UNIVERSIDAD NACIONAL AUTONOMA DE MEXICO
Doctorado en Ciencias Biomédicas
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Interacción entre el sistema circadiano y el control homeostático del metabolismo: procesos y mecanismos para el control de la temperatura

TESIS

Que para optar por el grado de:
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I DON'T KNOW HOW TO WRITE POETRY
OR BEAUTIFUL STORIES BUT THE LETTERS HERE ARE
DEDICATED TO ANGEL & JARL

"The most exciting phrase to hear in science, the one that heralds new discoveries is not; *eureka!*

But; *that's funny...-"*

-Isaac Asimov

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GENERAL ABSTRACT

Correct timing of our physiology is an essential condition for health. The Suprachiasmatic nucleus (SCN) as the master clock imposes its own neuronal rhythm on many target structures in the hypothalamus to ensure a correct synchronization between the light-dark cycle and physiological processes (Buijs *et al.*, 2003b; Kalsbeek *et al.*, 2006a). Lately attention has been given to the timing of metabolic processes since many studies have shown the deleterious effects of untimely activity and food intake on health (Salgado-Delgado *et al.*, 2010b), (Salgado-Delgado *et al.*, 2008; Hsieh *et al.*, 2014).

The arcuate nucleus of the hypothalamus (ARC) integrates information about nutrient availability from the bloodstream and cerebrospinal fluid (Krisch & Leonhardt, 1978; Broadwell *et al.*, 1983; Schaeffer *et al.*, 2013b), thus promoting homeostatic responses that influence energy balance and food intake (Sainsbury & Zhang, 2010) by means of neuronal connections to autonomic and endocrine centres (Elmquist *et al.*, 1998; Elias *et al.*, 1999; Fekete *et al.*, 2000; Niimi *et al.*, 2001). In the ARC two main neuronal populations are known as key players in the regulation of energy balance: proopiomelanocortin/cocaine amphetamine–regulated transcript neurons shown to inhibit food intake (Poggioli *et al.*, 1986; Fan *et al.*, 1997) and neuropeptide Y/agouti-related protein neurons, shown to be orexigenic (Clark *et al.*, 1984).

In the first part of this study we show in male rats that the SCN influences ARC daily neuronal activity by imposing a daily rhythm on the neurons with a peak in neuronal activity at the end of the dark phase (Guzman-Ruiz *et al.*, 2014). Bilateral SCN lesions showed a complete disappearance of ARC neuronal rhythms and unilateral SCN lesions showed a decreased activation in the ARC at the lesioned side. Moreover light exposure during the dark phase inhibited ARC

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and α-MSH neuronal activity. The daily inhibition of ARC neuronal activity occurred in light-dark (LD) conditions as well as in dark-dark (DD) conditions, demonstrating that the inhibitory effect is mediated by the increased activity of the SCN during the subjective day (Guzman-Ruiz *et al.*, 2014).

Neuronal tracer injections into the SCN and unilateral activation of α-MSH neurons after unilateral SCN lesions demonstrated that the SCN modulates α-MSH neurons in the ARC via direct neuronal input. The persistence of these activity patterns in fasted animals demonstrates that this SCN-ARC interaction is not necessarily satiety associated but may support physiological functions associated with changes in the sleep-wake cycle such as body temperature.

Thermoregulatory processes have a well-established central control site residing in several nuclei of the medial preoptic area whereby the Median preoptic nucleus (MnPO)(Nakamura, 2011; Nakamura & Morrison) is the main integration site of thermo-afferents (Romanovsky, 2007; Morrison *et al.*, 2008; Nakamura & Morrison, 2011; Clapham, 2012). In addition to the classic thermoregulatory cues (temperature of skin, viscera, environment and pro-inflammatory signals), time also plays an important role for the temperature set point. Shortly before the active phase of the organism, the temperature increases, while at least 30m before the resting phase the body temperature (Tb) is down-regulated (Refinetti & Menaker, 1992a) independent of the locomotor activity (Scheer *et al.*, 2005) and controlled by the SCN (Buijs & Kalsbeek, 2001).

Notably the circadian modulation of Tb also depends on the metabolic information conveyed to the brain to establish the correct amplitude in Tb rhythm in relation with the specific metabolic requirements of the organism. This multiple system interaction is shown when food deprivation induces an important Tb decrease during the resting phase. Interestingly SCN lesions prevent Tb response to fasting (Liu

et al., 2002; Scheer et al., 2005; Tokizawa et al., 2009) demonstrate that the Tb rhythm depends on the interplay between temporal signals from the SCN and the metabolic state of the organism, however none of the mechanisms are described.

In the second part of this study we hypothesized that not only the rhythm that the SCN imposes on the ARC α-MSH neurons would be important for the temperature rhythm but that the ARC and the SCN would also interact at the level of the preoptic area to influence the Tb.

A single CtB injection into the MnPO showed both vasopressin (AVP) neurons in the SCN and α-MSH neurons in the ARC.

Previous studies demonstrated the circadian profile of AVP from the SCN (Kalsbeek et al., 1995) with a release onset at the end of the dark period, this together with the demonstrated nocturnal profile of α-MSH neurons in the ARC (Guzman-Ruiz et al., 2014) prompted us to choose to investigate the involvement of these two neurotransmitters in temperature regulation in the MnPO at dawn onset.

We demonstrated by means of microdialysis in the MnPO that AVP infusion during the dark phase when Tb is high, resulted in a strong decrease in Tb. The physiological relevance was shown when the infusion of an antagonist of the AVP receptors (V1A) during the light phase when the Tb is low induced a higher Tb as compared to the vehicle, suggesting that indeed AVP release form the SCN in the MnPO induces a decrease in body temperature.

In addition we observed that infusion of an agonist of α -MSH receptors (MC3-4R) in the MnPO during the light phase when the Tb is low induced a clear maintenance in Tb instead of the normal Tb decrease associated with the onset of light. An antagonists of α-MSH infused during the night, induced a deep decrease in Tb, showing that indeed α-MSH in the MnPO increases Tb.

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In conclusion we demonstrated specifically with regard to the control of Tb rhythm, a neuronal interaction between SCN and ARC while their interaction in a common target area is essential to determine the temperature change at dawn. This together with the knowledge that both AVP arising from the SCN and α-MSH arising from the ARC share many target areas in the hypothalamus suggests that misalignment of the signals provided by the SCN with those arising from the ARC could lead to important physiological alterations that could go far beyond temperature regulation. For example obesity and diabetes in shift workers could be the result of untimely food intake resulting in the uncoupling of temporal (SCN) and metabolic information (ARC).

CHAPTER 1

PHYSIOLOGY;

UNDERSTANDING THE MECHANISMS OF LIFE

"I found the task so truly arduous... that I was almost tempted to think... that the movement of the heart was only to be comprehended by God. For I could neither rightly perceive at first when the systole and when the diastole took place by reason of the rapidity of the movement".

-William Harvey

The study of the internal mechanisms that sustain life are known as physiology and rises from one simple question; how?

How do camels survive in the desert without water? How does a pregnant female know when is time to give birth? How does a migratory bird know is time to go south? Without the understanding of how a behavior or a bodily function in the organism takes place, life is just an esoteric assumption.

In the 15th century no distinction between the description of the structure (anatomy) and the function (physiology) of the systems was made, therefore the role of a body part in every day physiology was assumed by how it looked like, without any understanding of the actual mechanisms.

A clear example was the assumption about the circulatory system, considered to be constituted of a series of tubes and a pump. However William Harvey's studies demonstrated that blood circulates in the body (1628) through unidirectional veins and arteries (Figure 1), he also described the systole and the diastole of the heart and for the first time a mechanistic process was reported for the explanation of a physiological phenomenon.
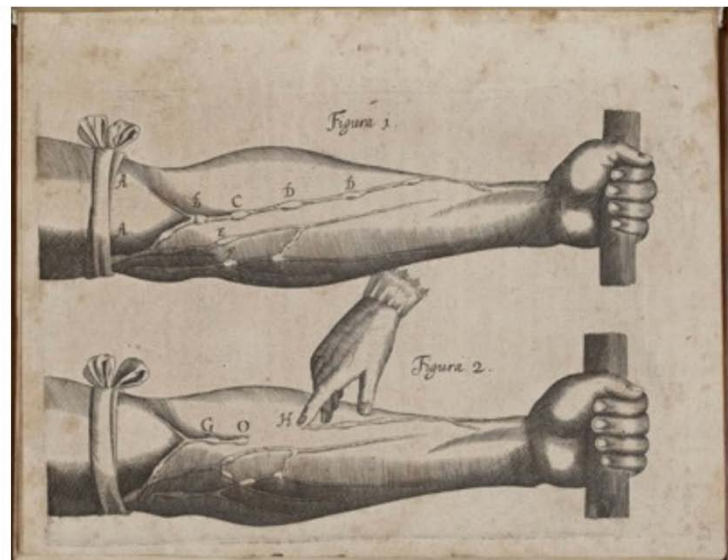
Figure 1. William Harvey's "Motv cordis" or "On the Motion of the heart and blood". Cover of the report (left) and (right) experiment where the physician tied a ligature onto the upper arm of a person. This cut off blood flow from the arteries and veins therefore below the ligature the arm was cool and pale, while above the ligature it was warm and swollen. When the ligature was slightly loosened, the veins were more visible, since they were full of blood. Harvey then noticed little bumps in the veins, the valves, structures that prevented the blood in the vein **to stream** down. On the contrary when Harvey tried to push the blood up the arm, it moved quite easy, thus he concluded that blood had an unidirectional flow within the veins and arteries modulated by valves.

HOMEOSTASIS

Later in the 19th century the study of medicine and the systems in the body went from an artistic to a scientific discipline. The first general physiologist, Claude Bernard (1813-1877), proposed that the stability of the *interior milieu*, the fluid surrounding the organs of the body, was necessary for the maintenance of the correct function of the organism. He also determined that the blood vessels of the body were controlled by specific nerves and responded to changes in the ambient temperature in order to sustain body temperature (Tb).

This compensatory capacity of the body, to deal with the changing environment was coined "homeostasis", term introduced by Walter

Cannon (1926) that refers to the auto-regulatory mechanisms necessary to sustain the internal conditions of the body within a certain range or "set point".

In order to sustain the internal set points, the organisms have developed mechanisms that integrate information about the changes inside and outside the system that in turn are able to respond by generating a compensatory mechanism. One clear example is the regulation of Tb in birds and mammals, where changes in environmental temperature and core Tb are transmitted via cold and warm thermo-receptors in the skin and viscera to the brain in order to promote either cold defensive or heat release mechanisms. These responses depend on the proper levels of Tb in each individual, that in turn are set in relationship to the metabolic rate of each individual and the environmental conditions.

In spite that the body needs a stable physiology, "homeostasis is not a single optimal control condition but rather a variety or continuum that changes with the animals circumstances" this notion was introduced by Donald C. Jackson (1987) and states that the set point of activity in the different organs is not always the same depending on the circumstances (temporal, metabolic, heath related).

For example during an infection, the Tb increases with 1-2°C in order to enable the proper activity of the immune system and to prevent the further development of microorganisms (Kanosue *et al.*, 2010; Nakamura, 2011), this temperature increase (fever) is not a constant phenomenon and its prolonged presence can induce heat shock syndrome or exhaustion of metabolic reserves, denaturalization of essential proteins, etc. In spite of these adverse effects the generation of fever is necessary to combat disease, hence the adaptation of the set points of the organism. Consequently the thermoregulatory set points are quite dynamic, depending on the requirements.

Mammals and birds are endothermic organisms that "maintain a relatively high and more or less constant Tb, whereby the main source of heat is a high resting (basal) metabolic rate" (Clarke & Portner, 2010).

The development of endothermy has enabled several *phyla* to occupy a wide variety of habitats (Crompton *et al.*, 1978) by developing the ability to sustain a more prolonged aerobic activity (Banet *et al.*, 1978) resulting in the capacity to remain active in spite of low ambient temperatures. Endothermy also offers an advantage after birth by enabling the maintenance of high temperatures during the postnatal stages, when the metabolic requirements of the organisms are also quite high. Moreover the endogenous generation of heat also confers a defense mechanism against pathogens through the generation of fever as mentioned before (Nakamura & Morrison, 2011).

Maintaining a constant temperature constitutes an evolutionary paradox; it implies an elevated energy cost to sustain high basal metabolic rates, paid mainly by high amount of food intake associated with a temporal modulation of the metabolic rate to reduce energy expenditure in the rest period. Consequently Tb is an indirect measurement of the metabolic rate of an organism (Figure 2), suggesting a tight relationship between energy intake and heat production.

The set point around which the Tb levels oscillate is narrow; this implies short tolerances to variation in endotherms. For example the daily variation in Tb is around 0.5-1°C between the active and the resting phase, on the other hand, during fever Tb increases with 1-2°C from the maximal Tb allowed for the organism (during activity). At the other hand in some species that undergo torpor Tb

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can descend several degrees depending on the environmental temperature. We can assume that depending on the condition a different set point exists in which the organism can function, depending on the seasonal, metabolic and health situation.
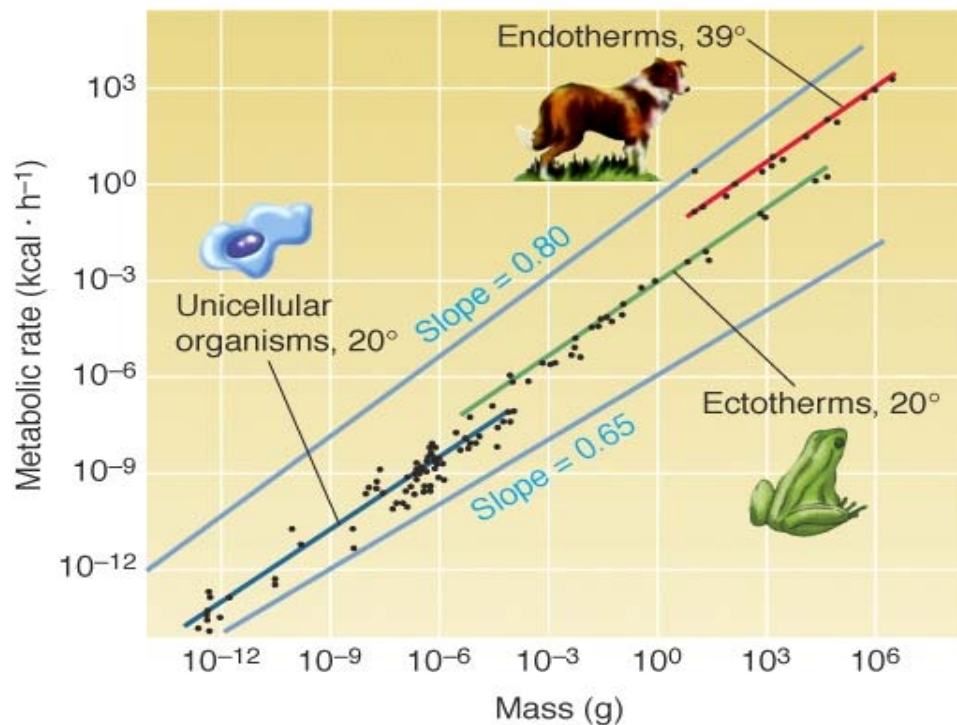
Figure 2. Metabolic rate of unicellular, ectotherms and endotherms in relationship with body mass. Ectotherms live at a lower metabolic rate than endotherms because their metabolic temperatures generally remain close to the environmental temperatures. The metabolic rates of endotherms are up to 20 times higher than in ectotherms, the price paid by endotherms for their high metabolic rates includes the intake of the correspondingly amount of food thus a 300g rodent needs 17 times more food per day as a 300g lizard.

CENTRAL CONTROL OF BODY TEMPERATURE; THE PREOPTIC AREA

Most of endothermic organisms are also homoeothermic, with the capacity to thermoregulate in order to sustain certain Tb set point, i.e., to control the amount and the moment of heat production and heat release. Hereto a variety of involuntary thermoregulatory

responses are controlled by the central nervous system, such as shivering and non-shivering thermogenesis, cutaneous vasomotion, sweating, panting and piloerection (Nakamura & Morrison, 2011).

The site in the central nervous system essential for the determination of the Tb set point is the preoptic area (POA). Several studies have demonstrated that the POA integrates thermo-afferent signals from the periphery in the Median Preoptic Nucleus (MnPO) (Boulant & Bignall, 1973; Boulant, 1981; 1998)

The MnPO is able to respond to changes in ambient (skin), core (viscera) and brain temperature as well as inflammatory signals, such as prostaglandins produced in the blood vessels of the circumventricular organs (CVOs)(Peruzzo *et al.*, 2000; Kanosue *et al.*, 2010).

When the ambient or core temperature is modified, thermoreceptors in respectively the skin or splanchnic nerve, transmit ascending sensory information to the dorsal-horn of the spinal cord where the discrimination between innocuous heat and cold takes place. Next this signal ascends via two pathways; the spino-thalamic entering the brain to the thalamus and ending in the cortex for cold and heat conscious discrimination and the spino-hypothalamic, entering the brain at the Lateral parabrachial nucleus (LPB) were information about cold enters through the lateral external LPB (LPBle) and heat information through the dorsal LPB (LPBd) (Saper & Loewy, 1980; Hylden *et al.*, 1989; Kobayashi & Osaka, 2003). Both areas of the LPB send glutamatergic projections to two different MnPO neuronal populations, the warm and cold sensitive neurons (Fulwiler & Saper, 1984).

The main immediate outputs of the MnPO to activate or inhibit thermoregulatory processes are efferents to the Dorsomedial hypothalamic nucleus (DMH) that in turn sends projections to the rostral reticular formation (RF) and the Raphé pallidus (rRPa). In

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cold environments or fever this system activates heat-producing pathways such as somatic outputs to skeletal muscle and sympathetic tone to Brown adipose tissue (BAT) respectively (Banet *et al.*, 1978; Imai-Matsumura & Nakayama, 1987).
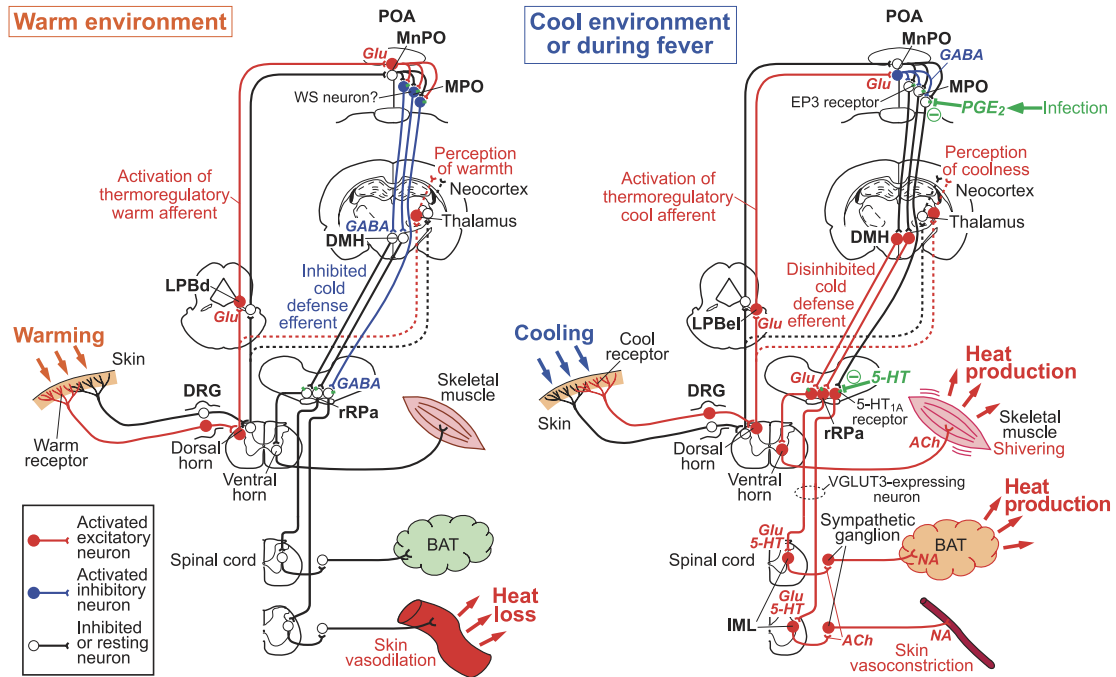
Figure 3. Autonomic and somatic effector responses for thermoregulation and fever. 5-HT, serotonin; ACh, acetylcholine; DRG, dorsal root ganglion; Glut, glutamate; NA, norepinephrine; WS neuron, warm-sensitive neuron; POA, preoptic area; BAT, brown adipose tissue; LPBd, lateral parabrachial nucleus, dorsal subregion; rRPa, rostral raphe pallidus nucleus; MnPO, median preoptic nucleus; MPO, medial preoptic area; DMH, dorsomedial hypothalamus; LPBel, lateral parabrachial nucleus, external lateral subregion; IML, intermediolateral cell column. (From....)

For vasomotion the MnPO sends inhibitory projections to the ventral tegmental area (VTA) inducing vasoconstriction, it also sends glutamatergic efferences to the periaqueductal grey matter (PAG) that induces skin vasodilatation (Figure 4). In addition several studies suggest that the caudal PAG could also play a role in transmitting a thermogenic signal from the DMH to the rRPa inducing BAT and shivering thermogenesis (Yoshida *et al.*, 2005).

Salivary secretion in rodents exposed to hot environments is mediated by projections from the MnPO to the Lateral hypothalamus (Nagashima *et al.*, 2000; Romanovsky, 2007; Nakamura & Morrison, 2011).

Pyrogenic efferents have been related to the communication between the CVOs; the subfornical organ (SFO) and the organum vasculosum lamina terminalis (OVLT) and neuronal inputs from the Nucleus of the solitary tract (NTS), that activate DMH thermogenic pathways that control shivering and non-shivering thermogenesis (Nagashima *et al.*, 2000).

Non-shivering thermogenesis is mediated by BAT activity caused by an increased sympathetic tone in response to cold exposure, infection or food intake (Bouillaud, 1999; Kozak & Anunciado-Koza, 2008).

The increased BAT sympathetic tone induces the expression of uncoupling protein 1 (UCP1) in the mitochondria to produce heat from glucose or lipid substrates (Morrison, 2003; McAllen, 2007; Nakamura, 2011). UCP1 causes the protons to by-pass the ATP synthase route of entry and the energy of the electrochemical gradient is dissipated as heat.

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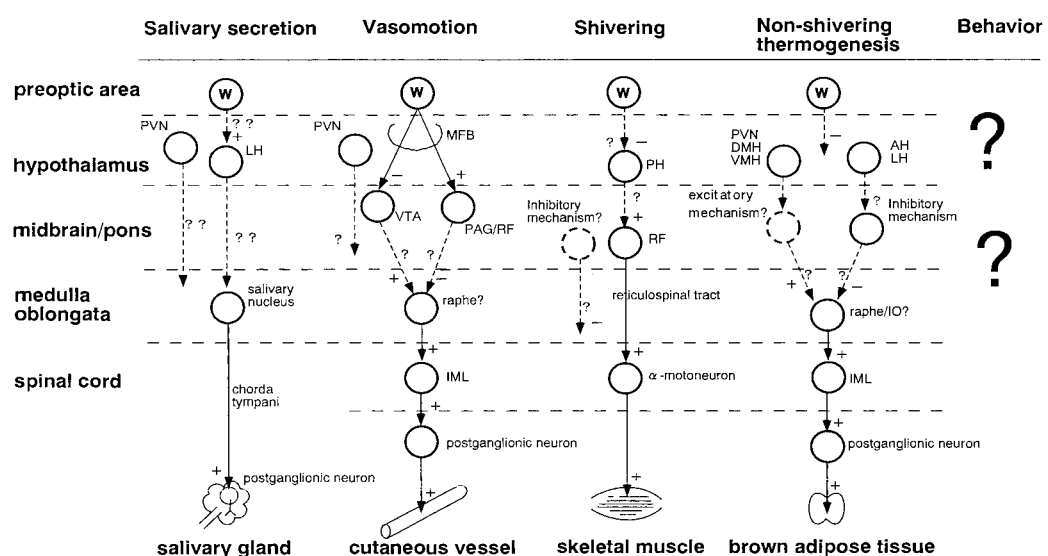
Figure 4. Neuronal circuits involved in thermoregulation. Scheme illustrating efferent pathways from the preoptic area thermoregulatory effectors. Continuous and broken lines indicate respectively identified and unidentified connections. Neuronal network for behavioral thermoregulation is unknown. W, warm-sensitive neuron; 1, facilitation; 2, inhibition; AH, anterior hypothalamus; DMH, dorsomedial hypothalamus; IML, intermediolateral cell column; IO, inferior olive; LH, lateral hypothalamus; MFB, medial forebrain bundle; PAG/RF, periaqueductal grey and reticular formation; PH, posterior hypothalamus; PVN, paraventricular hypothalamic nucleus; SN, salivary nucleus; VMH, ventromedial hypothalamus; VTA, ventral tegmental area. Taken from; (Nagashima *et al.*, 2000)

Besides changes in ambient, core temperature or infectious stimuli, several studies have demonstrated that the thermoregulatory processes are subjected to temporal metabolic control, in the next sections we will analyse how these two factors may contribute to the regulation of body temperature.

TEMPORAL AND METABOLIC REGULATION OF BODY TEMPERATURE

Every organism on earth is subjected to the cyclic changes of the environment, the most important being the day-night cycle. This alteration between 12h of light and 12h of darkness imposes a time-stamp to the physiology of almost every organism. Evolutive pressure has selected those organisms that are able to anticipate the cyclic changes of the environment.

The capacity to predict the day-night alternation is meticulously timed in mammals by the "biological clock", the Suprachiasmatic nucleus (SCN), localized in the anterior hypothalamus, which organizes the activity-rest rhythm of the body in ≈24h periods.

The SCN is known to establish different set points over the course of the day for the regulation of physiological and hormonal processes relative to the activity requirements (Stephan & Zucker, 1972;

Kalsbeek *et al.*, 2006a). The set point that the SCN imposes on Tb during the resting phase (day in rodents and night in humans) is lower than the set point established during the active phase (Refinetti & Menaker, 1992a; Benstaali *et al.*, 2001; Refinetti, 2003; 2010). Several studies have demonstrated that the SCN is necessary to sustain daily oscillations in Tb since SCN lesioned rats show constant Tb levels instead of a daily rhythm (Eastman *et al.*, 1984; Liu *et al.*, 2002; Scheer *et al.*, 2005).

Although it is not understood how the biological clock can establish day-night Tb set points, it seems that setting different temperature levels according to the time of the day represents a valuable strategy for the organisms to ratio their energetic reserves.

Furthermore, thermoregulatory set points are modulated also by the metabolic state of the body. As mentioned before, the development of endothermy is tightly coupled with the metabolic balance of the body therefore it has been generally accepted that Tb levels are good indicators of energy expenditure (Figure 2). For example diet-induced thermogenesis is a well-documented process that consists of a Tb increase during the postprandial phase of food intake (Acheson *et al.*, 1984; Diamond *et al.*, 1985). Postprandial thermogenesis is modulated by each of the components of food intake behavior (taste, stomach distension, digestion)(Diamond *et al.*, 1985; LeBlanc & Diamond, 1986; Diamond & LeBlanc, 1987a; Tittelbach & Mattes, 2002).

For a long time it was believed that the increases in Tb observed after food intake derive from chemical reactions taking place during the digestive process, however the increase in temperature elicited by eating appears several minutes before the animal has finished eating (Diamond *et al.*, 1985).

Later studies also demonstrated that postprandial thermogenesis consists of two phases. The first phase depends on both the

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sympathetic and the parasympathetic systems while the second phase depends mostly on the signals of the parasympathetic nervous system (Diamond & LeBlanc, 1987b). This diet-induced thermogenesis depends on the sympathetic activation of BAT (Cypess *et al.*, 2009).

Food deprivation or hypoglycaemia also modifies Tb set point, anorexic patients have decreased temperatures and fasted rodents tend to enter a torpid state that implies a decrease of Tb below the basal resting levels (Scalfi *et al.*, 1991; Scalfi *et al.*, 1992). Rats also show a torpor-like modulation of Tb during fasting; Tb decreases in relation with the time that the animals are without food intake (Liu *et al.*, 2002; Tokizawa *et al.*, 2009). It has been proposed that the modulation of Tb during fasting might be related with the sensing of metabolic signals; like thyroid hormones (Herrmann *et al.*, 1985; Silva & Larsen, 1986; Gupta & Chakrabarty, 1990; Silva, 2001), insulin (Marette & Bukowiecki, 1989; Schwartz *et al.*, 1992) , leptin (Ahima *et al.*, 1996), ghrelin (Gluck et al., 2006), and glucose levels. 2-Deoxy-glucose (Miselis & Epstein, 1975). An analogue of glucose that blocks glycolysis induces powerful Tb decreases, suggesting that sensing glucose or intracellular metabolic indicators like AMPK or the ADP/ATP ratio might be involved in the modulation of thermoregulatory areas of the brain (Dark & Pelz, 2008).

THE SUPRACHIASMATIC NUCLEUS; THE PEACEMAKER OF PHYSIOLOGY

The SCN has the endogenous capacity for a self-sustained 24h rhythm in neuronal activity. This allows it to transmit its rhythmic information to the rest of the body without any external time cues (Turek, 1981; Bos & Mirmiran, 1990).

Experiments that remove the changing light-dark (LD) cycle by placing subjects in constant darkness (DD) have demonstrated that the period of the activity–rest cycle persist within the endogenous

cycle imposed by the SCN (Usui, 2000). More over *in vitro* studies have demonstrated that organo-typic cultures of the SCN sustain their oscillations outside the body (Inouye & Kawamura, 1979; Bos & Mirmiran, 1990), meaning that the genetic program of the cells that constitute the biological clock have the molecular machinery to sustain their activity. The genes involved in this process are known as "clock genes" and interact with their products in positive and negative feedback loops that promote and inhibit their transcription with a period of about 24h (Field *et al.*, 2000; Bae *et al.*, 2001; Kalsbeek *et al.*, 2006b).

In spite the fact that each neuron of the SCN has the same clock genes, the oscillation of their neuronal activity is not the same (Figure 5), it has been demonstrated that different neurons have their own period and that the coordination of the cycles is arranged by the communication within the nucleus in order to release a clear an organized cycle (Welsh *et al.*, 1995; Saeb-Parsy & Dyball, 2003a; Bhumbra *et al.*, 2005).

Even though the SCN has the endogenous mechanism to ensure its own oscillation, it is also synchronized to the LD cycle by means of direct retinal projections called the retino-hypothalamic tract (RHT) (Altimus *et al.*, 2008; Altimus *et al.*, 2010; Nakamura *et al.*, 2011).

The RHT is formed by ganglion cells in the retina that are depolarized by light and send axonal projections to the ventral retino-recipient part of the SCN, enabling the biological clock to adjust its oscillation to the environmental LD cycle (Hattar *et al.*, 2002; Hattar *et al.*, 2006).
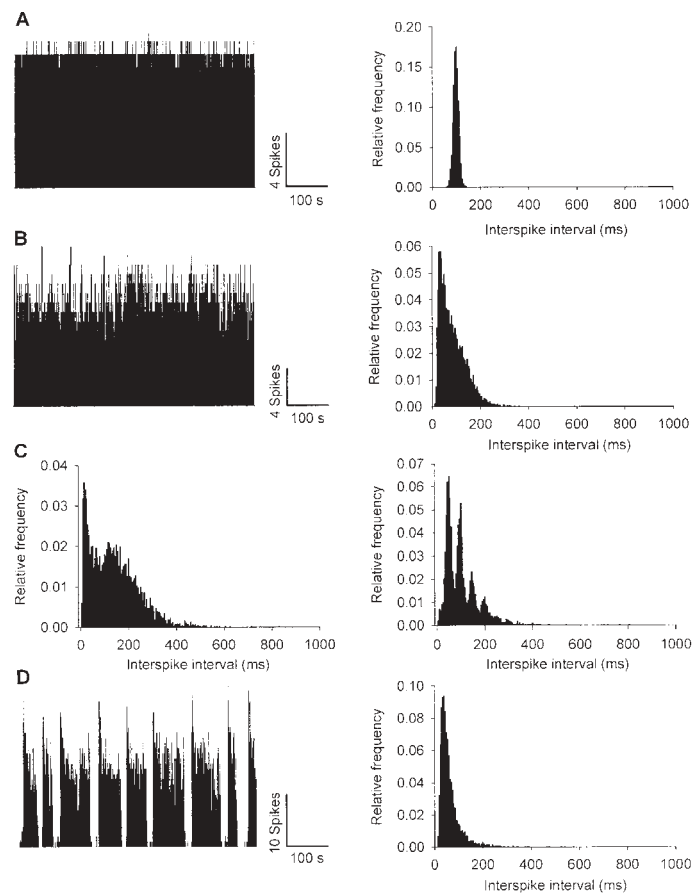
Figure 5. Electrical activity of distinct SCN neurons. (A and B) electrical activity in 100 seconds (left) and 1000 milliseconds (right) of two different neurons. (C and D) relative frequency in neuronal electrical activity of four different neurons in the SCN in 1000 milliseconds. Taken from: (Saeb-Parsy & Dyball, 2003a).

In addition it has been demonstrated that the SCN also receives information from other parts of the brain such as the intergeniculate leaflet (IGL) (Pickard *et al.*, 1987; Harrington, 1997; Morin & Allen, 2006; Saderi *et al.*, 2013), the nucleus of the solitary tract (NTS) (Buijs *et al.*, 2014), the medial raphé (mRPa) (Morin & Allen, 2006) and the metabolic arcuate nucleus (ARC) (Yi *et al.*, 2006). The SCN also has de capacity to activate its neuronal activity in response to a wide variety of signals such as ghrelin (Yi *et al.*, 2006), re-feeding after long term fasting (Saderi *et al.*, 2013), LPS infusions (Marpegan *et al.*, 2005; Guerrero-Vargas *et al.*, 2014) and changes in blood pressure (Buijs *et al.*, 2014). The implications of these inputs are

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still under investigation but suggest that the SCN is informed about the activity and metabolic state of the organism in order to adjust the output of the clock.

The SCN transmits its temporal information by means of humoral and neuronal communication; early studies demonstrated that SCN transplants to bilateral SCN lesioned animals (host) induce the acquirement of the locomotor activity rhythm of the donor though a diffusible signal (Ralph *et al.*, 1990; Silver *et al.*, 1996), furthermore encapsulation of the SCN preserves some aspects of the physiological rhythmicity but not of every rhythmic feature of physiology like glucose production, melatonin secretion or corticosterone (Meyer-Bernstein *et al.*, 1999), indicating that neuronal pathways from the SCN to hypothalamic nuclei are crucial for the arrangement of rhythmicity.

The SCN balances sympathetic and parasympathetic output to peripheral organs through separate preautonomic neurons through excitatory and inhibitory projections (Buijs *et al.*, 2003a; Buijs *et al.*, 2003b).

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βGAL-PRV   GFP-PRV

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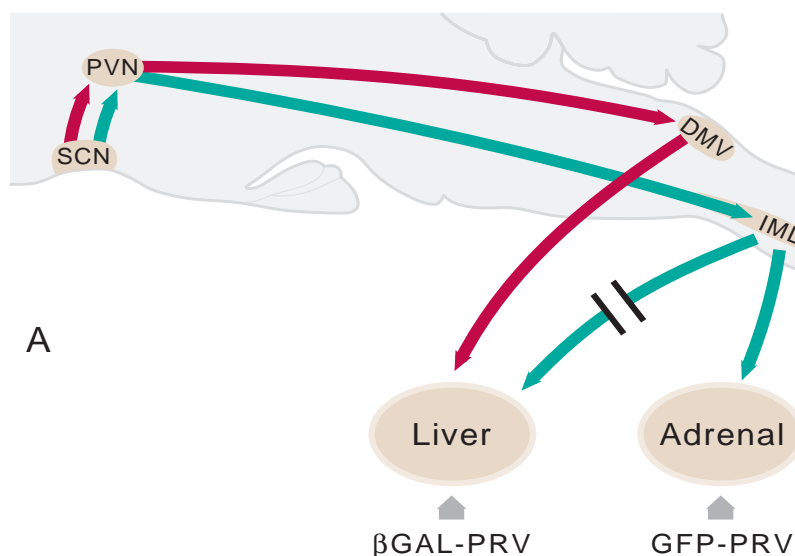
Figure 6. Separation of sympathetic and parasympathetic preautonomic neurons in the hypothalamus. Galactoside PRV (GAL-PRV) was injected into the sympathetic denervated liver, forcing the virus to infect the brain via the vagus nerve (red lines); simultaneously, the presympathetic neurons were labelled by an injection of green fluorescent protein (GFP)-PRV into the adrenal (green lines). After the labelling of the first-order neurons in the brainstem and spinal cord, this approach resulted in separate pre-parasympathetic and sympathetic neurons in the PVN (second order), followed by a similar separation of the third-order neurons in the SCN. Taken from; (Buijs *et al.*, 2003a).

This bimodal arrangement of the SCN neuronal pathways allows it to specifically increase or decrease the autonomic tone of the organs in the body, a characteristic that explains the variability of phases within the neurons of the SCN as well as the different phase in the distinct aspects of physiology under its temporal control (Figure 7).

In spite of the vast knowledge about the neuronal outputs of the SCN, there are many nuclei that receive SCN neuronal input like the Arcuate nucleus (ARC) whereby the functionality has not been determined.
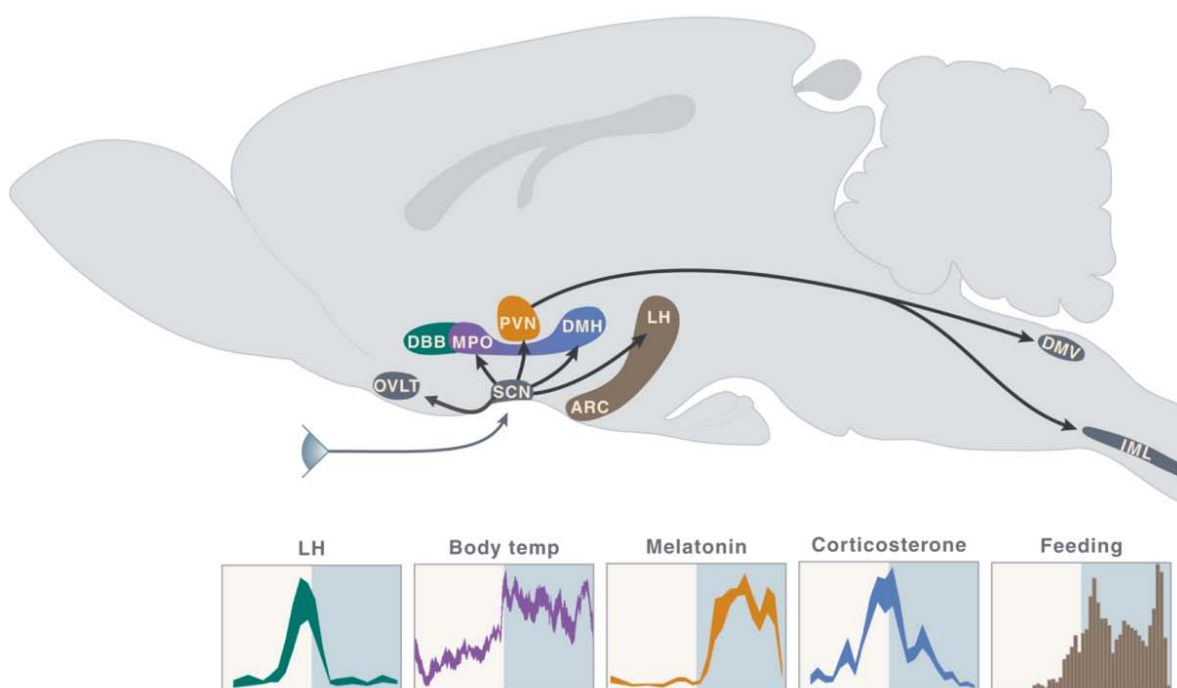
Figure 7. Neuronal outputs of the SCN. The Suprachiasmatic nucleus projects to hypothalamic nuclei in order to modulate daily hormonal and physiological set points. MPO; medial preoptic nucleus, OVLT; organum vasculosum of the lamina terminalis, DBB; diagonal band of broca, SCN; Suprachiasmatic nucleus, PVN; paraventricular nucleus, LH (brown); lateral hypothalamus, ARC; arcuate nucleus, DMH; Dorsomedial nucleus of the hypothalamus, DMV; dorsomotor nucleus of the vagus, IML; intermediolateral column, LH (green); luteinizing hormone.

ANATOMIC RELATIONSHIP BETWEEN THE SCN AND THE ARC

The main integration sites of the brain that receive metabolic information from the periphery are the Dorso-Vagal complex (DVC) and the Arcuate nucleus (ARC) (Schwartz *et al.*, 2000; Morton *et al.*, 2006; Schwartz, 2010).

The ARC is one of the most important humoral metabolic sensors of the brain. It is placed lateral to third ventricle and adjacent to the Median eminence (ME) allowing the metabolic information from the cerebrospinal fluid and the portal circulation to enter the ARC.

The ARC has two main neuronal populations Neuropeptide Y (NPY)/Agouti Related Protein (AgRP) and Proopiomelanocortin (POMC)/Cocaine Amphetamine Response Transcript (CART) producing neurons (Schwartz *et al.*, 2000). These neuronal populations respond to metabolites and hormones that indicate the energetic state of the body through a vast variety of receptors and transporters (see chapter 2).
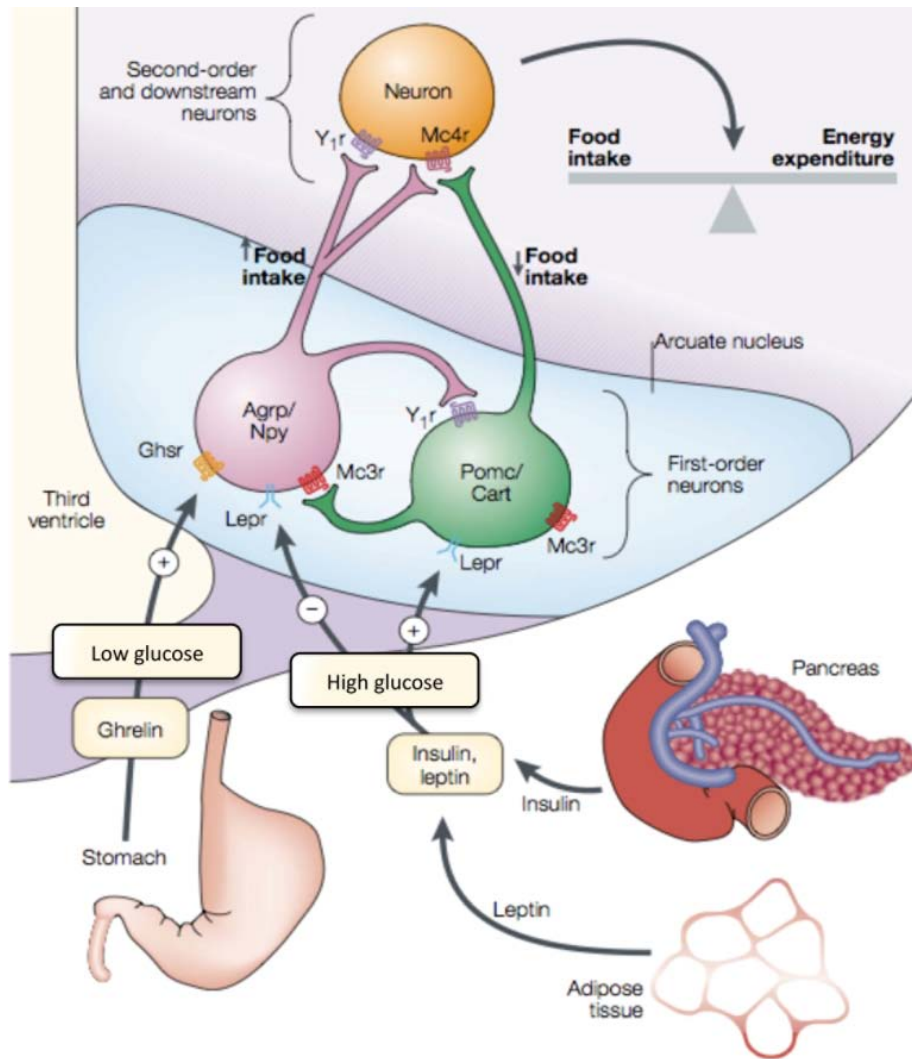
Figure 8. The ARC is a humoral sensor. The ARC is located adjacent to the median eminence and the third ventricle, a position that allows it to sense substances in the portal circulation such as glucose, leptin, insulin and ghrelin. These signals are perceived by the neurons of the ARC that project to second order nuclei like the paraventricular nucleus or the dorsomotor nucleus to induce changes in energy expenditure and food intake. AgRP/NPY are orexigenic and POMC/CART are anorexigenic peptides. Modified from; (Barsh & Schwartz, 2002).

The ARC controls food intake and energy balance (chapter 2) furthermore it was demonstrated that this nucleus also contributes to the modulation of the sleep-wake rhythm and the feeding rhythm (Wiater *et al.*, 2013).

Interestingly ARC and SCN lesions have similar effects in food intake rhythmicity (Figure 9) (Li *et al.*, 2012), suggesting that the

neurons in the ARC receive circadian information or provide metabolic information to the SCN and that this reciprocity is crucial to sustain some behaviors and physiology.
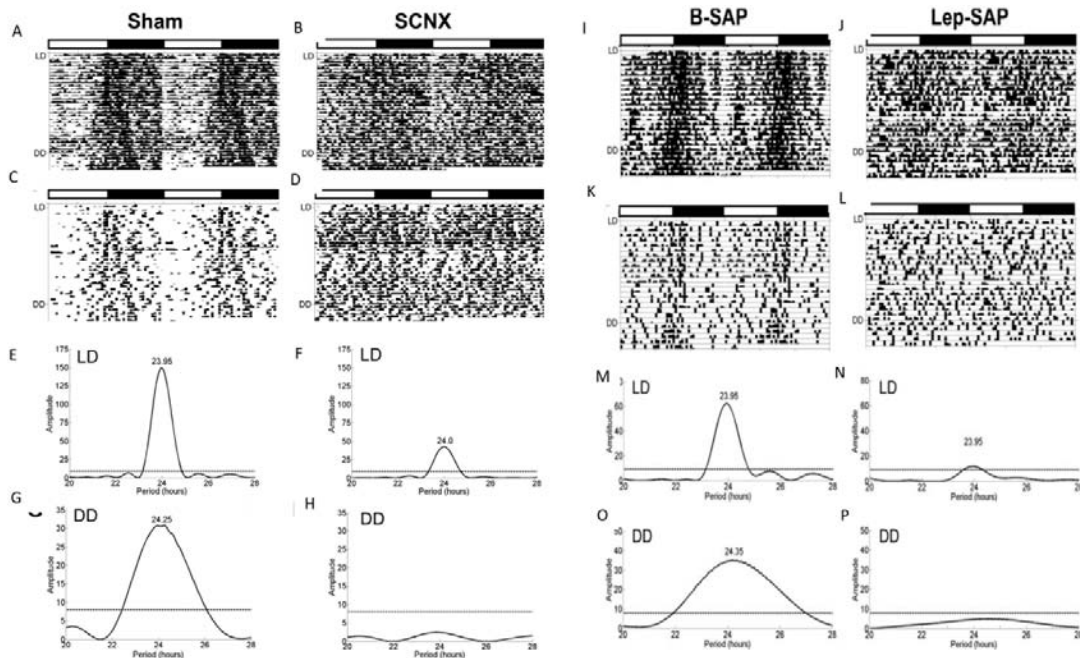
Figure 9. Eatograms comparing SCN lesioned and ARC Leptin-saporine lesioned rats. Food intake rhythmicity is lost after a SCN lesion (A-H) and the chemical lesion of the ARC leptin sensitive neurons via saporine-coupled Leptin (Femi-Pearse *et al.*). A and B are group eatograms, C and D single animal eatograms of Sham and SCN lesioned subjects in light-dark (LD) and constant darkness (DD) conditions, E-H represent the periodogram of B-saporine and ARC leptin-saporine lesioned subjects in LD and DD. I and J are group eatograms, K and L single animal eatograms of B-saporine and ARC leptin-saporine lesioned subjects in LD and DD, M-P represent the periodogram of B-saporine and ARC leptin-saporine lesioned subjects in LD and DD. Taken from; (Li *et al.*, 2012).

Interestingly the SCN and the ARC have direct neuronal reciprocal connections (Saeb-Parsy *et al.*, 2000; Yi *et al.*, 2006) (Figure 10,11), emphasizing their capacity to exchange temporal-metabolic information.
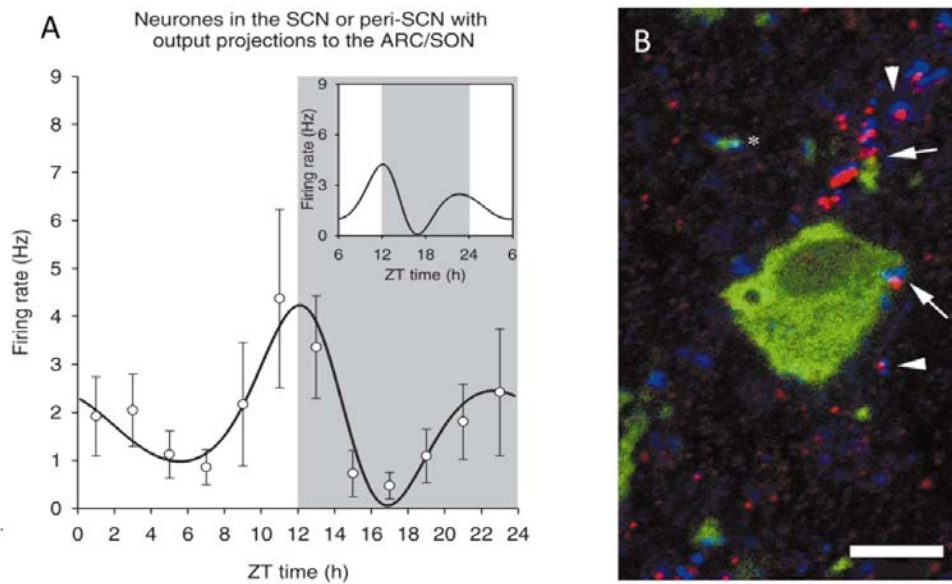
Figure 10. The SCN projects to the ARC. (A) Neuronal electrical activation of SCN neurons projecting to the ARC and (B) Dopaminergic (green) neurons in the ARC receiving VIP (red) synaptic contacts determined by synapsin (blue), Taken from; (Gerhold *et al.*, 2001; Saeb-Parsy & Dyball, 2003a).

In addition tracing studies have demonstrated the reciprocal connections between the SCN and the ARC, injections of the fraction B of the cholera toxin (CtB) in the ARC, shows clear fibers (anterograde) and somas (retrograde) tracing in the SCN demonstrating reciprocal connection between these two hypothalamic nuclei (Figure 11)(Yi *et al.*, 2006).
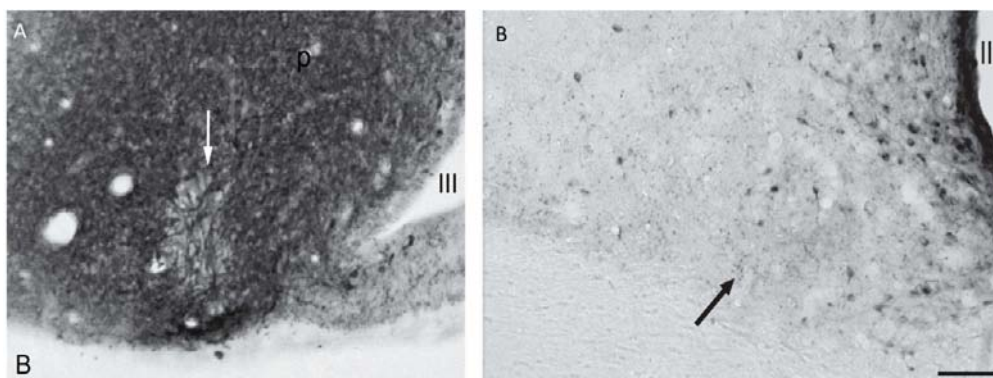
Figure 11. The SCN and the ARC share reciprocal direct neuronal connections. The SCN of animals injected with CtB in the ARC (A) show both cell bodies and fibers as result of a retro-anterograde tracing (B). Modified from (Yi *et al.*, 2006).

AIM OF THE STUDY

These observations stimulated us to investigate how temporal and metabolic information may influence the set point of temperature.

Hereto we first paid attention to the mechanisms used by the SCN to control the temporal changes in physiology and next how at the other hand the SCN may interact with metabolism to influence the temperature settings. In order to understand how metabolism may influence temperature we hypothesized that the arcuate nucleus (ARC), which is the main entrance for metabolic information to the hypothalamus, might be involved in temperature regulation.

SCOPE OF THE THESIS

In the present thesis we investigated the anatomical and functional basis for the circadian and metabolic control of Tb. Hereto we used anatomical tracing techniques to investigate the relationship between the different hypothalamic nuclei in the hypothalamus involved in the organization of circadian rhythms, energy balance and thermoregulation. We have used techniques to demonstrate neuronal activity in these nuclei and targeted lesioning procedures to investigate the relationships between these nuclei. Finally we used retro-microdialysis techniques using agonists and antagonists of SCN and ARC neurotransmitters to investigate the involvement of these nuclei in thermoregulation.

INVESTIGATION STAGES

FIRST PART

In the first part of this investigation we studied the functional relationship between the biological clock and a metabolic sensor of peripheral signals of the body the Arcuate Nucleus.

We hypothesized that the SCN confers an endogenous rhythm to neurons in the ARC by means of neuronal projections and especially to the α-MSH neuronal population, which has been demonstrated to induce energy expenditure and thermogenesis and to receive the main anatomical input from the SCN.

SECOND PART

In the second part of this study we investigated the involvement of the SCN and the ARC in the modulation of body temperature.

With the hypothesis that the main controller of thermoregulation the median preoptic nucleus (MnPO) might be the proper candidate to integrate temporal and metabolic information, we investigated whether the SCN and the metabolic ARC establish an anatomical and functional relationship with the MnPO.

CHAPTER 2

Introduction

Physiological and behavioral processes exhibit circadian fluctuations with peaks at different time points, with a clearly synchronized temporal order imposed by the Suprachiasmatic Nucleus (SCN) (Buijs *et al.*, 2006; Kalsbeek *et al.*, 2006c). Destruction of the SCN induces a complete loss of all these rhythmic functions even under LD conditions (Wise *et al.*, 1988; Silver *et al.*, 1996; Perreau-Lenz *et al.*, 2003; Ruiter *et al.*, 2003). Maintenance of these cycling patterns in physiology is due to the SCN ability to communicate information though neuronal projections to hypothalamic target nuclei (Guo *et al.*, 2005; Buijs *et al.*, 2006) resulting synchronization of hormonal and autonomic output with the rhythmic changes in behavior. In this respect also connections between the SCN and the ARC have been described (Saeb-Parsy *et al.*, 2000; Gerhold *et al.*, 2001; Yi *et al.*, 2006), with still little knowledge on the relevance of these connections.

The Arcuate nucleus (ARC) integrates information about nutrient availability from blood stream and cerebrospinal fluid (Krisch & Leonhardt, 1978; Broadwell *et al.*, 1983; Schaeffer *et al.*, 2013a) a characteristic that promotes homeostatic responses influencing energy balance and food intake (Sainsbury & Zhang, 2010) by

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means of neuronal connections to autonomic and endocrine centers, such as the Paraventricular Nucleus (PVN) and Lateral Hypothalamus (LH) (Elmquist *et al.*, 1998; Elias *et al.*, 1999; Fekete *et al.*, 2000; Niimi *et al.*, 2001). In the ARC two main neuronal populations are known as key players in the regulation of energy balance, proopiomelanocortin/Cocaine Amphetamine Regulated Transcript (POMC/CART) neurons shown to inhibit food intake (Poggioli *et al.*, 1986; Fan *et al.*, 1997) and Neuropeptide Y/Agouti-related protein (NPY/AgRP) neurons, shown to be orexigenic (Clark *et al.*, 1984; Stanley *et al.*, 1986; Corp *et al.*, 1990; Atasoy *et al.*, 2012).

Recently evidence from different sources, providing human epidemiological or experimental data and animal experimental data, has shown a strong relationship between metabolic health and the time of food consumption (Salgado-Delgado *et al.*, 2008; Salgado-Delgado *et al.*, 2010a). Earlier studies in addition have shown that the arcuate nucleus not only is involved in the control of food intake but has also an important contribution in cardiovascular regulation and energy expenditure both of which have an important circadian component. The fact that our society has such an increase in the occurrence of the metabolic syndrome, illustrated by the increase of cardiovascular and diabetes type2 diseases, has stimulated the interest in the interaction between circadian and metabolic regulated processes.

Therefore considering the essential role of the ARC in maintaining energy balance and the importance of the day-night rhythmicity in this respect, the aim of the present study was to investigate the functional relation between the SCN and the ARC by analyzing its connectivity, its diurnal neuronal activity pattern and to study the functional nature of this interaction. Our results show that α-MSH neurons have a daily pattern in neuronal activity, which is

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organized by the SCN whereby the SCN excites and probably inhibits α-MSH neuronal activity in a different temporal pattern.

MATERIAL AND METHODS

Animals

Adult male Wistar rats (Rattus norvegicus, 250–280g), obtained from the general bioterium of the Faculty of Medicine of Universidad Nacional Autónoma de México, were housed in individual cages under LD 12:12 conditions (light on at 7h considered ZT0). Water and regular laboratory rodent diet (Purina, Chow 5001; Woodstock, Ontario, Canada) were provided *ad libitum* throughout the study, unless otherwise stated. Rats were adapted one week to housing conditions before starting the experimental protocol. The Committee for Ethical Evaluation at Institute for Biomedical Research, UNAM, approved experiments that were carried out in strict accordance with the Mexican norms for animal handling (Norma Oficial Mexicana NOM-062-ZOO-1999).

Stereotactic surgeries

Bilateral (n=10) or unilateral (n=12) SCN lesions were performed under a mixture of Ketamine (40-80 mg/kg) with Xylazine (5-8 mg/kg) injected IM. For SCN lesions animals were placed in the stereotactic frame (tooth bar ±2.5 mm; arm: 4°; coordinates: -0.2mm from bregma; ±0.9mm lateral from midline; 8.2 to 8.6mm below brain surface), electrodes (0.2 mm diameter) were placed bilaterally in the SCN. A current of 0.35mA was applied for 45s for each electrode (Grass D.C. Constant Current Lesion Maker). For

unilateral SCN lesions only one lesioning electrode was used. Sham surgeries were performed as mentioned without current (sham bilateral n=4 and sham unilateral n=4).

The tracer Cholera Toxin B (CtB) 0.5% conjugated with Alexa Fluor-555 fluorescent dye (Molecular Probes, Eugene, OR, USA) was unilaterally injected (0.1ml) in the SCN of 12 rats with a Hamilton microsyringe using the same coordinates mentioned above. After the injection the syringe was left in place for 10 minutes to minimize leakage. Missed SCN injections were used as controls to verify tracing and target specificity.

Immunocytochemical staining

All rats were deeply anesthetized with a lethal dose of sodium pentobarbital and perfused intracardially with 0.9% saline followed by a solution of 4% paraformaldehyde in 0.1M Buffer Phosphate saline (PBS; pH 7.4). Brains were removed and kept in fixative at 4°C for overnight postfixation, equilibrated 48h with 30% sucrose in 0.1M PBS. Brains were cut in 40μm coronal sections with a cryostat and placed in 30% sucrose. Sections used for immune labeling were collected and rinsed in 0.1M PBS.

Six ARC sections per animal, 2 anterior, 2 medial and 2 posterior, were incubated with rabbit anti-c-Fos primary antibody (1:40,000, Calbiochem) over night under constant shaking at 4°C, subsequently sections were rinsed and incubated at room temperature with biotinylated donkey-antirabbit serum (1:200, Jackson) for 1h, rinsed and incubated in avidin-biotin complex (1:500 Vector Laboratories) for 1h, product visualization was obtained with; 0.01% diaminobenzidine, 0.05% nickel ammonium sulfate and 0.01% hydrogen peroxide for 6 min. A second staining was performed on the same sections with sheep anti-α-MSH primary antibody (1:10,000, Millipore) over night at constant shake at 4°C, followed by

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incubation with biotinylated donkey anti-sheep (1:200, Jackson) for 1h, then rinsed and incubated in avidin-biotin complex (1:500), same procedure was followed for the second staining without nickel.

6 (anterior-posterior) SCN sections of bilateral, unilateral lesioned and control rats were stained for Vasoactive Intestinal Peptide (VIP; 1:2000 (Buijs *et al.*, 1989). The SCN of CtB injected animals were also incubated with rabbit CtB primary antibody following the same immunohistochemical procedure described before to examine the position of the SCN lesions and CtB injections respectively.

Immunofluorescence staining

ARC sections of intact animals, were incubated with rabbit- anti- VIP or anti- GRP 1:2000 (Buijs *et al.*, 1989; Romijn *et al.*, 1998) together with sheep anti ɑ-MSH primary antibodies over night at 4°C. Sections were rinsed with PBS and incubated with secondary antibodies Alexa 555 conjugated AffiniPure Donkey anti-Rabbit and Alexa 484 or 647 conjugated AffiniPure Donkey anti-Sheep (1:200, Jackson Immuno Research) for 1h, then rinsed and placed on gelatinized glass slides, coverslipped in glycerol (50%)-PBS (50%) and analyzed with the LSM 5 Pascal confocal microscope (Zeiss, Jena, Germany).

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM), for each group; the results were considered statistically significant when $P<0.05$. To determine the presence of daily differences in ARC c-Fos neuronal activity and ɑ-MSH activation under both *ad libitum* and fasting conditions a series of non-repeated measures One-Way ANOVA were performed. If significant, ANOVA was followed by Tukey's *post hoc* to identify differences between individual group means. To compare the curves obtained for ARC c-Fos neuronal activity and ɑ-MSH activation under *ad libitum* and fasting

conditions, a two-Way ANOVA performed and followed by Bonferrioni's *post hoc* if required.

To determine the effect of SCN bilateral lesions at ZT22, unpaired Students t tests were performed.

Non-repeated measures One-Way ANOVAS were done to determine whether ARC activity of SCN sham and unilateral SCN lesions had significant differences. Since both ARC sides counted for either Sham or SCNx animals correspond to the same animal and section level, to compare the differences between sides we additionally performed a paired t tests to compare both ARC sides of the same brain in Sham and SCNx rats.

To determine if light affects ARC neuronal activity non-paired t tests were performed, finally differences between constant darkness at CT2 compared with ZT2 and 22 were examined through One-Way ANOVA.

EXPERIMENTAL PROCEDURE

Determination of ARC daily activity pattern

Rats were sacrificed at six time points along the LD cycle (ZT2, 6, 10, 14, 18 and 22, n=4 for each time point) in *ad libitum* or 48h fasting prior to sacrifice. Quantification of the total c-Fos IR neurons within the ARC (three sections per animal, anterior, medial and posterior) was performed with the Image J software by determining ARC area by free hand selection of the quantification area for each side of the ARC, background was subtracted and threshold was determined, particle analysis was set for particles of 1.0-2.0 circularity and 500-800 pixels.

The number of α-MSH neurons co-localizing with c-Fos was counted by two observers blind to the treatment, only those α-MSH neurons that presented a clear double mark of c-Fos (blue nuclei) and α-

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MSH staining (brown soma) were included as co-localizing neurons (Figure 1A).

ARC activation after SCN bilateral lesions

To ensure the completeness of the SCNxx, the locomotor activity of the animals was recorded during the next two weeks after surgery to discard the reappearance of the activity rhythm and only the animals with an arrhythmic activity profile were included in the experiments. The lack of rhythm in lesioned animals was determined in LD since small remnants of the SCN are known to inhibit the locomotor activity resulting in LD rhythmic masking locomotor activity profiles (Kalsbeek & Buijs). In addition VIP immunohistochemistry was performed to detect SCN remnants, animals that showed VIP-IR were discarded from further analysis (Figure 13B).

The brains of non-rhythmic animals (n=4) were collected at ZT22 for comparison purposes since intact animals showed the highest activation of the ARC at this time point, no other time point was taken into account for this experiment since previous studies showed that SCNxx rats present a complete lack of rhythms and show equal physiology and behavior at all time points also in LD (Scheer & Buijs, 1999). The ARC neuronal activation was quantified as described above.

ARC activation after SCN unilateral lesions

SCN unilateral lesions (SCNx) were performed using one electrode and analyzed for changes in c-Fos expression in the ARC depending on the lesion side. In order to ensure that SCNx comprise only one side of the SCN, we selected only animals presenting a locomotor activity rhythm under LD conditions and accepted only animals with

an intact SCN on one side and a complete lesioned SCN on the other side as determined by VIP staining (n=3).

ARC response to One-hour light pulse

1h light pulse of 100*lux* was given in animals after 48 hours of fasting during the dark phase from ZT21-22 (n=4). Animals were sacrificed at ZT22; analysis of the ARC activation was performed as described previously and compared to ZT22 48h fasted intact animals.

Endogenous inhibition of the ARC

ARC activation was determined in animals subjected to constant dark conditions (DD) for 2 days prior sacrifice at CT2 (n=3) and CT22 (n=3). ARC and α -MSH neuronal activity was compared animals obtained in LD at ZT2 and 22.

SCN projections to α-MSH neurons in the ARC

To determine SCN projections to α-MSH neurons, CtB was injected into the SCN; rats were sacrificed after 7 days. The ARC was analyzed for CtB positive fibers together with α-MSH staining. Moreover to investigate the possible transmitters involved, GRP and VIP innervation pattern on α-MSH neurons in the ARC was determined.

RESULTS

α-MSH NEURONS SHOW A PEAK OF ACTIVITY AT THE END OF THE DARK PERIOD

The number of c-Fos positive cells in the ARC of *Ad libitum* animals showed a daily peak (Figure 12C; time interaction, $F[5,18]=25.6$, $P<0.0001$), with a c-Fos IR maximum during the last part of the active phase at ZT22 and the lowest at ZT10 ($P<0.001$).

The present results showed at ZT22 the localization of c-Fos IR mainly in the dorso-lateral ARC coinciding with the presence of α-MSH neurons. Their activation was determined with double immunohistochemistry for c-Fos and α-MSH IR (Figure 12A, B left panel). Double-labeled c-Fos/α-MSH neurons showed a 24h activity pattern (Figure 12D; $F[5,18]=11.26$, $P<0.0001$), with the highest activation at ZT22 and the lowest al ZT10 ($P<0.05$). Since food intake in rats mainly occurs during the dark phase of the LD cycle, and α-MSH neurons are activated by food intake associated signals (Morton *et al.*, 2006), the observed activation of α-MSH neurons could be triggered by feeding and not by direct inputs from the SCN. Therefore we examined c-Fos expression in the ARC after 48hr fasting; in spite of the lack of food intake the ARC c-Fos-IR pattern persisted (time interaction, $F[5,18]=17.61$, $P<0.0001$) with differences between ZZ10 y ZT22 ($P=0.001$). Even when differences between *ad libitum* and fasting were found (interaction between conditions, $F[1,5]= 3.24$ $P<0.05$) at ZT14 ($P<0.01$) and ZT22 ($P<0.01$) with fasted animals presenting lower c-Fos levels (Figure 12C) the daily activation pattern was conserved even in the absence of food intake. In addition no differences between *ad libitum* and fasted rats in α-MSH cells expressing c-Fos were observed (Figure 12D; $F[1,5]= 0.02$ $P= 0.89$).

The persistence in the peak of c-Fos expression in α-MSH neurons at ZT22 during fasting conditions suggests that food intake is not the only factor involved in the rhythm in α-MSH neuronal activity. However fasting does not abolish the rhythm of other important

signals for ARC activity such as ghrelin, leptin, glucose and corticosterone (Ruiter *et al.*, 2003; Espelund *et al.*, 2005), which could represent an important humoral pathway for the SCN to promote a rhythm of neuronal activity in the ARC.
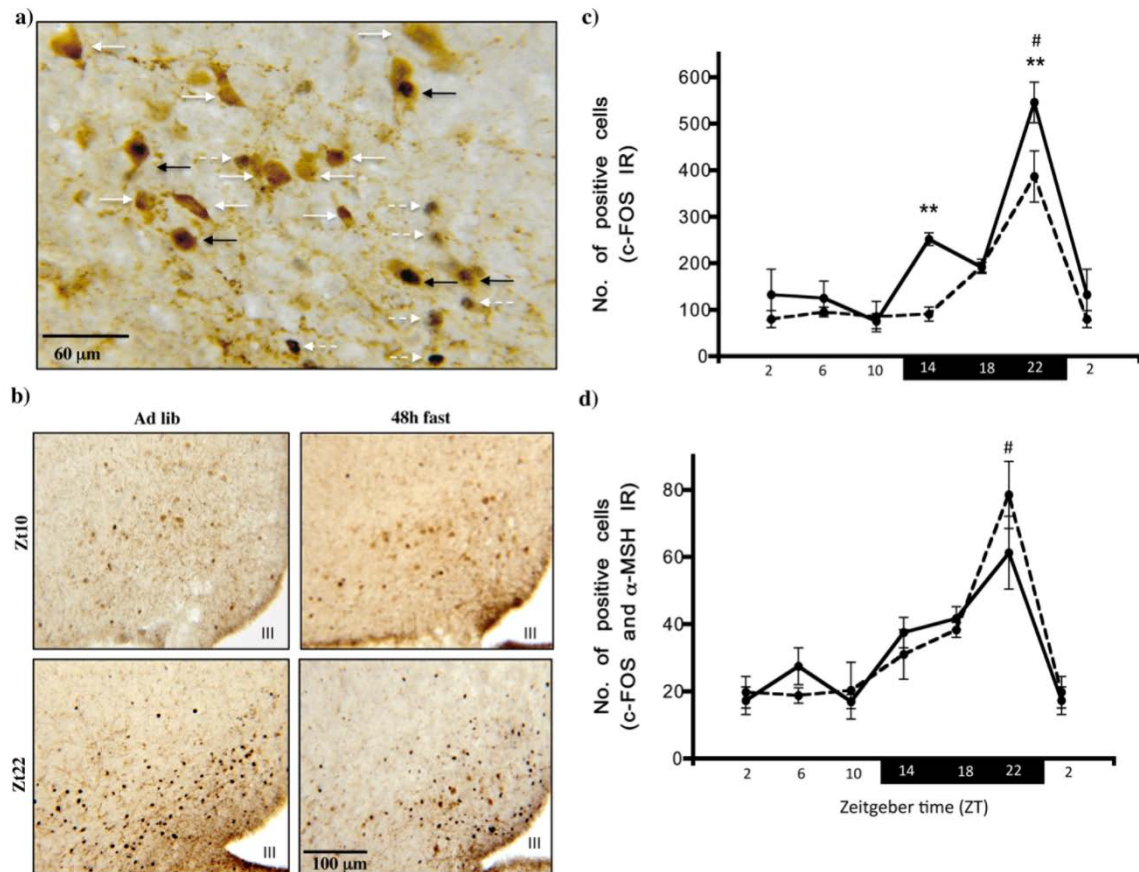
Figure 12. c-Fos is rhythmically expressed in α-MSH neurons in the Arcuate Nucleus. c-Fos in the ARC shows a daily activity pattern under ad libitum and 48h fasting conditions. (a) Representative ARC section at ZT18 under fasting conditions showing c-Fos (DAB+Ni = blue) and α-MSH (DAB= brown). White dashed arrows point to single c-Fos IR neurons; white solid arrows show single α-MSH IR neurons and black arrows designate neurons positive for both c-Fos and α-MSH. (b) Displays two representative time points from ad libitum and 48h Fasting conditions, which represent the highest and the lowest point during the LD cycle, ZT10 and 22 respectively. (c) ARC daily c-Fos activity pattern under ad libitum (solid line) and after a 48h fast period (dashed line), these curves represent total ARC neuronal activity under both conditions, (d) Daily curves from ARC c-Fos and α-MSH co-localization under ad libitum conditions (solid line) and after a 48h fast period (dashed line).

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** Illustrates significant difference between fasted and non-fasted time point (P<0.001), # denotes acrophases. ARC (Arcuate nucleus) ZT (Zeitgeber time), ɑ-MSH (α-Melanocite Stimulating Hormone), III (Third ventricle) LD (Light-Dark).

Bilateral SCN lesions (SCNxx) eliminate ARC activity peak at ZT22

To further determine whether the peak in ARC activation at ZT22, observed under *ad libitum* and fasting conditions was SCN dependent, we performed SCN bilateral lesions and examined the neuronal activation of the ARC. Only complete lesioned animals as determined by actograms and by post mortem analysis of VIP staining, were included (Figure 13A).

Since SCNxx (n=4) rats do not have a clear feeding pattern and eat during the entire 24h, our animals were fasted 48h prior to sacrifice to prevent ARC activity changes due to random food intake. After the SCNxx, the animals were sacrificed at ZT22 showing a nearly complete disappearance of c-Fos staining in the ARC (Figure 13C P=0.0004 and 2D P=0.0003), demonstrating that the presence of the SCN is essential to drive ARC activity.

ZT22 was the only sampled time point of SCNxx animals since several studies have demonstrated that SCN lesioned animals lack all rhythms including feeding (Abe *et al.*, 1979; Atasoy *et al.*, 2012), indicating that the ARC also lacks rhythmicity, and that the maximal activation at ZT22 is dependent on the SCN integrity.

The ARC is also responsive to humoral signals that oscillate, these rhythms have been demonstrated to be coordinated in a circadian pattern by the SCN (Kalsbeek *et al.*, 2001; Perreau-Lenz *et al.*, 2003; Cailotto *et al.*, 2005), whether the present diminishment in c-Fos in

the ARC and in α-MSH neurons is due only to the lack of rhythmicity in humoral signals or due to the loss of direct innervation from the SCN to the ARC remains not clear.
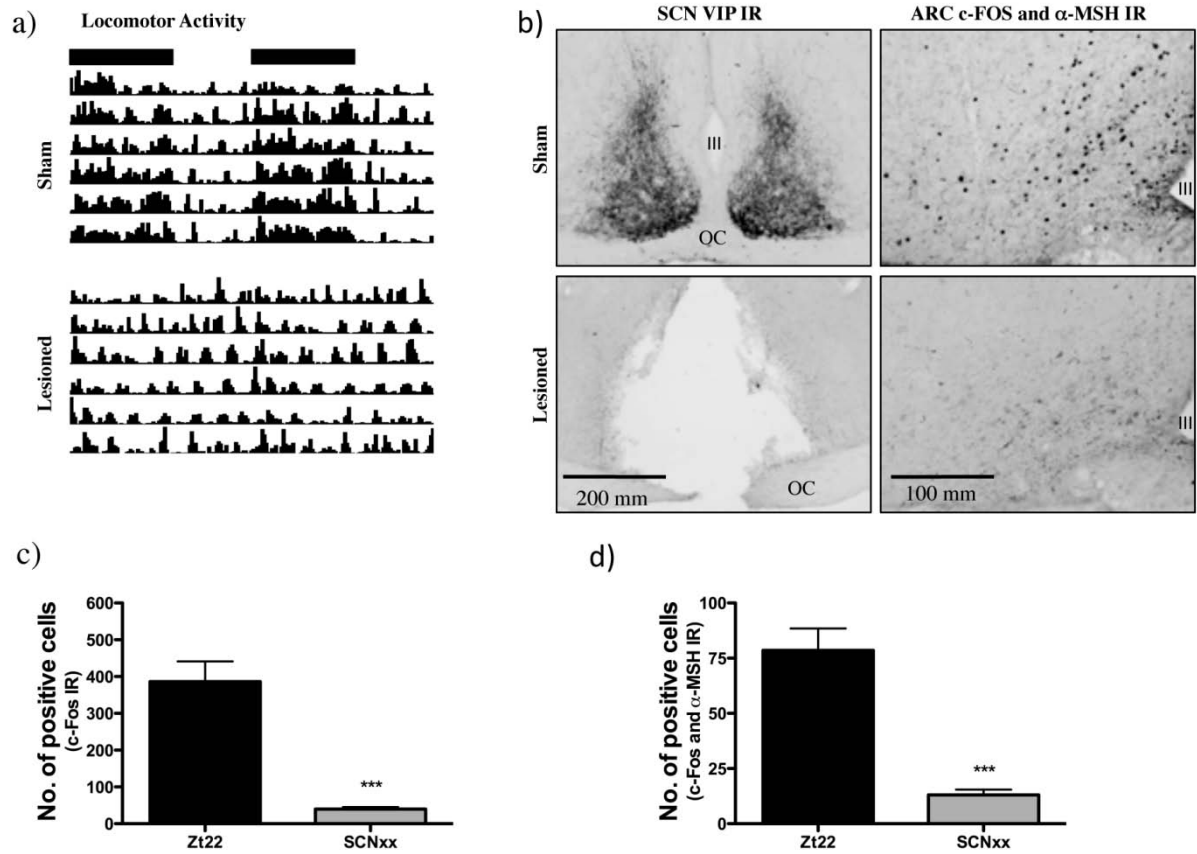
Figure 13. Bilateral lesions of the SCN prevent ARC activity at ZT22. (a) Actograms representing locomotor activity of sham (top) and lesioned (bottom) animals, (b) SCN VIP staining for sham and lesioned rats (left boxes). ARC c-Fos and α-MSH staining for sham and lesioned animals (right boxes) show that sham ARC staining present black dense dots of c-Fos together with α-MSH staining while SCNxx brain present only light gray α-MSH only somas. (c) Total ARC activation (c-Fos IR counts) at ZT22 of sham and SCN lesioned animals. (d) ARC α-MSH activation (c-Fos and α-MSH co-localization counts) at ZT22 of shams and SCN lesioned animals. The SCN lesioned animals showed significantly less c-Fos ($P<0.05$). All animals were fasted for 48h. VIP (Vasoactive Intestinal Peptide), ARC (Arcuate Nucleus of the Hypothalamus), IR (Immunoreactivity), SCN (Suprachiasmatic Nucleus), SCNxx (Bilateral SCN lesion), ZT (Zeitgeber time), OC (Optic Chiasma), III (Third ventricle).

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To determine whether the communication between the SCN and the ARC is at least partly neuronal, unilateral lesions of the SCN (SCNx, n=3) were performed. The efficiency of the lesion was determined in two ways, first the animals needed to present locomotor activity rhythm under LD conditions (Figure 14A) and the SCN needed to be only unilateral ablated as determined by VIP staining (Figure 14B).

After SCNx both sides of the ARC showed a significant decrease in neuronal activation as compared to sham animals at ZT22 (Figure 14C and D; $F[3,8]=9.42$, $P=0.005$ for total c-Fos and $F[3,8]=12.47$, $P=0.002$ for c-Fos/α-MSH). However the side of the ARC corresponding with the SCN lesion side presented even less c-Fos ($t[2,3]=23.63$, $P=0.0009$) and less α-MSH activated neurons ($t[2,3]=8.5$, $P=0.006$) as compared to the intact side of the same animal, indicating that the neuronal input from the SCN indeed is at least partly responsible for the ARC c-Fos peak.

Interestingly the onset of SCN activity seems to have an inhibitory effect on the ARC as observed by the loss of c-Fos activation in the ARC at ZT2 (Figure 12). This observation suggests that not only the SCN is necessary for the induction of c-Fos expression in the ARC during the night but that activity of the SCN might be responsible for its inhibition at the beginning of the resting phase. Therefore in the next series of experiments we investigated whether exposure of the animals to light might change ARC neuronal activity.
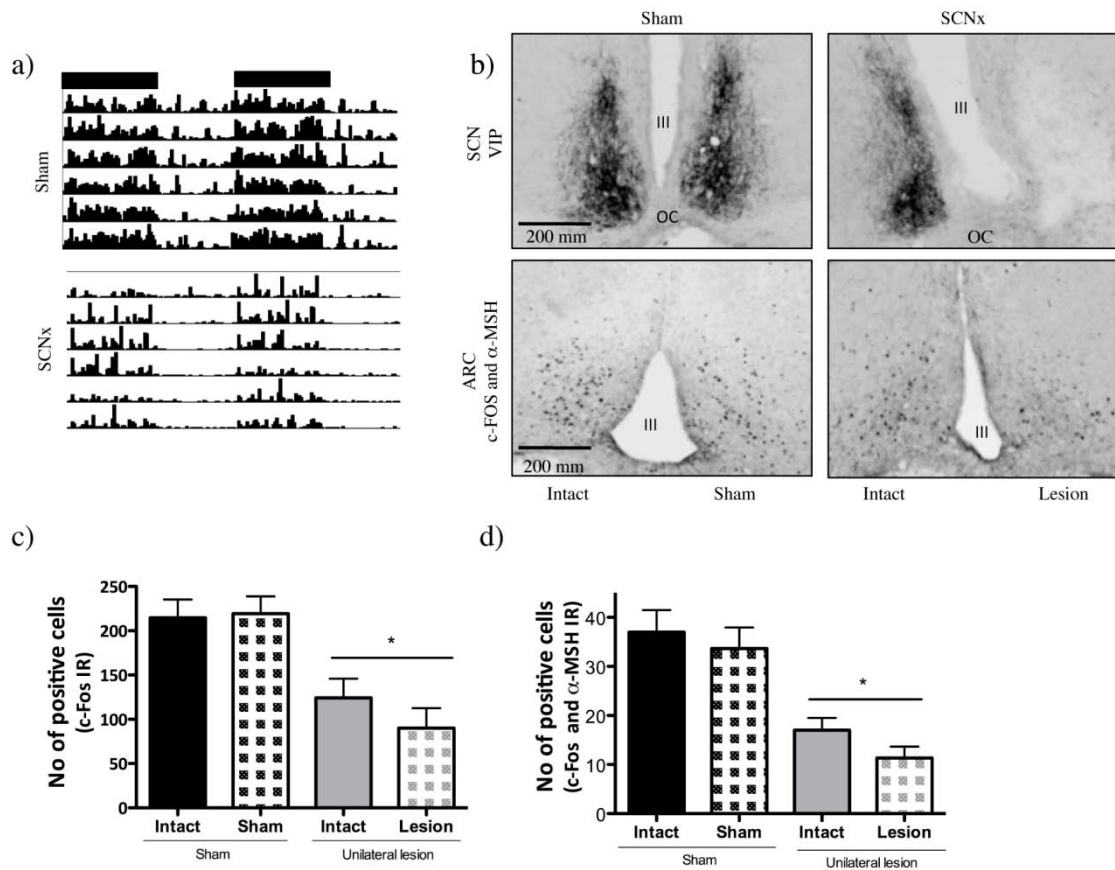
Figure 14. SCN unilateral lesions result in a diminished ARC activation at the lesioned side. (a) Actograms representing locomotor activity of sham (n=4) and unilateral SCN lesioned animals (top boxes). (b) SCN VIP staining for sham and lesioned rats (middle boxes), ARC c-Fos staining for sham and lesioned animals (bottom boxes). (c) ARC activation (c-Fos IR counts) at ZT22 at intact or sham or SCN lesioned side. (d) ARC ɑ-MSH activation (c-Fos and ɑ-MSH co-localization counts) at ZT22 of shams and SCN unilateral lesioned animals. Each bar represents one side of the ARC compared to the other. The lesioned side showed significant less c-Fos ($P<0.05$). Experiments were conducted under 48h fasting conditions. VIP (Vasoactive Intestinal Peptide), ARC (Arcuate Nucleus of the Hypothalamus), IR (Immunoreactivity), SCN (Suprachiasmatic Nucleus), SCNx (Unilateral SCN lesion), ZT (Zeitgeber time), OC (Optic Chiasma), III (Third ventricle).

LOSS OF ARC ACTIVITY BY LIGHT AT ZT 22

Photic signals especially at night are known to activate SCN neurons (Earnest *et al.*, 1993; Castel *et al.*, 1997); to phase shift SCN activity and to change its neuronal output resulting for example in the inhibition of melatonin secretion by GABA-ergic signaling (Perreau-Lenz *et al.*, 2003; Perreau-Lenz *et al.*, 2004). Since we observed that the SCN induced maximal neuronal activity in the ARC at the end of the dark period and we observed that the ARC showed an immediate decrease in neuronal activity after light onset (Figure 12), we examined whether light exposure during the night would be able to change ARC neuronal activity.

1h light pulse from ZT21-22 immediately decreased ARC c-Fos expression (P=0.001) and the number of the α-MSH cells co-localizing with c-Fos (P=0.0007) as compared to ZT22 without light (Figure 15). The observed diminishment of c-Fos in the ARC by light suggests that the decreased ARC c-Fos expression at ZT2 is provoked by light induced SCN neuronal activity resulting in a similar inhibition as observed at ZT2. It seems unlikely that hypothetical direct retinal projections to the ARC are responsible for this inhibition since it is known that the retino-hypothalamic projections are excitatory (Saeb-Parsy & Dyball, 2004).
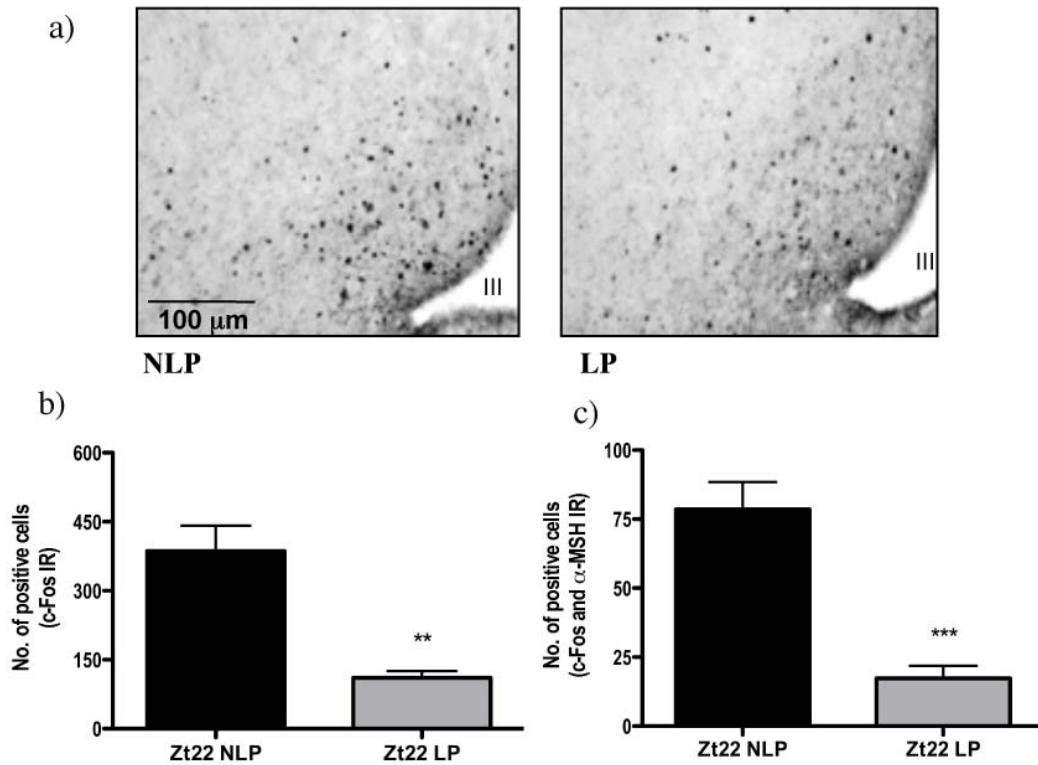
Figure 15. One hour light pulse form ZT21-22 decreases ARC activation.

(C) Representative ARC c-Fos and α-MSH sections at ZT22 without light and after a one-hour light pulse (right) it is clear that the c-Fos black dots are strongly diminished after the light pulse which was shown after quantification in (b) Total ARC activation (c-Fos counts) after the light pulse (ZT22) and (c) ARC α-MSH activation, (c-Fos and α-MSH co-localization counts) after light pulse (ZT22). Experiment conducted after a 48h fast for all groups. NLP (No Light Pulse), LP (Light Pulse), ZT (Zeitgeber time).

To demonstrate that ARC inhibition at ZT2 is associated with SCN endogenous activity, we quantified ARC activation in animals sacrificed after 2 days in constant darkness (DD) and fasted at CT2 and 22 and compared with their respective fasted controls sacrificed in LD.

Irrespective whether animals were sacrificed under DD or LD conditions a marked difference was observed between (subjective) day and night time sacrificed animals (Figure 16A; $F_{[3,10]}=13.84$

P=0.0007), with no differences between ZT or CT sacrificed animals.

Furthermore α-MSH activation also persisted in DD (Figure 16B; F[3,11]=17.91 P=0.0002), with no differences between ZT or CT sacrificed animals. The difference between CT2 vs CT22 (P<0.05) remained, though the difference was somewhat smaller than observed between ZT22 and ZT2 (P<0.01) indicating greater amplitude in LD condition. Yet the persistence of the rhythm in DD conditions demonstrates it to be a true circadian phenomenon.
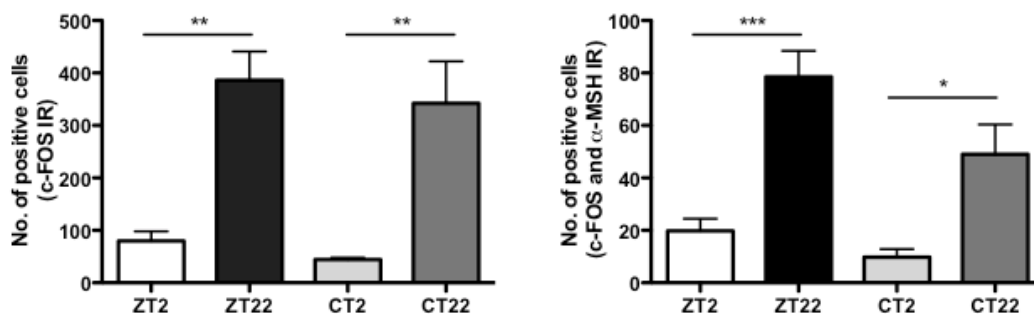
Figure 16. The ARC activity pattern persists in constant dark conditions. (a) ARC total activity (c-Fos counts) at ZT22, ZT2 (LD) CT2 and CT22 (DD) and (b) ARC α-MSH activity (c-Fos and α-MSH co-localization counts) at ZT22, ZT2 (LD) CT2 and CT22 (DD) of 48h fasted animals. Significant differences are represented with *. ZT (Zeitgeber time), CT (Circadian time), LD (Light-Dark conditions), DD (Dark-Dark conditions).

SCN TERMINALS CONTACT ARC α-MSH NEURONS

The SCN features a wide range of neuronal populations; the two most important for photic reception are VIP and GRP neurons (Romijn *et al.*, 1996), making them good candidates for neuronal populations involved in the transmission of light to the α-MSH neurons.

VIP (Figure 17A) terminals are in close apposition with most α-MSH neurons in the ARC nucleus. VIP innervation in the ARC was less dense than that observed for GRP, for GRP (Figure 17B) we

observed that all α-MSH neurons received contacts with GRP fibers. However lesioning the SCNxx removed completely all VIP innervation from the ARC suggesting that all VIP in the ARC is derived from the SCN; this however was not the case for GRP fibers, the GRP innervation in the ARC was diminished after SCNxx but did not disappear suggesting that also GRP from other sources may influence the ARC. To provide further evidence for the direct neuronal interaction of the SCN with ARC neurons we injected the neuronal tracer CtB in the SCN (Figure 17C and D) and determined its projections to the ARC with emphasis on the analysis of α-MSH neurons. Injections were considered successful when they were inside the SCN (n=3).

Injections into the SCN resulted in relatively dense projections to the lateral ARC exhibiting frequent neuronal contacts with α-MSH neurons (Figure 6E), while injections that were just above or lateral of the SCN did not show such projections. The SCN input was most dense at the same side of the injection but also contra-lateral projections were present, indicating that the neuronal connections between the SCN and the ARC are in majority but not limited to one side. We also observed co-localization with CtB traced fibers in the ARC for GRP indicating the GRP origin from the SCN (Figure 17F).
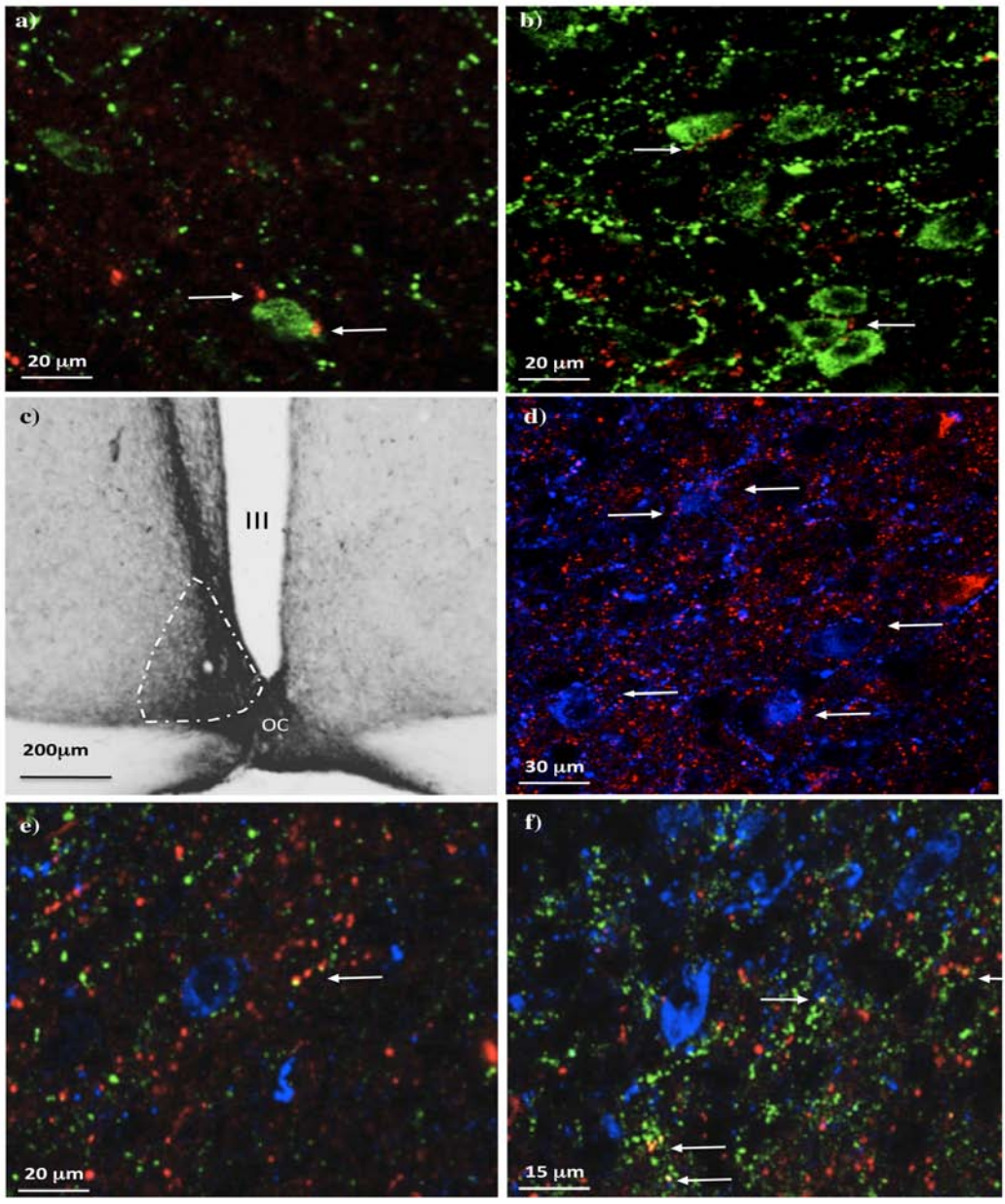
Figure 17. SCN fibers contact **ɑ**-MSH neurons. (a) VIP and (b) GRP IR fibers (red) in the ARC contact **ɑ**-MSH (green) neurons in the ARC. (c) DAB-Ni section of the SCN with CtB injection, (d) CtB traced fibers (red) originating from the SCN in close relation with **ɑ**-MSH IR cells (blue), (e and f) CtB (red) and GRP (green) co-localizing (yellow) in the ARC (arrows) n=4. VIP (Vasoactive Intestinal Peptide), GRP (Gastric Releasing Peptide), III (third ventricle), OC (optic chiasm), **ɑ**-MSH (a-Melanocite stimulating hormone) and CtB (Cholera toxin fraction B).

The present data demonstrates that the SCN has the capacity to stimulate neuronal activity of the ARC (ZT22) and probably to inhibit it during the day (ZT2); in addition it shows that the α-MSH neuronal activity follows the same pattern. Furthermore because unilateral lesions of the SCN resulted in the decrease of ARC α-MSH activity mainly at the side ipsilateral to the lesion we conclude that the rhythm in ARC neuronal activity is largely derived from neuronal inputs from the SCN. In addition the observation that neuronal tracing from the SCN results not only in ipsilateral but in some contra lateral staining as well, may explain the despaired diminishment of c-Fos after unilateral lesion of the SCN. However since c-Fos also decreases at the contra lateral site of the lesion it cannot be excluded that also circulating signals such as the hormones corticosterone or melatonin may contribute to this rhythm in activity. Further evidence of SCN-ARC projections was revealed by CtB injections showing a marked association of VIP and GRP with α-MSH cell bodies and CtB tracer co-localization with GRP fibers indicating that SCN-ARC neuronal pathways are at least partly responsible for the observed activation.

Studies have evaluated GRP effects in the ARC, and shown that bombesin-like peptides increase the firing rate of both NPY and POMC neurons (Lin & Pan, 1994; van den Pol et al., 2009) suggesting that GRP originating from the SCN may activate ARC α-MSH neurons. A major problem for this hypothesis is that GRP-VIP neurons in the SCN are activated by light pulses (Castel et al., 1997) a condition whereby the ARC α-MSH neurons are inhibited. Moreover in the SCN, VIP and GRP mRNA levels present a peak in the middle of the light phase (Shinohara et al., 1993; Miller et al., 2006) also providing the

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possibility for the inhibitory effect observed during the onset of the subjective day, as previously demonstrated for VIP inhibiting dopamine neurons in the ARC (Gerhold *et al.*, 2002).

In view of reports that demonstrate the possibility of the ARC to show oscillations in vitro (Guilding *et al.*, 2009) it is important to consider whether in SCNxx animals the ARC may show a free running rhythm that prevents us to see its activation. Considering that the SCNxx resulted in the loss of all rhythmicity included food intake (Abe *et al.*, 1979) and considering that food intake is importantly driven by the ARC (Cone, 2005) we assume that also the ARC in SCNxx animals loses its rhythmicity. Moreover all our SCNxx animals showed the same low activity of the ARC, if the ARC were free running one would expect at least one animal to show a higher activity of c-Fos. Still the combination of these studies demonstrating the rhythmic properties of the ARC and the present study showing the SCN-ARC interaction give a fascinating picture of the importance of the presence of rhythms in the ARC. Nonetheless it has also been demonstrated that ARC lesions also induce arrhythmic food intake when animals underwent constant darkness or constant light (LL) (Bugarith *et al.*, 2005; Li *et al.*, 2012) suggesting that communication between the SCN and the ARC is important in both directions since even when the SCN is able to mimic a rhythm in food intake in LD, it is incapable to sustain it in DD or LL when the ARC has been lesion, thus the information that the ARC nucleus is also crucial for the SCN activity (Saeb-Parsy *et al.*, 2000; Yi *et al.*, 2006) to sustain food intake when synchronizing photic signals are absent.

Notably the only other identified SCN targets that up till now show a similar activation and inhibition pattern are the pre-autonomic PVN neurons that control melatonin secretion in a multi-synaptic circuit (Perreau-Lenz *et al.*, 2003; Perreau-Lenz *et al.*, 2004). These PVN neurons are activated at night by SCN glutamatergic terminals that

stimulate melatonin secretion and are inhibited by a GABA-ergic SCN projection that is activated by light suggesting that the activation and inhibition as presently seen in a similar time frame in the ARC may be induced by comparable signals from the SCN. Although we cannot exclude that the loss of ARC activation at ZT2 or at ZT 22 induced by light is induced by lack of stimulatory input from the SCN, we would like to propose that the SCN projections to the ARC provide a similar activation inhibition pattern to the ARC as they offer to the PVN. At the other hand in principle it is possible that also other retinal targets within or outside the hypothalamus (Horvath, 1998; Saeb-Parsy *et al.*, 2000; Hattar *et al.*, 2006) may provide an indirect inhibitory input to the ARC. The fast disappearance of c-Fos after the light exposure may also favor an SCN or retinal target mediated inhibition rather than simple lack of excitation; future studies will need to clarify this interaction.

In addition to these ARC neuronal activity changes imposed directly by the SCN, circulating metabolites or hormones can activate or inhibit ARC neurons in a circadian manner (van den Top & Spanswick, 2006). Many of these compounds also have a clear 24h rhythm directly or indirectly driven by the SCN (Kalsbeek et al., 2011) with receptors in the ARC (Guan *et al.*, 1997; Li *et al.*, 2012; Plum *et al.*, 2012) indicating that the temporal ARC activation can also be promoted by humoral pathways or through multi-synaptic neuronal relays such as via the DMH (ter Horst & Luiten, 1986).

Independent of the feeding condition 60% of the ARC α-MSH neurons are activated at ZT22 illustrating the involvement of these α-MSH neurons in functions especially associated with the end of the activity period, probably supporting the circadian control of energy balance. Since these α-MSH neurons are also activated after food intake, this activation by the SCN in absence of food shows that the SCN will activate the ARC in agreement with the daily physiology to support

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functions that as a rule are associated with satiety. It is known that when food intake occurs outside the time indicated by the SCN this may lead in the long run to obesity and diabetes (Kreier *et al.*, 2003; Spiegel *et al.*, 2009), one of the explanations that now surges from our study is that in such situation the ARC is not suitably prepared for the ingestion of food which may result in inadequate autonomic and hormonal responses associated with the food intake. Also the co-expression of a wide variety of transmitters like glutamate, GABA, dynorphin or acetylcholine in the α-MSH neurons(Messina *et al.*, 2005), suggests their possible involvement in a wide variety of physiological functions other than the direct inhibition of food intake.

The present study concurs with earlier studies that also have shown rhythmicity in certain populations of ARC neurons (Jamali & Tramu, 1999; Lu *et al.*, 2002; Ellis *et al.*, 2008; Guilding *et al.*, 2009); together these data show that the SCN not only provides an output to synchronize behavioral, hormonal and autonomic functions but also may gate and prepare the ARC for the access of sensory circulating information. Thus the activity of the ARC is at the one hand driven by circulating metabolic information and at the other hand directly driven by the SCN. At the other hand it is known that the ARC is also able to influence the functionality of the SCN, its neuronal projections influence not only the neuronal activity of the SCN but also its circadian phase. It is tempting to speculate that this ARC-SCN reciprocal interaction is essential to maintain a well balanced metabolic circadian profile, which might be another explanation that desynchronization of circadian and metabolic signals for example by food intake outside the time indicated by the SCN may result in the metabolic syndrome.

CHAPTER 3

Guzmán-Ruiz M, Ramirez-Corona A, Ramirez-Plascencia O, Sabath SE[1], Basualdo CM, Guerrero-Vargas NN, León-Mercado L, Durón-Javier C, Fuentes R, Escobar C and Buijs RM

INTRODUCTION

Thermoregulatory processes have a well-established centre of control that resides in several nuclei of the preoptic area whereby the Median preoptic nucleus (MnPO) is the main integration nucleus of thermo-afferents (Romanovsky, 2007; Morrison *et al.*, 2008; Nakamura & Morrison, 2011; Clapham, 2012). In addition to the classic thermoregulatory cues (temperature of skin, temperature of viscera and pro-inflammatory signals), the temporal order also plays an important role in the establishment of the temperature set point (Eastman *et al.*, 1984; Benstaali *et al.*, 2001; Liu *et al.*, 2002; Scheer *et al.*, 2005). Shortly before the active phase of the organism, the core temperature increases, while just before the beginning of the resting phase the body temperature (Tb) drops (Refinetti & Menaker, 1992a) independently of the locomotor activity (Scheer *et al.*, 2005). This anticipatory regulation is controlled by the Suprachiasmatic nucleus (SCN) in the hypothalamus that functions as the biological clock and imposes temporal organization to physiology (Scheer et al., 2005, Liu et al., 2002,(Buijs & Kalsbeek, 2001).

The circadian control of resting Tb is adjusted depending on the feeding state of the animal, whereby the set point (amplitude) is established in relation with the metabolic requirements of the

organism. This multiple system interaction is revealed when food intake elicits a higher thermogenesis during the active phase of the organism (Romon *et al.*, 1993; LeBlanc & Soucy, 1996) and when food deprivation induces a Tb decrease during the beginning of the resting phase. This system interaction becomes evident when SCN lesions prevent such fasting induced Tb decrease (Liu *et al.*, 2002; Scheer *et al.*, 2005; Tokizawa *et al.*, 2009). The mechanisms why SCN lesions prevent such metabolic related temperature decrease are not understood.

In view of the observed interaction between the SCN and the ARC (Saeb-Parsy & Dyball, 2003b; Yi *et al.*, 2006; Guzman-Ruiz *et al.*, 2014) whereby the ARC also expresses a daily rhythm in neuronal activation of the α-MSH (Guzman-Ruiz et al., 2014) we hypothesized that the Tb rhythm depends on the interplay between temporal signals from the SCN and metabolic signals arising from the ARC. Thus we sought to determine whether the ARC as a main hypothalamic integrator of metabolic information (Williams *et al.*, 2000; Seeley *et al.*, 2004; Cone, 2005; van den Top & Spanswick, 2006) interacting with the SCN at the level of the MnPO could influence Tb. Hereto we investigated first by means of neuronal tracing the possible input of both ARC and SCN to the MnPO. After observing that with a single tracer injection into the MnPO both vasopressin neurons in the SCN as well as α-MSH cells in the ARC were labelled we demonstrated by means of microdialysis that an alternating release pattern of these neurotransmitter molecules the decreased Tb at dawn.

MATERIALS AND METHODS

Animals

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Adult male Wistar rats (Rattus norvegicus) weighing 250–280 g were housed in individual cages, under LD 12:12 conditions (light on at 7h considered ZT0 and light off at 19h considered ZT12). Room temperature was maintained controlled within thermo-neutrality. Water and regular laboratory rodent diet (Purina, Chow 5001; Woodstock, Ontario, Canada) were provided *ad libitum* throughout the study unless otherwise stated in the experimental procedures.

Rats were acclimatized to bioterium conditions for one week before surgeries. The Committee for Ethical Evaluation at Institute for Biomedical Research, UNAM, approved experiments in strict accordance with the Mexican norms for animal handling (Norma Oficial Mexicana NOM-062-ZOO-1999).

Tracing with CtB in the MnPO
Rats (n=12) were deeply anesthetized under a mixture of Ketamine (40-80 mg/kg) with Xylazine (5-8 mg/kg) injected IM. Rats were placed in the stereotactic frame (tooth bar -2.5 mm; arm angle of 98°) and the retrograde tracer Cholera Toxin B (CtB) 0.25% conjugated with the Alexa Fluor-555 fluorescent dye (Molecular Probes, Eugene, OR, USA), was injected 0.1ul in the MnPO with a Hamilton micro-syringe (coordinates: +0.3 mm anterior from bregma; ±0.10 mm lateral from midline; 5.5 to 6 mm below brain surface depending on weight variation), the injection syringe was left for 10 minutes in place to minimize leakage. Missed MnPO or injections with leakage were used as controls to verify tracing specificity (n=6).

Microdialysis probes in the MnPO.
Microdialysis (MD) probes were constructed according to procedures described previously (Kalsbeek et al., 1995, 1996). The

dialysis probes (1.5 mm long, 0.7 mm wide, and 0.2 mm thick) were implanted adjacent to the MnPO to avoid lesions within the nucleus (coordinates: +0.3 mm anterior from bregma; ±0.11 mm lateral from midline; 5.4 to 5.9 mm below brain surface depending on weight variation).

After implantation of the microdialysis probe and iButtons, the animals were allowed to recover from surgery for 7 days before the microdialysis infusions. During this period, the animals became accustomed to the experimental conditions in Plexiglas cages, designed to allow infusions under unrestrained conditions. All experiments were performed in the animal's own home cage. The input port of the dialysis probe was not connected.

Inlet and outlet tubes for the probe were threaded through a stainless steel support spring attached to the skull. The entire assembly was suspended from a counter-balanced beam and did not influence the animal's posture or motion.

The syringe contained Ringer's (NaCl 0.85%, KCl 0.04%, CaCl$_2$ 0.034%, Milli Q H$_2$O) fluid with or without the respective agonist or antagonist (5ng/ml except for V1A antagonist that was used in 2.5ng/ml). Dialysates were changed by connecting the polyethylene tube from the appropriate syringe to the fluid swivel. The dead space between the fluid swivel and dialysis membrane was 40ml. Therefore, dialysate containing the respective drug reached the membrane 15-20 min after the change of syringes, this time was taken into account for all analysis.

Body temperature determination

To monitor Tb sterilized temperature sensors (iButton Sensor-Temperature Logger; Maxim Integrated Products, Dallas Semiconductor, Dallas, TX) were inserted IP through a small

incision cut in the abdominal wall. iButtons were programmed to collect temperature data every 10 min during the experimental procedure.

Tb change was determined by obtaining the mean of the hour before the light pulse or the last hour of the 2h stabilizing ringer infusion period performed before each treatment of the microdialysis experiments, then we obtained the difference between the last hour mean and the Tb value every ten minutes for the entire light pulse or the time that the MD infusion lasted.

Immunocytochemistry

All rats were deeply anesthetized with a lethal dose of sodium pentobarbital and perfused intracardially with 0.9% saline solution followed by paraformaldehyde 4% in 0.1M Phosphate saline buffer (PBS; pH 7.4). Brains were removed and kept in fixative at 4° C for overnight postfixation, followed by 48h with 30% sucrose in 0.1M PBS. Brains were cut in 40µm coronal sections with a cryostat. Sections used for either Nissel staining, immune labeling or *in situ* hybridization were collected and rinsed in 0.1M PBS RNAase free.

To determine the CtB injection site, 6 MnPO sections (2 anterior, 2 medial and 2 posterior) from the CtB injected animals were incubated over night with rabbit anti CtB primary antibody 1:4000, (Calbiochem) at constant shake at 4°C. After primary antibody incubation, sections were rinsed and incubated at room temperature with a biotinylated donkey anti-rabbit serum 1:400, (Jackson labs) for 1h, then rinsed and incubated in avidin-biotin complex 1:500, (Vector Laboratories) for 1h, and the reaction product was visualized by incubation in 0.01% diaminobenzidine with 0.01% hydrogen peroxide 30Vol. and 0.05% nickel ammonium sulfate was added to the diaminobenzidine solution to darken the reaction product.

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To determine the identity of the neurons traced with CtB, SCN and ARC sections were incubated with the primary antibody for rabbit Arginine Vasopressin 1:2000 (Buijs *et al.*, 1989) or sheep a-Melanocite Stimulating Hormone Polyclonal Antibody 1:1600, (Millipore) respectively and Cy2-conjugated Affinity Pure Donkey Anti-rabbit or Anti-Sheep secondary antibodies 1:400, (Jackson labs.) then rinsed and placed on gelatinized glass slides, coverslipped in glycerol-PBS (1:1), and analyzed with the Laser Scanning Microscope 5 Pascal confocal microscope (Zeiss).

To determine the position of the MD canula 3 sections of the MnPO (anterior, medial and posterior) were stained with cresyl violet 1%.

In addition sections of the MnPO of rats (4 sections) that received a light pulse and the dorsomedial nucleus (DMH, 4 sections) and the rostral Raphe (rRPa, 4 sections) of MD treated rats were incubated with rabbit anti-c-*FOS* primary antibody 1:40 000; (Calbiochem) for 72 h at 4°C, sections were rinsed and incubated 1 h at room temperature with biotinylated donkey-antirabbit serum 1:400 (Jackson labs) and incubated in avidin-biotin complex 1:500 (Vector Laboratories) for 1 hour. Product visualization was obtained with 0.01% diaminobenzidine (DAB), 0.05% nickel ammonium sulfate, and 0.01% hydrogen peroxide 30Vol. Quantification of the c-FOS positive cells was performed by obtaining the mean of the 4 sections of each brain.

In situ hybridization

Brains of intact rats (n=6) were also sectioned in 40mm slices and collected in PFA4% to preserve RNA, 4-6 MnPO sections were selected and washed 3 times in PBS RNAase free-tween 20 0.05%, sections where then incubated in 0.1% (diethyl-pyrocarbonate) DEPC active in PBS for 30 min at room temperature, rinsed three

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times with PBS-tween20 0.05%, and incubated with H_2O_2 0.3% rinsed two times with PBS-tween20 0.05% and once with DEPC water, incubated in a 1% trietanolamine 0.25% acetic acid in DEPC treated MilliQ water solution for 10min, rinsed twice with PBS-tween20 0.05%, then sections were incubated in hybridizing buffer for 1h at 63°C, after that, 400 ng/ml of RNA probe for either Gad65 was added and incubated over night at 63°C then rinsed with 5x saline-sodium citrate (SSC) buffer for 5min, at room Temperature , rinsed with subsequent dilution at 63°C for 30min each of 5xSSC with formamide 50% , 2xSSC with formamide 50% and 0.2x with formamide 50%. Rinse once with 1XSSC without formamide at room temperature for 10 min. After that the section was blocked with blocking buffer (Roche) 30 min at room temperature and, incubated with digoxigenin HRP in sheep (1:1000) (Roche) in blocking buffer 1h rinsed three times with PBS Tween20 0.05%, incubated with TSA (1:200) for 15min, rinsed three times with PBS Tween20 0.05% and incubated with streptavidin with Cy3 (1:1000) rinsed three times with PBS Tween20 0.05% and start the immunocytochemistry incubating for either AVP in rabbit or a-MSH in sheep primary antibodies and continuing as described previously.

The percentage of GABAergic neurons associated with AVP or a-MSH fibers in the MnPO was determined by counting the amount of GAD65 positive neurons and the GAD65 positive neurons that showed close association with AVP or α-MSH. Neurones were only considered once even if they showed many fibers associated to their soma.

BAT q-PCR

Total RNA was extracted from the BAT using trizol reagent (Life Technologies) as recommended by the manufacturer.. Total RNA (2500ng) was reverse transcribed to single strand cDNA using

SuperScript III First-strand synthesis kit (Invitrogen) as recommended by the manufacturer. For relative quantification by real-time PCR (q-PCR), 50 ng of the 1st strand cDNA sample was mixed with 5µl Master Mix Sybr select master mix (Applied Biosystems), 2 µl milli Q water, and 1 µl primers mix 10mM, HPRT Fwd 5′-ACATTGTGGCCCTCTGTGTG-3′, Rev 5′-GGGCTGTACTGCTTGACCAA-3′, Rev-erb-a Fwd 5′-TAAAGTGTGTGGGGACGTGG-3′, Rev 5′-TGCCAACGGAGAGACACTTC-3′, UCP1 Fwd 5′-GCCGGCGATCCGGGCTTAAA-3′, Rev 5′-GGCTCGGAGGGCAGAGACCA-3′) (Turek *et al.*). An ABI Prism 7000 Sequence Detection System (Applied Biosystems) was used to amplify the genes from each BAT sample in triplicate. on a 96-well reaction plate. The relative amounts of mRNA were calculated by using the ΔΔCt method with an efficiency adjustment according to the Pfaffl equation (Pfaffl, 2001). The HPRT gen was used as an endogenous control for data normalization.

EXPERIMENTAL PROTOCOL

Neuronal activity of the MnPO in response to a light pulse during the night

Since light induces large temperature decreases at the end of the dark period, rats (n=6) were implanted with ip temperature probes. Two groups were formed; one killed under normal LD conditions at ZT22 (control n=3) and a group that received an hour light pulse (LP) from ZT21 to 22, both groups were transcardically perfused and the ip temperature buttons were collected and their brains processed for c-FOS immunohistochemistry of the MnPO.

Functional determination of the role of α-MSH and AVP signals in the MnPO

Microdialysis experiments were conducted whereby dialysis canulas were placed into the MnPO together with an ip temperature iButton.

Two time points were selected for the microdialysis protocols; the first at the onset of the resting phase, associated with the increase in AVP release from the SCN (Kalsbeek et al., 1995), coinciding with the diurnal Tb decrease and with the decrease in α-MSH activation in the ARC (Guzmán-Ruiz et al., 2014). Ringer's perfusion (3ml/min) was performed from (ZT0-2) to ensure the stabilization of the Tb before the perfusion agonist or antagonist and to establish a baseline from the same individual. After the Ringer infusion, the syringe was exchanged for; Melanotan II acetate salt (MTII) (Sigma–Aldrich) a MC3-4R agonist (5ng/ml, perfusion rate; 3ml/min, n=4), [deamino-Pen[1], O-Mer-Tyr[2], Arg[8]]-Vasopressin (V1A-ant) (Sigma–Aldrich) a V1A receptor antagonist (V1a-ANT, 2.5ng/ml, n=4) or V1aANT+MTII (5ng/ml and 2.5ng/ml perfusion rate; 3ml/min respectively) for three hours (ZT2-5), followed by a second 2h Ringer perfusion, unless the animals were killed for brain and BAT collection. As control we performed the same protocol including changing the syringes, but instead of agonist or antagonist, Ringer was perfused during the entire experiment (n=5).

The second time point at the end of the dark phase, corresponds with low AVP release from the SCN (Schwartz et al., 1983; Kalsbeek et al., 1995) and the peak of a-MSH neuronal activation in the ARC (Guzman-Ruiz et al., 2014). Ringer's perfusion (perfusion rate; 3ml/min) was performed from (ZT16-18). After the Ringer infusion, the syringe was exchanged for; [Arg[8]]-Vasopressin Acetate Salt (AVP) (Sigma–Aldrich) (5ng/ml, n=4) or Acetyl-[Nle[4], Asp[5], D-2-Nal[7], Lys[10]]-cyclo-a-Melanocyte Stimulating (SHU9119) MC3-4 antagonist (5ng/ml, n=6) for 2.3h (ZT18-20.3), followed by a second 2h Ringer perfusion, unless the animals were killed for brain and BAT collection. As control we performed the same protocol including

changing the syringes, but instead of agonist or antagonist, Ringer was perfused during the entire experiment (n=7).

Activation of pre-autonomic outputs and peripheral thermo-effectors after stimulation of the MnPO

To investigate the possible thermoregulatory pathways used by the MnPO to increase the temperature after V1Aant+MTII or to decrease the temperature after SHU9119 infusion., changes in neuronal activity in the brain of MD rats was also examined (n=16) by means of c-FOS in hypothalamus (DMH) and mesencephalon (Raphé), in this case the animals were sacrificed immediately after the agonist of antagonist infusion. Similarly after dialyses animals were sacrificed (n=14) to measure Ucp1 and Rev-erba in BAT to investigate possible mechanisms of temperature increase.

Statistic analysis

c-FOS expression in the MnPO in response to light pulse was compared with non-paired t-test and non-repeated measures one-way ANOVAs and Tukey's *posthoc* tests for c-FOS expression of the MD treated animals. Ucp1 and Rev-erba gene expression was compared with non-paired t-test.

The analysis of the Tb curves was performed with non-repeated measures two-way ANOVA, followed by Bonferrioni's or Dunnett's *posthoc* if significant. The differences were considered to be significant if $P < 0.05$. Data are expressed as means ±s.e.m.

RESULTS

THE MEDIAN PREOPTIC NUCLEUS RESPONDS TO LIGHT

Since light especially at the end of the dark period was shown to decrease Tb (Scheer *et al.*, 2005) we investigated whether the MnPO responds to a photic stimulus and thus is responsive to SCN input. Animals were subjected to a light pulse from ZT21 to 22 under *ad libitum* conditions, resulting in a Tb decrease, the two way ANOVA indicated a significant effect of light ($F[1,32]= 34.42$, $P<0.0001$, Figure 18A). This stimulus promoted the increase in c-FOS expression in the MnPO ($t[4]=4.128$; $p= 0.0073$, Figure 18B) indicating that this nucleus is responsive to photic information.
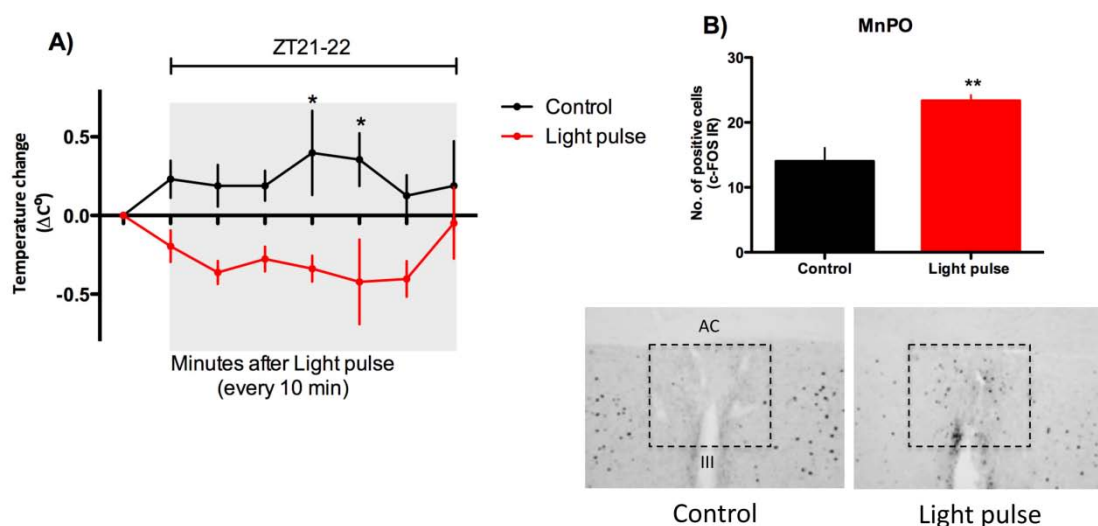
Figure 18.Light induces core temperature decrease and c-FOS expression in the MnPO. (A) Temperature response in animals that received a light pulse during the night from ZT21-22 as compared to control animals without a light pulse (n=3 controls and n= 3 LP), where significance between time points is indicated with (*p<0.05) as evaluated with Bonferroni's multiple comparisons *posthoc* test. (B) Number of c-FOS expressing cells in the MnPO in response to light and representative sections of the MnPO c-FOS-IR (dashed rectangles) during the dark phase at ZT22, ($t[4]=4.128$; **p= 0.0073). MnPO, median preoptic nucleus, AC, anterior commissure; III, third ventricle.

Retrograde tracing from the MnPO

Retrograde tracer CtB injections in the MnPO in the area where increased c-FOS to light was observed were performed. The injection site was considered correct when the extension of the

injection was within the dorsal or ventral MnPO limits (Figure 19 A), injections with leakage to the septal area or the bed nucleus of the stria terminalis were excluded, these and injections outside the MnPO were used as control injections to verify the specificity of the projections. Successful injections in the MnPO always resulted in retrograde labeled neurons both in the in the SCN and the ARC (Figure 19 B-C). All successful injections into the MnPO resulted in the labeling of AVP neurons in the SCN (Figure 19 D-F) simultaneous with α-MSH neurons in the ARC (Figure 19 G-I). VIP neurons in the SCN never showed evidence of the presence of CtB while GRP showed important co-localization in addition NPY neurons from the ARC were also labeled with CtB but not CART neurons never showed any co-localization with the tracer (Supplemental Figure 18).
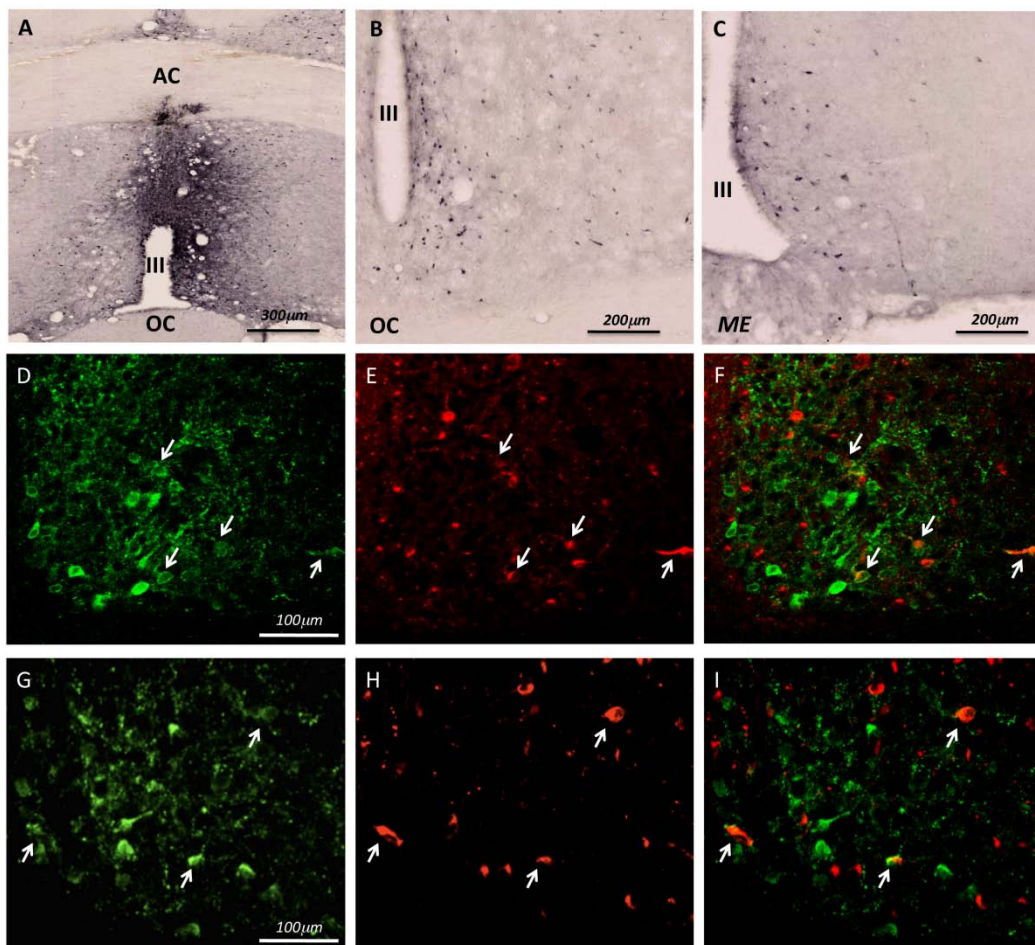
Figure 19. CtB injections in the MnPO result in retrograde filled neurons in ARC and SCN. (A) Shows the correct injection site in the ventral MnPO, (B) shows the retrograde tracing to the SCN, (C) represents the retrograde tracing to the ARC, (D) demonstrates SCN AVP-IR in green, (E) presents CtB-IR retrograde tracing from the MnPO to the SCN in red and (F) merge demonstrating the co-localization between AVP and CtB. (G) shows α-MSH-IR neurons in the ARC in green, (H) shows CtB retrograde tracing from the MnPO to the ARC and (I) merge demonstrating the co-localization between α-MSH and CtB. CtB; B fraction of the Cholera Toxin, SCN; Suprachiasmatic nucleus, AVP; arginine-vasopressin, IR; immunoreactivity, a-MSH; alpha-Melanocite stimulating hormone, AC; anterior commissure, OC; optic chiasma, III; third ventricle, ME; median eminence.

α-MSH SIGNALING IN THE MnPO INDUCES A TEMPERATURE INCREASE

The identified neurons of the SCN and the ARC that project to the MnPO have a clear circadian rhythm (Schwartz *et al.*, 1983; Kalsbeek *et al.*, 1995; Guzman-Ruiz *et al.*, 2014).

a-MSH neuronal activity is especially high at the end of the dark phase and inhibited at the beginning of the light period (Guzman-Ruiz *et al.*, 2014)), therefore we first examined the effect of the infusion of an MC3-4 receptor agonist, Melanotan II acetate salt (MTII) in the MnPO early in the light phase with the hypothesis that this would prevent the decrease in temperature observed during the day.

MTII infusion prevented the diurnal drop in Tb in comparison to the Ringer infused rats that showed a clear Tb decrease associated with the onset of light, the two way ANOVA showed a significant effect of MTII (F [1,166]=66.35, $P<0.0001$, Figure 20 A). One important observation was that MTII did not induce thermogenesis since the animals did not show a temperature increase instead Tb maintained the levels of the base line instead of the normal diurnal decrease.

Next we hypothesized that the high activity of a-MSH neurons in the ARC keeps the body temperature high. Therefore we investigated the effect of the antagonist of the MC3-4R Acetyl-[Nle[4], Asp[5], D-2-Nal[7], Lys[10]]-cyclo-a-Melanocyte Stimulating (SHU9119).

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Infusion of SHU9119 from ZT18 to 20.3, during the reported peak of the a-MSH neurons in the ARC induced a deep decrease in Tb after an hour of infusion (F [1,240]=76.21, P<0.0001, Figure 20 B). This SHU9119 mediated Tb decrease was so fast and steep that the infusion needed to be terminated 40 minutes before the anticipated time in order to allow the animals to recover from the induced hypothermia.

AVP SIGNALING IN THE MNPO INDUCES A DECREASE IN BODY TEMPERATURE

AVP secretion from the SCN has a diurnal pattern, starting to increase at end of the dark and the beginning of the light period (Schwartz *et al.*, 1983; Kalsbeek *et al.*, 1995). Therefore we infused AVP in Tb in the MnPO during the dark phase (ZT18-21) when Tb is high.

AVP infusion from ZT18 to 21, the second half of the dark phase, resulted in a strong decrease in Tb in comparison to Ringer infusion, two-way ANOVA showed that the interaction between the effect of AVP and time was significant (F[24, 208]= 4.98, P<0.0001, Figure 20C). When the same infusion was executed in the beginning of the light phase (ZT2-4) a profound hypothermia was observed that one animal died and only switching to ringer infusion could revert the hypothermic state of the other animal, therefore no further experiments were performed using AVP at this time point (Supplemetal Figure 3A, illustrates the surviving animal). This observation prompted the hypothesis that AVP in the MnPO is involved in the daily decrease in temperature during the day. In order to investigate this hypothesis we infused the antagonist of the V1A receptor [deamino-Pen[1], O-Mer-Tyr[2], Arg[8]]-Vasopressin (V1a-ANT) in the MnPO during the first part of the light phase when the

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diurnal temperature decreases. Blocking the V1A receptors in the MnPO clearly maintains a higher temperature during the light phase (ZT2-5) as compared to the Ringer infusion at the same time point, two-way ANOVA showed significant differences in the effect of V1a-ANT (F [1,166]=159.55, P< 0.0001, Figure 20 D). The same experiment was performed during the dark phase from ZT18 to 21, before the onset of AVP release (Schwartz et al., 1983), and two-way ANOVA did not showed an effect of time and V1a-ANT infusion on Tb (Supplemental Figure 3B, F [24,179]= 0.64, P=0.9012).

BALANCE BETWEEN AVP AND α-MSH RELEASE IN THE MNPO DETERMINES DIURNAL TB

The onset of the light phase is characterized by a steep Tb decrease, suggesting that this decrease might be due to the loss of a-MSH input to the MnPO, which was indicated by the infusion of MTII (Figure 20A) that did not induce a temperature increase but instead prevented the temperature decrease. Consequently we considered that the lack of temperature increase by MTII infusion could be due to a simultaneous occurring hypothermic effect of AVP release, as suggested by the outcome of the AVP infusions during the night and light phase. The infusion of the V1a-ANT with MTII, confirmed the latter hypothesis since the combination of these compounds resulted in a marked increase in Tb as compared to Ringer or the single infusion of either V1Aant or MTII, the two-way ANOVA showed a significant differences elicited by the different drug infusions (F[3,332]= 126.67 P<0.0001, Figure 20 E) Dunnett's compares each curve vs. V1a-ANT+MTII, demonstrating that V1a receptors need to be blocked in order to allow a-MSH to induce thermogenesis. Consequently the lack of temperature increase at ZT2-3 using MTII alone was due to the presence of diurnal AVP that prevents thermogenesis.

a-MSH neurons are most active at the end of the dark period (Guzman-Ruiz *et al.*, 2014) thus a-MSH might be released to the MnPO during that period, blocking the a-MSH receptor after ZT19 will induce a decrease in temperature (Figure 20 B), suggesting an additional signal that activates heat-loss mechanisms and thus temperature decrease. In view of our results in the beginning of the light period where the powerful temperature reducing effects of AVP were demonstrated, we hypothesized that blocking the a-MSH receptors may result in a decrease in Tb if at the same moment AVP is released in the MnPO as a hypothermic signal.

To test this hypothesis we antagonized both AVP receptors and MC3-4R during the dark phase observing that adding the V1a-ANT to the SHU9119 completely prevented the hypothermia, the two way ANOVA demonstrated significant differences in the interaction between time and the effect of the drugs ($F_{[48, 314]}$ = 1.590, P= 0.01, Figure 20 F) Dunnett's *posthoc* tests only shows significant differences between Tb of V1a-ANT+SHU9119 vs. SHU9119 and not Ringer demonstrating that the balance between the release of AVP and a-MSH determines the decrease of body temperature at the end of the dark phase.
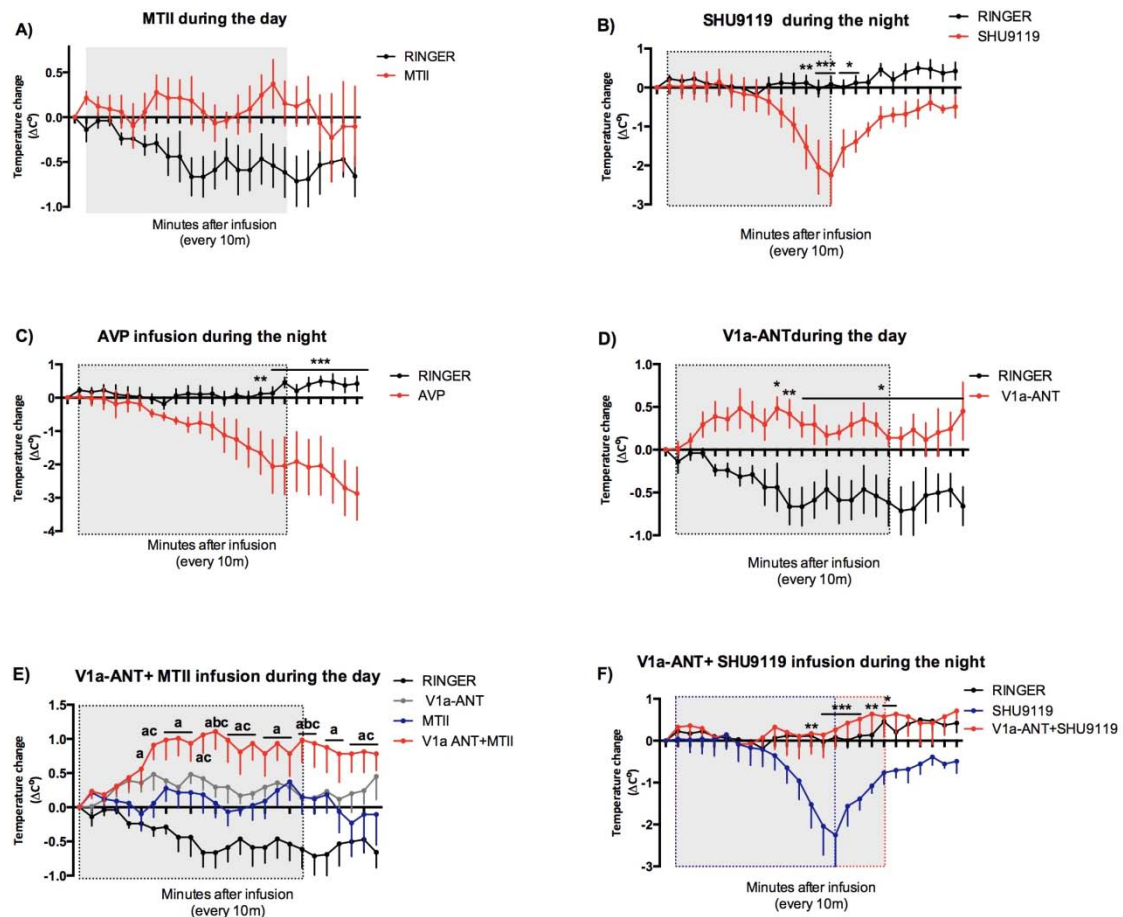
Figure 3. Balance between AVP and a-MSH in the MnPO determines temperature. Change in core temperature of animals infused in the MnPO with (A) MTII (n=4) vs. Ringer (n=5) at ZT2-5, $F[1,166]=66.35$, $P<0.0001$, (B) SHU9119 (n=6) vs. Ringer (n=7) at ZT18-20.3, $F[1,240]=76.21$, $P<0.0001$, (C) AVP (n=4) vs. Ringer (n=7) at ZT18-21, $F[24,208]=4.98$, $P<0.0001$, (D) V1a-ANT (n=4) vs. Ringer (n=5) at ZT2-5, $F[1,166]=159.55$, $P<0.0001$, significant differences obtained from the Bonferroni's *posthoc* for individual time points are indicated with (*). Simultaneous infusion of (E) V1A-ant+MTII (n=5) at ZT2 (n=5) as compared to the respective single infusions.

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$F[3,332]=126.67$ $P<0.0001$, Dunnett's *posthoc* analysis compares every curve vs. V1a-ANT+MTII, significant differences between V1a-ANT+MTII vs. Ringer are represented as (a), V1a-ANT+MTII vs. MTII are noted as (b) and V1a-ANT+MTII vs. V1a-ANT as (c), (F) V1a-ANT+ SHU9119 at ZT18 (n=4) as compared to the SHU9119 single infusion, $F[48,314]=1.590$, $P=0.01$), Dunnett's *post-hoc* analysis compares Ringer and SHU9119 vs. V1A ant+SHU9119, significant differences between V1a-ANT+ SHU9119 vs. SHU9119 are

indicated with (*), no significant differences between V1a-ANT+ SHU9119 vs. Ringer were found. Temperature data was collected every 10m, Grey rectangles indicate the infusion period. (*) for $P<0.05$, (**) for $P<0.01$ and (***) for $P<0.0001$.

AVP AND α-MSH SIGNAL TO GABA-ERGIC NEURONS IN THE MnPO

The MnPO has GABA-ergic neurons that receive sensory information about ambient temperature and body temperature; in turn they project to other thermoregulatory nuclei to modulate thermo-effectors in the periphery (Nakamura & Morrison, 2011). To investigate whether AVP and α-MSH interact with these neuronal populations in the MnPO we performed the *in situ* hybridization for GAD65 and immunohistochemistry of AVP or α–MSH in the MnPO.

We determined that GAD65 expressing neurons were associated with both, AVP (24%, ±4.33 SEM, Figure 4) and α-MSH terminals (65%, ±4.36 SEM, Figure 21).
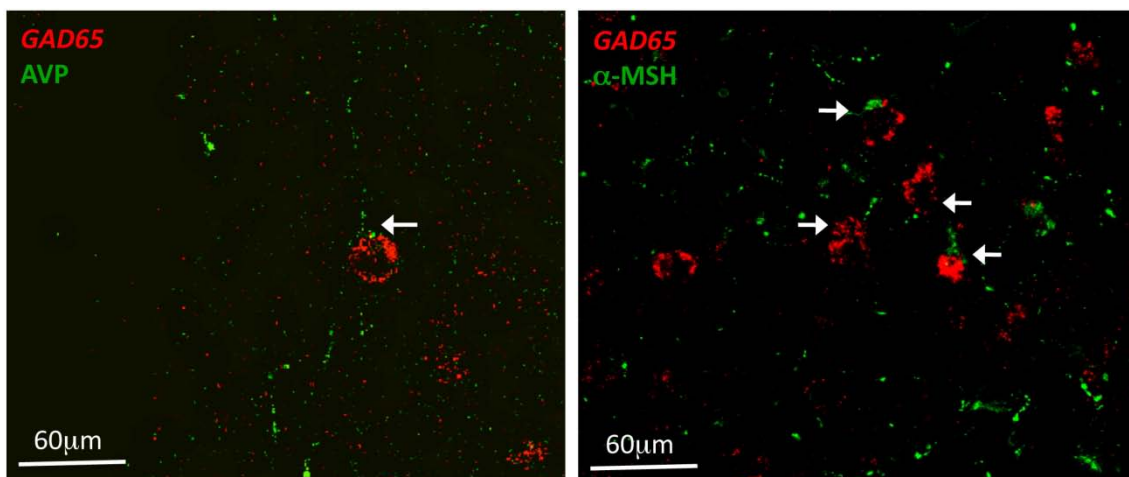

Figure 21. AVP and a-MSH fibers contact GABA-ergic neurons in the MnPO. In situ hybridization for Gad65 expressing neurons (red) and AVP or α–MSH immunoreactive fibers (green) of the MnPO. Arrows indicate close association between fibers and somas.

AVP AND α-MSH SIGNALS IN THE MnPO ACTIVATE PRE-AUTONOMIC OUTPUTS AND PERIPHERAL THERMO-EFFECTORS

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To investigate the possible thermoregulatory pathways used by the MnPO to increase the temperature after V1Aant+MTII or to decrease the temperature after SHU9119 infusion, we quantified the neuronal activation with the c-FOS marker in the DMH and the Raphe, two classical nuclei involved in the thermogenic output.

The DMH showed clear activation in response to the V1a-ANT+MTII ($p<0.001$, Figure 22 A) thermogenic infusion as compared with Ringer, while no induction of c-FOS was observed after the SHU9119 hypothermic treatment, interestingly no differences were observed between Ringer at ZT2-5 and Ringer at ZT18-21.

The Raphe was activated after infusion of V1a-ANT+MTII in comparison with Ringer infusion at ZT2 ($p<0.001$, Figure 22 B) and also showed an increase in c-FOS expression ($P<0.05$, Figure 22 B) as compared with Ringer infusion after the SHU9119 hypothermic treatment.

To determine if the pharmacological treatments in the MnPO modulate the activity of thermo-effectors in the periphery we evaluated the effect of V1a-ANT+MTII and SHU9119 in BAT gene expression, we observed the induction of Ucp1 gene expression in BAT relative to Ringer of animals treated with V1a-ANT+MTII as compared to SHU9119 Ucp1 expression relative to Ringer ($t[6]=3.787$ $p<0.0046$, Figure 22 C). At the other hand rats infused with SHU9119 showed an important increase in Rev-erba as compared with the animals that received V1Aant+MTII ($t[6]=7.354$ $P<0.0002$, Figure 22 D) consistent with a decrease in Tb.
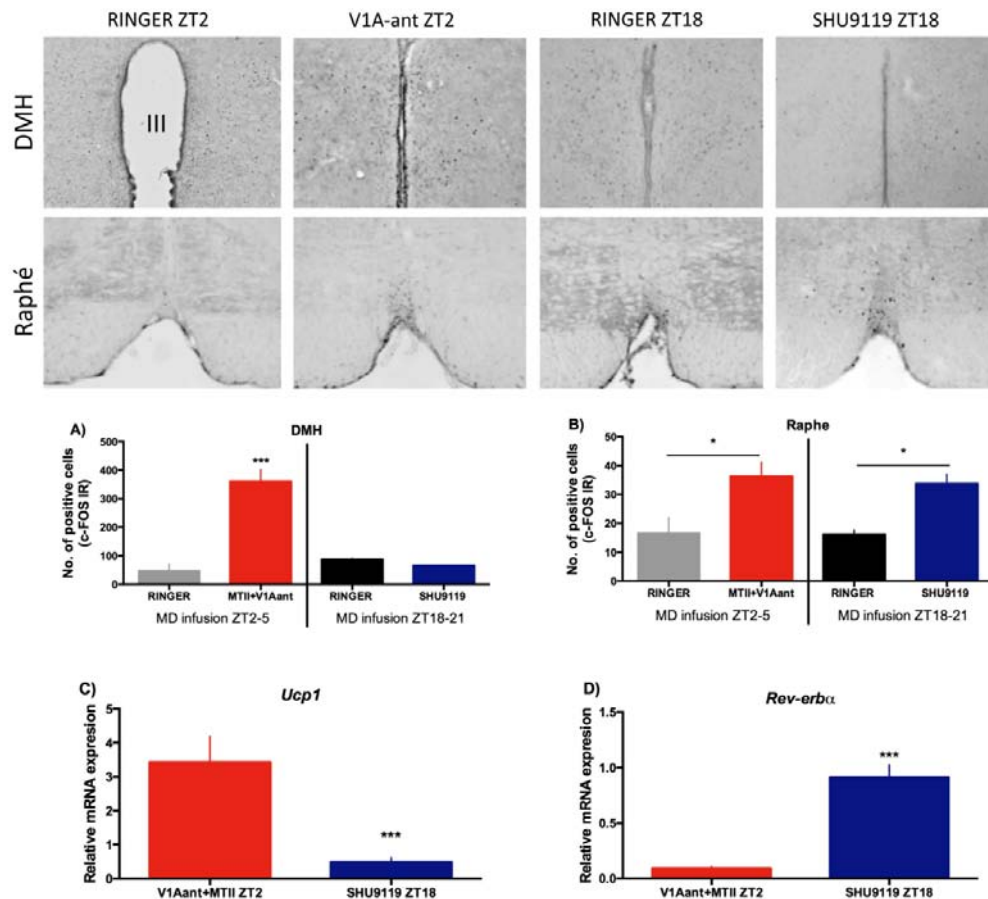
Figure 22. Activation of thermoregulatory pathways. c-FOS-IR of thermoregulatory nuclei (A) DMH (One-way ANOVA F[3, 12]= 42.69, P= 0.0001) and (B) Raphe (One-way ANOVA F[3, 12]= 8.795, P = 0.0023), Ringer ZT2 n=3, V1a-ANT+ MTII n=4, Ringer ZT18 n=5, SHU9119 n=4. Significant differences obtained from the Tukey's *posthoc* are indicated with (*) for p<0.05, and (***) for p<0.0001. (C) BAT Ucp1 (t[6]= 3.787 ***p=0.0046) and (D) Rev-Erba (t[6]= 7.354 ***p=0.0002) gene expression relative to their respective Ringer infusion, MTII+V1Aant (n=4) and SHU9119 (n=4). Dorsomedial nucleus of the hypothalamus (DMH), brown adipose tissue (BAT) and uncoupling protein 1 (Ucp1).

DISCUSSION

The daily rhythm in temperature is set by the SCN (Eastman *et al.*, 1984; Refinetti & Menaker, 1992b) but its amplitude, especially in the transition from dark to light, is influenced by metabolic conditions (Liu *et al.*, 2002; Scheer *et al.*, 2005). Here we have investigated the contribution of the ARC to these phenomena and demonstrated that the MnPO the main thermoregulatory area in the brain receives direct projections not only from the SCN but also from the ARC, one of the key nuclei involved in the regulation of metabolism.

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Since previous studies have shown that both AVP (Schwartz *et al.*, 1983; Kalsbeek *et al.*, 1995) and α-MSH neurons (Guzman-Ruiz *et al.*, 2014) show a clear circadian rhythm we chose to investigate the involvement of these two neuropeptides in temperature regulation in the MnPO. The results demonstrate that AVP is involved in the early morning temperature decrease while α-MSH is involved in the maintenance of high temperature observed at nigh. Moreover we demonstrated that a daily alternation of AVP and α-MSH release is essential to precisely time and regulate the drop in temperature at the onset of the light period (Figure S4). The demonstration that the interaction between circadian and metabolic system derived stimuli is essential to determine the diurnal temperature drop, in accordance with the importance of keeping circadian and metabolic stimuli aligned for the synchronization of physiological processes.

DAILY MODULATION OF THE DIURNAL TEMPERATURE DECREASE

The present study shows that thermoregulatory activity of the MnPO is not only modulated by Tb changes in the periphery, this nucleus also integrates temporal information, conveyed in the coordination of two signals, from the SCN (AVP) and the ARC (a-MSH) neurons.

The time dependent effect of the infusion of agonist and antagonists suggest a rhythmic release pattern determined by the own rhythms of AVP and α-MSH in the SCN and ARC respectively (Schwartz et al., 1983(Guzman-Ruiz *et al.*, 2014).

The onset of AVP release from the SCN takes place during the late dark period and increases further in the beginning of the light period (Schwartz *et al.*, 1983). At the other hand we recently observed an opposite pattern of α-MSH neuronal activity with increased expression from the beginning until the last part of the dark period. In addition we demonstrated that this α–MSH neuronal activity disappears after light exposure (Guzman-Ruiz *et al.*, 2014). These alternating

patterns correlate quite closely with the present observations of antagonist induced temperature changes demonstrating that the circadian release pattern of AVP and α-MSH are related with the diurnal temperature changes.

α-MSH fibers in the hypothalamus are mainly provided by the ARC, even when POMC neurons in the NTS also express α-MSH, their projections only cover lateral parabraquial nucleus (LPB) and the Reticular formation (Fan *et al.*, 2004). Although AVP is found in the SON, BNST, PVN and the SCN, our CtB injections did not show any labeling of the SON and no neurons in the PVN nor the BNST were positive for AVP, in addition previous studies have demonstrated that AVP from the BNST does not project to the MPA since castration in males induces a disappearance of the entire AVP in the BNST without decreasing the AVP fibers in the MPA (de Vries *et al.*, 1986).

Our infusions fail to answer the question of how the increase in temperature during end of the resting phase is mediated, since a-MSH levels during the first part of the active phase are quite low, it is not possible for this neuropeptide to be involve in the nocturnal temperature increase during the first half of the night.

The decrease of AVP at the end of the light period coincides with the increase in temperature previous to the dark phase. It has also been demonstrated that the rhythm of NPY in the ARC and the SCN present a peak just before the onset of light in accordance with its reported hypothermic effect through inhibition of the humoral outputs in the PVN that modulate BAT (Jhanwar-Uniyal *et al.*, 1990; Gluck *et al.*, 2006; Shi *et al.*, 2013). In addition it has also demonstrated that ghrelin responsive neurons in the ARC induces a clear inhibition of the SCN (Yi *et al.*, 2006) coinciding with a reported peak of NPY-ir both in the SCN and the ARC just before the dark onset, suggesting that the inhibition of the SCN through NPY can also induce Tb increase during the night by the removal of the hypothermic effect of AVP.

An alternative hypothesis is that orexins receive MnPO projections and have outputs to pre-autonomic BAT and induce thermogenesis (Yoshimichi *et al.*, 2001; Madden *et al.*, 2012) furthermore a peak in orexin neuronal activity is observed during the onset of the activity phase and is mediated by SCN signals (Marston *et al.*, 2008) suggesting that orexins might be responsible for the temperature increase at the end of the light period.

BODY TEMPERATURE RHYTHM IS REGULATED THROUGH A PREOPTIC PATHWAY

The MnPO has well-established outputs via DMH and the Raphe to modulate peripheral thermo-effectors as BAT, shivering and vasoconstriction (Nakamura, 2011). In the present study we demonstrated neuronal activation of the DMH and the Raphe in response to a thermogenic treatment in the MnPO (V1A-ant+MTII). In addition the increase of Ucp1 expression in BAT after V1A-ant+ MTII demonstrates that at least part of the thermogenic effect observed in these animals is elicited via the activation of BAT thermogenesis. This increase in Ucp1 coincides with a decrease expression of Rev-erb alpha, in agreement with the knowledge that Rev-erb alpha is a repressor of Ucp1 in BAT and that its down regulation precedes the physiological induction of Ucp1 (Gerhart-Hines et al., 2013).

Interestingly the infusion of SHU9119 (hypothermic) did not induce activation of the DMH in agreement with previous reports indicating that tonic inhibition of the DMH prevents BAT thermogenesis (Tupone *et al.*, 2014). SHU9119 infusion induced a clear decrease in UCP1 and increased Rev-erb-a expression, which is known to correlate with BAT inactivity (Gerhart-Hines *et al.*, 2013). Interestingly this hypothermic treatment also showed the activation of the Raphe, also implicated in vasodilatation, though it has been previously demonstrated that

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inhibition of the raphe is necessary for vasodilatation of the tail and therefore heat release, consecuently this activity patter during hypothermia might not be related to thermoeffectors but to other functions of the nuclei. We were unable to demonstrate activation or inhibition of the sympathetic tone to the tail we can only assume that other thermoregulatory pathways are involved in the modulation of diurnal temperature decrease in response to AVP signals to the MnPO.

These results suggest that the previously described thermoregulatory pathways that modulate Tb via BAT also transmit the circadian fluctuations in body temperature.

AVP and α-MSH fibers contact GABAergic neurons in the MnPO

It is known that the MnPO receives peripheral and central signals to activate both heat release and thermogenic mechanisms. The activation of GABA-ergic neurons in the MnPO promotes thermogenic mechanisms in response to cold, while the activation of Glutamatergic neurons results in heat loss (Nakamura, 2011; Morrison *et al.*, 2012). In the present study we demonstrated that a-MSH fibers are in close contact with 65% of the GABAergic neurons in the MnPO suggesting that the release of a-MSH to this neurons may induce thermogenesis and BAT heat generation. On the other hand 24% of GABAergic neurons showed close association with AVP fibers suggesting an inhibitory role on GABAergic interneurons within the MnPO. Unfortunately we were not able to determine clear associations between vGlut2 expressing neurons in the MnPO and AVP or a-MSH fibers.

Physiological Implications of the metabolic and temporal control on Tb

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Mammalian endothermy is an expensive adaptation that permits activity of the organism during low ambient temperatures. Therefore body temperature is tightly regulated in relation with the energy requirements of the individual (i.e. allowing lower body temperatures in periods of inactivity). Previous studies have shown that the fluctuations of daily temperature is SCN dependent and that the Tb rhythm amplitude responds to the metabolic state of the individual and the presence of the SCN (Refinetti & Menaker, 1992b; Liu *et al.*, 2002; Scheer *et al.*, 2005). Furthermore daily temperature changes are mainly driven by vasodilatation and BAT thermogenesis (Ootsuka *et al.*, 2009; Gerhart-Hines *et al.*, 2013). Recent studies have demonstrated that BAT thermogenic activity is important for metabolic health since the absence of this tissue induces obesity (Heikens *et al.*, 2011; Vijgen *et al.*, 2011; Vijgen *et al.*, 2012), this observation together with the present data suggests point out the relevance of a well organized control of BAT thermogenesis. While previously we demonstrated that the SCN drives the daily activity of the ARC, the present study shows that the coordination of these two signals in the MnPO is essential to control body temperature and BAT thermogenesis.

Individuals subjected to jet lag, shift-work or food intake outside the normal feeding pattern, exhibit clear metabolic alterations such as an increase in body weight and interestingly eating during the resting phases shifts the temperature rhythm to the phase where food is presented, exhibiting low Tb during the last part of the night and a very high level during at the beginning of the light phase, a time stamp that doesn't follow the rhythm that the SCN imposes (Salgado-Delgado *et al.*, 2008; Scheer *et al.*, 2009; Salgado-Delgado *et al.*, 2010a; Salgado-Delgado *et al.*, 2013), suggesting that the temporal and metabolic information reaching the MnPO respectively from the SCN and the ARC is not synchronized, and thus is altering the

sympathetic output to BAT leading to important physiological alterations with as consequence obesity.

GENERAL DISCUSSION

In the present thesis we have investigated the relationship between the SCN and the ARC. Our results indicate that the demonstrated SCN-ARC interaction has a major role in the regulation of physiology. We demonstrated not only that the SCN drives the rhythm of α-MSH neurons in the ARC (chapter 2), but also that this rhythm in α–MSH neuronal activity is essential for temperature regulation (chapter 3). These observations suggest that for adequate body temperature control, the SCN is required to transmit oscillatory information to at least two hypothalamic nuclei the ARC and the MnPO. The advantage of this circuit is evident, the SCN still maintains control over the daily change in temperature, however, the incorporation of the ARC in the net enables to include metabolic information in the temperature set point at the same time. The reach of this SCN-ARC interaction might be broader than temperature regulation since both nuclei share the regulations of many processes that we will discuss later.

The clock machinery and its implication in metabolic health

The mechanisms involved in the temporal modulation of every day physiology have been widely studied. Many of the pathways involved include several nuclei (mainly hypothalamic) that receive and integrate information from peripheral sensors all over the body that are tuned by the biological clock in order to modulate their responses to sensory information or to generate the initiation of the respective rest or activity phase. This temporal information is sent to (pre) autonomic outputs that generate the appropriate response, in the case of body temperature the responses include the regulation of the metabolic rate of BAT, skin vasoconstriction, vasodilatation,

etc. In the present work we paid special attention to the possible mechanisms used by the SCN to influence the daily temperature variations.

In recent years chronobiologists have stressed the importance of the synchronization between body functions and behaviours that modulate energy balance such as food intake on one side and the environmental signals like light, that synchronize the biological clock, on the other (de Castro, 2004; Salgado-Delgado *et al.*, 2008; Salgado-Delgado *et al.*, 2010b; Salgado-Delgado *et al.*, 2013; Hsieh *et al.*, 2014).

Studies have demonstrated that the uncoupling of metabolic and temporal signals such as consuming food or being active during the resting period, makes the organism prone to developing metabolic problems like obesity and pro-inflammatory profiles and even to develop diseases like cancer (Guerrero-Vargas et al, submitted).

Notably the results of these studies, show that the majority of the body functions modified by the internal desynchronization between temporal an metabolic cues are related with outputs of the autonomic nervous system, such as white adipose tissue (WAT) accumulation, decreased glucose tolerance, corticosterone secretion, increased blood pressure and impairment of BAT thermogenic activity. However up till now the areas in the brain and the mechanisms involved in the uncoupling of circadian and metabolic systems have not been demonstrated.

One important evidence that points to the implication of the biological clock in metabolic health is the fact that animals under a HFD have a less responsive SCN to light pulses and their locomotor activity rhythm is lost under DD conditions (Mendoza *et al.*, 2008; Pendergast *et al.*, 2013). Moreover both the SCN and the ARC nucleus have important outputs to pre-autonomic nuclei like the MnPO (Chapter 3), PVN (De Vries & Buijs, 1983; Buijs *et al.*, 1993; Kalsbeek *et al.*,

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2004) and the DMH (ter Horst & Luiten, 1986; Kalsbeek *et al.*, 1992) that in turn are involved in the sympathetic and parasympathetic output to WAT, BAT and liver (Kalsbeek *et al.*, 2006d; Kreier *et al.*, 2006; Morrison & Nakamura, 2011) major players in the development of metabolic diseases.

It is well known that the peripheral glucose uptake and production is time dependent driven by the SCN (glucose tolerance) (la Fleur *et al.*, 2001; Kalsbeek *et al.*, 2006d; Kalsbeek *et al.*, 2008; Kalsbeek *et al.*, 2014). Since the ARC is a major area for sensing circulating glucose levels (Herrera-Moro et al., in prep) and shares reciprocal connections with the SCN (Saeb-Parsy *et al.*, 2000; Yi *et al.*, 2006), we have determined in chapter 2 that the SCN afferents to the ARC increase its neuronal activity at night and inhibit the ARC during the day. In addition a recent study in our group has demonstrated that the relationship between the ARC and the SCN are essential for central glucose sensing and its peripheral regulatory role in circulating glucose levels (Herrera-Moro et al; In prep). Furthermore we and others have demonstrated that the ARC is part of the circadian machinery and lesions of this nucleus or severing the connections between the SCN and the ARC modify circadian patterns in food intake, temperature and locomotor activity (Wiater *et al.*, 2011). This relationship also impacts energy expenditure as can be concluded from the studies showing that light pulses decrease Tb time dependently (Scheer *et al.*, 2005), and fasting induces torpor states only in the light period (Liu *et al.*, 2002). More importantly here we also have determined that signals from both the SCN and the ARC modulate the expression of UCP1 in BAT, an important player in thermoregulation and energy expenditure (Chapter 3). These observations suggest that the uncoupling of autonomic outputs of the

brain from the driving force of the SCN and metabolic integration sites like the ARC may be an important factor for the development of metabolic disease.

AUTONOMIC UNCOUPLING AS A HYPOTHESIS FOR METABOLIC DISEASE

Given the nature of the physiological disturbances induced by the uncoupling of circadian and metabolic signals in general physiology, the desynchronization of sympathetic and parasympathetic outputs can be hypothesized when an animal acquires an obese phenotype. For example the high glucose and insulin levels may be due to higher parasympathetic output to the pancreas and higher sympathetic output to the liver. This autonomic desynchronization may extend to e.g. muscle and heart tissue and consequently lead to the development of the metabolic syndrome and diabetes.

A disturbance in the relationship between the SCN and the ARC can also be assumed in obese models, since HFD rats or mice show a spontaneous disarrangement of food intake which shifts towards the resting phase, in addition they present metabolic disorders related to the deregulation of autonomic outputs (Kohsaka *et al.*, 2007; Pendergast *et al.*, 2013).

Studies suggest that the ARC conveys metabolic information to the SCN (Yi *et al.*, 2006) and to pre-autonomic output areas like the PVN, DMH or integration sites like the MnPO (Chapter 3), suggesting that the information integrated at the ARC might be important for the overall physiology, in accordance with the powerful effects not only on glucose, but also on blood pressure, BAT modulation and circadian rhythmicity in high fat diets (HFD), but also the observed turnover of ARC cellular populations such as the decrease of POMC neurons or the proliferation of glial populations (Horvath *et al.*, 2010; Thaler *et al.*, 2012).

Moreover the coordinated output of the ARC and the SCN to the MnPO (chapter 3), demonstrates the importance of the synchronization between the activity of these nuclei to modulate temperature and perhaps other features of physiology. Our results demonstrate the close relationship between ARC and SCN may provide the basis on which autonomic disturbances in metabolic syndrome can be explained (Figure 23).
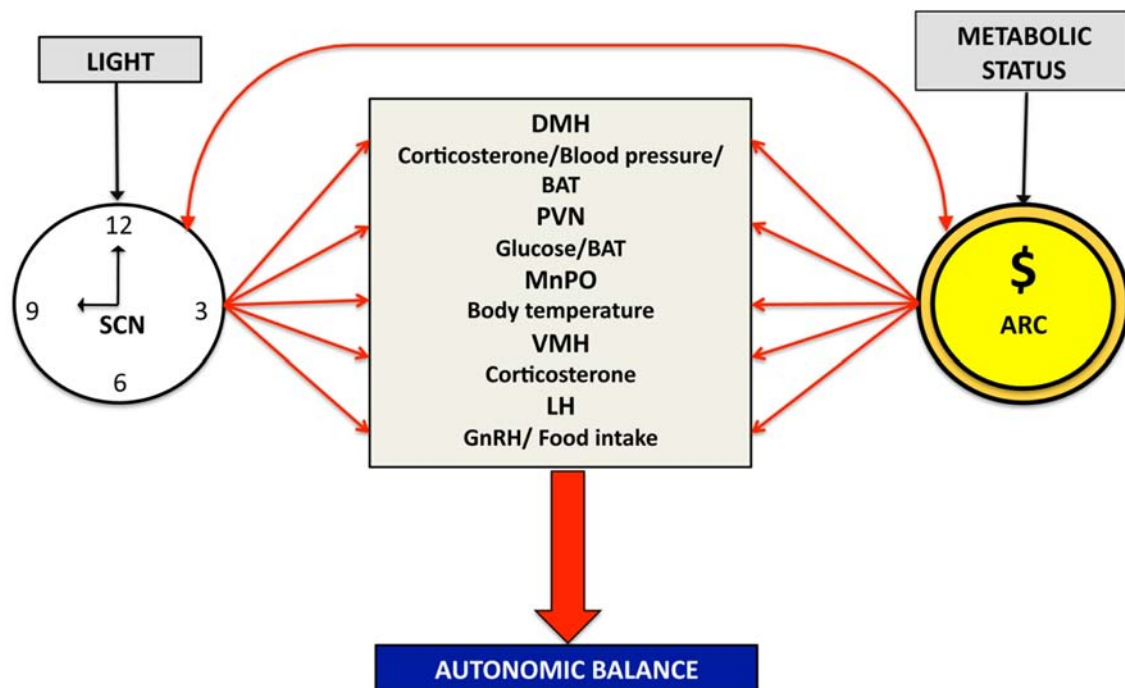
Figure 23. Shared outputs of the SCN and the ARC known to modulate autonomic output. Both SCN and ARC project to several hypothalamic nuclei that control autonomic outputs, this interaction balances between the correct time-stamp and the necessary energy cost to perform a certain physiological function.

BAT MODULATION AN IMPORTANT FACTOR FOR METABOLIC HEALTH

In chapter 3 we demonstrated that the interaction between the SCN and the ARC modulates BAT UCP1 expression, an uncoupling protein used by brown adipocytes to generate heat. Since HFD changes the activity of the ARC, our observation in chapter 3 suggests changes in the autonomic output to BAT under HFD due to ARC activity

modification. Normally food intake induces BAT activity through the sympathetic activation of UCP1 transcription after eating, postprandial thermogenesis (Argyropoulos & Harper, 2002), studies have demonstrated instead, that overfeeding or HFD does not induce sympathetic activation of BAT as food intake normally does (LeBlanc & Soucy, 1996). This loss of postprandial activation of BAT (Tupone *et al.*, 2014) might be one of the clues to understand obesity. In addition there is an important difference in the induction of diet induced thermogenesis depending on the time of the day when food is consumed, suggesting a possible target site for the circadian and metabolic influence on energy expenditure. Interestingly signals like leptin act both on ARC and MnPO and induce a clear induction of BAT sympathetic tone, while this induction decreases when the subjects overeat or become obese (Schlogl *et al.*, 2013). Together with the notion of decreased BAT tissue in obese subjects these observations strongly suggest that disturbances in food intake, not only affect ARC and SCN activity but also induce the deregulation of the autonomic tone reflected in BAT loss.

Consequently the present thesis provides a first step to understand how brain areas involved in the temporal and metabolic organization of the body interact in order to produce a coherent arrangement of physiology. The implication that the loss of coherence between the temporal and the metabolic system might lead to metabolic disease and understanding the mechanisms involved in the correct arrangement of physiology could in turn give the basis for therapies that prevent or control these type of diseases.

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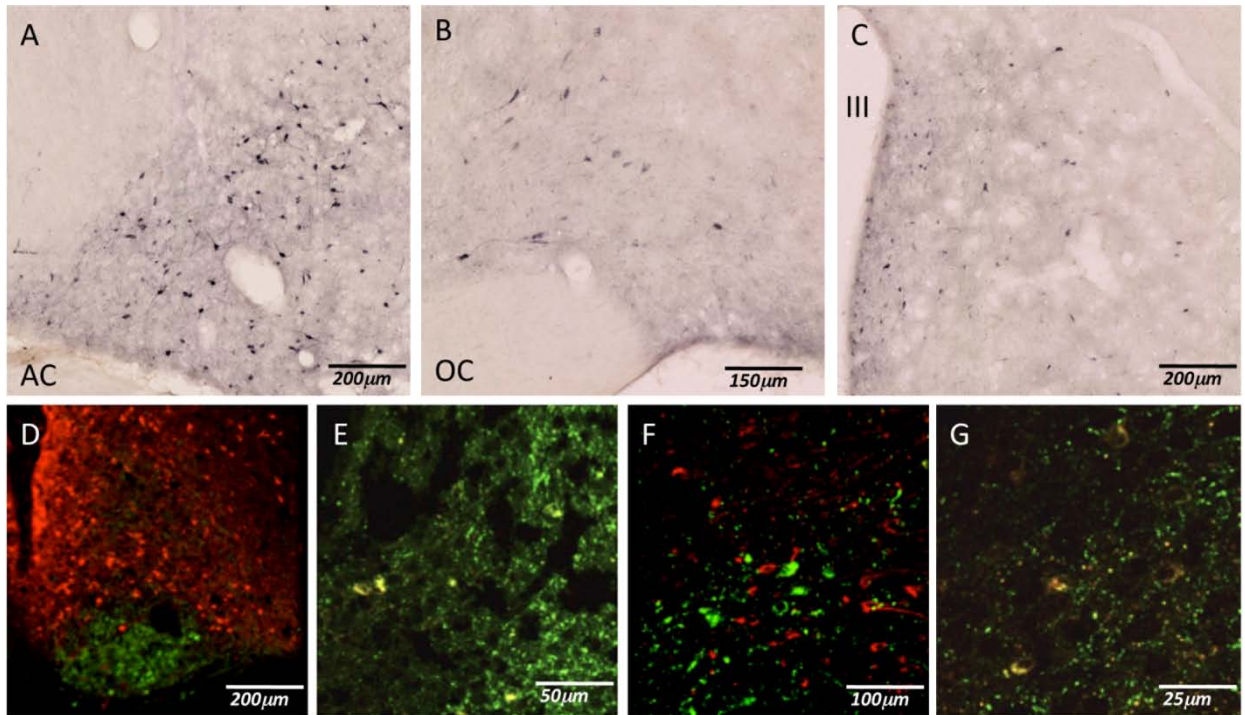
SUPLEMENTAL INFORMATION

Figure S1. Other projections to the MnPO and co-localization with peptides from the ARC and the SCN. (A) Bed nucleus of the stria terminallis (B) Supraoptic nucleus and (C) Paraventricular nucleus sections that show CtB retrograde tracing from the MnPO. (D) SCN section with VIP-IR (green) CtB-IR (red) without co-localization, (E) shows co-localization between in SCN GRP-IR neurons (green) and CtB-IR (red) retrograde labelled cells, (F) ARC section stained for CART (green) and CtB (red) showing no co-localization and (G) shows NPY-IR (green) and CtB-IR (red) co-localizing. CtB; B fraction of the Cholera Toxin, SCN; Suprachiasmatic nucleus, VIP; vasointestinal peptide, GRP; gastrointestinal peptide, IR; immunoreactivity, CART; cocaine amphetamine related transcript, NPY; neuropeptide Y OC; optic chiasma, III; third ventricle.
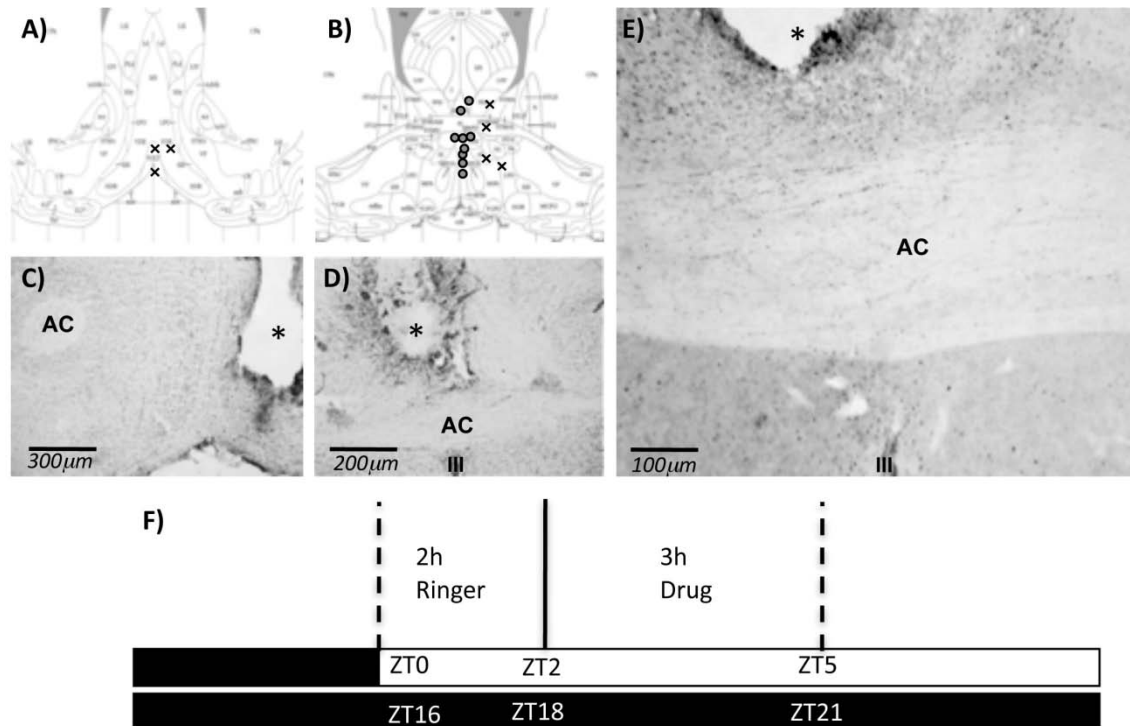
Figure S2. Effective and non-effective sites of microdialysis in the MnPO. (A) Paxinos schemes and Nissel stainings for the determination of the canula placement. The sites where infusion had an effect in body temperature (o) and the areas where probe placements did not have any effect on temperature (x) are indicated in the top schemes from Paxinos rat brain atlas, (B) represents the time points where infusions where done, black bars indicate the dark phase and the white bar indicates light phase. AC, anterior commissure, OC optic chiasm and ZT, Zeitgeber time.
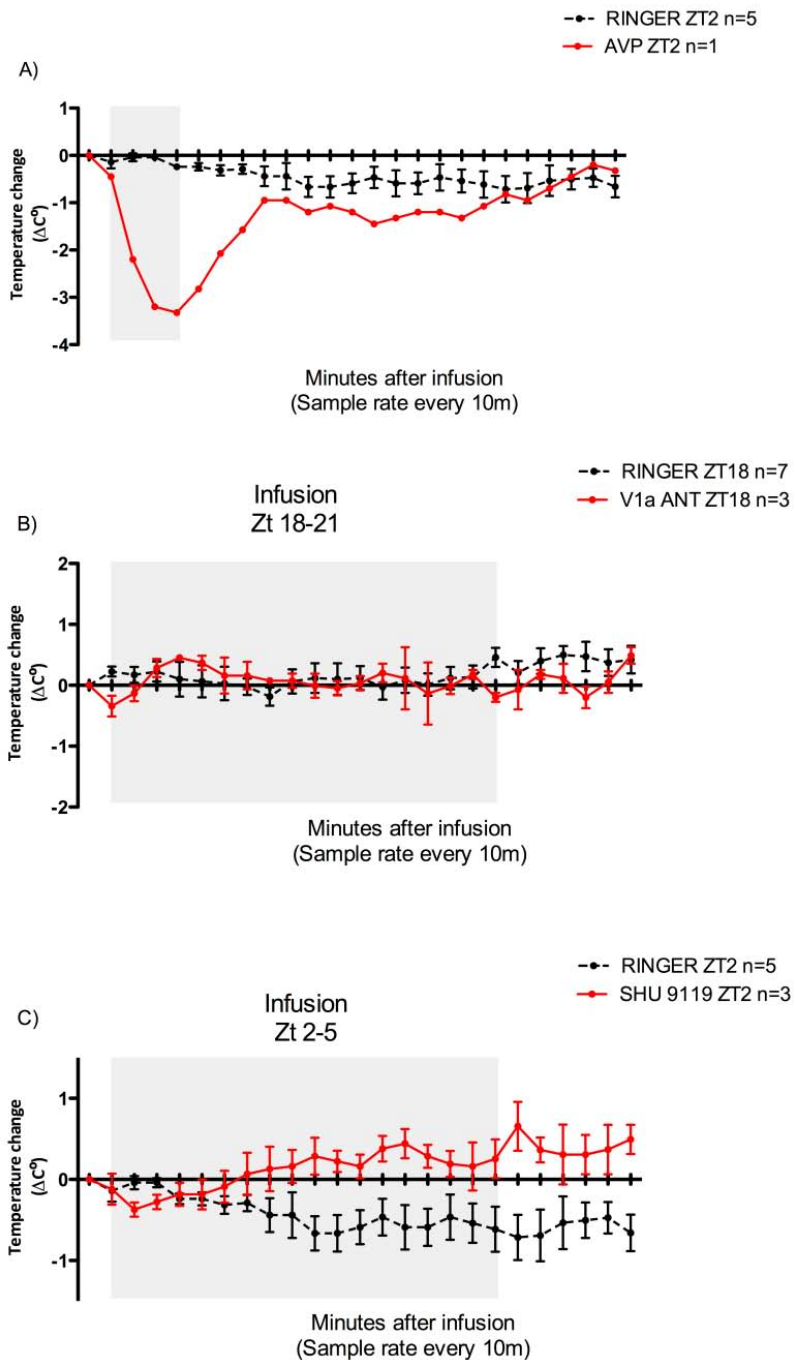
Figure S3. MnPO microdialysis control infusions. (A) AVP infusion in the MnPO during the day (Zt2) induce an immediate Tb decrease, therefore the infusion was stopped after only 40m, (B) V1Aant infusion in the MnPO during the dark phase (Zt18) does not induce any change in Tb (F [1,179]= 1.78, P=0.1843), (C) MC3-4R antagonist (SHU9119) infusion in the MnPO during the day induces the opposite effect than the observed during the night infusion (Zt2) (F[1,135]= 47.32 P<0.0001).

ABREVIATIONS

SCN: Suprachiasmatic nucleus

ARC: arcuate nucleus

α-MSH: alpha-melanocyte stimulating hormone

DD: constant darkness

MnPO: median preoptic nucleus

Tb: body temperature

Ta: ambient temperature

AVP: arginine-vasopressin

V1A: vasopressin 1 A receptor

MC3-4R: melanocortin 3-4 receptor

DMH: Dorsomedial hypothalamic nucleus

LPB: lateral parabrachial nucleus

MPA: medial preoptic area

MPO: medial preoptic nucleus

DMV: dorsomotor nucleus of the vagus

RF: reticular formation

rRPa: rostral raphe anterior nucleus

mRPa medial raphe anterior nucleus

BAT: brown adipose tissue

VTA: ventral tegmental area

PAG: periacueductal grey matter

CVO: circinvetricula organ

SFO: subfornical nucleus

OVLT: organum vasculosum of the lamina terminallis

NTS: nucleus of the solitary tract

DRG: dorsal root gangion

5-HT: seronotnin

ACh: acetil choline

Glut: glutamate

IML: intermediolateral column

LH: lateral hypothalamus or luteinizing hormone

PH: posterior hypothalamus

VMH: Ventromedial hypothalamus

LD: light-dark

DBB: diagonal band of broca

AgRP: agouti related peptide

NPY: neuropeptide Y

POMC: proopiomelanocortin

CART: cocaine amphetamine related transcript

PUBLICATIONS

1) Saderi N, Cazarez-Marquez F, Buijs FN, Salgado-Delgado RC, Guzman-Ruiz MA, del Carmen Basualdo M, Escobar C, Buijs RM (2013) The NPY intergeniculate leaflet projections to the suprachiasmatic nucleus transmit metabolic conditions. Neuroscience; 246:291-300.

2) Guerrero-Vargas NN, Salgado-Delgado R, Basualdo Mdel C, Garcia J, Guzman-Ruiz M, Carrero JC, Escobar C, Buijs RM (2014) Reciprocal interaction between the suprachiasmatic nucleus and the immune system tunes down the inflammatory response to lipopolysaccharide. J Neuroimmunol; 273:22-30.

3) Guzman-Ruiz M, Saderi N, Cazarez-Marquez F, Guerrero-Vargas NN, Basualdo MC, Acosta-Galvan G, Buijs RM (2014) The suprachiasmatic nucleus changes the daily activity of the arcuate nucleus alpha-MSH neurons in male rats. Endocrinology; 155:525-535.

4) Sabath E, Salgado-Delgado R, Guerrero-Vargas NN, Guzman-Ruiz MA, del Carmen Basualdo M, Escobar C, Buijs RM (2014) Food entrains clock genes but not metabolic genes in the liver of suprachiasmatic nucleus lesioned rats. FEBS Lett; 588:3104

5) Guzmán-Ruiz M, Ramirez-Corona A, Ramirez-Plascencia O, Sabath SE, Basualdo CM, Guerrero-Vargas NN, León-Mercado L, Durón-Javier C, Fuentes R, Escobar C and Buijs RM. (2015) The Suprachiasmatic and Arcuate nucleus orchestrate the diurnal temperature decrease in the rat. SUBMITTED.

6) Natalí N. Guerrero-Vargas, Rebeca Fuentes, Joselyn García, Roberto Salgado-Delgado, Mara Guzmán-Ruiz, María del Carmen Basualdo, Carolina Escobar, Regina P Markus and Ruud M. Buijs. (2015) Shift work in rats results in a higher inflammatory response to Lipopolysaccharide and enhanced tumor development. SUBMITTED.

7) Herrera Moro Chao D, León Mercado L, Escobar Briones C, Acosta Galván G, Foppen E, Guzmán-Ruiz M, Basualdo MC, Buijs RM. (2015) The biological clock gates sensory information about negative metabolic conditions in the ARC. SUBMITTED.

8) Guzmán-Ruiz M, Guerrero-Vargas NN, Buijs F, Basualdo CM and Buijs R. (2015) Daily modulation of GFAP expressing cells in the Arcuate nucleus, IN PREPARATION.