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# Impaired leukocyte trafficking and skin inflammatory responses in hamsters lacking a functional circadian system

Brian J. Prendergast [a,b,*], Erin J. Cable [a], Priyesh N. Patel [a], Leah M. Pyter [a], Kenneth G. Onishi [a], Tyler J. Stevenson [a], Norman F. Ruby [c], Sean P. Bradley [a]

[a] Department of Psychology, University of Chicago, Chicago, IL 60637, USA
[b] Committee on Neurobiology, University of Chicago, Chicago, IL 60637, USA
[c] Department of Biological Sciences, Stanford University, Palo Alto, CA 94305, USA

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ABSTRACT

The immune system is under strong circadian control, and circadian desynchrony is a risk factor for metabolic disorders, inflammatory responses and cancer. Signaling pathways that maintain circadian rhythms (CRs) in immune function *in vivo*, and the mechanisms by which circadian desynchrony impairs immune function, remain to be fully identified. These experiments tested the hypothesis that the hypothalamic circadian pacemaker in the suprachiasmatic nucleus (SCN) drives CRs in the immune system, using a non-invasive model of SCN circadian arrhythmia. Robust CRs in blood leukocyte trafficking, with a peak during the early light phase (ZT4) and nadir in the early dark phase (ZT18), were absent in arrhythmic hamsters, as were CRs in spleen clock gene (*per1*, *bmal1*) expression, indicating that a functional pacemaker in the SCN is required for the generation of CRs in leukocyte trafficking and for driving peripheral clocks in secondary lymphoid organs. Pinealectomy was without effect on CRs in leukocyte trafficking, but abolished CRs in spleen clock gene expression, indicating that nocturnal melatonin secretion is necessary for communicating circadian time information to the spleen. CRs in trafficking of antigen presenting cells ($CD11c^+$ dendritic cells) in the skin were abolished, and antigen-specific delayed-type hypersensitivity skin inflammatory responses were markedly impaired in arrhythmic hamsters. The SCN drives robust CRs in leukocyte trafficking and lymphoid clock gene expression; the latter of which is not expressed in the absence of melatonin. Robust entrainment of the circadian pacemaker provides a signal critical to diurnal rhythms in immunosurveilliance and optimal memory T-cell dependent immune responses.



## 1. Introduction

In periodic environments, circadian clocks permit synchrony of the internal milieu and anticipation of predictable changes in the environment (Pittendrigh, 1960; Bronson, 1989). Daily temporal organization of physiology and behavior is provided by entrainment of the circadian pacemaker in the hypothalamic suprachiasmatic nucleus (SCN) to the environmental light:dark cycle. The mammalian circadian system is composed of a network of hierarchically-interacting tissue-specific circadian clocks (Baggs et al., 2009). In order to generate coherent physiological rhythms, the phases of these clocks are regularly reset by the output of the master circadian pacemaker in the SCN (Schibler, 2007). Perhaps the most conspicuous circadian rhythm (CR) driven by the SCN is the activity/rest cycle, but scores of physiological processes occur in a circadian manner, including endocrine function, cellular and organismal metabolism, body temperature, and countless behaviors (Takahashi et al., 2008).

Immune function is under strong circadian control. Virtually all immunological variables investigated to date exhibit circadian cycles. CRs are evident in circulating leukocyte numbers and phenotypes, lymphocyte metabolism and function, and cytokine production *in vitro* and *in vivo* (Esquifino et al., 2007). Brain-immune interactions that govern the expression of infection-induced sickness behaviors fluctuate across the circadian cycle (Arjona and Sarkar, 2005, 2006; Guan et al., 2005; Marpegan et al., 2005), and mortality from sepsis varies markedly with time-of-day (Franklin et al., 2003, 2007; Liu et al., 2006; Marpegan et al., 2009; Morrow and Opp, 2005; Weil et al., 2009). Furthermore, immunocompetence varies with the stability of the circadian system: extensive nighttime shift work or transmeridian travel induces chronic circadian disruption, leading to higher rates of various cancers (Pukkala et al., 2002; Reynolds et al., 2002; Schernhammer et al., 2001,

* Corresponding author. Address: Institute for Mind and Biology, University of Chicago, 940 E. 57th St., Chicago, IL 60637, USA. Tel.: +1 773 702 8441; fax: +1 773 702 6898.
*E-mail address:* prendergast@uchicago.edu (B.J. Prendergast).

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2003), and stably-entrained behavioral and endocrine rhythms predict improved survival time in cancer patients (Mormont et al., 2000; Sephton et al., 2000). Mice with disrupted circadian phenotypes exhibit diverse immunological disorders, including enhanced tumorigenesis (Fu et al., 2002), disrupted lymphoid development (Kurebayashi et al., 2000; Sun et al., 2000), impaired T cell function and autoimmune disease (Seimiya et al., 2004), and exacerbated innate inflammatory responses (Castanon-Cervantes et al., 2010).

One major obstacle to addressing the functional significance of the circadian system in immunity has been the lack of an adequate and appropriate model system. SCN ablation reliably eliminates rhythms, but also damages substantial adjacent hypothalamic tissue, and causes nonspecific production of stress hormones (Bittman et al., 1991; Buijs et al., 1993), along with glial scars and CNS immune responses that continue for months after the insult (Logan et al., 1992; Silver and Miller, 2004). Constant light (LL) can induce arrhythmia, but such effects are transient and chronically elevate glucocorticoid secretion (Daan and Pittendrigh, 1975; Eastman and Rechtschaffen, 1983; Welberg et al., 2006). Combinations of clock gene knockouts eliminate CRs (Reppert and Weaver, 2002), but clock genes are present in all tissues, and are pleiotropic in function (Baggs et al., 2009; Greenspan, 2001; Meyer-Bernstein and Sehgal, 2001). The pleiotropy issue is of major logical importance for understanding reports of disease states in clock gene knockout mice: such effects may be due to circadian disruption (centrally or at tissue level), but may instead be due to direct roles of clock genes in cellular metabolic processes (Baggs et al., 2009; Bur et al., 2009; Kohsaka and Bass, 2007; Male et al., 2012). In a single relevant lesion study, diurnal rhythms in blood leukocytes were moderately dampened (not abolished) and growth rates of implanted tumors were accelerated in mice with complete bilateral lesions of the SCN, suggesting that in the absence of circadian organization mechanisms inhibiting tumor growth are impaired (Filipski et al., 2003).

Here we use a model of chronic SCN arrhythmia generated noninvasively, thereby avoiding the pitfalls and confounds of CNS lesions, bright LL, and genetic mutations (Grone et al., 2011; Ruby et al., 2009). CRs in sleep/wake, body temperature, melatonin secretion and locomotor activity of hamsters can be eliminated within a few days in Siberian hamsters by light treatments administered once (see Section 2). This noninvasive method for permanently eliminating CRs has the distinct advantage of allowing animals to remain undisturbed in the presence of a standard light:dark cycle and was used to investigate functional consequences of complete circadian desynchrony on multiple aspects of immune function.

## 2. Materials and methods

### 2.1. Animals

Siberian hamsters were born and raised in a 15L:9D (lights off at 18:00 h CST) breeding colony maintained at the University of Chicago. Hamsters were housed in polypropylene cages, with food (Harlan, Teklad) and filtered tap water provided *ad libitum*; cotton nesting material was also available in the cage. Ambient temperature and relative humidity were held constant at 19 ± 2 °C and 53 ± 10%, respectively. All procedures conformed to the USDA Guidelines for the Care and Use of Laboratory Animals and were approved by the Institutional Animal Care and Use Committee of the University of Chicago.

### 2.2. Experiment 1. Effects of circadian and ultradian arrhythmia on blood leukocyte trafficking

Male and female hamsters (3–5 months of age; ($n$ = 60; 24 females, 36 males) were single-housed and transferred to 16L:8D (lights off at 18:00) for 4 weeks prior to the implementation of a circadian disrupting phase-shift (DPS) protocol (Ruby et al., 2004; see Section 2.6.1). A group of control hamsters ($n$ = 8) was subjected to a sham-DPS procedure. CRs in locomotor activity were monitored over 10 consecutive days in all hamsters 1–3 months after the phase-shift was administered; this allowed identification of the presence/absence of significant CRs, and measurement of quantitative aspects of the CR waveform (see Section 2.6.2).

#### 2.2.1. Circadian leukocyte trafficking

Rhythmic aspects of blood leukocyte trafficking were assessed in 25 μl blood samples collected at 4 time points (ZT4, ZT11, ZT18 and ZT23) across the circadian cycle, over the course of 3 successive days, according to methods described elsewhere (Prendergast et al., 2003).

#### 2.2.2. Stress-induced leukocyte trafficking

To elicit stress responses, hamsters (circadian entrained, "ENTR", $n$ = 8; circadian arrhythmic, "ARR", $n$ = 8) were placed in restraint stress tubes (RST; dimensions; Bilbo et al., 2002) at ZT3 (3 h after light onset); hamsters remained in the RST for 2 h, thus the midpoint of the stressor occurred at ZT4. Control (non-stressed) hamsters remained in their home cages in a separate room. Blood (150 μl) was obtained from RS hamsters via the retro-orbital sinus under isoflurane anesthesia prior to the onset of the stress/control procedure (baseline), and again 15, 60 and 120 min later; blood was obtained from controls at baseline and again 120 min later. Following each blood draw, hamsters were administered 0.2 ml warm sterile physiological saline (s.c.) to facilitate rehydration. Leukocyte concentrations were determined in a 25 μl aliquot of heparinized whole blood (see Section 2.9).

### 2.3. Experiment 2. Effects of circadian arrhythmia on leukocyte trafficking and lymphoid clock gene expression under constant conditions

Female hamsters ($n$ = 93) were subjected to the DPS procedure; 25 (27%) reentrained to the shifted photocycle, whereas 56 (60%) became behaviorally-arrhythmic; the remainder exhibited free-running locomotor activity and were excluded from subsequent investigation. From this population, 24 ENTR and 24 ARR hamsters were subjected to an Aschoff Type II manipulation (Aschoff, 1965), which eliminates masking effects of the light–dark cycle by allowing rhythms to persist in constant conditions (continuous darkness; DD) for >2 cycles prior to sample collection. At the onset of darkness (ZT16), ENTR and ARR hamsters were transferred from 16L:8D to DD. A dim (1 lux) red light remained on at all times in DD. Retroorbital blood (25 μl) was obtained for blood leukocyte determination, and hamsters were killed by rapid decapitation at projected ZT17 (49 h after the onset of DD) or projected ZT1 (58 h after the onset of DD). Spleens were rapidly dissected (<2 min) onto dry ice and stored at −80 °C until RNA extraction.

### 2.4. Experiment 3. Effects of pinealectomy on blood leukocyte trafficking and lymphoid clock gene expression

Adult female hamsters ($n$ = 36) were pinealectomized (PINx) or sham-pinealectomized according to methods described by Carter and Goldman (1983; see Section 2.10). Six weeks after surgery, retroorbital blood was collected at projected ZT1 and ZT17 using an Aschoff Type II design, as described above. Blood samples were obtained under isoflurane anesthesia in a pseudo-random order and leukocytes were enumerated from a 25 μl sample of whole heparinized blood. Hamsters were killed by rapid decapitation, and spleens were rapidly dissected as described for Experiment 2.