For example, the PVN projects to the liver via the spinal cord; activating this pathway promotes gluconeogenesis (Yi et al., 2012).

Other hypothalamic nuclei regulate feeding behavior through their projections to the PVN or the ARC. The LH, for example, projects to the PVN, and activating this pathway promotes feeding behavior (Wu et al., 2015) . Conversely, the projections from the VMH to the ARC inhibit feeding behavior (Sternson, Shepherd, & Friedman, 2005). The VMH also projects to other preautonomic regions of the brainstem that project to peripheral organs to regulate seveghreliral metabolic processes, like heat production by the brown adipose tissue (Martínez-Sánchez et al., 2017; Orozco-Solís et al., 2016) and glucose utilization in the periphery (Coutinho et al., 2017; Fujikawa et al., 2016; Shiuchi et al., 2009). In this way, the hypothalamus integrates multiple metabolic processes including feeding.

Because the ARC has direct access to blood-borne molecules, the hypothalamus orchestrates feeding behavior depending on the organism's needs. For example, low

energy stores promote the release of acylated ghrelin from the stomach; this signal is detected in the ARC to promote feeding (Mondal et al., 2005; Wang et al., 2014). Importantly, feeding behavior is not only triggered by a lowering in storage molecules, but it has a clear circadian rhythm, which is controlled by another hypothalamic area, the suprachiasmatic nucleus (SCN) (Challet, 2019; Yi et al., 2006).

1.2 The circadian component of metabolism

1.2.1 Circadian rhythms and the SCN

Circadian rhythms are oscillations in behavior and physiology that are completed in approximately 24 hours, i.e., one day. These rhythms are controlled by the suprachiasmatic nucleus (SCN) of the hypothalamus, considered the master clock of the body (Buijs et al., 2019). At the cellular level, circadian oscillations are supported by a molecular network of rhythmically expressed genes —called clock genes—which form a feedback loop of gene transcription and translation. The core clock consists of the proteins CLOCK, BMAL PER and CRY. CLOCK and BMAL form a dimer that promotes the transcription of *per* and *cry*. As *per* and *cry* are expressed, they dimerize to form a complex that represses CLOCK and BMAL, inhibiting their own transcription (Hastings, Maywood, & Brancaccio, 2019). In SCN neurons, circadian oscillations in clock genes translate into circadian oscillations in neuronal activity. Furthermore, intercellular communication within the SCN ensures that these circadian oscillations are coupled among neurons, producing a robust endogenous oscillation of the SCN as a whole (Hastings, Brancaccio, & Maywood, 2014). In addition to its capacity to sustain endogenous oscillations, the SCN

receives information from intrinsically photosensitive retinal ganglion cells (ipRGCs) that detect environmental light. In this way, light serves as the SCN's main synchronizer, ensuring that circadian rhythms are in phase with the cycling environmental conditions (Golombek & Rosenstein 2010; Buijs, Escobar, & Swaab 2013). Therefore, integrating its endogenous oscillations with the changes in the environment, the SCN imposes the circadian time on other brain regions and peripheral organs, generating whole-body circadian rhythms.

1.2.2 The circadian rhythm in feeding

All organisms show circadian oscillations in behaviors like feeding and sleeping and in many physiological variables, like circulating glucose. In rats, for example, most of the food consumption (approximately 80%) is associated with the period when animals are active (night) (Strubbe & Van Dijk, 2002). These rhythms exploit the capacity of the organism to store energy reserves and optimize energy use for the time when it is most needed. Because the SCN regulates all circadian rhythms, lesioning the SCN results in the loss of all rhythms, including feeding patterns (Nagai et al., 1978; Stoynev, Ikonomov, & Usunoff, 1982).

1.2.3 Basal circadian oscillations in metabolites

Since feeding behavior oscillates in a circadian manner, it seems logical that circulating metabolites show a day-night pattern in consequence. Nevertheless, these oscillations are not just a reflection of the postprandial state, instead, they are directly controlled by the circadian system (Kalsbeek et al., 2011). For example, glucose oscillates in the blood in a circadian fashion, even during fasting conditions

or when animals eat several times throughout the day (Escobar et al., 1998; La Fleur, et al., 1999) Furthermore, SCN lesioning also results in a loss of the blood glucose rhythm (La Fleur et al., 1999), sustaining the role of the SCN in the circadian regulation of glycemia.

1.2.4 Circadian postprandial responses

In addition, the postprandial responses to food vary depending on the time of the day. Insulin sensitivity is higher when food is expected, i.e., in the active phase (la Fleur, 2003), and the capture of fatty acids by the muscle is also higher at this timepoint (Morán-Ramos et al. 2017). These differences indicate that the hypothalamus primes metabolic organs to process energetic molecules differently depending on the time of the day, which has metabolic repercussions when people eat when they normally should not, as will be discussed later.

1.2.5 Communication of the hypothalamus with sensory systems

The hypothalamus' capacity to regulate peripheral organ function according to the time of the day arises from the input it receives from sensory systems. As previously mentioned, it receives input from the retina, providing information about cycling light-dark conditions. The hypothalamus also receives input from the blood via the circumventricular organs (CVOs); specialized sensory centers devoid of a blood-brain barrier where circulating molecules and ions are detected. These areas include the *organum vasculosum laminae terminalis* (OVLT), the subfornical organ (SFO), and the median eminence (ME); they project to multiple hypothalamic areas (Cottrell & Ferguson, 2004; Duvernoy & Risold, 2007; Sisó, Jeffrey, & González, 2010). As

previously mentioned, the ARC also detects blood-borne information, and it is considered to have similar sensory functions to the CVOs (Ciofi, 2011; Morita & Miyata, 2013). Many different substances can be sensed in the CVOs, including glucose, fatty acids, and leptin, a hormone that plays an important role in energy balance (further discussed in Chapter 4). Therefore, sensory information conveyed from the CVOs to the hypothalamus is essential for integrating the organism's metabolic state.

In addition to these sensory pathways, the hypothalamus receives inputs from spinal and vagal nerves that detect metabolically relevant signals within peripheral organs. Information from spinal and vagal sensory nerves reaches the hypothalamus through multi-synaptic pathways (Buijs et al. 2019). For example, GLP-1, a molecule secreted in the stomach after feeding, can be detected by the vagus nerve and signal to the hypothalamus to inhibit food intake and modulate glycemia (Krieger, Langhans, & Lee, 2015). In this sense, the hypothalamus integrates information from multiple sensory systems to regulate metabolism.

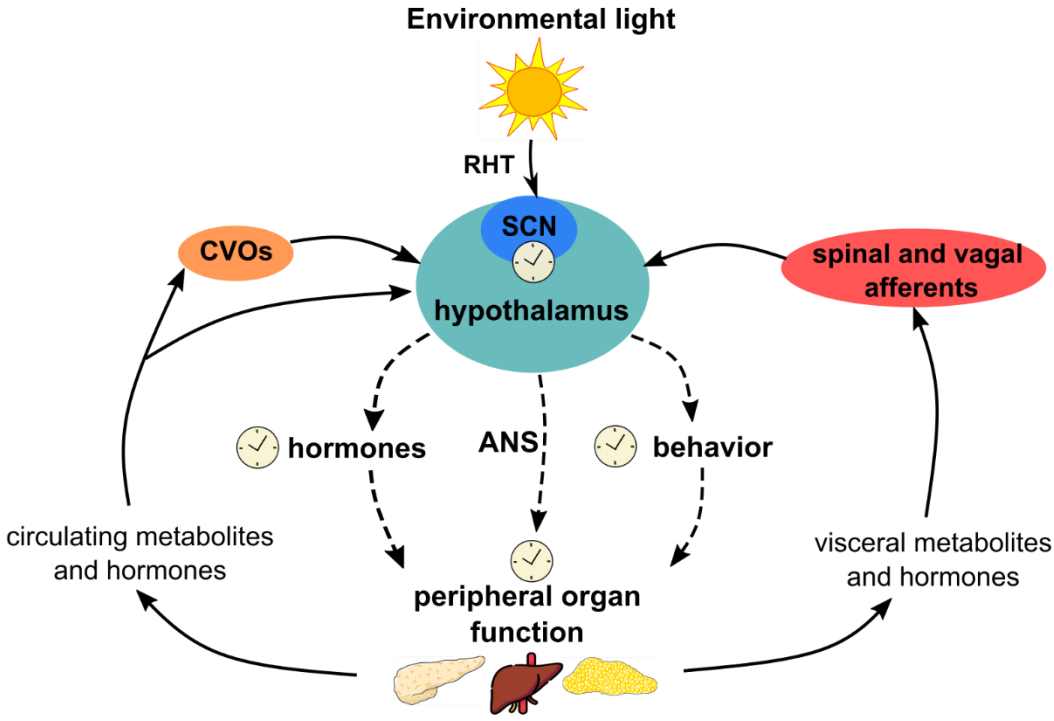

Figure 1.1. The hypothalamus as the integrator of the circadian system. The SCN, located in the hypothalamus, has an endogenous circadian clock that oscillates in cycles of approximately 24-hours. This clock is adjusted to the environmental daily light cycles through the information received from the retino-hypothalamic tract (RHT). In addition, the hypothalamus receives input from sensory systems like the circumventricular organs (CVOs) and spinal and vagal afferents and is capable of directly sensing circulating molecules through the hypothalamic arcuate nucleus. The integration of this information generates circadian rhythms in peripheral organs through hormones (e.g. corticosterone), the autonomic nervous system (ANS), and behavior (such as feeding). Finally, the different hormones and metabolites produced in the periphery serve as feedback signals, so the hypothalamus can adjust circadian physiology according to the internal state of the organism.

1.2.6 Communication of the hypothalamus with endocrine systems and the ANS

Once the hypothalamus has received information regarding the organism's metabolic state, it changes its output to adjust physiology accordingly. This regulation is carried out by hormonal and nervous systems (Buijs et al. 2019).

The hypothalamus regulates many hormonal systems through different pathways; one example is the neuroendocrine pathways known as hypothalamus-pituitary-organ (HPX) axes. For this, several hypothalamic nuclei secrete neuropeptides into the portal pituitary system, which reach the anterior pituitary gland to regulate the secretion of pituitary hormones. Pituitary hormones are then released into the circulation and reach their target areas, which produce different hormones that affect whole-body metabolism (Clarke, 2015). For example, the PVN has a subset of neurons that release corticotropin-releasing hormone (CRH) into the portal system. CRH stimulates corticotrope cells of the anterior pituitary to secrete adrenocorticotropin hormone (ACTH), which is released from the circulation and reaches the adrenal cortex, promoting corticosterone release; this is known as the hypothalamus-pituitary-adrenal (HPA) axis. Similarly, the hypothalamus regulates the secretion of thyroid hormones, and gonadal hormones, among others.

Importantly, many of these hormones oscillate in a circadian manner. These circadian oscillations are fine-tuned thanks to the hypothalamic control of the autonomic nervous system (ANS), which “prepares” the organs for the coming hormones. For example, corticosterone increases slightly before waking; this increase is necessary to energetically prepare the organism for the activity period

(Kalsbeek et al. 2006). Therefore, in preparation for the activity period, the hypothalamus activates the sympathetic branch of the ANS that innervates the adrenal gland; this allows the adrenal gland to be more responsive to ACTH and thus secrete corticosterone more efficiently (Buijs 1999; Leon-Mercado et al. 2017). Therefore, the ANS provides another communication pathway between the hypothalamus and the periphery to regulate circadian physiology.

The ANS is subdivided into two branches distinguished by their anatomy and function: the sympathetic system (SNS) and the parasympathetic system (PSNS). The SNS arises from motor neurons located in the intermediolateral column of the spinal cord (IML) projecting to different ganglia in the abdominal cavity. In turn, these ganglia innervate organs using noradrenaline as a neurotransmitter. Regarding metabolic regulation, the activation of the SNS has been generally related to a catabolic state, e.g., gluconeogenesis in the liver, burning of fatty acids by the organs, and inhibition of insulin secretion by the pancreas. In contrast, the PSNS arises from the dorso-motor nucleus of the vagus (DMV) of the brainstem, which branches in the abdominal cavity to innervate different organs using, among others, acetylcholine as a neurotransmitter. The activation of the PSNS is related to digestive and postprandial functions, e.g., glycogen synthesis by the liver and insulin production by the pancreas (Marino, Xu, & Hill, 2011; Wehrwein, Orer, & Barman, 2016). Since both branches of the ANS have seemingly opposing functions, metabolic homeostasis results from the balance of the activity of these two branches (Buijs, 2013). In contrast, different conditions that promote an unbalanced ANS are

related to metabolic disease (Kreier et al., 2003), emphasizing the importance of autonomic balance for health.

1.2.7 Metabolic rhythms in tissues

As discussed, the hypothalamus communicates with metabolically relevant organs via multiple endocrine and neural pathways to maintain circadian oscillations following the changing environment. In addition, cells throughout the body express the molecular clock machinery, although clock gene oscillations in peripheral tissues are not self-sustained like in the SCN (Kaeffer & Pardini, 2005). Thus, daily hormonal and neural signals conveyed by the SCN can synchronize clock gene oscillations in the tissues and thus regulate cellular functions in a circadian manner. However, some clock genes in peripheral organs can also be synchronized to food-related cues (Mukherji et al., 2015). This is especially relevant when feeding behavior occurs in contradiction to the expected time (i.e., eating during the resting phase), which will be further discussed below.

1.3 Circadian disruption and metabolic disease

1.3.1 Shift work

As reviewed above, the hypothalamus plays a central role in metabolic regulation by adjusting behavior and physiology to the time of the day. In contrast, a major part of the modern human population displays behaviors that do not match what their circadian system dictates. One clear example is shift workers, who change their regular activity and feeding patterns depending on their working hours. In many cases, shift workers need to change their activity period to the night, when they

should be sleeping. Many epidemiological studies have shown that this condition increases the risk for different diseases, including dyslipidemias, hypertension, and obesity, all hallmarks of metabolic syndrome (Bonham, Bonell, & Huggins, 2016; Sun et al., 2018). This increased risk may be explained by different factors present in night workers, such as eating more calories, feeding at night, and sleeping less. Several animal models have been developed to dissect the role of each of these factors in metabolic disease (Opperhuizen et al., 2015), which will be addressed below.

1.3.2 Feeding schedules

Food intake is typically associated with the active phase of organisms, in humans, corresponding to the day. Furthermore, how organisms manage food-related components also depends on the time of the day; for example, hyperglycemia lasts longer after an evening meal than the same morning meal (Van Cauter, Polonsky, & Scheen, 1997). Since shift workers eat during the period when they normally wouldn't (reverse feeding), two situations occur: 1) food-related components like glucose represent an unexpected signal to the hypothalamus and other metabolic organs, and 2) the longer presence of circulating metabolites represents a persistent signal to the hypothalamus and other organs. These two cases could lead to metabolic disease, but the exact mechanisms remain unknown.

Several animal studies have shown that feeding-related components synchronize peripheral organs like the liver and muscle (Opperhuizen et al., 2016; Salgado-Delgado et al., 2013). In this sense, reverse feeding decouples clock gene

oscillations in peripheral organs from the central oscillator, the SCN, which is mainly synchronized to the light/dark cycle (Mukherji et al., 2015). This has led to the hypothesis that the metabolic disease observed in reverse feeding is associated with internal desynchronization, where circadian oscillators are not synchronized amongst each other (Oosterman, Wopereis, & Kalsbeek, 2020). Although this hypothesis has been widely supported, it is still not clear whether internal desynchronization leads to disease and how that process occurs. One study challenged this hypothesis by generating genetically modified mice that had a different oscillation period in the SCN (approximately 27 hours) than in the rest of the organs (24 hours) (van der Vinne, Swoap, Vajtay, & Weaver, 2018). These mice had an uncoupling of approximately 6 hours between central and peripheral clock gene oscillations; nevertheless, they did not show any metabolic issues. Moreover, another study questioned the capacity of food-related cues to synchronize circadian rhythms in organs when food intake occurs in natural patterns, i.e. not restricted to a period of 12 hours or less, commonly used in reverse-feeding studies (Xie et al., 2020). These studies raise the question of whether indeed internal desynchrony promotes metabolic issues in reverse feeding or other mechanisms are involved. Chapters 2 and 4 of this thesis will discuss this matter extensively.

1.3.3 Constant light

Light is a crucial synchronizing signal for the circadian system. The SCN receives direct innervation from light-sensitive cells in the retina and adjusts its endogenous rhythmicity according to the external light-dark cycle (Golombek & Rosenstein 2010; Buijs, Escobar, & Swaab 2013). Since shiftwork increases exposure to light at night,

light would activate the SCN in a period where it should not be active. Thus, light at night impedes the SCN's endogenous synchronization and, consequently, affects its output to the rest of the body.

Several animal studies have shown that constant light desynchronizes clock genes in the SCN and the periphery (Challet et al., 2003; Yamamuro et al., 2020). In the long term (4 weeks), this affects organ function, promoting fat accumulation and glucose intolerance (Coomans et al., 2013; Fonken & Nelson, 2014). Importantly, the effects of constant light on metabolism can be diminished by exogenously reestablishing some rhythmic hormonal signals such as corticosterone and melatonin, along with restricting food to a 12-hour period (Báez-Ruiz et al., 2017). This reinforces the idea that reestablishing timely hypothalamic signals can improve synchronization among the periphery and thus partially reestablish metabolic homeostasis.

One issue that has not been addressed sufficiently is how light at night could interact with conflicting feeding schedules. In diurnal humans, feeding, activity, and light usually coincide in the same period — the daytime. However, in shift workers, these variables might not fully coincide, or they might do so in the nighttime, a period where the circadian system would not anticipate light and food. Therefore, the contribution of light to the metabolic impact of reverse feeding will be fully addressed in Chapter 2 of this thesis.

1.4 Aim and scope

The present thesis explores the relationship between the circadian system and metabolism. Since different studies have already provided evidence to link these two systems, the current work is focused on the mechanisms that underlie this relationship. In this sense, different hypotheses will be presented along with experimental data to support them.

Chapter 2 explores a previously overlooked relationship in the field of chrono-disruption in rodents: the conflict between light and food. On the one hand, feeding during the day is associated with metabolic disease (Salgado-Delgado et al., 2013); on the other, light exposure can acutely change metabolism (Opperhuizen et al., 2017). Thus, we aimed to determine if these two conflicting signals could be involved in the appearance of metabolic disease by the experimental approach of a skeleton photoperiod. This chapter has been published in the Journal *Advanced Biology*.

Chapter 3 explores the relationship between leptin and the circadian system. Leptin is an adipokine, traditionally related to the inhibition of feeding behavior. However, we present evidence beyond its traditional role regarding the circadian temperature regulation. We also discuss some of the implications of these data for circadian physiology and obesity.

Chapter 4 includes a general conclusion and final remarks regarding the relationship between the circadian system and metabolism in the context of metabolic disease.

In addition, other publications generated during my doctoral studies are attached at the end of the thesis for reference.

Chapter 2. Small shifts in daily rhythms are associated with adiposity and glucose intolerance in rats

Published as:

Méndez-Hernández, R., Rumanova V., Guzmán-Ruiz, M.A., Foppen, E., Moreno-Morton, R., Hurtado-Alvarado, G., Escobar, C., Kalsbeek, A., & Buijs, R.M. (2022). Minor Changes in Daily Rhythms Induced by a Skeleton Photoperiod Are Associated with Increased Adiposity and Glucose Intolerance. Advanced Biology Jul 11:e2200116. https://doi.org/10.1002/adbi.202200116 Epub ahead of print.

2.1 Abstract

Eating during the rest phase is associated with metabolic syndrome, proposed to result from a conflict between food consumption and the energy-saving state imposed by the circadian system. However, in nocturnal rodents, eating during the rest phase (day-feeding, DF) also implies food intake during light exposure. To investigate whether light exposure contributes to DF-induced metabolic impairments, animals receive food during the subjective day without light. A skeleton photoperiod (SP) is used to entrain rats to a 12:12 cycle with two short light pulses framing the subjective day. DF-induced adiposity is prevented by SP, suggesting that the conflict between light and feeding stimulates fat accumulation. However, all animals under SP conditions develop glucose intolerance regardless of their feeding schedule. Moreover, animals under SP with ad libitum or night-feeding have increased adiposity. SP animals show a delayed onset of the daily rise in body

temperature and energy expenditure and shorter duration of nighttime activity, which might contribute to the metabolic disturbances. These data emphasize that metabolic homeostasis can only be achieved when all daily cycling variables are synchronized. Even small shifts in the alignment of different metabolic rhythms, such as those induced by SP, may predispose individuals to metabolic disease.

2.2 Introduction

Metabolic syndrome is a complex, multifactorial disease that affects a growing number of people worldwide. The underlying causes are related to modern lifestyle, including lack of physical activity, consumption of highly caloric western diets, and disruption of the circadian system. Shift workers, who decouple their behavior from the external circadian cycles, have an increased risk for obesity, dyslipidemias, and impaired glucose regulation, all associated with diabetes and metabolic syndrome. In shift workers, at least two factors represent a risk for circadian disruption: exposure to light at night and food intake during the rest phase (Oosterman et al., 2020; Pickel & Sung, 2020).

These two factors can be modeled with rodents in a laboratory setting (Morán-Ramos et al., 2016; Opperhuizen et al., 2015). Rats under constant light display increased adiposity and glucose intolerance after several weeks (Báez-Ruiz et al., 2017; Guerrero-Vargas et al., 2017; Wideman & Murphy, 2009) and even an acute light pulse in the middle of the night can change glucose tolerance (Opperhuizen et al., 2017). Since light at night disturbs the normal functioning of the brain master clock, the suprachiasmatic nucleus (SCN), the altered metabolism might be caused

by light-induced changes in the output of the SCN to peripheral organs, ultimately resulting in circadian desynchrony among them.

Rodents that are forced to eat during their rest phase (day-feeding, DF) also develop metabolic impairments. DF does not only cause a misalignment between the central brain clock and the peripheral clocks, but also desynchronizes clock gene rhythms among different organs and leads to increased adiposity and glucose intolerance (Bechtold, Gibbs, & Loudon, 2010; Salgado-Delgado et al., 2008; Salgado-Delgado et al., 2013; Wang et al., 2017) . It has been suggested that DF provokes an internal conflict between food consumption and the energy-saving state imposed by the SCN. However, in nocturnal rodents, DF also implies food intake during light exposure, which may exert metabolic changes *per se*. From this perspective, the metabolic impairments derived from DF could also arise from a conflict between light exposure and food intake, which normally occur separately in nocturnal rodents.

To test the hypothesis that metabolic disease arises from the conflict between light and feeding, we exposed animals to DF in the absence of light. To prevent animals from free-running under constant darkness, we used a skeleton photoperiod (SP), where animals are maintained in complete darkness except for two 30-min light pulses indicating the beginning and end of the subjective day (Pittendrigh & Daan, 1976). The SP is supposed to mimic an animal's natural behavior in the burrow, i.e., emerging from the darkness of the burrow to sample the presence or absence of environmental light. It is well documented that SP entrains locomotor activity rhythms in several rodent species (Daan & Pittendrigh, 1976; Flôres & Oda, 2020; Strubbe, Spiteri, & Alingh Prins, 1986).

Under SP or a regular 12:12 light-dark photoperiod (LD), we restricted food intake to the subjective day (DF), subjective night (NF), or allowed animals to eat ad libitum (AL). After four weeks, we assessed glucose tolerance, adiposity, and temperature rhythms. Since we found unexpected effects of the SP, we performed a second experiment to further evaluate these effects on metabolic rhythms under AL feeding conditions. Finally, we tested the metabolic effects of the lack of light during the day by exposing rats to constant darkness. Taken together, the present data suggest that metabolic impairments are not only induced by a complete reversal of the timing of food intake, but minor shifts in metabolic rhythms induced by an SP also cause metabolic disturbances.

2.3 Hypothesis

- The absence of light during day-feeding in rats will prevent the day-feeding-induced metabolic impairments.

2.4 Objectives

- Evaluate glucose tolerance and adiposity after four weeks under a skeleton or normal 12:12 photoperiod and three different feeding regimes: day feeding, night feeding or ad libitum feeding.

- Assess metabolic rhythms (temperature, energy expenditure, locomotor activity and respiratory exchange quotient) in rats exposed to a skeleton or normal photoperiod and ad libitum feeding.

- Evaluate the effect of total lack of light on glucose tolerance and adiposity.

2.5 Methods

2.5.1 Animals.

This study was conducted in accordance with the guidelines and requirements of the World Medical Association Declaration of Helsinki (1964), approved by the local ethics committee, and performed following the guidelines on animal experimentation of the Mexican Official Norm NOM-062-ZOO-1999 and the Netherlands Institute for Neuroscience (NIN) to minimize animal suffering. Male Wistar rats of 270-300 g and approximately nine weeks of age from the *Unidad Académica Bioterio*, Faculty of Medicine (Universidad Nacional Autonoma de Mexico, Mexico City) (Experiment 1 and 3), and from Charles River (Sulzfeld, Germany) (Experiment 2) were housed individually at room temperature (20-23 °C) under a standard 12:12 light-dark cycle, (lights on at 7:00 am) with water and food ad libitum, unless stated otherwise.

2.5.2. Experiment 1. Day-feeding in the absence of light and the use of the skeleton photoperiod.

To evaluate the contribution of light to the metabolic impairments in DF, we subjected rats to a standard 12:12 photoperiod (LD), or a skeleton photoperiod (SP), which consisted in exposing animals to complete darkness except for two 30-min light pulses at the beginning and end of the subjective day. Standard laboratory fluorescent lighting (Clough, 1982) of approximately 380 lux was used for both LD and SP groups. After one week in the respective photoperiod (baseline, week 0), both groups were subjected to food restriction during the day (DF) for 11 hours (from ZT 0.5 to ZT 11.5). In addition, we used four other control groups with ad libitum

feeding (AL) or night-restricted feeding (NF) under LD or SP conditions. NF animals were also subjected to food restriction for 11 hours in the subjective night (from ZT 12.5 to ZT 23.5). This experimental design gave a total of 6 experimental groups: LD-AL, SP-AL, LD-DF, SP-DF, LD-NF, and SP-NF (Figure 1.1a). Each group included eight animals (n = 8). Rats were subjected to the respective photoperiod for one week (baseline, week 0) and then placed under AL, DF or NF for 11 hours (ZT 12.5 to ZT 23.5) for four weeks. Food intake and body weight were measured weekly on the same day, during the evening light pulse. Four weeks after food restriction or ad libitum feeding, animals underwent a glucose tolerance test. One week later, animals were euthanized to collect their retroperitoneal (pWAT), epididymal (eWAT), and inguinal subcutaneous (scWAT) fat.

2.5.2.1 Glucose tolerance test.

Glucose tolerance tests were performed after four weeks of the experiment. Animals were transferred to a separate experimental room to avoid stressful conditions and fasted for 12 hours overnight. At ZT 0, they were administered intraperitoneally with 1 mg kg^{-1} of glucose (1 mL), and blood was sampled via tail puncture at 0, 15, 30, 60, and 120 minutes after the injection. Glucose was measured with the commercial glucometer Accucheck®.

2.5.2.2 Euthanasia and tissue collection.

Rats were sacrificed using an overdose of sodium pentobarbital (215 mg kg^{-1}). Retroperitoneal, epididymal, and subcutaneous inguinal fat pads were dissected and

weighed. Fat mass was calculated as the percentage with respect to total body weight.

2.5.2.3. Temperature measurements

To monitor core body temperature, rats were implanted with sterilized intraperitoneal temperature sensors (iButton Sensor- Temperature Logger; Maxim Integrated Products), which collected temperature data every 30 min. Animals were allowed to recover for a week before starting experimental procedures. Incomplete or failed temperature recordings were not considered for the analysis. Therefore, the sample sizes for the temperature data are the following: LD-AL, n = 5; SP-AL, n = 6; LD-DF, n = 6; SP-DF, n = 5; LD-NF, n = 6; SP-NF, n = 7.

2.5.3 Experiment 2. Skeleton photoperiod and metabolic rhythms.

To further evaluate how SP modifies metabolic rhythms, we performed indirect calorimetry analysis on two groups of animals subjected to either LD (n = 6) or SP (n = 6) and ad libitum feeding. These animals were first subjected to one week of LD (week 0) to determine their baseline measures of food intake, locomotor activity, energy expenditure (EE), and respiratory exchange ratio (RER). Then, they were maintained in LD or transferred to SP, and the same measures were obtained for weeks 2 and 4 after placement in the respective photoperiod. Total body mass and fat mass were measured using EchoMRI. These measurements were performed weekly (on the same day of the week) during the daytime.

2.5.3.1 Indirect calorimetry.

Animals were placed in metabolic cages (TSE systems) that allowed the continuous monitoring of food intake, locomotor activity, oxygen consumption, and carbon dioxide production. Energy expenditure and RER measurements were obtained from the TSE system.

2.5.4 Experiment 3. Metabolic parameters after four weeks of constant darkness.

To determine if the metabolic issues in SP animals were due to the absence of light, we exposed animals to constant darkness for four weeks (DD-AL group). Food intake and body weight were measured weekly under dim red lighting (2 lux); the red incandescent lamp was only turned on briefly during the measurement, which was performed at the end of the subjective day (ZT11). Four weeks later, animals underwent a glucose tolerance test. One week later, animals were euthanized to measure their retroperitoneal (pWAT), epididymal (eWAT), and inguinal subcutaneous (scWAT) fat mass.

2.5.5 Data analysis

2.5.5.1 Cosinor analysis

Cosinor analysis was performed with Python, using the code available at https://github.com/rodrigo-moreno/Cosinor_Analysis. For each variable (Experiment 1, temperature; Experiment 2, activity, food intake, energy expenditure and RER), we calculated the 3-day average of each time point for each animal, considering three days where the animals were left undisturbed. The data was adjusted to a cosine curve; adjustment to the curve (r^2), amplitude, mesor, and acrophase were

calculated for each variable. In addition, we performed a serial analysis using the Heaviside function as described in Díez-Noguera, 2013 (Díez-Noguera, 2013) to calculate the onset and offset of all variables. Briefly, this procedure estimates the timepoint at which the values of the measured variable surpass the daily mean (onset) or decrease below the daily mean (offset). Then, the alpha was calculated by subtracting offset-onset values.

2.5.5.2 Statistical analysis

All statistical analyses were performed with GraphPad Prism 5 software. Results are expressed as the mean ± the standard error of the mean (S.E.M.) unless stated otherwise. For all statistical analyses, significance was set at an alpha of 0.05 or lower.

For experiment 1, the data was tested for normality using the Kolmogorov-Smirnov test and for homogeneity of variances using the Brown-Forsythe test. If the data had a normal distribution and homogeneity of variances, two-way ANOVAs were performed (*Feeding* × *Photoperiod*). When significant effects were found, Dunnet's test was used to compare each group against the LD-AL control. The data regarding initial weight and food intake (Figure S1) were analyzed with a one-way ANOVA, considering that the food restriction had not been implemented yet. For the GTT (Figure 2.2d-i, 7b), the difference between each timepoint and the basal glucose level was plotted, and the area under the curve (AUC) was calculated for each animal considering all peaks above the baseline. Since the values for the temperature onset, offset, acrophase, and adjustment to the cosine curve did not

show homogeneity of variances (Figure 2.3g,h, Table S1), two-way ANOVAs were not performed in these datasets.

For experiments 2 and 3, the data was tested for normality using the Kolmogorov-Smirnov test and for homogeneity of variances using the Brown-Forsythe test. Then, unpaired t-tests were performed. When the data showed significantly different variances, an unpaired t-test with Welch's correction was performed. For fat mass, body mass, daily food intake and daily activity (Figure 2.4a,b), a two-way ANOVA for repeated measures (*Group* × *Time*) was performed, followed by Sidak's test comparing both groups in each time point.

2.6 Results

2.6.1 The effect of day-feeding and skeleton photoperiod on adiposity, weight gain, and glucose tolerance.

Rats were subjected to either a skeleton photoperiod (SP) or a standard 12:12 light-dark cycle (LD). After one week in the photoperiod, rats were subdivided into ad libitum feeding (AL), day-feeding (DF), or night-feeding (NF) (Figure 2.1a). Four weeks after remaining in the corresponding feeding schedule, rats were subjected to a glucose tolerance test. Finally, the retroperitoneal (pWAT), epididymal (eWAT), and subcutaneous (scWAT) fat pads were dissected and measured.

Under AL conditions, rats exposed to the SP had higher scWAT (approximately 33% more) compared to their LD counterparts (Figure 2.1c). As expected, LD-DF had increased scWAT (approximately 41% more) compared to the LD-AL control (Figure 2.1c); but the SP-DF group, which was not exposed to light during the feeding period,

did not increase its adiposity (Figure 2.1b-d). The SP-NF group also displayed higher adiposity affecting the three fat pads (approximately 53% more for pWAT, 50% for eWAT, and 56% for scWAT) (Figure 2.1b-d), while the LD-NF group showed normal adiposity. The two-way ANOVA indicated a significant interaction of *Photoperiod* × *Feeding* for pWAT ($F_{(2,41)}$=5.862, P= 0.0058), eWAT ($F_{(2,41)}$=5.566, P=0.0073), and scWAT ($F_{(2,40)}$=9.582, P=0.0004).

The increased adiposity observed in SP-AL and SP-NF groups was not associated with body weight gain or weekly food intake (Figure 2.1e-f) since these two groups had similar measures to those of the LD-AL and LD-NF controls. Conversely, LD-DF rats, which showed increased adiposity, exhibited lower body weight gain compared to the LD-AL control group (Figure 2.1e). However, SP-DF rats had similar body weight gain to those in the other groups. Food intake in the last week of the experiment was significantly lower in the LD-DF (approximately 79% of the food consumed by the control group) and SP-DF groups (approximately 81% of the food consumed by the control group) compared to the LD-AL control (Figure 2.1f). The two-way ANOVA confirmed a significant effect of *Feeding* on weekly food intake ($F_{(2,37)}$=12.09, P<0.0001). In agreement with this, feed efficiency was lower in the LD-DF group compared to the LD-AL control (P=0.0106) (Figure 2.1g). Of note, body weight and food intake at the beginning of the experiment were similar among groups (Figure S1), indicating that the differences in body weight gain and food intake at the end of the experiment were associated with the food restriction.

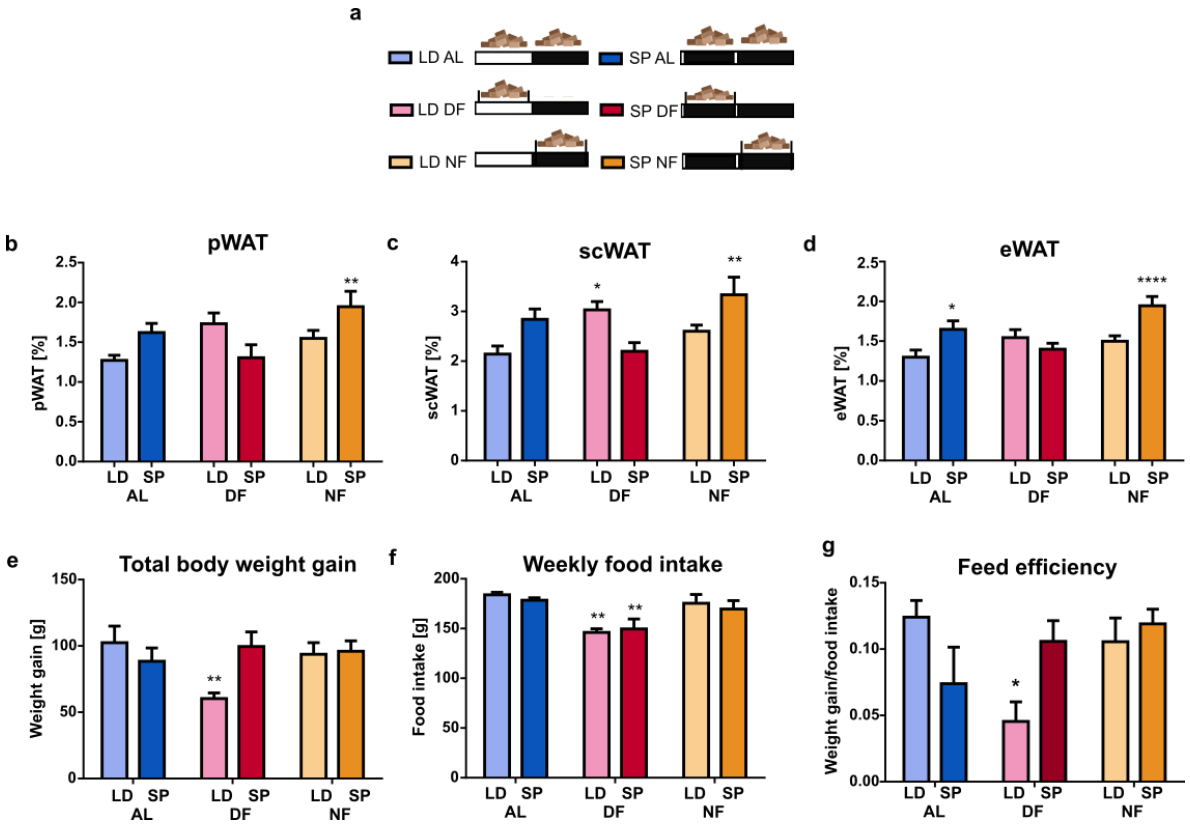

Figure 2.1. Skeleton photoperiod increased adiposity except during day-feeding. a) a) Schematic representation of the experimental design. Rats were assigned to a 12:12 light-dark cycle (LD) or a skeleton photoperiod (SP) with food available ad libitum (AL), restricted feeding for 11 hours during the subjective day (DF), or restricted feeding for 11 hours during the subjective night (NF). b-d) Percentage of retroperitoneal (pWAT, b), inguinal subcutaneous (scWAT, c), and epididymal (eWAT, d) adipose tissue respective to body weight after four weeks of experimental treatment. d) Body weight gain after the four weeks of experimental treatment. e) Total food intake of the last week of the experiment (Week 4). g) Feed efficiency (weight gain · food intake-1) in the last week of the experiment (Week 4). n =8 for all groups. Data are represented as mean ± S.E.M. *P<0.05, **P<0.01, ****P<0.0001 with respect to the LD-AL control group after Dunnet's test.

To further evaluate the metabolic state of the different groups, we subjected animals to a glucose tolerance test (GTT) at ZT 0 (Figure 2.2). Basal glucose levels did not differ significantly among groups (Figure 2.2a). All groups reached the highest blood glucose levels 15 min after the glucose injection (Figure 2.2d-i). The two-way ANOVA showed a significant effect of *Feeding* on circulating glucose at this timepoint ($F_{(2,40)}$=3.559, P=0.0378); the change in glucose levels in LD-DF animals at this time point was significantly higher than in the LD-AL group (approximately 89% more than the control group) (Figure 2.2b). In addition, the two-way ANOVA for the area under the curve (AUC) of the GTT (Figure 2.2c) confirmed a significant effect of *Photoperiod* on the AUC ($F_{(1,40)}$=4.507, P=0.040), indicating that the SP induces glucose intolerance independently of the feeding schedule.

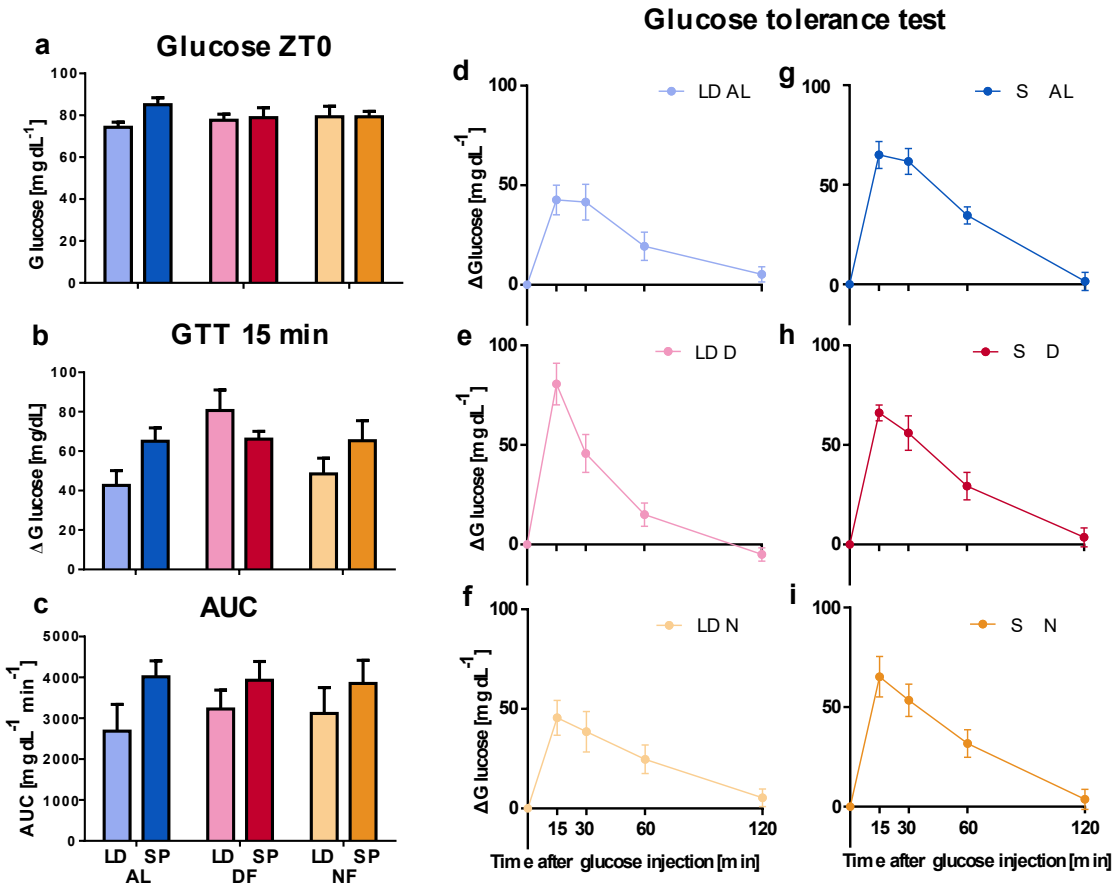

Figure 2.2 Day-feeding and skeleton photoperiod induce glucose intolerance. a) Circulating glucose levels at the start of the glucose tolerance test (GTT) performed at ZT 0 after four weeks of experimental treatment. b) Change in circulating glucose after 15 min of intraperitoneal glucose administration. c) Area under the curve (AUC) of the GTT for each experimental group. Two-way ANOVA (photoperiod × feeding) indicated a significant effect of the photoperiod on AUC. d-i) Change in circulating glucose after glucose injection in LD-AL (d), LD-DF (e), LD-NF (f), SP-AL (g), SP-DF (h), and SP-NF (i) groups. n =8 for all groups. Data are presented as mean ± S.E.M. **P<0.01 compared to the LD-AL control after Dunnet's test.

2.6.2 Both day-feeding and skeleton photoperiod change body temperature.

DF protocols are known to alter the daily temperature rhythm, shifting the temperature acrophase earlier into the day (Roberto Salgado-Delgado, Angeles-Castellanos, Saderi, Buijs, & Escobar, 2010). Thus, we analyzed the body temperature rhythm of all groups. All groups showed a rhythm in body temperature (Table S1, Figure 2.3a-f). The SP groups showed an apparent delay in the temperature onset (i.e. the moment when body temperature rises in preparation for the activity period) compared to their LD counterparts (Figure 2.3g). However, the values for the temperature onset had significantly different variances among groups as detected with the Brown-Forsythe test (P=0.0057) (Figure 2.3g), so further statistical analysis to compare the means was not performed. Similarly, the temperature offset (Figure 2.3h) and acrophase (Table S1) showed different variances among groups (offset, P=0.0022; acrophase, P=0.0004), indicating that the treatments affect intra-group variability in the temperature rhythm. Therefore, the description of these three variables (temperature onset, offset and acrophase) is merely qualitative.

In the LD-DF group, the temperature onset, offset, and acrophase were shifted to an earlier moment of the LD cycle (Figure 2.3b, Table S1), indicating that the overall temperature rhythm shifts towards the period of food intake. In contrast, the temperature onset and acrophase of SP-DF animals was closer to that of LD-AL animals (Figure 2.3e,g and Table S1), and only the temperature offset was shifted earlier like the LD-DF group (Figure 2.3h). Consequently, the duration of high temperature during the nighttime (alpha=offset-onset) was significantly shorter in

SP-DF compared to LD-AL (P<0.0001 after two-way ANOVA and Dunnet's test). A significantly shorter temperature alpha was also observed in SP-AL animals (P = 0.0265) (Figure 2.3i).

Taken together, these apparent changes in the temperature rhythms could mean that the SP affects metabolic rhythms *per se*, which is further described in the next section.

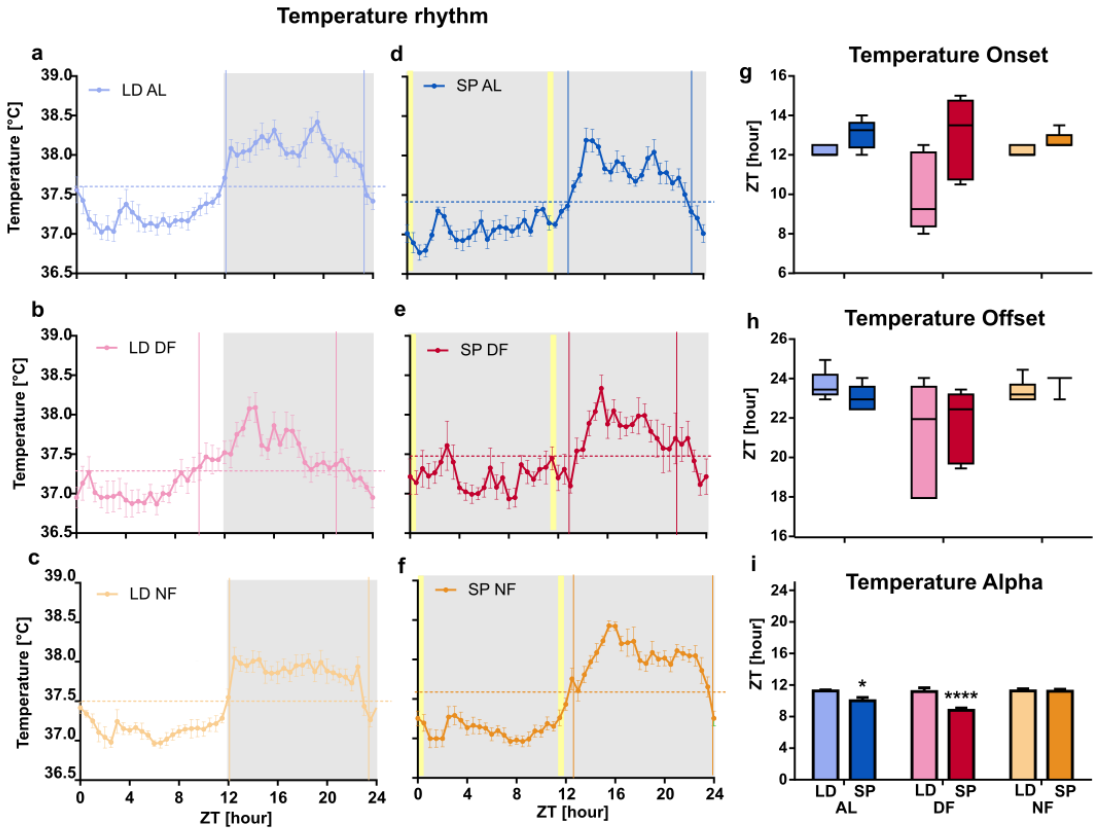

Figure 2.3. Day-feeding and Skeleton photoperiod change the temperature rhythm. a-f) Daily temperature rhythm of LD-AL (a), LD-DF (b), LD-NF (c), SP-AL (d), SP-DF (e), and SP-NF (f) animals, obtained as the average of 3 days of the last week of the experiment. Horizontal dotted lines indicate the overall daily average, and vertical lines indicate the onset and offset of the temperature rhythm. Shaded

areas indicate “lights-off,” and yellow lines represent light pulses. g-i) Temperature onset (g) offset (h), and alpha (i, offset-onset) during the last week of the experiment. LD-AL, n = 5; SP-AL, n = 6; LD-DF, n = 6; SP-DF, n = 5; LD-NF, n = 6; SP-NF, n = 7. The data in a-f and i are presented as mean ± S.E.M. *P<0.05 ****P<0.0001 compared to the LD-AL control after Dunnet’s multiple comparison test. The data in g and h are presented as box plots (median with minimal and maximal values), since the temperature onset and offset showed significantly different variances after Brown-Forsythe test (onset, P=0.0057; offset, P =0.0022).

2.6.3 Skeleton photoperiod changes metabolic rhythms in specific moments of the circadian cycle.

In the previous sections, we described that SP induces glucose intolerance and higher adiposity, suggesting that SP may promote an anabolic state. Since no previous study had reported metabolic effects of an SP, we performed a new experiment with LD and SP animals under ad libitum conditions to better understand these effects. We measured the fat mass and whole-body metabolic rhythms of LD and SP animals under AL feeding for 4 weeks. Confirming our previous results, SP animals significantly increased their fat mass in the fourth week in SP (Figure 2.4a). Two-way ANOVA for repeated measures showed a significant *Week* × *Group* interaction ($F_{(4,40)}$ = 4.396, P=0.0049). These changes were not accompanied by differences in total body mass, which remained similar between both groups throughout the experiment (Figure 2.4b).

Total food intake was not different between both groups (Figure 2.4c). However, food intake during the subjective day significantly increased after the second week in SP,

although the overall day-night difference in this behavior clearly remained (Figure 2.4c). Similarly, the amount of locomotor activity (as measured by infrared light beams) also increased during the subjective day in SP animals, but the total amount of locomotor activity did not differ from LD animals (Figure 2.4d).

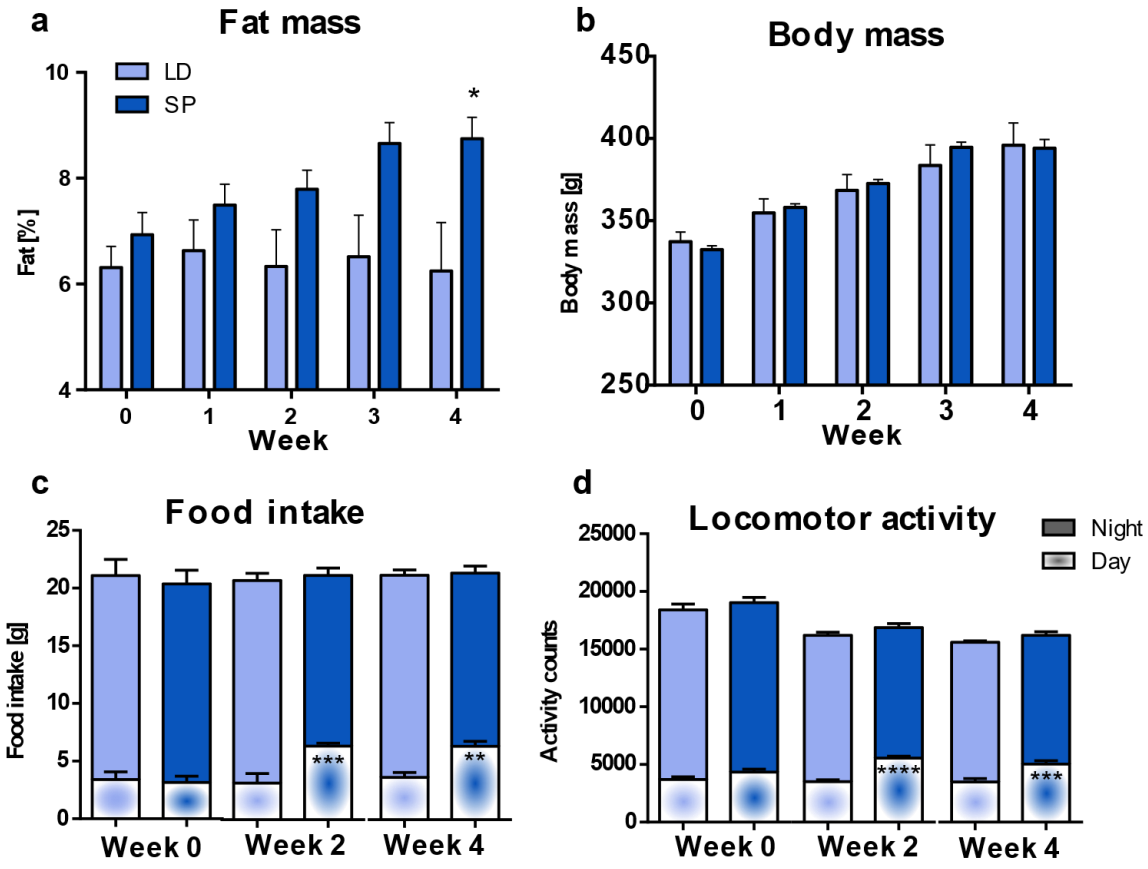

Figure 2.4. Skeleton photoperiod increases adiposity without changing total food intake or body mass in animals fed ad libitum. a) Percentage of fat mass with respect to body weight and b) Total body mass of LD and SP animals through the four weeks of experimental treatment. c) Average 24-hr food intake and d) locomotor activity of LD and SP animals in weeks 0 (baseline), 2, and 4, including day (light shade) and night (dark shade) intake and activity. n = 6 in both groups.

Data are presented as mean ± S.E.M. * P<0.05, **P<0.01, ***P<0.001, ****P<0.0001 with respect to the LD control group in the same week.

By analyzing locomotor activity every 30 min, we observed that SP animals increased their activity in the early morning (ZT 2-ZT 3.5) and decreased it at the transition from the subjective day to the subjective night (ZT 12) (Figure 2.5a,e). Similarly, food intake showed several bouts during the day and decreased at the end of the night (ZT 23) (Fig 5b,f).

Indirect calorimetry analysis showed that 24-h energy expenditure (EE) of SP animals was not significantly different from the LD control (Figure 2.5c,g), but the amplitude of the rhythm was significantly lower (Table S2). Conversely, SP animals had a lower respiratory exchange ratio (RER) enduring the major part of the active period and the first half of the resting period (Figure 2.5d,h). The amplitude of the RER rhythm was also decreased in SP animals compared to LD animals (Table S2). Taken together, these data suggest that SP shows lower metabolic flexibility for switching between the use of fat or carbohydrates as a fuel source. In addition, the changes in EE and RER rhythms appeared in the second week of SP exposure (Figure 2.5 c,d; Table S2), before the significant increase in fat mass (Figure 2.4), supporting the idea that the changes in the daily rhythms of RER and EE are responsible for the increased fat accumulation.

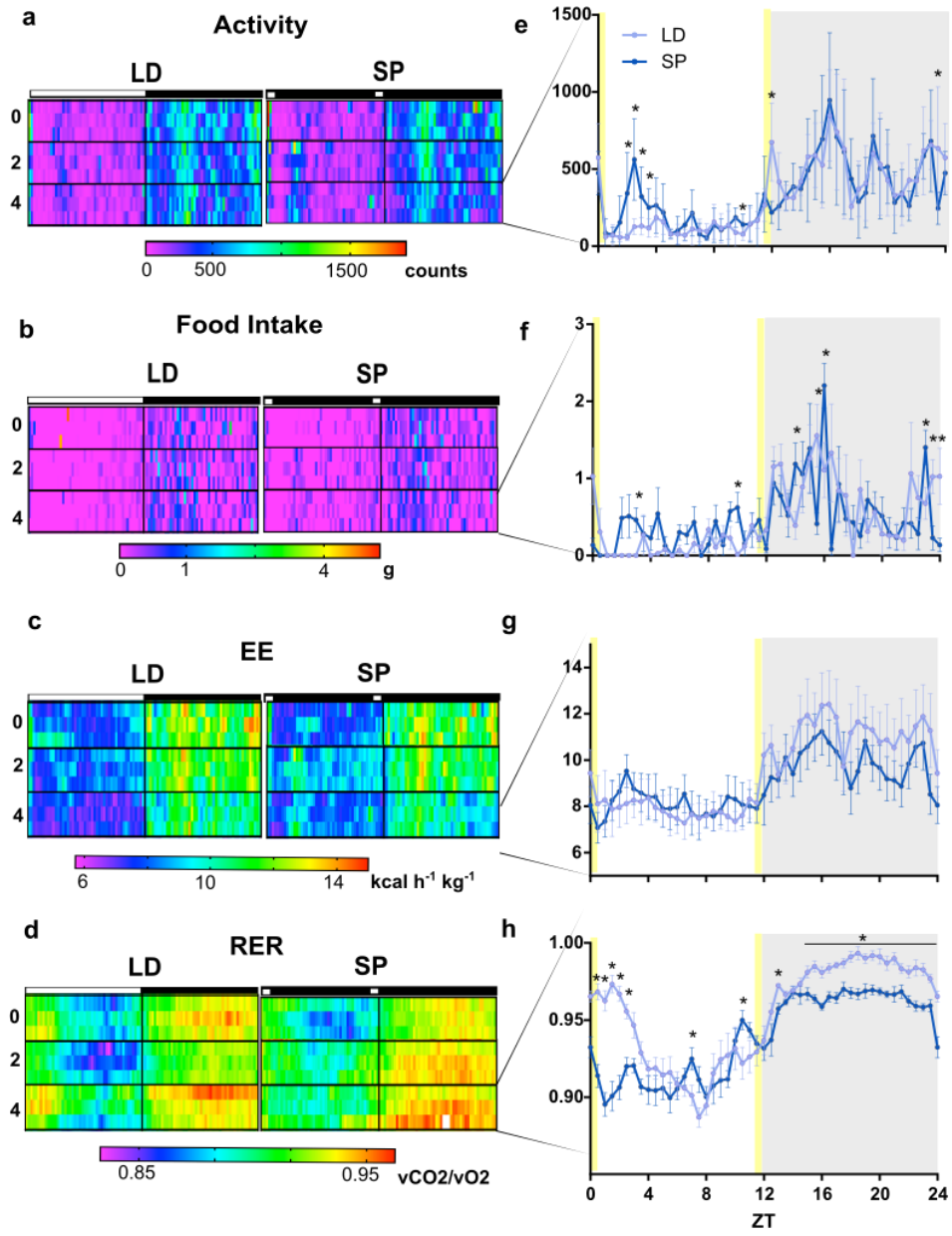

Figure 2.5. Skeleton photoperiod changes metabolic rhythms at specific moments of the circadian cycle. a-d) Average heat maps of a) locomotor activity, b) food intake, c) energy expenditure (EE), and d) respiratory exchange ratio (RER), in LD and SP animals fed ad libitum during weeks 0 (baseline), 2, and 4. e-h) Daily profile of locomotor activity (e) food intake (f), EE (g), and RER (h) representing the mean temperature obtained from 3 days of week 4 in both experimental groups. Shaded areas indicate “lights-off,” and yellow lines represent light pulses for the S

group. n = 6 for all measures in both groups. Data are presented as mean ± S.E.M., * P<0.05 in each timepoint after multiple t-tests.

2.6. 4 Skeleton photoperiod uncouples the different metabolic rhythms

Further analysis of the locomotor activity rhythm showed no significant differences in the onset, acrophase, and offset between the SP and LD groups (Figure 2.6a). However, because the onset was slightly shifted later into the subjective night in the SP group (Figure 2.6a), there was a significant decrease in the duration of nighttime activity, i.e. alpha (approximately, a 10% decrease) (Figure 2.6b) compared to the LD control.

SP animals also shifted the acrophase of food intake earlier than the LD control. Furthermore, in SP animals, the EE onset shifted later into the subjective night (Figure 2.6a), similar to what was observed for the daily rhythm in body temperature (Figure 2.3), and the EE offset shifted earlier (Figure 2.6a). Together these changes resulted in a significantly shorter EE alpha (approximately, a 14% decrease) (Figure 2.6b), which could explain the increased adiposity observed in SP animals. The onset, acrophase, and offset of the RER were shifted earlier (Figure 2.6a), but no differences were found in RER alpha between the SP and LD groups (Figure 2.6b).

In LD animals, the onset of activity roughly coincided with the onset of food intake, EE, and RER because these variables all increase in preparation for the activity period. However, in SP animals, these onsets were more dispersed; the standard deviation between them was higher in SP than in LD animals ($t_{(10)}$ =2.353, P=0.0404)

(Figure 2.6c). Such a dispersion was not observed for the acrophases or the offset (Figure 2.6d,e), indicating specificity for the anticipation of the incoming activity period. These changes suggest that although SP does not affect the overall synchronization of each of the metabolic rhythms to the photoperiod, it does cause a slight uncoupling of these rhythms, especially for the anticipation of the upcoming activity period (evidenced by the significantly increased dispersion of the onsets, which doubled in the SP group), which might have an impact on energy metabolism.

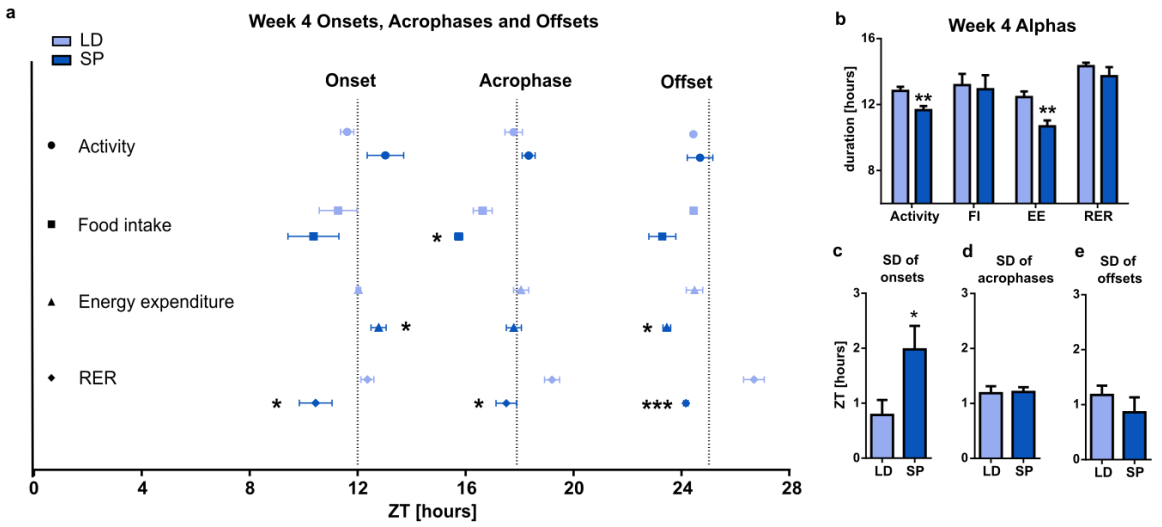

Figure 2.6. Skeleton photoperiod uncouples metabolic rhythms at the day-night and night-day transition periods. a) Onset, acrophase, and offset of LD-AL and SK-AL groups. Dotted vertical lines indicate the average of all onsets, acrophases, or offsets for the LD-AL group. b) Alpha of locomotor activity, food intake (FI), energy expenditure (EE), and respiratory exchange ratio (RER) of both groups. c-e) Standard deviation (SD) of the averaged onsets (c), acrophases (d), and offsets (e) of all variables for each group. n = 6 for all variables of both groups. Data are presented as mean ± S.E.M. *P<0.05, **P<0.01, ***P<0.001 after unpaired t-test. ZTs above 24 hours are included for observational purposes, being ZT24=ZT0 and ZT28=ZT4.

2.6.5 The metabolic effects of the SP appear not related to the conflict between food and light.

Finally, we investigated whether the metabolic effects of the SP could be due to the lack of light during the day, since previous studies suggest that constant darkness promotes an energy-saving state in mice (Zhang et al., 2006). Therefore, rats were exposed to constant darkness for four weeks under ad libitum feeding conditions (DD-AL group). Their food intake, weight gain, glucose tolerance, and adiposity were compared to the LD group. We did not find any significant differences in these parameters between DD-AL and LD-AL animals (Figure 2.7a-e), indicating that the complete absence of light for four weeks does not seem to impact metabolism in rats.

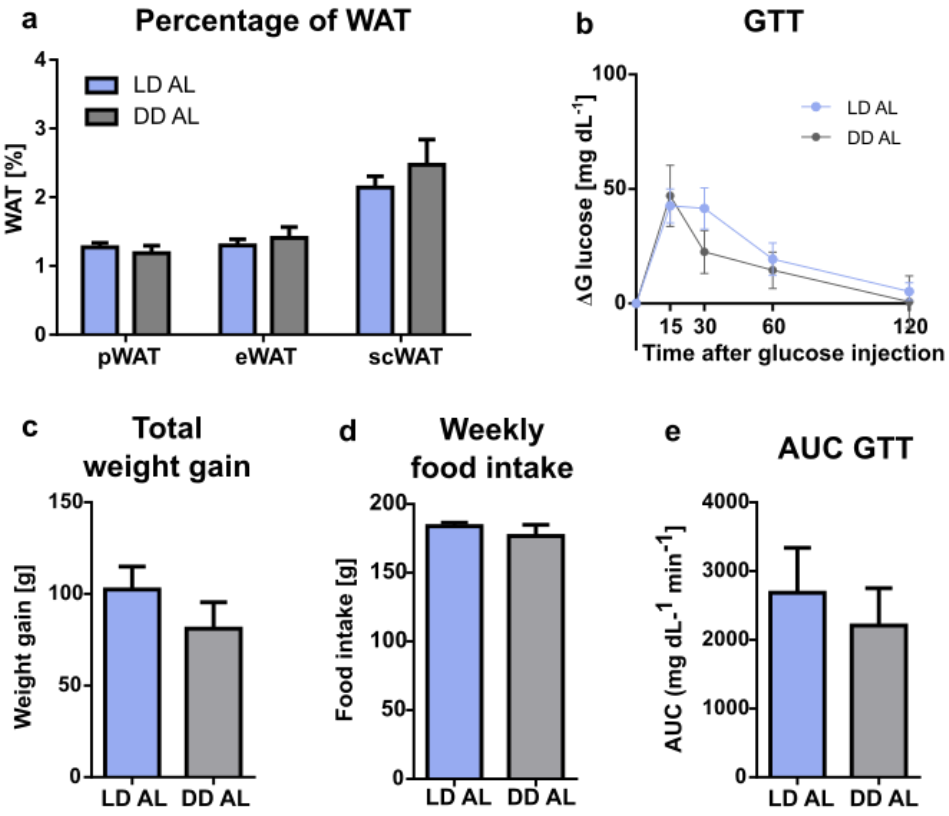

Figure 2.7. Exposure to four weeks of constant darkness does not change adiposity and glucose tolerance. a) Percentage of retroperitoneal (pWAT), epididymal (eWAT), and inguinal subcutaneous (scWAT) adipose tissue respective to body weight after four weeks of constant darkness (DD) or a 12:12 light-dark cycle (LD) under ad libitum feeding (AL). b) Intraperitoneal glucose tolerance test, and c) total weight gain after four weeks in the respective photoperiod. d) Food intake during the fourth week of the experiment for each group. e) Area under the curve of the GTT for both experimental groups. LD-AL, n = 8; DD-AL, n = 4. Data are represented as mean ± S.E.M. No significant differences were found after unpaired t-tests.

2.7 Discussion

2.7.1 The conflict between light and food during the day.

In nocturnal animals, feeding during the rest phase coincides with light exposure. In this sense, the present study aimed to resolve a critical issue in chronobiology: “What is the contribution of light to metabolic disturbances observed under daytime feeding in rats?” Using S , we observed that the absence of light prevents D -induced adiposity, but glucose intolerance persisted in D . This suggests that “incorrect” timing of food intake is not the sole mechanism driving metabolic disease, but that the conflict between light and food is also involved. Reinforcing the importance of the presence or absence of light, SP increased adiposity and decreased glucose tolerance even without time-restricted feeding, indicating the strong effects of SP on energy metabolism.

Although SP and DF both impair glucose tolerance, the mechanism underlying their effect on metabolism might be different. This is nicely illustrated by the body temperature rhythm of the SP-DF group, which shows an offset around ZT22 (closer to the LD-DF group) but an onset around ZT12 (closer to the SP-AL group) (Figure 2.3). Another indication of the differential effects of DF and SP is that LD-DF animals show a lower body weight gain than LD-AL animals, but SP-DF animals do not (Figure 2.1e). In agreement with this, feed efficiency was decreased in the LD-DF group but not in the SP-DF group (Figure 2.1g). The fact that the LD-DF has high adiposity despite lower feed efficiency is a clear indication of the profound metabolic reorganization induced by DF, while the normal adiposity in SP-DF could be explained by SP-driven mechanisms that, under DF conditions, defend normal body

weight gain at the expense of fat accumulation. In addition, the presence of light in LD-DF animals could inhibit feed efficiency under DF conditions; therefore, the lack of light in SP might have prevented the decreased feed efficiency in the SP-DF group.

Many studies have reported that DF uncouples daily clock gene rhythms among peripheral tissues (Damiola et al., 2000; Opperhuizen et al., 2016; Salgado-Delgado et al., 2013), but DF also promotes a circadian conflict in energy-regulating centers in the brain (Ramírez-Plascencia et al., 2017). It is still unclear if the peripheral disturbance precedes the central disturbance or if they both appear simultaneously. In this sense, glucose intolerance in the LD-DF group could appear as a consequence of internal desynchronization (Mukherji et al., 2015). In contrast, it is unlikely that SP acutely affects peripheral organs without the involvement of the brain, since the nervous system is responsible for light sensing. Therefore, the SP could change glucose tolerance via a neural pathway, as described below.

2.7.2 Possible mechanisms for the metabolic effects of SP.

The effects of SP on metabolism could be mediated by the SCN, the central clock regulating circadian rhythms. This nucleus receives direct input from the retina and is responsible for entraining circadian rhythms to the light-dark cycle. A previous study found that the rhythmic expression of c-fos (a neuronal activity marker) in the ventrolateral SCN is less robust in rats exposed to an SP compared to a full LD photoperiod (Schwartz et al., 1994). In addition, animals lacking the ion channel

Nav1.1, which have impaired intra-SCN communication, show a delayed onset of locomotor activity despite general maintenance of circadian rhythms (Han et al., 2012). The metabolic state of Nav1.1 KO animals has not been assessed, but other studies have shown that impaired intra-SCN communication may induce metabolic syndrome in rats (Luo et al., 2021). Thus, if SP modifies SCN activity, its output to other hypothalamic areas might also be changed, for instance, those that regulate metabolism in preparation for the activity period. However, the precise contribution of SP to SCN activity has yet to be determined.

Another interesting observation is that SP animals increase their daytime feeding when they are allowed to eat AL, which could account for their increased adiposity, but restricting food intake to the day (SP-DF group) does not produce greater adiposity. This observation indicates that the effects observed in SP-AL cannot be attributed to increased feeding during the subjective day. In addition, coupling food intake with the period of high activity and temperature under SP conditions (the SP-NF group) does not alleviate the metabolic problems. This result contrasts with other studies where restricting food to the appropriate period in nocturnal rodents mitigates the negative effects of a high-fat diet (Duncan et al., 2016; Hatori et al., 2012; Sherman et al., 2012), which further supports the idea that SP changes metabolism through a different mechanism independent of feeding behavior.

2.7.3 Anticipation of day-night and night-day transitions is needed for metabolic balance.

One of our most interesting findings is that SP-AL animals show time-specific changes in metabolic rhythms before the appearance of fat accumulation. SP animals fail to correctly anticipate the upcoming period of nocturnal activity, as demonstrated by the delayed onset of increased body temperature and energy expenditure. Delayed onset of activity has been observed in several other experimental setups that have increased adiposity and hyperglycemia, including *clock*-mutant mice (Han et al., 2012; Turek et al., 2005). The authors mainly attributed these effects to increased daytime feeding, but food restriction experiments were not performed (Turek et al., 2005). Therefore, the delayed preparation for the activity period might contribute to the metabolic impairments, but this possibility remains to be tested.

The night-day transition period also shows important changes in SP animals because the offset of both EE and RER occur earlier. This contrasts with other studies using normal LD photoperiods showing that RER is still high after the feeding period has finished (Opperhuizen et al., 2016). Together with the fact that the onset of EE is also changed, it seems that SP affects the circadian system in such a way that the day-night and night-day transition phases are not anticipated properly. Since a high RER is related to a higher oxidation of carbohydrates, it seems that SP animals do not oxidize them sufficiently at the end of the subjective night and beginning of the subjective day, which could explain their increased adiposity.

2.7.4 Light during the day is needed to maintain metabolic health.

SPs have been amply used to study circadian physiology in the absence of light, based on the “natural” sampling of light that rodents supposedly carry out in their burrows (Flôres & Oda, 2020; Jud, et al., 2005; Stephan, 1983) and the phase response curve of light, which shows only small or no induction of phase shifts during the light period (Honma, Katabami, & Hiroshige, 1978). The present study also confirmed the entraining effect of SP even when animals are fed during the subjective day. However, clearly, overall entrainment to a 12:12 cycle may not be sufficient to maintain metabolic homeostasis.

The SP combines light pulses with darkness during the day. This raises the question: Are the metabolic changes in SP animals due to the light pulses or the lack of light during the day? One study showed that exposing mice to complete darkness leads to an energy-saving state (Zhang et al., 2006). Furthermore, impairing the SCN´s oscillatory function produced a loss of peripheral rhythms, fat accumulation, and glucose intolerance, but only in constant darkness conditions (Kolbe et al., 2019). However, we did not observe glucose intolerance or increased adiposity in intact rats after four weeks of constant darkness (Figure 2.7). Thus, the metabolic impairments observed in SP animals must be related to the light-dark-light combination, emphasizing the need for a robust light signal to maintain metabolic fluctuations adequately aligned with the circadian time. Moreover, light pulses are known to promote activity and sleep acutely (Muindi et al., 2013; Pilorz et al., 2016). Since we found decreased activity at ZT12 in the SP group compared to the LD control (Figure 2.5a,e), it is possible that the light pulse from ZT11.5 to ZT12 inhibited activity at this

time point. Light also increases corticosterone secretion (Ishida et al., 2005) and changes liver metabolism (Cailotto et al., 2009; Opperhuizen et al., 2019) via the autonomic nervous system. Therefore, the SP-associated light pulses could modify metabolic rhythms by changing behavior or the autonomic output to the organs. However, it is worth noting that most studies evaluating the effects of light pulses have been performed during the nighttime, so the contribution of subjective-daytime light pulses to nocturnal rodent physiology needs to be studied further.

The present study supports the idea that developing a metabolic syndrome-like phenotype may already be caused by only minor changes in rhythmicity. Delays in the day-night and night-day transitions —such as those promoted by SP— may promote metabolic disease. This may be relevant for some night workers exposed to an SP-like schedule (two pulses of higher-intensity light at the beginning and end of the day) that have a shifted melatonin rhythm (Dumont, Benhaberou-Brun, & Paquet, 2001). In addition, people that sleep less during weekdays typically delay their activity on the weekend to recover from sleep loss, which has been associated with metabolic disease (Depner et al., 2019). Moreover, the preference to carry out one's activities later in the day (evening chronotype) is also associated with metabolic diseases (Vera et al., 2018; Yu et al., 2015), which further supports the hypothesis that delaying locomotor activity from the expected period of the day may contribute to metabolic impairments.

Overall, our study indicates that a robust light-dark cycle is crucial to maintain metabolic homeostasis, in addition to the appropriate timing of food intake. Optimal energy homeostasis involves not only day-night differences in locomotor activity, but

also proper coordination of all cycling physiological variables at every moment of the circadian cycle, especially during day-night and night-day transition phases. Failure to maintain this synchronization, either by a light disturbance such as a skeleton photoperiod or by food intake during the rest phase, will result in metabolic changes that predispose individuals to disease.

Chapter 3. Leptin acts on the hypothalamus regulating circadian temperature rhythms.

3.1 Abstract

Leptin is an adipokine strongly implicated in metabolic regulation. Leptin *knock-out* mice show excessive weight gain associated with increased feeding behavior; therefore, leptin has been considered an important anorectic signal. However, recent studies using other experimental approaches have shown that leptin has many physiological functions beyond the inhibition of feeding behavior. In this chapter, we dissect the physiological functions of leptin regarding the circadian regulation of body temperature. We found that leptin has different effects on temperature depending on the moment of administration; while leptin administration at night (CT14) has no effect, leptin administration in the morning (CT2) increases the body temperature. These effects occur simultaneously with the activation of leptin's signaling cascade in the brain; leptin administration at CT2 induces greater p-STAT3 immunoreactivity in the arcuate nucleus of the hypothalamus than CT14 administration. Furthermore, the presence of leptin might be an "ok" signal to the brain, helping maintain the normal temperature rhythm, since administering leptin to fasted animals prevents the fasting-induced decrease in temperature at the beginning of the day. These data identify a role for leptin in normal physiology beyond food intake, emphasizing that its action on temperature rhythms depends on the time of the day.

3.2 Introduction

Leptin is a hormone produced by the adipose tissue (adipokine). After its discovery in the 1980s, it was proposed as an important satiety signal because leptin *knock-out* mice showed increased feeding behavior and obesity, which could be counteracted by applying exogenous leptin (Friedman & Halaas, 1998). Furthermore, administrating exogenous leptin to non-obese mice decreased food intake and body fat mass (Halaas et al., 1997). The brain mediates the effects of leptin on food intake. Leptin receptors are located in several hypothalamic nuclei that regulate food intake, including the arcuate nucleus (ARC), the ventromedial nucleus (VMH), and the dorsomedial nucleus (DMH) (Fei et al., 1997).

Interestingly, circulating leptin levels oscillate in a circadian manner (Kalsbeek et al. 2015). Since the classical function of leptin is to inhibit food intake, it seems logical that the rhythm in circulating leptin could be driving the circadian rhythm in food intake. However, this does not seem to be the case, since the peak in circulating leptin levels in rats has been reported at the beginning of the active period (i.e., night), the phase when rats eat the most (Kalsbeek et al. 2015). Thus, leptin is unlikely to act as a satiety signal during this period.

Furthermore, many studies have shown that leptin correlates with weight and adipose tissue mass in humans and rodents (Caron et al., 2018; Mastronardi, Yu, & McCann, 2002a; Van Dielen et al., 2001). Thus, people with obesity have higher circulating leptin, but, at the same time, they show increased food intake. Therefore, some authors have argued that people with obesity could have leptin resistance.

This proposal was supported by experiments where exogenous leptin applied to hyperleptinemic obese mice did not activate leptin's signalling cascade as efficiently as in lean mice (El-Haschimi et al., 2000; Halaas et al., 1997). However, other studies using leptin antagonists have shown that endogenous leptin retains its feeding-supressing effect in mice with diet-induced obesity (Ottaway et al., 2015), indicating that excessive food intake in obesity conditions might not be related to leptin resistance. Since the concept of leptin resistance in obesity is still controversial (Flier & Maratos-Flier, 2017; Myers et al., 2012), we aimed to understand further the physiological role of leptin to comprehend its role in disease.

In physiological conditions, leptin decreases acutely after fasting, and food restriction experiments change the phase of circulating leptin (Bodosi et al., 2004; Martínez-Merlos et al., 2004). Because of this correlation with energy reserves, some authors have proposed that, instead of a satiety factor, leptin signals that energy reserves are not under threat, and thus no adaptations to a negative metabolic balance need to be implemented (Rexford S. Ahima, 2008; Ravussin, Leibel, & Ferrante, 2014). In particular, since leptin abruptly decreases in fasting, this signal could promote the metabolic adaptations to fasting. i.e., increased drive to eat, increased lipolysis, and higher corticosterone secretion (Ahima et al. 1996; Perry et al. 2019).

One adaptation to fasting that has not yet been explained mechanistically is the temperature decrease observed at the beginning of the resting period (Liu et al. 2002). Many reports using exogenous leptin administration have shown that it affects body temperature through its action on the hypothalamus (Bell et al., 2018; Fischer et al., 2016; Wiater et al., 2013). However, it is unclear whether this occurs in

physiological conditions since the doses of administered leptin usually surpass circulating leptin levels in an order of magnitude of 10^6. Provided that endogenous leptin does influence temperature, the drop in leptin levels under fasting conditions could be related to the fasting-induced drop in temperature. Since this temperature drop is only observed at the beginning of the resting period, the effect of leptin on temperature must be time-dependent. Therefore, we aimed to determine if leptin is detected differently in the ARC depending on the time of the day. Hereby, the ARC was chosen because it has leptin receptors (Caron et al., 2018), provides the first passage of leptin into the hypothalamus (Balland et al., 2014), and influences the circadian temperature rhythm (Guzmán-Ruíz et al., 2015b).

3.3 Hypotheses

- Leptin is detected differently in the hypothalamus, depending on the time of the day.
- Leptin influences temperature depending on the time of the day.

3.4 Objectives

- Determine the basal daily rhythm of leptin in circulation and its corresponding detection by the hypothalamus.
- Evaluate the effect of leptin on the temperature at different moments of the circadian cycle.

3.5 Methods

3.5.1 Animals.

Male Wistar rats of 270-300 g from the *Unidad Académica Bioterio*, Faculty of Medicine, UNAM were housed under a standard 12:12 light-dark cycle, (lights on at 7:00 am) with water and food ad libitum, unless stated otherwise. Room temperature was maintained at thermoneutrality. All experiments were conducted according to the Mexican norms for animal handling (NOM 062 ZOO 199) and all measures were taken to minimize animal use and suffering.

3.5.2 Experiment 1. Determination of the rhythm of circulating leptin and its detection by the hypothalamus

To determine the basal rhythm of leptin in circulation and its detection by the brain, blood and brain samples were taken at six different periods of the circadian cycle: CT 2, 6, 10, 14, 18, and 22. Three rats were sampled per timepoint for a total of 18 animals. Rats were separated into cages containing three rats per cage (one cage per timepoint) and were transferred to a separate experimental room 24 hours before the experiment. On the day of the experiment, the lights remained off to eliminate the possible effects of lights on the measurements. A dim red light was used for handling and sampling under the darkness. Rats pertaining to the same cage were blood sampled via tail puncture and then injected with pentobarbital for perfusion.

3.5.2.1 Blood sampling

Approximately 500 µL of blood was obtained via tail puncture and stored in microfuge tubes with EDTA. The samples were maintained in ice until sera separation. To

obtain the sera, we centrifuged the samples at 8000 rpm for 10 minutes; the sera were separated by manual pipetting and stored at -20°C for further processing.

3.5.2.2 Perfusion and brain processing

Before perfusion, the body weight of all rats was measured. Then, rats were administered with an overdose of pentobarbital and perfused intracardially with ice-cold saline solution followed by 4% paraformaldehyde (PFA). Brains were extracted and post-fixed for 24 hours in PFA. Then, brain tissue was cryoprotected in a 30% sucrose solution for 24 hours. Subsequently, brains were frozen and cut into 30 µm sections with a cryostat. Brain sections were collected in 12-well plates with phosphate-buffered saline solution (PBS) for immunohistochemical processing.

3.5.2.3 Immunohistochemistry for p-STAT3

STAT-3 is a transcription factor that is part of the signaling cascade of leptin in the brain (Belgardt et al., 2009). Upon the binding of leptin to its receptor, STAT3 becomes phosphorylated and translocates to the nucleus to promote the expression of other targeted genes (Belgardt et al., 2009). Thus, the presence of p-STAT3 in neurons indicates that those neurons responded directly to leptin; therefore, we measured p-STAT3 in the ARC, VMH, and SCN. Sections corresponding to the hypothalamus (-0.48 to -3.6 mm with respect to the bregma according to axinos' Brain Atlas (Paxinos & Watson, 2005)) were incubated in antigen retrieval buffer (TRIS base 10 mM, EDTA 1 mM, Tween 0.5% in deionized water) for 20 min at 80°C. Afterward, the sections were rinsed three times with PBS and blocked with incubation buffer (PBS with 1% bovine albumin) for one hour at room temperature.

The sections were then incubated in the primary rabbit antibody for p-STAT3 (Cell signalling ®) diluted 1:500 in incubation buffer overnight at 4°C. The next day, sections were rinsed three times with PBS and incubated in a secondary biotinylated antibody (donkey anti-rabbit, Sigma ®) for 2 h. The sections were then rinsed three times with PBS and incubated for 5 min with revealing buffer (DAB 2%, $NiSO_4$ 0.6 %, H_2O_2 0.012% in Tris-buffered saline solution). After triplicate rinsing, the sections were mounted on gelatin-covered glass slides and left to dry.

The next day, the sections were dehydrated by placing them for 3 minutes in the following solutions: ethanol 10%, ethanol 90%, ethanol 100%, ethanol- xylene 1:1, and xylene 100%. Then, the sections were covered with Entellan ® covering medium and a glass coverslip. The sections were left to dry for several hours before observation in the microscope.

3.5.2.4 Image processing

The sections stained for p-STAT3 were observed in a light microscope under 10X magnification. Three sections corresponding to the arcuate nucleus and ventromedial hypothalamus (VMH) were selected for microphotography and counting of immunopositive cells. Digital images were processed using ImageJ software. The area corresponding to the arcuate nucleus or VMH was manually outlined, the background was substracted by 50%, and the bilateral number of positive cells were counted with the object counting function.

3.5.2.5 Leptin measurement

Serum samples were unfrozen, and 100 µL of serum were processed for leptin measurement by ELISA (Biovendor ®) according to the manufacturer's instructions.

3.5.3 Experiment 2. Injection of leptin at different timepoints.

To determine the time-dependent effect of leptin, rats were implanted with an intraperitoneal temperature sensor and an intravenous jugular cannula. After recovery, the animals were transferred to the experimental room and fasted overnight. On the day of the experiment, the lights remained off to eliminate the possible effects of lights on the measurements. A dim red light was used for handling and sampling under the darkness. Rats were administered leptin via a remote cannula (100 µL/kg dose) at different time points: CT 2 and 14. One hour after leptin administration, animals were euthanized with an overdose of pentobarbital and perfused as described in section 4.5.2.2. Then, the brains were processed for p-STAT3 immunohistochemistry as described in section 4.5.2.3.

3.5.3.1 Cannula implantation surgery.

The cannulation procedure was carried out according to Harms and Ojeda (1974) and Heiser (2007). Briefly, animals were anesthetized with i.p. ketamine (40-80 mg/kg) and i.m. xylazine (5-8mg/kg). All surgical materials were sterilized with benzalkonium chloride. The left neck area of the rat was shaved and cleaned with ethanol, and a 1 cm incision was performed with a scalpel. The jugular vein was located and punctured. A silicone catheter with filling medium (glycerol 80%, heparin 10%, antibiotic mixture 10%) was inserted in the vein and secured with suture thread.

The other end of the catheter was placed subcutaneously to exit through the back of the animal. The distal tip of the catheter was held in place with a leather vest, as shown in Figure 3.1.

Figure 3.1. Intravenous cannula support system. After intravenous cannulation, a leather vest is placed onto the animal to hold the intravenous sampling system in place. On the day of the experiment, the intravenous catheter is connected to a system to withdraw blood and administer substances (in this case, leptin) into the vein remotely.

3.5.3.2 Implantation of an intraperitoneal temperature sensor.

After cannulation, rats were implanted with an intraperitoneal temperature sensor. An area of approximately 2 cm^2 within the lower abdomen was shaved and cleaned with ethanol. A skin incision of approximately 2 cm was performed with a scalpel.

The underlying muscle tissue was carefully ripped with dissecting scissors. The sensor was carefully introduced into the abdominal cavity. Then, the muscle and skin were sutured, and the area was cleaned with ethanol. Rats were administered antibiotics for post-operative care and left to recuperate for seven days before the experiments.

3.5.3.3 C-fos immunohistochemistry

Sections corresponding to the hypothalamus were collected and processed for immunohistochemistry. The sections were incubated in the primary rabbit antibody for c-fos (Cell signaling) diluted 1:500 in incubation buffer overnight at 4°C. The next day, the sections were rinsed three times with PBS and incubated for two hours at room temperature with biotinylated donkey anti-rabbit antibody (Sigma), followed by two hours of incubation with avidin-peroxidase complex (Vector Laboratories) diluted 1:500 in incubation buffer. Sections were then rinsed three times with PBS and incubated for 5 min with revealing buffer (DAB 2%, $NiSO_4$ 0.6 %, H_2O_2 0.012% in Tris-buffered saline solution). After triplicate rinsing, the sections were mounted on gelatin-covered glass slides and left to dry.

3.5.4 Experiment 3. Leptin in the temperature adaptation to fasting.

To determine whether leptin is necessary for the circadian temperature adaptations to fasting, we recorded the temperature of a group of rats for three days with the intraperitoneal sensor (section 3.5.3.1). The day before the experiment, animals were transferred to the experimental room, and the lights remained off to eliminate the possible effects of lights on the measurements. A dim red light was used for

handling and sampling under the darkness. On the first day of the experiment, rats received food ad libitum; on the second day, they were fasted starting at CT0; on the third day, they remained under fasting conditions but also received i.v. leptin at CT22. Four hours later, animals were euthanized to recover the i.p. temperature sensors.

3.5.5 Statistical analysis.

All statistical analyses were performed in GraphPad Prism 5. Full statistical details are provided for each experiment.

3.6 Results

3.6.1 Leptin is detected by the hypothalamus at different circadian times.

Circulating leptin levels correlated with the weight of the animals regardless of the moment of sacrifice (Figure 3.2, Pearson Correlation P = 0.0425). No significant differences were found among circulating leptin levels at the different time points of the circadian cycle (Figure 3.3, one-way ANOVA P = 0.2256).

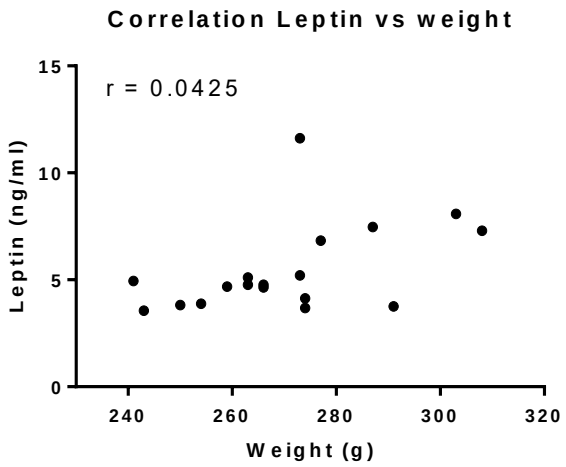

Figure 3.2. Leptin concentration as a function of body weight. r = 0.0425 after earson's correlation, n=18.

Although p-STAT3 levels appeared lower at night, the number of p-STAT-3 positive cells in the ARC did not show a significant circadian pattern, probably due to the small sample size (Figure 3.3, one-way ANOVA P = 0.7988). No correlation was found between the amount of p-STAT-3 positive cells in the ARC and the concentration of circulating leptin (data not shown). The number of p-STAT-3-positive cells in the VMH was also seemingly lower at night but did not show a significant difference between circadian timepoints (Figure 3.3 one-way ANOVA P = 0.1200). The SCN did not show any p-STAT3 staining (data not shown), suggesting that leptin does not signal directly on this nucleus.

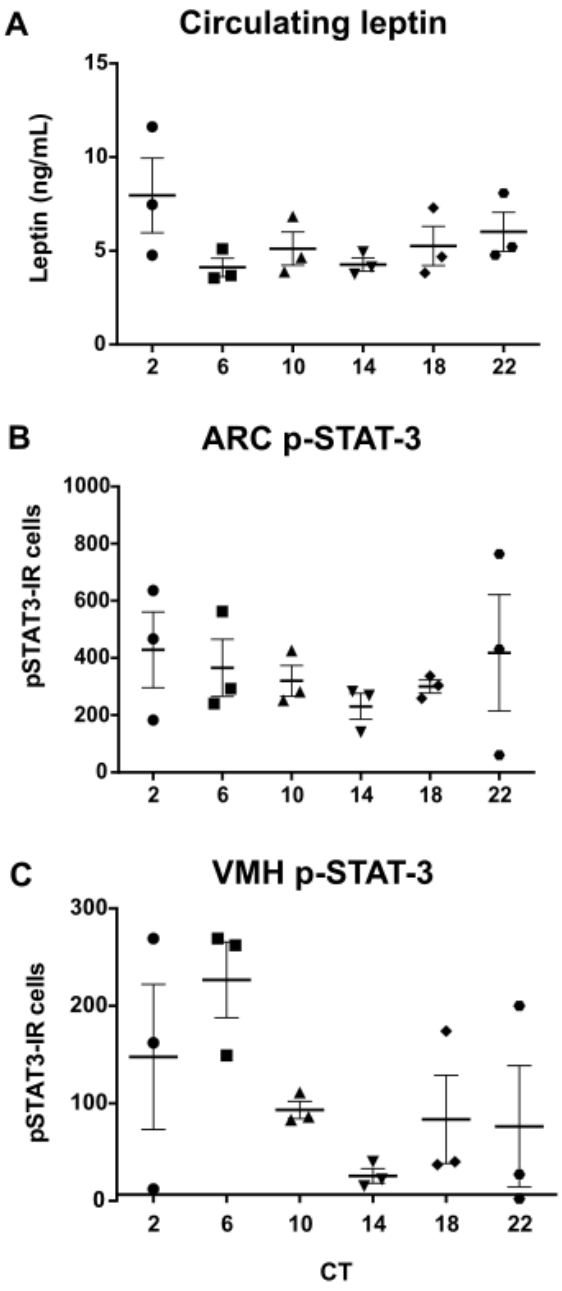

Figure. 3.3. Circulating leptin and leptin signaling under basal conditions. A) Basal leptin levels throughout the circadian cycle in animals under ad libitum feeding. B,C) Number of p-STAT-3 positive cells in the ARC (B) and VMH (C) at different moments of the circadian cycle. Mean ± S.E.M are shown. n = 3 per time point.

3.6.2 The same dose of leptin has different effects on the hypothalamus and temperature depending on the time of the day.

In fasted animals, leptin administration increased p-STAT3 immunoreactivity both at ZT2 (isher's LSD test P = 0.0015) and ZT14 (isher's LSD test P = 0.0112) compared to their respective saline control. However, the magnitude of the p-STAT3 response was different depending on the time of the day: leptin administration at CT2 induced significantly higher p-STAT3 immunoreactivity in the ARC than at CT14 2 hours after administration (Figure 3.4, i sher's LSD test = 0.0031). This suggests that, unlike in basal conditions, fasting might induce circadian differences in leptin access into the brain or its detection by the ARC.

In contrast, the number of c-fos positive cells in the ARC (Figure 3.5) did not change significantly after leptin administration regardless of the time of administration (two-way ANOVA showed no effect of time, treatment, or interaction). The SCN showed the expected difference between day and night c-fos (two-way ANOVA revealed a significant effect of time, $F_{(1, 11)} = 8.346$, P = 0.0147). Still, there was no difference in c-fos between saline- and leptin-administered animals (Figure 3.6).

Furthermore, leptin administration increased temperature one hour after administration at CT2 compared to the saline control; but the administration at CT14 did not affect temperature (Figure 3.7). This result, together with the fact that p-STAT3 was increased in the ARC after leptin administration at CT2, suggests that the detection of leptin in the ARC could mediate the effects of leptin on temperature at CT2.

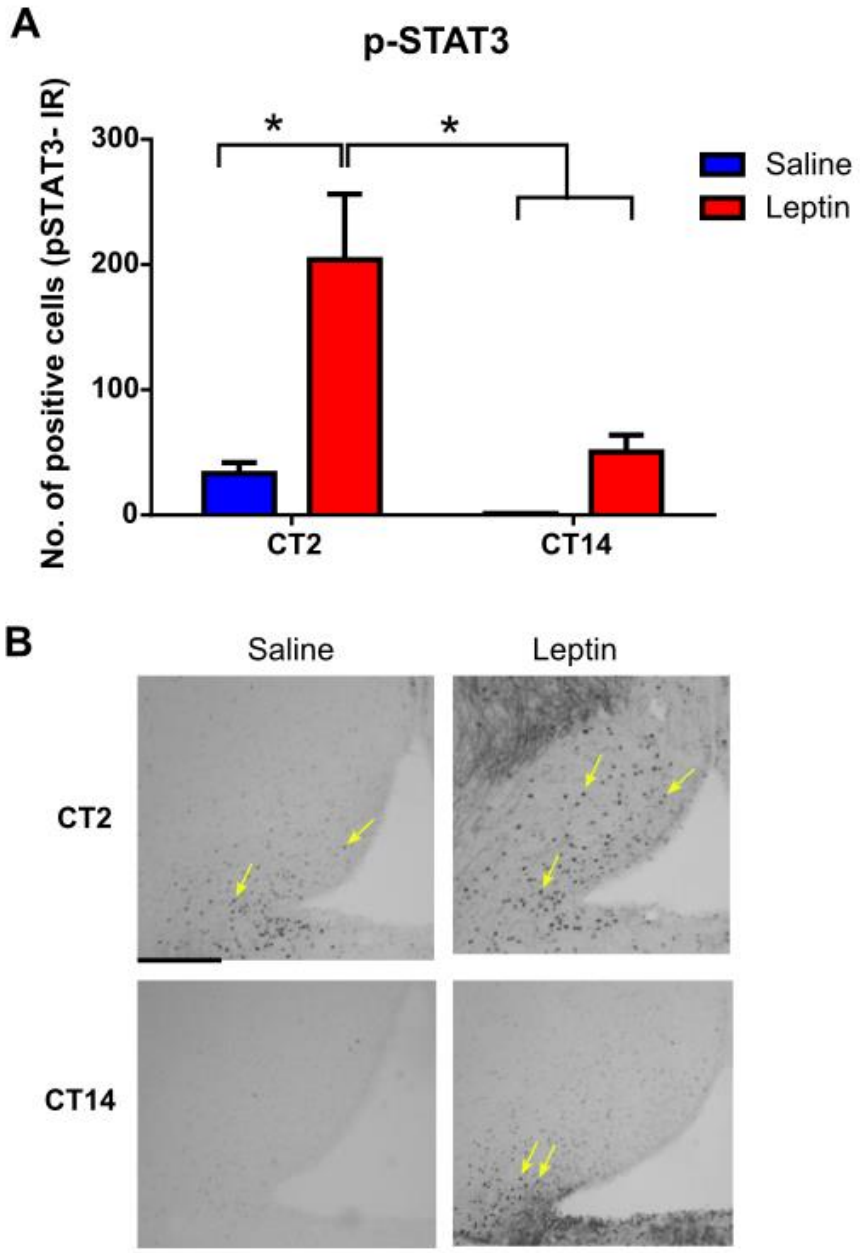

Figure 3.4 p-STAT3 in the ARC in response to leptin in fasted animals. A) Number of p-STAT3 positive cells in the ARC two hours after leptin administration. B) Representative images of p-STAT-3 ARC staining. CT2 saline, n=4; CT2 Leptin, n=5; CT14 saline, n=5; CT14 leptin, n=4. Data are presented as mean ± S.E.M. <0.05 after fisher's LSD test. The yellow arrows indicate examples of positive cells, and the black line indicates the scale of 100 μm.

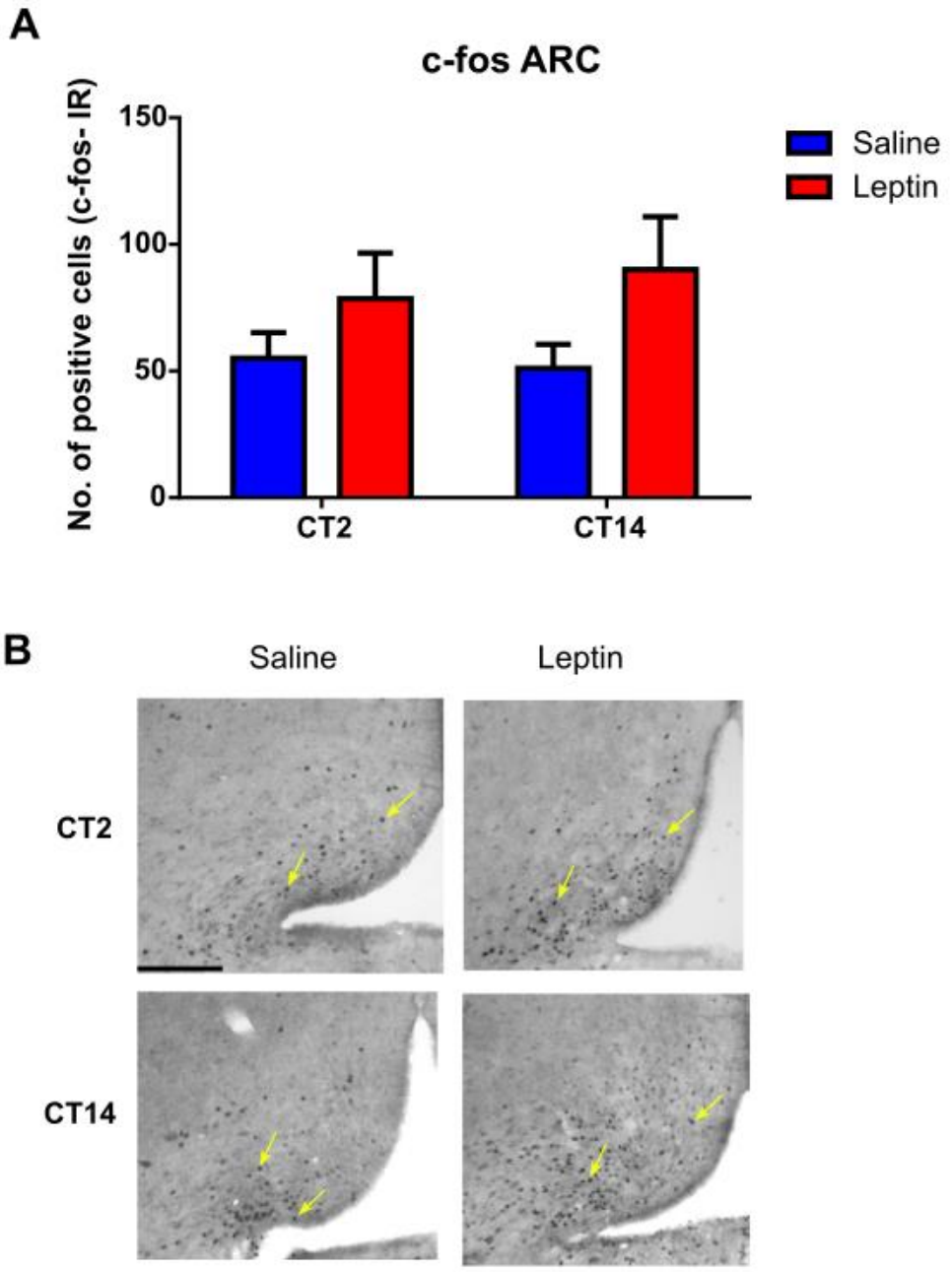

Figure 3.5 c-fos in the ARC in response to leptin in fasted animals. A) Number of c-fos positive cells in the ARC two hours after leptin administration. B) Representative images of c-fos ARC staining. No significant differences were found. CT2 saline, n=4; CT2 Leptin, n=5; CT14 saline, n=5; CT14 leptin, n=4. Data are presented as mean ± S.E.M. The yellow arrows indicate examples of positive cells, and the black line indicates the scale of 100 μm.

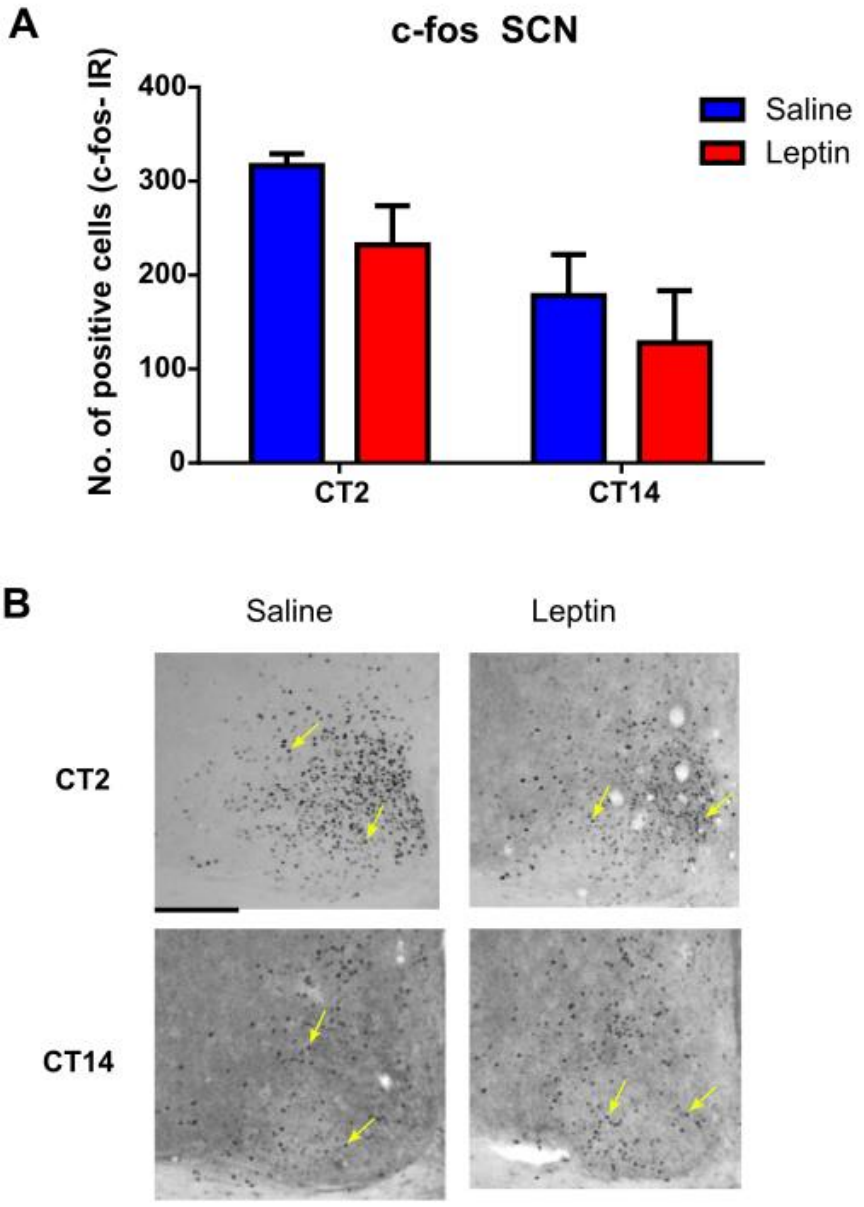

Figure 3.6 c-fos in the SCN in response to leptin in fasted animals. A) Number of c-fos positive cells in the SCN two hours after leptin administration. B) Representative images of c-fos SCN staining. No differences were found between rats administered with leptin and saline. CT2 saline, n=4; CT2 Leptin, n=5; CT14 saline, n=5; CT14 leptin, n=4. Data are presented as mean ± S.E.M. The yellow arrows indicate examples of positive cells, and the black line indicates the scale of 100 µm.

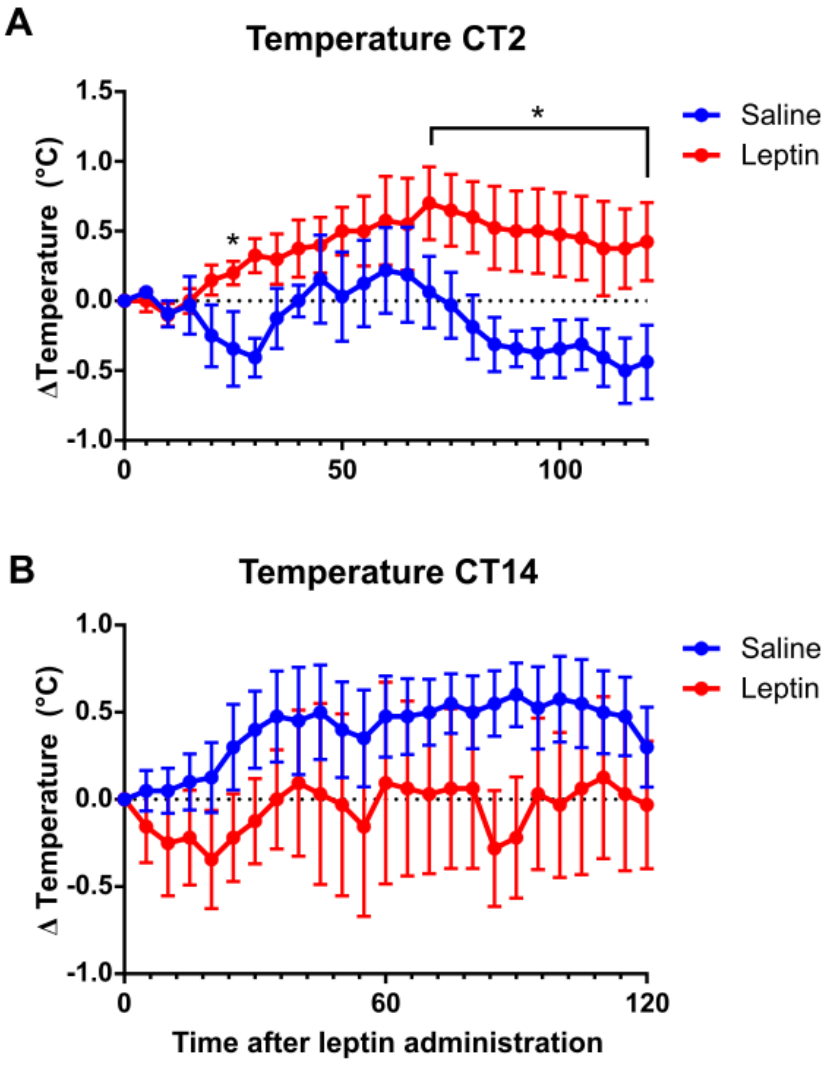

Figure 3.7. The effect of leptin on temperature at different time points in fasted animals. Temperature change after leptin administration at CT2 (A) and CT14 (B). CT2 saline, n=4; CT2 Leptin, n=5; CT14 saline, n=5; CT14 leptin, n=4. Data are presented as mean ± S.E.M. < 0.05 after LSD's ish er's test.

3.6.3 Leptin prevents the decrease in temperature associated with fasting

Since the first experiments in ad libitum animals showed a seemingly higher p-STAT3 in the ARC at CT22-2 and fasting produced circadian differences in p-STAT3 in the ARC, we hypothesized that leptin could be relevant for the body temperature adaptations to fasting that occur at the night-day transition period (CT 23-1). Animals under fasting conditions decrease their body temperature in this period, and each day of fasting lowers the temperature further (Liu et al., 2002). Indeed, one day of fasting significantly reduced the mean temperature in the period between CT 23-1 compared to the same rats on the previous non-fasting day ($p= 0.0249$ after Dunnet's test, Figure 3.8). This temperature difference was 0.5403 ± 0.06941 °C lower in the 1-day fast compared to non-fasting. Leptin administration at CT22 the second day after fasting reestablished body temperature to pre-fasting levels ($p= 0.8006$ after Dunnet's test, Figure 3.8) despite the fact that 2-day-fasting animals decrease their temperature further than a 1-day fast (Liu et al., 2002). These observations suggest that leptin prevents the fasting-induced temperature decrease observed in the night-day transition.

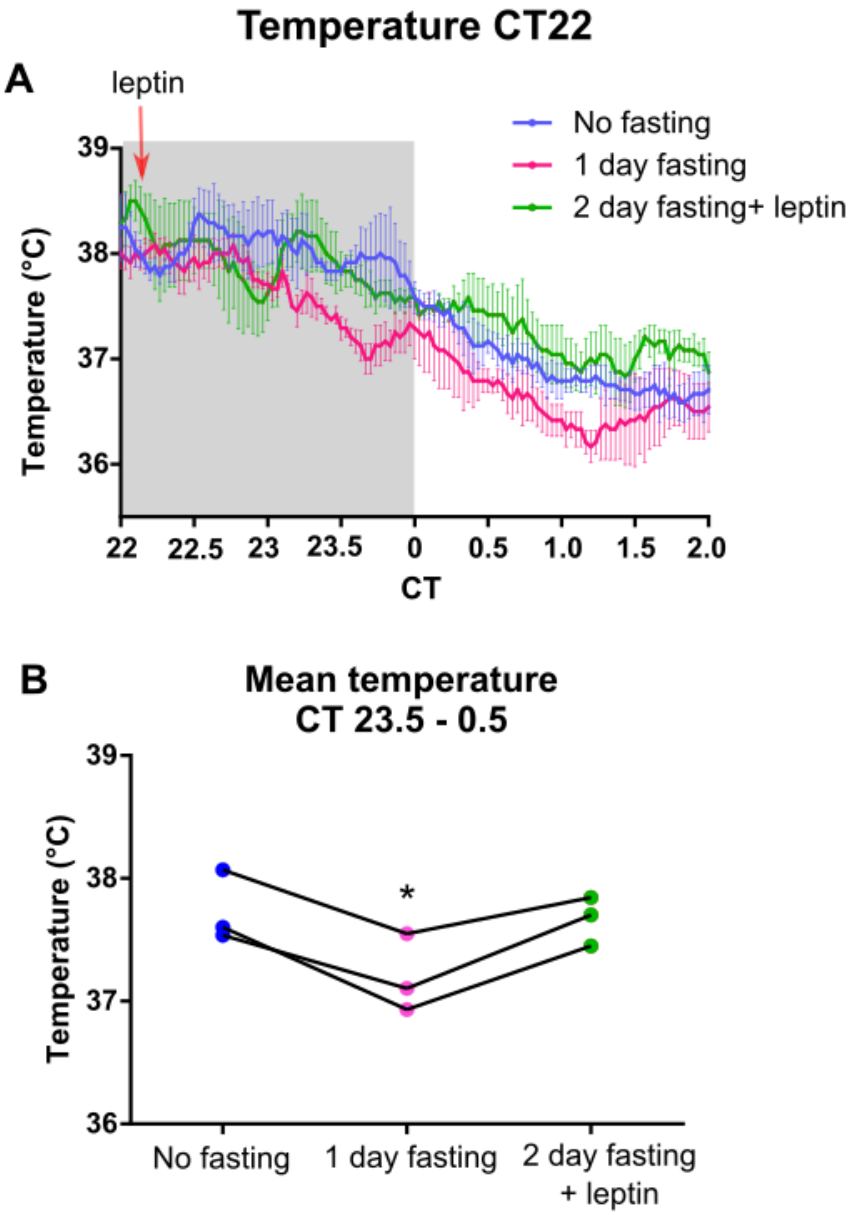

Figure 3.8 Leptin administration prevents the fasting-induced temperature decrease. A) Body temperature starting at CT22 in the same animals under different conditions: under ad libitum feeding (no fasting), after 24 hours of fasting (day 1 fasting), or after 2 days of fasting and leptin administration at CT22 (day 2 fasting + leptin). The moment of leptin administration is indicated by the red arrow. Shaded gray area indicates the subjective night, and clear area indicates the subjective day. B) Mean temperature in the period between CT 23.5 to 0.5. All data are presented

as mean ± S.E.M. < 0.05 after Dunnet's multiple comparisons test against "no fasting" day, n=3.

3.7 Discussion.

3.7.1 Circadian differences in circulating leptin

Our first experiment analyzed circulating leptin levels under ad libitum conditions. Leptin did not show a significant rhythm in the circulation, which contrasts with some studies showing that leptin peaks at night in rats (Kalsbeek et al., 2015; Mastronardi, Yu, & McCann, 2002b). However, other studies in different species have found higher leptin levels during the day (Pan & Kastin, 2001), and others have found no rhythm (Rynders et al., 2020). Even if leptin does have a rhythm in the circulation, the fact that body weight correlates with circulating leptin levels independently of the moment of sacrifice (Figure 3.2) indicates that fat reserves are more important to determine leptin levels than the circadian time.

3.7.2 Circadian differences in leptin signaling: access into the brain or sensitivity?

Regardless of its circulating concentration, leptin exerts most of its effects through the hypothalamus. Therefore, we measured pSTAT-3, part of leptin's signaling cascade, in the ARC at different circadian times; we found more pSTAT-3 immunoreactivity after leptin administration in the morning than at night (Figure 3.3). Importantly, these circadian differences were only significant after administering leptin in fasting conditions, although a trend (not significant probably due to small sample size) was also observed under basal *ad libitum* conditions.

The circadian difference in leptin signalling in the ARC could be explained by at least two different processes that will be discussed below: 1) leptin has a different access into the brain depending on the time of the day, or 2) leptin elicits higher signaling, (i.e., leptin neurons are more sensitive) depending on the time of the day.

The entry of leptin into the ARC is known to be mediated by tanycytes, a population of glial cells that transport leptin into the brain parenchyma through leptin receptors (Balland et al., 2014). These tanycytes mediate the entry of glucose into the ARC, and their barrier function is regulated in a circadian manner by SCN-derived signals like vasopressin (Rodriguez-Cortes et al., 2022). Thus, tanycytes could modulate leptin entry into the brain to promote a higher access at ZT22-2. Moreover, fasting conditions are known to increase the permeability of substances into the ARC through tanycytes (Langlet et al., 2013). In agreement with this, we observed that fasting followed by leptin administration boosted the circadian differences in p-STAT3 in the ARC between CT2 and CT14 (Figure 3.4), suggesting that these differences might indeed be mediated through the differential access of leptin into the ARC. Interestingly, rats administered with leptin at CT14 show increased p-STAT3 staining in the region close to the ventricle, where tanycytes are located (Figure 3.4). This could indicate that tanycytes are detecting circulating leptin but not allowing its entrance into the brain parenchyma at this time point; however, this hypothesis remains to be tested.

Conversely, the circadian differences in leptin signaling could be due to differences in the sensitivity of leptin receptors (LepRs). One study in mice (Cedernaes et al., 2019) evaluated the expression of LepRs in AgRP neurons at two different

timepoints and found no circadian differences in LepR expression. However, in the same study, they observed that LepRs were upregulated in these neurons after 24 hours of fasting, indicating that fasting can change leptin sensitivity. This result agrees with the observation that fasting also changes leptin entry into the brain, indicating that both processes could mediate the circadian differences observed in leptin signaling in the ARC.

Furthermore, a recent study showed the access of Evans Blue dye into the ARC is different depending on the time of the day; in this study, the penetration of Evans Blue was measured in relation to the distance from the ventricle (Rodriguez-Cortes et al., 2022). With this method, they observed that Evans Blue entered further into the ARC (i.e., more distance from the ventricle) when administered at ZT22 and ZT2 compared to ZT11. This distance is critical because the two main neuronal populations in the ARC, AgRP/NPY and POMC neurons, have different localizations within the nucleus: while AgRP/NPY neurons are closer to the ventricle, POMC neurons are located ventrolaterally in the ARC (Chronwall, 1985). Therefore, if a molecule such as leptin enters further into the ARC at a specific timepoint, it might reach POMC neurons and thus have different effects. Future studies should address p-STAT3 in distinct neuronal populations to understand if leptin can activate other ARC neurons depending on the time of the day.

3.7.3 The effect of leptin on hypothalamic circuits regulating temperature

Leptin administration in fasted animals at CT2 did not only produce higher p-STAT3 than at CT14, but it also had a differential effect on temperature: while it increased

temperature at CT2, it did not do so at CT14. Importantly, the effects of leptin were not immediate: the change in body temperature at CT2 occurred more than one hour after leptin administration. This observation suggests that the influence of leptin on temperature needs to be integrated in the different hypothalamic regions. This is supported by the observation that despite a clear induction of p-STAT3 in response to leptin, there was no significant change in c-fos, a marker of neuronal activity. This also suggests leptin does not increase temperature by directly activating all ARC neurons. In the ARC, leptin's signaling cascade culminates in the hyperpolarization of NPY neurons (Baver et al., 2014; Spanswick et al., 1997) and, to a lesser extent, with the depolarization of POMC neurons (Cowley et al., 2001). Furthermore, since NPY neurons have inhibitory projections to POMC neurons, the hyperpolarization of NPY neurons may further increase POMC activity (Cowley et al., 2001).

POMC neurons are important for temperature regulation because they produce the peptide α-MSH (Guzmán-Ruíz et al., 2015), which is released in the median preoptic area (MnPO to increase body temperature. Therefore, leptin could increase the temperature at CT2 by activating the POMC-MnPO pathway. The VMH also has leptin receptors (Caron et al., 2018), and has been associated with circadian changes in body temperature in mice (Orozco-Solís et al., 2016b). This nucleus has extensive projections to the ARC (Sternson et al., 2005), providing another pathway whereby leptin can regulate temperature. Unfortunately, we did not study the effects of leptin on the VMH nor in specific subpopulations within the ARC; thus, the circuits underlying the circadian effects of leptin on temperature need to be investigated further.

3.7.4 Leptin's role in the adaptation to fasting

As previously discussed, fasting triggers a series of physiological changes that promote the mobilization of fat reserves, including a sharp drop in circulating leptin levels. These changes also include the activation of the Hypothalamus-Pituitary-Adrenal (HPA) axis, which culminates with the release of corticosterone from the adrenal gland (Ahima, 2000). Recently, Perry et al. (2019) have shown that the decrease in leptin under fasting conditions triggers HPA-axis activation in mice. As a result, increased corticosterone secretion activates AgRP/NPY neurons to promote feeding behavior (Perry et al., 2019). Furthermore, the rise in circulating leptin after refeeding is essential for the postprandial temperature increase (Perry et al., 2020). Consistent with this, we observed that, under fasting conditions, leptin administration reestablishes normal temperature in the period between CT23.5 to CT 0.5, i.e., the transition from the subjective night to the subjective day (Figure 3.8). Our result supports the hypothesis that leptin decrease is essential for the fasting-induced temperature decrease at this time point, but further studies are needed to carefully dissect the brain regions involved in this process.

As indicated previously, α-MSH release from POMC neurons maintains temperature high, particularly at the end of the night period (Guzmán-Ruiz et al., 2015). Therefore, leptin's action on OMC neurons at CT22 could drive α-MSH release, thus maintaining temperature at high levels. In contrast, the absence of leptin under fasting conditions would no longer signal to POMC neurons, which could trigger the temperature decrease at this timepoint. However, since the activation of α-MSH

neurons at CT22 is not diminished under fasting conditions (Guzmán-Ruiz et al., 2014), this is probably not the pathway through which leptin maintains temperature.

An alternative hypothesis is that leptin influences temperature through the SCN-ARC axis (Méndez-Hernández, Escobar, & Buijs, 2020). Since the SCN does not detect leptin directly (section 3.6.1), leptin detection by the ARC could signal this information to the SCN. The SCN regulates temperature regulation in a circadian manner by releasing vasopressin into the MnPO to decrease temperature in preparation for the resting period, i.e., the day (Guzmán-Ruiz et al., 2015). Therefore, under fasting conditions, the absence of leptin could trigger exacerbated vasopressin release, decreasing temperature further. This hypothesis is supported by the observation that AVP concentrations in the hypothalamus increase under fasting conditions (Poplawski et al., 2010). Furthermore, there is a negative correlation between hypothalamic AVP and circulating leptin under fasting conditions (Poplawski et al., 2010). More research is necessary to test whether leptin indeed acts on the SCN-ARC axis to regulate temperature adaptations to fasting.

3.7.5 Implications of leptin´s thermogenic effect on obesity

Previous studies have shown that both rats (obese Zucker rats) and mice (ob/ob) with leptin deficiency have a lower average body temperature than mice with normal leptin signaling (Dubuc, Wilden, & Carlisle, 1985; Murakami, Horwitz, & Fuller, 1995). These studies support a role for leptin in temperature regulation, as observed in the present study. Moreover, ob/ob mice under fasting conditions decrease their

temperature more than lean controls (Dubuc et al., 1985), indicating that the total absence of leptin impacts temperature under fasting conditions.

A study in women with obesity (presumably leptin-resistant) showed that average wrist temperature was lower in these women, and their temperature rhythm was blunted (Corbalán-Tutau et al., 2011). Another study also found a correlation between body temperature and body mass in human subjects (Adam, 1989). These observations indicate that changes temperature rhythms are associated with obesity, but it is still unclear if lower temperature causes obesity or if it is only a consequence of other changes in basal metabolism (Landsberg et al., 2009).

Overall, our study suggests that leptin has a role in the circadian regulation of body temperature rhythms, most likely mediated by differential access of this hormone into distinct hypothalamic regions depending on the time of the day. Studying these hypothalamic circuits in more detail is necessary to gain a deeper understanding of the leptin system in physiological and pathological conditions.

Chapter 4. General discussion and conclusions

4.1 Metabolic syndrome: a circadian disease?

As discussed throughout the present thesis, circadian disruption is closely associated with fat accumulation, glucose intolerance, dyslipidemias, and cardiovascular complications, all hallmarks of metabolic syndrome. The relationship between the circadian system and metabolism is such that some authors have even proposed the term “Circadian syndrome” to refer to the idea that metabolic syndrome directly arises from disrupting the circadian system (Zimmet et al., 2019).

As reviewed in Chapters 1 and 2, constant light and day-feeding in rats can disturb the circadian system and promote glucose intolerance and fat accumulation. Furthermore, in Chapter 2, we described a novel condition promoting metabolic impairments: a skeleton photoperiod. Importantly, the different environmental conditions (constant light, food intake during the resting phase, and the skeleton photoperiod) seem to disrupt the circadian system in different ways (Figure 4.1). While constant light abolishes all circadian rhythms (i.e., food intake, locomotor activity, clock genes) (Guerrero-Vargas et al., 2017; Nováková et al., 2011; Wideman & Murphy, 2009), food during the day shifts temperature rhythms and uncouples peripheral clocks but does not change activity rhythms (Opperhuizen et al., 2016; Salgado-Delgado et al., 2010). Conversely, the skeleton photoperiod maintains a general synchronization of most rhythms and only delays activity and temperature at the beginning of the active period (Chapter 2). These observations support the idea that any circadian disturbance —big or small— can impact metabolism.

Nevertheless, some questions remain: 1) how can different circadian disturbances have a similar metabolic effect? 2) what mechanisms drive metabolic dysfunction as a consequence of circadian disruption? These questions will be discussed below.

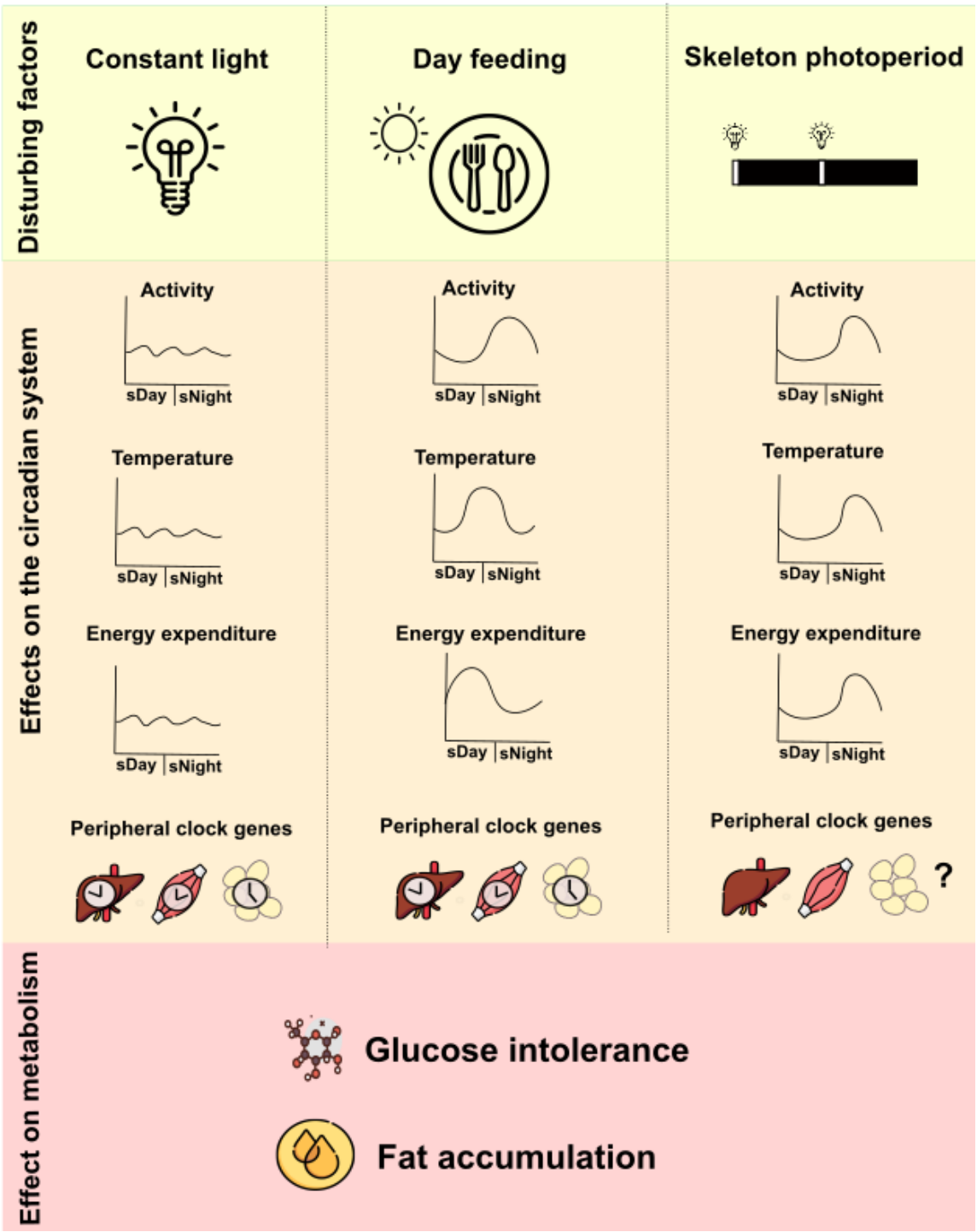

Figure 4.1. The effects of constant light, food during the day, and a skeleton photoperiod on the circadian system and metabolism in rats. Constant Light desynchronizes activity, temperature, and energy expenditure rhythms; it also promotes desynchrony among peripheral organs. Day-feeding shifts temperature and energy expenditure into the day without changing activity patterns. Furthermore, a skeleton photoperiod delays activity, temperature, and energy expenditure, without affecting overall synchronization. The effect of a skeleton photoperiod on peripheral clock genes has not been assessed yet. Despite different changes in the circadian system, the three conditions result in glucose intolerance and fat accumulation.

4.2 Mechanisms for circadian disruption and metabolic syndrome

4.2.1 Constant light, day-feeding, and skeleton photoperiod: different pathways to metabolic syndrome

As discussed, the suprachiasmatic nucleus (SCN) of the hypothalamus coordinates the circadian rhythms of the whole body. This coordination takes place via its influence on other hypothalamic structures that regulate different processes such as food intake, energy balance, body temperature, and locomotor activity. Therefore, disrupting optimal functioning of the hypothalamus by means of constant light, day feeding, or a skeleton photoperiod could underlie the metabolic disturbances observed in those conditions. Furthermore, because the three conditions affect circadian rhythms differentially, the affected hypothalamic pathways should be different, or they might be affected in a different measure (Figure 4.2).

4.2.1.1 Constant light

Since light directly activates the SCN through the retino-hypothalamic tract, constant light activates the SCN, eventually leading to the loss of clock gene oscillations in this nucleus (Coomans et al., 2013; Nováková et al., 2011). Consequently, the rhythm in hormones like corticosterone and melatonin, which serve as a time signal for peripheral organs, is also lost (Báez-Ruiz et al., 2017). Therefore, no “time signal” is conveyed to the rest of the body, and the peripheral organs cannot optimize the use of resources according to the time of the day. The lack of oscillating functions, i.e., “flexible” use of energy, could eventually lead to a reprogramming of the tissues into a non-flexible, anabolic state (Guerrero-Vargas et al., 2017), promoting fat accumulation and glucose intolerance. More experiments are necessary to test whether the lack of oscillating functions is the triggering factor that promotes a “non-flexible” anabolic state in organs like the liver and adipose tissue.

4.2.1.2 Skeleton photoperiod

Because the skeleton photoperiod maintains general synchronization of daily rhythms, there is no reason to assume a complete loss of SCN clock gene oscillations under this condition. Indeed, one study found oscillating clock genes in the SCN of rats exposed to a 1-hour-light-pulse skeleton photoperiod (Challet et al., 2003). However, in the same study, *Per1* and *Per2* genes had a slightly decreased amplitude in the skeleton photoperiod compared to a full light-dark period (Challet et al., 2003). Moreover, the rhythm of c-fos (a marker of neuronal activity) in the SCN is less robust under skeleton photoperiod conditions compared to a full light-dark condition (Schwartz et al., 1994). Since the skeleton photoperiod induces slight

delays in temperature, energy expenditure, and activity (Chapter 2), these data suggest that robust SCN activity is necessary to maintain robust oscillations in hormones and the ANS to ensure proper anticipation of each circadian period.

It is still necessary to evaluate how hormones such as corticosterone and melatonin change under skeleton photoperiod conditions in rats. However, some evidence suggests acute changes in the ANS after daytime light pulses; light pulses given in the daytime increase sympathetic nerve activity in the visceral compartment (splachnic nerve) (Niijima et al., 1993). Activation of sympathetic efferents in the liver is known to promote glucose production and inhibit glycogenesis (Mizuno & Ueno, 2016; Yi et al., 2010). Therefore, sympathetic activation by a light pulse could acutely decrease the liver's capacity to capture glucose, maintaining circulating glucose levels high during the glucose tolerance test (as observed in this study), but this possibility remains to be tested.

Interestingly, the circadian increase in body temperature is also associated with sympathetic activation (Orozco-Solís et al., 2016a). Thus, the fact that rats under skeleton photoperiod do not show the expected temperature increase at the beginning of the active phase indicates that either light pulses in the day do not promote sympathetic activation, or that the light-induced sympathetic activation is not generalized, but different in each organ. In agreement with the latter, hypothalamic SCN neurons communicate with peripheral tissues through separate pre-autonomic neurons (Buijs et al., 2003), providing an anatomical framework to modulate the ANS outflow to each organ in a differential manner.

Future studies should address how hypothalamic circuits are modified under skeleton photoperiod conditions and how these modifications change ANS outflow to each organ. This is especially important because some authors have proposed that metabolic syndrome may arise from a disbalance between the two branches of the ANS, the SNS and the PSNS, in several body compartments (Kreier et al., 2003). The proposal states, for example, that there is an overall increase in the PSNS tone to the abdominal organs (promoting higher insulin release by the pancreas and higher glucose uptake in the adipose tissue) and, at the same time, a higher SNS tone to other organs like the heart (promoting hypertension). Therefore, studying ANS changes under the skeleton photoperiod could provide insight into the mechanisms promoting glucose intolerance and fat accumulation in a model that does not induce complete loss of circadian rhythms.

4.2.1.3 Day feeding

Finally, day feeding affects the circadian system differently. It has already been reported that food-related signals can entrain clock genes in some organs but not in the SCN (Mukherji et al., 2015). Since the SCN imposes its rhythm on peripheral organs, the contradiction of SCN-derived and food-derived signals in each organ might impede proper clock gene synchronization. Indeed, clock gene oscillations in different organs are either lost or not synchronized to the master clock's rhythm (Bechtold et al., 2010; Salgado-Delgado et al., 2008; Salgado-Delgado et al., 2013; Wang et al., 2017). Since organs also communicate via hormones and metabolites, the lack of synchronization in each organ could further affect the others, generating a feed-forward desynchronization phenomenon. In addition, although food-related

signals do not change SCN clock genes, they provide feedback signals integrated within the hypothalamus. Therefore, other nuclei within the hypothalamus could also lose their rhythmic functions, as discussed in Méndez-Hernández, Escobar, & Buijs, (2020) (added in the supplementary information). Overall, this process would also impede energetic flexibility in metabolic tissues, similar to what occurs in constant light conditions. These aspects are illustrated in Figure 4.2.

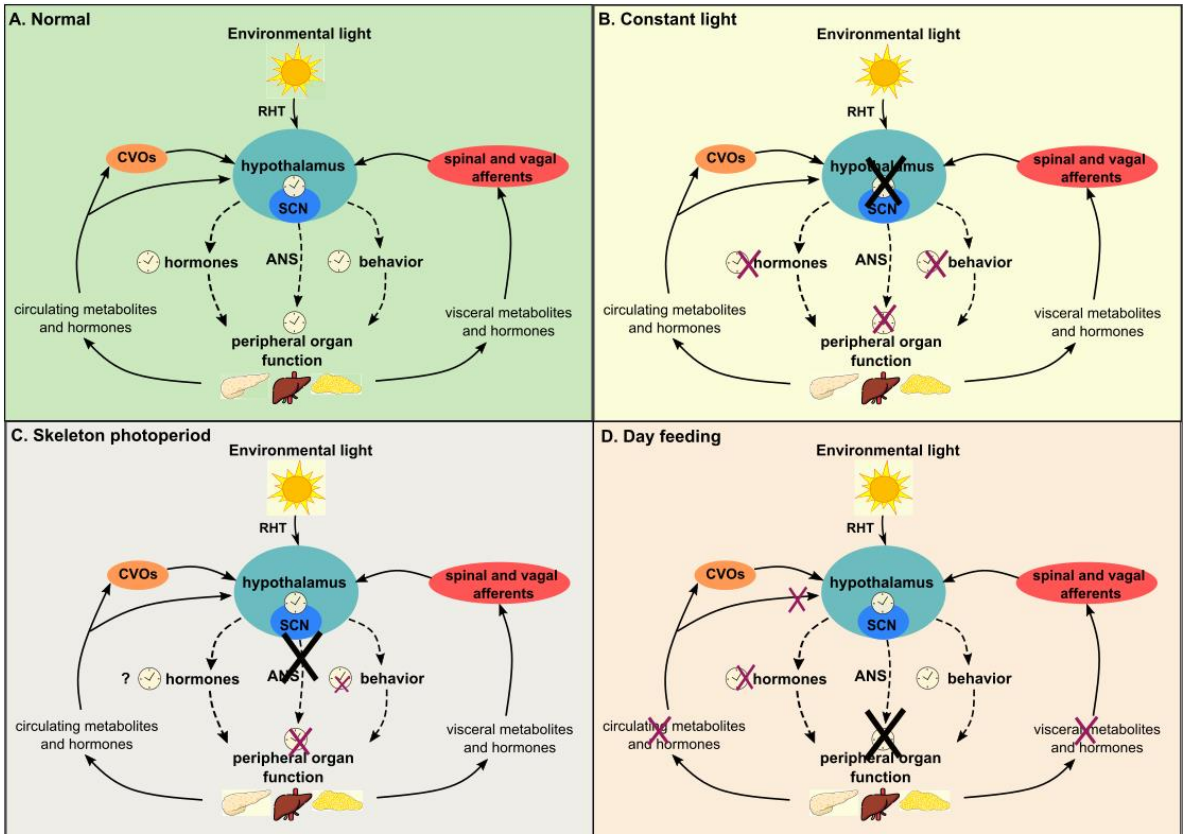

Figure 4.2. Proposed mechanisms for metabolic impairments due to different types of circadian disruption. A) Normal functioning of the circadian system involves the integration of external and internal stimuli in accordance with the time of the day (for further detail, see Figure 1.1). B) Constant light Initially affects the capacity of the SCN to synchronize to a circadian cycle, consequently affecting the synchronization of the whole body. C) The light pulses of a skeleton photoperiod

mainly affect the ANS, promoting a disbalance in sympathetic and parasympathetic input to the organs affecting their functioning. The effects of the skeleton photoperiod on hormones and feedback to the brain were not assessed. D) Day-feeding affects proper clock gene synchronization among peripheral organs, producing untimely feedback signals to the brain and consequently affecting whole-body homeostasis. Black “X” marks indicate the systems that are initially affected, and red “X” marks indicate systems that are affected in consequence.

4.3 Modulating hormones and the ANS: an approach for treating metabolic syndrome?

As discussed in the previous section, constant light, skeleton photoperiod, and day-feeding promote metabolic disruption in rats through different but overlapping mechanisms. One of the most important overlapping mechanisms is that the communication pathways between the hypothalamus and the periphery (i.e., hormones and the ANS) are modified in these different conditions due to hypothalamic changes. Therefore, it is interesting to investigate whether some of these changes could be prevented by applying hormones at the proper time or by modulating autonomic activity.

4.3.1 Hormonal changes in circadian disruption.

Many hormones change in conditions of circadian disruption, including corticosterone, melatonin, leptin, ghrelin, and growth hormone, among others (Bedrosian, Fonken, & Nelson, 2016; Kim, Jeong, & Hong, 2015). Here, we will discuss a few that directly impact metabolism: corticosterone, melatonin, and leptin.

4.3.1.1 Corticosterone and melatonin

The importance of hormones in reestablishing metabolic homeostasis is illustrated by a recent study using rats under constant light conditions (Báez-Ruiz et al., 2017). In this study, animals under a light-dark cycle showed the expected corticosterone peak at the day-night transition, but animals under constant light showed constant low levels of this hormone (i.e., no corticosterone rhythm). Also, animals under constant light did not show the expected nocturnal peak in circulating melatonin; rather, melatonin production was suppressed by the light. These changes in melatonin and corticosterone were accompanied by hyperglycemia, hyperinsulinemia, glucose intolerance, and fat accumulation. However, hyperglycemia and glucose tolerance were prevented when the animals under constant light were subjected to a 12-hour food restriction and supplementation with corticosterone and melatonin at the proper phase. These data reinforce the idea that proper synchronization of hormones and behavior can reestablish glucose homeostasis in conditions of circadian desynchrony. This conclusion is supported by the observation that when the hormones were given out of phase with the food, all the beneficial effects of the hormones disappeared (Báez-Ruiz et al., 2017). The contributions of other hormones in reestablishing metabolic homeostasis under circadian desynchronization conditions have not been investigated.

4.3.1.2 Leptin

As discussed in Chapter 3, leptin is another hormone for metabolic regulation, but the contribution of leptin to the pathogenesis of the metabolic syndrome is still not understood. Data from the present thesis (Chapter 3) indicate that leptin's access

into the hypothalamus shows a circadian pattern, with a higher entrance of leptin in the night-day transition period (ZT22-2). In addition, leptin's detection in the arcuate nucleus (ARC) during this period seems important to signal that energy reserves are not under threat, i.e., that the animal is not under fasting conditions and does not need to implement energy-saving adaptations. In constant light (Maroni et al., 2018) and day-feeding conditions (Martínez-Merlos et al., 2004), leptin levels during the nighttime are lower. Therefore, it is possible that, under these conditions, the lower leptin levels at night are insufficient to signal that energy reserves are not under threat. Consequently, the hypothalamus would implement the necessary mechanisms to adapt to a fasting condition that is not actually present. Since one of the leptin-mediated adaptations to fasting is the temperature decrease at the night-day transition period (Chapter 3), the fact that day-fed animals show a lower temperature in this period (Chapter 2) further supports this hypothesis. In addition, as discussed in Méndez-Hernández, Escobar, & Buijs (2020), metabolic syndrome has been associated with an incapacity of the brain to properly sense circulating glucose levels; therefore, a similar situation could be happening with leptin. In view of this explanation, leptin resistance could be interpreted as an incapacity of the hypothalamus to detect leptin in the night-day transition period, promoting an anabolic, fasting-like phenotype. More research is necessary to understand hypothalamic leptin signaling under conditions of circadian disruption and to evaluate whether reestablishing leptin signaling in these circuits could improve metabolism.

4.3.2 ANS changes in metabolic syndrome

In addition to hormonal changes, disrupting hypothalamic function impacts ANS balance. Autonomic disbalance has been proposed as a cause of metabolic syndrome, where visceral organs receive an increased parasympathetic stimulation that promotes an anabolic phenotype (Kreier et al., 2003). Several epidemiological studies (Boer-Martins et al., 2011; Wulsin et al., 2015) and studies in animals (Kreier et al., 2002, 2006; Lehnen et al., 2013) support this hypothesis; however, because of the difficulty in targeting autonomic efferents specifically in each organ, reestablishing autonomic balance proves difficult. Nevertheless, recent studies in humans using vagus nerve stimulation have reported a decrease in body weight after several weeks of treatment (de Lartigue, 2016). Importantly, the vagus carries both motor and sensory nerves, so the effect cannot be attributed only to parasympathetic activation, but sensory nerves might also be involved.

4.3 Conclusion

As discussed throughout this thesis, the circadian system integrates information from the environment and the internal milieu to optimize efficient use of energy throughout the daily cycle. Metabolic homeostasis depends on the proper alignment of all circadian variables. Conditions like constant light, food during the resting phase, and a skeleton photoperiod, as described in this thesis for the first time, can all disrupt the circadian system. Although the different conditions disrupt the circadian system to different degrees and through various mechanisms, they all eventually lead to glucose intolerance and fat accumulation. More research is necessary to understand how these conditions change hypothalamic functioning and the role of hormones like

leptin in this process. Given the worldwide obesity epidemic, gaining knowledge of these mechanisms is essential for establishing a strategy to prevent metabolic syndrome among the population.

References

Adam, K. (1989). Human body temperature is inversely correlated with body mass. *European Journal of Applied Physiology and Occupational Physiology*, *58*(5), 471–475. https://doi.org/10.1007/BF02330699

Ahima, R. S., Prabakaran, D., Mantzoros, C., Qu, D., Lowell, B., Maratos-Flier, E., & Flier, J. S. (1996). Role of leptin in the neuroendocrine response to fasting. *Nature*, Vol. 382, pp. 250–252. https://doi.org/10.1038/382250a0

Ahima, Rexford S. (2000). Leptin and the neuroendocrinology of fasting. *Frontiers in Hormonal Research*, *26*(3), 42–56. Retrieved from http://www.scopus.com/inward/record.url?eid=2-s2.0-0346957344&partnerID=40&md5=2ec14e606f5151a5660d2cf65c6569f5

Ahima, Rexford S. (2008). Revisiting leptin's role in obesity and weight loss. *Journal of Clinical Investigation*, *118*(7), 2380–2383. https://doi.org/10.1172/JC136284

Báez-Ruiz, A., Guerrero-Vargas, N. N., Cázarez-Márquez, F., Sabath, E., Basualdo, M. D. C., Salgado-Delgado, R., … Buijs, R. M. (2017). Food in synchrony with melatonin and corticosterone relieves constant light disturbed metabolism. *The Journal of Endocrinology*, *235*(3), 167–178. https://doi.org/10.1530/JOE-17-0370

Balland, E., Dam, J., Langlet, ., Caron, E., Steculorum, S., Messina, A., … révot, V. (2014). Hypothalamic tanycytes are an ERK-gated conduit for leptin into the brain. *Cell Metabolism*, *19*(2), 293–301. https://doi.org/10.1016/j.cmet.2013.12.015

Baver, S. B., Hope, K., Guyot, S., Bjorbaek, C., Kaczorowski, C., & O'Connell, K. M. S. (2014). Leptin Modulates the Intrinsic Excitability of AgRP/NPY Neurons in the Arcuate Nucleus of the Hypothalamus. *Journal of Neuroscience*, *34*(16), 5486–5496. https://doi.org/10.1523/JNEUROSCI.4861-12.2014

Bechtold, D. a, Gibbs, J. E., & Loudon, A. S. I. (2010). Circadian dysfunction in disease. *Trends in Pharmacological Sciences*, *31*(5), 191–198. https://doi.org/10.1016/j.tips.2010.01.002

Bedrosian, T. A., Fonken, L. K., & Nelson, R. J. (2016). Endocrine Effects of Circadian Disruption. *Annual Review of Physiology*, *78*, 109–131. https://doi.org/10.1146/annurev-physiol-021115-105102

Belgardt, B. F., Okamura, T., & Brüning, J. C. (2009). Hormone and glucose signalling in POMC and AgRP neurons. *Journal of Physiology*, *587*(22), 5305–5314. https://doi.org/10.1113/jphysiol.2009.179192

Bell, B. B., Harlan, S. M., Morgan, D. A., Guo, D. F., & Rahmouni, K. (2018). Differential contribution of POMC and AgRP neurons to the regulation of regional autonomic nerve activity by leptin. *Molecular Metabolism*, *8*, 1–12.

https://doi.org/10.1016/j.molmet.2017.12.006

Bodosi, B., Gardi, J., Hajdu, I., Szentirmai, E., Obal, F., & Krueger, J. M. (2004). Rhythms of ghrelin, leptin, and sleep in rats: effects of the normal diurnal cycle, restricted feeding, and sleep deprivation. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *287*(5), R1071–R1079. https://doi.org/10.1152/ajpregu.00294.2004

Boer-Martins, L., Figueiredo, V. N., Demacq, C., Martins, L. C., Consolin-Colombo, ., i gueiredo, M. J., … Moreno, H. (2011). Relationship of autonomic imbalance and circadian disruption with obesity and type 2 diabetes in resistant hypertensive patients. *Cardiovascular Diabetology*, *10*(1), 24. https://doi.org/10.1186/1475-2840-10-24

Bonham, M. P., Bonell, E. K., & Huggins, C. E. (2016). Energy intake of shift workers compared to fixed day workers: A systematic review and meta-analysis. *Chronobiology International*, *33*(8), 1086–1100. https://doi.org/10.1080/07420528.2016.1192188

Buijs, R. M., Escobar, C., & Swaab, D. F. (2013). The circadian system and the balance of the autonomic nervous system. In *Handbook of Clinical Neurology* (Vol. 117, pp. 173–191). https://doi.org/10.1016/B978-0-444-53491-0.00015-8

Buijs, R.M., Guzmán Ruiz, M. A., Méndez Hernández, R., & Rodríguez Cortés, B. (2019). The suprachiasmatic nucleus; a responsive clock regulating homeostasis by daily changing the setpoints of physiological parameters. *Autonomic Neuroscience: Basic and Clinical*, *218*. https://doi.org/10.1016/j.autneu.2019.02.001

Buijs, Ruud M. (1999). Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. *European Journal of Neuroscience*, *11*(5), 1535–1544. https://doi.org/10.1046/j.1460-9568.1999.00575.x

Buijs, Ruud M. (2013). The autonomic nervous system; a balancing act. In *Handbook of Clinical Neurology Autonomic Nervous System* (1st ed., Vol. 117, pp. 1–11). https://doi.org/10.1016/B978-0-444-53491-0.00001-8

Buijs, Ruud M., La Fleur, S. E., Wortel, J., Van Heyningen, C., Zuiddam, L., Mettenleiter, T. C., … Niijima, A. (2003). The suprachiasmatic nucleus balances sympathetic and parasympathetic output to peripheral organs through separate preautonomic neurons. *Journal of Comparative Neurology*, *464*(1), 36–48. https://doi.org/10.1002/cne.10765

Buijs, Ruud M, Escobar, C., & Swaab, D. F. (2013). The circadian system and the balance of the autonomic nervous system. In *Handbook of clinical neurology* (1st ed., Vol. 117, pp. 173–191). https://doi.org/10.1016/B978-0-444-53491-0.00015-8

Cailotto, C., Lei, J., van der Vliet, J., van Heijningen, C., van Eden, C. G., Kalsbeek, A., … Buijs, R. M. (2009). Effects of nocturnal light on (clock) gene expression

in peripheral organs: A role for the autonomic innervation of the liver. *PLoS ONE*, *4*(5), 1–12. https://doi.org/10.1371/journal.pone.0005650

Caron, A., Lee, S., Elmquist, J. K., & Gautron, L. (2018). Leptin and brain-adipose crosstalks. *Nature Reviews Neuroscience*, *19*(3), 153–165. https://doi.org/10.1038/nrn.2018.7

Cedernaes, J., Huang, W., Ramsey, K. M., Waldeck, N., Cheng, L., Marcheva, B., … Bass, J. (2019). Transcriptional Basis for Rhythmic Control of Hunger and Metabolism within the AgRP Neuron. *Cell Metabolism*, *29*(5), 1078-1091.e5. https://doi.org/10.1016/j.cmet.2019.01.023

Challet, E. (2019). The circadian regulation of food intake. *Nature Reviews Endocrinology*, *15*(7), 393–405. https://doi.org/10.1038/s41574-019-0210-x

Challet, E., Poirel, V. J., Malan, A., & Pévet, P. (2003). Light exposure during daytime modulates expression of Per1 and Per2 clock genes in the suprachiasmatic nuclei of mice. *Journal of Neuroscience Research*, *72*(5), 629–637. https://doi.org/10.1002/jnr.10616

Chronwall, B. M. (1985). Anatomy and Physiology of the Neuroendocrine Arcuate Nucleus. *Peptides*, *6*(2), 1–11.

Ciofi, P. (2011). The arcuate nucleus as a circumventricular organ in the mouse. *Neuroscience Letters*, *487*(2), 187–190. https://doi.org/10.1016/j.neulet.2010.10.019

Clarke, I. J. (2015). Hypothalamus as an endocrine organ. *Comprehensive Physiology*, *5*(1), 217–253. https://doi.org/10.1002/cphy.c140019

Clough, G. (1982). Environmental effects on animals used in biomedical research. *Biological Reviews*, *57*(3), 487–523. https://doi.org/10.1111/j.1469-185X.1982.tb00705.x

Coomans, C. P., van den Berg, S. a a, Houben, T., van Klinken, J.-B., van den Berg, R., ronk, A. C. M., … Meijer, J. H. (2013). Detrimental effects of constant light exposure and high-fat diet on circadian energy metabolism and insulin sensitivity. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology*, *27*(4), 1721–1732. https://doi.org/10.1096/fj.12-210898

Corbalán-Tutau, M. D., Madrid, J. A., Ordovás, J. M., Smith, C. E., Nicolás, F., & Garaulet, M. (2011). Differences in daily rhythms of wrist temperature between obese and normal-weight women: Associations with metabolic syndrome features. *Chronobiology International*, *28*(5), 425–433. https://doi.org/10.3109/07420528.2011.574766

Cottrell, G. T., & Ferguson, A. V. (2004). Sensory circumventricular organs: Central roles in integrated autonomic regulation. *Regulatory Peptides*, *117*(1), 11–23. https://doi.org/10.1016/j.regpep.2003.09.004

Coutinho, E. A., Okamoto, S., Ishikawa, A. W., Yokota, S., Wada, N., Hirabayashi,

T., … Minokoshi, Y. (2017). Activation of S 1 neurons in the ventromedial hypothalamus by DREADD technology increases insulin sensitivity in peripheral tissues. *Diabetes*, *66*(9), 2372–2386. https://doi.org/10.2337/db16-1344

Cowley, M. A., Smart, J. L., Rubinstein, M., Cerdán, M. G., Diano, S., Horvath, T. L., … Low, M. J. (2001). Leptin activates anorexigenic OMC neurons through a neural network in the arcuate nucleus. *Nature*, *411*(6836), 480–484. https://doi.org/10.1038/35078085

Daan, S., & Pittendrigh, C. S. (1976). A Functional analysis of circadian pacemakers in nocturnal rodents - II. The variability of phase response curves. *Journal of Comparative Physiology ■ A*, *106*(3), 253–266. https://doi.org/10.1007/BF01417857

Damiola, F., Le Minli, N., Preitner, N., Kornmann, B., Fleury-Olela, F., & Schibler, U. (2000). Restricted feeding uncouples circadian oscillators in peripheral tissues from the central pacemaker in the suprachiasmatic nucleus. *Genes and Development*, *14*(23), 2950–2961. https://doi.org/10.1101/gad.183500

de Lartigue, G. (2016). Role of the vagus nerve in the development and treatment of diet-induced obesity. *The Journal of Physiology*, *0*, 1–25. https://doi.org/10.1113/JP271538

Depner, C. M., Melanson, E. L., Eckel, R. H., Stothard, E. R., Morton, S. J., Wright, K. ., … erreault, L. (2019). Ad libitum Weekend Recovery Sleep a ils to Prevent Metabolic Dysregulation during a Repeating Pattern of Insufficient Sleep and Weekend Recovery Sleep Article Ad libitum Weekend Recovery Sleep Fails to Prevent Metabolic Dysregulation during a Repeating Pa. *Current Biology*, *29*(6), 957-967.e4. https://doi.org/10.1016/j.cub.2019.01.069

Díez-Noguera, A. (2013). Methods for serial analysis of long time series in the study of biological rhythms. *Journal of Circadian Rhythms*, *11*(1), 1. https://doi.org/10.1186/1740-3391-11-7

Dubuc, P. U., Wilden, N. J., & Carlisle, H. J. (1985). Fed and Fasting Thermoregulation in ob/ob mice. *Annals of Nutrition and Metabolism*, *29*, 358–365.

Dumont, M., Benhaberou-Brun, D., & Paquet, J. (2001). Profile of 24-h light exposure and circadian phase of melatonin secretion in night workers. *Journal of Biological Rhythms*, *16*(5), 502–511. https://doi.org/10.1177/074873001129002178

Duncan, M. J., Smith, J. T., Narbaiza, J., Mueez, ., Bustle, L. B., Qureshi, S., … Legan, S. J. (2016). Restricting feeding to the active phase in middle-aged mice attenuates adverse metabolic effects of a high-fat diet. *Physiology and Behavior*, *167*, 1–9. https://doi.org/10.1016/j.physbeh.2016.08.027

Duvernoy, H. M., & Risold, P. Y. (2007). The circumventricular organs: An atlas of comparative anatomy and vascularization. *Brain Research Reviews*, *56*(1), 119–147. https://doi.org/10.1016/j.brainresrev.2007.06.002

El-Haschimi, K., Pierroz, D. D., Hileman, S. M., Bjørbæk, C., & Flier, J. S. (2000). Two defects contribute to hypothalamic leptin resistance in mice with diet-induced obesity. *Journal of Clinical Investigation*, *105*(12), 1827–1832. https://doi.org/10.1172/JCI9842

Escobar, C., Díaz-Muñoz, M., Encinas, F., & Aguilar-Roblero, R. (1998). Persistence of metabolic rhythmicity during fasting and its entrainment by restricted feeding schedules in rats. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*, *274*(5), R1309–R1316.

Fei, H., Okano, H. J., Li, C., Lee, G. H., Zhao, C., Darnell, R., & Friedman, J. M. (1997). Anatomic localization of alternatively spliced leptin receptors (Ob-R) in mouse brain and other tissues. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(13), 7001–7005. https://doi.org/10.1073/pnas.94.13.7001

Fischer, A. W., Hoefig, C. S., Abreu-Vieira, G., de Jong, J. M. A., Petrovic, N., Mittag, J., … Nedergaard, J. (2016). Leptin Raises Defended Body Temperature without Activating Thermogenesis. *Cell Reports*, *14*(7), 1621–1631. https://doi.org/10.1016/j.celrep.2016.01.041

Flier, J. S., & Maratos- l ier, E. (2017). Leptin's hysiologic Role: Does the Emperor of Energy Balance Have No Clothes? *Cell Metabolism*, *26*(1), 24–26. https://doi.org/10.1016/j.cmet.2017.05.013

Flôres, D. E. F. L., & Oda, G. A. (2020). Quantitative Study of Dual Circadian Oscillator Models under Different Skeleton Photoperiods. *Journal of Biological Rhythms*, *35*(3), 302–316. https://doi.org/10.1177/0748730420901939

Fonken, L. K., & Nelson, R. J. (2014). The effects of light at night on circadian clocks and metabolism. *Endocrine Reviews*, *35*(4), 648–670. https://doi.org/10.1210/er.2013-1051

Friedman, J. M., & Halaas, J. L. (1998). Leptin and the regulation of body weight in mammals. *Nature*, *395*(6704), 763–770. https://doi.org/10.1038/27376

Fujikawa, T., Castorena, C. M., Pearson, M., Kusminski, C. M., Ahmed, N., Battiprolu, . K., … Elmquist, J. K. (2016). SF-1 expression in the hypothalamus is required for beneficial metabolic effects of exercise. *ELife*, *5*(NOVEMBER2016), 1–17. https://doi.org/10.7554/eLife.18206

Golombek, D. A., & Rosenstein, R. E. (2010). Physiology of Circadian Entrainment. *Physiological Reviews*, *90*, 1063–1102. https://doi.org/10.1152/physrev.00009.2009.

Guerrero-Vargas, N. N., López-Bago, A., Navarro-Espíndola, R., Espitia-Bautista, E., Basualdo, M. del C., Buijs, R. M., … Lascurain, R. (2017). Circadian disruption promotes tumor growth by anabolic host metabolism; experimental evidence in a rat model. *BMC Cancer*, *17*(1), 1–13. https://doi.org/10.1186/s12885-017-3636-3

Guzmán-Ruiz, M. A., Ramirez-Corona, A., Guerrero-Vargas, N. N., Sabath, E., Ramirez-Plascencia, O. D., Fuentes-Romero, R., … Buijs, R. M. (2015). Role of the Suprachiasmatic and Arcuate Nuclei in Diurnal Temperature Regulation in the Rat. *The Journal of Neuroscience*, *35*(46), 15419–15429. https://doi.org/10.1523/JNEUROSCI.1449-15.2015

Guzmán-Ruiz, M., Saderi, N., Cazarez-Márquez, F., Guerrero-Vargas, N. N., Basualdo, M. C., Acosta-Galván, G., & Buijs, R. M. (2014). The suprachiasmatic nucleus changes the daily activity of the arcuate nucleus α-MSH neurons in male rats. *Endocrinology*, *155*(2), 525–535. https://doi.org/10.1210/en.2013-1604

Halaas, J. L., Boozer, C., Blair-West, J., Fidahusein, N., Denton, D. A., & Friedman, J. M. (1997). Physiological response to long-term peripheral and central leptin infusion in lean and obese mice. *Proceedings of the National Academy of Sciences of the United States of America*, *94*(16), 8878–8883. https://doi.org/10.1073/pnas.94.16.8878

Han, S., Yu, . H., Schwartz, M. D., Linton, J. D., Bosma, M. M., Hurley, J. B., … De La Iglesia, H. O. (2012). Na V1.1 channels are critical for intercellular communication in the suprachiasmatic nucleus and for normal circadian rhythms. *Proceedings of the National Academy of Sciences of the United States of America*, *109*(6), 1–10. https://doi.org/10.1073/pnas.1115729109

Harms, P. G., & Ojeda, S. R. (1974). A rapid and simple procedure for chronic cannulation of the rat jugular vein. *Journal of Applied Physiology*, *36*(3), 391–392. https://doi.org/10.1152/jappl.1974.36.3.391

Hastings, M H, Brancaccio, M., & Maywood, E. S. (2014). Circadian pacemaking in cells and circuits of the suprachiasmatic nucleus. *Journal of Neuroendocrinology*, *26*(1), 2–10. https://doi.org/10.1111/jne.12125

Hastings, Michael H., Maywood, E. S., & Brancaccio, M. (2019). The mammalian circadian timing system and the suprachiasmatic nucleus as its pacemaker. *Biology*, *8*(1), 1–22. https://doi.org/10.3390/biology8010013

Hatori, M., Vollmers, C., Zarrinpar, A., Ditacchio, L., Bushong, E. a, Gill, S., … Panda, S. (2012). Time-Restricted Caloric Intake Prevents Metabolic Diseases in Mice Fed a High-Fat Diet. *Cell Metabolism*, *15*, 848–860. https://doi.org/10.1016/j.cmet.2012.04.019

Heiser, A. (2007). Rat Jugular Vein and Carotid Artery Catheterization for Acute Survival Studies. In *Springer*. https://doi.org/10.1007/0-387-49416-2

Honma, K., Katabami, F., & Hiroshige, T. (1978). A phase response curve for the locomotor activity in the rat. *Specialia*, 1602–1603.

Ishida, A., Mutoh, T., Ueyama, T., Bando, H., Masubuchi, S., Nakahara, D., … Okamura, H. (2005). Light activates the adrenal gland: timing of gene expression and glucocorticoid release. *Cell Metabolism*, *2*(5), 297–307. https://doi.org/10.1016/j.cmet.2005.09.009

Jud, C., Schmutz, I., Hampp, G., Oster, H., & Albrecht, U. (2005). A guideline for analyzing circadian wheel-running behavior in rodents under different lighting conditions. *Biological Procedures Online*, *7*(1), 101–116. https://doi.org/10.1251/bpo109

Kaeffer, B., & Pardini, L. (2005). Clock genes of mammalian cells: Practical implications in tissue culture. *In Vitro Cellular and Developmental Biology - Animal*, *41*(10), 311–320. https://doi.org/10.1290/0506044.1

Kalsbeek, a, Fliers, E., Romijn, J. a, & Wortel, J. (2015). *The Suprachiasmatic Nucleus Generates the Diurnal Changes in Plasma Leptin Levels*. *142*(6).

Kalsbeek, a, Palm, I. F., La Fleur, S. E., Scheer, F. a J. L., Perreau-Lenz, S., Ruiter, M., … Buijs, R. M. (2006). SCN outputs and the hypothalamic balance of life. *Journal of Biological Rhythms*, *21*(6), 458–469. https://doi.org/10.1177/0748730406293854

Kalsbeek, A., Scheer, F. A., Perreau-Lenz, S., La Fleur, S. E., Yi, C.-X., Fliers, E., & Buijs, R. M. (2011). Circadian Disruption and SCN Control of Energy Metabolism. *FEBS Letters*, *585*(10), 1412–1426. https://doi.org/10.1016/j.febslet.2011.03.021.CIRCADIAN

Kim, T. W., Jeong, J. H., & Hong, S. C. (2015). The impact of sleep and circadian disturbance on hormones and metabolism. *International Journal of Endocrinology*, *2015*. https://doi.org/10.1155/2015/591729

King, B. M. (2006). The rise, fall, and resurrection of the ventromedial hypothalamus in the regulation of feeding behavior and body weight. *Physiology and Behavior*, *87*(2), 221–244. https://doi.org/10.1016/j.physbeh.2005.10.007

Kolbe, I., Leinweber, B., Brandenburger, M., & Oster, H. (2019). Circadian clock network desynchrony promotes weight gain and alters glucose homeostasis in mice. *Molecular Metabolism*, *30*(October), 140–151. https://doi.org/10.1016/j.molmet.2019.09.012

Kreier, F., Fliers, E., Voshol, P. J., Van Eden, C. G., Havekes, L. M., Kalsbeek, A., … Buijs, R. M. (2002). Selective parasympathetic innervation of subcutaneous and intra-abdominal fat - Functional implications. *Journal of Clinical Investigation*, *110*(9), 1243–1250. https://doi.org/10.1172/JCI200215736

Kreier, F., Kap, Y. S., Mettenleiter, T. C., Van Heijningen, C., Van Der Vliet, J., Kalsbeek, A., … Buijs, R. M. (2006). Tracing from fat tissue, liver, and pancreas: A neuroanatomical framework for the role of the brain in type 2 diabetes. *Endocrinology*, *147*(3), 1140–1147. https://doi.org/10.1210/en.2005-0667

Kreier, F., Yilmaz, A., Kalsbeek, A., Romijn, J. a., Sauerwein, H. P., Fliers, E., & Buijs, R. M. (2003). Hypothesis: Shifting the Equilibrium from Activity to Food Leads to Autonomic Unbalance and the Metabolic Syndrome. *Diabetes*, *52*(11), 2652–2656. https://doi.org/10.2337/diabetes.52.11.2652

Krieger, J. P., Langhans, W., & Lee, S. J. (2015). Vagal mediation of GLP-1's effects

on food intake and glycemia. *Physiology and Behavior*, *152*, 372–380. https://doi.org/10.1016/j.physbeh.2015.06.001

la Fleur, S. E. (2003). Daily rhythms in glucose metabolism: Suprachiasmatic nucleus output to peripheral tissue. *Journal of Neuroendocrinology*, *15*(3), 315–322. https://doi.org/10.1046/j.1365-2826.2003.01019.x

La Fleur, S. E., Kalsbeek, A., Wortel, J., & Buijs, R. M. (1999). A suprachiasmatic nucleus generated rhythm in basal glucose concentrations. *Journal of Neuroendocrinology*, *11*(8), 643–652. https://doi.org/10.1046/j.1365-2826.1999.00373.x

Landsberg, L., Young, J. B., Leonard, W. R., Linsenmeier, R. A., & Turek, F. W. (2009). Is obesity associated with lower body temperatures? Core temperature: a forgotten variable in energy balance. *Metabolism: Clinical and Experimental*, *58*(6), 871–876. https://doi.org/10.1016/j.metabol.2009.02.017

Langlet, F., Levin, B. E., Luquet, S., Mazzone, M., Messina, A., Dunn-Meynell, A. A., … Dehouck, B. (2013). Tanycytic VEG -A boosts blood-hypothalamus barrier plasticity and access of metabolic signals to the arcuate nucleus in response to fasting. *Cell Metabolism*, *17*(4), 607–617. https://doi.org/10.1016/j.cmet.2013.03.004

Lehnen, a M., Leguisamo, N. M., Casali, K. R., & Schaan, B. D. (2013). Progressive cardiovascular autonomic dysfunction in rats with evolving metabolic syndrome. *Autonomic Neuroscience : Basic & Clinical*, *176*(1–2), 64–69. https://doi.org/10.1016/j.autneu.2013.02.011

Leon-Mercado, L., Herrera Moro Chao, D., Basualdo, M.-C., Kawata, M., Escobar, C., & Buijs, R. M. (2017). The arcuate nucleus: A site of fast negative feedback for corticosterone secretion in male rats. *ENeuro*, *4*(1), 1–14. https://doi.org/10.1523/ENEURO.0350-16.2017

Li, M. M., Madara, J. C., Steger, J. S., Krashes, M. J., Balthasar, N., Campbell, J. N., … Lowell, B. B. (2019). The Paraventricular Hypothalamus Regulates Satiety and Prevents Obesity via Two Genetically Distinct Circuits. *Neuron*, *102*(3), 653-667.e6. https://doi.org/10.1016/j.neuron.2019.02.028

Liu, S., Chen, X. M., Yoda, T., Nagashima, K., Fukuda, Y., & Kanosue, K. (2002). Involvement of the suprachiasmatic nucleus in body temperature modulation by food deprivation in rats. *Brain Research*, *929*(1), 26–36. https://doi.org/10.1016/S0006-8993(01)03374-1

Luo, S., Ezrokhi, M., Cominos, N., Tsai, T. H., Stoelzel, C. R., Trubitsyna, Y., & Cincotta, A. H. (2021). Experimental dopaminergic neuron lesion at the area of the biological clock pacemaker, suprachiasmatic nuclei (SCN) induces metabolic syndrome in rats. *Diabetology and Metabolic Syndrome*, *13*(1), 1–17. https://doi.org/10.1186/s13098-021-00630-x

Marino, J. S., Xu, Y., & Hill, J. W. (2011). Central insulin and leptin-mediated autonomic control of glucose homeostasis. *Trends in Endocrinology and*

Metabolism, *22*(7), 275–285. https://doi.org/10.1016/j.tem.2011.03.001

Maroni, M. J., Capri, K. M., Cushman, A. V., Monteiro De Pina, I. K., Chasse, M. H., & Seggio, J. A. (2018). Constant light alters serum hormone levels related to thyroid function in male CD-1 mice. *Chronobiology International*, *35*(10), 1456–1463. https://doi.org/10.1080/07420528.2018.1488259

Martínez-Merlos, M. T., Ángeles-Castellanos, M., Díaz-Muñoz, M., Aguilar-Roblero, R., Mendoza, J., & Escobar, C. (2004). Dissociation between adipose tissue signals, behavior and the food-entrained oscillator. *Journal of Endocrinology*, *181*(1), 53–63. https://doi.org/10.1677/joe.0.1810053

Martínez-Sánchez, N., Seoane-Collazo, P., Contreras, C., Varela, L., Villarroya, J., Rial- ensado, E., … López, M. (2017). Hypothalamic AM K-ER Stress-JNK1 Axis Mediates the Central Actions of Thyroid Hormones on Energy Balance. *Cell Metabolism*, *26*(1), 212-229.e12. https://doi.org/10.1016/j.cmet.2017.06.014

Mastronardi, C. A., Yu, W. H., & McCann, S. M. (2002a). Resting and circadian release of nitric oxide is controlled by leptin in male rats. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(8), 5721–5726. https://doi.org/10.1073/pnas.082098499

Mastronardi, C. A., Yu, W. H., & McCann, S. M. (2002b). Resting and circadian release of nitric oxide is controlled by leptin in male rats. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(8), 5721–5726. https://doi.org/10.1073/pnas.082098499

Méndez-Hernández, R., Escobar, C., & Buijs, R. M. (2020). Suprachiasmatic Nucleus–Arcuate Nucleus Axis: Interaction Between Time and Metabolism; Essential for Health. *Obesity*, *28*(S1), S10–S17. https://doi.org/10.1002/oby.22774

Mizuno, K., & Ueno, Y. (2016). Autonomic Nervous System and the Liver. *Hepatology Research*, (May), 1–6. https://doi.org/10.1111/hepr.12760

Mondal, M. S., Date, Y., Yamaguchi, H., Toshinai, K., Tsuruta, T., Kangawa, K., & Nakazato, M. (2005). Identification of ghrelin and its receptor in neurons of the rat arcuate nucleus. *Regulatory Peptides*, *126*(1-2 SPEC. ISS.), 55–59. https://doi.org/10.1016/j.regpep.2004.08.038

Moran-Ramos, S., Baez-Ruiz, A., Buijs, R. M., & Escobar, C. (2016). When to eat? The influence of circadian rhythms on metabolic health: are animal studies providing the evidence? *Nutrition Research Reviews*, 1–14. https://doi.org/10.1017/S095442241600010X

Moran-Ramos, S., Guerrero-Vargas, N. N., Mendez-Hernandez, R., Basualdo, M. del C., Escobar, C., & Buijs, R. M. (2017). The suprachiasmatic nucleus drives day–night variations in postprandial triglyceride uptake into skeletal muscle and brown adipose tissue. *Experimental Physiology*, *102*(12), 1584–1595. https://doi.org/10.1113/EP086026

Morita, S., & Miyata, S. (2013). Accessibility of low-molecular-mass molecules to the median eminence and arcuate hypothalamic nucleus of adult mouse. *Cell Biochemistry and Function*, *31*(8), 668–677. https://doi.org/10.1002/cbf.2953

Muindi, F., Zeitzer, J. M., Colas, D., & Heller, H. C. (2013). The acute effects of light on murine sleep during the dark phase: Importance of melanopsin for maintenance of light-induced sleep. *European Journal of Neuroscience*, *37*(11), 1727–1736. https://doi.org/10.1111/ejn.12189

Mukherji, A., Kobiita, A., Damara, M., Misra, N., Meziane, H., Champy, M.-F., & Chambon, P. (2015). Shifting eating to the circadian rest phase misaligns the peripheral clocks with the master SCN clock and leads to a metabolic syndrome. *Proceedings of the National Academy of Sciences*, *112*(48), E6691–E6698. https://doi.org/10.1073/pnas.1519807112

Murakami, D. M., Horwitz, B. A., & Fuller, C. A. (1995). Circadian rhythms of temperature and activity in obese and lean Zucker rats. *American Journal of Physiology - Regulatory Integrative and Comparative Physiology*, *269*(5 38-5). https://doi.org/10.1152/ajpregu.1995.269.5.r1038

Myers, M. G., Heymsfield, S. B., Haft, C., Kahn, B. B., Laughlin, M., Leibel, R. L., … Yanovski, J. A. (2012). Challenges and opportunities of defining clinical leptin resistance. *Cell Metabolism*, *15*(2), 150–156. https://doi.org/10.1016/j.cmet.2012.01.002

Nagai, K., Nishio, T., Nakagawa, H., Nakamura, S., & Fukuda, Y. (1978). Effect of bilateral lesions of the suprachiasmatic nuclei on the circadian rhythm of food-intake. *Brain Research*, *142*(2), 384–389. https://doi.org/10.1016/0006-8993(78)90648-0

Niijima, A., Nagai, K., Nagai, N., & Akagawa, H. (1993). Effects of light stimulation on the activity of the autonomic nerves in anesthetized rats. *Physiology and Behavior*, *54*(3), 555–561. https://doi.org/10.1016/0031-9384(93)90249-F

Nováková, M., Polidarová, L., Sládek, M., & Sumová, a. (2011a). Restricted feeding regime affects clock gene expression profiles in the suprachiasmatic nucleus of rats exposed to constant light. *Neuroscience*, *197*, 65–71. https://doi.org/10.1016/j.neuroscience.2011.09.028

Nováková, M., Polidarová, L., Sládek, M., & Sumová, A. (2011b). Restricted feeding regime affects clock gene expression profiles in the suprachiasmatic nucleus of rats exposed to constant light. *Neuroscience*, *197*, 65–71. https://doi.org/10.1016/j.neuroscience.2011.09.028

Oosterman, J. E., Wopereis, S., & Kalsbeek, A. (2020). The Circadian Clock, Shift Work, and Tissue-Specific Insulin Resistance. *Endocrinology*, *161*(12), 1–11. https://doi.org/10.1210/endocr/bqaa180

Opperhuizen, A.-L., Stenvers, D. J., Jansen, R. D., Foppen, E., Fliers, E., & Kalsbeek, A. (2017). Light at night acutely impairs glucose tolerance in a time-, intensity- and wavelength-dependent manner in rats. *Diabetologia*, *60*(7), 1333–

1343. https://doi.org/10.1007/s00125-017-4262-y

Opperhuizen, A.-L., van Kerkhof, L. W. M., Proper, K. I., Rodenburg, W., & Kalsbeek, A. (2015). Rodent models to study the metabolic effects of shiftwork in humans. *Frontiers in Pharmacology*, *6*(March), 1–20. https://doi.org/10.3389/fphar.2015.00050

Opperhuizen, A. L., Foppen, E., Jonker, M., Wackers, P., Van Faassen, M., Van Weeghel, M., … Kalsbeek, A. (2019). Effects of light-at-night on the rat liver – A role for the autonomic nervous system. *Frontiers in Neuroscience*, *13*(JUN), 1–14. https://doi.org/10.3389/fnins.2019.00647

Opperhuizen, A. L., Wang, D., Foppen, E., Jansen, R., Boudzovitch-Surovtseva, O., de Vries, J., … Silver, R. (2016). eeding during the resting phase causes profound changes in physiology and desynchronization between liver and muscle rhythms of rats. *European Journal of Neuroscience*, *44*(10), 2795–2806. https://doi.org/10.1111/ejn.13377

Orozco-Solis, R., Aguilar-Arnal, L., Murakami, M., Peruquetti, R., Ramadori, G., Coppari, R., & Sassone-Corsi, P. (2016a). The circadian clock in the ventromedial hypothalamus controls cyclic energy expenditure. *Cell Metabolism*, *23*(3), 467–478. https://doi.org/10.1016/j.cmet.2016.02.003

Orozco-Solis, R., Aguilar-Arnal, L., Murakami, M., Peruquetti, R., Ramadori, G., Coppari, R., & Sassone-Corsi, P. (2016b). The circadian clock in the ventromedial hypothalamus controls cyclic energy expenditure. *Cell Metabolism*, *23*(3), 467–478. https://doi.org/10.1016/j.cmet.2016.02.003

Ottaway, N., Mahbod, ., Rivero, B., Norman, L. A., Gertler, A., D'Alessio, D. A., & Perez-Tilve, D. (2015). Diet-induced obese mice retain endogenous leptin action. *Cell Metabolism*, *21*(6), 877–882. https://doi.org/10.1016/j.cmet.2015.04.015

Pan, W., & Kastin, A. J. (2001). Diurnal variation of leptin entry from blood to brain involving partial saturation of the transport system. *Life Sciences*, *68*(24), 2705–2714. https://doi.org/10.1016/S0024-3205(01)01085-2

Paxinos, G., & Watson, C. (2005). The Rat Brain in Stereotaxic Coordinates. In *Elsevier*. https://doi.org/10.1017/CBO9781107415324.004

Perry, R. J., Lyu, K., Rabin-Court, A., Dong, J., Li, X., Yang, Y., … Shulman, G. I. (2020). Leptin mediates postprandial increases in body temperature through hypothalamus-adrenal medulla-adipose tissue crosstalk. *Journal of Clinical Investigation*, *130*(4), 2001–2016. https://doi.org/10.1172/JCI134699

Perry, R. J., Resch, J. M., Douglass, A. M., Madara, J. C., Rabin-Court, A., Kucukdereli, H., … Shulman, G. I. (2019). Leptin's hunger-suppressing effects are mediated by the hypothalamic–pituitary–adrenocortical axis in rodents. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(27), 13670–13679. https://doi.org/10.1073/pnas.1901795116

Pickel, L., & Sung, H. K. (2020). Feeding Rhythms and the Circadian Regulation of Metabolism. *Frontiers in Nutrition*, *7*(April), 1–20. https://doi.org/10.3389/fnut.2020.00039

Pilorz, V., Tam, S. K. E., Hughes, S., Pothecary, C. A., Jagannath, A., Hankins, M. W., … eirson, S. N. (2016). Melanopsin Regulates Both Sleep-Promoting and Arousal-Promoting Responses to Light. *PLoS Biology*, *14*(6), 1–24. https://doi.org/10.1371/journal.pbio.1002482

Pittendrigh, C. S., & Daan, S. (1976). A functional analysis of circadian pacemakers in nocturnal rodents IV. Entrainment:Pacemaker as Clock. *Journal of Comparative Physiology A*, *106*(3), 223–252. https://doi.org/10.1007/BF01417856

Poplawski, M. M., Mastaitis, J. W., Yang, X. J., & Mobbs, C. V. (2010). Hypothalamic responses to fasting indicate metabolic reprogramming away from glycolysis toward lipid oxidation. *Endocrinology*, *151*(11), 5206–5217. https://doi.org/10.1210/en.2010-0702

Ramirez-Plascencia, O. D., Saderi, N., Escobar, C., & Salgado-Delgado, R. C. (2017). Feeding during the rest phase promotes circadian conflict in nuclei that control energy homeostasis and sleep–wake cycle in rats. *European Journal of Neuroscience*, *45*(10), 1325–1332. https://doi.org/10.1111/ejn.13563

Ravussin, Y., Leibel, R. L., & Ferrante, A. W. (2014). A missing link in body weight homeostasis: The catabolic signal of the overfed state. *Cell Metabolism*, *20*(4), 565–572. https://doi.org/10.1016/j.cmet.2014.09.002

Rodriguez-Cortes, B., Hurtado-Alvarado, G., Martinez-Gomez, R., Leon-Mercado, L. A., Prager- Khoutorsky, M., & Buijs, R. M. (2022). Suprachiasmatic nucleus-mediated glucose entry into the arcuate nucleus determines the daily rhythm in blood glycemia. *Current Biology*, *32*, 1–10.

Rodríguez, E. M., Blázquez, J. L., & Guerra, M. (2010). The design of barriers in the hypothalamus allows the median eminence and the arcuate nucleus to enjoy private milieus: The former opens to the portal blood and the latter to the cerebrospinal fluid. *Peptides*, *31*(4), 757–776. https://doi.org/10.1016/j.peptides.2010.01.003

Rynders, C. A., Morton, S. J., Bessesen, D. H., Wright, K. P., & Broussard, J. L. (2020). Circadian Rhythm of Substrate Oxidation and Hormonal Regulators of Energy Balance. *Obesity*, *28*(S1), S104–S113. https://doi.org/10.1002/oby.22816

Salgado-Delgado, R., Ángeles-Castellanos, M., Buijs, M. R., & Escobar, C. (2008). Internal desynchronization in a model of night-work by forced activity in rats. *Neuroscience*, *154*(3), 922–931. https://doi.org/10.1016/j.neuroscience.2008.03.066

Salgado-Delgado, R. C., Saderi, N., Basualdo, M. D. C., Guerrero-Vargas, N. N., Escobar, C., & Buijs, R. M. (2013). Shift work or food intake during the rest

phase promotes metabolic disruption and desynchrony of liver genes in male rats. *PloS One*, *8*(4), e60052. https://doi.org/10.1371/journal.pone.0060052

Salgado-Delgado, Roberto, Angeles-Castellanos, M., Saderi, N., Buijs, R. M., & Escobar, C. (2010). Food intake during the normal activity phase prevents obesity and circadian desynchrony in a rat model of night work. *Endocrinology*, *151*(3), 1019–1029. https://doi.org/10.1210/en.2009-0864

Schwartz, W. J., Takeuchi, J., Shannon, W., Davis, E. M., & Aronin, N. (1994). Temporal regulation of light-induced c-fos and fos-like protein expression in the ventrolateral subdivision of the rat suprachiasmatic nucleus. *Neuroscience*, *58*(3), 573–583.

Shah, B. ., Vong, L., Olson, D. ., Koda, S., Krashes, M. J., Ye, C., … Lowell, B. B. (2014). MC4R-expressing glutamatergic neurons in the paraventricular hypothalamus regulate feeding and are synaptically connected to the parabrachial nucleus. *Proceedings of the National Academy of Sciences of the United States of America*, *111*(36), 13193–13198. https://doi.org/10.1073/pnas.1407843111

Sherman, H., Genzer, Y., Cohen, R., Chapnik, N., Madar, Z., & Froy, O. (2012). Timed high-fat diet resets circadian metabolism and prevents obesity. *The FASEB Journal*, *26*(8), 3493–3502. https://doi.org/10.1096/fj.12-208868

Shiuchi, T., Haque, M. S., Okamoto, S., Inoue, T., Kageyama, H., Lee, S., … Minokoshi, Y. (2009). Hypothalamic Orexin Stimulates Feeding-Associated Glucose Utilization in Skeletal Muscle via Sympathetic Nervous System. *Cell Metabolism*, *10*(6), 466–480. https://doi.org/10.1016/j.cmet.2009.09.013

Sisó, S., Jeffrey, M., & González, L. (2010). Sensory circumventricular organs in health and disease. *Acta Neuropathologica*, *120*(6), 689–705. https://doi.org/10.1007/s00401-010-0743-5

Spanswick, D., Smith, M. A., Groppi, V. E., Logan, S. D., & Ashford, M. L. J. (1997). Leptin inhibits hypothalamic neurons by activation of ATP-sensitive potassium channels. *Nature*, *390*(6659), 521–525. https://doi.org/10.1038/37379

Stephan, F. K. (1983). Circadian rhythms in the rat: Constant darkness, entrainment to T cycles and to skeleton photoperiods. *Physiology and Behavior*, *30*(3), 451–462. https://doi.org/10.1016/0031-9384(83)90152-X

Sternson, S. M., Shepherd, G. M. G., & Friedman, J. M. (2005). Topographic mapping of VMH → arcuate nucleus microcircuits and their reorganization by fasting. *Nature Neuroscience*, *8*(10), 1356–1363. https://doi.org/10.1038/nn1550

Stoynev, A. G., Ikonomov, O. C., & Usunoff, K. G. (1982). Feeding pattern and light-dark variations in water intake and renal excretion after suprachiasmatic nuclei lesions in rats. *Physiology and Behavior*, *29*(1), 35–40. https://doi.org/10.1016/0031-9384(82)90362-6

Strubbe, J. H., Spiteri, N. J., & Alingh Prins, A. J. (1986). Effect of skeleton photoperiod and food availability on the circadian pattern of feeding and drinking in rats. *Physiology and Behavior*, *36*(4), 647–651. https://doi.org/10.1016/0031-9384(86)90348-3

Strubbe, J. H., & Van Dijk, G. (2002). The temporal organization of ingestive behaviour and its interaction with regulation of energy balance. *Neuroscience and Biobehavioral Reviews*, *26*(4), 485–498. https://doi.org/10.1016/S0149-7634(02)00016-7

Sun, M., eng, W., Wang, ., Li, ., Li, Z., Li, M., … Tse, L. A. (2018). Meta-analysis on shift work and risks of specific obesity types. *Obesity Reviews: Am Official Journal of the International Associaton for the Study of Obesity*, *19*(1), 28–40. https://doi.org/10.1111/obr.12621

Turek, F. W., Joshu, C., Kohsaka, A., Lin, E., Ivanova, G., McDearmon, E., … Bass, J. (2005). Obesity and metabolic syndrome in circadian Clock mutant nice. *Science*, *308*(5724), 1043–1045. https://doi.org/10.1126/science.1108750

Van Cauter, E., Polonsky, K. S., & Scheen, A. J. (1997). Roles of circadian rhythmicity and sleep in human glucose regulation. *Endocrine Reviews*, *18*(5), 716–738. https://doi.org/10.1210/er.18.5.716

van der Vinne, V., Swoap, S. J., Vajtay, T. J., & Weaver, D. R. (2018). Desynchrony between brain and peripheral clocks caused by CK1δ/e disruption in GABA neurons does not lead to adverse metabolic outcomes. *Proceedings of the National Academy of Sciences of the United States of America*, *115*(10), E2437–E2446. https://doi.org/10.1073/pnas.1712324115

Van Dielen, . M. H., Van't Veer, C., Schols, A. M., Soeters, P. B., Buurman, W. A., & Greve, J. W. M. (2001). Increased leptin concentrations correlate with increased concentrations of inflammatory markers in morbidly obese individuals. *International Journal of Obesity*, *25*(12), 1759–1766. https://doi.org/10.1038/sj.ijo.0801825

Vera, B., Dashti, H. S., Gómez-Abellán, P., Hernández-Martínez, A. M., Esteban, A., Scheer, . A. J. L., … Garaulet, M. (2018). Modifiable lifestyle behaviors, but not a genetic risk score, associate with metabolic syndrome in evening chronotypes. *Scientific Reports*, *8*(1), 1–11. https://doi.org/10.1038/s41598-017-18268-z

Wang, D., Opperhuizen, A.-L., Reznick, J., Turner, N., Su, Y., Cooney, G. J., … Kalsbeek, A. (2017). Effects of feeding time on daily rhythms of neuropeptide and clock gene ex- pression in the rat hypothalamus Effects of feeding time on daily rhythms of neuropeptide and clock gene expression in the rat hypothalamus. *Brain Research*, *1671*, 93–101. https://doi.org/10.1016/j.brainres.2017.07.006

Wang, Q., Liu, C., Uchida, A., Chuang, J. C., Walker, A., Liu, T., … Zigman, J. M. (2014). Arcuate AgRP neurons mediate orexigenic and glucoregulatory actions

of ghrelin. *Molecular Metabolism*, *3*(1), 64–72. https://doi.org/10.1016/j.molmet.2013.10.001

Wehrwein, E. A., Orer, H. S., & Barman, S. M. (2016). Overview of the Anatomy, Physiology, and Pharmacology of the Autonomic Nervous System. *Comprehensive Physiology*, *6*(3), 1239–1278. https://doi.org/10.1002/cphy.c150037

Wiater, M. F., Li, A.-J., Dinh, T. T., Jansen, H. T., & Ritter, S. (2013). Leptin-sensitive neurons in the arcuate nucleus integrate activity and temperature circadian rhythms and anticipatory responses to food restriction. *AJP: Regulatory, Integrative and Comparative Physiology*, *305*(8), R949–R960. https://doi.org/10.1152/ajpregu.00032.2013

Wideman, C. H., & Murphy, H. M. (2009). Constant light induces alterations in melatonin levels, food intake, feed efficiency, visceral adiposity, and circadian rhythms in rats. *Nutritional Neuroscience*, *12*(5), 233–240. https://doi.org/10.1179/147683009X423436

Wu, Z., Kim, E. R., Sun, H., Xu, Y., Mangieri, L. R., Li, D. ., … Tong, Q. (2015). Gabaergic projections from lateral hypothalamus to paraventricular hypothalamic nucleus promote feeding. *Journal of Neuroscience*, *35*(8), 3312–3318. https://doi.org/10.1523/JNEUROSCI.3720-14.2015

Wulsin, L. R., Horn, P. S., Perry, J. L., Massaro, J. M., & D'Agostino, R. B. (2015). Autonomic Imbalance as a Predictor of Metabolic Risks, Cardiovascular Disease, Diabetes, and Mortality. *The Journal of Clinical Endocrinology and Metabolism*, *100*(6), 2443–2448. https://doi.org/10.1210/jc.2015-1748

Xie, X., Kukino, A., Calcagno, H. E., Berman, A., Garner, J. P., & Butler, M. P. (2020). Natural food intake patterns do not synchronize peripheral clocks. *BMC Biology*, *18*(160), 1–11.

Yamamuro, D., Takahashi, M., Nagashima, S., Wakabayashi, T., Yamazaki, H., Takei, A., … Ishibashi, S. (2020). eripheral circadian rhythms in the liver and white adipose tissue of mice are attenuated by constant light and restored by time-restricted feeding. *PLoS ONE*, *15*(6), 1–16. https://doi.org/10.1371/journal.pone.0234439

Yi, C. X., o ppen, E., Abplanalp, W., Gao, Y., Alkemade, A., La l eur, S. E., … Kalsbeek, A. (2012). Glucocorticoid signaling in the arcuate nucleus modulates hepatic insulin sensitivity. *Diabetes*, *61*(2), 339–345. https://doi.org/10.2337/db11-1239

Yi, C. X., la Fleur, S. E., Fliers, E., & Kalsbeek, A. (2010). The role of the autonomic nervous liver innervation in the control of energy metabolism. *Biochimica et Biophysica Acta - Molecular Basis of Disease*, *1802*(4), 416–431. https://doi.org/10.1016/j.bbadis.2010.01.006

Yi, C. X., Van Der Vliet, J., Dai, J., Yin, G., Ru, L., & Buijs, R. M. (2006). Ventromedial arcuate nucleus communicates peripheral metabolic information to the

suprachiasmatic nucleus. *Endocrinology*, *147*(1), 283–294. https://doi.org/10.1210/en.2005-1051

Yu, J. H., Yun, C. H., Ahn, J. H., Suh, S., Cho, H. J., Lee, S. K., … Kim, N. H. (2015). Evening chronotype is associated with metabolic disorders and body composition in middle-aged adults. *Journal of Clinical Endocrinology and Metabolism*, *100*(4), 1494–1502. https://doi.org/10.1210/jc.2014-3754

Zhang, J., Kaasik, K., Blackburn, M. R., & Cheng, C. L. (2006). Constant darkness is a circadian metabolic signal in mammals. *Nature*, *439*(7074), 340–343. https://doi.org/10.1038/nature04368

Zimmet, P., Alberti, K. G. M. M., Stern, N., Bilu, C., El-Osta, A., Einat, H., & Kronfeld-Schor, N. (2019). The Circadian Syndrome: is the Metabolic Syndrome and much more! *Journal of Internal Medicine*, *286*(2), 181–191. https://doi.org/10.1111/joim.12924

Supplementary information

1. Abbreviations

Abbreviation	Meaning
AgRP	agouti-related peptide
AL	ad libitum
ANOVA	Analysis of Variance
ANS	autonomic nervous system
ARC	arcuate nucleus
AUC	area under the curve
AVP	arginine vassopresin
BMAL	Brain and Muscle ARNT-Like 1
CART	cocaine and amphetamine-regulated transcript
CCK	cholecystokinin
CLOCK	Circadian Locomotor Output Cycles Kaput
CRY	cryptochrome
CSF	cerebrospinal fluid
CT	circadian time
CTB	B subunit of the cholera toxin
CVOs	circumventricular organs
Cy5	cyanine 5
DAB	diaminobenzidine
DD	Constant darkness
DF	day-feeding
DF	day feeding
DMH	dorsomedial hypothalamus
DMV	dorsomotor nucleus of the vagus
EE	energy expenditure
ELISA	enzyme-linked immunosorbent assay
eWAT	epididymal white adipose tissue

GABA	gamma amino-butyric acid
GRP	gastrin-releasing peptide
GTT	glucose tolerance test
HPA	hypothalamic-pituitary-adrenal
i.g.	intragastric
i.m.	intramuscular
i.p	intraperitoneal
i.v.	intravenous
IML	intermediolateral column of the spinal cord
ipRGCs	intrinsically photosensitive retinal ganglion cells
LD	12:12 light-dark photoperiod
LH	lateral hypothalamus
ME	median eminence
MnPO	median preoptic area
NF	night feeding
NPY	neuropeptide Y
NTS	nucleus of the solitary tract
OVLT	*organum vasculosum laminae terminalis*
PBN	parabraquial nucleus
PBS	phosphate-buffered saline solution
PER	Period Circadian Regulator
PFA	paraformaldehyde
PK-2	prokinecitin-2
POMC	proopiomelanocortin
PRV	pseudorabies virus
PSNS	parasympathetic nervous system
PVN	paraventricular nucleus
pWAT	retroperitoneal white adipose tissue
RER	respiratory exchange quotient
REV-ERBs	nuclear receptor subfamily of intracellular transcription factors
ROREs	Retinoic acid-related Orphan Receptors

SCN	suprachiasmatic nucleus
scWAT	inguinal subcutaneous white adipose tissue
SEM	standard error or the mean
SFO	subfornical organ
SP	skeleton photoperiod
SNS	sympathetic nervous system
STAT-3	signal transducer and activator of transcription 3
VIP	vasoactive intestinal peptide
VMH	ventromedial hypothalamus
WAT	white adipose tissue
ZT	zeitgeber time
α-MSH	α-melanocyte stimulating hormone

2. Figure index

3. Supporting figures and tables

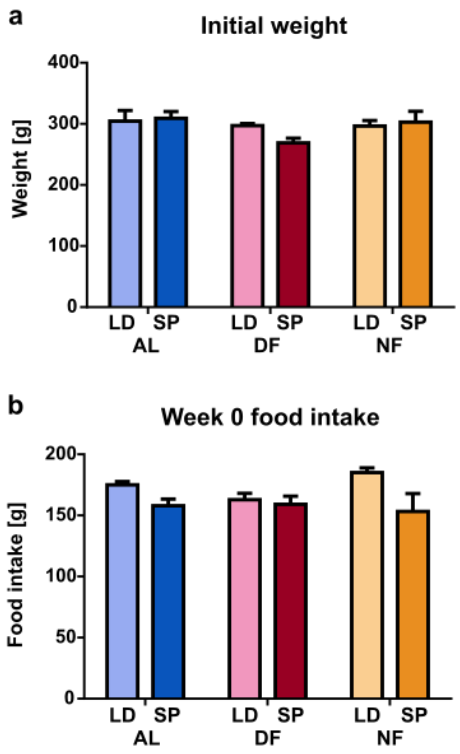

FIGURE S1. INITIAL WEIGHT AND FOOD INTAKE. A) WEIGHT OF THE DIFFERENT ANIMAL GROUPS STARTING THE EXPERIMENT. B) FOOD INTAKE DURING THE FIRST WEEK OF THE EXPERIMENT, BEFORE THE FOOD RESTRICTION (WEEK 0). N =8 FOR ALL GROUPS. DATA ARE REPRESENTED AS MEAN ± S.E.M. NO DIFFERENCES WERE FOUND AMONG GROUPS AFTER ONE-WAY ANOVA.

Cosinor parameter	LD-AL	SP-AL	LD-DF	SP-DF	LD-NF	SP-NF
Acrophase[a)]	17.45 ± 0.1998	17.18 ± 0.2297	15.01± 0.7653	17.51 ± 0.5241	17.35 ± 0.2361	17.82 ± 0.2190
Amplitude [b)]	0.6000 ± 0.0507	0.5050 ± 0.0299	0.4450 ± 0.0548	0.4320 ± 0.0609	0.5167 ± 0.0462	0.631 ± 0.0103
Mesor [b)]	37.64 ± 0.0882	37.39 ± 0.0655	37.32 ± 0.0836*	37.48 ± 0.0673	37.50 ± 0.0593	37.56 ± 0.0678
Adjustment (r^2)[a)]	0.7837 ± 0.0430	0.6379 ± 0.0462	0.5630 ± 0.8160	0.4362 ± 0.0611	0.7283 ± 0.0355	0.7572 ± 0.0187

Table S1. Cosinor values for the temperature rhythm of all groups during the fourth week of treatment. a) Brown-Forsythe test indicated significantly different variances among groups in the acrophase and adjustment. b) Two-way ANOVA revealed a significant effect of feeding on amplitude (P=0.0084),and an interaction on mesor (P=0.032). * P<0.05 compared to the LD-AL control after Dunnet's test. LD-AL, n = 5; SP-AL, n = 6; LD-DF, n = 6; SP-DF, n = 5; LD-NF, n = 6; SP-NF, n = 7.

Variable	Cosinor parameter	Week 0		Week 2		Week 4	
		LD-AL	SP-AL	LD-AL	SP-AL	LD-AL	SP-AL
Activity [counts]	Amplitude	319.0 ± 13.35	318.1 ± 12.75	248.5 ± 6.395	203.4 ± 9.831 **	250.5 ± 4.862	211.1 ± 14.09 **
	Acrophase	18.03 ± 0.2802	18.46 ± 0.3918	18.01 ± 0.2267	18.92 ± 0.3788	17.78 ± 0.3240	18.34 ± 0.2430
	Mesor	386.20 ± 15.41	401.2 ± 10.6900	338.2 ± 7.613	354.7 ± 12.30	319.0 ± 4.221	333.8 ± 10.48
	Adjustment	0.4084 ± 0.02669	0.3947 ± 0.03120	0.3615 ± 0.01516	0.2262 ± 0.01969 ***	0.4062 ± 0.01187	0.2335 ± 0.02160 ****
	Onset	13.03 ± 0.6379	12.78 ± 0.5270	12.28 ± 0.4014	13.61 ± 0.3073 **	11.61 ± 0.2472	13.03 ± 0.6760 $
	Offset	24.37 ± 0.07838	24.37 ± 0.06949	24.24 ± 0.09324	24.19 ± 0.2500	24.44 ± 0.0	24.69 ± 0.4761
	Alpha	11.34 ± 0.6283	11.60 ± 0.5094	11.96 ± 0.3948	10.58 ± 0.2007 *	12.83 ± 0.2472	11.66 ± 0.2487 ** $
Food intake [g]	Amplitude	0.4630 ± 0.02773	0.4541 ± 0.03353	0.4598 ± 0.02303	0.2942 ± 0.009949 ****	0.4214 ± 0.01983	0.2860 ± 0.01243 ***
	Acrophase	16.37 ± 0.2340	16.30 ± 0.2316	16.63 ± 0.3528	15.75 ± 0.1421 *	16.63 ± 0.3528	15.75 ± 0.1472 *
	Mesor	0.4394 ± 0.01863	0.4242 ± 0.01687	0.4300 ± 0.02038	0.4666 ± 0.008754	0.4448 ± 0.009864	0.4682 ± 0.005421
	Adjustment	0.2267 ± 0.0374	0.2593 ± 0.04361	0.2778 ± 0.04513	0.1125 ± 0.008518 **	0.1974 ± 0.02798	0.1006 ± 0.003832 **
	Onset	11.36 ± 0.2713	10.44 ± 0.5323	11.53 ± 0.3270	8.941 ± 1.457 $	11.28 ± 0.7032	10.36 ± 0.9435
	Offset	24.37 ± 0.1483	24.21 ± 0.1052	23.37 ± 0.5399	22.69 ± 1.109	24.46 ± 0.1187	23.29 ± 0.4983 $
	Alpha	13.01 ± 0.3387	13.76 ± 0.5707	12.17 ± 0.4613	13.75 ± 0.8145	13.18 ± 0.6785	12.93 ± 0.8494
Energy Expenditure [kcal h^{-1} kg^{-1}]	Amplitude	2.412 ± 0.4231	2.212 ± 0.3892	2.160 ± 0.3497	1.340 ± 0.1142 $	2.235 ± 0.3940	1.188 ± 0.1412 * $
	Acrophase	18.17 ± 0.1503	18.11 ± 0.1201	17.67 ± 0.2244	18.05 ± 0.2643	18.06 ± 0.2794	17.79 ± 0.2788
	Mesor	10.38 ± 1.546	10.02 ± 1.246	10.26 ± 1.433	9.787 ± 0.7886	9.512 ± 0.9655	8.968 ± 0.8355
	Adjustment	0.6169 ± 0.0268	0.6574 ± 0.0330	0.6541 ± 0.0354	0.4149 ± 0.0370 ***	0.6281 ± 0.0289	0.3574 ± 0.0299 ****
	Onset	12.44 ± 0.4282	12.94 ± 0.4082	11.94 ± 0.2582	13.28 ± 0.6009	12.03 ± 0.0833	12.78 ± 0.2789 * $
	Offset	24.64 ± 0.3706	24.37 ± 0.0695	24.10 ± 0.0695	24.05 ± 0.2373$	24.49 ± 0.3034	23.46 ± 0.1401 *
	Alpha	12.19 ± 0.1620	11.43 ± 0.4306	12.15 ± 0.2674	10.78 ± 0.7180$	12.46 ± 0.3380	10.68 ± 0.3603 **
Respiratory Exchange Ratio [$vCO_2 \cdot v\,vO_2^{-1}$]	Amplitude	0.04883 ± 0.0051	0.04896 ± 0.0032	0.04793 ± 0.0053	0.03104 ± 0.0013 *	0.04361 ± 0.0035	0.03549 ± 0.0006 *
	Acrophase	19.36 ± 0.2618	19.71 ± 0.4676	19.00 ± 0.2679	17.94 ± 0.3028 *	19.20 ± 0.2795	17.51 ± 0.3808 **
	Mesor	0.9487 ± 0.006285	0.9367 ± 0.006725	0.9313 ± 0.003253	0.9425 ± 0.002653 *	0.9547 ± 0.00363	0.9386 ± 0.00215 **
	Adjustment	0.8314 ± 0.0198	0.8500 ± 0.0261	0.8477 ± 0.0255	0.7715 ± 0.0233	0.8300 ± 0.0178	0.7247 ± 0.0209 **
	Onset	12.86 ± 0.4167	12.94 ± 0.5477	11.94 ± 0.4472	10.78 ± 0.8233	12.36 ± 0.2386	10.44 ± 0.6055 *
	Offset	26.52 ± 0.3956	26.10 ± 0.4925	26.02 ± 0.4339	24.45 ± 0.1253 * $	26.69 ± 0.3812	24.17 ± 0.0879 ** $
	Alpha	13.66 ± 0.2812	13.16 ± 0.4194	14.08 ± 0.4707	13.17 ± 0.7189	14.33 ± 0.2113	13.72 ± 0.5522

Table S2. Cosinor values for Activity, Food intake, Energy Expenditure, and Respiratory Exchange Ratio of LD-AL and SP-AL animals. n = 6 for both groups. * P<0.05, ** P<0.01 ***, P<0.001, ****P<0.0001 after unpaired t-test for each week. ($) unpaired t-test with Welch's correction due to significantly different variances.

Other publications generated during PhD

Cited in this thesis:

Méndez-Hernández, R., Escobar, C., & Buijs, R. M. (2020). Suprachiasmatic Nucleus–Arcuate Nucleus Axis: Interaction Between Time and Metabolism; Essential for Health. *Obesity, 28*(S1), S10–S17. https://doi.org/10.1002/oby.22774 ***Pdf attached at the end for reference.***

Buijs, R. M., Guzmán Ruiz, M. A., **Méndez Hernández, R.**, & Rodriguez Cortés, B. (2019). The suprachiasmatic nucleus; a responsive clock regulating homeostasis by daily changing the setpoints of physiological parameters. *Autonomic Neuroscience, 218*(February), 43–50. https://doi.org/10.1016/j.autneu.2019.02.001

Moran-Ramos, S., Guerrero-Vargas, N. N., **Mendez-Hernandez, R.**, Basualdo, M. del C., Escobar, C., & Buijs, R. M. (2017). The suprachiasmatic nucleus drives day–night variations in postprandial triglyceride uptake into skeletal muscle and brown adipose tissue. *Experimental Physiology 102*(12), 1584–1595. https://doi.org/10.1113/EP086026

Others:

Guzmán-Ruiz, M. A., Guerrero-Vargas, N. N., Lagunes-Cruz, A., García-Aviles, J. E., Hurtado-Alvarado, G., **Mendez-Hernández, R**., … Guevara-Guzmán, R. (2022). Circadian modulation of microglial physiological processes and immune responses. *Glia,* (August), 1–13. https://doi.org/10.1002/glia.24261

García-Aviles, J. E., **Méndez-Hernández, R.**, Guzmán-Ruiz, M. A., Cruz, M., Guerrero-Vargas, N. N., Velázquez-Moctezuma, J., & Hurtado-Alvarado, G. (2021). Metabolic Disturbances Induced by Sleep Restriction as Potential Triggers for Alzheimer's Disease. *Frontiers in Integrative Neuroscience, 15*(September), 1–13. https://doi.org/10.3389/fnint.2021.722523

Review Article
OBESITY BIOLOGY AND INTEGRATED PHYSIOLOGY

Obesity

Suprachiasmatic Nucleus–Arcuate Nucleus Axis: Interaction Between Time and Metabolism Essential for Health

Rebeca Méndez-Hernández[1], Carolina Escobar[2], and Ruud M. Buijs [1]

In mammals, time and metabolism are tightly coupled variables; this relationship can be illustrated by numerous examples, such as the circadian variation in food intake or the circadian response to a glucose bolus. We review evidence that the interaction between the suprachiasmatic nucleus and the arcuate nucleus plays a key role in the execution of these functions. The nuclei are reciprocally connected via different projections, and this interaction provides an ideal anatomical framework to modify the temporal output of the hypothalamus to metabolic organs as a consequence of the feedback from the periphery. The suprachiasmatic nucleus–arcuate nucleus relationship is essential to integrate metabolic information into the circadian system and thus adapt circadian rhythms in core body temperature, locomotor activity, food intake, and circulating molecules such as glucose and corticosterone. With the rise in obesity-associated diseases in the world population, gaining knowledge about this relationship, and the consequences of disturbing this liaison, is essential to understand the pathogenesis of obesity.

Obesity (2020) **0**, 1-8.

Study Importance

What is already known?

- The arcuate nucleus is a well-known hypothalamic nucleus regulating metabolism; here we pay special attention regarding the regulation of its functions according to the time of the day.

What does this review add?

- We review evidence supporting that the suprachiasmatic nucleus and arcuate nucleus of the hypothalamus are at the core of the interaction between circadian timing and metabolic regulation, emphasizing their role in food intake, locomotor activity, temperature, and circulating glucose and glucocorticoid rhythms, as well as current evidence for the contribution of the suprachiasmatic nucleus–arcuate nucleus axis in obesity and metabolic dysregulation.

Introduction: The Relationship Between Time and Metabolism

Perhaps the first and most important indication that timing is crucial for metabolic regulation in mammals is the predictable observation that food intake follows a day-night pattern, with higher food intake associated with the activity period (1,2). Moreover, the fact that the response to food-related components varies depending on the time of the day indicates intrinsic mechanisms linking metabolic functions with time. Examples of this are the differential postprandial insulin response to glucose (3), differential triglyceride uptake after a lipid bolus (4), and differential corticosterone secretion after a hypoglycemic challenge (5). Enzymes and hormones that are important in regulating metabolism also follow a day-night pattern of expression and activity, illustrating the importance of timing in mammalian metabolism (6,7).

The importance of the association between time and metabolism can be inferred from recent data pointing to metabolic disruption and obesity as a consequence of circadian disruption because of jet lag, shift work, light at night, and short sleep. This review explores the mechanisms for this circadian-metabolism relationship, both in a physiological condition and in the context of obesity.

Herein, we propose two major players, the suprachiasmatic nucleus (SCN) and the arcuate nucleus (ARC), both located in the ventral hypothalamus.

Suprachiasmatic Nucleus as the Master Clock

SCN, a multiple oscillator

Since the 1970s, the SCN has been identified as the master clock in mammals; it possesses several unique characteristics that will be briefly discussed. It functions as an autonomous clock, with the electrical activity of its neurons oscillating in cycles of approximately 24 hours, even *in vitro* (8-10). The basis for this is a molecular machinery of clock genes and complex neuron-glia interaction unique for the SCN and essential for maintaining its endogenous function (11,12). Because clock genes are present in all tissues (peripheral

[1] Instituto de Investigaciones Biomédicas, Universidad Nacional Autónoma de México, Ciudad Universitaria, Mexico City, Mexico. Correspondence: Ruud M. Buijs (ruudbuijs@gmail.com) [2] Departamento de Anatomía, Facultad de Medicina, Universidad Nacional Autónoma de México, Ciudad Universitaria, Mexico City, Mexico.

 Received: 11 November 2019; **Accepted:** 15 January 2020; **Published online XX Month 2020. doi:10.1002/oby.22774**

oscillators) but most do not have the autonomous capacity of the SCN, the main function of the SCN is to impose its rhythm to other structures in the brain and from there to the whole body. The autonomous clock in the SCN can be synchronized to the light-dark cycle via the retino-hypothalamic tract (13,14). This allows the peripheral oscillations to be in synchrony with external time cues such as light and provides a pathway by which light can modify several physiological parameters. Importantly, not all neurons in the SCN show the same rhythm; most, but certainly not all, are active during the daytime, and regional differences in activity can also be found (15). The SCN contains several neurotransmitters, including Gamma-aminobutyric acid and glutamate, but also a wide range of peptides, including arginine vasopressin (AVP), vasoactive intestinal peptide (VIP), gastrin-releasing peptide, and prokinecitin-2 (PK-2), to name a few (13,16).

SCN communicates with various hypothalamic and sensory areas to integrate behavior and physiology with changes in environment

The master clock, via its extensive projections to hypothalamic and extrahypothalamic nuclei, synchronizes behavior with hormone secretion and with the adequate autonomic output to the organs (14,17). We propose that this orchestration in the output of the SCN, whereby behavior is synchronized with physiology, is essential for health, and a desynchrony of these elements may lead to disease. This balance is of such importance that the SCN has the capacity to transmit information to different tissues and organs by means of different pathways, allowing it to give dissimilar signals to the organs. For example, separate neurons in the SCN have multi-synaptic connections with the motor neurons of the sympathetic or parasympathetic system or with different tissues (17,18). Through these multi-synaptic pathways, the organs are warned of impending changes in hormones or the coming food. In this organization, all hypothalamic structures play a crucial role. For the moment, the paraventricular nucleus (PVN) has the clearest role, with its preautonomic neurons receiving direct input from the SCN while transmitting circadian information to autonomic motor neurons (19,20). Notably, PVN neuroendocrine neurons only receive indirect SCN input, via the ARC, medial preoptic nucleus, or dorsomedial hypothalamus (18,21). Even more striking, the influence of the SCN on behavior is still hardly understood and is probably achieved by its simultaneous input to multiple hypothalamic areas.

The SCN also receives direct innervation from many sensory areas, such as the nucleus of the solitary tract, the circumventricular organs, and the ARC (22). This information may be important for adjusting circadian physiology to changes in the environment such as food availability. Several studies have shown that, indeed, physiological changes in the periphery in response to environmental stressors, such as hunger-related metabolites, blood pressure, and glucose levels, may influence the activity of SCN neurons (22). Here we will focus on the interaction between the SCN and the ARC (Figure 1).

Arcuate Nucleus as the Metabolic Integrator

ARC has access to blood-borne substances

There is ample evidence that the ARC has an important role in monitoring circulating metabolites (23). Its anatomical position is essential for this function, as it is located lateral to the third ventricle and forms a complex with the median eminence, which is a site partially devoid of blood-brain barrier. This allows peripheral substances to access the ARC in a relatively fast way and provides a pathway for feedback from the circulation. Indeed, its neurons express leptin, ghrelin, cortisol, and insulin receptors and are sensitive for glucose, free fatty acids, etc. (23,24). Circulating leptin, for example, enters the median eminence, is transported to the third ventricle, and is subsequently transported into the ARC in a matter of minutes (25); this allows this nucleus to be the first and main leptin sensor. Tanycytes, particular glial cells located in the border of the ventricle, are essential for leptin transport into the ARC and are also important in the regulation of hormonal passage into the median eminence and vice versa (26,27). Thus, tanycytes represent an interesting target when studying passage of molecules into the Median Eminence–ARC complex and sensing of circulating molecules.

Corticosterone is also detected in the ARC before it reaches other brain areas, functioning as the primary target of this hormone for the fast negative feedback of the hypothalamus-pituitary-adrenal system (28). Interestingly, the detection of corticosterone by the ARC also follows a circadian pattern that depends on glucocorticoid and mineralocorticoid receptors (28). This probably indicates that the sensory function of the ARC is in some way circadian gated.

ARC regulates energy expenditure via autonomic nervous system

The ARC regulates metabolism, in part, by modulating peripheral responses to food-related components via the autonomic nervous system. It projects to the PVN, ventromedial nucleus, and lateral hypothalamus, which contain preautonomic neurons that can modulate both sympathetic and parasympathetic branches of the autonomic system (29,30). In addition, cocaine and amphetamine regulated transcript (CART) neurons in the ARC project to the thoracic intermediolateral column of the spinal cord and can be activated by leptin (31). It has been shown that changing the activity of agouti-related peptide (AgRP) and proopiomelanocortin (POMC) neurons in the ARC by leptin can increase the firing of sympathetic or parasympathetic nerves that innervate different organs, including the liver (32,33). Also, leptin via the ARC can modulate heart rate and blood pressure by increasing sympathetic firing (34,35). Thus, the ARC is the main brain region serving the autonomic effects of leptin. Other hormones can also change the output of the autonomic system via the ARC; for example, dexamethasone (a glucocorticoid receptor agonist) infusion in the ARC modulates hepatic insulin sensitivity via the sympathetic nervous system (36). In this way, the ARC works as an integrative output for metabolic regulation.

ARC regulates food intake

Many studies have given the ARC a central spot in the regulation of food intake. The general view is that neurons coexpressing neuropeptide Y (NPY) and AgRP relate to functions associated with energy need (such as food intake) and neurons that coexpress POMC/CART with energy excess (such as inhibition of food intake) (37,38). Considering that circulating hormones such as leptin and ghrelin can modify NPY/AgRP and POMC/CART neuronal activity when applied directly into the ARC, they also have been given a direct role in food intake (39). However, the fact that the highest activity of alpha melanocyte stimulating hormone (α-MSH) neurons (a subset of POMC neurons) occurs at the end of

the dark phase even in fasted animals (40) indicates that the view that these neurons serve to inhibit food intake is too simple. Furthermore, it neglects the observation that the peak of circulating leptin is at the beginning of the eating period in rodents, which also challenges the view of leptin as a satiety hormone. Consequently, another level of integration is necessary that includes the fact that food intake is regulated in a circadian manner. Thereby, information about time must be integrated in the ARC or its target areas; the SCN-ARC axis may be involved in the circadian control of food intake, which will be discussed below.

Anatomical Evidence for SCN-ARC Bidirectional Communication

SCN projections to ARC

Many studies characterizing the SCN efferent have consistently shown that it projects to the ARC (41-43) (Figure 2), yet little is known about which transmitters are involved in this communication. VIP fibers coming from the SCN have been found in the ARC, many of them contacting α-MSH neurons (40). This may be the pathway by which the SCN drives the rhythm in α-MSH activity. VIP fibers have also been found near to the ARC-Median eminence interphase. The fact that these fibers disappear after cutting the communication between the SCN and the ARC supports that this peptide is derived from the SCN (44). Likewise, some traced fibers from the SCN terminate on NPY neurons in the ARC (45), although the identity of the SCN fibers is still unknown. This could have an implication for feeding rhythms (discussed below).

Injection of a retrograde tracer in the ARC aimed at mapping the input to kisspeptin neurons showed labeled neurons in the SCN, some of which corresponded to AVP neurons (46). Interestingly, this innervation to kisspeptin neurons was only found in female but not male rats and thus could be specific for the regulation of female-specific functions.

The ARC is also a target of PK-2 afferent fibers from the SCN (47). The function of this pathway has not been assessed; nevertheless, the ARC does express PK-2 receptors (48), and this peptide has been proposed to be important for the SCN output, especially for locomotor activity rhythms (49).

ARC projections to SCN

Injection of the retrograde tracer Cholera toxin subunit B into the SCN resulted in labeled neurons in the ARC, some of which were AgRP neurons (30,40); this was confirmed later by demonstration of AgRP fibers in the SCN (44). Also, some kisspeptin neurons in the ARC were shown to project to the SCN (50). Interestingly, these fibers increase their abundance according to the time of the day, with more abundant fibers corresponding to the night phase (Buijs et al.,

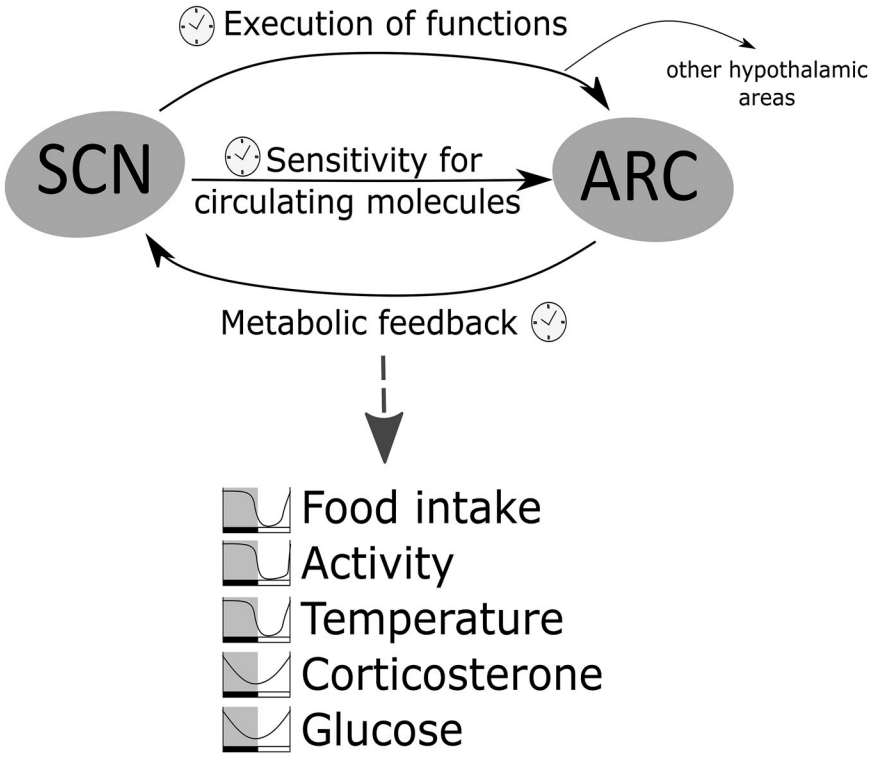

Figure 1 Interaction between SCN and ARC regulates the rhythmicity of metabolic functions. The SCN communicates time-related information to the ARC in order to execute metabolic functions at the correct circadian time. At the same time, the SCN influences the sensitivity of the ARC for circulating molecules, thus allowing it to respond according to the time of the day. As the ARC senses hormones and metabolites from the periphery, it conveys this information back to the SCN, allowing the SCN to adapt its output and completing the feedback that the SCN needs to adjust physiology. This interaction between the SCN and ARC is necessary to maintain rhythms in food intake, locomotor activity, temperature, and circulating corticosterone and glucose.

unpublished data). This illustrates the reciprocity of SCN-ARC communication (Figure 2).

Electrical stimulation along with recordings of SCN and ARC demonstrated bidirectional communication

Probably the first experiments that tested the communication between the SCN and ARC were experiments done by Saeb-Parsy et al. (51), demonstrating the existence of SCN neurons that can inhibit or activate ARC neurons. Because of their patterns of orthodromic and antidromic activation, they reported that "in some cases the same suprachiasmatic neurones which projected to the arcuate region were influenced by the activity of their target region" (51). In a subsequent study (52), they recorded the daily activity of SCN neurons and found that, as expected, there was a rhythm in their activity, with the highest peak corresponding to the end of the day. However, some SCN neurons that projected to the ARC had two peaks of activity, at ZT10-12 and at ZT22-24. This last peak in activity could have a functional relevance in the activation of α-MSH neurons, which is higher in this time point (40).

Is there ventricular communication from SCN to ARC?

Although neuronal projections between SCN and ARC have been reported, there is also evidence suggesting that there might be ventricular communication between the SCN and the ARC. AVP has a rhythm in cerebrospinal fluid (CSF) (53); whether it is released from the SCN remains to be shown, but classic experiments showing that SCN explants in the ventricle can restore some rhythms in SCN-lesioned animals support the idea that SCN can exert some synchronizing functions via AVP or other peptides released into the CSF (54-56). In hypothalamic PVN slices, which do not have any clock gene oscillations because of disconnection from the SCN, adding an explanted SCN to the medium restores the rhythm in the PVN. Furthermore, the addition of AVP into the medium could synchronize PVN oscillations, and the previous application of an AVP receptor antagonist prevented this synchronization (57). This also suggests that AVP released into the CSF can exert some synchronizing properties, which is especially interesting considering that the ARC has access to substances in the CSF because of the tanycyte populations located in the line of the ventricle. It is tempting to propose that ventricular AVP could modulate tanycytic function. In addition, some VIP projections to the ARC innervate the area close to the ventricle (44), which could provide another pathway for SCN-tanycyte communication.

SCN-ARC Axis and Integration of Physiological Functions

Changes in ARC and SCN activity under different metabolic conditions

In general, the electrical activity of the SCN is higher during the day than during the night. Moreover, the ARC has an overall rhythm in activity (as measured by c-fos, an immediate early gene produced shortly after neuronal activation immunoreactivity), which is inverse to SCN activation. Furthermore, SCN lesion removes the rhythm of c-fos in the ARC, while fasting amplifies the day-night difference (40). Fasting also has effects on both the ARC and the SCN, as it increases NPY immunoreactivity in the ARC (58) and decreases the activity of SCN neurons as measured by c-Fos immunoreactivity; the latter is probably mediated by inputs coming from the intergeniculate leaflet (59). Other experiments have shown that when the ARC is activated by stimuli such as peripheral administration of a ghrelin analog (30) or oral glucose administration (44), the SCN diminishes its activity. Taken together, these data suggest a tight relationship between the activity of these two nuclei. The question arises, how does SCN-ARC axis regulate physiology depending on the time of the day?

SCN-ARC axis in food intake

The SCN is essential for driving rhythms in food intake, as SCN-lesioned animals show arrhythmic feeding behavior (60). However, the pathways underlying the temporal organization of feeding behavior are still poorly understood. When leptin-responsive neurons in the ARC were ablated using the injection of saporin toxin bound to leptin, the animals almost lost their rhythm in food intake, and they did so completely when put in constant darkness conditions (61). In this condition, both α-MSH and AgRP neurons were diminished, suggesting a role for both peptides in the circadian regulation of food intake. Injections of the toxin in the ventromedial hypothalamus (which also has leptin-responsive neurons) showed no effects on food intake, indicating the specificity of this function to the ARC. Still, the SCN was intact and showed normal AVP immunoreactivity, indicating that regardless of an intact SCN, the disruption of the ARC-SCN axis disturbs feeding rhythms.

The PVN of the hypothalamus also has a role in food intake and receives projections from both the SCN and the ARC. NPY/AgRP release into the PVN promotes food intake (62). NPY concentration in the PVN and also in the ARC has a circadian rhythm, which peaks in the onset of the activity phase, that is, when animals eat (63). Furthermore, ablating NPY/AgRP neurons in the ARC disrupted circadian feeding patterns (64). These data suggest that rhythmic NPY/AgRP could drive rhythmic food intake, but how the SCN drives rhythmic NPY is still unanswered. Considering that efferent fibers from the SCN colocalize with NPY neurons in the ARC (45), it is possible that the SCN directly influences NPY release from the ARC, although this remains to be shown. Moreover, circulating corticosterone follows a circadian pattern, which is mediated by the SCN, and is considered to be an important synchronizer of circadian functions (65). Because glucocorticoids can be detected by the ARC (28) and have been shown to modulate NPY/AgRP expression in this nucleus (66), they could provide a pathway by which the output of the SCN mediates feeding rhythms. More work is necessary to integrate other hormones in this system as well as other hypothalamic areas, including the dorsomedial hypothalamus, which also receives NPY/AgRP projections from the ARC (67).

SCN-ARC axis in locomotor activity rhythms

Several studies have raised the possibility that the ARC has a role in the regulation of the daily rhythms of locomotor activity, especially through leptin. Mice with ablated leptin receptors have diminished locomotor activity but only during the night; reactivation of this receptor in the ARC reestablished nighttime activity (68). Also, constitutive expression of Signal transducer and activator of transcription 3 in AgRP neurons increases night activity in mice (69). Furthermore, deletion of Rho kinase1 factor in AgRP neurons, which induces leptin resistance, also decreases nighttime locomotor activity (70). Together, these data point to a specific time frame in which leptin,

probably via AgRP neurons, can increase locomotor activity. Leptin receptor expression in AgRP neurons does not seem to be expressed in a circadian manner in mice (71); thus, one could hypothesize that the sensitivity to leptin during this time frame could be due to information conveyed by the SCN.

The most compelling evidence that the SCN-ARC interaction is necessary to regulate daily locomotor activity patterns is the study in which the SCN and ARC were disconnected via a retrochiasmatic cut (44). This manipulation disrupted SCN-ARC fibers, leaving both nuclei intact and the SCN displaying *period1* rhythmicity similar to the sham control. These animals display normal locomotor rhythms when placed in a normal light-dark cycle but lose their rhythms completely when placed in constant darkness. The latter suggests that SCN-ARC direct connection is necessary for endogenous locomotor activity rhythm, while the maintenance of the rhythm in light-dark conditions could be mediated by another pathway from the SCN, inducing light-mediated suppression of locomotor activity during the day.

SCN-ARC axis in temperature

The complexity of how the SCN and ARC interact to regulate circadian physiology is well illustrated by the control of body temperature. In rats, core body temperature rhythm is low during the day and high during the night. This circadian control of temperature is dependent on the SCN, as SCN-lesioned animals lose this rhythm (72,73). Also, interrupting SCN-ARC communication eliminates this rhythm in constant darkness (44), again suggesting that SCN-ARC direct communication is important for the endogenous component of this rhythm. Furthermore, the median preoptic area (MnPO), which is essential for temperature regulation (74), receives projections from the SCN (AVP) and from the ARC (α-MSH) (75). During the light period, AVP released from the SCN to the MnPO induces a decrease in temperature. Moreover, at the end of the dark phase (ZT 22), when α-MSH neurons have their highest activity because of SCN influence (40), the release of α-MSH in the MnPO maintains a high temperature (75). Thus, by influencing the MnPO directly and through the ARC, the SCN can maintain the circadian temperature rhythm. Also, because the ARC is essential for the integration of metabolic information, its interaction with the SCN gives the possibility to integrate this metabolic information into the circadian thermoregulatory circuit. Indeed, the temperature rhythm can be modified by fasting, during which temperature decreases further than in fed animals but only at the beginning of the light phase. Furthermore, the fasting-induced decrease in temperature is lost in SCN-lesioned animals (76). Thus, it is possible that, at this time point, fasting-related information reaches the SCN via the ARC in order to adjust the output of the SCN to the MnPO, decreasing temperature in a time-specific way. Hence, the circadian thermoregulatory circuit composed of SCN and ARC is ideally positioned to modify temperature rhythms according to the metabolic condition of the animal.

SCN-ARC in integration of reproductive functions

Kisspeptin neurons in the ARC play a role in orchestrating reproductive functions (77,78). As discussed previously, these neurons receive projections from the SCN (46). When kisspeptin ARC neurons were silenced in female mice, they lost their normal estrous cycle, remaining in diestrus (50). Interestingly, they also displayed a disorganization of the timing of food intake with increased daytime feeding, resulting in weight gain. Other rhythms, such as locomotor activity and temperature, were also lost. These effects seemed to be independent of the SCN, which displayed a normal clock gene rhythm (50), again suggesting that these neurons in the ARC represent an immediate output of the SCN for the generation of rhythms. In this sense, kisspeptin neurons represent a common substrate for the integration of timing and metabolism with reproductive functions and could provide a target for the study of the mechanisms behind circadian and reproductive dysfunction observed in animals fed a high-fat diet (79-82). Indeed, kisspeptin-1 gene expression in the ARC is downregulated by high-fat feeding (83), although the implications of this have not been assessed.

SCN-ARC axis in glucose and glucocorticoid control

Circulating glucose levels follow a circadian rhythm, which is driven by the SCN (84). The SCN, via projections to the PVN and the sympathetic system, can increase glucose production by the liver in preparation for the activity phase (85). Moreover, the ARC can contribute to this regulation by sensing peripheral glucose levels, thus completing the feedback mechanisms necessary to maintain glycemia. The ARC can convey this information to the SCN as well, as illustrated by the fact that an oral glucose bolus increases the activity of the ARC and at the same time decreases the activity of the SCN (44). In addition, hypoglycemia induced by peripheral 2-deoxyglucose injection resulted in a more powerful activation of ARC neurons at ZT11 than at ZT2 (45). In unilateral SCN-lesioned animals, this activation was diminished at the lesioned side, indicating that the SCN changes the sensitivity of the ARC to hypoglycemia, making it more sensitive to this challenge at the time of the expected circadian peak in glucose (ZT11).

Similar to glycemic control, the circadian control of corticosterone release in preparation for the active phase is mediated by the SCN, via the PVN, hypophysis and sympathetic system to the adrenal (21,86). The autonomic projections to the adrenal gland are essential for the stimulation of corticosterone secretion, but these neurons lack glucocorticoid receptors (28); thus, the ARC has an important role in the feedback to those autonomic PVN neurons. The ARC has abundant glucocorticoid receptors and is the first site sensing peripheral glucocorticoid concentration changes, and it provides that information to the PVN to adjust circulating corticosterone levels. Similar to glucose regulation, the ARC has a different sensitivity for glucocorticoids depending on the time of the day (28), probably imposed by the SCN.

Together, these data support the idea that the SCN conveys time-related information to the sensory ARC and at the same time receives input from it. This reciprocal interaction would allow the SCN to set the sensitivity for circulating molecules according to the time of the day and would also provide it with feedback information from the ARC on how to adapt its output when a challenge such as hypoglycemia occurs (Figure 1). These time-of-day–dependent counterregulatory responses are very well known in relation to hypoglycemic responses after insulin injections in people with type 1 diabetes (87).

SCN-ARC Axis in Disease

Recent postmortem observations of anatomical changes in the ARC and SCN of patients with type 2 diabetes (88,89) raise the following question: are these changes cause or consequence of the disease?

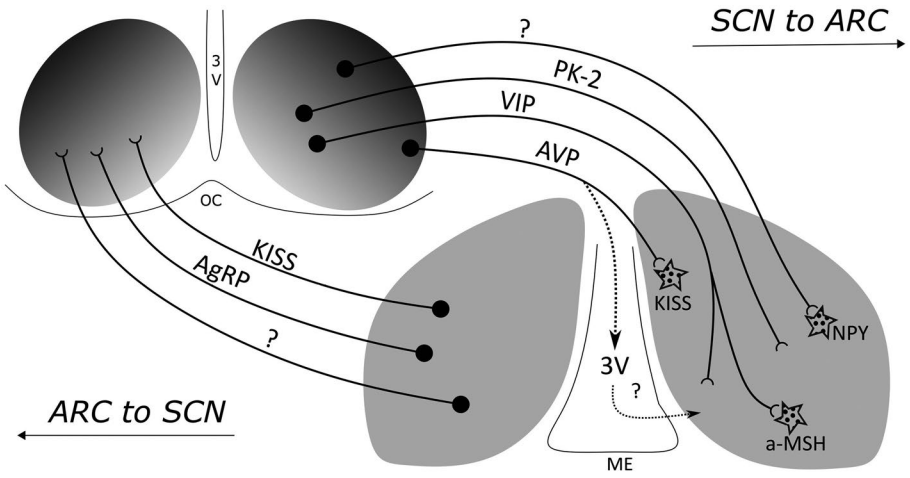

Figure 2 Communication between SCN and ARC. The SCN sends extensive projections to the ARC using different transmitters. Some SCN neurons of unknown identity reach NPY neurons in the ARC, while PK-2 fibers also project to this nucleus. SCN VIP neurons project to α-MSH neurons in the ARC and also innervate the area close to the ME-ARC interphase. SCN AVP neurons contact KISS neurons in the ARC in female rats. AVP that is released into the ventricle could also serve as a communication pathway between the SCN and ARC. Moreover, the ARC sends some projections of unknown identity to the SCN. Some ARC AgRP neurons project to the ventrolateral region of the SCN, which is also a target of KISS projections that change their abundance according to the time of the day. 3V, third ventricle; AgRP, agouti-related peptide; α-MSH, alpha melanocyte stimulating hormone; ARC, arcuate nucleus; AVP, arginine-vasopressin; KISS, kisspeptin; ME, median eminence; OC, optic chiasm; PK-2, prokinecitin-2; SCN, suprachiasmatic nucleus; VIP, vasoactive intestinal peptide.

Several studies indicate that a disturbed circadian lifestyle because of exposure to "light at night" is a risk factor to develop overweight and obesity (90,91). In view of what we have reviewed, it seems clear that disrupting the activity of the SCN by means of altered light-dark cycles would change the output to the ARC as well, probably losing its rhythmic capacity to sense circulating molecules or to drive the rhythms it is involved in. Indeed, acute light exposure during the dark can inhibit α-MSH activity in the ARC (40), but the contribution of long-term light at night in ARC function remains to be assessed

Moreover, long-term exposure to a high fat–high sucrose diet in rodents results in obesity and metabolic dysfunction, which is accompanied by the loss of rhythms in circulating hormones, such as luteinizing hormone, melatonin, and corticosterone (92). Interestingly, exposure to high-fat diet immediately modifies feeding behavior, whereby food intake is shifted toward the resting phase (79,80). Restricting high-fat feeding to the active phase ameliorates most of the effects of the high-fat diet despite eating the same amount of calories (93-95). In addition, restricting normo-caloric food intake to the resting phase of animals also promotes fat accumulation (96). Thus, it appears that food at the "wrong time" has a larger contribution to the development of obesity than the diet per se. In view of what we have reviewed above, there are two explanations for this phenomenon: (1) out-of-time food intake serves as a mistimed feedback to the SCN, resulting in a disturbed output of the SCN, which initially is mainly directed to adjust the circulating glucose levels but in the long term results in a loss of other rhythms; and (2) food-related signals such as glucose reach the ARC at a time when, because of SCN influence, this nucleus is incapable of giving an appropriate response. In both cases, mistimed signaling would directly affect the interaction between the SCN and the ARC. Thus, in the long term, the ARC would no longer perform its circadian output functions nor respond to circulating molecules in a time-appropriate manner. Indeed, reverse feeding induces loss of the a-MSH neuronal activity rhythm (97) along with changes in clock gene expression in the ARC (98). Furthermore, patients with diabetes present a sharpened "dawn phenomenon," in which glucose levels before waking increase even though their glycemia is already high (99); this could be interpreted as the ARC not being able to sense glucose levels properly, hence increasing glycemia despite it being already elevated. This hypothesis is further supported by the fact that in postmortem brains of diabetic patients, NPY is upregulated in this nucleus, similar to what happens in a healthy individual that has been fasted (88). In addition, glucose uptake in the brain of patients with diabetes is enhanced (10) as a further indication of the incapacity of the brain (the ARC) of diabetic patients to sense the actual glucose concentration.

The enormous increase in the consumption of sugar, especially at the inappropriate time (i.e., the resting phase), leads to frequent spikes in glucose in the circulation that may contribute to the disruption of the ARC-SCN axis. Consequently, early induced miscommunication in ARC-SCN axis by untimely increased glucose levels could be involved in a permanent disruption of this system, resulting in dysregulated peripheral glucose levels and, ultimately, circadian and metabolic dysfunction.O

Acknowledgments

The authors would like to thank Dr. Mara Guzman-Ruiz and Dr. Ranier Gutierrez for their input.

Funding agencies: This work was supported by grants DGAPA-PAPIIT IG-200417, CONACyT 1802, and CONACyT 279293.

Disclosure: The authors declared no conflict of interest.

References

1. Strubbe JH, Van Dijk G. The temporal organization of ingestive behaviour and its interaction with regulation of energy balance. *Neurosci Biobehav Rev* 2002;26:485-498.
2. Challet E. The circadian regulation of food intake. *Nat Rev Endocrinol* 2019;15:393-405.
3. Kalsbeek A, Strubbe JH. Circadian control of insulin secretion is independent of the temporal distribution of feeding. *Physiol Behav* 1998;63:553-560.
4. Moran-Ramos S, Guerrero-Vargas NN, Mendez-Hernandez R, Basualdo MDC, Escobar C, Buijs RM. The suprachiasmatic nucleus drives day–night variations in postprandial triglyceride uptake into skeletal muscle and brown adipose tissue. *Exp Physiol* 2017;102:1584-1595.
5. Kalsbeek A, Ruiter M, la Fleur SE, Van Heijningen C, Buijs RM. The diurnal modulation of hormonal responses in the rat varies with different stimuli. *J Neuroendocrinol* 2003;15:1144-1155.
6. Stenvers DJ, Jonkers CF, Fliers E, Bisschop PHLT, Kalsbeek A. Nutrition and the circadian timing system. *Prog Brain Res* 2012;199,359-376.
7. Asher G, Sassone-Corsi P. Time for food: the intimate interplay between nutrition, metabolism, and the circadian clock. *Cell* 2015;161:84-92.
8. Inouye ST, Kawamura H. Persistence of circadian rhythmicity in a mammalian hypothalamic "island" containing the suprachiasmatic nucleus. *Proc Natl Acad Sci U S A* 1979;76:5962-5966.
9. Green DJ, Gillette R. Circadian rhythm of firing rate recorded from single cells in the rat suprachiasmatic brain slice. *Brain Res* 1982;245:198-200.
10. Groos G, Hendriks J. Circadian rhythms in electrical discharge of rat suprachiasmatic neurones recorded in vitro. *Neurosci Lett* 1982;34:283-288.
11. Colwell CS. Linking neural activity and molecular oscillations in the SCN. *Nat Rev Neurosci* 2011;12:553-569.
12. Hastings MH, Brancaccio M, Maywood ES. Circadian pacemaking in cells and circuits of the suprachiasmatic nucleus. *J Neuroendocrinol* 2014;26:2-10.
13. Golombek DA, Rosenstein RE. Physiology of circadian entrainment. *Physiol Rev* 2010;90:1063-1102.
14. Buijs RM, Escobar C, Swaab DF. The circadian system and the balance of the autonomic nervous system. *Handb Clin Neurol* 2013;117:173-191.
15. Silver R, Schwartz WJ. The suprachiasmatic nucleus is a functionally heterogeneous timekeeping organ. *Methods Enzymol* 2005;393:451-465.
16. Ibata Y, Okamura H, Tanaka M, et al. Functional morphology of the suprachiasmatic nucleus. *Front Neuroendocrinol* 1999;20:241-268.
17. Buijs RM, La Fleur SE, Wortel J, et al. The suprachiasmatic nucleus balances sympathetic and parasympathetic output to peripheral organs through separate preautonomic neurons. *J Comp Neurol* 2003;464:36-48.
18. Buijs RM, Chun SJ, Niijima A, Romijn HJ, Nagai K. Parasympathetic and sympathetic control of the pancreas: a role for the suprachiasmatic nucleus and other hypothalamic centers that are involved in the regulation of food intake. *J Comp Neurol* 2001;431:405-423.
19. La Fleur SE, Fliers E, Kalsbeek A. Neuroscience of glucose homeostasis. *Handb Clin Neurol* 2014;126:341-351.
20. O'Hare JD, Zsombok A. Brain-liver connections: role of the preautonomic PVN neurons. *Am J Physiol Endocrinol Metab* 2016;310:E183-E189.
21. Buijs RM. Anatomical and functional demonstration of a multisynaptic suprachiasmatic nucleus adrenal (cortex) pathway. *Eur J Neurosci* 1999;11:1535-1544.
22. Buijs RM, Guzmán Ruiz MA, Méndez Hernández R, Rodríguez Cortés B. The suprachiasmatic nucleus; a responsive clock regulating homeostasis by daily changing the setpoints of physiological parameters. *Auton Neurosci Basic Clin* 2019;218:43-50.
23. Rodríguez EM, Blázquez JL, Guerra M. The design of barriers in the hypothalamus allows the median eminence and the arcuate nucleus to enjoy private milieus: the former opens to the portal blood and the latter to the cerebrospinal fluid. *Peptides* 2010;31:757-776.
24. Haddad-Tóvolli R, Dragano NRV, Ramalho AFS, Velloso LA. Development and function of the blood-brain barrier in the context of metabolic control. *Front Neurosci* 2017;11:1-12.
25. Balland E, Dam J, Langlet F, et al. Hypothalamic tanycytes are an ERK-gated conduit for leptin into the brain. *Cell Metab* 2014;19:293-301.
26. Müller-Fielitz H, Stahr M, Bernau M, et al. Tanycytes control the hormonal output of the hypothalamic-pituitary-thyroid axis. *Nat Commun* 2017;8:484.
27. García-Cáceres C, Balland E, Prevot V, et al. Role of astrocytes, microglia, and tanycytes in brain control of systemic metabolism. *Nat Neurosci* 2019;22:7-14.
28. Leon-Mercado L, Herrera Moro Chao D, Basualdo MDC, Kawata M, Escobar C, Buijs RM. The arcuate nucleus: a site of fast negative feedback for corticosterone secretion in male rats. *eNeuro* 2017;4:1-14.
29. Uyama N, Geerts A, Reynaert H. Neural connections between the hypothalamus and the liver. *Anat Rec A Discov Mol Cell Evol Biol* 2004;280:808-820.
30. Yi CX, Van Der Vliet J, Dai J, Yin G, Ru L, Buijs RM. Ventromedial arcuate nucleus communicates peripheral metabolic information to the suprachiasmatic nucleus. *Endocrinology* 2006;147:283-294.
31. Elias CF, Lee C, Kelly J, et al. Leptin activates hypothalamic CART neurons projecting to the spinal cord. *Neuron* 1998;21:1375-1385.
32. Bell BB, Harlan SM, Morgan DA, Guo DF, Rahmouni K. Differential contribution of POMC and AgRP neurons to the regulation of regional autonomic nerve activity by leptin. *Mol Metab* 2018;8:1-12.
33. Tanida M, Yamamoto N, Morgan DA, Kurata Y, Shibamoto T, Rahmouni K. Leptin receptor signaling in the hypothalamus regulates hepatic autonomic nerve activity via phosphatidylinositol 3-kinase and AMP-activated protein kinase. *J Neurosci* 2015;35:474-484.
34. Rahmouni K, Morgan DA. Hypothalamic arcuate nucleus mediates the sympathetic and arterial pressure responses to leptin. *Hypertension* 2007;49:647-652.
35. Simonds SE, Pryor JT, Ravussin E, et al. Leptin mediates the increase in blood pressure associated with obesity. *Cell* 2014;159:1404-1416.
36. Yi CX, Foppen E, Abplanalp W, et al. Glucocorticoid signaling in the arcuate nucleus modulates hepatic insulin sensitivity. *Diabetes* 2012;61:339-345.
37. Timper K, Brüning JC. Hypothalamic circuits regulating appetite and energy homeostasis: pathways to obesity. *Dis Model Mech* 2017;10:679-689.
38. Roh E, Song DK, Kim M-S. Emerging role of the brain in the homeostatic regulation of energy and glucose metabolism. *Exp Mol Med* 2016;48:e216. doi:10.1038/emm.2016.4
39. Belgardt BF, Okamura T, Brüning JC. Hormone and glucose signalling in POMC and AgRP neurons. *J Physiol* 2009;587:5305-5314.
40. Guzmán-Ruiz M, Saderi N, Cazarez-Márquez F, et al. The suprachiasmatic nucleus changes the daily activity of the arcuate nucleus α-MSH neurons in male rats. *Endocrinology* 2014;155:525-535.
41. Swanson LW, Cowan WM. The efferent connections of the suprachiasmatic nucleus of the hypothalamus. *J Comp Neurol* 1975;160:1-12.
42. Berk ML, Finkelstein JA. An autoradiographic determination of the efferent projections of the suprachiasmatic nucleus of the hypothalamus. *Brain Res* 1981;226:1-13.
43. Stephan FK, Berkley KJ, Moss RL. Efferent connections of the rat suprachiasmatic nucleus. *Neuroscience* 1981;6:2625-2641.
44. Buijs FN, Guzmán-Ruiz M, León-Mercado L, et al. Suprachiasmatic nucleus interaction with the arcuate nucleus; essential for organizing physiological rhythms. *eNeuro* 2017;4:1-14.
45. Herrera Moro Chao D, León-Mercado L, Foppen E, et al. The suprachiasmatic nucleus modulates the sensitivity of arcuate nucleus to hypoglycemia in the male rat. *Endocrinology* 2016;157:3439-3451.
46. Yeo SH, Kyle V, Blouet C, Jones S, Colledge WH. Mapping neuronal inputs to Kiss1 neurons in the arcuate nucleus of the mouse. *PLoS One* 2019;14:1-23.
47. Zhang C, Truong KK, Zhou QY. Efferent projections of prokineticin 2 expressing neurons in the mouse suprachiasmatic nucleus. *PLoS One* 2009;4:e7151. doi:10.1371/journal.pone.0007151
48. Cheng MY, Bullock CM, Li C, et al. Prokineticin 2 transmits the behavioural circadian rhythm of the suprachiasmatic nucleus. *Nature* 2002;417:405-410.
49. Da Li J, Hu WP, Zhou QY. The circadian output signals from the suprachiasmatic nuclei. *Prog Brain Res* 2012;199:119-127.
50. Padilla SL, Perez JG, Ben-hamo M, Bussi IL, Palmiter RD, De Iglesia HO. Kisspeptin neurons in the arcuate nucleus of the hypothalamus orchestrate circadian rhythms and article kisspeptin neurons in the arcuate nucleus of the hypothalamus orchestrate circadian rhythms and metabolism. *Curr Biol* 2019;29:1-13.
51. Saeb-Parsy K, Lombardelli S, Khan FZ, McDowall K, Au-Yong ITH, Dyball REJ. Neural connections of hypothalamic neuroendocrine nuclei in the rat. *J Neuroendocrinol* 2000;12:635-648.
52. Saeb-Parsy K, Dyball REJ. Defined cell groups in the rat suprachiasmatic nucleus have different day/night rhythms of single-unit activity in vivo. *J Biol Rhythms* 2003;18:26-42.
53. Reppert SM, Artman HG, Swaminathan S, Fisher DA. Vasopressin exhibits a rhythmic daily pattern in cerebrospinal fluid but not in blood. *Science* 1981;213:1256-1257.
54. Sawaki Y, Nihonmatsu I, Kawamura H. Transplantation of the neonatal suprachiasmatic nuclei into rats with complete bilateral suprachiasmatic lesions. *Neurosci Res* 1984;1:67-72.
55. Lehman MN, Silver R, Gladstone WR, Kahn RM, Gibson M, Bittman EL. Circadian rhythmicity restored by neural transplant. Immunocytochemical characterization of the graft and its integration with the host brain. *J Neurosci* 1987;7:1626-1638.
56. Silver R, LeSauter J, Tresco PA, Lehman MN. A diffusible coupling signal from the transplanted suprachiasmatic nucleus controlling circadian locomotor rhythms. *Nature* 1996;382:810-813.
57. Tousson E, Meissl H. Suprachiasmatic nuclei grafts restore the circadian rhythm in the paraventricular nucleus of the hypothalamus. *J Neurosci* 2004;24:2983-2988.
58. Saderi N, Buijs FN, Salgado-Delgado R, et al. A role for VGF in the hypothalamic arcuate and paraventricular nuclei in the control of energy homeostasis. *Neuroscience* 2014;265:184-195.
59. Saderi N, Cazarez-Márquez F, Buijs FN, et al. The NPY intergeniculate leaflet projections to the suprachiasmatic nucleus transmit metabolic conditions. *Neuroscience* 2013;246:291-300.
60. Stoynev AG, Ikonomov OC, Usunoff KG. Feeding pattern and light-dark variations in water intake and renal excretion after suprachiasmatic nuclei lesions in rats. *Physiol Behav* 1982;29:35-40.
61. Li AJ, Wiater MF, Oostrom MT, et al. Leptin-sensitive neurons in the arcuate nuclei contribute to endogenous feeding rhythms. *Am J Physiol Regul Integr Comp Physiol* 2012;302:R1313-R1326.
62. Stanley BG, Leibowitz SF. Neuropeptide Y injected in the paraventricular hypothalamus: a powerful stimulant of feeding behavior. *Proc Natl Acad Sci U S A* 1985;82:3940-3943.
63. Jhanwar-Uniyal M, Beck B, Burlet C, Leibowitz SF. Diurnal rhythm of neuropeptide Y-like immunoreactivity in the suprachiasmatic, arcuate and paraventricular nuclei and other hypothalamic sites. *Brain Res* 1990;536:331-334.
64. Wiater MF, Mukherjee S, Li AJ, et al. Circadian integration of sleep-wake and feeding requires NPY receptorexpressing neurons in the mediobasal hypothalamus. *Am J Physiol Regul Integr Comp Physiol* 2011;301:1569-1583.
65. Kalsbeek A, Palm IF, La Fleur SE, et al. SCN outputs and the hypothalamic balance of life. *J Biol Rhythms* 2006;21:458-469.

66. Shimizu H, Arima H, Watanabe M, et al. Glucocorticoids increase neuropeptide Y and agouti-related peptide gene expression via adenosine monophosphate-activated protein kinase signaling in the arcuate nucleus of rats. *Endocrinology* 2008;149:4544-4553.
67. Bai FL, Yamano M, Shiotani Y, et al. An arcuato-paraventricular and -dorsomedial hypothalamic neuropeptide Y-containing system which lacks noradrenaline in the rat. *Brain Res* 1985;331:172-175.
68. Coppari R, Ichinose M, Lee CE, et al. The hypothalamic arcuate nucleus: a key site for mediating leptin's effects on glucose homeostasis and locomotor activity. *Cell Metab* 2005;1:63-72.
69. Mesaros A, Koralov SB, Rother E, et al. Activation of Stat3 signaling in AgRP neurons promotes locomotor activity. *Cell Metab* 2008;7:236-248.
70. Huang H, Lee SH, Ye C, et al. ROCK1 in AgRP neurons regulates energy expenditure and locomotor activity in male mice. *Endocrinology* 2013;154:3660-3670.
71. Cedernaes J, Huang W, Ramsey KM, et al. Transcriptional basis for rhythmic control of hunger and metabolism within the AgRP neuron. *Cell Metab* 2019;29:1078-1091.e5.
72. Refinetti R, Kaufman CM, Menaker M. Complete suprachiasmatic lesions eliminate circadian rhythmicity of body temperature and locomotor activity in golden hamsters. *J Comp Physiol A* 1994;175:223-232.
73. Scheer FA, Pirovano C, Van Someren EJW, Buijs RM. Environmental light and suprachiasmatic nucleus interact in the regulation of body temperature. *Neuroscience* 2005;132:465-477.
74. Mckinley MJ, Yao ST, Uschakov A, McAllen RM, Rundgren M, Martelli D. The median preoptic nucleus: front and centre for the regulation of body fluid, sodium, temperature, sleep and cardiovascular homeostasis. *Acta Physiol* 2015;214:8-32.
75. Guzman-Ruiz MA, Ramirez-Corona A, Guerrero-Vargas NN, et al. Role of the suprachiasmatic and arcuate nuclei in diurnal temperature regulation in the rat. *J Neurosci* 2015;35:15419-15429.
76. Liu S, Chen XM, Yoda T, Nagashima K, Fukuda Y, Kanosue K. Involvement of the suprachiasmatic nucleus in body temperature modulation by food deprivation in rats. *Brain Res* 2002;929:26-36.
77. Harter CJL, Kavanagh GS, Smith JT. The role of kisspeptin neurons in reproduction and metabolism. *J Endocrinol* 2018;238:R173-R183.
78. Williams WP, Kriegsfeld LJ. Circadian control of neuroendocrine circuits regulating female reproductive function. *Front Endocrinol (Lausanne)* 2012;3:1-14.
79. Kohsaka A, Laposky AD, Ramsey KM, et al. High-fat diet disrupts behavioral and molecular circadian rhythms in mice. *Cell Metab* 2007;6:414-421.
80. Pendergast JS, Branecky KL, Yang W, Ellacott KLJ, Niswender KD, Yamazaki S. High-fat diet acutely affects circadian organisation and eating behavior. *Eur J Neurosci* 2013;37:1350-1356.
81. Sánchez-Garrido MA, Ruiz-Pino F, Manfredi-Lozano M, et al. Obesity-induced hypogonadism in the male: premature reproductive neuroendocrine senescence and contribution of Kiss1-mediated mechanisms. *Endocrinology* 2014;155:1067-1079.
82. Skaznik-Wikiel ME, Swindle DC, Allshouse AA, Polotsky AJ, McManaman JL. High-fat diet causes subfertility and compromised ovarian function independent of obesity in mice. *Obstet Gynecol Surv* 2016;71:532-533.
83. Liu RR, Li H, Zhang DM, Lv YF, Lin XY, Zhang HQ. Expression of kisspeptin/kiss1r system is down-regulated in the hypothalamic arcuate nucleus of pubertal male rats with high-fat-diet. *J Reprod Contracept* 2014;25:1-11.
84. Kalsbeek A, La Fleur S, Fliers E. Circadian control of glucose metabolism. *Mol Metab* 2014;3:372-383.
85. Kalsbeek A, La Fleur S, Van Heijningen C, Buijs RM. Suprachiasmatic GABAergic inputs to the paraventricular nucleus control plasma glucose concentrations in the rat via sympathetic innervation of the liver. *J Neurosci* 2004;24:7604-7613.
86. Leliavski A, Dumbell R, Ott V, Oster H. Adrenal clocks and the role of adrenal hormones in the regulation of circadian physiology. *J Biol Rhythms* 2015;30:20-34.
87. Cryer PE. Mechanisms of hypoglycemia-associated autonomic failure and its component syndromes in diabetes. *Diabetes* 2005;54:3592-3601.
88. Saderi N, Salgado-Delgado R, Avendaño-Pradel R, et al. NPY and VGF immunoreactivity increased in the arcuate nucleus, but decreased in the nucleus of the tractus solitarius, of type-II diabetic patients. *PLoS One* 2012;7:1-16.
89. Hogenboom R, Kalsbeek MJ, Korpel NL, et al. Loss of arginine vasopressin- and vasoactive intestinal polypeptide-containing neurons and glial cells in the suprachiasmatic nucleus of individuals with type 2 diabetes. *Diabetologia* 2019;62:2088-2093.
90. Fonken LK, Nelson RJ. The effects of light at night on circadian clocks and metabolism. *Endocr Rev* 2014;35:648-670.
91. Reid KJ, Santostasi G, Baron KG, Wilson J, Kang J, Zee PC. Timing and intensity of light correlate with body weight in adults. *PLoS One* 2014;9:e92251. doi:10.1371/journal.pone.0092251
92. Cano P, Jiménez-Ortega V, Larrad Á, Toso CFR, Cardinali DP, Esquifino AI. Effect of a high-fat diet on 24-h pattern of circulating levels of prolactin, luteinizing hormone, testosterone, corticosterone, thyroid-stimulating hormone and glucose, and pineal melatonin content, in rats. *Endocrine* 2008;33:118-125.
93. Sherman H, Genzer Y, Cohen R, Chapnik N, Madar Z, Froy O. Timed high-fat diet resets circadian metabolism and prevents obesity. *FASEB J* 2012;26:3493-3502.
94. Sutton EF, Beyl R, Early KS, Cefalu WT, Ravussin E, Peterson CM. Early time-restricted feeding improves insulin sensitivity, blood pressure, and oxidative stress even without weight loss in men with prediabetes. *Cell Metab* 2018;27:1212-1221.e3.
95. Hatori M, Vollmers C, Zarrinpar A, et al. Time-restricted caloric intake prevents metabolic diseases in mice fed a high-fat diet. *Cell Metab* 2012;15:848-860.
96. Salgado-Delgado RC, Saderi N, Basualdo MDC, Guerrero-Vargas NN, Escobar C, Buijs RM. Shift work or food intake during the rest phase promotes metabolic disruption and desynchrony of liver genes in male rats. *PLoS One* 2013;8:e60052. doi:10.1371/journal.pone.0060052
97. Ramirez-Plascencia OD, Saderi N, Escobar C, Salgado-Delgado RC. Feeding during the rest phase promotes circadian conflict in nuclei that control energy homeostasis and sleep–wake cycle in rats. *Eur J Neurosci* 2017;45:1325-1332.
98. Wang D, Opperhuizen A-L, Reznick J, et al. Effects of feeding time on daily rhythms of neuropeptide and clock gene expression in the rat hypothalamus. *Brain Res* 2017;1671:93-101.
99. Radziuk J, Pye S. Diurnal rhythm in endogenous glucose production is a major contributor to fasting hyperglycaemia in type 2 diabetes. Suprachiasmatic deficit or limit cycle behaviour? *Diabetologia* 2006;49:1619-1628.
100. Boersma GJ, Johansson E, Pereira MJ, et al. Glucose uptake in muscle, visceral adipose tissue, and brain strongly predict whole-body insulin resistance in the development of type 2 diabetes. *Diabetes* 2018;67:1790.