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"INTERACCIÓN ENTRE EL SISTEMA INMUNE Y EL SISTEMA CIRCADIANO"

TESIS

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PRESENTA

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RESUMEN

El sistema circadiano sincroniza y coordina los procesos fisiológicos del cuerpo de acuerdo con los cambios dinámicos en el ambiente. En mamíferos, el núcleo supraquiasmático (NSQ) ubicado en el hipotálamo anterior es considerado el reloj biológico, éste se sincroniza al ciclo luz-oscuridad y se encarga de la organización temporal de toda la fisiología a través de la generación de los ritmos circadianos en la ingestión de alimentos, la secreción de hormonas, el ciclo sueño-vigilia y la temperatura entre otros.

Los ritmos biológicos se observan en múltiples frecuencias en el sistema inmune. En condiciones basales se ha demostrado que el número de células inmunes así como sus productos presentan fluctuaciones diarias a lo largo del ciclo luz-oscuridad. Aunque se desconoce como el NSQ comunica la información temporal a los órganos y células del sistema inmune, se especula que podría utilizar vías autonómicas y neuroendocrinas tal como se ha reportado para otros órganos y sistemas.

Por otra parte, se ha demostrado que las alteraciones en los ritmos circadianos, provocadas por la reducción del tiempo de sueño, mayor exposición a la luz brillante durante la noche, irregularidades diarias en el ciclo sueño - vigilia (jet-lag, trabajo nocturno o por turnos), así como a un elevado consumo de alimentos durante la fase de inactividad, se asocian con una mayor vulnerabilidad a desarrollar ciertas enfermedades. Lo anterior sugiere que el sistema circadiano afecta la funcionalidad el sistema inmune, sin embargo, la participación del NSQ en la regulación de la respuesta inmune aún no ha sido explorada de forma sistemática.

En esta tesis, investigamos la interacción entre el sistema circadiano y el sistema inmune, a través de: 1. la evaluación de la actividad neuronal del NSQ y otras áreas dentro del cerebro en respuesta a un estímulo inmune periférico 2. la evaluación de la respuesta inmune en condiciones de alteración de la ritmicidad circadiana 3. la evaluación de las posibles vías de comunicación entre el sistema inmune y el NSQ y viceversa para determinar los mecanismos por medio de los cuales el NSQ podría regular el funcionamiento del sistema inmune.

Nuestros resultados demuestran que las señales de temporalidad del NSQ son esenciales para regular la magnitud de la respuesta inflamatoria. La administración de lipopolisacárido intravenoso a ratas macho de la cepa Wistar, induce una respuesta

inflamatoria cuya magnitud depende de la actividad neuronal del NSQ. De forma importante, las ratas sometidas a un protocolo de desincronización circadiana, por medio de la actividad forzada o la ingestión de alimento durante el periodo de inactividad, presentan una respuesta inflamatoria exacerbada en repuesta a la administración de LPS y una mayor propensión a desarrollar tumores después de la inoculación de células tumorales.

Para estudiar los mecanismos por medio de los cuales el NSQ podría informarse del estado inmunológico del cuerpo y así regular la respuesta inmune, evaluamos la activación neuronal de distintas áreas dentro del cerebro así como la respuesta inflamatoria en animales cuyas conexiones neuronales entre el hígado (un órgano clave en la respuesta inmune innata) y el NSQ habían sido destruidas por medio de procesos quirúrgicos de denervación selectiva de las ramas simpática y parasimpática que inervan al hígado.

En esta parte del trabajo demostramos que el NSQ puede recibir información inmune desde la periferia a través de vías sensoriales espinales. Del mismo modo el NSQ parece utilizar primordialmente al sistema autónomo simpático para regular la magnitud de la respuesta inflamatoria ya que en las ratas cuyas conexiones simpáticas entre el hígado y el NSQ habían sido removidas, se observó una respuesta inflamatoria exacerbada.

En conclusión, este trabajo demuestra la importancia del NSQ en la regulación de la respuesta inflamatoria. Diversas condiciones que involucran alteraciones en la comunicación del NSQ con la periferia, inducen respuestas inflamatorias exacerbadas. El mecanismo por medio del cual el NSQ regula la respuesta inflamatoria aún no se conoce a detalle, sin embargo, el presente trabajo sugiere que el NSQ está incorporado en un circuito inmunológico el cual involucra al sistema nervioso autónomo particularmente la rama simpática, en la regulación de la respuesta inflamatoria.

SUMMARY

The circadian system synchronizes and coordinates physiological process in the body according to dynamical changes in the environment. In mammals the hypothalamic Suprachiasmatic nucleus (SCN) considered the master circadian clock, organizes circadian rhythms and coordinates the efficiency and timing of physiological processes – hormone secretion, sleep-wake cycle, temperature changes, behavior and autonomic outflow to body organs– according to the light-dark cycle.

Biological rhythms are observed at multiple frequencies in the immune system. Under basal conditions, the number of immune cells and their products showed daily fluctuations along the light-dark cycle. Little is know about how the SCN target organs and cells of the immune system. Though it may be assumed that similarly as other systems, autonomic and neuroendocrine pathways are involved.

Circadian rhythms disruptions, induced by reduced sleep time, bright light exposition at night, sleep-wake cycle abnormalities (*jet-lag*, shift and night workers) and high consumption of food during the inactive phase are associated with increased vulnerability to certain diseases. The above suggest that the circadian system affects the functionality of the immune system, however the particular role of the master circadian clock (SCN) in the regulation of immune responses has not been studied systematically.

In this thesis we investigated the interaction mechanisms between the immune and circadian system through: 1. The evaluation of SCN neuronal activity after peripheral LPS administration 2. The evaluation of the inflammatory response under circadian rhythms disruption conditions 3. The evaluation of possible communication pathways between the immune system and the SCN and viceversa.

Our results demonstrate that the timing of the SCN is essential for the magnitude of the inflammatory response. Lypoolysscharide (LPS) administration to male Wistar rat induced an inflammatory response which magnitude depends on SCN neuronal activity. Importantly, circadian desynchronization induced by our shift-work protocol ("forced day activity") or food consumption during the inactive phase resulted in increased inflammatory response to LPS. These animals also developed bigger tumors after tumor cells inoculation.

In order to study the pathways used by the SCN to be inform about the inflammatory status of the body and regulate the immune response, we evaluated neuronal activation of different brain areas including the SCN as well as the inflammatory response to LPS after hepatic denervation procedures. Our results demonstrated that the SCN is able to received immune information by spinal sensory pathways. In order to regulate the immune response, the SCN use autonomic pathways specifically sympathetic, since sympathetic hepatic denervation procedures induced increased inflammatory response to LPS.

In conclusion, our work demonstrates the importance of the SCN for the regulation of the inflammatory response. Different conditions that involved circadian desynchronization induced heightened inflammatory responses. The precise pathways and mechanisms via which the SCN and other brain areas are incorporated in this immunological circuit still need further investigation. However, our results highlight the role of the autonomic nervous system as one of the pathways used by the SCN to regulate the inflammatory response.

CHAPTER 1

Introduction

The immune system

In living organisms a defense system exists in order to protect them from pathogens or other foreign particles or substances that invade the body. This system, known as the immune system is composed of a variety of organs, cells and molecules capable of recognize and respond to any threat that could perturb body homeostasis. In order to achieve this, the immune system has precise mechanisms that allow the discrimination between self and non-self molecules. In mammals the immune system is divided into two major interconnected components the innate and the adaptive immune response. For the purpose of this thesis we will only review the innate immune response.

The innate immune response

The innate immune response is considered the first line of defense of the body, preventing most of the infections to become dangerous for the health of the individual and also contributes to tissue repair and removal of dying and senescent cells. It is not specific for individual pathogens but recognizes highly conserved components of microorganisms called pathogen associated molecular patterns (PAMP´s) such as the lipopolysaccharide (LPS), peptidoglycan, bacterial DNA and RNA of glycan's expressed in the external membrane of certain pathogens (Medzhitov and Janeway, 2000). Immune cell types such as monocytes, macrophages, neutrophils, dendritic cells (DCs), mast cells natural killer cells (NK) and eosinophil's orchestrate the innate immune response through the recognition of specific PAMP´s via germ line encoded receptors referred as pattern recognition receptors (PRRs) (Medzhitov, 2007). The cells of the innate immune system are able to detect different classes of pathogens and abnormal or transformed cells through this PRRs that are expressed on their cell surface (Kawai and Akira, 2010).

Cytokines

The inflammatory process is mainly mediated by cytokines; small soluble proteins secreted from different cells types not necessarily belonging to the immune system, the cytokines can exert autocrine, paracrine or endocrine effects by interacting with cells surface receptors. Cytokines are produced very early in response to pathogens or danger signals and stimulate a host response aimed to control cellular stress and minimize

cellular damage; they can directly or indirectly affect immune cells to induce an adaptive immune response. In this sense, cytokines are considered as a bridge between the innate and the adaptive immune response. In fact a strong cytokine response is a requirement for a strong adaptive immune response. Besides their major role in inflammatory processes they also influence other physiological process via their action on the central nervous system (Vitkovic et al., 2000). Depending on their function during the immune response, cytokines are classified as pro-inflammatory or anti-inflammatory. Pro-inflammatory cytokines include interleukin (IL)-1, IL-6, IL-8, IL-12, interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) promote inflammation and are produced rapidly after pathogen encounter (Janeway CA., 2003). Anti-inflammatory cytokines such as IL-10, IL-4, IL-22, IL-11 and TGF-β regulate immune responses in order to avoid exaggerated activation of the immune system and pro-inflammatory cytokines production. In this sense the adequate balance between pro-inflammatory and anti-inflammatory cytokines is necessary to maintain tissue homeostasis after an insult and determines the outcome of disease.

Innate immune activation via lipopolysaccharide administration (LPS)

The endotoxin LPS is the most frequently used and most powerful stimulus to trigger the signaling pathways involved in innate immunity and inflammation. LPS is an important component of the outer membrane of Gram-negative bacteria. Mainly macrophages and other immune cell types such as dendritic cells (DCs) express the specific receptor that recognizes the immunogenic part of LPS, the lipid A (Gronow and Brade, 2001). This specific receptor for LPS is the toll like receptor 4 (TLR4). In the circulation, LPS interacts with the LPS binding protein (LBP, a soluble protein), this interaction facilitates the association of LPS to the co-receptor CD14 and the subsequent transfer of LPS to the TLR4/myeloid differentiation protein 2-receptor complex (MD-2) in the cellular membrane. The LPS TLR4 interaction activates myeloid differentiation primary response 88 (MyD88) dependent or independent pathways. In MyD88 independent pathways, TLR4 engages the adaptor protein Toll/IL-1 receptor (TIR)-domain-containing adaptor inducing interferon-β protein (TRIF), TRAM (TRIF-related adaptor molecule) facilitates this binding leading to the activation of the transcription factor interferon regulatory factor 3 (IRF-3) and type 1 interferon production (Lu et al., 2008). The activation of MyD88 dependent pathways involve the interaction of TLR4 with the adaptor protein MyD88, this association is facilitated by TIRAP (TIR domain-containing adaptor protein

or MyD88- adapter-like) and culminates in nuclear translocation of the nuclear factor-kappa B (NF-κB) and activating protein-1 (AP-1) both transcription factors that activate the transcription of numerous pro-inflammatory genes, which encodes cytokines, chemokines, antimicrobial peptides between others and triggers the inflammatory process (Raetz and Whitfield, 2002).

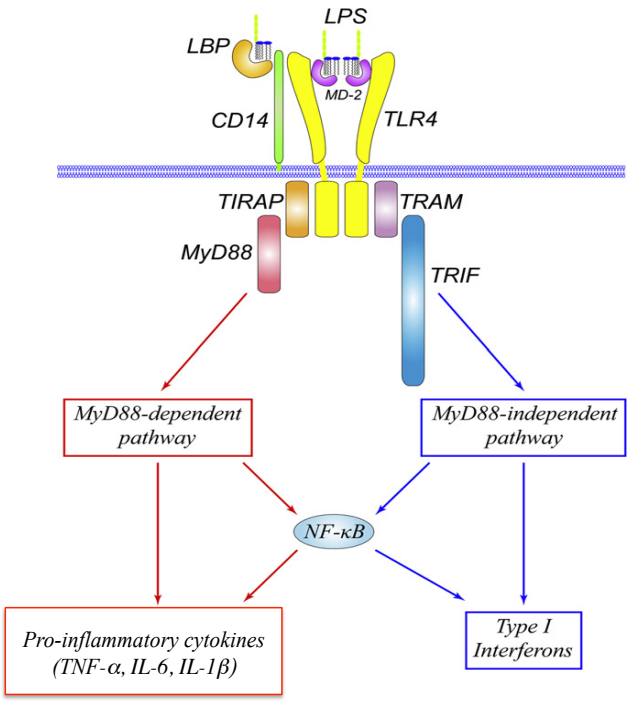

Figure 1. LPS/TLR4 signaling pathway. LBP and CD14 facilitate LPS recognition by the TLR4/MD-2 receptor complex. LPS/TLR4 signaling pathway induce the activation of MyD88-dependent and MyD88 independent pathways, which mediate the activation of pro-inflammatory genes and Type I interferons genes respectively. Modified from (Lu et al., 2008).

For a long time it has been recognized that the inflammatory response has important effects on the central nervous system, where mainly the hypothalamus is considered to play an important role in the response to inflammation. Because most physiological functions organized by the hypothalamus are under circadian control, also the defense of the body to infection can be considered to vary under the influence of the sleep activity cycle.

The suprachiasmatic nucleus drives circadian rhythms in physiology.

In mammals, the hypothalamic suprachiasmatic nucleus (SCN) is considered to be the master circadian pacemaker, which is entrained by environmental variables such as the light-dark cycle and is therefore synchronized with the solar day. As the master circadian

clock, the SCN generates circadian (approximately 24h) rhythms of biological processes even in the absence of environmental cues. The SCN transmits this circadian message to other regions in the brain and to organs in the body, considered peripheral oscillators, because they also exhibit daily fluctuations, however their temporal order is regulated by the SCN. In this sense, the main task of the SCN is to maintain a temporal organization of our physiology synchronized to environmental cycles.

The SCN is a bilaterally paired nucleus consisting of tightly compacted, small-diameter neurons; it is located in the anterior hypothalamus above the optic chiasm and lateral to the third ventricle. The SCN is commonly divided into two regions: a ventral region receiving retinal input and a dorsal region with mainly Arginine vasopressin (AVP) and somatostatin (SS), as well as GABA and glutamate containing cells (Moore et al., 2002). Via direct synaptic connections with ganglion cells of the retina, the ventral neurons receive information about the light intensity in the environment and integrate this external input in order to adapt the phase of the SCN to the photoperiod. Ventral neurons communicate this environmental information to the rest of the SCN, through the release of neuropeptides like vasoactive intestinal peptide (VIP) or gastrin-releasing peptide (GRP), as well as the neurotransmitter GABA or glutamate (Aida et al., 2002).

The tightly packed SCN cells have extensive connections within the SCN and form a dense network. Individual SCN neurons have an intrinsic oscillator capacity that consists of interlinked auto-regulatory transcriptional–translational feedback loops that drive rhythmic, ~24-h expression patterns of core clock proteins, which are necessary for the generation, and regulation of circadian rhythms within individual cells (Lowrey and Takahashi, 2004; Reppert and Weaver, 2002). In mammals, the members of the basic helix-loop-helix (bHLH)-PAS (Period-Arnt-Single-minded) transcription factor family *Clock (circadian locomotor output cycles kaput)* and *Bmal1 (brain and muscle ARNT like protein1)* are part of the positive limb of the feedback circuitry. The negative limb consists of PER: CRY, heterodimers that translocate back to the nucleus to repress their own transcription by acting on the CLOCK: BMAL1 complex (Brown and Schibler, 1999; Shearman et al., 2000). Additional loops are formed with the help of retinoic acid-related orphan nuclear receptor Rev-Erbα and RORs, which fine tune the oscillations generated by the main loops and contribute to the strength of this molecular clock circuitry. Besides regulating their own transcription, clock proteins regulate other target genes that encode for various products as well. Together such genes are known, as clock-

controlled genes (CCGs). These genes have in common the element E-box on its promoter region, which means that their transcription is dependent on the components of the biological clock molecular machinery (CLOCK: BMAL1) and therefore show circadian rhythm variations in expression. Although the individual SCN cells are supposed to have an autonomous oscillator capacity (Welsh et al., 1995) recently it was suggested that only the network of SCN cells is able to maintain this for long periods of time (Webb et al., 2009).

The SCN coordinates circadian rhythms in our body by sending projections to at least four different neuronal targets (Buijs et al., 2003a):

1. Endocrine neurons (such as corticotropin-realeasing hormone, CRH, and gonadotropin–releasing hormone, GnRH).

2,3. Pre-autonomic neurons of the paraventricular nucleus of the hypothalamus (PVN), specifically to separate neurons that are the origin of long descending projections to pre-ganglionic parasympathetic and sympathetic neurons in the brainstem and spinal cord, respectively.

4. Inter-neurons that reside in other hypothalamic structures that transmit the circadian signal to other brain regions, and areas outside the hypothalamus.

Signals originating from the SCN can regulate output pathways that control physiological functions, such as proper timing of hormone release (for example, corticosterone and melatonin secretion) cardiovascular activation, feeding behavior, cell growth, body-temperature fluctuations, renal filtration, nutrient mobilization and xenobiotic metabolism, etc. (Hastings et al., 2003a). The main function of the SCN is to synchronize all these physiological functions in a way that the body is optimally prepared for the changes in activity and rest periods.

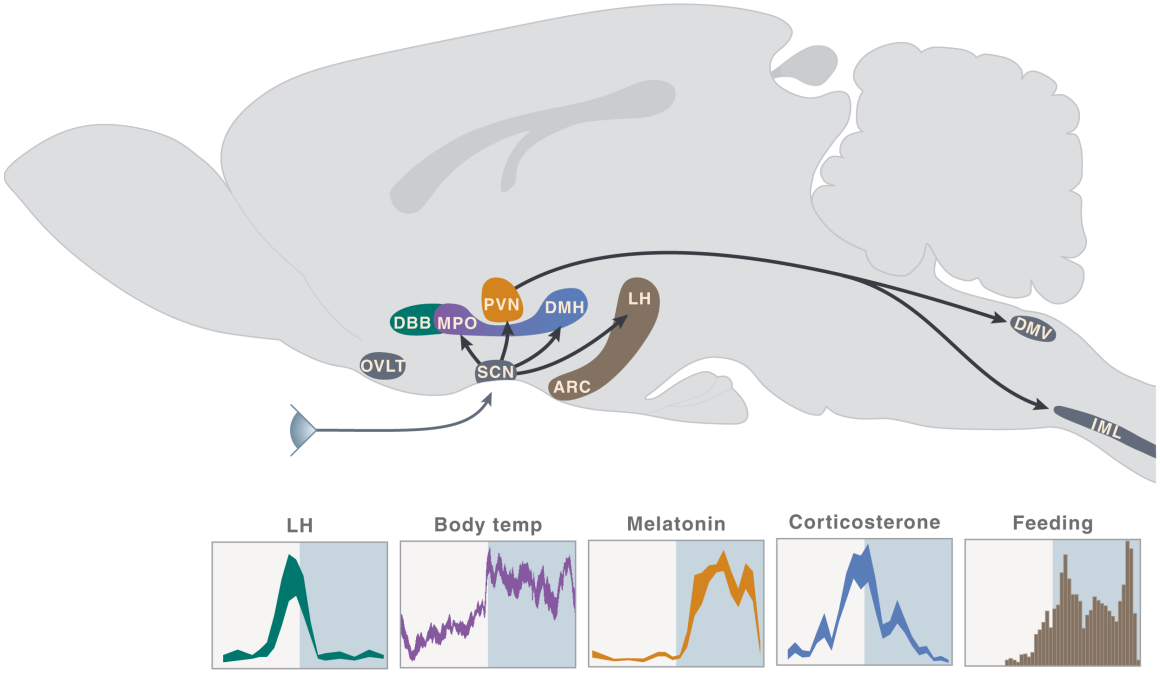

Figure 2. SCN outputs to several hypothalamic areas are associated with the circadian control of specific physiological functions, such as rhythmic release of luteinizing hormone (LH), melatonin, corticosterone and behavioral variables like feeding behavior and body temperature rhythms. ARC, arcuate nucleus; DBB, diagonal brand of Broca; DMH, dorsomedial hypothalamus; DMV, dorsal motor nucleus of the vagus; IML, intermediolateral columns; LH, lateral hypothalamus; MPO, medial preoptic area; OVLT, organum vasculosum of lamina terminalis; PVN, paraventricular nucleus of the hypothalamus; SCN, suprachiasmatic nucleus. Modified from (Buijs and Kalsbeek, 2001).

Circadian rhythms in the immune system under basal conditions

Humans

The correct timing of our physiological functions is set by the SCN; consequently the expression of circadian rhythms in the immune system is not an exception. In humans, circadian rhythmicity has been found in the abundance and function of different immune cell types. As they can be easily isolated by non-invasive protocols from blood, circulating leukocytes (white blood cells) belonging to the innate or adaptive immune system represent the most studied human immune cell population. In healthy humans, higher numbers of circulating leukocytes such as B and T lymphocytes have been found during the night (Ackermann et al., 2012; Kirsch et al., 2012; Miyawaki et al., 1984).

In contrast the peak of circulating innate immune cells such as NK cells and granulocytes (neutrophils, eosinophils and basophils) as well as monocytes (macrophages and dendritic cells) was found during the day period (Bourin et al., 1993; Haus and Smolensky, 1999; Kirsch et al., 2012). The levels of certain immune cell products such as cytokines also vary according to the light-dark cycle. For example, plasma levels of IL-2 are higher at noon whereas IFN-γ, TNF-α, IL-12 and IL-10, peak during the day but also during the night showing a biphasic pattern (Petrovsky and Harrison, 1998; Young et al., 1995). In contrast, higher concentrations of IL-6 and IL-β are found in the blood during the sleeping time (Gudewill et al., 1992). Besides the above-presented data, circadian variations in immune parameters under basal conditions are small and sometimes difficult to observe because of the small quantities of cytokines normally present in healthy individuals as compared to inflammatory conditions. Importantly the significance of these circadian variations in the response to an immune challenge is not known.

Rodents

Similar as in humans circadian rhythmicity in rodent immune system has been investigated in the blood where, higher counts of total leukocytes, lymphocytes (T or B lymphocytes) and granulocytes isolated from rats were found, opposed to humans, in the first half of the resting period whereas NK cell counts, as in humans, were higher at the active period (Depres-Brummer et al., 1997; McNulty et al., 1990; Zhao et al., 2012). Blood monocytes counts peak at the beginning of the dark period and lower levels were found at the beginning of the light period (McNulty et al., 1990). Also circulating cytokines such as TNF-α, IL-1-α and IL-6 show 24h rhytmicity under physiological conditions with peaking values during the light as well as during the dark phase (Luna-Moreno et al., 2009). The TLRs, a type of PRRs that are expressed in innate immune cells such as macrophages and DCs (Medzhitov and Janeway, 2000) and are essential to detect pathogens show circadian variations in their expression levels (Froy and Chapnik, 2007; Silver et al., 2012b). This might be related to the need to detect circulating pathogens that can arrive in the circulation as the result of food intake and the capturing of these pathogens by the uptake process in the gut. These observations warrant the question: If the inflammatory molecules vary over the day-night cycle does that mean that the inflammatory response can also be regulated in a circadian fashion?

Does the immune response vary along the circadian cycle?

Humans

Clinical evidence supports the correlation between time of the day and inflammatory illness manifestation, for example, allergic rhinitis episodes, as well as symptoms of asthma, are worse at night or early in the morning (Smolensky et al., 2007), while rheumatoid arthritis symptoms such as pain, functional disability and stiffness are mainly present early in the morning, associated with increase levels of the pro-inflammatory cytokine IL-6 (Gibbs and Ray, 2013; Haus et al., 2012). While these clearly suggests that the time of inflammation affect disease outcome, the involved mechanisms still not known. Although *in vitro* studies have shown that NK cells capacity of killing infected cells or malignant cells (cytotoxicity) as well as the phagocytic activity of granulocytes, is maximal in cells isolated at the end of the night of early in the morning (Gatti et al., 1988; Melchart et al., 1992). In agreement with this, night isolated human whole blood cells produce more TNF-α in response to *ex vivo* immune stimulation (Aguillon et al., 2001). Likewise, in blood monocytes, the up-regulation of costimulatory (CD80 and CD86) as well as antigen presenting molecules (MHC-II) after immune stimulation is higher in evening collected monocytes in comparison with morning collected monocytes (Lancaster et al., 2005). These observations suggest that in humans the immune response can be influenced by the circadian system and may correspond with observed susceptibility to inflammation; however most of these observations were made *in vitro* and hardy resemble physiological conditions. In this sense, rodents offer a much better opportunity to investigate this relationship.

Rodents

As we already mention, LPS is commonly used to examine experimentally the innate immune response in rodents and even in humans. Circadian variations in innate immune responses have been mainly described after large or lethal doses of inflammatory substances. In mice, pioneer studies demonstrated a day-night rhythm in the susceptibility to lethal doses of LPS (Halberg et al., 1960) that was later correlated with an overproduction of pro-inflammatory cytokines (Marpegan et al., 2009). Mortality is greater when the LPS challenge is given during the night at a time that coincides with increased neutrophils migration to the liver (Schernhammer et al., 2001). These experiments consequently, investigate the response of the body to septic shock; however

the contribution of circadian rhythms in the response to lower, non-lethal or even physiological concentrations of circulating endotoxins is less documented. More importantly the mechanisms via which the circadian system may exert its influence in the immune system has not been investigated yet. This will be the topic of investigation in chapter 2 in which we injected low doses of LPS at different times of the day to investigate the innate immune response of the rat. In addition, in order to study the involvement of the SCN in the time dependent regulation of the immune response, we analyze the activation of different brain areas (including the SCN) after the administration of a low dose of LPS.

How can the immune information reach the SCN?

In spite of the evidence pointing to a role of the SCN in the timing of the immune response, a mechanistic explanation still missing. Therefore we investigated first in chapter 2 the response of the brain including the SCN to an inflammatory stimulus. It is well known that a systemic inflammatory stimulus like the LPS or cytokines induce neuronal activation in different areas of the brain including the SCN (Buijs et al., 2008; Elmquist et al., 1996; Konsman et al., 1999; Marpegan et al., 2005) moreover such stimulus also increases the levels of interleukins, nitric oxide, prostaglandins, and other substances in the brain (Larson and Dunn, 2001; Singh and Jiang, 2004). Since LPS and cytokines minimally pass the murine Blood Brain Barrier (BBB) (Banks and Robinson, 2010), at least two possibilities exist for the entrance of the immune information to the brain. One of the possibilities is that they act first on brain cells that are outside the BBB such as the Circumventricular Organs (CVO´s); importantly these cells are able to communicate with other brain areas. Here the Area Postrema (AP) and the Organum Vasculosum of Lamina Terminalis (OVLT) appear to be the initial sites of activation (Katsuura et al., 1990; Lee et al., 1998; Xia and Krukoff, 2001) and where specific receptors for LPS (Chakravarty and Herkenham, 2005; Laflamme and Rivest, 2001) and cytokines (Robertson et al., 2000) have been reported and also local synthesis of cytokines may occur (Klir et al., 1993). These CVO's could be important sensory structures for pathogens via which the brain could be warned for circulating infectious substances. Noteworthy is that the CVO's project directly to the SCN (Lind et al., 1982). In this way circulating inflammatory information may reach the SCN by direct neuronal projections. The second possibility is related to the activation of peripheral sensory nerves that innervate immune organs, and are supposed to be sensory fibers in the vagus

nerve or their cells in the nodose ganglion (Dantzer, 1994; Watkins et al., 1995). Indeed afferent neurons of the vagus nerve express IL1R and prostaglandins (Ek et al., 1998) also mRNA and protein for TLR4 have been described in the nodose ganglion of the afferent vagus nerve suggesting that local inflammatory molecules could effectively signal to the brain via the sensory vagus nerve. The ascending vagus nerve signals activate neurons within the AP and the Nucleus of the Tractus Solitarius (NTS) (Smith et al., 1998) who sends neuronal projections to the SCN (Buijs et al., 2014). The analysis of the possible entrance routes of immune information to the brain suggests that regardless the route used to convey immune information to the brain, this information may also be transmitted to the master circadian clock in order to inform it about the immunological state of the body. The outcome of this communication, we think, will allow the SCN to integrate the immune information in order to organize diurnal rhythms in the inflammatory response and sickness related behaviors via different outputs.

SCN outputs to the immune system

As with every organ in our body, the SCN may communicate its rhythmic signals to immune organs and their clock genes via hormones and by both branches (sympathetic and parasympathetic) of the autonomic nervous system (Kalsbeek et al., 2006a) in order to regulate physiology. Whether the communication between the master circadian clock and the immune organs is able to regulate the immune response is one of the main questions that have been investigated in the present thesis.

The expression of clock genes has been documented in immune organs and cells (Arjona and Sarkar, 2005; Mazzoccoli et al., 2011; Silver et al., 2012a). Genetic manipulations of circadian clock genes have evidence their role in the control of immunity mainly in the innate immune response (Gibbs et al., 2012; Liu et al., 2006; Narasimamurthy et al., 2012). In addition humoral (corticosterone and melatonin) and autonomic outputs (sympathetic and parasympathetic) of the SCN also seem to contribute to the regulation of body defenses (Gibbs et al., 2014; Schernhammer et al., 2001; Tracey, 2002). In chapter 5 we will study the relevance of the autonomic output of the SCN to the liver for the regulation of the inflammatory response.

It has been proposed that in order to regulate the timing of physiological processes, besides being informed about the external light-dark cycles, the SCN also needs to be informed about the state of body organs (Buijs et al., 2006), so that it can adapt its output

in order to adjust physiology. The changes in neuronal activity of the SCN in response to an immune challenge strongly support an active role of the SCN in the control of the immune response. Since SCN lesions (SCNxx) remove all rhythmicity in the animal both behavioral and physiological SCN (Eastman et al., 1984) we used SCNxx as a tool to investigate the involvement of the SCN in the immune response (Chapter 2). The electrolytic ablation of the SCN (SCNxx), demonstrated increased production of pro-inflammatory cytokines in response to LPS stimulation (Chapter 2), which suggest an important inhibitory role of the SCN in the inflammatory response.

The role of the brain in the organization of the immune response

The observation that many areas of the brain including the SCN are activated after an immune challenge suggests that these areas in one way or another are implicated in the response of the brain to such a challenge. Indeed it is known that the brain drives many of the processes that occur after an immune challenge, such as the "sickness behavior", a series of physiological an behavioral changes (depression, weakness, lethargy, loss of appetite, etc) considered as an initial host defense response during the course of inflammation, infection or injury. This gives the brain an important function in the regulation of body defenses against bacterial invasion. One of these brain driven defenses is a change in thermoregulation after an immune insult. A fever response (elevation of body temperature) helps the immune system to fight infections by increasing activity of white blood cells and inhibiting growth of many microorganisms. Fever is controlled by thermoregulatory nuclei inside the central nervous system (CNS), the medial preoptic nucleus (MnPO) is one of the main thermoregulatory centers which is located in the rostral part of the hypothalamus. Interestingly, the MnPO receives an important input from the SCN and is also implicated in the circadian control of body temperature (Guzman- Ruiz M *et al*, in progress). In rodents an endogenous circadian rhythm in body temperature independent of locomotor activity is characterized by high temperature levels during the dark phase, while low temperature levels are observed during the light phase. Circadian rhythm of SCN-neuronal activity is in anti-phase with circadian rhythm of body temperature, suggesting that SCN neuronal activity is regulating temperature under physiological conditions (Scheer et al., 2005). The role of the SCN in thermoregulation after an immune challenge has not been studied. It is well known that moderate doses of LPS induce a fever response (Rudaya et al., 2005), LPS has been extensively used for the study of fever response; nevertheless there are very few studies where day-night

differences in temperature response after an LPS challenge are evaluated. For example, *(Sugimoto et al., 1996)* studied this phenomenon however they fail to demonstrate day-night differences in temperature response after an LPS challenge. As part of the present thesis that investigates the involvement of the biological clock in the immune response we also have investigated this process (Chapter 2) and in contrast to Sugimoto´s findings, our results demonstrated that LPS administration during the early night induce a severe temperature response characterized by an intense decrease in body temperature (hypothermic response) that was not observed after early day LPS challenge (Guerrero-Vargas et al., 2014a). Thus we observed important changes to moderate doses of LPS depending on the time of the immune challenge and we aimed to investigate the possible mechanisms of these changes.

Circadian desynchronization and disease

Modern lifestyles and work schedules have changed e.g. our sleep, wake and food consumption patterns. In humans, activity and food intake during the dark phase is in contradiction with the signals that are send by the master circadian clock that are aimed to synchronize the activity and physiology of the individual to the light dark cycle. When we neglect the signals of the SCN and are active and eat at the moment the SCN signals for rest and fasting, the result is that the feedback the SCN receives from the periphery is in desynchrony with the signals it sends and do not coincide with its own rhythm. This phenomenon is known as internal desynchronization. Another common cause of desynchronization is the one experience by trans-meridional workers or travelers who rapidly change from one time zone to another. Hereby the SCN needs to be re-synchronized to the new time schedule; however this will take several days and generally these people suffer from the well-known jet *lag* syndrome. Recent data in human and animal literature suggest that circadian disruptions, mainly caused by the exposure to non-traditional work schedules or *jet lag*, may be important for the development of serious pathologies such as autoimmune disorders (Magrini et al., 2006) cancer (Hansen, 2001; Pukkala et al., 2002; Reynolds et al., 2002; Schernhammer et al., 2001; Schernhammer et al., 2003) reproductive disorders (Nurminen, 1998), obesity (Karlsson et al., 2001) diabetes (Karlsson et al., 2001) stroke (Karlsson et al., 2005) and cardiovascular diseases between others. The mechanisms underlying the effects of circadian desynchronization on the development on disease are not understood. Therefore in view of the important inhibitory role of the SCN on the immune response we

hypothesized that circadian disruption could affect the development of adequate immune responses and make individuals more prone to disease. Therefore in chapter 3 we have investigated the consequences of circadian desynchronization on the immune response and we also investigated the possible mechanisms behind this changes. Hereto we have used the "shift-work" model in rodents that was previously developed in our laboratory (Salgado-Delgado et al., 2008) to study these relationships. This study demonstrates that shift work increased pro-inflammatory cytokines production after an LPS challenge, while importantly tumor development in these animals was also accelerated, suggesting that the observed increased immune responses to LPS could be a signal of a pro-inflammatory environment, which is a well-known carcinogenic promoter (Grivennikov and Karin, 2011). In order to investigate whether food intake during the normal rest phase (a common practice of modern society and shift-workers) could also affect the immune response, we measured cytokines production after LPS administration, as well as the function of a specific immune cell type, in a group of rats that had restricted food access to the rest period. This study demonstrates that mistime food consumption increased the inflammatory response to LPS (chapter 4).

Altogether these observations indicate the important role of the brain and the SCN in the organization of the immune response, which will be discussed in more detail in chapter 5.

SCOPE OF THE THESIS

The aim of the present thesis was to investigate:

- The existence of a daily rhythm in the inflammatory response to LPS (cytokines production and temperature response).
- The capacity of SCN to respond to an LPS challenge (evaluation of c-Fos expression).
- The contribution of the SCN in the regulation of the inflammatory response to LPS (SCN lesion studies).

After establishing the existence of a daily rhythm in the inflammatory response to LPS and the role of the SCN in the control of this response, we focused on the effects of circadian desynchronization process on the inflammatory response and disease, as well as in the possible mechanism behind the SCN control of the inflammatory response. The aim of the follow up studies was to investigate:

- Whether circadian desynchronization, using a rat model of "shift-work", affect the daily response of the immune system and increased the sensitivity of the body and cells to an immune challenge.
- Whether shift-work rats are more prone to develop tumors after tumor cells inoculation in order to relate the inflammatory response with cancer development.
- Whether altered food schedules, commonly observed in shift-workers, could also affect the inflammatory response to LPS.
- The possible pathways of immune-SCN communication.
- Possible outputs from the SCN to the immune system to regulate the immune response.

CHAPTER 2

Reciprocal interaction between the Suprachiasmatic nucleus and the immune system tunes down the inflammatory response to lipopolysaccharide

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Abstract

Several studies have shown circadian variations in the response of the immune system suggesting a role of the Suprachiasmatic nucleus (SCN). Here we show that Lipopolysaccharide (LPS) administration in the beginning of the active period induced more severe responses in temperature and cytokines than LPS given in the rest period. Moreover night administered LPS increased SCN basal neuronal activity indicating a direct influence of inflammation on the SCN. Bilateral lesions of the SCN resulted in an increased inflammatory response to LPS demonstrating that an interaction between the SCN and the immune system modulates the intensity of the inflammatory response.

Introduction

In mammals, the Suprachiasmatic nucleus (SCN) organizes circadian rhythms and coordinates the efficiency of physiological processes according to the light-dark cycle. The correct timing of essential functions such as hormone secretion, behavior, temperature changes and autonomic outflow to body organs is set by the SCN (Buijs et al., 2013) and circadian rhythmicity of the immune system is not an exception (Arjona et al., 2004; Bourin et al., 1993; Pelegri et al., 2003; Petrovsky and Harrison, 1997; Suzuki et al., 1997; Takane et al., 2002; Young et al., 1995). Already before the discovery of the SCN, pioneer studies in this field demonstrated a day-night rhythm in the susceptibility to

lethal doses of lipopolysaccharide (LPS) a component of gram-negative bacteria (Halberg et al., 1960), and this response was later correlated with an overproduction of proinflammatory cytokines in rodents (Marpegan et al., 2009). Clinical studies have shown a correlation between time of the day and illness manifestation, for example, allergic rhinitis episodes are worse at night and early in the morning (Smolensky et al., 2007). The interaction between the immune system and the circadian system is illustrated by studies where the administration of LPS alters the expression of several circadian clock genes in the SCN and in other tissues (Okada et al., 2008; Takahashi et al., 2001; Yamamura et al., 2010). Likewise turpentine administration was shown to change clock gene expression in the liver in a time dependent manner (Kalsbeek et al., 2006b). That inflammation may affect directly the SCN is suggested by experiments showing that LPS administration during the subjective night induces phase delays (Marpegan et al., 2005). In agreement with this observation recently a role of TNF-α in the circadian response to LPS was demonstrated (Paladino et al., 2014).

Further evidence implicating the circadian system in the control of inflammation is provided in experiments whereby circadian disruption reduced the survival rate after sub-lethal doses of LPS (Castanon-Cervantes et al., 2010). In addition human and animal studies suggest that circadian disruption may be important for the development of serious pathologies such as cancer (Filipski et al., 2004; Hansen, 2001; Logan et al., 2012; Manikkam et al., 2012; Schernhammer et al., 2003; Sephton and Spiegel, 2003).

In spite of all this evidence, the contribution of the SCN in the response to non-lethal concentrations of circulating endotoxins is not documented. Consequently in the present study we examined the involvement of the SCN in the inflammatory response using moderate doses of LPS. At two time points, one coinciding with the beginning of the rest phase (Zeitgeber Time 2, ZT2) and the other coinciding with the beginning of the active phase (ZT14), the production of TNF-α, IL-6 and body temperature response to LPS was investigated. The influence of the immune response on the SCN was examined by analysis of SCN neuronal activity by means of c-Fos immunohistochemistry after LPS administration. With bilateral lesions of the SCN we explored the dependence of the inflammatory response to SCN modulation. Present results confirm a strong circadian regulation in the immune response whereby the SCN exerts an inhibitory influence over inflammatory processes.

Methods

Animals and general housing conditions

Adult male Wistar rats weighing 280 to 300g at the beginning of the experiments were obtained from the animal facility of the Faculty of Medicine of the UNAM. Animals were housed in individual cages and maintained in a soundproof monitoring room with a 12:12 h light-dark cycle (LD), lights-on at 7:00, defined as Zeitgeber time 0 (ZT0) and lights off at 19:00 defined as Zeitgeber time 12 (ZT12). The room was maintained at a regulated temperature of ranging between 22-25 °C and with continuous air flow. All rats were housed under specific pathogen free conditions and given free access to water and food (Rodent Laboratory Chow 5001, Purina, Minnetanka, MN, USA). Experimental procedures used in this study were in strict accordance with the Mexican norms for animal handling Norma Oficial Mexicana NOM-062-ZOO-1999, which conforms to international guidelines for animal handling, and were approved by the Ethics Committee in the Instituto de Investigaciones Biomédicas UNAM. All efforts were made to minimize the number of animals and their suffering.

Experimental Design

For this study, a total of 117 Wistar rats were used.

Experiment 1: *effect of LPS administration time on: A) proinflammatory cytokines production, B) Temperature response and C) SCN neuronal activity.*

Rats were cannulated in the external jugular vein and assigned to one of two conditions, administration at ZT2 or administration at ZT14 (n= 6/group). These administration time-points were chosen in order to have a day and a night sample. Each rat received on day 1 an administration of iv vehicle (0.9% sterile pyrogen-free saline; 1 ml/kg) and on day 3 administration of iv LPS (100mg/kg) at the assigned time point (ZT2 or ZT14). After each administration blood samples were collected from the jugular cannula at 0, 40 and 80 minutes post LPS or vehicle. From the obtained serum IL-6 and TNF-α were determined.

Another series of animals were cannulated in the external jugular vein and were implanted with intra-abdominal temperature sensors. Rats assigned to one of the two groups (ZT2 or ZT14). Rats (n= 6 /group) received iv, vehicle (on day 1) and LPS (100

μg/kg; on day 3) at the same time point. Following both vehicle and LPS, body temperature was continuously monitored in 5 min intervals for 24h.

Other animals were cannulated in the external jugular vein and were randomly assigned to one of four groups, LPS ZT2, LPS ZT14, vehicle ZT2 or vehicle ZT14. Rats (*n*= 4-6/group) received iv, vehicle (300 mg of 0.9% sterile pyrogen-free saline) or LPS (100 μg /kg) and were perfused 90 minutes later in order to obtain the brains for immunohistochemistry analysis to determine c-Fos expression in the SCN.

Experiment 2: effect of a low dose of LPS on A) proinflammatory cytokines production and B) Temperature response in intact and SCN lesioned animals.

Since in a pilot experiments, the dose of 100 μg /kg of LPS resulted in severe lethal of nearly lethal responses in SCN lesioned animals (SCNxx), we needed to lower the dose of LPS to study the effect of LPS in SCNxx animals. Therefore in an additional control group of intact animals we used a dose of 1.6 μg of LPS and confirmed first the diurnal variations in cytokines production after the administration of this very low dose of LPS.

Intact rats were cannulated in the external jugular vein in order to inject iv LPS in LD conditions at either ZT2 or ZT14. Rats (*n*= 5/group) were injected with 1.6 μg/kg of LPS. After iv, LPS administration blood samples were collected from the jugular cannula at 0, 40, 80 minutes post LPS.

Rats bearing bilateral SCN lesions (*n*= 5/group) and Sham surgery animals (*n*= 5/group) were cannulated in the external jugular vein and challenged with iv vehicle (300ml) and 2 days later with iv LPS (1.6 μg/kg) at ZT2, after administration blood samples were collected from the jugular cannula at 0, 40 and 80 minutes post LPS or vehicle. From the obtained serum IL-6 and TNF-α were determined.

Another group of rats bearing bilateral SCN lesions (*n*=5) and Sham surgery rats (*n*=6) received intra-abdominal temperature sensor and were cannulated in the external jugular vein in order to be challenged with iv administration of LPS (1.6 μg/kg). Following LPS administration, body temperature was continuously monitored in 5 min interval for 24h. At the end of experiments A and B rats were perfused to obtain their brains for confirming the SCN lesion with Vasoactive Intestinal Peptide staining (VIP) and to recover the temperature sensors.

Surgical procedures

All surgeries were performed using aseptic procedures and under deep anesthesia using a combination of ketamine (90 mg/kg Cheminova, México) and xylazine (10 mg/kg, Procin^{R}, Pisa, México) anesthesia.

Intra-jugular cannula insertion

A polyethylene silicon tube cannula (0.025 in. i.d. and 0.047 in. o.d.; Silastic Laboratory tubing; Dow Corning Corp., Midland, MI, USA) was inserted in the internal jugular vein. The cannula was filled with heparin (500 U/ml) as anti-coagulant. The outer end of the cannula was fixed in the back between both shoulder blades and plugged with a small nail. Rats were allowed to recover during 7 days before experiments.

Intra-abdominal temperature sensors

A small incision was made in the abdominal cavity and a sterilized iButton (iButton Maxim integrated^{TM} San Jose, CA, U.S.A.) was introduced in the rat peritoneum. Anterior abdominal muscles were sutured with black braided silk (Tramat^{TM}, México). Rats were left to recover for 7 days. The iButtons were programmed to collect core temperature data every 5 minutes for all experiments. Temperature data were collapsed into 15 minutes intervals and the means were calculated for each group. To determine changes in body temperature (T_b) the mean for a particular 15 minutes period on the day of LPS injection was subtracted from the mean of the hour prior to treatment.

Stereotactic surgeries

In order to perform SCN lesions, animals were placed in a stereotactic frame (tooth bar $\pm$ 2.5 mm). Electrodes (0.2 mm diameter) were placed bilaterally in the SCN (arm angle of 4°; coordinates: -0.2 mm from bregma; $\pm$ 0.9 mm lateral from midline; 8.2 to 8.6 mm below brain surface depending on weight variation) and to each side current of 0.35 mA was transmitted for 45 seconds (Grass D.C. Constant Current Lesion Maker). Sham surgery animals were performed in the same way with the insertion of two electrodes without current. After surgical procedures rats were allowed to recover for at least 4 weeks before starting monitoring of general activity and body temperature to verify arrhythmicity in LD conditions. Only rats that showed arrhythmic activity together with a loss of temperature rhythm were included in the SCN lesion group (SCNxx). At the end of experiments, animals were sacrificed and the lesion of the SCN was confirmed by

staining Vasoactive Intestinal Peptide (VIP), since earlier studies have indicated that small remnants of the SCN can be easy detected by VIP staining (*see* (Hosoi et al., 2005). Animals that showed VIP cells were also discarded from the analysis (n= 10); in this way only complete SCN lesion animals that showed an arrithymic pattern in behavior or temperature irrespective of the time of testing were selected.

Treatment Solutions and Reagents

For all experiments, inflammation was induced by a single intravenous (iv) injection of lyophilized LPS (100 mg, *Escherichia coli* serotype 0127:B8, Sigma-Aldrich, St. Louis, MO, USA Lot No. 051M4004) reconstituted with 0.9% sterile pyrogen-free saline (Baxter, México) to derive a 1mg/ml stock solution that was aliquoted and stored at -20°C. For the administration, aliquots were thawed and diluted with 0.9% sterile pyrogen-free saline yielding a final concentration of 100mg/kg or 1.6mg/kg.

Rats in the control groups were injected with the vehicle (0.9% sterile pyrogen-free saline; 1 ml/kg) at the same time-points.

Sample collection and ELISA

Blood samples (300ml) were collected in Microvette®/500 tubes (Sarsted, Nümbrecht Germany) before LPS or vehicle infusion (0 min) and post-infusion times 40, and 80 minutes. Samples were centrifuged at 7000 rpm, during 7 minutes. Plasma was stored in 60ml aliquots at -80 °C until assay. IL-6 and TNF-α were determined by ELISA according to the manufacturer's recommendations (ThermoFisher Scientific, Rockford, USA, #ER3IL65 for IL-6 and # ER3TNFA5 for TNF-α). Detection limits of the assays in plasma were <16pg/mL for IL-6 and < 15pg/mL for TNF-α. Standard curves were used to determine cytokines levels. Cytokine levels are expressed as picogram /mL.

Immunohistochemistry

Rats were deeply anesthetized with an overdose of sodium pentobarbital (Pisabental, PiSa, Atilalaquia, Hgo, México; 65 mg/mL) and perfused transcardially with 150 mL 0.9% sterile pyrogen-free saline, followed by 150 mL 4% paraformaldehyde diluted in phosphate buffer (PBS, 0.1 M, pH 7.2).

Brains were removed, post fixed and kept in fixative for 24h, at 4°C, and cryo-protected in 30% sucrose for 3 to 4 days. Brains were frozen and cut in coronal sections of 40mm

at –20°C. Sections were serially collected and processed for c-Fos immunohistochemistry. Free-floating SCN sections were incubated for 72 hours under constant shaking at 4° with rabbit c-Fos antibody (1:40000; Calbiochem, Cat. No. PC38. CA, U.S.A.). Subsequently, sections were rinsed and incubated (1 hour), at room temperature with biotinylated donkey-antirabbit serum (1:200, Jackson, ImmunoResearch Laboratories inc., Baltimore, U.S.A.) rinsed and incubated in avidin-biotin complex (1:500 Vector Laboratories) for 1 hour; product visualization was obtained with 0.01% diaminobenzidine, 0.05% nickel ammonium sulfate and 0.01% hydrogen peroxide for 6 minutes. SCN sections of bilateral lesion animals and Sham rats were stained for VIP (VIP; 1:2000, (Sorrells et al., 2009)) using the same immunohistochemical procedure described before, to examine the position and completeness of the SCN lesions. Brain sections were mounted, dehydrated, and cover-slipped with microscopy Entellan New (Merck, Whitehouse Station, NJ).

Cell Count

In order to quantify immunoreactivity of c-Fos (Fos-IR) in the SCN, three representative sections in the antero-posterior axis were selected in accordance with the stereotaxic atlas (Paxinos G, 1998) (0.92, 1.20, and 1.40 anterior/posterior level to bregma) as is indicated in the stereotactic atlas. Images were examined under a light microscope and captured with a 20x ocular. Immunoreactive-positive nuclei in the SCN were counted bilaterally using a computerized image analysis system (Image J, 1.42q, National Institutes of Health Bethesda, MD) by determining the SCN area by free hand for each side of the SCN. The background was subtracted and threshold was determined, particle analysis was set for particles of 1.0-2.0 circularity and 500-800 pixels. Background optic density was established in a nearby region lacking Fos-IR.

Data analysis

All data are presented as mean ± standard error of the mean (s.e.m). IL-6 and TNF-α plasma levels after LPS or vehicle administration at ZT2 or ZT14 were compared with a two-way (ZT X time after LPS) ANOVA for repeated measures for time after LPS administration followed by Bonferroni´s post-hoc test for multiple comparisons. IL-6 and TNF-α plasma levels after LPS in Sham and SCNxx animals were compared with a two-way (time x condition) ANOVA for time after LPS administration as repeated measure followed by Bonferroni´s post-hoc test for multiple comparisons. Temperature

data were analyzed with a two way (treatment x time) ANOVA for repeated measures by two factors (treatment and time) followed by Bonferroni´s post-hoc test for multiple comparisons. Fos-IR in the SCN was analyzed with a two way (ZT x treatment) ANOVA, followed by Bonferroni´s port-hoc test for multiple comparisons. Statistical comparisons were performed using Graphpad Prism version 6 for Mac. Threshold for statistical significance was set at a= 0.05.

Results

Proinflammatory cytokine release depends on the time of LPS administration

In order to study temporal differences in the production of IL-6 and TNF-α as a response to an immune challenge; vehicle or LPS was administrated at ZT2 or ZT14. The two way ANOVA indicated a significant interaction between time of administration (ZT) and time after LPS administration ($F_{[2, 14]}$ =11.33; P= 0.0012). As shown in Figure 1A, LPS administration at ZT14 triggered significantly higher plasma levels of IL-6 80 minutes after the LPS challenge as compared to the ZT2 challenge (P < 0.001). No effects where found for vehicle administration at ZT2 and ZT14 ($F_{[1, 16]}$ = 0.01422; P= 0.909*) (Data not shown).*

For the response of TNF-α the two way ANOVA indicated a significant interaction between the ZT groups and time after LPS administration ($F_{[2, 16]}$= 6.131; P= 0.010). Forty minutes after LPS administration a significant rise of TNF-α in plasma levels was observed at ZT2 and ZT14. However after 80 minutes of LPS administration at ZT14 a significant increase of TNF-α plasma levels was observed as compared to the ZT2 group (Figure 1B, P < 0.001). No effects on TNF-α plasma levels where found after vehicle administration at ZT2 and ZT14 ($F_{[1, 8]}$ = 0.00; P= 0.99) *(Data not shown).*

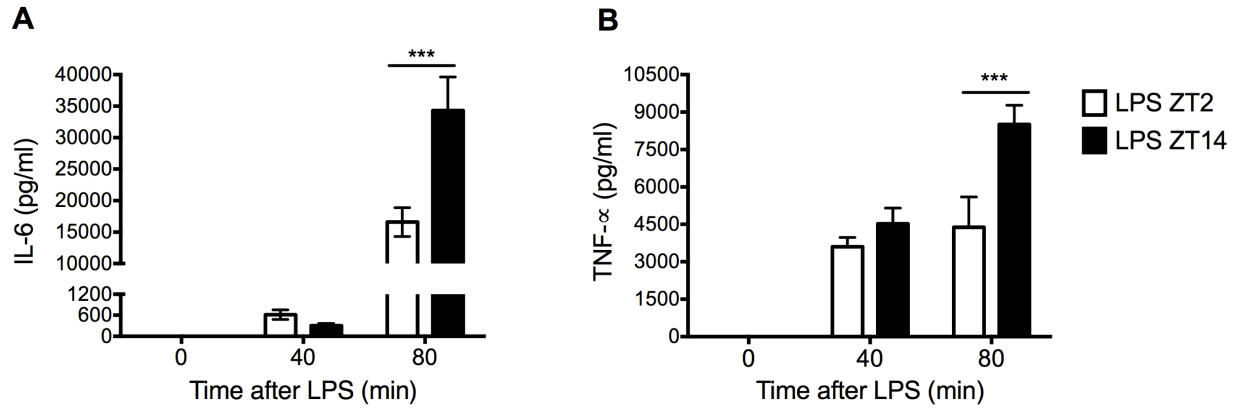


Figure 1. LPS administration at ZT14 triggered higher IL-6 (panel A) and TNF-α (panel B) plasma levels as compared to ZT2**.** IL-6 and TNF-α plasma concentrations were measured before and after iv, administration of LPS (100 μg/kg) at ZT2 or ZT14. Values are expressed as means ± s.e.m. (*n= 4-6*/group). ***P < 0.001 significance of differences vs. LPS ZT2.

Thermoregulatory response to LPS varies according to the administration time

Analysis of body temperature indicated that rats given LPS at ZT2 exhibit a hyperthermic response that was significantly different from vehicle administration (P< 0.001). 6 hours after LPS treatment, temperature levels increased up to 0.8 °C above basal values (Figure 2A). The two way ANOVA indicated a significant effect for the interaction of treatment and time after treatment administration, both as factors of repeated measures ($F_{[41, 164]}$ =2.21; P= 0.0002).

LPS administration at ZT14 triggered 2 hours after the treatment a pronounced decrease in core temperature (hypothermic response) that was significantly different from vehicle administration, reaching minimum decrease of 1.32 °C below control values 3 hr after the LPS treatment (P<0.001; Figure 2B). The two way ANOVA indicated a significant effect for the interaction of treatment and time after treatment administration, both as factors of repeated measures ($F_{[41, 205]}$ = 3.194; P< 0.0001). Vehicle administration at ZT14 triggers a significant (P<0.01) increase in core body temperature almost 2 hours after. This could be seen as an effect of manipulation during the rat active phase associated with increase in activity.

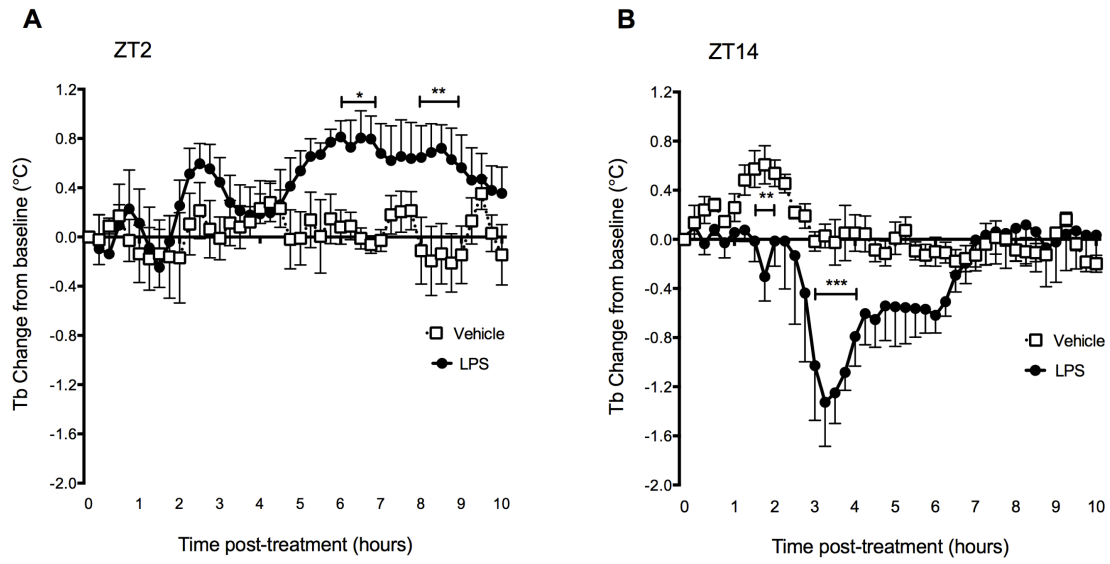


Figure 2. LPS administration early in the rest phase ZT2 (panel A) induced hyperthermia, in contrast, LPS administration early in the active phase ZT14 (panel B) induced hypothermia. All values are expressed as means ± s.e.m. (*n*= 6/group). Results are shown as change from pre-administration temperature values to determine changes in body temperature (T_b) the mean for a particular 15 minutes period on the day of LPS injection was subtracted from the mean of the hour prior to treatment. * P < 0.05; ** P < 0.01; *** P < 0.001, significance of differences vs vehicle group.

LPS challenge activates the SCN

The daily variations in TNF-α, IL-6 and temperature in response to LPS administration, led us to postulate a direct influence of the master circadian clock (SCN) on the inflammatory response. We therefore investigated whether SCN neuronal activity as demonstrated by c-Fos staining was changed after LPS administration. Analysis of c-Fos expression in the SCN was performed in rats exposed to vehicle or LPS at ZT2 or ZT14. No significant changes in c-Fos expression in the SCN were observed between LPS or vehicle injected rats at ZT2 (P =0.05; Figure 3). In contrast, LPS administration at ZT14, which is a time of low c-Fos expression in the SCN, triggered a significant increase of c-Fos expression as compared to vehicle administration at the same time point (P<0.05). The two way ANOVA indicated a significant effect for administration time ($F_{[1, 16]}$ =31.6; P< 0.0001) and treatment among groups ($F_{[1, 16]}$ = 6.510; P= 0.0212) but no significant effect due to their interaction.

LPS administration also induced a clear expression of c-Fos in the paraventricular nucleus (PVN) as compared to vehicle (Supplementary Figure 3). No differences in c-Fos expression were observed between LPS ZT2 and LPS ZT14 injected rats

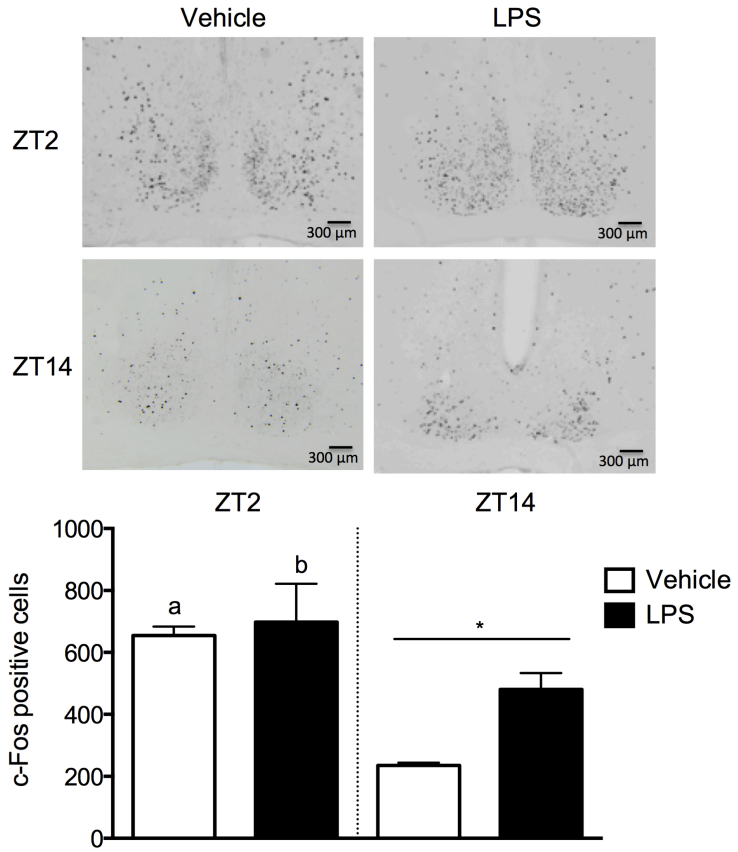


Figure 3. Nocturnal LPS challenge induces activation of SCN neurons. SCN photomicrographs after treatments (panel A) show that during the day the SCN shows high expression of c-Fos protein while at night only the LPS injected animals show an increase in c-Fos especially in the ventrolateral part. Scale bar = 300 µm. The number of c-Fos positive cells in the SCN was counted bilaterally in 3 representative sections (panel B). All values are expressed as means ± s.e.m (*n= 4*-6/group). * P < 0.05 significance of differences LPS against vehicle. aP < 0.001 vehicle ZT2 against vehicle ZT14. bP < 0.05 LPS ZT2 against LPS ZT14.

SCN lesions augment fever response and the production of TNF-α and IL-6 after LPS administration

The day/night difference in normal SCN neuronal activity (active during the day vs inactive during the night) and the day/night difference in cytokines production and thermoregulatory response induced by LPS administration, led us to the hypothesis that SCN neuronal activity, may influence the intensity of proinflammatory cytokines production. Therefore the effect of bilateral lesions of the SCN (SCNxx) on the production of TNF-α, IL-6 and on the temperature response after LPS administration at

ZT2 was investigated. Experiments in SCNxx animals were done at one time point since the ablation of the master circadian clock induces a loss of rhythmicity in all measured physiological variable e.g. temperature and locomotor activity (Eastman et al., 1984) and induced responses in SCNxx animals are the same in the light as well as in the dark period (Angeles-Castellanos et al., 2010; la Fleur et al., 2001; Terazono et al., 2003).

Animals to be included in the SCNxx group were chosen by observing the loss of rhythmicity in the temporal distribution of activity (Supplementary Figure 1A) and the observation of the loss of daily body temperature patterns (Supplementary Figure 1B*)*. The complete lesion was further verified after the experiment with immunohistochemistry staining for VIP (Supplementary Figure 1C). Only rats complying with the 3 criteria were included in the analysis.

An LPS challenge (100 μg/kg) to SCNxx animals proved lethal or nearly lethal as illustrated by the strong decrease in temperature (Supplementary Figure 2). Therefore in order to allow the detection of the hypothesized increased inflammatory response in SCNxx animals we chose a low dose of LPS (1.6 μg/kg). We first analyzed diurnal variation in cytokines production between ZT2 and ZT14 after this low LPS challenge in intact animals (Figure 4A). LPS administered at ZT14 triggered significantly higher IL-6 plasma levels after 40 ($P < 0.05$) and 80 minutes after the challenge ($P < 0.01$) as compared to LPS administration at ZT2. The two way ANOVA indicated a significant effect for the interaction of ZT administration with the time after LPS ($F_{[2,16]} = 34.11$, $P < .0001$). With this low dose TNF-α plasma levels reached highest levels 40 minutes after LPS in both groups, however at ZT14 the response was significantly higher as compared with LPS administration at ZT2 (Figure 4B; $P < 0.01$). The two way ANOVA indicated a significant effect for administration time (ZT) ($F_{[1,8]} = 5.470$, $P = 0.04$). After the confirmation that cytokines production after both high (100 μg/kg) and low (1.6 μg/kg) doses of LPS is under circadian regulation, we decided to use 1.6 μg/kg of LPS to evaluate the role of the SCN in the inflammatory response.

SCNxx rats given LPS exhibit an initial increase in temperature for 1h followed by a second phase of hyperthermia that was significantly different from Sham rats. Main effects of LPS were observed 7 hrs after treatment, reaching 1.06°C above their basal temperature values (Figure 5A). The two way ANOVA indicated a significant effect for the interaction of groups and time after LPS administration ($F_{[41,410]} = 1.820$; $P=0.002$).

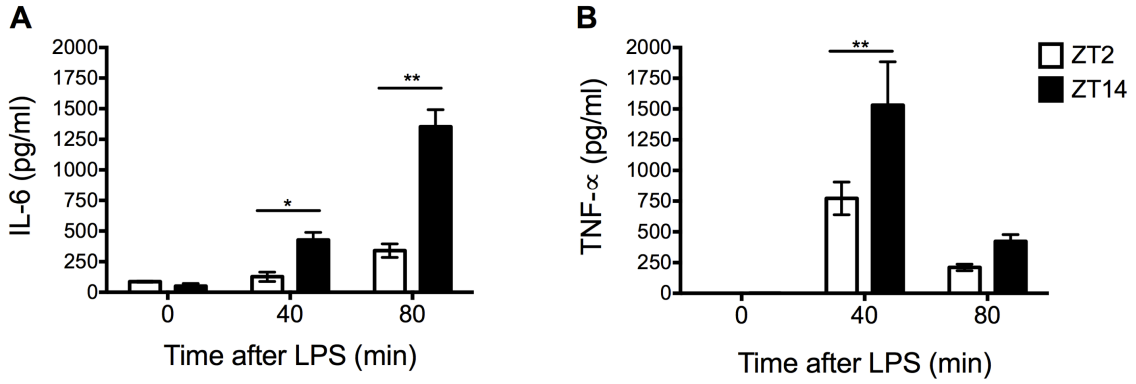
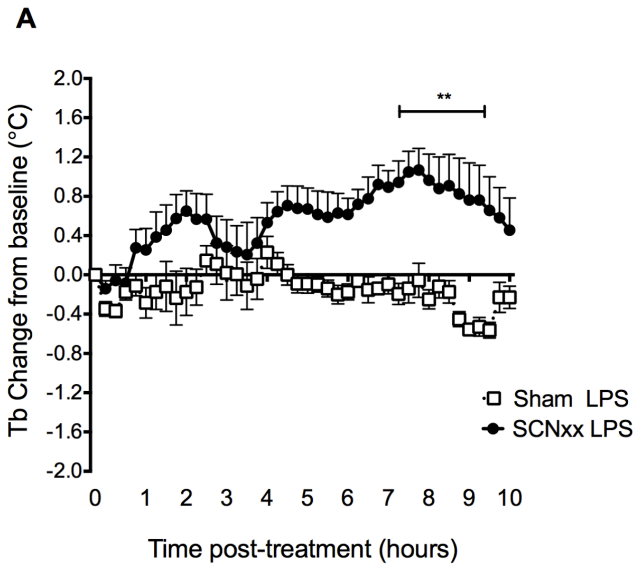
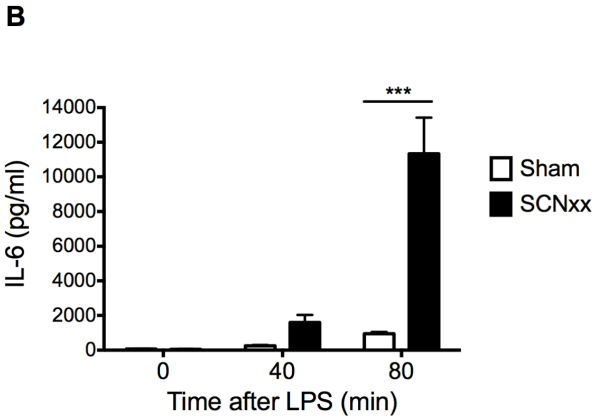


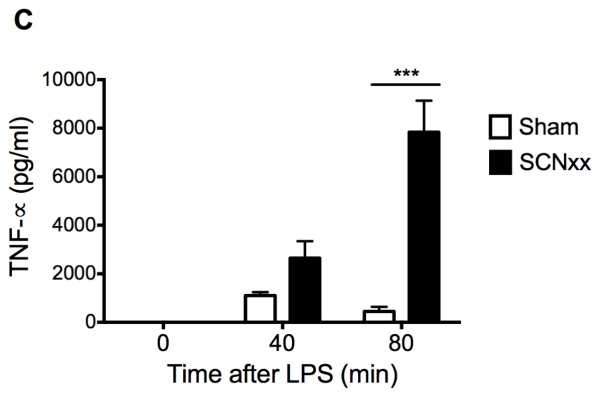
Figure 4. Administration of 1.6 μg/kg of LPS to intact rats at ZT14 triggered higher IL-6 (panel A) and TNF-α (panel B) plasma levels as compared to ZT2 LPS administration**.** IL-6 and TNF-α plasma concentrations were measured before and after iv, administration of LPS at ZT2 or ZT14. Values are expressed as means ± s.e.m. (*n*= 5/group) . *P < 0.05; **P< 0.01 significance of differences vs. ZT2.

LPS administration to SCNxx rats resulted in 10 fold higher IL-6 (Figure 5B; *P*< 0.001) and TNF-α plasma concentration in comparison to Sham animals 80 minutes after the LPS challenge (Figure 5C; P< 0.001). The two way ANOVA indicated a significant interaction of the condition (SCNxx or Sham) and time after LPS administration among groups for IL-6 production *($F_{[2,16]}$* = 18.32; *P*< 0.0001) and TNF-α *($F_{[2,18]}$* = 25.54; P< 0.0001). No significant differences where found for vehicle administration between Sham and SCNxx animals neither in IL-6 ($F_{[2, 16]}$ = 1.44; P= 0.264*)* nor in TNF-α plasma levels ($F_{[2, 16]}$ = 0.0; P= 0.999*) (Data not shown)*.

Figure 5. SCN lesion amplifies the temperature and cytokine response. Temperature response after iv, administration of LPS (1.6 μg/kg) at ZT2 to SCNxx and Sham surgery rats (panel A). All values are expressed as means ± s.e.m. (*n*= 6/group). Results are shown as change from pre-administration temperature values to determine changes in body temperature (T_b) the mean for a particular 15 minutes period on the day of LPS injection was subtracted from the mean of the hour prior to treatment. ** P < 0.01; *** P < 0.001, significance of differences vs Sham LPS. IL-6 (panel B) and TNF-α (panel C) plasma levels before and after LPS (1.6 μg/kg) administration to SCNxx and Sham surgery animals at ZT2. Values are expressed as means ± s.e.m. (*n*= 6/group) . *P < 0.05; ***P < 0.001 significance of differences vs. Sham


A
Tb Change from baseline (°C)
2.0
1.6
1.2
0.8
0.4
0.0
-0.4
-0.8
-1.2
-1.6
-2.0
**
Sham LPS
SCNxx LPS
0 1 2 3 4 5 6 7 8 9 10
Time post-treatment (hours)


B
IL-6 (pg/ml)
14000
12000
10000
8000
6000
4000
2000
0
***
Sham
SCNxx
0 40 80
Time after LPS (min)


C
TNF-∝ (pg/ml)
10000
8000
6000
4000
2000
0
***
Sham
SCNxx
0 40 80
Time after LPS (min)

Discussion

The present study demonstrates that the production of TNF-α, IL-6 and the temperature response after an inflammatory stimulus is strongly influenced by the SCN. The production of proinflammatory cytokines after LPS challenge is 2 times higher during the active phase as compared to the rest phase, while SCNxx results in at least 10 fold increased IL-6 and TNF-α plasma levels. Several explanations are possible for this effect: First, it is known that the output of the SCN sets a daily rhythm in hormones secretion as well as in autonomic output (Buijs and Kalsbeek, 2001) known to be important for the inflammatory response (Buijs et al., 2008; Kapcala et al., 1995; Logan et al., 2011; Schernhammer et al., 2001; Tracey, 2002). Thus the output of the SCN may set the sensitivity of the immune system to LPS, explaining why normal activity of the SCN may lead to a lower inflammatory response and lesioning of the SCN in enhanced responses. Second, the SCN synchronizes clock genes and other rhythmic processes in the periphery (Reppert and Weaver, 2002). It has been demonstrated that clock geens are expressed in immune cells such as macrophages (Hayashi et al., 2007; Keller et al., 2009; Silver et al., 2012a) lymphocytes (Du et al., 2005), mast cells (Baumann et al., 2013; Wang et al., 2011), NK cells (Arjona and Sarkar, 2005), eosinophils (Baumann et al., 2013), dendritic cells and B cells (Silver et al., 2012a) as well as in the spleen, lymph nodes (Grivennikov et al., 2010; Keller et al., 2009) and in the bone marrow (Chen et al., 2000). Manipulations of the circadian clock expression in immune cells have shown their role in immune functions (Arjona and Sarkar, 2006a, b; Gibbs et al., 2012; Liu et al., 2006; Logan et al., 2013; Oishi et al., 2006). Circadian expression of clock genes in the periphery is tightly controlled by humoral and autonomic outputs of the SCN and it has been proposed that in order to regulate the timing of physiology the SCN also need to be informed about the state of body organs (Buijs et al., 2006). Hence during the daytime the phase of the rhythm of the certain clock genes may determine a low response while at night the opposite phase of the clock genes may induce a high response (Gibbs et al., 2012). SCN lesion (SCNxx) may then result in a complete disorganization of central as well as peripheral clock genes rhythms (Akhtar et al., 2002), causing an enhanced response of the immune cells or other immune response elements, since these peripheral clock genes are involved in the response of the immune system.

The third option is a combination of these two possibilities giving the SCN an important role in actively suppressing the immune response via changing molecular rhythms in the

periphery as well as by direct changes in the autonomic output of the brain. For example it has been demonstrated that SCN ablation results in impaired circadian rhythms of circulating leukocytes and accelerated tumor growth (Filipski et al., 2002; Filipski et al., 2006).

The involvement of the SCN in the inflammatory response

Also previous studies suggested an involvement of the SCN in the immune response; an increase in mortality and cytokines production was reported after the administration of 20 mg/kg of LPS at the end of the rest phase (ZT11), while similar injections at the end of the active phase (ZT19) were less harmful (Halberg et al., 1960; Marpegan et al., 2009). In our study we investigated the direct involvement of the SCN in the regulation of the inflammatory response and therefore used non-lethal, and low doses of LPS (100 and 1.6 μg/kg). In addition we used the early light or early dark period in order not to examine the effects of LPS injection in the transition period between rest and activity. Herein our ZT14 time-point is more comparable to the ZT11 time-point used in previous studies because in fact at ZT11, although in the light phase, the animal is preparing for activity and thus its physiology is more comparable with the ZT14 time point than with the ZT19 (Buijs et al., 2013). Furthermore recent studies also used time points that are more comparable to the time points we have used and similarly observed higher responses of the immune system in the dark phase (Gibbs et al., 2012; Schernhammer et al., 2001).

Regarding the direct involvement of the SCN in the inflammatory response, it is knows that c-Fos expression levels in the SCN oscillate throughout the day-night cycle under basal conditions (Aronin et al., 1990). We also observed, higher c-Fos during the light phase and lower expression in the dark period. The neuronal activation of the SCN induced by LPS was especially evident during the dark phase and in the ventro-lateral SCN; an area that plays an important role in light reception and photo-transduction (Mikkelsen et al., 1995), suggesting that peripheral injection of LPS may mimic the reception of light to the SCN. During the light phase when the normal activity of the SCN is already high, no additional activation could be observed.

Lateral cerebral ventricle injection of cytokines mixture (TNF-α and IFN-γ) also induced higher c-Fos ventro-lateral SCN expression only after early night administration (Sadki et al., 2007); suggesting that LPS induced increased in TNF-α as shown in the present study may be responsible for the observed activation in the SCN. The present

observation may explain why earlier it was found that peripheral injected LPS could induce phase changes in the SCN or change the expression of clock genes in the SCN (Marpegan et al., 2005; Okada et al., 2008; Takahashi et al., 2001).

The involvement of the SCN in the temperature response

In the present study we observed a different temperature response pattern after an LPS challenge during the active phase as compared to the rest phase. The temperature response after 100 μg/kg LPS at ZT14 was characterized by a hypothermic response, which was not observed after the ZT2 challenge. It is well known that large doses of LPS induce hypothermia (Derijk and Berkenbosch, 1994; Derijk et al., 1994; Romanovsky et al., 1996), while moderate doses induce a hyperthermia (Almeida et al., 2006). Consequently hypothermia has been related to severe inflammation processes and is proposed to be a physiological response aimed to save energy and decrease blood pressure during severe infection (Romanovsky et al., 1996). Since the hypothermic response observed at ZT14 was measured after a non-lethal LPS challenge that only induced hyperthermia at ZT2; we suggest an increased sensitivity and/or increased response to LPS during the active phase in nocturnal rodents. The higher TNF-α and IL-6 plasma levels measured after the LPS challenge during the active phase confirmed this and might be –partially– responsible for the severe temperature response observed during this phase. Since the SCN influences the daily temperature changes (Scheer et al., 2005), the presence of the SCN in intact animals may also influence the temperature pattern after LPS. Interestingly the SCNxx animals that received the similar 100 μg/kg dose of LPS also developed, a delayed, hypothermia but reached lower temperature values than intact rats injected with the same dose of LPS at ZT14 (*Supplementary Figure 2*). SCNxx animals that received a lower dose of LPS reach higher temperature levels in comparison to Sham animals; an explanation for this response might be the LPS-induced elevated proinflammatory cytokine production in SCNxx, as they are known to initiate fever pathways. In this sense, IL-6 has been recognized as an important fever mediator that could activate COX-2 pathway in cerebral microvasculature (Rummel et al., 2006).

SCN activity is associated with a lower inflammatory response.

The present study demonstrates that the high neuronal activity of the SCN observed during the day coincides with a diminished inflammatory response while the low night activity of the SCN at ZT14 is associated with an increased inflammatory response.

Together with the observations that an LPS injection increases nighttime –low– neuronal activity and that SCN ablation increased the inflammatory response to LPS suggests that SCN activity is associated with a diminished inflammatory response.

Consequently the question arises how the SCN might be activated by a peripheral LPS injection and how such SCN activation may lead to a diminished inflammatory response.

It is well known that a systemic inflammatory stimulus like LPS activates several different areas of the brain (Buijs et al., 2008; Elmquist et al., 1996; Goehler et al., 2006; Konsman et al., 1999; Marpegan et al., 2005). Since LPS passes minimally the murine Blood Brain Barrier (BBB) (Banks and Robinson, 2010) one entrance for LPS might be the Circumventricular Organs (CVO´s) where LPS may trigger neuronal activation through its interaction with its specific receptor TLR4 (Elmquist et al., 1996; Goehler et al., 2006; Laflamme and Rivest, 2001). Neuronal projections of the CVO's not only reach numerous areas in the hypothalamus and brain stem but also may reach the SCN providing a neuronal pathway by which LPS can modulate SCN activity (Gross P.M., 1990; Gross et al., 1990; Lind et al., 1982). An additional neuronal entry would be through the activation of vagal afferents by circulating cytokines, this signal would be relayed through the Nucleus of the Tractus Solitarius (Clements-Jewery et al.) (Maier et al., 1998) onwards to the hypothalamus and the SCN (Buijs et al., 2014; Ter Horst et al., 1989). Consequently, SCN neurons activated by LPS may stimulate, or inhibit important pre-autonomic centers in the hypothalamus (Buijs and Kalsbeek, 2001) and may not only modify the temperature but also the cytokine response.

The observation of c-Fos expression in the PVN after LPS administration (*Supplementary Figure 3*) especially in the medial parvocellular part of the nucleus, where the CRH are located, agrees with previous observations (Belevych et al., 2010)

Next, SCN neurons activated by LPS may stimulate important pre-autonomic centers in the hypothalamus (Buijs and Kalsbeek, 2001) and may not only modify the temperature but also the cytokine response. The above is corroborated with studies showing that the autonomic output of the brain importantly affects the immune response (Borovikova et al., 2000; Buijs et al., 2008; Martelli et al., 2014; Schernhammer et al., 2001; Watkins et al., 1995).

Two other studies have examined the effect of LPS on inflammatory responses in SCNxx animals; (Wachulec et al., 1997) observed, as in the present study, that SCNxx animals

initially develop a relatively small fever response after non-lethal doses (100 mg/kg, i.p.) of LPS, similar to ZT2 injected intact animals but did not follow the temperature of the animals long enough to observe the dramatic temperature changes as observed in the present study (Supplementary *Figure 2*), that prompted us to decrease the LPS dose in order to allow for detection of differences in temperature response between Sham and SCNxx animals.

Conclusions

Previous data on the importance of the rhythm of clock genes together with the present data on the importance of the SCN for the regulation of the inflammatory response show that the SCN as the master clock has several different possibilities to modify and influence the response to an inflammatory challenge. The present results highlight the role of the SCN; however one needs to consider that also many other brain regions, such as the amygdala, are also activated after the LPS challenge (Prager et al., 2013), and may also have the capacity to influence the immune response. The precise pathways and mechanisms via which the different brain structures and the SCN are incorporated in this immunological circuit still need further investigation. One of the major findings of the present study is the severity of the inflammatory response in SCNxx animals. Together with the observations that after forced phase shifting animals show a higher mortality after an LPS challenge (Castanon-Cervantes et al., 2010), suggests that many health problems associated with shift work (Evans and Davidson, 2013) might be due to increased inflammatory responses because of a desynchronized SCN. Future studies will need to clarify this.

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Natalí N. Guerrero-Vargas, Roberto Salgado-Delgado, María del Carmen Basualdo, Joselyn García and Mara Guzmán contributed to the acquisition of the data. Natalí N. Guerrero-Vargas, Ruud M. Buijs, Carolina Escobar and Julio C. Carrero, contributed to

the analysis and interpretation of the data. Ruud M. Buijs and Natalí N. Guerrero-Vargas initiated and designed the study. Natalí N. Guerrero-Vargas, Carolina Escobar Mara Guzmán performed temperature and statistical analyses.

Natalí N. Guerrero-Vargas and Ruud M. Buijs wrote the paper. All authors declare that there are no conflicts of interest.

Supplemental data

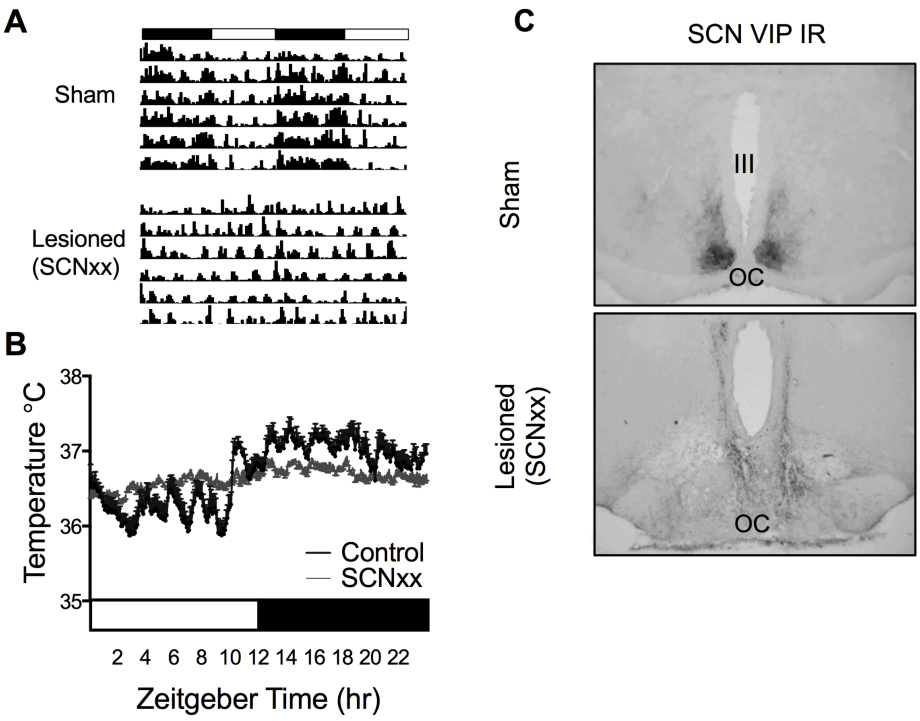

Figure S1.Verification for the completeness of the SCN bilateral lesion. Locomotor activity of the animals was examined at least three weeks after the lesion in order to determine arhythmicity in LD. In panel (A) actograms representing locomotor activity of Sham (top) and Lesioned (bottom) animals are shown. Core temperature in sham animals exhibit a robust daily pattern while SCNxx animals show an arhythmic pattern (panel B). SCN VIP staining for sham rats (top) evidences an intact SCN while in the SCN lesioned rats the absence of VIP staining confirmed a complete lesion (panel C). VIP (Vasoactive Intestinal Peptide), IR (Immunoreactivity), SCN (Suprachiasmatic Nucleus), SCNxx (Bilateral SCN lesion), OC (Optic Chiasma), III (Third ventricle).

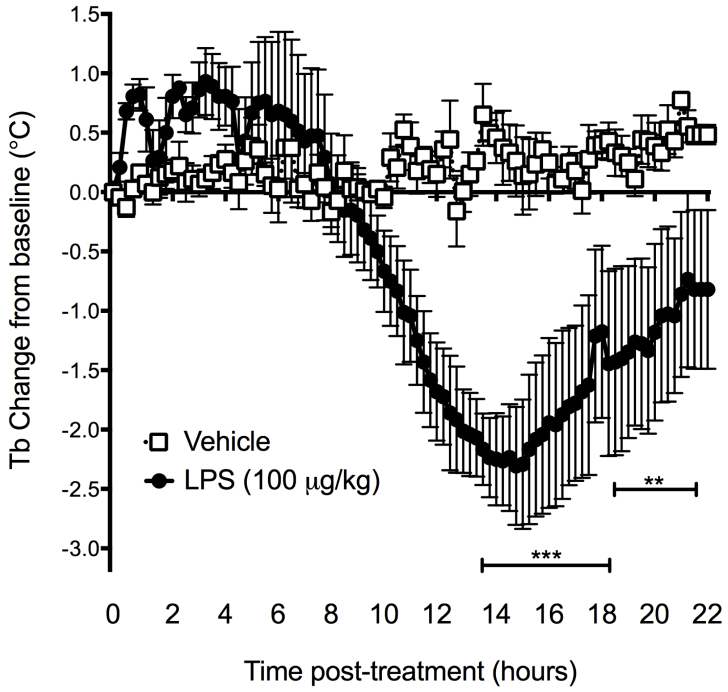

Figure S2. An LPS challenge of 100 mg/kg to SCN lesion rats (SCNxx) proved lethal of nearly lethal. From our first group of SCNxx rats (100 mg/kg, *n*=5), 2 animals died, not immediately after LPS injection but in the following 24 h indicating the severity of the response. Supporting this, are the temperature recordings of them and the surviving animals. The administration of 100 μg/kg of LPS to SCN lesion rats (SCNxx) induce an initial increase in temperature followed only after 10-18 hours by a strong decrease in temperature from which some animals did not recover. All values are expressed as means ± s.e.m. (*n*= 3/group). Results are shown as change from pre-administration temperature values to determine changes in body temperature (T_b) the mean for a particular 15 minutes period on the day of LPS injection was subtracted from the mean of the hour prior to treatment. ** $P < 0.01$; *** $P < 0.001$, significance of differences vs vehicle group. The two way ANOVA indicated a significant effect for the interaction of treatment and time after administration, both as repeated measures factor ($F_{90, 180} = 10.59$; $P < 0.0001$).

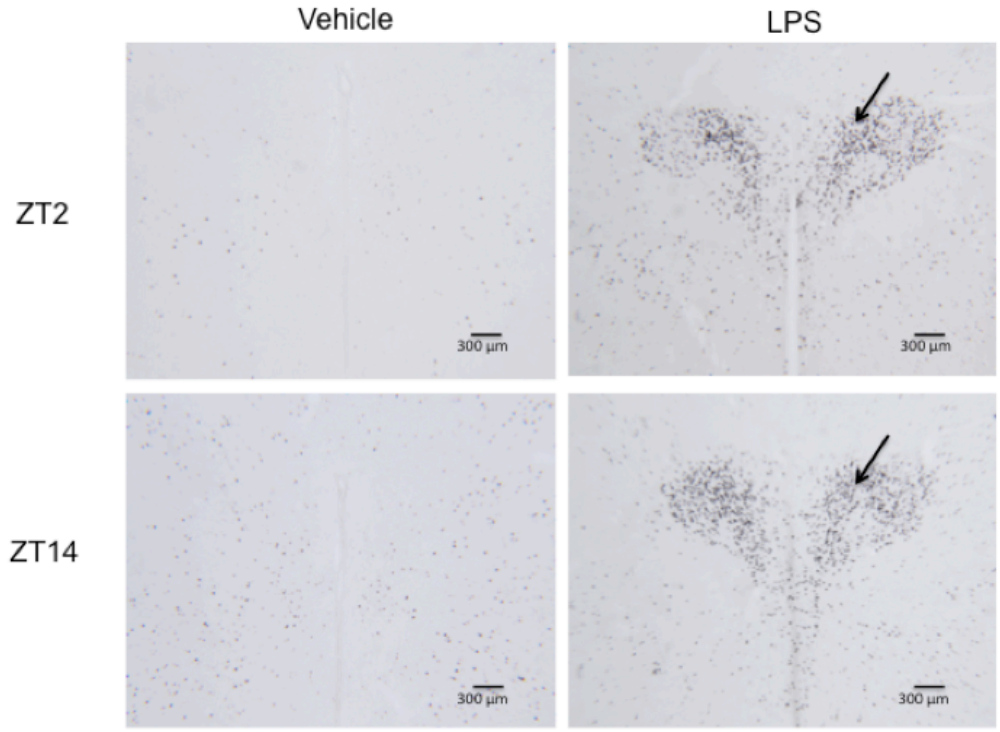

Figure S3. LPS challenge induces activation of PVN neurons. PVN photomicrographs after treatments show that LPS induce similar expression of c-Fos protein after day of night administration. Arows indicate the position of the parvocellular neurons in the medial PVN. Scale bar = 300 mm.

CHAPTER 3

Shift work in rats results in increased inflammatory response after lipopolysaccharide administration; a role for food consumption

Natalí N. Guerrero-Vargas, Mara Guzmán, Rebeca Fuentes, Joselyn García, Roberto Salgado-Delgado, María del Carmen Basualdo, Carolina Escobar, Regina P Markus and Ruud M. Buijs

Submitted

Abstract

The Suprachiasmatic nucleus (SCN) drives circadian rhythms in behavioral and physiological variables including the inflammatory response. Shift work is known to disturb circadian rhythms and is associated with increased susceptibility to develop disease. In rodents, circadian disruption due to shifted light schedules (*jet lag*) induced increased innate immune responses. To gain more insight in the influence of circadian disruption on the immune response, here we characterize the inflammatory response in a model of rodent shift work and demonstrated that circadian disruption affect the inflammatory response to lipopolysaccharide (LPS) both *in vivo* and *in vitro*. Since food consumption is a main disturbing element in the shift-work schedule we also evaluated the inflammatory response to LPS in a group of rats that had no access to food during their working hours.

Our results demonstrated that the shift work schedule did not affect basal TNF-a levels neither in blood nor in the liver. In spite of this, we observed that shift-work induced increased cytokines response after LPS stimulation in comparison to control rats. Also Kupffer cells (liver macrophages) isolated from shift-work rats produced more TNF-a in response to *in vitro* LPS stimulation suggesting important effects of circadian desynchronization on the functionality of this cell type. Importantly, shift-work effects on the inflammatory response to LPS were prevented when food access was not available during the working schedule. Together these results show that dissociating behavior and food intake from the synchronizing drive of the SCN severely disturbs the immune response.

Introduction

In mammals, the hypothalamic suprachiasmatic nucleus (SCN) is considered the master circadian pacemaker, which is entrained mainly by the light-dark cycle. The biological clock drives 24-h (circadian) rhythms in most physiological and behavioral variables, including hormone secretion, body temperature, locomotor activity and sleep (Hastings et al., 2003b). Modern lifestyle, night and shift-work as well as light schedules have changed our activity period and food consumption towards the night promoting the disruption of circadian rhythmicity.

Recent data in human and animal literature suggest that circadian desynchronization, mainly caused by the exposure to non-traditional work schedules or to *jet lag*, increase the susceptibility to develop pathologies (Evans and Davidson, 2013) such as cancer (Filipski et al., 2006; Schernhammer et al., 2001; Schernhammer et al., 2003; Takane et al., 2002), reproductive disorders (Nurminen, 1998) overweight (Barclay et al., 2012; Karlsson et al., 2001; Salgado-Delgado et al., 2013), diabetes (Karlsson et al., 2005; Morikawa et al., 2005), stroke (Karlsson et al., 2005) and cardiovascular disease (Portaluppi et al., 2012; Tenkanen et al., 1998; Tuchsen et al., 2006).

The effects of circadian disruption on the immune system have been analyzed in rodents subjected to experimental *jet lag* (chronic shifts of the light-dark cycle). Such protocol resulted in increased cytokines production and lethality to high doses of lipopolysaccharide (LPS), a component of gram-negative bacteria (Castanon-Cervantes et al., 2010). The rhythms of leukocyte recruitment to tissues under physiological and inflammatory conditions are also disrupted after experimental *jet lag* (Schernhammer et al., 2001). In mice, shifted light schedules modified circadian expression of clock genes and cytolytic factors in natural killer (NK) cells, as well as their cytotoxic activity, which is associated with cancer development (Logan et al., 2013).

Recently we demonstrated a direct inhibitory role of the SCN on the inflammatory response to LPS (Guerrero-Vargas et al., 2014a) previously our group demonstrated that in a rat model of shift-work, the activation of the SCN is reduced (Salgado-Delgado et al., 2010b). Therefore we hypothesized that the cause for the development of certain pathologies in shift-workers might be a disturbed response of the immune system due to circadian desynchrony. To test this idea we have used an experimental model of shift-work model in Wistar rats (forced activity during the rest phase) that was developed in

our laboratory (Salgado-Delgado et al., 2008). Animals subjected to this protocol exhibit disrupted circadian rhythmicity, consume more food during the rest period, gain more body weight, (Salgado-Delgado et al., 2010a) and show clear signs of metabolic disturbances, as demonstrated by lipid accumulation. Moreover after this protocol, the liver showed to be importantly affected presenting a loss of rhythmic expression in clock and metabolic genes in the liver (Salgado-Delgado et al., 2013), an organ that plays an important role in the elimination of systemic bacterial infection (Benacerraf et al., 1959; Gregory et al., 1996; Holub et al., 2009) and is the main filter of circulating LPS in the body (Jirillo et al., 2002) as it contains one of the largest macrophage reservoirs (Kupffer cells) capable of recognize and respond to circulating LPS (Wu et al., 2010a).

Here we evaluated the effects of the "shift-work" schedule on TNF- a and IL-6 production after LPS stimulation at two different time-points. Basal TNF-a protein content in the liver was also analyzed. The effects of desynchronization on the liver macrophages (Kupffer cells) response to LPS were evaluated *in vitro.* Finally the contribution of shifted food consumption in shift-work animals was also examined on the increased inflammatory response to LPS.

Methods

Animals and general housing conditions

Adult male Wistar rats weighing 210 to 220g at the beginning of the experiments were obtained from the animal facility of the Faculty of Medicine of the UNAM. Animals were housed in individual cages and maintained in a monitoring room with a 12:12 h light-dark cycle (LD), lights-on at 7:00, defined as Zeitgeber time 0 (ZT0) and lights off at 19:00 defined as Zeitgeber time 12 (ZT12). The room was maintained at a controlled temperature of 22 $\pm$1 °C and with continuous air flow. All rats were housed under specific pathogen free conditions and given free access to food (Rodent Laboratory Chow 5001, Purina, Minnetanka, MN, USA) and water, unless otherwise stated. Experimental procedures used in this study were in strict accordance with the Mexican norms for animal handling Norma Oficial Mexicana NOM-062-ZOO-1999, which conforms to international guidelines for animal handling, and were approved by the Ethics Committee in the Instituto de Investigaciones Biomédicas UNAM. All efforts were made to minimize the number of animals and their suffering.

Desynchronization Protocol (Shift-work)

Rats were placed from Monday to Friday for 8 h (stars at ZT2 end ZT10) in slow rotating drums that are used for sleep deprivation (33 cm diameter 633 cm wide). Each rotating drum has four concentric subdivisions, which allows individual housing of four rats. Drums rotate with a speed of one-revolution/3 min to force the rats to stay awake. Due to the slow movement of the drums, rats do not need to walk all the time; they can sit, groom and even lie down. In addition, they can drink and eat freely from a small bottle and pellets hanging from the middle tube, *see (Salgado-Delgado et al., 2008).* This procedure was carried out for 5 weeks from Monday to Friday. During weekends all rats remained undisturbed in their home cages.

Experimental Design

For acclimation, all rats were monitored in their home cages for 8 days in LD conditions.

After this period, rats were randomly assigned to one of 2 groups: Control rats (CTRL), were left undisturbed in their home cages during 5 weeks; Shift work (SW) rats were exposed for 5 weeks to the shift-work protocol. Surgeries were performed after rats concluded 4 weeks of shift-work protocol. Rats were allowed to recover during the weekend, and the following Monday, the working protocol was restarted for a fifth week. All experiments were performed at the end of the 5th week of the shift-work protocol. For this study, a total of 86 Wistar rats were used.

Experiment 1: *Effect of circadian desynchronization (shift-work) on:*

A) Basal TNF-a protein content in the liver

B) Pro-inflammatory cytokines blood levels after LPS administration

A) CTRL and SW rats (*n*= 4-5/group) were randomly sacrificed at one of two time-points: Zeitgeber time 2 (ZT2; 2 hours after light onset) or Zeitgeber time 14 (ZT14; 2 hours after light offset) in order to obtain the liver and determine TNF-a protein levels using an ELISA kit.

B) Other series of CTRL and SW rats were cannulated in the external jugular vein and were assigned to one of two groups, LPS administration at ZT2 or LPS administration at ZT14 (*n*= 5-7/group). These administrations time-points were chosen based on previous studies (Guerrero-Vargas et al., 2014a). At the end of the 5th week of the shift-work protocol, each rat received an iv LPS (100 mg/kg).

Blood samples were collected from the jugular cannula at 0, 40 and 80 min post LPS. In the obtained serum IL-6 and TNF-a were determined.

Experiment 2: *Effect of shift-work on the response of liver macrophages (Kupffer cells) to LPS in vitro.*

The liver of CTRL (*n=5*) and SW (*n=9*) rats was perfused at ZT2 through the portal vein with HBSS solution containing 0.2% IV Collagenase from *Clostridium histolyticum.* Kupffer cells were separated using two-step Percoll according to (Froh et al., 2003) *for details see section 2.7.* Kupffer cells isolated from CTRL and SW rats were incubated with fresh medium containing 1mg/mL of LPS or vehicle (HBSS) for 0, 1, 2, 4, 6, and 24 h at 37°C. TNF-a was measured in the conditioned medium samples of all incubation times using an ELISA kit.

Experiment 3: *Effect of feeding schedule on the inflammatory response to LPS of desynchronized rats.*

A group of rats (*n=7*) were enrolled to the shift-work protocol. However throughout the 5 weeks protocol, food was not available in the activity wheels, *i.e.* from ZT2-ZT10.

Animals had free access to food at end of the daily shift-work schedule, *i.e.* from ZT10-ZT2. On the weekends, they remained undisturbed in individual cages and had free access to food and water. A group of shift-work animals with no food in the activity wheel (SW-NFW, *n=7*) and CTRL (*n=7*) rats were cannulated in the external jugular vein and after recovery received LPS (100 mg/kg) at ZT2. Blood samples were collected from the jugular cannula at 0, 40 and 80 minutes post LPS. From the obtained serum IL-6 and TNF-a were determined.

Surgical procedures, intra-jugular cannula insertion

All surgeries were performed using aseptic procedures. Rats were anesthetized with a combination of ketamine (90 mg/kg Cheminova, México) and xylazine (10 mg/kg, Procin^{R}, Pisa, México) anesthesia. A polyethylene silicon tube cannula (0.025 in. i.d. and 0.047 in. o.d.; Silastic Laboratory tubing; Dow Corning Corp., Midland, MI, USA) was inserted in the internal jugular vein. The cannula was filled with heparin (500 U/ml) as anti-coagulant. The outer end of the cannula was fixed in the back between both shoulder blades and clotted with a small needle. Rats were allowed to recover during 7 days before experiments.

Treatment Solutions and Reagents

For all experiments, inflammation was induced by a single iv injection of lyophilized LPS (100 mg, *Escherichia coli* serotype 0127:B8, Sigma-Aldrich, St. Louis, MO, USA Lot No. 051M4004) reconstituted with 0.9% sterile pyrogen-free saline (Baxter, México) to derive a 1mg/ml stock solution that was aliquoted and stored at -20. For the administration, aliquots were thawed and diluted with 0.9% sterile pyrogen-free saline yielding a final concentration of 100 μg/kg.

Sample collection and ELISA

Blood samples (300 μl) were collected in Microvette®/500 tubes (Sarsted, Nümbrecht Germany) before LPS or vehicle infusion (0) and post-infusion times 40, and 80 minutes. Samples were centrifuged at 7000 rpm, during 7 minutes. Plasma was stored in 60μl aliquots at -80 °C until assay. IL-6 and TNF-a were determined by ELISA according to the manufacturer's recommendations (Invitrogen, Frederick, USA, #KRC3012, LOT 1350343A for TNF-a and # KRC0061C, LOT 1358388B for IL-6). Samples were analyzed in duplicate. Detection limits of the assays in plasma were <4pg/mL for TNF-a and <5pg/mL for IL-6. The intra-assay coefficient of variation (%CV) was 6.3% for TNF-a and 7.8% for IL-6. Inter-assay %CV was 2.6% for TNF-a and 3.93% for IL-6. Cytokine levels are expressed as picogram per ml.

Tissue Collection

After 5 weeks in the shift-work protocol, SW and CTRL rats were randomly sacrificed at ZT2 or ZT14. Rats were deeply anesthetized with an overdose of sodium pentobarbital (Pisabental, PiSa, Atilalaquia, Hgo, México; 65 mg/mL) and part of the left lobule of the liver was quickly removed and immediately frozen at -80 °C.

TNF-a determination in the liver

Frozen tissue samples were weighed and placed in saline at a ratio of 250mg tissue/0.5ml of 0.09% sterile pyrogen-free saline. Samples were homogenized. The final homogenates were centrifuged (12,000 rpm 10 minutes) and supernatants were immediately assayed for TNF-a using an ELISA kit. Data was obtained as pg/ml and then transformed to pg/mg tissue.

Kupffer cells isolation and culture

Kupffer cells were isolated from the livers of SW and CTRL rats using the method of Froh *et al* (Froh et al., 2003). Briefly, the liver was perfused through the portal vein with Ca^{2+}- and Mg^{2+}- free Hanks' balanced salt solution (HBSS) at 37 °C at rate of 20ml/min, (200ml total volume) then with complete HBSS solution containing 0.2% IV Collagenase from *Clostridium histolyticum* (Sigma Aldrich, St Louis MO, USA, C5138-1G, LOT 061M8628V) at a rate of 20ml/min, (200 ml total volume). The liver was removed from the animal, placed in sterile petri dish containing HBSS solution containing 0.2% IV Collagenase and sliced with scissors. Sliced tissue was filtered through a 100mm nylon sterile cell strainer (BD Falcon™, USA, 352360, LOT 1293837). Kupffer cells were separated using two-step Percoll gradient (GE Healthcare, Bio-sciences AB, Uppsala, Sweden). After 15 min of centrifugation at 1800 g, middle layers were collected, washed with HBSS and re-suspended in RPMI-1640 (Gibco®, USA, 1879-020, LOT 901692) containing 10% of Fetal Bovine Serum (FBS, Gibco®, USA). The purity of the Kupffer cells was determined to be approximately 90% by CD163 (ED-2) staining (Santa Cruz biotechnology inc. USA, SC-5865) and viability was found to be 98% using the trypan blue exclusion test (Supplementary Figure 1*)*. Kupffer cells were incubated at 37°C for 24 h under 5% CO2 in 24 well cell culture plates (Santa Cruz biotechnology inc. USA, SC-204444) at a density of $5X10^5$/ ml and cultured in RPMI-1640 containing 10% of FBS.

Kupffer cells treatment with LPS

Kupffer cells were incubated overnight in RPMI-1640 containing 10% of FBS. On the following day, the cells were incubated with fresh medium containing 1mg/mL of LPS for 0, 1, 2, 4, 6, and 24 h at 37°C. Supernatants were then harvested and kept at -70 °C until assayed. TNF-a in the culture medium was measured using an ELISA kit.

Data analysis

All data are presented as mean ± standard error of the mean (s.e.m). IL-6 and TNF-a plasma levels after LPS administrations at ZT2 or ZT14 rats were compared with a two-way ANOVA for repeated measures for Condition (CTRL vs SW) and time after LPS as a factor of repeated measures. This was followed by Bonferroni´s post-hoc test for multiple comparisons. TNF-a plasma levels after LPS stimulation in isolated Kupffer cells from CTRL and SW were compared with a two-way ANOVA for Condition (CTRL

vs SW) and time after LPS administration as a factor of repeated measures. IL-6 and TNF-a plasma levels after LPS administrations at ZT2 in CTRL, SW-NFW and SW rats were compared with a two-way ANOVA for Condition (CTRL, SW-NFW or SW) and time after LPS administration as factor for repeated measures followed by Tukey´s post-hoc test for multiple comparisons. Statistical comparisons were performed using Graphpad Prism version 6 for Mac. Threshold for statistical significance was set at a= 0.05.

Results

Shift-work does not affect basal TNF-a protein levels in the liver

In order to investigate the effects of the experimental shift-work schedule on basal TNF-a protein levels in the liver, an organ that plays an important role in the immune response, the livers of CTRL and SW rats were recovered at ZT2 or at ZT14.

In the livers of SW and CTRL rats similar TNF-a protein levels were measured at both ZT2 and ZT14 time-points (p=0.05; Fig. 1), however the two way ANOVA indicated a significant difference between condition (CTRL vs SW; $F_{[1, 13]}$ =4.38; P=.046) with lower values for SW condition. Moreover no significant interaction between condition and ZT were obtained ($F_{[1, 13]}$ =0.010; P=0.918).

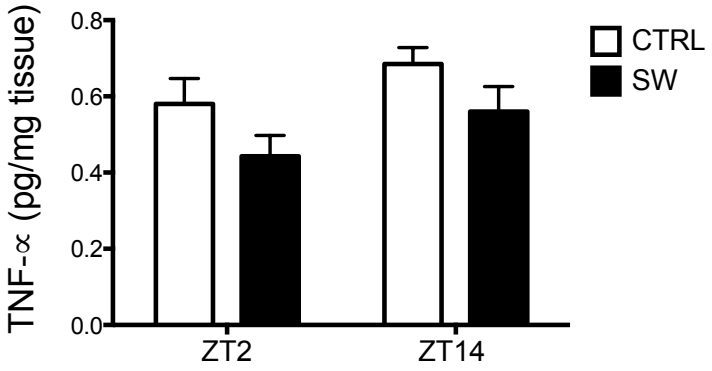

Figure 1. Basal TNF-a protein levels in the livers of CTRL and SW rats at ZT2 and ZT14 (*n=4-5/group*). Values are expressed as means ± s.e.m. Two-way ANOVA and *post hoc* Bonferroni.

Shift work (SW) increased cytokine production after LPS administration at ZT2.

In order to study the effect of shift-work on TNF-a and IL-6 production after an inflammatory stimulus, LPS was given iv, at ZT2 or ZT14 to CTRL and SW rats. Basal TNF-a and IL-6 plasma levels, as measured by the time 0, were very low or undectable and not different between SW and CTRL rats in all cases. In SW rats LPS administration at ZT2 triggered significantly higher TNF-a plasma levels ($p < 0.001$) at both 40 and 80 minutes after LPS as compared with CTRL rats (Fig. 2A). The two way ANOVA indicated a significant interaction between condition and time after LPS administration ($F_{[2, 18]} = 11.68$; P= 0.0006). In SW rats also IL-6 plasma levels were significantly elevated 80 minutes after LPS administration at ZT2 ($p < 0.001$) as compared with CTRL rats (Fig. 2B). The two way ANOVA indicated a significant interaction between the condition and time after LPS administration ($F_{[2, 18]} = 29.92$; P< 0.0001).

In contrast, at ZT14 LPS administration resulted in similar TNF-a (Fig. 2C) and IL-6 (Fig. 2D) plasma levels in SW and CTRL rats at 40 and 80 minutes after the stimulus. The two way ANOVA indicated no significant interaction between condition and time after LPS administration (TNF-α: $F_{[2, 20]} = 0.112$; P= 0.29; IL-6: ($F_{[2, 20]} = 0.190$; P= 0.827).

As we have previously shown (Guerrero-Vargas et al., 2014a), LPS administration to CTRL (synchronized) rats at ZT14 triggered higher TNF-α and IL-6 plasma levels in comparison to LPS administrated at ZT2. Here we confirm this time dependent response and report in CTRL rats increased cytokines plasma levels at 80 min after the LPS administration at ZT14 ($p < 0.001$; Supplementary Figure 2). In contrast, SW rats, with their increased response at ZT2 did not show this daily variation of TNF-α (Fig 2E) or IL-6 (Fig 2F) in response to LPS. The two way ANOVA indicated no significant effect of ZT (TNF-a: $F_{[1, 9]} = 3.486$; P= 0.0.094; IL-6 : $F_{[1, 10]} = 0.021$; P= 0.8863) and no significant interaction between time after LPS administration and ZT (TNF-a: $F_{[2, 18]} = 0.894$ P= 0.426; IL-6 : $F_{[2, 20]} = 0.306$; P= 0.739). Therefore in the next series of experiments, aimed to study the cause for this disparate inflammatory response we studied changes at ZT2 where the differences between SW and CTRL rats were the largest.

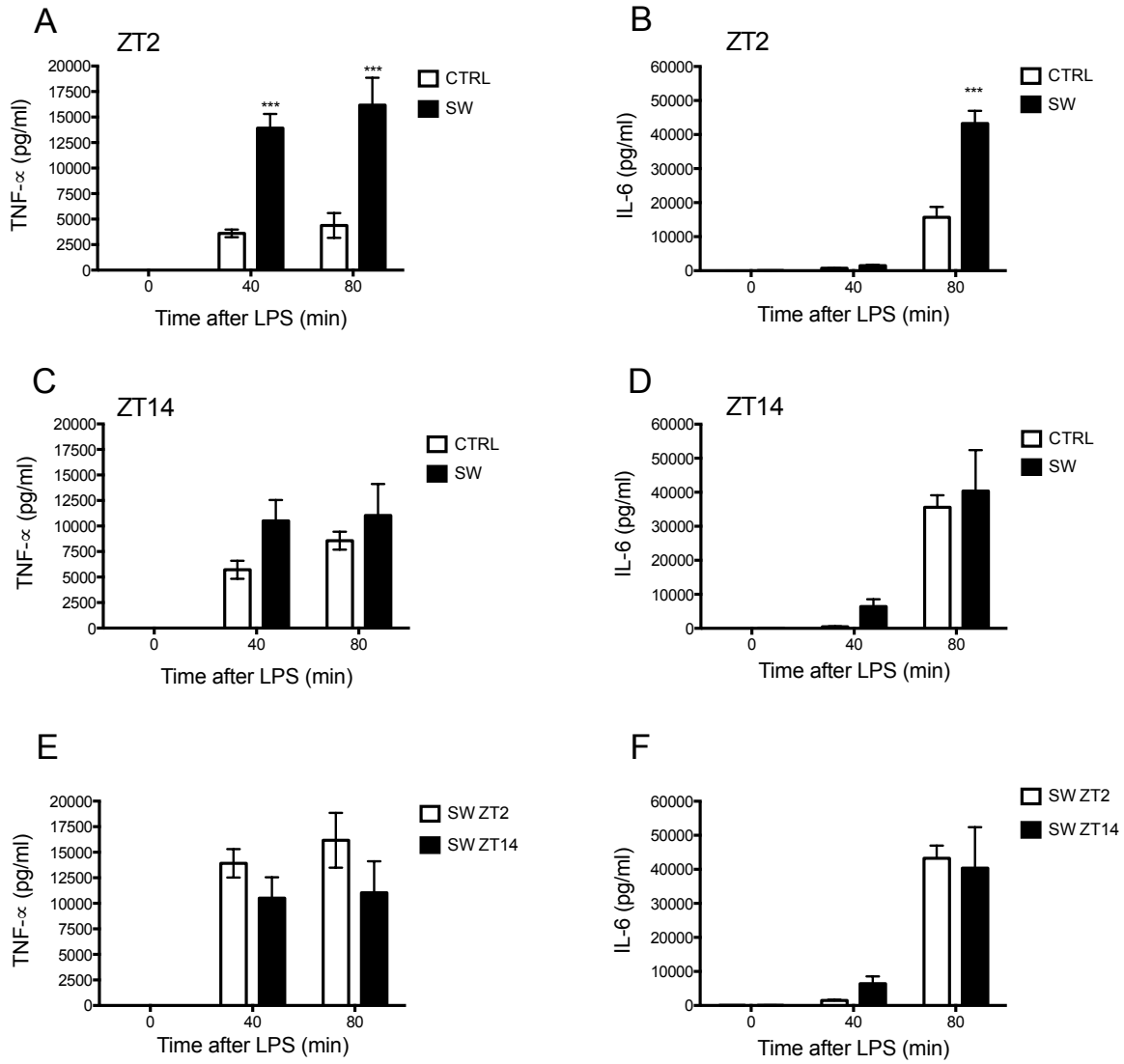

Figure 2. SW increased the inflammatory response to LPS. TNF-a (A) and IL-6 (B) plasma levels were higher in SW rats after LPS stimulation at ZT2 in comparison to CTRL rats (*n= 6-7*/group). However TNF-a (C) and IL-6 (D) plasma levels in SW and CTRL were similar after LPS administration at ZT14 (*n= 5-7*/group). Similar TNF-a (E) and IL-6 plasma levels (F) were measured after LPS administration at ZT14 or at ZT2 in SW rats (*n= 6-7*/group). TNF-a and IL-6 plasma concentrations were measured before and after iv, administration of LPS (100 μg/kg) at ZT2 or ZT14. Values are expressed as means ± s.e.m. Two-way ANOVA and *post hoc* Bonferroni ***$P < 0.001$ significance of difference vs. CTRL

Kupffer cells isolated from shift-work rats produced more TNF-a in response to LPS stimulation.

The liver is one of the main sources of cytokines after LPS is injected into the circulation, therefore we evaluated the response to LPS after 5 weeks of the shift-work protocol in a specific immune cell type, the liver macrophages (Kupffer cells) *in vitro.* Based on our *in vivo* results demonstrating an increased immune response of SW at ZT2, Kupffer cells

were isolated from SW and CTRL rats at this time point. Kupffer cells isolated from SW rats produced more TNF-a after LPS stimulation *in vitro* in comparison to CTRL Kupffer cells, this was significantly different between groups at 2, 6 and 24 h (p <0.05; Fig. 3). The two way ANOVA indicated significant interaction between time after LPS administration and condition ($F_{[4, 48]}$ = 2.695; P= 0.0418). No differences in TNF-a supernatant levels were found between CTRL and SW after HBSS (vehicle) administration ($F_{[4,40]}$ = 1.180; P= *0.334; data not shown).*

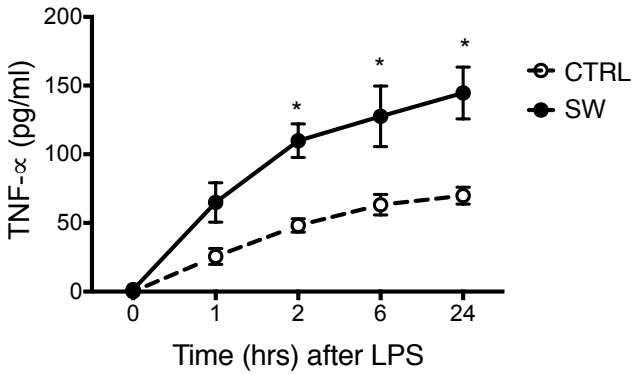

Figure 3. TNF-a was measured in Kupffer cells conditioned medium containing LPS (1μg/mL). Open circles represent TNF-α levels from CTRL isolated Kupffer circles and solid circles respresent TNF-α levels from SW isolated Kupffer cells. Values are expressed as means ± s.e.m. Each point represents the average ± s.e.m. of 5-9 animals per group. Two-way ANOVA and *post hoc* Bonferroni *P < 0.05 significance of differences vs. CTRL

Avoiding food intake during the working hours prevents increased inflammatory response

Previously it was demonstrated that desynchronization and weight gain induced by this shift work protocol, were prevented if food intake was restricted to the normal activity period of the rat (Salgado-Delgado et al., 2010a). In order to investigate whether food intake during the rest phase was contributing to the heightened inflammatory response to LPS observed in SW rats, cytokines production after LPS administration at ZT2 was evaluated in a group of SW rats that had no acces to food in the activity wheels (SW-NFW) during the 5 weeks protocol and consequently were eating mainly during the dark phase. As observed previously, LPS administration at ZT2 to SW rats triggered higher TNF-a plasma levels at 40 and 80 minutes as compared to CTRL rats. This response was prevented in SW-NFW rats that were only able to eat in their homecages (ZT10-ZT2), which corresponds to their normal activity phase (Fig. 4A). TNF-a plasma levels in SW

rats were also significantly higher as compared to SW-NFW animals at 40 and 80 minutes after LPS administration. The two way ANOVA indicated a significant interaction between time after LPS administration and condition ($F_{[4,36]} = 4.034$; P= 0.0084). No significant differences between the SW-NFW and CTRL groups were found after post hoc test.

IL-6 plasma levels were also significantly increased in SW rats 80 minutes after LPS administration as compared to CTRL and SW-NFW rats (Fig. 4B). Restricting food intake to SW rats (SW-NFW) toward to night prevented this response (Fig 4B). The two way ANOVA indicated a significant interaction between time after LPS administration and condition ($F_{[2, 38]} = 10.83$; P< 0.0001). No signicant differences between SW-NFW and CTRL groups were found after post hoc test.

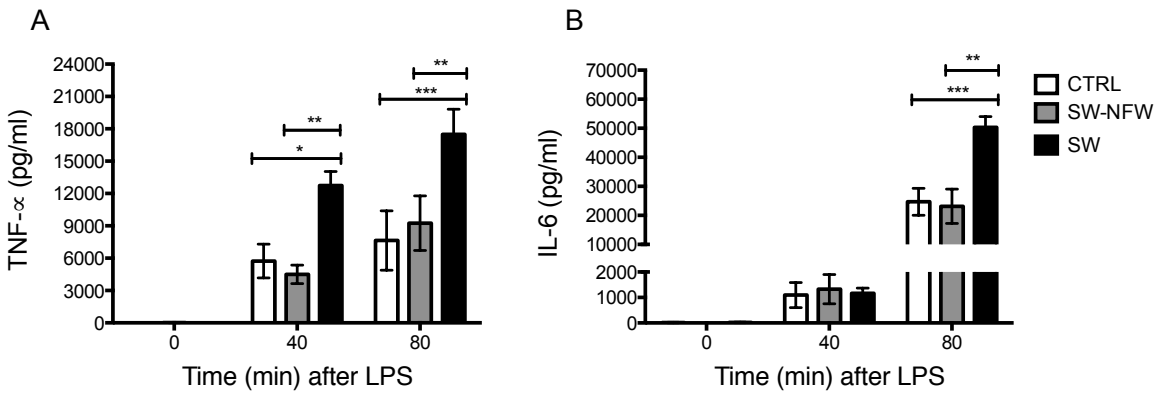

Figure 4. LPS administration at ZT2 to SW-NFW triggered similar TNF-a (A) and IL-6 (B) plasma levels in comparison to CTRL rats. TNF-a and IL-6 plasma concentrations were measured before and after iv, administration of LPS (100 µg/kg) at ZT2. Values are expressed as means ± s.e.m. (*n= 5-7*/group). Two-way ANOVA and *post hoc* Tuckey; *P < 0.05, **P < 0.01, ***P < 0.001 significance of differences vs. SW.

Discussion

Shift work is associated with increased vulnerability to develop disease (Evans and Davidson, 2013). However the mechanisms underlying the effects of circadian desynchronization on the development of disease are not fully understood. Here we demonstrate that circadian desynchronization induced by an experimental model of "shift-work" (SW) in Wistar rats leads to enhanced TNF-a and IL-6 production after LPS

stimulation. Since no changes in basal TNF-a content in the liver or blood were observed, the enhanced response of SW rats is, at least partly, based on the increased inflammatory response of the liver, the main organ of the body involved in detoxification of the blood. This was demonstrated by the higher response of Kupffer cells that even after being isolated from the liver showed a higher response to LPS. Notably, preventing food intake during the working hours reduced to control levels the inflammatory response to LPS observed in SW rats, illustrating that the enhanced food intake in the day period is the main event leading to desynchronization and enhanced inflammatory response.

These observations agree with other studies showing that circadian desynchronization induced by shifted light schedules decrease the survival of rodents after high doses of LPS (Adams et al., 2013; Castanon-Cervantes et al., 2010).

Circadian desynchronization induced by shift-work increased the inflammatory response to an LPS challenge

Here we report that LPS administration to SW rats during the light phase (ZT2) resulted in almost 4 times higher TNF-a and 3 times higher IL-6 plasma levels as compared to the response of CTRL rats. Importantly, LPS administration at ZT14 induced similarly high cytokines levels in SW and CTRL rats. The explanation for this can be found in our earlier studies demonstrating that high cytokines production in response to LPS is associated with low SCN neuronal activity, a feature of the dark period (ZT14), while low cytokine production is associated with high SCN neuronal activity, a feature of the day period (ZT2) (Guerrero-Vargas et al., 2014a). Salgado-Delgado et al., (2010b) demonstrated that shift-work rats have low SCN neuronal activity at ZT2 as compared to control rats and in line with the previous observations this suggests that in shift-work animals the SCN does not diminish the cytokines response as efficiently as in control rats. Hereby it is also important to note that the cytokine response of SW at ZT2 is increased in a similar way as in SCN lesioned rats (Guerrero-Vargas et al., 2014a), which strengthens the point that SCN neuronal activity may play an important role in diminishing the inflammatory response to LPS.

Previous studies have demonstrated that the central nervous system influences the immune response via the autonomic nervous system (Felten and Felten, 1988; Martelli et al., 2014; Rosas-Ballina and Tracey, 2009). Since the output of the SCN determines a daily balance of the autonomic output to the organs (Buijs et al., 2013) a misbalance in

the autonomic output to immune organs, such as the liver, in SW rats may also promote an increased the inflammatory response to LPS.

In addition to a misbalance in the autonomic output of the brain, the increased inflammatory response of Kupffer cells to LPS indicates that shift work also changes the capacity of isolated cells to respond. In SW rats the disrupted rhythm of liver clock genes and other genes as demonstrated by (Salgado-Delgado et al., 2013) may also be partly responsible for the increased inflammatory response, because it is well documented that clock genes influence immune functions (Arjona and Sarkar, 2006a, b; Gibbs et al., 2012; Keller et al., 2009; Liu et al., 2006; Logan et al., 2013; Oishi et al., 2006).

Since glucocorticoids are recognized as modulators of the inflammatory response, their role in the inflammation of SW is quite complex and deserves to be addressed. We have previously shown that the present shift work schedule does not change circadian rhythmicity of corticosterone plasma levels. However a peak in corticosterone is observed at ZT3 just after the animals are placed in the rotating wheels. (Salgado-Delgado et al., 2008; Salgado-Delgado et al., 2010a). Besides inducing inflammation, LPS administration induces a high corticosterone response (Kalsbeek et al., 2012), which most accepted function is to decrease cytokines over-production (Kapcala et al., 1995; Sternberg, 1997). Interestingly however, SCN ablation induces high corticosterone levels (Kalsbeek et al., 2012) and is associated with an increased inflammatory response to LPS in the same manner as SW rats (Guerrero-Vargas et al., 2014a) indicating that high corticosterone levels alone are not sufficient to suppress the cytokine response.

This makes clear that in order to consider the effects of glucocorticoids one needs to distinguish the effects of endogenous corticoids like corticosterone or cortisol, or the effect of highly active glucocorticoids like dexamethasone. Even the effects of dexamethasone on the immune response depend on several aspects such as its concentration as well as the time of exposure, *i.e.* before of after the immune challenge. For example, dexamethasone administration 1h after the LPS stimulus is associated with attenuated inflammatory responses in the central nervous system as well as in the periphery, while its administration prior the immune challenge is associated with an increase pro-inflammatory response mainly in the central nervous system (Frank et al., 2010; Munhoz et al., 2006; Sorrells et al., 2009). In contrast, exposure to stress resulting in endogenous glucocorticoid production prior to an LPS challenge is also associated

with increased cytokines production in the periphery (Johnson et al., 2002) and increased expression of inflammatory markers in the liver (Frank et al., 2010).

Considering our shift work experiment, it is important that time restricted feeding like in our SW-NFW group (Shift-work, no food in the wheel), did not change corticosterone levels as compared with our SW group that had *ad libitum* food access (Salgado-Delgado et al., 2010a), yet this group showed a strong decrease in cytokine response as compared to SW rats. Altogether this suggest that the increased inflammatory response of shift-work rats is not likely to be driven by changes in the HPA axis activation and corticosterone production and points to a more complex mechanism whereby desynchronization or disorganization of the physiological systems involved in the regulation of the immune response may be responsible for the observed increased inflammatory response.

Shift work induces desynchronized immune responses

Our shift work protocol does not change the basal level of inflammatory molecules like TNF-a or IL-6 in the circulation since similar undetectable plasma levels of these cytokines were measured in both conditions (SW and CTRL). Surprisingly in spite of the fact that shift work animals at ZT2 responded with higher cytokine levels after the LPS challenge, their basal TNF-a protein levels in the liver tended to be diminished as compared to CTRL animals and therefore suggest an amplified inflammatory response capacity or reaction of these animals to an immune challenge.

This observation is of relevance for human studies where only certain inflammatory markers in the circulation are reported to be increased such as leukocyte counts (Sookoian et al., 2007), C reactive protein levels (CRP) (Puttonen et al., 2011) and IL-6 levels (Khosro et al., 2011). Other studies fail to find effects of shift work on inflammatory markers such as TNF-a (Khosro et al., 2011) and some even report lower basal levels of circulating TNF-a and IL-b among shift-workers (Copertaro et al., 2011). These conflicting data may be related to the heterogeneity of the populations, but may also be explained by the observations of the present work, where only under challenging conditions a higher sensitivity for inflammation can be detected. Our observations of enhanced inflammatory response may explain why shift-work and circadian desynchronization are strongly associated with negative health consequences indicating that the normal defense responses are disrupted. This is also suggested by the outcome of

other studies where phase-shift schedules exacerbate the development of experimental colitis in mice (Preuss et al., 2008), reduced the survival of animals with cardiomyopathic heart disease (Penev et al., 1998) and resulted in increased tumor development (Filipski et al., 2003; Filipski et al., 2009; Logan et al., 2012).

Different from the chronic phase-shift schedules, our desynchronization protocol focuses on the effects of activity and food consumption in contradiction with SCN signals aimed to set physiology for the rest period. In this sense, our shift-work protocol does not involve changes in the lighting schedule and thus resembles more to human shift-work. Here we confirm that circadian desynchronization, whether induced by *jet lag* or shift-work, provoked exacerbated immune responses, as demonstrated by our study and others.

Metabolic changes in shift-work and the immune response

Interestingly, shift-work rats not only show increased inflammatory response to LPS but also have increased body weight, adiposity, triglycerides levels in plasma as well as liver steatosis together with damped expression of metabolic genes in the same organ (Salgado-Delgado et al., 2008; Salgado-Delgado et al., 2010a; Salgado-Delgado et al., 2013). The immune system and energy metabolism are known to be well connected and recently the term immunometabolism has emerged in order to describe and study their interactions (Mathis and Shoelson, 2011). Some studies indicate that the innate immune response is regulated through the crosstalk between the nuclear transcription factor NF-kB (responsible for the expression of several inflammatory genes), the histone deacetylase Sirtuin 1 (SIRT1) (Fernandes et al., 2012; Yang et al., 2012) and the Peroxisome proliferator-activated receptors (PPARs) reviewed in (Daynes and Jones, 2002). It has been demonstrated that SIRT activation inhibits TNF-a production (Li et al., 2013; Yang et al., 2012) whereas SIRT downregulation leads to increased cytokines production from macrophages (Fernandes et al., 2012) and increased TNF-a production from cultured Kupffer cells after LPS stimulation (Shen et al., 2009). A previous study by (Salgado-Delgado et al., 2013) described that in shift-work rats SIRT-1 and PPAR´s are deregulated and diminished, suggesting that shift work may increase the response to an LPS challenge at ZT2 via the downregulation of SIRT-1 and PPAR protein levels in the liver.

Food consumption during the "working hours" promotes the inflammatory response to LPS

Altered food consumption patterns observed in shift work rats are considered the basis of the desynchronization effects of shift-work (Salgado-Delgado et al., 2010a). As one of the major findings of the present study we showed that the increased inflammatory response to LPS in shift work rats was prevented when food intake during the working hours was avoided and animals were only allowed to eat at the end of their working protocol. This indicates that food consumption during the normal rest phase is one of the major factors contributing to the exacerbated inflammatory response to LPS observed in shift work rats and possibly also for other desynchronization protocols such as *jet lag,* since sleep loss is not associated with the increased immune response observed in *jet lag* rodents (Brager et al., 2013).

In support of these observations, we showed that low doses of LPS administrated to rats that were only allowed to eat during their normal rest period (daytime feeding), resulted in 5 times higher TNF-a and 8 times higher IL-6 plasma levels as compared to control rats with *ad libitum* access to food (Guerrero-Vargas et al., *in preparation*).

Similar as shift work, daytime feeding desynchronizes clock gene expression in peripheral organs (Damiola et al., 2000; Salgado-Delgado et al., 2010b), consequently these desynchronizing effects could also disturb the response of immune organs and cells to an immune challenge.

The potentiation of the inflammatory responses by the mistiming of food intake has important implications also for human health since altered feeding schedules have been reported in shift workers (de Assis et al., 2003; Lennernas et al., 1995; Pasqua and Moreno, 2004).

In conclusion the present results show that shift work in rats lead to an augmented inflammatory response, which cannot be associated to increased basal levels of inflammatory markers. Maintaining food intake patterns associated with the normal activity period may help to regulate the inflammatory response.

Competing Interests

The authors have declared that no competing interests exist.

Acknowledgments

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Natalí N. Guerrero-Vargas, Joselyn García, Rebeca Fuentes, Roberto Salgado-Delgado, María del Carmen Basualdo, and Mara Guzmán contributed to the acquisition of the data. Natalí N. Guerrero-Vargas, Ruud M. Buijs, Carolina Escobar and Regina P Markus, contributed to the analysis and interpretation of the data. Ruud M. Buijs and Natalí N. Guerrero-Vargas initiated and designed the study.

Natalí N. Guerrero-Vargas and Ruud M. Buijs wrote the paper. All authors declare that there are no conflicts of interest.

Supplemental Data

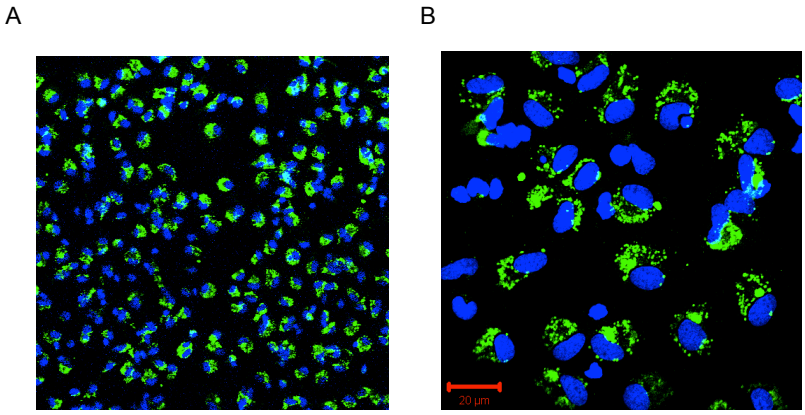

Figure S1. Liver macrophages (Kupffer cells) were stained positively for ED2 (*green*) and counterstained with nuclear stain DAP (*blue*). The purity was ≥ 90%. A (x 20); B (x 60). Scale bar= 20mm.

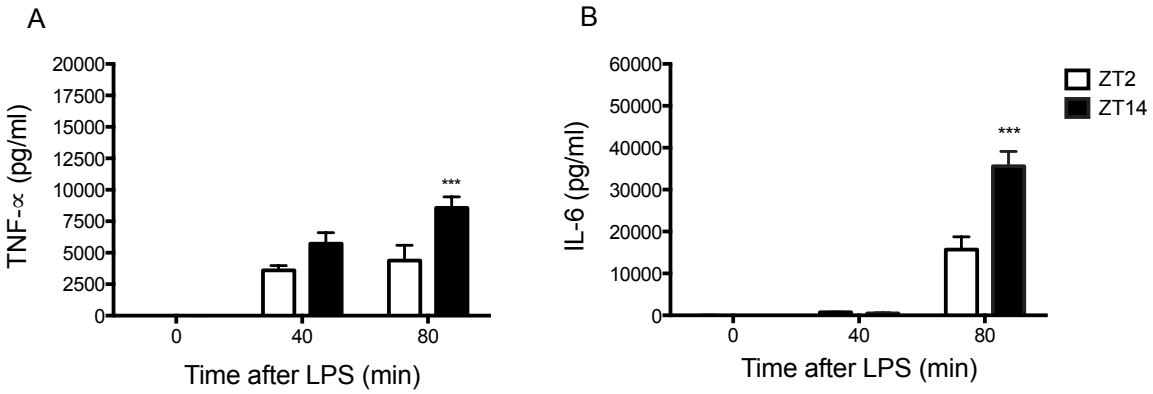

Figure S2. TNF-α (A) and IL-6 (B) plasma levels before and after LPS (100 µg/kg) administration. Values are expressed as means ± s.e.m. (*n*= 5-7/group). The two way ANOVA indicated a significant effect of ZT (TNF-α: $F_{[1, 10]}$ =10.51; P=0.008; IL-6: $F_{[1, 9]}$ =16.88; P= 0.002) and a significant interaction between time after LPS administration and ZT (TNF-α: $F_{[2, 20]}$ =4.177; P=0.030; IL-6: $F_{[2, 18]}$ =17.56; P< 0.0001) *post hoc* Bonferroni; ***P < 0.001 significance of differences vs. ZT2.

CHAPTER 4

Food consumption during the normal inactive phase of the rat increased the inflammatory response to Lipopolysaccharide

Natalí N. Guerrero-Vargas, Elizabeth Sabath, Mara Guzmán, María del Carmen Basualdo, Carolina Escobar, and Ruud M. Buijs

To be submitted

Abstract

Modern lifestyles and work schedules have changed our sleep-wake and food consumption patterns to the normal rest phase, which is in contradiction with the signals that are send by the master circadian clock (the suprachiasmatic nucleus, SCN) aimed to modify metabolic function in order to prepare us for sleep. When we are active or eating instead of sleeping, the SCN receives an "active" message from the periphery that does not coincide with its own communication to the periphery. This phenomenon is known as internal desynchronization and has been related with increased susceptibility to develop disease. In the present study we analyzed the effects of food consumption during the normal resting phase of Wistar rats (*food during the day*, FD), on the inflammatory response to an LPS challenge. Intravenous LPS administration to FD rats triggered higher circulating cytokines levels as compared to control rats. In order to analyse a possible source for these increased cytokines, liver macrophages (Kupffer cells) were isolated and exposed to LPS. Kupffer cells obtained from FD rats produce more TNF-α in response to LPS stimulation indicating a major disturbance of liver macrophages following such desyncFhronized food protocol.

Consequently the time of food consumption is an important factor determining the magnitude of the inflammatory response by increased sensitivity of Kupffer cells. This suggests that maintance of normal food intake patterns could help to prevent exacerbated inflammatory responses and possibly the development of diseases as observed in shift workers.

Introduction

Nowadays people used to be more active during their normal sleeping period either for work (as a demand of the developing world economy), study or fun (nocturnal lifestyle). As a result of this extendend or sometimes inverted active period the exposure to altered food schedules is a comon practice and together with delayed sleep onset could be considered a feature of modern lifestyle.

Since the suprachiasmatic nucleus (SCN), organizes circadian rhythms on behavior and physiology according to the light-dark cycle, abnormal cycles of food intake and/or activity, generate conflicting signals out of phase with its temporal message. In consequence, this conflicting temporal information promotes circadian desynchronization.

Several studies have demonstrated that altered circadian rhythms as observed in human shift workers are associated with metabolic disruption, resulting in obesity, diabetes (Karlsson et al., 2005; Morikawa et al., 2005; Zhao et al., 2012) and cardiovascular disease (Tenkanen et al., 1998), while other diseases with an important inflammatory component, such as cancer are also importantly increased (Davis and Mirick, 2006; Gassmann et al., 2014; Schernhammer et al., 2001; Schernhammer et al., 2003).

Importantly, altered feeding schedules as observed in shift workers (de Assis et al., 2003; Lennernas et al., 1995; Pasqua and Moreno, 2004) and in humans with the night eating syndrome (NES) are associated with increased body weight and obesity (Colles et al., 2007; Gallant et al., 2012; Gluck et al., 2008; Muhlhans et al., 2009).

In rats, limiting food acces to their normal resting phase (light period) results in body weight gain (Arble et al., 2009; Salgado-Delgado et al., 2010b), increased fat accumulation and altered rhythms in circulating glucose and tryglicerides as well as decreased glucose tolerance (Salgado-Delgado et al., 2010b; Salgado-Delgado et al., 2013). Several studies have also demonstrated that inverted food consumption importantly affects the metabolism of the liver, where fat accumulation is observed (Damiola et al., 2000; Glad et al., 2011; Salgado-Delgado et al., 2013; Satoh et al., 2006; Wu et al., 2010b). The observation that the circadian expression of clock and metabolic genes is inverted or even abolished (Salgado-Delgado et al., 2013) shows that restricted daytime food acces has the capacity to desynchronize liver rhythms from the SCN, since

under these conditions no changes in the expression of clock genes in the central pacemarker are observed (Damiola et al., 2000).

Giving the growing appreciation that obesity is linked with inflammation (Bastard et al., 2006; Emanuela et al., 2012; Lumeng and Saltiel, 2011) and that altered food patterns lead to obesity and metabolic disease, we hypothesized that mistimed food consumption could also disturb the inflammatory response to LPS. Therefore in the present study we examined the influence of the timing of feeding on the response to an inflammatory challenge with Lypopolysaccharide (LPS). We previously demonstrated an SCN driven day-night difference in the inflammatory response to LPS (Guerrero-Vargas et al., 2014a) suggesting that altered food consumption could also desynchronize the inflammatory response. Hereto we examined the effect of food restriction to the normal resting phase of the rat on TNF-α and IL-6 production before and after the administration of a low dose of LPS at two different time-points.

Since the liver is an important target for the effects of altered food schedules, while it also a key organ mediating inflamation (Gao et al., 2008) functions as the main filter for circulating LPS in the body (Gregory et al., 1996; Holub et al., 2009; Jirillo et al., 2002) by its resident macrophages (Kupffer cells) we also studied the response of liver isolated Kupffer cells to *in vitro* LPS stimulation.

Matherial and Methods *Animals and general housing conditions*

Adult male Wistar rats weighing 210 to 220g at the beginning of the experiments were obtained from the animal facility of the Faculty of Medicine of the UNAM. Animals were housed in individual cages and maintained in a monitoring room with a 12:12 h light-dark cycle (LD), lights-on at 7:00, defined as Zeitgeber time 0 (ZT0) and lights off at 19:00 defined as Zeitgeber time 12 (ZT12). The room was maintained at a controlled temperature of 22 $\pm$1 °C and with continuous air flow. All rats were housed under specific pathogen free conditions and given free access to food (Rodent Laboratory Chow 5001, Purina, Minnetanka, MN, USA) and water, unless otherwise stated. Experimental procedures used in this study were in strict accordance with the Mexican norms for animal handling Norma Oficial Mexicana NOM-062-ZOO-1999, which conforms to international guidelines for animal handling, and were approved by the Ethics Committee in the Instituto de Investigaciones Biomédicas UNAM. All efforts were made to minimize the number of animals and their suffering.

Experimental Design

For the experimental phase 31 rats were randomly assigned to the following groups:

1. Control (CTRL) rats feed *ad libitum* were left undisturbed for 5 weeks and received LPS (2 μ g/kg) at Zeitgeber time 2 (ZT2; 2 hours after light onset; n=8) or LPS (2 μ g/kg) Zeitgeber time 14 (ZT14; 2 hours after light offset; n=6).

2. Food during the day (FD) rats were provided with food exclusively during 12h/day during the light phase for 5 weeks and were randomly assigned to one of the two groups: LPS (2 μ g/kg) administration at ZT2 (n=5) or LPS (2 μ g/kg) administration at ZT14 (n=6).

3. A separate group of CTRL (n=8) and FD (n=6) rats was sacrificed at ZT2 for Kupffer cells isolation. All experiments were performed at the end of the 5-week schedule.

Treatment Solutions and Reagents

For all experiments, inflammation was induced by a single iv injection of lyophilized LPS (100 mg, *Escherichia coli* serotype 0127:B8, Sigma-Aldrich, St. Louis, MO, USA Lot No. 051M4004) reconstituted with 0.9% sterile pyrogen-free saline (Baxter, México) to derive a 1mg/ml stock solution that was aliquoted and stored at -20. For the administration, aliquots were thawed and diluted with 0.9% sterile pyrogen-free saline yielding a final concentration of 2 μg/kg.

Sample collection and ELISA

Blood samples (300 μl) were collected in Microvette®/500 tubes (Sarsted, Nümbrecht Germany) before LPS or vehicle infusion (0) and post-infusion times 40, and 80 minutes. Samples were centrifuged at 7000 rpm, during 7 minutes. Plasma was stored in 60μl aliquots at -80 °C until assay. IL-6 and TNF-α were determined by ELISA according to the manufacturer's recommendations (Invitrogen, Frederick, USA, #KRC3012, LOT 1350343A for TNF-α and # KRC0061C, LOT 1358388B for IL-6). Detection limits of the assays in plasma were <4pg/mL for TNF-α and <5pg/mL for IL-6. Cytokine levels are expressed as picogram per ml.

Kupffer cells isolation and culture

Kupffer cells were isolated from the livers of FD and CTRL rats using the method of Froh *et al* (Froh et al., 2003). Briefly, the liver was perfused through the portal vein with Ca^{2+}- and Mg^{2+}- free Hanks' balanced salt solution (HBSS) at 37 °C at rate of 20ml/min, (200ml total volume) then with complete HBSS solution containing 0.2% IV Collagenase from *Clostridium histolyticum* (Sigma Aldrich, St Louis MO, USA, C5138-1G, LOT 061M8628V) at a rate of 20ml/min, (200 ml total volume). The liver was removed from the animal, placed in sterile petri dish containing HBSS solution containing 0.2% IV Collagenase and sliced with scissors. Sliced tissue was filtered through a 100mm nylon sterile cell strainer (BD Falcon™ , USA, 352360, LOT 1293837). Kupffer cells were separated using two-step Percoll gradient (GE Healthcare, Bio-sciences AB, Uppsala, Sweden). After 15 min of centrifugation at 1800 g, middle layers were collected, washed with HBSS and re-suspended in RPMI-1640 (Gibco®, USA, 1879-020, LOT 901692) containing 10% of Fetal Bovine Serum (FBS, Gibco®, USA). The purity of the Kupffer cells was determined to be approximately 90% by CD163 (ED-2) staining (Santa Cruz biotechnology inc. USA, SC-5865) and viability was found to be 98% using the trypan blue exclusion test. Kupffer cells were incubated at 37°C for 24 h under 5% CO2 in 24 well cell culture plates (Santa Cruz biotechnology inc. USA, SC-204444) at a density of $5X10^5$/ ml and cultured in RPMI-1640 containing 10% of FBS.

Kupffer cells treatment with LPS

Kupffer cells were incubated overnight in RPMI-1640 containing 10% of FBS. On the following day, the cells were incubated with fresh medium containing 1mg/mL of LPS for 0, 1, 2, 6, and 24 h at 37°C. Supernatants were then harvested and kept at -70 °C until assayed. TNF-a in the culture medium was measured using an ELISA kit.

Data analysis

All data are presented as mean ± standard error of the mean (s.e.m). IL-6 and TNF-α plasma levels after LPS administrations at ZT2 or ZT14 rats were compared with a two-way ANOVA for repeated measures for Condition (CTRL vs FD) and time after LPS as a factor of repeated measures. This was followed by Bonferroni´s post-hoc test for multiple comparisons. TNF-α plasma levels after LPS stimulation in isolated Kupffer cells from CTRL and FD were compared with a two-way ANOVA for Condition (CTRL vs FD) and time after LPS administration as a factor of repeated measures. Statistical

comparisons were performed using Graphpad Prism version 6 for Mac. Threshold for statistical significance was set at a= 0.05.

Results

Food intake during the normal rest phase increased the inflammatory response to LPS.

All animals that received their food ad libitum (CTRL) or during the day (FD) showed very low or undectable basal TNF-α and IL-6 plasma levels at ZT2 as well as at ZT14. In contrast LPS administration at ZT2 induces significantly higher TNF-α (Fig. 1A) and IL-6 (Fig. 1B) plasma levels in FD rats as compared to CTRL animals at 40 (p <0.01) and 80 (p <0.01) minutes after LPS administration. The two way ANOVA indicated a significant interaction between time after LPS administration and condition (TNF-α-: $F_{[2, 20]}$ =7.339; P= 0.003; IL-6: ($F_{[2, 18]}$ =9.892; P= 0.0013). LPS administration at ZT14 also induces higher TNF-α plasma levels in FD rats at 40 min (p <0.05; Fig 1C) and higher IL-6 plasma levels at 80 min (p <0.05; Fig 1D) as compared to CTRL rats. The two way ANOVA indicated a significant effect of time after LPS administration for both cytokines (TNF-α-: $F_{[2, 18]}$ =6.403; P= 0.007; IL-6: ($F_{[2, 20]}$ =10.08; P= 0.0009).

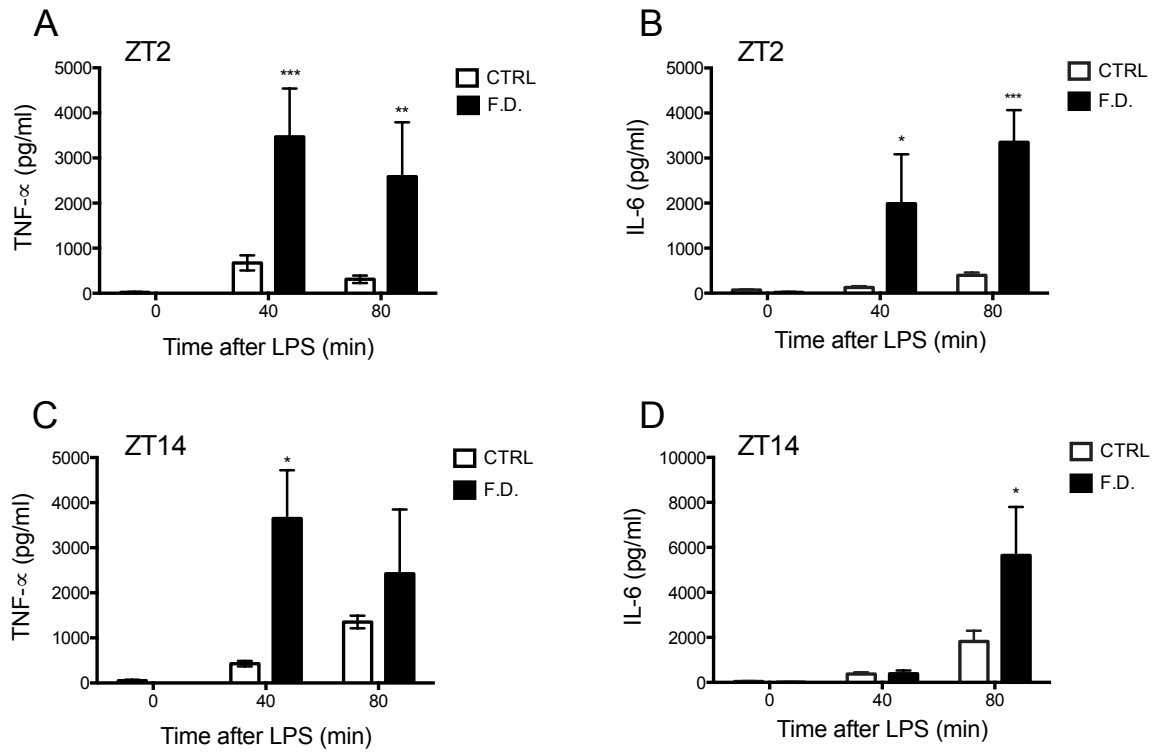

Figure 1. LPS administration at ZT2 to animals fed during the day (FD) triggered higher TNF-α (A) and IL-6 (B) plasma levels in comparison to CTRL rats. TNF-α (C) and IL-6 (D) plasma levels in CTRL and FD rats after LPS administration at ZT14. Cytokines concentrations were measured before and after iv, administration of LPS (2 μg/kg) at ZT2 or ZT14. Values are expressed as means ± s.e.m. (*n= 5-7*/group). Two way ANOVA and post hoc Bonferroni; *P < 0.005, **P < 0.01, ***P < 0.001 significance of differences vs. CTRL

Day-Night variations of IL-6 and TNF-α in response to LPS are lost in FD rats.

We have previously shown that LPS administration to CTRL rats at ZT14 triggered higher TNF-α and IL-6 plasma levels in comparison to LPS administrated at ZT2 (Guerrero-Vargas et al., 2014a). Here we confirm these findings and observed increased cytokines levels at 80 after the LPS administration at ZT14 ($p < 0.001$; Suplementary Figure 1). Importantly these normal day-night variations in TNF-α and IL-6 plasma levels after LPS administration were lost in FD animals. LPS administration at ZT2 to FD rats triggered similar plasma levels of TNF-α (Fig. 2A) and IL-6 (Fig. 2B) as LPS given at ZT14. The two way ANOVA indicated no significant interaction between time after LPS administration and ZT (TNF-α : $F_{[2, 16]}$ =0.019; P=0.980; IL-6: $F_{[2, 16]}$ =1.219 P= 0.321).

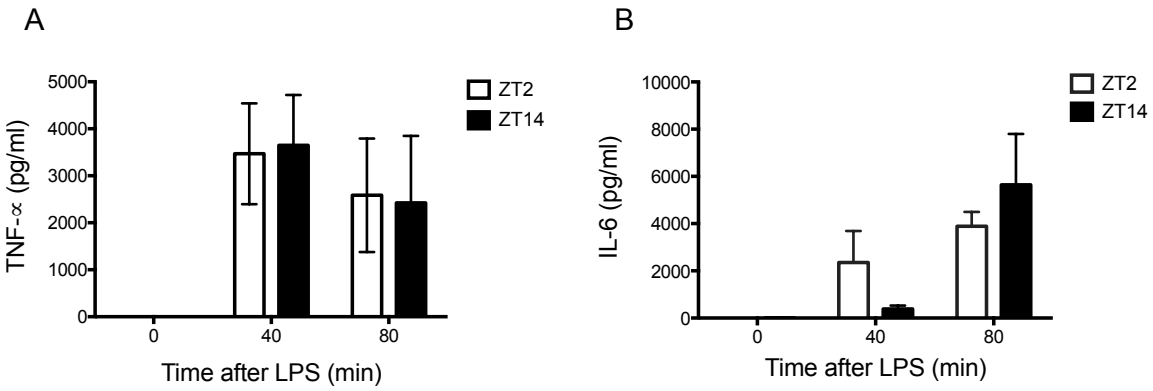

Figure 2. Similar TNF-α (A) and IL-6 plasma levels (B) were observed after LPS administration at ZT14 or at ZT2 in animals fed during the day (FD). TNF-α and IL-6 plasma concentrations were measured before and after iv, administration of LPS (2 μg/kg) at ZT2 or ZT14. Values are expressed as means ± s.e.m. (*n= 6-7*/group). Two way ANOVA and post hoc Bonferroni.

Food during the day increased the response of Kupffer cells to LPS stimulation.

Kupffer cells were isolated from FD and CTRL rats at ZT2. Kupffer cells isolated from FD rats produce more TNF-α at 6 and 24 h after LPS *in vitro* stimulation in comparison to Kupffer cells isolated from CTRL rats (p <0.01; Fig. 2). The two way ANOVA indicated significant interaction between time after LPS administration and condition ($F_{[4, 60]}$ = 5.005; P= 0.0015). No differences in TNF-α supernatant levels where found between CTRL and FD after HBSS (vehicle) administration ($F_{[4,40]}$ = 0.2067; P= 0.933) *(Data not shown)*.

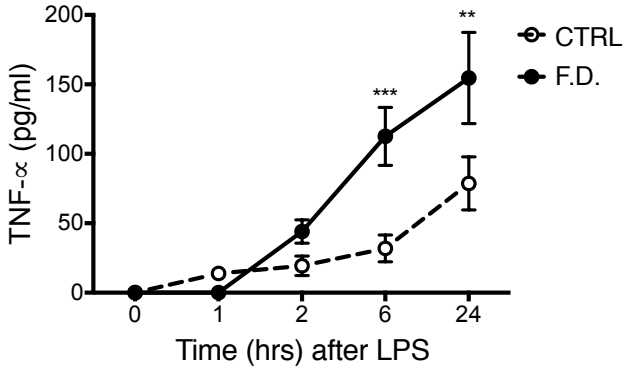

Figure 3. Kupffer cells isolated from rats fed during the day (FD) produce more TNF-α in response to LPS stimulation as compared to Kupffer cells isolated from CTRL rats. TNF-α was measured in Kupffer cells conditioned medium containing LPS (1μg/mL). Values are expressed as means ± s.e.m. Each point represents the average ± s.e.m. of 6-8 animals per group. Two-way ANOVA and *post hoc* Bonferroni; **P < 0.01, ***P < 0.001, significance of difference vs. CTRL.

Discussion

The results of the present study indicate that changing the normal food consumption pattern of nocturnal rats increased the inflammatory response to an LPS challenge. In addition to the increased inflammatory response observed in FD animals, also the normal circadian cytokines production in response to an LPS challenge disappeared. These changes could be based on the observed increased sensitivity of liver macrophages to LPS stimulation. Consequently food restriction to the normal rest phase induces enhanced and arrhythmic cytokines production in response to LPS administration.

Food consumption during the day increased the inflammatory response to LPS.

Besides using a small dose of LPS for all our experiments, FD schedule induced 8 times higher TNF-α and 3 times higher IL-6 plasma levels in comparison to CTRL rats in reponse to LPS adminstration at ZT2. Importantly increased cytokines levels were also measured in FD rats after LPS administration at ZT14. We have previously shown that the magnitude of TNF-α and IL-6 response after the administration of a low of LPS is time dependent (Guerrero-Vargas et al., 2014a). Here we confirm these findings and showed that the day-night differences in cytokines response after LPS administration are lost when animals eat only in the normal resting phase. Altogether these results point to an important role for the time of food consumption in the magnitude of the inflammatory response to LPS.

That FD increased cytokines production in response to an LPS challenge may be explained by the already reported effects of this protocol on the daily rhythms of certain variables in peripheral circadian clock expression. Several studies demonstrated that clock genes expressed in immune organs and cells play an important role in immune functions (Arjona and Sarkar, 2006a; Gibbs et al., 2014; Keller et al., 2009; Liu et al., 2006; Logan et al., 2013); recently we and others demonstrated that restriction of food to only the resting part of the day inverts or abolishes the rhythm of clock as well as metabolic genes in the liver (Damiola et al., 2000; Glad et al., 2011; Salgado-Delgado et al., 2013; Wu et al., 2010b). The liver, besides its metabolic role is also considered a key organ mediating inflammation and plays an important function in the elimination of systemic bacterial infection (Benacerraf et al., 1959; Gao et al., 2008; Gregory et al., 1996; Holub et al., 2009) mainly through the Kupffer cells capacity to recognize and respond to circulating LPS (Wu et al., 2010a). In this sense the effects of FD on the inflammatory response to LPS may be partially explained via the disruption of clock genes expression in the liver.

Another study reported that FD schedule exacerbated the immune response after cecal ligation and puncture procedure (CLP), a well-known model of sepsis via host barrier disruption. In this study, the authors reported decreased survival of FD rats after CLP, which was correlated with increased circulating cytokines levels (Oyama et al., 2014). An important difference between this and our study is the severity of the immune stimulus; although LPS could induce sepsis, this is only achieved after the administration of large doses of the endotoxin that are far from the dose used in our study (2μg/kg) that is about

50 times lower than the usual dose of LPS and 500 times lower than a septic dose. This suggests that small doses of LPS comparable with doses that might be liberated from the intestines after a fat meal (Cani et al., 2007) may induce a strong inflammatory response.

It is worth noting that in humans, the night eating syndrome (NES) is related with mood disorders such as depression (Colles et al., 2007; Gluck et al., 2001; Striegel-Moore et al., 2010), which has been suggested to have an inflammatory component (McNamara and Lotrich, 2012; Schiepers et al., 2005). Regarding our findings, it would be important to investigate whether humans with NES are also more prone to develop certain inflammatory diseases.

Food intake in the normal resting phase sensitized Kupffer cells to LPS.

That FD increased the sensitivity of Kupffer cells to LPS stimulation emphasizes that this altered food schedule not only induces liver steatosis but also Kupffer cell functioning. Our observation also concurs with studies showing that disrupted clock genes expression affect the immune function of other macrophages populations (Hayashi et al., 2007; Silver et al., 2012b). Since the FD protocol changes the clock gene expression in the whole liver, it is likely that also the clock gene expression in Kupffer cells isolated from FD rats is affected. Inverted food schedules may also affect only certain Kupffer cells subpopulations in the liver, for example especially the population located in the periportal area that showed increased production of pro-inflammatory cytokines and phagocytic activity as compared to Kupffer cells located in other areas of the liver (Laskin et al., 2001).

Does the SCN contribute to the enhanced inflammatory response to LPS of rats fed during the day?

We have previously demonstrated the important role of the SCN in the regulation of the inflammatory response to LPS (Guerrero-Vargas et al., 2014a). Restricted feeding changes clock gene expression in peripheral tissues, but does not change them in the SCN (Damiola et al., 2000; Oyama et al., 2014; Schibler et al., 2003). That the brain could regulate the immune response via the autonomic nervous system has been suggested by several studies (Felten et al., 1993; Martelli et al., 2014; Rosas-Ballina and Tracey, 2009; Tracey, 2002). Another explanation for the increased inflammatory response observed in FD rats may be a failure not only in SCN activity itself but in the communication with

other brain areas for example with the PVN an important autonomic output to immune organs, such as the liver. More studies are necessary to clarify this.

In summary, our study indicates that altered food consumption during the normal resting phase increased and desynchronized the inflammatory response to an LPS challenge. Importantly FD also affects the function of Kupffer cells.

Since altered feeding schedules (de Assis et al., 2003; Lennernas et al., 1995; Pasqua and Moreno, 2004) and increased risk of develop certain metabolic and inflammatory related diseases have been reported in human shift workers, and possibly in humans with NES, we suggest that food pattern consumption plays an important role in the regulation of the immune response and possibly on the development of obesity-related inflammatory diseases. Therapies that involved the maintance of normal food intake patterns may prevent the development of these diseases.

Competing Interests

The authors have declared that no competing interests exist.

Acknowledgments

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Supplemental Data

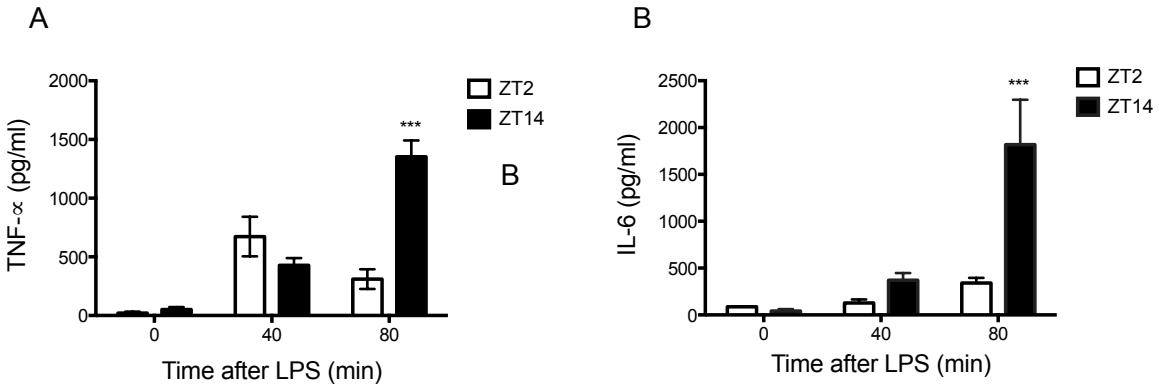

Figure S1. Administration of 2 μg/kg of LPS to intact rats at ZT14 triggered higher (A) TNF-a and (B) IL-6 plasma levels as compared to ZT2 LPS administration**.** IL-6 and TNF-a plasma concentrations were measured before and after iv, administration of LPS at ZT2 or ZT14. Values are expressed as means ± s.e.m. (*n*= 5/group) . ***P< 0.001 significance of differences vs. ZT2. The two way ANOVA indicated a significant interaction between time after LPS administration and ZT (TNF-α: $F_{[2, 22]}$ =22.25; P=<0001; IL-6: $F_{[2, 18]}$ =6.008; P= 0.01).

CHAPTER 5

A hepatic spinal sensory reflex pathway regulates the inflammatory response to Lipopolysaccharide

Natalí N. Guerrero-Vargas, Fernando Cazerez-Márquez, María del Carmen Basualdo and Ruud M. Buijs

To be submitted

Abstract

The brain is able to influence the response of the immune system via humoral and neuronal pathways, yet how immune information is transmitted to the brain is not completely understood. In the present study we analyzed possible immune sensory pathways between the liver (the main filter of circulating bacteria in the body) and the brain. Hereto we investigated, by selective hepatic denervation procedures, the importance of the autonomic innervation of the liver in sensing lipopolysaccharide (LPS) and for the regulation of the inflammatory response. Intravenous LPS administration to male Wistar rats induces neuronal activity (measured by c-Fos expression) e.g. in the dorsal horn (DH) of the spinal cord (T5-T7), which disappeared after specific sympathetic denervation of the liver. In contrast, c-Fos expression in the DH after LPS stimulation was not affected in rats with hepatic parasympathetic denervation. Hepatic sympathetic denervation also resulted in a 3 times increased TNF-α plasma levels in comparison to Sham and rats with hepatic parasympathetic denervation.

These data show that the liver is able to communicate inflammatory information to the brain via a spinal sensory pathway and points to an important role of the hepatic sympathetic branch in the regulation of the inflammatory response to LPS. Together our results provide evidence for the existence of a "sympathetic spinal sensory reflex" that serves to inhibit the inflammatory response.

Introduction

The brain plays an important role in the regulation of peripheral immune responses through hormonal (neuroendocrine) and neuronal pathways. Several studies have demonstrated a role for the vagal output of the brain in the regulation of the immune response to LPS using subdiaphragmatic denervation procedures or vagal stimulation (Bernik et al., 2002a; Borovikova et al., 2000; Martelli et al., 2014)Recently that view was challenged and a major role for the sympathetic nerves in the inhibition of the immune response was proposed (Martelli et al., 2014). Also the mechanisms (immuno-sensory mechanisms) whereby peripheral inflammatory information may reach the brain are still controversial. Two possible communication pathways have been described, a neural sensory route represented by the sensory neurons of the nodose ganglion of the vagus innervating mainly organs of the abdominal cavity, and a humoral pathway that involves the action of immune mediators in the circumventricular organs (CVOs) and choroid plexus, followed by the propagation of these immune signals into the brain parenchyma (Dantzer et al., 2000).

The liver is considered as the main metabolic organ of the body (Kmiec, 2001) while it also plays an important role by the elimination of systemic bacterial infections (Benacerraf et al., 1959; Gregory et al., 1996; Holub et al., 2009). Importantly it is a main filter for circulating lipopolysaccharide (LPS, a component of gram-negative bacteria) in the body (Jirillo et al., 2002) and contains one of the largest resident macrophages populations (Kupffer cells) that are able to recognize and respond to LPS (Wu et al., 2010a) producing pro-inflammatory cytokines such as TNF-α. The liver as other visceral organs communicates with the brain by sympathetic and parasympathetic nerves, both containing afferent as well as efferent fibers (Craig, 2002) as well as by hormones or growth factors that may directly pass the blood brain barrier (Bookout et al., 2013). The neurons in the nodose ganglion receiving sensory input from the liver transmit this information to the nucleus of the solitary tract (NTS) in order to be distributed over numerous areas in the fore an hind brain. Spinal afferent input from the liver enters the brain via dorsal root ganglion neurons with axons terminating in the dorsal horn (DH) of the spinal cord. Spinal sensory pathways to the DH from the abdominal cavity have been described for their capacity to transmit pain signals mainly from the gastrointestinal tract (Almeida et al., 2004) and recently for transmitting metabolic information from the liver

portal system (Fujita and Donovan, 2005). Their possible role in sensing peripheral immune information has not yet been investigated

Here we tested the hypothesis that immune information can reach the brain by hepatic spinal afferents activating an autonomic reflex influencing the inflammatory response to LPS. Hereto we analyzed the dependence of this reflex on hepatic sympathetic and parasympathetic input/output via selective denervation procedures. Inflammation was induced by the administration of a low dose of LPS during the dark period. We have previously shown that LPS administration during the night (ZT14) is associated with increased TNF-α plasma levels and brain activation as compared to day-time LPS administration (ZT2) (Guerrero-Vargas et al., 2014a). Rats were sacrificed 50 min after the challenge in order to be able to analyze the effects of LPS *per se* on neuronal activation of the DH. As a marker of neuronal activity in the DH we analyzed the product of the early gene c-Fos; which can be detected at the earliest within 50-60 min after stimulation (Hoffman et al., 1993). The results of the present study clearly illustrate that the spinal sensory nerves play an important role in the signaling pathway to the central nervous system and that without the sympathetic innervation of the liver an enhanced immune response is evince.

Methods

Animals and general housing conditions

Adult male Wistar rats weighing 200 to 220 g at the beginning of the experiments were obtained from the animal facility of the Faculty of Medicine of the UNAM. Animals were housed in individual cages and maintained in a soundproof monitoring room with a 12:12 h light-dark cycle (LD), lights-on at 7:00, defined as Zeitgeber time 0 (ZT0) and lights off at 19:00 defined as Zeitgeber time 12 (ZT12). The room was maintained at a regulated temperature of ranging between 22-25 °C and with continuous air flow. All rats were housed under specific pathogen free conditions and given free access to water and food (Rodent Laboratory Chow 5001, Purina, Minnetanka, MN, USA). Experimental procedures used in this study were in strict accordance with the Mexican norms for animal handling Norma Oficial Mexicana NOM-062-ZOO-1999, which conforms to international guidelines for animal handling, and were approved by the Ethics Committee in the Instituto de Investigaciones Biomédicas UNAM. All efforts were made to minimize the number of animals and their suffering.

Treatment Solutions and Reagents

For all experiments, inflammation was induced by a single intravenous (iv) injection of lyophilized LPS (100 mg, *Escherichia coli* serotype 0127:B8, Sigma-Aldrich, St. Louis, MO, USA Lot No. 051M4004) reconstituted with 0.9% sterile pyrogen-free saline (Baxter, México) to derive a 1mg/ml stock solution that was aliquoted and stored at -20°C. For the administration, aliquots were thawed and diluted with 0.9% sterile pyrogen-free saline yielding a final concentration of 2 μg/kg.

Surgical procedures

All surgeries were performed using aseptic procedures and under deep anesthesia using a combination of ketamine (90 mg/kg Cheminova, México) and xylazine (10 mg/kg, Procin^{R}, Pisa, México) anesthesia.

Hepatic sympathectomy

A laparotomy was performed in the midline. The liver lobes were gently pushed up, and ligaments around the liver lobes were severed to free the bile duct and portal vein complex, which were isolated from each other. At the level of the hepatic portal vein, the hepatic artery breaks up into the hepatic artery proper and the gastroduodenal artery. This division occurs on the ventral surface of the portal. At this point, the arteries were separated via blunt dissection from the portal vein. Nerve bundles running along the hepatic artery proper were visualized and were removed using microsurgical instruments under an operation microscope (25X magnification). Any connective tissue attachments between the hepatic artery and portal vein were also broken, eliminating any possible nerve crossings. The effectiveness of the denervation was checked by immunohistochemistry of the liver for tyrosine hydroxylase (TH) and Neuropeptide Y (NPY), both markers for sympathetic efferent fibers (Akiyoshi et al., 1998; el-Salhy et al., 1993). A sympathetically denervated rat was included in the analysis only if NPY and TH staining was less than 10%.

Hepatic parasympathectomy

A laparotomy was performed in the midline. The fascia containing the hepatic branch was stretched by gently moving the stomach and the esophagus. The hepatic branch is revealed as it separates from the left vagal trunk. With a binocular-operating microscope, the neural tissue was transected between the ventral vagus trunk and the liver. Some other

small branches running in the fascia between the stomach and the liver were also transected. Particular care was taken not to damage the dorsal and ventral trunks innervating the stomach and abdominal tissues or blood vessels that run along the hepatic vagus branches. We have previously validated our method for selective hepatic parsympathectomy by using retrograde tracing (Kalsbeek et al., 2004).

Total liver denervation

A total liver denervation was achieved by cutting both the sympathetic and parasympathetic branches to the liver. Rats with sham denervation surgery, as described above except for cutting the nerve, served as the control group.

Intra-jugular cannula insertion

Three weeks after denervation procedures, a polyethylene silicon tube cannula (0.025 in. i.d. and 0.047 in. o.d.; Silastic Laboratory tubing; Dow Corning Corp., Midland, MI, USA) was inserted in the internal jugular vein. The cannula was filled with heparin (500 U/ml) as anti-coagulant. The outer end of the cannula was fixed in the back between both shoulder blades and plugged with a small nail. Rats were allowed to recover during 7 days before experiments.

Sample collection and ELISA

Blood samples (300µl) were collected in Microvette®/500 tubes (Sarsted, Nümbrecht Germany) before LPS or vehicle infusion (0 min) and post-infusion times 20, and 50 minutes. Samples were centrifuged at 7000 rpm, during 7 minutes. Plasma was stored in 60µl aliquots at -80 °C until assay. IL-6 and TNF-α were determined by ELISA according to the manufacturer's recommendations (ThermoFisher Scientific, Rockford, USA, #ER3IL65 for IL-6 and # ER3TNFA5 for TNF-α). Detection limits of the assays in plasma were <16pg/mL for IL-6 and < 15pg/mL for TNF-α. Standard curves were used to determine cytokines levels. Cytokine levels are expressed as picogram /mL.

Immunohistochemistry

Rats were deeply anesthetized with an overdose of sodium pentobarbital (Pisabental, PiSa, Atilalaquia, Hgo, México; 65 mg/mL) and perfused transcardially with 150 mL 0.9% sterile pyrogen-free saline, followed by 150 mL 4% paraformaldehyde diluted in phosphate buffer (PBS, 0.1 M, pH 7.2). Brains, the spinal cord and a part of the left

lobule of the liver were removed, post fixed and kept in fixative for 24h, at 4°C, and cryo-protected in 30% sucrose for 3 to 4 days. The spinal cords and the livers were frozen and cut in coronal sections of 40μm at –20°C. DH sections were serially collected and processed for c-Fos immunohistochemistry. Free-floating DH sections were incubated overnight under constant shaking at 4° with rabbit c-Fos antibody (1:4000; Calbiochem, Cat. No. PC38. CA, U.S.A.) diluted in PBS with 0.5% Triton X-100 (Sigma-Aldrich), 0.025% BSA (ROCHE). Subsequently, sections were rinsed and incubated (90 min), at room temperature with biotinylated donkey-antirabbit serum (1:500, Jackson, ImmunoResearch Laboratories inc., Baltimore, U.S.A.) rinsed and incubated in avidin-biotin complex (1:500 Vector Laboratories) for 1 hour; product visualization was obtained with 0.025% diaminobenzidine, 0.05% nickel ammonium sulfate and 0.01% hydrogen peroxide in TBS (0.01M) for 12 minutes. DH sections were mounted, dehydrated, and cover-slipped with microscopy Entellan New (Merck, Whitehouse Station, NJ).

Immunohistochemistry of TH and NPY in the liver

Livers sections from Sham, Psx, Sx and CD rats were processed for TH and NPY immunohistochemistry separately. In order to diminish liver intrinsic peroxidase reactivity, sections were first incubated with hydrogen peroxidase 3% diluted in PBS for 10 min under constant shaking at room temperature. Subsequently, sections were rinsed and blocked in PBS solution containing, 0.5% fetal bovine serum and 0.5% bovine serum albumin for 10 min at room temperature. Free-floating liver sections were incubated independently for 1 hour at room temperature and overnight at 4° with rabbit TH antibody (1:300; Millipore U.S.A.) or NPY (Buijs, 1989) diluted in TBS with gelatin 0.25%, Triton X-100 at room. Subsequently, sections were rinsed and incubated (1 hour), at room temperature with biotinylated donkey-anti-rabbit serum (1:500, Jackson, ImmunoResearch Laboratories inc., Baltimore, U.S.A.) rinsed and incubated in avidin-biotin complex (1:500).

Cell Count

In order to quantify immunoreactivity of c-Fos (Fos-IR) in the DH, six sections were used and pictures were taken using an Axioplan microscope (Zeiss, Jena, Germany) equipped with a digital color camera (Olympus, DP25, Japan). Six pictures were taken from one side on each section.with a 20x ocular. Immunoreactive-positive nuclei in the

DH were counted from lamina I to IV in thoracic levels 5 to 7 (T5-T7) using a computerized image analysis system (Image J, 1.42q, National Institutes of Health Bethesda, MD). The background was subtracted and threshold was determined, particle analysis was set for particles of 0.5-1.0 circularity and 20-200 pixels. Background optic density was established in a nearby region lacking Fos-IR.

Data analysis

All data are presented as mean ± standard error of the mean (s.e.m). TNF-α plasma levels after LPS administrations were compared with a two-way (Time after LPS x Condition (Sham, Psx, Sx and CD) ANOVA for repeated measures for time after LPS followed by Tukey´s post-hoc test for multiple comparisons. Fos-IR in the DH was analyzed with a one-way ANOVA, followed by Tukey´s port-hoc test for multiple comparisons. Statistical comparisons were performed using Graphpad Prism version 6 for Mac. Threshold for statistical significance was set at $\alpha = 0.05$.

Results

LPS administration activates Dorsal horn neurons; hepatic sympathectomy prevents this

We analyzed neuronal activity as demonstrated by c-Fos staining in the DH of Sham, Psx, Sx and CD rats after LPS administration. C-Fos expression was also analyzed in Sham rats that received vehicle. LPS administration to Sham and Psx rats triggered a significant increase of c-Fos expression in the DH as compared to Sham vehicle administration ($p < 0.05$). Importantly, in Sx and CD rats, LPS administration fails to induce c-Fos expression in the DH as compared to Sham and Psx rats ($p<0.05$). C-Fos levels in the DH of Sx and CD were not different from Sham animals that received vehicle administration (*Data not shown*). The one-way ANOVA indicated a significant effect for condition ($F_{[4, 22]} = 6.686$; $P = 0.0011$; Fig. 1)

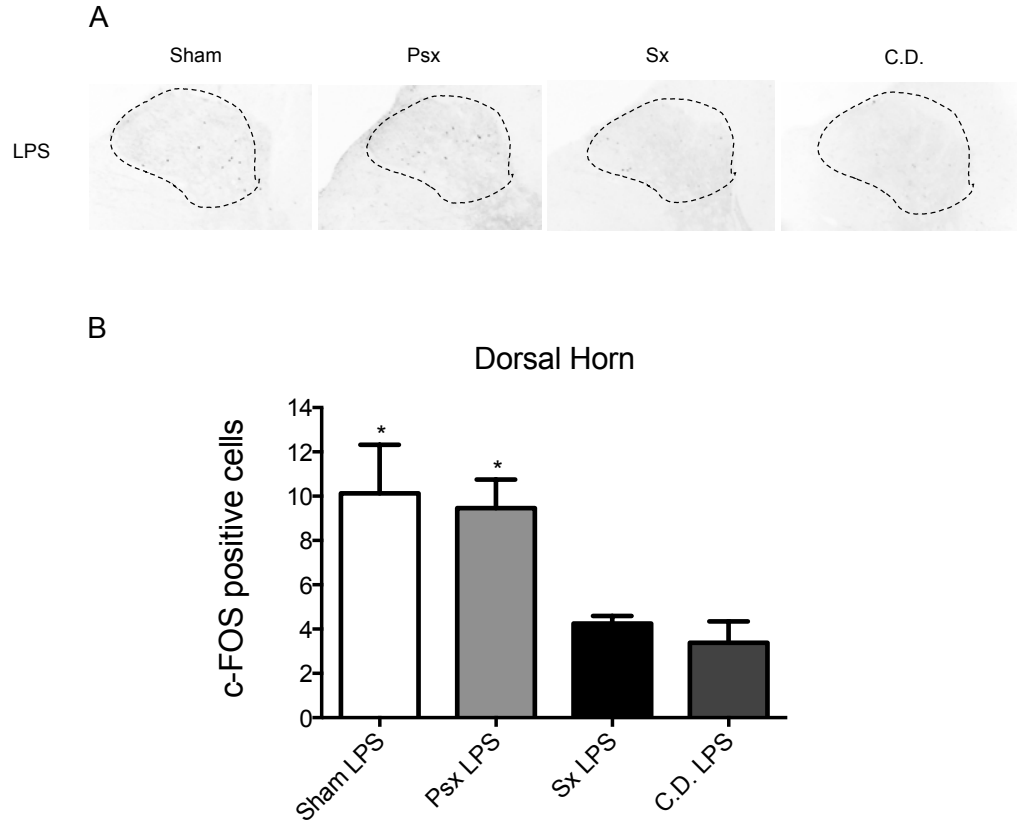

Figure 1. The dorsal horn (DH) in the spinal cord receives immune information from the liver after an LPS challenge. (A) DH photomicrographs (T5-T7). LPS administration induces higher expression of c-Fos protein in laminae I-IV in sham and in rats with hepatic parasympathetic denervation (Psx) in comparison to rats with hepatic sympathetic denervation (Sx) and rats with complete liver denervation (CD). Scale bar = 300 μm. (B) The number of c-Fos positive cells in the DH was counted bilaterally in 3 representative sections. The enclosed area corresponds to laminae I-IV in each picture. Animals received iv LPS (2μg/kg) or vehicle. All values are expressed as means ± s.e.m (*n= 5*-8/group). One-way ANOVA and *post hoc* Tukey; *P < 0.05 significance of difference vs Sx LPS and CD LPS.

Hepatic sympathetic denervation increased TNF-α production after LPS administration

LPS given iv, to Sham , Px, Sx and CD rats resulted in very low or undectable basal TNF-α plasma levels, as measured by the time 0 and were not different between groups. TNF-α plasma levels were similar in all groups 20 min after LPS administration. However 50 minutes after LPS administration higher TNF-α plasma levels were measured in Sx and CD rats at ($p < 0.001$) in comparison Sham and Psx rats (Fig. 2). The two way ANOVA indicated a significant interaction between condition and time after LPS administration ($F_{[6, 30]} = 7.199$; $P < 0.0001$).

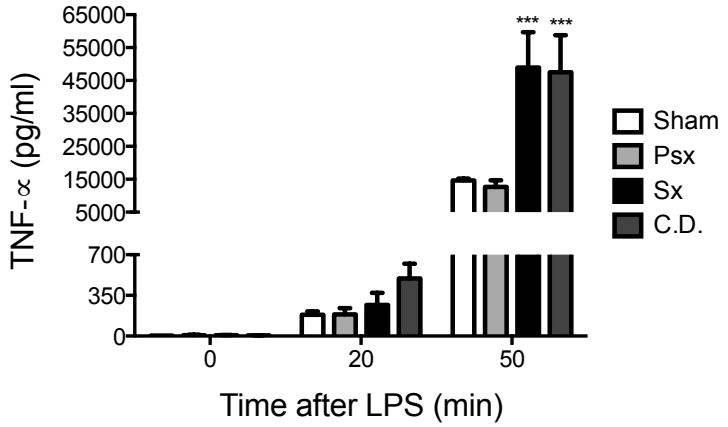

Figure. 2. Sympathetic (Sx) and complete hepatic denervation (CD) increased TNF-α production after an LPS challenge. Higher TNF-α plasma levels were measured in Sx and CD rats 50 min after LPS stimulation in comparison to Sham and rats with hepatic parasympathetic denervation (Psx) rats. TNF-α plasma levels plasma concentrations were measured before and after iv, administration of LPS (2μg/kg). Values are expressed as means ± s.e.m. (*n= 4-5*/group). Two-way ANOVA and *post hoc* Tukey ***P < 0.001 significance of differences vs. Sham and Psx.

Discussion

In the present study we provide for the first time evidence that an immune challenge with LPS is sensed by the liver (possibly Kupffer cells) moreover, this sensory information is not only transmitted by vagal sensory nerves but importantly by spinal sensory nerves activating neurons in the laminae I-IV of the DH of the spinal cord segments T5-T7. At the same time we demonstrated that the removal of the liver sympathetic innervation increases substantially the inflammatory response after LPS administration. This suggests the involvement of this sensory pathway in a sympathetic reflex aimed to decrease the inflammatory response to LPS. That such effect is indeed due to the removal of the liver sympathetic innervation is demonstrated by a similar enhanced inflammatory response after complete liver denervation and its absence by selective parasympathetic denervation.

It is necessary to determine if other areas of the brain, besides the DH, are activated after the administration of a low dose of LPS; however some of our previous observations indicate that the administration of this dose of LPS is capable to induce c-Fos expression in the NTS (the major sensory recipient from both vagal and spinal afferents that

innervates the viscera), Area Postrema (AP; a circumventricular organ capable of detect circulating immune information) and in the suprachiasmatic nucleus (SCN).

Using the current low dose of LPS, we did not observe any indication of intermediolateral nucleus (IML) neuronal activity or activity of the DMV; this can be explained by the fact that the used dose was too small for such activation. At the other hand previous observation using a 50 times higher dose of LPS showed indeed IML activation and not DMV activation which may agree with earlier studies showing that LPS induce c-Fos expression in IML indicating activation of sympathetic efferent (Tkacs and Strack, 1995). Since connections between the sensory neurons in the DH and the IML have been demonstrated (Buijs, 2013) these data indeed suggest the existence of a sympathetic immune reflex that could play a role in the regulation of the inflammatory response.

Hepatic sympathetic innervation regulates the inflammatory response to LPS

Specific and complete denervation procedures allow us to study the role of the autonomic innervation of the liver in the regulation of the immune response.

Hepatic parasympathectomy (Psx) does not change the TNF-α response after LPS administration, since similar TNF-α plasma levels were found in Psx and Sham animals at every measured time-point. Although an inhibitory role of the vagus nerve on the inflammatory response as been suggested (Tracey, 2002), other studies have demonstrated that severing all vagal input below the diaphragm (subdiaphragmatic denervation) does not affect the production of TNF-α after LPS administration (Caldwell et al., 1999; Martelli et al., 2014). These studies are in line with our results and suggest that the inflammatory response to LPS is not regulated via the parasympathetic branch.

Other studies suggest that the immune information after an LPS could reach the brain at the level of the NTS through the vagus nerve (Gaykema et al., 1998; Goehler et al., 1998), however subdiaphragmatic vagal denervation diminish but does not eliminate c-Fos expression in the NTS. As we already mentioned the NTS receives sensory input from both vagal and spinal afferents, importantly projections from neurons located in laminae I and IV of the DH have also been demonstrated (Esteves et al., 1993; Gamboa-Esteves et al., 2001). This together with our results demonstrating neuronal activation of the DH after LPS administration, suggest that immune information could be conveyed to the brain via spinal sensory pathways.

In contrast to Psx, hepatic sympathetic denervation (Sx) and complete denervation (CD) were associated with decreased neuronal activity in the DH as compared to Sham and Psx rats. Since c-Fos expression in the DH of Sx rats was similar to that observed in the DH of Sham animals that received vehicle, this suggests that the immune information could be conveyed to the brain via spinal sensory pathways and that the lack of hepatic sympathetic innervation interferes with this communication pathway.

Recently a role for the sympathetic nervous system in the regulation of the inflammatory response to LPS has been suggested (Martelli et al., 2014). After the section of the splanchnic sympathetic nerve increased TNF-α plasma levels in response to LPS were measured. However the splanchnic nerve innervates many visceral organs including the liver, which does not allow to determine a specific role for each organ in the regulation of this response. Our results extend these findings and demonstrate that the liver is able to sense immune information and send it to the brain through spinal sympathetic afferent in order to regulate TNF-α production.

The reflex efferent arm deserved more investigation, however our results suggest that must be sympathetic and not vagal, indeed sympathetic neuropeptides release could play an important role since for other tissues their anti-inflammatory actions had been demonstrated (Kees et al., 2003; Straub et al., 2006). Future analysis will be aimed to understand how hypothalamic structures will be activated especially since recently we have demonstrated that the SCN regulates the magnitude of the inflammatory response to LPS (Guerrero-Vargas et al., 2014a). The anatomical basis for the SCN control of the liver via autonomic pathways has also been demonstrated (la Fleur et al., 2000). This together with the present results suggest that the SCN could be informed of the inflammatory status of the body via this spinal sensory pathway and in turn could regulate the intensity of the inflammatory response via sympathetic output to the liver. More studies are necessary to clarify this.

Competing Interests

The authors have declared that no competing interests exist.

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Natalí N. Guerrero-Vargas, Fernando Cázerez-Marquez and María del Carmen Basualdo, contributed to the acquisition of the data. Natalí N. Guerrero-Vargas, Fernando Cázerez-Marquez and Ruud M. Buijs contributed to the analysis and interpretation of the data. Ruud M. Buijs and Natalí N. Guerrero-Vargas initiated and designed the study.

Natalí N. Guerrero-Vargas, Fernando Cázerez-Marquez and Ruud M. Buijs wrote the paper. All authors declare that there are no conflicts of interest.

CHAPTER 6

General Discussion

Contents

I. THE SCN REGULATES THE INFLAMMATORY RESPONSE

The inflammatory response is highly organized, aimed to protect the body from foreign agents (virus or bacteria) and damaged cells. However at the other hand exaggerated inflammatory responses could result in the development of disease (Kuek et al., 2007). Therefore a balanced regulation of the inflammatory response is essential to maintain body homeostasis. As every physiological function also the inflammatory response is subject to circadian regulation; we demonstrated that the inflammatory response to LPS is higher at night as compared to the day and dependents on SCN activity (Guerrero-Vargas et al., 2014b). This is also supported by our finding that constant light exposure, considered as an artificial lesion of the SCN suppressing circadian rhythms in almost all physiological variables (Eastman and Rechtschaffen, 1983; Honma and Hiroshige, 1978; Wideman and Murphy, 2009), also exacerbates the inflammatory response to LPS (Fig. 1).

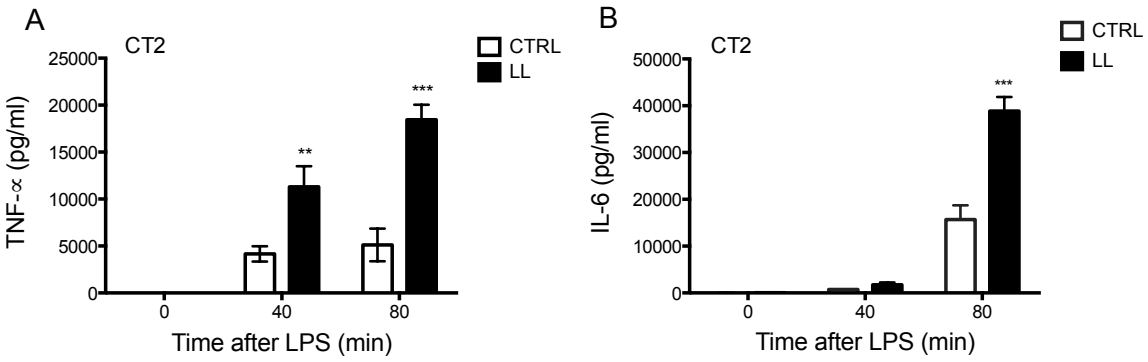

Figure 1. Rats exposed to constant light conditions (LL, 24 hours of light) during four weeks, produced more TNF-α and IL-6 after LPS administration as compared to control rats (A) TNF-α and (B) IL-6 plasma levels were higher in LL rats (n=4) after LPS stimulation at CT2 (Circadian time 2) in comparison to control rats (CTRL *n= 6-7*/group). The two way ANOVA indicated a significant interaction between time after LPS administration and condition (TNF-α: $F_{[2, 18]}$ =13.75; P=0.0002; IL-6: $F_{[2, 20]}$ =27.43; P< 0.0001). Post hoc Bonferroni. **P < 0.01, ***P < 0.001 significance of differences vs. CTRL.

Prolonged light exposure disrupts the organization of SCN neurons resulting in overall decreased neuronal activity (Ohta et al., 2005). These data, together with our observations that low or lack of SCN neuronal activity is associated with increased inflammatory response (chapter 2, chapter 3), strengthens the role of the SCN in the regulation of the of the inflammatory response to LPS. In principle there are two possibilities for the SCN to regulate the immune response. 1. It may set the day night response level of the immune system. 2. It may actively regulate the immune response.

It is important to note that in order to have a regulatory role on the immune response, the SCN need to be informed about the immunological state of the body. In chapter 2 we demonstrated that after intravenous LPS administration, the SCN show increased neuronal activation (Guerrero-Vargas et al., 2014b) suggesting that indeed the SCN might have a regulatory role in the immune response of the peripheral organs. Although we have not studied in detail the nature of the activated neurons, c-Fos expression in response to LPS administration was clearly visible in the ventro-lateral part of the SCN where mainly vasoactive intestinal peptide (VIP) and vasopressin containing neurons are present (Card et al., 1981). Further analysis characterizing the involved cell population, could help to design future experiments aimed to test the inhibitory role of the SCN in the

inflammatory response using techniques that allow us to silence or activate specific SCN neurons at certain times of the day coupled to LPS administration.

As reviewed in the introduction, at least two different pathways (humoral and neural) are proposed to be available for immune molecules and cells to communicate with the brain. In chapter 5 we have explored possible communication pathways and demonstrated that the liver, an important organ for LPS clearance and detection (Jirillo et al., 2002), is able to communicate immune information to the brain including the SCN via a spinal a sensory pathway that will be discussed in the next section.

In order to better understand the possible pathways available for the SCN to regulate the immune response, it is important to consider that the SCN transmits its temporal message to every organ in the body using hormonal and neuronal pathways. This suggests that these pathways could also be used by the SCN to target organs and cells of the immune system.

HORMONAL PATHWAYS

Glucocorticoids

Humoral factors such as corticosteroids and melatonin that are under circadian control are classically viewed as major regulators of the immune response. One of the best studied glucocorticoids, corticosteroid, is secreted in a circadian manner by the adrenal gland (Krieger, 1975) and is proposed to drive circadian rhythms of immune cells numbers and cytokine levels in the circulation under basal conditions (Petrovsky et al., 1998). However next to the normal circadian levels of the hormone, after LPS administration a surge in corticosterone plasma levels can be observed (Buijs et al 2008).

The increased release of corticosteroids in the course of an inflammatory response is seen as an important regulatory mechanism to counteract the over production of cytokines (Besedovsky and del Rey, 1992; Rivier and Rivest, 1993). These ideas are based on the powerful immune suppressive effects of artificial glucocorticoids such as dexamethasone but are not supported by evidence following induced inflammation, for example by LPS. Indeed, *in vitro* and *in vivo* studies have shown that glucocorticoids (such as dexamethasone) administration at high doses regulates cytokine production (Chensue et al., 1991; Coelho et al., 1995; Doherty et al., 1992). Similarly, glucocorticoids are also the mainstay therapy for diverse diseases that involve increased activation of the immune

system such as allergic and chronic inflammatory diseases. Apart from the higher affinity of dexamethasone for the glucocorticoid receptor, the levels reached by these treatments are far above the concentrations of the hormone as response to inflammation. Consequently the arguments that the corticosterone increase after inflammation has an anti-inflammatory effect are not supported yet by experimental evidence. In fact, others and our experiments strongly argue for the absence of such connection. For example LPS administration to SCN lesioned animals (SCNxx) triggers higher corticosterone levels than in intact animals (Kalsbeek et al., 2012), nevertheless these high levels do not diminish the cytokines production since we observed that SCNxx animals produced 10 fold higher cytokines after LPS stimulation as compared to control animals (chapter 2), indicating that corticosterone is not used to diminish the immune response. In agreement with this, recent evidence showed that glucocorticoids are necessary neither for rhythmic LPS-induced macrophage cytokine secretion nor for daily resetting by the master clock (Keller et al., 2009).

Melatonin

Immune-modulatory actions for the pineal produced hormone melatonin have also been described. Melatonin production is under strong circadian control and is considered as a biological signal of darkness.

In both diurnal and nocturnal animals, the secretion of melatonin reaches maximum levels in the middle of the night and gradually decreases by the morning. Nighttime melatonin levels have been related with the number and activity of immune cells in both human and rodents (Guerrero and Reiter, 2002). Early studies where surgical or functional removal of the pineal gland was performed (pinealectomy) showed that loss of pineal gland correlates with weight loss of thymus and spleen and with a decrease of their immune cells content and function (Brainard et al., 1988; Maestroni, 1996; McKinney et al., 1975). In agreement, melatonin administration improves the survival of rodents from lethal doses of LPS that normally cause septic shock and death (Maestroni, 1996; Requintina and Oxenkrug, 2003; Wu et al., 2001). The mechanism is thought to be via the inhibition of TNF-α and IL-6 production (Sullivan et al., 1996; Wu et al., 2001) as well as a decrease of nitric oxide NO levels (Escames et al., 2003).

Most of the reported actions of melatonin point to an anti-inflammatory role of the hormone, which could seem to contradict our findings of an increased inflammatory

response after night LPS administration (chapter 2). However at the time of our night LPS challenge (ZT14), melatonin plasma levels are hardly increased and are indeed comparable to those observed by daytime (Perreau-Lenz et al., 2005). In this sense, day-night difference in melatonin production does not seem to explain the time dependent response to LPS in our experimental design.

An interesting observation is that intravenous LPS administration during the nocturnal surge of melatonin decreases plasma levels of the hormone (Tamura et al., 2009) leading to the suggestion that the physiological relevance of melatonin reduction during inflammation could be related with the full mounting of the defense responses. Since melatonin reduces the rolling and adhesion of leukocytes (Lotufo et al., 2001; Lotufo et al., 2006), therefore indeed this suggests that the nocturnal melatonin surge should be suppressed in order to optimize immune responses. Since this contrasts with the observations that melatonin administration enhances the survival after high doses of LPS as cited above, it is clear that more work needs to be done to clarify the possible mechanisms.

NEURONAL PATHWAYS

Within the hypothalamus are different neurons projecting to the Dorsal motor nucleus of the vagus (DMV) or to the sympathetic motor neurons located in the intermediolateral column of the spinal cord (IML) (Buijs et al., 2003b). The SCN targets also with different neurons these pre-autonomic neurons in the hypothalamus directly or indirectly. Via these autonomic connections, the SCN is able to influence the functionality of all organs in the body including immune organs (Buijs et al., 2003a; Buijs et al., 2008). Considering this, we will discuss the evidence for a neuronal influence on the inflammatory response focusing on the effects of the SCN. Accepting the possibility that the brain may play a role in the immune response brings as a consequence the acceptance that the brain needs to be informed about the inflammatory status of the body. Therefore in the following discussion of the autonomic pathways that may play a role in the response of the brain towards the immune system we will discuss also the putative importance of the sensory autonomic pathways for informing the brain about the inflammatory status.

Parasympathetic innervation

Parasympathetic innervation of immune organs such as the thymus (Fatani et al., 1986; Micic et al., 1992) and the bone marrow (Artico et al., 2002) is documented. In the case

of the spleen, some studies fail to demonstrate parasympathetic innervation (Bellinger et al., 1993; Nance and Burns, 1989), whereas we have demonstrated parasympathetic input (Buijs et al., 2008). In order to study the role of parasympathetic pathways in the regulation of the immune response, several studies have used surgical procedures to remove the vagus nerve; the longest nerve of the parasympathetic system that innervates most of the peripheral organs including immune organs.

Sensory pathways

Afferent neurons of the vagus nerve express IL1R and prostaglandins (Ek et al., 1998) also mRNA and protein for TLR4 have been described in the nodose ganglion of the afferent vagus nerve (Hosoi et al., 2005) suggesting that local inflammatory molecules could be effectively signal the brain via the sensory vagus nerve.

Studies focusing on the sensory role of parasympathetic nerves have demonstrated that sub-diaphragmatic vagotomy, surgical cutting of the vagus nerve, attenuates the central effects it diminishes neuronal activation after intraperitoneal injections of moderate doses of LPS (Wan et al., 1994), suggesting a role of the sensory vagus nerve in the transmission of neuro-immune afferent information from the abdominal cavity and viscera to the brain (Maier et al., 2008). However when LPS is given intravenously, vagotomy minimally affects the neuronal activation of LPS (Wan et al., 1994), which suggests that the pathway used to transmit immune information to the brain may depend on the administration route and the strength of the stimulus.

Other studies where total vagotomy is performed, fail to block the increase in IL-1β or IL-6 (Bluthe et al., 1994; Hansen et al., 2000; Laye et al., 1995) in response to LPS administration suggesting the involvement of other neuronal pathways in the regulation of the inflammatory response to LPS.

Autonomic pathways

The currently most accepted theory for brain control of the inflammatory response is the neuronal parasympathetic reflex, according to this theory, efferent vagus nerve stimulation diminishes systemic and localized inflammatory processes (Tracey, 2009). However the mechanism of the reflex is not purely parasympathetic and is suggested to involve the activation of splenic nerves (sympathetic) (Rosas-Ballina et al., 2008) and the subsequent release of noradrenaline that activates a specific population of T cells in the

spleen that produce Acetylcholine (Ach), which interacts with specific receptors (α-nicotinic ACh receptors) present in macrophages and in this way inhibits TNF-α production (Rosas-Ballina et al., 2011). Despite the anti-inflammatory reflex has been widely accepted as the neuronal pathway that control the inflammatory response, some studies fail to demonstrate clear stimulatory effects of vagotomy on the inflammatory response to LPS (Bernik et al., 2002b; Fuentes et al., 2005; Martelli et al., 2014; Mihaylova et al., 2012). Similarly we also have observed that hepatic parasympathetic denervation do not influence cytokines production in response to an LPS challenge (chapter 5). Importantly we (chapter 5) and others (Martelli et al., 2014) have demonstrated that the sympathetic input to immune organs play an important role in the regulation of the inflammatory response to LPS.

Sympathetic pathways

The sympathetic nervous system (SNS) is proposed to be the main contributor of neural modulation of immune function in the thymus gland (Nance et al., 1987; Trotter et al., 2007) spleen (Cano et al., 2001; Nance and Burns, 1989), lymph nodes (Felten and Felten, 1988; Romeo et al., 1994) the bone marrow (Felten and Felten, 1988) and the liver (chapter 5).

Sensory pathways

The sensory role of sympathetic nerves in the transmission of immune information to the brain is not documented. We have studied the effects of selective sympathetic denervation of the liver on the induction of c-Fos in the brain after LPS administration and demonstrated that hepatic sympathetic denervation diminished neuronal activation of the DH, suggesting that sympathetic (sensory) innervation of the liver plays an important role in immune signaling from the liver to brain (chapter 5). This neuronal circuitry deserves further characterization and analysis, however we propose that at least including the activation of neurons in the dorsal horn (DH) of the spinal cord, the immune message from the liver may be transmitted to the brain through the NTS. It has been shown that the NTS receives projections from DH neurons located in laminae I and IV (Esteves et al., 1993; Gamboa-Esteves et al., 2001) and we and others have demonstrated also NTS neuronal activation after LPS administration (Elmquist et al., 1996). Importantly, NTS projections (mainly glutamatergic) to the ventrolateral part of the SCN have been recently described (Buijs et al., 2014). Therefore our demonstration of c-Fos expression in the

ventrolateral SCN after LPS administration (Guerrero-Vargas et al., 2014b) suggests that the NTS may transmit immune information from liver to SCN. Since the NTS receives sensory information both from the DH but also from the sensory vagus, we cannot distinguish yet from which sensory pathway the SCN may get its information. The diminishment of SCN activation after complete denervation and not after sympathetic or parasympathetic denervation suggests that both branches may contribute to the information reaching the SCN.

Because sympathetic denervation procedures remove both sympathetic afferent and efferent pathways it would be interesting to specifically remove sympathetic afferent neurons and investigate whether the immune to brain message transmission is also disrupted.

A sympathetic inhibitory reflex?

In chapter 5 we studied the contribution of liver autonomic innervation on the inflammatory response to LPS. Our results demonstrate that surgical removal of the sympathetic innervation of the liver results in exacerbated TNF-α production in response to LPS, which suggests an inhibitory role for liver sympathetic input in the inflammatory response to LPS. This is supported by a recent study demonstrating that cutting the sympathetic splanchnic nerve that provides sympathetic innervation to the spleen, liver, gastro-intestinal tract and adrenal glands enhanced TNF-α plasma levels after LPS administration (Martelli et al., 2014).

The inhibitory effects of the SNS in the inflammatory response may be mediated via the release of Norepinephrine (NE), the primary transmitter released from sympathetic nerve terminals. Receptors for NE (alpha-adrenergic receptors, αAR, and beta-adrenergic receptors βAR) are expressed in immune cells of both humans and rodents, indicating that SNS effects on immune cells can be direct (Kin and Sanders, 2006; Kohm and Sanders, 2001). Some studies demonstrated that adrenergic input to the spleen induces NK cells, leukocytes and macrophages activity via the release of NE (Bellinger et al., 1993; Nance and Burns, 1989). *In vitro* NE treatment inhibits the production of TNF-α in response to LPS (Yu et al., 2014). At the other hand NE input to the spleen shows a strong circadian rhythm and selectively sympathetic denervation disrupts daily variations in cytokines and cytolytic factors in NK cells and splenocytes (Logan et al., 2011).

In this sense, several studies suggest that increased sympathetic activity and β-adrenergic receptor activation regulate the production of pro-inflammatory cytokines after immune stimulation (Cole et al., 1998; Izeboud et al., 1999; O'Connor et al., 2007; Wahle et al., 2005).

Importantly, as we already mentioned, the communication between the SCN and peripheral tissues may occur through both branches of the autonomic nervous system. The anatomical pathways that demonstrate autonomic connections between the SCN and the liver, have been demonstrated (la Fleur et al., 2000), as well as the role of sympathetic nerves in the transmission of timing information from the SCN to the liver (Terazono et al., 2003).

Regarding the inhibitory effects of the SNS in the inflammatory response to LPS (chapter 5) and the above-mentioned evidence for SCN-liver communication via autonomic pathways, it is worth to speculate that the SCN may convey a regulatory message to the liver via the sympathetic nervous system. In this sense, it has been demonstrated that SCN lesions flattened the daily NE content in the liver (Terazono et al., 2003), which may contribute to explain the exacerbated inflammatory response of these animals after LPS administration (chapter 2). Nevertheless we could not discard the contribution of other peptides that are co-released with the classic neurotransmitters from sympathetic terminals such as NPY, substance P or somatostatin .

In the same way it is important to note that the autonomic nervous system is one of the main pathways used by the SCN to convey rhythmic signals to the rest of the body, although endocrine humoral pathways are also involved. Regarding our observations, we would like to suggest that the SCN is a critical modulator of the immune system by balancing sympathetic and parasympathetic outflow to immune organs such as the liver.

II. A MODEL FOR THE INFLUENCE OF THE SCN IN THE REGULATION OF THE INFLAMMATORY RESPONSE TO LPS

Based on the results of the present thesis we propose a model for the influence of the SCN in the regulation of the inflammatory response to LPS (Fig. 2). We demonstrated that the SCN, as well as other brain areas, are able to respond to the peripheral

administration of LPS (chapter 2). That LPS induces different immune responses depending on the administration time can be explained by the normal fluctuating neuronal activity of the SCN. Low SCN neuronal activity, a feature of the dark period, is associated with a high inflammatory response, whereas its high neuronal activity, a feature of the light period, is associated with low inflammatory response. Support for this proposal is the exacerbated inflammatory response to LPS observed in animals with SCN lesions (chapter 2) and after circadian desynchronization processes -induced by shift work and mistimed food consumption (chapter 3 and chapter 4). Importantly these conditions are also associated with low/disturbed SCN activity (Salgado-Delgado et al., 2010b).

In spite that night LPS administration induces neuronal activity in the SCN and is associated with an increased inflammatory response, we proposed that the neuronal activity induced by LPS may be aimed to counteract cytokines overproduction, in fact, our hepatic denervation-experiments reveals that when the SCN is not responding to a night LPS challenge, the inflammatory response is further increased.

Altogether the present evidence indicates that the interaction between the SCN and the immune system inhibits the inflammatory response. We propose that in order to regulate the inflammatory response, the SCN is integrated in an immune axis in which: 1) it receives immune information from the liver via humoral and neuronal vagal/spinal sensory pathways. Hereby sympathetic afferent projections from the liver to the SCN appear to play an important role in the spinal sensory pathway, since sympathetic denervation procedures diminish neuronal activation of the DH in response to LPS (chapter 5). 2) The SCN integrates immune information and conveys it to other hypothalamic areas such as the PVN (chapter 2). Importantly direct SCN input to autonomic neurons both vagal and sympathetic has been demonstrated (Buijs et al., 2003a; Cui et al., 2001; Teclemariam-Mesbah et al., 1997), 3) Because selective denervation of the sympathetic but not the parasympathetic input to the liver prevented the inhibitory actions of the SCN (chapter 5) and results in a increased inflammatory response, the inhibitory SCN message may reach the liver most likely through the activation of sympathetic pre-autonomic neurons in the PVN.

That circadian desynchronization processes induce exacerbated inflammatory responses similar to those observed in SCNxx animals may be explained by disturbed SCN activity

after desynchronization that could result in disrupted SCN output to immune organs. This will be discussed in the next section.

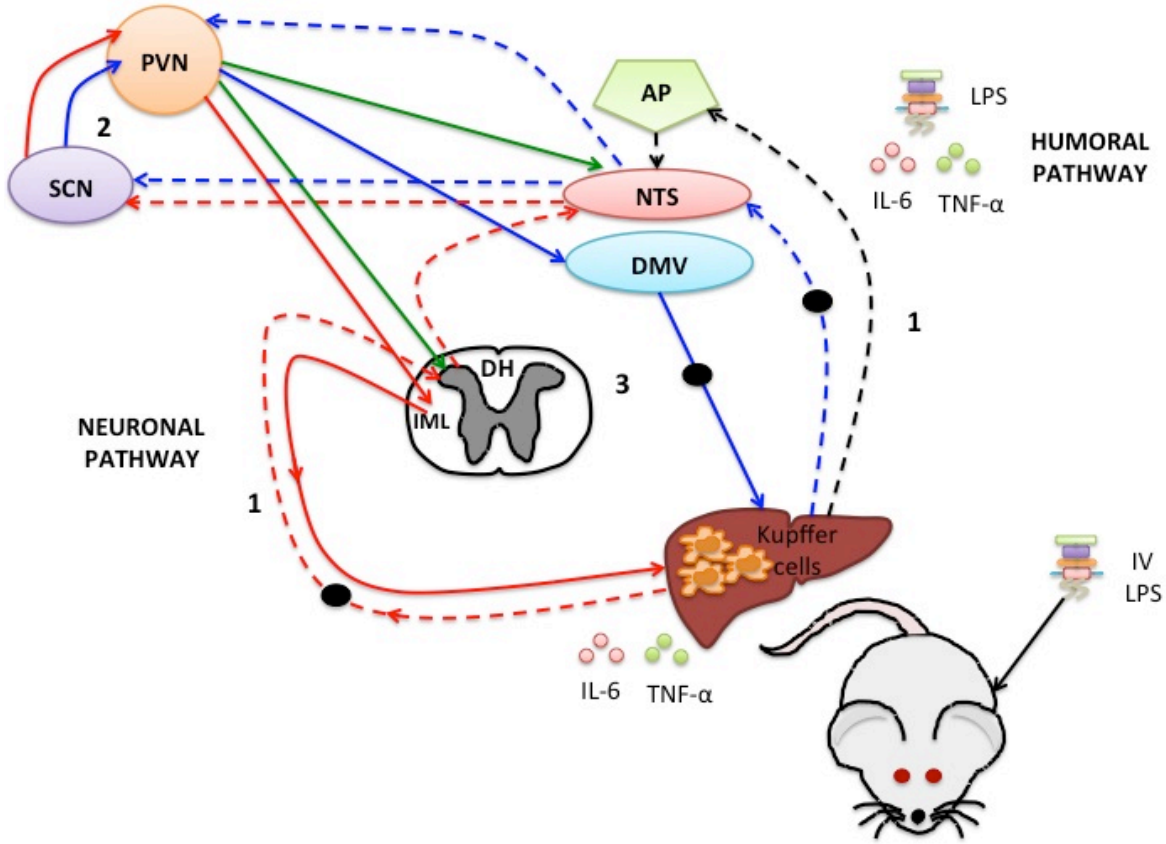

Figure 2. A model for the influence of the SCN in the regulation of the inflammatory response to LPS. Red lines represent sympathetic output, blue lines parasymphatetic output. Red dotted lines represent sympathetic brain input and dotted blue lines vagal input. Black dotted lines represent the humoral pathway. Green lines represent outputs from the PVN that modulate sensory inputs. AP, area postrema; DH, dorsal horn; DMV, dorsal motor nucleus of the vagus; IML, intermediolateral columns; IV, intravenous; NTS, nucleus of the tractus solitarium; PVN, paraventricular nucleus of the hypothalamus; SCN, suprachiasmatic nucleus.

III. CIRCADIAN DESYNCHRONIZATION AND DISEASE

Several epidemiologic studies suggest that people exposed to circadian desynchronization due to shift-work (SW), noctural work or even jet lag, are more prone to develop certain diseases some of which have an important inflammatory component. In chapter 3, we demonstrated that circadian desynchronization induced by shift-work in rats is associated

with an exacerbated inflammatory response to LPS, as well as increased sensitivity of Kupffer cells. Interestingly, inoculated tumor cells grew faster in SW rats in comparison to control rats (Fig. 3), suggesting that the increased sensitivity to LPS could be extrapolated to other pathologies such as cancer.

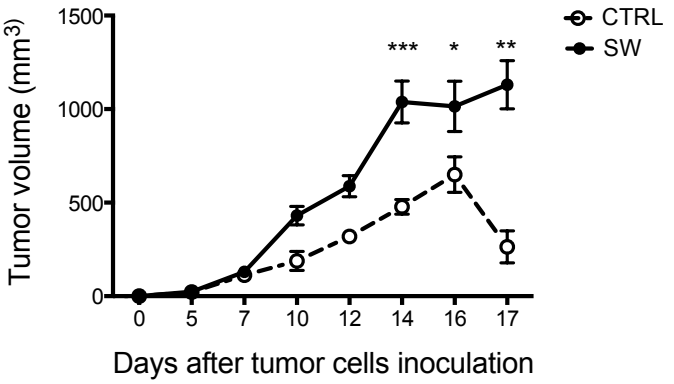

Figure 3. Shift work rats (SW) developed bigger tumors after tumor cells inoculation. Control rats (CTRL, *n=6*) are represented by open circles, SW rats (*n=10*) by solid circles. Values are expressed as means ± s.e.m. Two-way ANOVA indicated a significant interaction between time after tumor cells inoculation and condition ($F_{[7, 98]} = 8.77$; P< 0.0001), *post hoc* Bonferroni; ***P < 0.001, **P < 0.01 and *P < 0.05 significance of differences vs. CTRL.

We propose that the increased tumor development might be related to differences at cellular level similar to those as we observed in the Kupffer cells isolated from SW rats. Our observation of increased tumor cell growth together with increased inflammatory response of (isolated) Kupffer cells indicates that such higher inflammatory reaction may facilitate the increased tumor growth. This is supported by the proposal that a pro-inflammatory environment is a carcinogenic promoter (Grivennikov et al., 2010; Grivennikov and Karin, 2011; Mantovani et al., 2008).

Recent evidence has pointed out that shift-work is associated with fatigue, irritability, depression and anxiety (Arruda et al., 2014; Haus and Smolensky, 2006). Importantly several studies have indicated that altered circadian rhythms in rodents also lead to depressive-like symptoms, like anxiety and anhedonia as expressed by reduced sucrose intake and high corticosterone levels. Since our shift-work model in rats is related to circadian desynchronization as well as increased inflammatory responses, both of them associated with psychological manifestations, we evaluated depressive-like behaviors in

shift-work in an attempt to further evaluate disease susceptibility after circadian desynchronization. Interestingly shift-work rats exhibited high number of grooming events (Fig. 4A) as compared to control rats, indicating high levels of anxiety. The number of steps to the center of the open field was higher in CTRL rats as compared to SW (Fig. 4B). For the fecal boli and exploration measurements, CTRL and SW exhibited similar values (Fig. 4C,D).

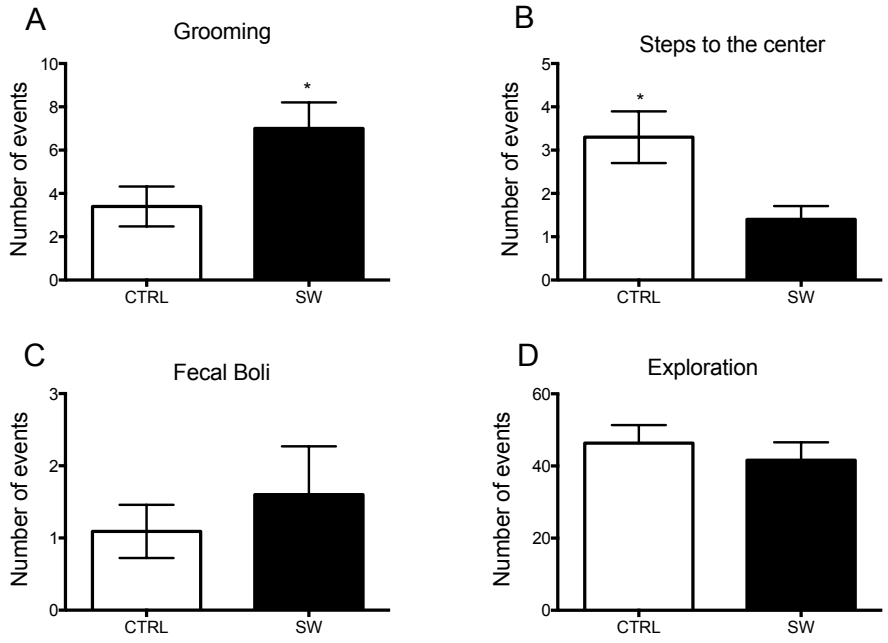

Figure 4. Open field behavior: (A) grooming, (B) steps to the center, (C) fecal boli and (D) exploration were evaluated in CTRL (*n=10*, white bars), or in shift-work rats (SW *n=10*, black bars). Values are expressed as means ± s.e.m. T test; *P < 0.05 significance of differences vs. CTRL. Measurements were performed during the dark phase, when animals are normally active and less susceptible to be stressed.

In the anhedonia test, CTRL rats consume more sugar than SW rats (Fig. 5A), suggesting a depressive like state of SW rats. Plasma melatonin exhibited a clear circadian rhythm in both CTRL and SW rats (Fig. 5B), however ZT17 melatonin levels in SW were lower as compared to ZT17 melatonin levels in CTRL rats suggesting a lower sympathetic output, at least to the pineal. Such disrupted SCN output may be extrapolated to other organs in the body such as the liver, in fact circadian rhythms in metabolic variables and clock gene expression are changed in SW rats (Salgado-Delgado et al., 2013). Moreover the contradictory signals from the periphery to the brain as a result of activity and food consumption during the normal resting phase may further disturb SCN activity (Salgado-

Delgado et al., 2010b) and hence the autonomic outflow. All these together may promote exacerbated inflammatory responses that could be associated with disease development.

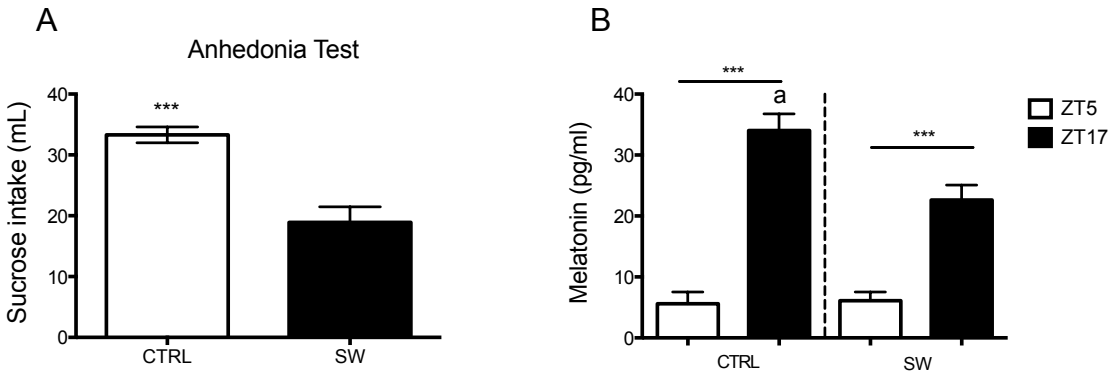

Figure 5. (A) Sugar ingestion in the anhedonia test remained low in shift-work rats (SW, *n=10* black bar) in comparison to control (CTRL *n=10*, open bar) indicating an anhedonic response. Values are expressed as means ± s.e.m. T test; ***P < 0.001 significance of differences vs. CTRL. (B) Night melatonin plasma levels (Zeitgeber time 17, ZT17) are lower in SW rats (*n=10/group*) as compared with CTRL rats (*n=6-7/group*). One way ANOVA and *post hoc* Tuckey; ***P < 0.001, significance of differences vs. ZT5 (Zeitgeber time 5); aP<. 01, significance of difference CTRL ZT17 vs. SW ZT17.

The present observations indicate that circadian disruption due to shift-work in rats induces depression and anxiety like behaviors, which highlight this circadian desynchronization model as a good model to study depressive like pathologies. The increased inflammatory response to LPS as well as the increased tumor development together with the depressive like behavior observed in SW rats strengthens the role of the circadian system and the role of the SCN in the regulation of the immune response. In this sense, we have clearly demonstrated that circadian rhythms disruptions result in increased susceptibility to develop tumors but maybe this could be extended to other diseases with an important inflammatory component.

CONCLUSION

In this thesis we showed the importance of the SCN for the regulation of the inflammatory response. The lack of SCN or desynchronization from its timing message induces increased inflammatory responses. The precise pathways and mechanisms via which the SCN and other brain areas are incorporated in this immunological circuit still need further investigation. However, our results highlight the role of the autonomic nervous system as one of the systems used by the SCN to inhibit the inflammatory response.

Moreover, we have shown that ignoring the message of the biological clock by behavior i.e. activity during the rest phase, makes animals more sensitive to LPS and to inflammatory processes such as cancer. The present study suggest that many health problems associated with shift work might be due to increased inflammatory responses because of a desynchronized SCN.

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List of abbreviations

AP	Area postrema
BBB	Blood brain barrier
CD	Complete hepatic denervation
CLP	Cecal ligation and puncture procedure
COX2	Ciclooxigenase 2
CTRL	Control rat
CRP	C reactive protein
CVOs	Circumventricular Organs
DH	Dorsal horn
DMV	Dorsal motor nucleus of the vagus
FD	Food during the day
HBSS	Hank Balanced Salt Solution
IFN γ	Interferon γ
IL-1β	Interleukin 1β
IL-6	Interleukin 6
IML	Intermediolateral nucleus
iv	Intravenous
LD	Light dark
LPS	Lipopolysaccharide
NES	Night eating syndrome
NK	Natural killer cells

NF-κB	Nuclear factor kappa B
NPY	Neuropeptide Y
NTS	Nucleus of the solitary tract
PPARs	Peroxisome proliferator-activated receptors
Psx	Parasympathetic hepatic denervation
PVN	Paraventricular nucleus
SCN	Suprachiasmatic nucleus
SCNxx	Suprachiasmatic Nucleus lesion animals
SIRT	Sirtuin
SW	Shift-work rat
SW-NFW	Shift-work rat no food in the wheel
Sx	Sympathetic hepatic denervation
TBS	Tris buffered saline
TH	Tyrosine hydroxylase
TLR4	Toll- like Receptor 4
TNF-α	Tumor Necrosis Factor a
ZT	Zeitgeber Time

List of Publications

1. Reciprocal interaction between the suprachiasmatic nucleus and the immune system tunes down the inflammatory response to lipopolysaccharide.

Guerrero-Vargas NN, Salgado-Delgado R, Basualdo Mdel C, García J, Guzmán-Ruiz M, Carrero JC, Escobar C, Buijs RM.J Neuroimmunol. 2014 Aug 15;273(1-2):22-30. doi: 10.1016/j.jneuroim.2014.05.012. Epub 2014 May 29. PMID:24916044.

2. O Relógio Biológico e os ritmos circadianos de mamíferos: uma contextualização

Histórica. The Biological Clock and the circadian rhythms of mammals: a history contextualization

Leila Eliza Barbosa Lima, **Natalí Nadia Guerrero Vargas**. Revista da Biologia (2014) 12(2): 1–7 DOI: 10.7594/revbio.12.02.01

3. Food entrains clock genes but not metabolic genes in the liver of suprachiasmatic nucleus lesioned rats.

Sabath E, Salgado-Delgado R, **Guerrero-Vargas NN**, Guzman-Ruiz MA, del Carmen Basualdo M, Escobar C, Buijs RM. FEBS Lett. 2014 Aug 25;588(17):3104-10. doi: 10.1016/j.febslet.2014.06.045. Epub 2014 Jun 28.PMID:24983496

4. The suprachiasmatic nucleus changes the daily activity of the arcuate nucleus α-MSH neurons in male rats.

Guzmán-Ruiz M, Saderi N, Cazarez-Márquez F, **Guerrero-Vargas NN**, Basualdo MC, Acosta-Galván G, Buijs RM. Endocrinology. 2014 Feb;155(2):525-35. doi: 10.1210/en.2013-1604. Epub 2013 Nov 21. PMID: 24265453.

5. Shift work or food intake during the rest phase promotes metabolic disruption and desynchrony of liver genes in male rats.

Salgado-Delgado RC, Saderi N, Basualdo Mdel C, **Guerrero-Vargas NN**, Escobar C, Buijs RM. PLoS One. 2013;8(4):e60052. doi: 10.1371/journal.pone.0060052. Epub 2013 Apr 2.PMID:23565183.