Published in final edited form as:

Physiol Behav. 2017 March 15; 171: 165–174. doi:10.1016/j.physbeh.2016.12.019.

Circadian rhythms accelerate wound healing in female Siberian hamsters

Erin J. Cable^{1,*}, Kenneth G. Onishi¹, and Brian J. Prendergast^{1,2}

¹Department of Psychology, University of Chicago, Chicago, IL, 60637 USA

²Committee on Neurobiology, University of Chicago, Chicago, IL, 60637 USA

Abstract

Circadian rhythms (CRs) provide temporal regulation and coordination of numerous physiological traits, including immune function. CRs in multiple aspects of immune function are absent in rodents that have been rendered circadian-arrhythmic through various methods. In Siberian hamsters, circadian arrhythmia can be induced by disruptive light treatments (DPS). Here we examined CRs in wound healing, and the effects of circadian disruption on wound healing in DPS-arrhythmic hamsters. Circadian entrained/rhythmic (RHYTH) and behaviorally-arrhythmic (ARR) female hamsters were administered a cutaneous wound either 3 h after light onset (ZT03) or 2 h after dark onset (ZT18); wound size was quantified daily using image analyses. Among RHYTH hamsters, ZT03 wounds healed faster than ZT18 wounds, whereas in ARR hamsters, circadian phase did not affect wound healing. In addition, wounds healed slower in ARR hamsters. The results document a clear CR in wound healing, and indicate that the mere presence of organismal circadian organization enhances this aspect of immune function. Faster wound healing in CR-competent hamsters may be mediated by CR-driven coordination of the temporal order of mechanisms (inflammation, leukocyte trafficking, tissue remodeling) underlying cutaneous wound healing.

Keywords

biological rhythms; circadian disruption; innate immune function; injury; skin

1. Introduction

The immune system and the central nervous system (CNS) engage in multiple interactions (e.g., Maier & Watkins, 1998; Bilbo & Schwarz, 2012; Rivest, 2009). Although classically viewed as composed of substrates separate from those that reside in the CNS, the immune system and the brain consist of interacting units that regulate host defense in context (Louveau et al., 2015).

*Address correspondence to: Erin J. Cable, Institute for Mind and Biology, University of Chicago, 940 E. 57th St., Chicago, IL 60637 USA, Phone: 773-834-3317, erinca@uchicago.edu.

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Temporal context is important to behavior, metabolism, and immune function. Circadian timing markedly affects the immune system, a connection that has been revealed both under steady-state conditions and following disruptions to the circadian timing system. Several aspects of immunity are dependent on the output of the circadian system (Evans & Davidson, 2013). Leukocyte trafficking from the blood to peripheral tissues exhibits a strong circadian rhythm (CR), with concentrations of blood leukocytes high during the inactive period, and low during the active period (Dhabhar et al., 1994). The magnitude of infection-induced acute inflammatory response and subsequent expression of sickness behaviors is dependent on the time of day (Arjona & Sarkar, 2005, 2006; Guan et al., 2005; Marpegan et al., 2005). The magnitude of sickness responses to a simulated bacterial infection (treatment with lipopolysaccharide [LPS]) is dependent on the circadian time of exposure (Marpegan et al., 2009).

Cytokine and sickness responses are also altered following disruption of the circadian timing system (lesions: Wachulec et al., 1997; Guerrero-Vargas et al., 2014; disruptive light treatments: Prendergast et al., 2015; simulated shift work: Guerrero-Vargas et al., 2015). CRs in immunity are not limited to innate immune responses: T cell-dependent inflammation (a measure of the adaptive immune response) is greater when antigen exposure occurs during the rest as compared to the active phase (Pownall et al., 1979).

Circadian regulation of the immune system becomes readily apparent following disruption of CRs. Chronic CR disruption is correlated with a higher rate of cancer (Pukkala et al., 2002; Reynolds et al., 2002; Schernhammer et al., 2001), whereas stable CR entrainment is associated with increased quality of life and survival in cancer patients (Innominato et al., 2012; Mormont et al., 2000; Sephton et al., 2000). Repeated shifts in the light-dark cycle robustly increase LPS-induced mortality in mice (Castanon-Cervantes et al., 2010). In addition, dim light during the dark phase is sufficient to suppress adaptive and innate immune responses in Siberian hamsters (Bedrosian et al., 2011). Light treatments that induce a loss of behavioral CRs also eliminate CRs in leukocyte trafficking in hamsters (Prendergast et al., 2013). CRs in leukocyte trafficking may be of particular significance for innate immune responses to tissue damage and subsequent wound healing (Mercado et al., 2002; Rojas et al., 2002; Viswanathan & Dhabhar, 2005). In mice, loss of the protein NONO, which is crucial to normal circadian clock function, results in disorganization of cellular proliferation and remodeling following a dermal incision (Kowalska et al., 2013).

Wound healing is an innate immune response that reflects the coordinated activity of the immune system (e.g., inflammation, leukocyte and cell migration, tissue remodeling; Guo & DiPietro, 2010). The phases of wound healing are overlapping, but each is defined by specific characteristics: an initial inflammatory response rapidly follows tissue damage and hemostasis. Neutrophils act via phagocytosis and secretion of antimicrobial molecules during the early inflammatory response to clear bacteria and foreign material from wounds, which is critical for subsequent healing (Sylvia, 2003). Macrophage and lymphocyte infiltration in the inflammatory and proliferative phases continue the removal of foreign material, generate collagen, and recruit fibroblasts to the wound site for formation of an extracellular matrix and tissue repair (Adamson, 2009; Diegelmann & Evans, 2004; Van Linthout et al., 2014). Finally, epithelial remodeling completes the process of wound

healing; this stage may occur over long timescales (i.e., months to years; Velnar et al., 2009). To our knowledge, the effects of circadian time-of-day on wound healing processes have not been directly examined in a non-human animal model.

To better understand the effects of CRs on wound healing, we used a technique for inducing circadian disruption that differs from more common models (jet lag, clock gene mutations, bright constant light, brain lesions). In Siberian hamsters, a combination of light treatments (a nocturnal light pulse followed by a phase shift in the light-dark cycle; “DPS treatment”) eliminates CRs in sleep, temperature, and activity (Ruby et al. 1996; Ruby et al., 2004) in a subset of individuals. DPS treatment also renders mediobasal hypothalamic clock gene expression (*per1*, *per2*, *bmal1*, and *cry1*) arrhythmic and suppressed in amplitude (Grone et al., 2011). DPS has the advantage of allowing arrhythmic hamsters to remain undisturbed in a standard light-dark cycle, without the necessity of lesions or genetic mutations.

Using this non-invasive method of inducing behavioral arrhythmia, these experiments examined the functional relevance of coherent behavioral CRs in the integrative immune process of cutaneous wound healing. This was accomplished using two convergent approaches: (1) examining healing rates of wounds delivered at different circadian phases in circadian competent hamsters, and (2) examining the effects of circadian disruption/arrhythmia on wound healing rates.

2. Methods

2.1 Animals

Female Siberian hamsters (*Phodopus sungorus*) were derived from a breeding colony maintained in a long-day, 15L:9D photoperiod (LD) 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 available in the cages. Ambient temperature and relative humidity were held constant at $19 \pm 2^\circ\text{C}$ and $53 \pm 10\%$, respectively. All procedures were approved by the Institutional Animal Care and Use Committee of the University of Chicago. Hamsters were subjected to the circadian disruptive phase shift procedure (‘DPS’; see section 2.2) at 2–4 months of age.

2.2 Circadian rhythm disruption (DPS procedure)

The DPS manipulation that destabilizes the hamster circadian pacemaker employs phase-resetting light stimuli that render a proportion of hamsters behaviorally circadian arrhythmic (“ARR”; Ruby et al., 2004). Hamsters for this experiment were drawn from a larger cohort (n=206) of female hamsters that were subjected to the DPS procedure and then were assigned to different experiments. For the DPS procedure, hamsters were first housed for 4 weeks in a 16L:8D photoperiod. Then, on a single night, a 2 h light pulse was administered during the 5th through 7th h of the dark phase. On the next day, the 16L:8D photocycle was phase-delayed by 3 h, via extension of the light phase. Of the hamsters selected for the present study, 35 were subjected to the full DPS protocol, and 8 hamsters received a control light treatment: they were subjected to the 3 h phase-delay, but were not given the 2 h light

pulse on the prior night; this control manipulation does not lead to high rates of circadian disruption (Ruby et al., 2004).

After DPS treatment, home-cage locomotor activity (LMA) was assessed using passive infrared motion detectors positioned outside the cage (22 cm above the cage floor). Motion detectors registered activity when 3 of 27 zones were crossed. Activity triggered closure of an electronic relay recorded by a computer running ClockLab software (Actimetrics, Evanston, IL). Cumulative activity counts were collected at 1 min intervals. Activity data for circadian chronotyping (see below) were collected in a single 10 day interval occurring 10–11 months after the DPS treatment, and 12–30 days before cutaneous wound healing was assessed (cf. Ruby et al., 1998).

2.3 Circadian Chronotyping following DPS

Criteria for assessing the presence/absence of CRs were similar to those in prior reports of DPS-induced CR disruption (Ruby et al., 2004; Ruby et al., 1998). χ^2 periodogram and Lomb-Scargle periodogram (LSPs) were used to detect presence/absence of CRs. LSP analyses were implemented to compliment χ^2 analyses, due to the increased sensitivity of the LSP in detecting CRs in non-sinusoidal data (e.g., ‘square-wave’ data, typical of circadian-entrained hamsters) and an increased propensity for χ^2 periodograms to indicate false peaks in noisy data (as is typical of DPS-induced ARR hamsters; Ruf, 1999; Refinetti et al., 2007). χ^2 and LSP analyses were performed on a 10 day block of activity data (cf. Ruby et al., 1998). CR amplitude was quantified in all hamsters, regardless of chronotype, as the Qp value at 24.0 h from the χ^2 periodogram.

Hamsters were designated as entrained (RHYTH) if they exhibited: (1) significant ($P < 0.001$) circadian (22 – 26 h) activity peaks in both the χ^2 periodogram and the LSP, (2) consistently clear daily activity onsets and offsets upon visual inspection of the actogram, and (3) activity predominantly restricted to the dark phase of the LD cycle.

Hamsters were designated as arrhythmic (ARR) if they exhibited: (1) the absence of clear and significant ($P < 0.001$) circadian peaks in χ^2 periodogram or the LSP, (2) an absence of consistent and clear daily activity onsets and offsets, and (3) LMA distributed throughout the light and dark phases of the LD cycle.

Three hamsters exhibited activity patterns that were not easily classifiable. One hamster exhibited a temporally-narrow (< 1.8 min bandwidth) ‘spike’ in the χ^2 periodogram which did not resemble a normal circadian peak, but nevertheless exceeded the χ^2 significance level of $p < 0.0001$, indicative of power at a restricted frequency. This χ^2 ‘spike’ exhibited no significant power in the surrounding frequency band (atypical of hamsters with normal CRs) and reached a peak amplitude of 8.9 Qp units above the significance level (cf., RHYTH hamsters exhibit peaks with a mean of 269.0 Qp units above the significance level). This animal also lacked clear circadian activity onsets and offsets, exhibited activity throughout the day and night, and exhibited almost no detectable power in the LSP (PN = 0.21) or the FFT (relative power = 0.001). For these reasons the hamster was classified as ARR. A second hamster exhibited modest but significant peaks in the χ^2 periodogram and the LSP, but lacked clear and consistent activity onsets and offsets, and exhibited locomotor activity

throughout the light and dark phases on 4 of 10 assessment days, but temporal order in daily activity onsets and offsets on 6 of 10 days. A third hamster, although lacking significant peaks in the χ^2 periodogram and LSP, exhibited a free-running activity pattern ($\tau \sim 25$ h) with clear onsets and offsets on 7–8 of the assessment days. Due to their ambiguous and free-running chronotypes, respectively, these latter two hamsters were excluded from all analyses. 26 RHYTH and 14 ARR hamsters exhibited clear chronotypes and were used in these experiments.

2.4 Skin wounding procedure

Hamsters were administered a circular cutaneous wound using a 3.5 mm punch biopsy tool according to methods detailed elsewhere (Kinsey et al., 2003). Briefly, hamsters were lightly anesthetized with isoflurane, and a patch of fur approximately 900 mm² on the dorsal surface was shaved with electric clippers. The shaved region was disinfected using Betadine solution (Purdue Fredrick, Stamford, CT), and two uniform, circular wounds 3.5 mm in diameter were made simultaneously in the dorsal skin using a sterile, disposable punch biopsy tool (Miltex, York, PA). Wounds (entrance and exit) were bilateral, but only data generated from analyses of the entrance wound were used in statistical analyses (Kiecolt-Glaser et al., 1995).

Immediately following wounding (“Day 0”), and at 24 h intervals thereafter for the next 17 days, each wound was photographed using a digital camera (Canon Powershot ELPH110HS, Melville, NY); a reference standard (a printed black 3 mm diameter circle on a white paper background) was included in every photograph. In each digital image, entrance wounds and reference standards were traced at 150× magnification using graphic design software (Adobe Illustrator, San Jose, CA), and their respective areas were calculated. The area of each wound was divided by the area of the reference standard in each photograph. The ratio of these values provided a measure of standardized wound size (SWS) for each wound (Kiecolt-Glaser et al., 1995). This technique has been previously used in this species (Kinsey et al., 2003).

All skin wounding was performed by the same experimenter, as was all subsequent skin photography and image analysis. In order to consolidate skin wounding and daily skin photography into the specified 1 hour circadian time interval, skin wounding was performed in two separate rounds, separated by 20 days. In Round 1, 16 RHYTH hamsters were randomly assigned to receive skin wounds either three hours after lights on, (ZT03; n=8), or two hours after lights off (ZT18; n=8) in order to determine if CRs in skin wounding manifest. RHYTH hamsters with the subjectively most robustly entrained activity patterns were included in Round 1.

In the second round of wounding (Round 2), the remaining 10 RHYTH hamsters (ZT03: n=5; ZT18: n=5), and all 14 ARR (ZT03: n=8; ZT18: n=6) hamsters received skin wounds. Hamsters were randomly assigned to ZT groups. All wounding procedures and analyses were performed blind to chronotype.

2.5 Wound healing metrics

Percent change in wound size for each animal was calculated on each day as the ratio of SWS of the wound on a given day to the SWS of the same individual's wound on Day 0, thus providing a measure of relative wound size (RWS). Wounds were considered 50% healed on the first day that wounds were 50% reduced in size from day 0 values and remained so upon all measurements thereafter. Analogous calculations were performed to determine the day on which wounds were 100% healed. A minority of hamsters (n=2 of 40) did not achieve the criteria for 100% healing by day 17 and were assigned a default value of day 17 for 100% healing.

2.6 Validation of wound assessments during the dark and light phases

Pilot studies determined that the focal length of the digital camera would automatically change when photographs were taken under dim red light (at ZT18) as compared to under normal room illumination (at ZT03). In order to ensure that any differences in image quality that resulted from these changes in focal length did not affect wound size measurements, we performed a validation experiment. In this experiment, (n=6 wounds) a dorsal cutaneous wound was performed and photographed under normal room illumination; immediately thereafter, the lights were turned off, and the same wound was photographed in the darkness under dim red light. The same reference standard was present in every photograph. For the purposes of increasing sample sizes for this validation procedure, entrance and exit wounds were measured, thus SWS values were calculated for a total of 12 wounds. There was a strong, positive, linear relationship between a SWS obtained in the light and in the dark ($R^2=0.98$, $P<0.0001$), indicating that wounds could be precisely and accurately measured under both illumination conditions.

2.7 Locomotor activity analyses

Relations between patterns of locomotor activity during the interval shortly following wounding and rates of wound healing were also assessed. To expedite photography, hamsters were not housed under activity monitoring lids after they were wounded, therefore, measures of activity were obtained from activity records generated 12–30 days prior to wounding (i.e., locomotor activity files used for circadian chronotyping). Mean total activity counts (over 10 days) were quantified during two separate 8 hour intervals: ZT03-ZT11 (for ZT03-wounded hamsters) and ZT18-ZT02 (for ZT18-wounded hamsters), corresponding to the 8 h following ZT03 and ZT18 wounds, respectively.

2.8 Statistical analyses

CR amplitude measures (χ^2 periodogram, Lomb-Scargle periodogram and FFT values) were compared using t-tests. Standardized wound sizes (SWS) on Day 0 were compared using ANOVA, and pairwise comparisons were performed using t-tests. Longitudinal analyses of relative wound size (RWS) values were performed using repeated measures ANOVAs with wounding time as a between-subject variable. Group means for the day on which wounds were 50% and 100% healed were compared using ANOVA, and pairwise comparisons were performed using t-tests. Partial eta-squared was used to compare the magnitude of the effect of wounding time on healing rates. Linear regression analyses were used to validate

photography in the light and dark phases, to assess relations between CR amplitude measures (Qp) and wound healing rates, and to assess relations between locomotor activity levels and wound healing. The level of statistical significance was set at $\alpha=0.05$.

3. Results

3.1 Circadian responses to the DPS procedure

26 hamsters yielded RHYTH chronotypes, and 14 hamsters yielded ARR chronotypes and were included in subsequent procedures and analyses. Of the 8 hamsters that were not subjected to the 2 h light pulse on the night before the 3 h phase shift, all 8 yielded RHYTH chronotypes and were also included in these experiments.

3.2 CR amplitude measures in Rounds 1 and 2

Relative to Round 1 RHYTH hamsters, RHYTH hamsters in Round 2 exhibited markedly lower amplitude CRs, as determined by mean ($\pm$ SD): [1] Qp values at 24.0 h in the χ^2 periodogram (Round 1: 526 ± 107 ; Round 2: 384 ± 140 ; $t_{24}=2.93$, $p<0.01$), [2] PN values at 24.0 h in the LSP (Round 1: 121 ± 44 ; Round 2: 78 ± 59 ; $t_{24}=2.13$, $p<0.05$). ARR values for these CR metrics were substantially lower in ARR hamsters (χ^2 : 151 ± 31 ; LSP: 3.63 ± 2.86) relative to RHYTH hamsters ($p<0.0001$, ARR vs. Round 1 or Round 2 RHYTH values, all comparisons). Thus, despite being categorically similar in circadian chronotype, the Round 1 and Round 2 populations of RHYTH hamsters differed markedly in CR power. Consequently, in analyses of the effect of ZT on wound healing rates, data in RHYTH hamsters were analyzed separately for these two rounds, as were data for ARR hamsters in Round 2.

3.3 Day 0 standardized wound sizes (SWSs)

ANOVA indicated significant differences in Day 0 SWS across treatment groups ($F_{5,34}=3.22$, $p<0.05$). This effect appeared to be driven by: [1] smaller Day 0 wound sizes in Round 1 RHYTH hamsters wounded at ZT18 as compared to ARR hamsters wounded at ZT03 ($t_{14}=3.24$, $p<0.01$) and ZT18 ($t_{12}=2.84$, $p<0.05$), and [2] smaller Day 0 wound sizes in Round 2 RHYTH hamsters wounded at ZT18 as compared to ARR hamsters wounded at ZT03 ($t_{11}=2.50$, $p<0.05$). Pairwise differences were not evident in Day 0 SWS between any other treatment group ($p>0.05$, all comparisons).

3.4 Effects of time-of-day on wound healing, Round 1

Among the robustly RHYTH hamsters in Round 1, there was a significant interaction between the time of skin wounding and the change in RWS over the next 17 days ($F_{17,238}=2.89$, $p<0.0005$; Fig. 1A), characterized by a pattern of slower healing in hamsters wounded at ZT18. Wound sizes of ZT18 hamsters tended to be greater than those of ZT03 hamsters on Day 2 ($p<0.07$), and were significantly greater than those of ZT03 hamsters on Days 6–10 ($p<0.05$, all comparisons). Wounds of ZT03 hamsters achieved the 50% healing criterion >1 day sooner than those of ZT18 hamsters ($F_{1,14}=7.18$, $p<0.05$; Fig. 1B); but 100% healing rate did not differ between ZT groups ($F_{1,14}=0.74$, $p>0.40$; Fig. 1C).

3.5 Effects of time-of-day on wound healing, Round 2

Among the RHYTH hamsters in Round 2, there was a similar trend towards a significant interaction between wounding time and change in RWS ($F_{17,136}=1.65$, $p<0.06$; Fig. 1D), again characterized by a pattern of ZT18 wounds tending to heal slower than ZT03 wounds. RWS of ZT18 hamsters were significantly greater than those of ZT03 hamsters on Day 3 ($p<0.05$), tended to be greater on Day 4 ($p<0.07$), and were significantly larger on Days 10–14 ($p<0.05$, all comparisons). Wounds of ZT03 hamsters also exhibited non-significant trends towards achieving the 50% and 100% healing criteria sooner than those of ZT18 hamsters ($F_{1,8}>3.52$, $p<0.10$; both comparisons; Fig. 1E, 1F).

Among ARR hamsters in Round 2, time-of-wounding did not affect the pattern of change in wound size over the next 17 days ($F_{17,204}=0.92$, $p>0.50$; Fig. 1G). Wounds of ZT18 ARR hamsters did not differ from those of ZT03 hamsters on any day post-wounding ($p>0.05$, all comparisons). Wounding time affected neither 50% ($F_{1,12}=0.34$, $p>0.50$) nor 100% ($F_{1,12}=0.36$, $p>0.50$) healing rates among ARR hamsters (Fig. 1H, 1I).

3.6 Magnitude of treatment effects

Partial eta-squared was used to compare the magnitude of the treatment effect (wounding ZT) across the three chronotype groups (robustly-RHYTH hamsters in Round 1, RHYTH hamsters in Round 2, and ARR hamsters in Round 2). Partial eta-squared was calculated as the quotient of: ($SS_{\text{between}} / (SS_{\text{between}} + SS_{\text{error}})$) from the repeated measures ANOVA table for each analysis (Levine & Hullett, 2002). Partial eta-squared values were 0.171, 0.171, and 0.071, for Round 1 RHYTH, Round 2 RHYTH, and Round 2 ARR hamsters, respectively.

3.7 Effects of circadian chronotype on wound healing

In order to consider the influence of circadian power on healing among RHYTH hamsters, wound healing was compared between Round 1 and Round 2 RHYTH hamsters. RHYTH hamsters wounded at ZT03 exhibited a significant effect of Round ($F_{17,187}=4.38$, $p<0.0001$; Fig 2A) on wound size, such that Round 1 hamsters had significantly smaller wounds on Days 7–11 ($p<0.05$), and tended to have smaller wounds on Days 12 and 13 ($p<0.06$). In contrast, RHYTH hamsters wounded at ZT18 exhibited no significant difference between Round ($F_{17,187}=1.17$, $p>0.20$; Fig 2B). Round 1 RHYTH hamsters tended to reach 50% healing faster than Round 2 hamsters for wounds at ZT03 ($F_{1,11}=3.89$, $p<0.07$; Fig 2C), and Round 1 RHYTH females wounded at ZT18 achieved this criterion significantly faster ($F_{1,11}=8.04$, $p<0.02$; Fig 2D). There was no effect of Round or wound ZT on 100% healing rate. Because there was a significant effect of Round in the RHYTH hamsters, these two RHYTH populations were not collapsed in the following analyses with ARR hamsters.

Among Round 1 RHYTH and ARR animals, there was a significant effect of chronotype at both wounding times (ZT03: $F_{17,238}=5.57$, $p<0.0001$; ZT18: $F_{17,238}=2.06$, $p<0.02$, Fig. 3A, 3D), characterized by ARR hamsters healing slower than Round 1 RHYTH hamsters. Among hamsters wounded at ZT03, ARR hamsters had larger wounds than Round 1 RHYTH hamsters on Days 7–14 ($p<0.04$); among ZT18 wounded hamsters, ARR hamsters had larger wounds on Days 10–14 ($p<0.002$, all comparisons). In hamsters wounded at ZT03, ARR hamsters achieved 50% and 100% healing benchmarks slower than Round 1

RHYTH hamsters (50%: $F_{1,14}=10.59$, $p<0.006$, Fig 3B; 100%: $F_{1,14}=8.57$, $p<0.02$, Fig 3C). In hamsters wounded at ZT18, ARR hamsters reached 50% healing slower than RHYTH hamsters ($F_{1,12}=5.79$, $p<0.05$, Fig 3E) and tended to reach 100% healing slower than RHYTH hamsters ($F_{1,12}=4.3$, $p<0.07$, 3F).

When comparing Round 2 RHYTH and ARR hamsters, there was no significant effect of chronotype on wound size over the course of healing for either ZT03 ($F_{17,187}=0.61$, $p>0.8$; Fig 4A) or ZT18 wounds ($F_{17,153}=1.04$, $p>0.4$; Fig 4B). In Round 2, chronotype did not affect healing to 50%, but did influence healing to 100% in animals wounded at ZT03: ARR hamsters reached the 100% healing benchmark slower than RHYTH hamsters ($F_{1,11}=5.37$, $p<0.05$; Fig 4C). This effect was not evident in animals wounded at ZT18 ($F_{1,9}=0.59$, $p>0.4$; Fig 4D).

3.8 Relations between locomotor activity and healing rates

We next examined whether wound healing rate was associated with diurnal differences in locomotor activity (recorded prior to wounding) typical of that which would occur during the 8 h interval after wounding was performed (ZT03 - ZT11 [light phase] and ZT18 - ZT02 [majority (6 h) of the dark phase and 2 h of the light phase]). Across experimental rounds and ZTs, there were predictably significant between-group differences in activity during these 8 h epochs ($F_{5,34}=12.7$, $p<0.0001$; Fig. 5A). In Round 1, among RHYTH hamsters, mean activity was markedly lower between ZT03 and ZT11 (in ZT03-wounded hamsters) as compared to activity between ZT18 and ZT02 (in ZT18-wounded hamsters; $t_{14}=5.84$, $p<0.0001$); similar relations were obtained among RHYTH hamsters in Round 2 ($t_8=3.47$, $p<0.01$), although the magnitude of the difference in activity was quantitatively smaller. Among ARR hamsters, mean activity between ZT03 and ZT11 (in ZT03 hamsters) did not differ from activity between ZT18 and ZT02 (in ZT18 hamsters; $t_{12}=0.57$, $p>0.50$; Fig. 5A).

Separate linear regressions were performed for Round 1-RHYTH, Round 2-RHYTH, and Round 2-ARR hamsters (collapsed across wounding ZT), to assess relations between locomotor activity (typical of that which would occur during the 8 h interval after wounding was performed) and wound healing. Among Round 1 RHYTH hamsters, activity typical of that which would occur during the 8 h interval after wounding was a significant positive predictor of the interval of time required to achieve 50% wound healing ($R^2=0.37$, $df=15$, $p<0.05$; Fig. 5B). A categorically similar relation was obtained among RHYTH hamsters in Round 2 ($R^2=0.41$, $df=9$, $p<0.05$; Fig. 5C). For both groups of RHYTH hamsters, increased locomotor activity counts during the interval following wounding was associated with a greater number of days to achieve the 50% healing benchmark. Among ARR hamsters, however, no relation between these variables was observed ($R^2<0.01$, $df=13$, $p>0.90$; Fig. 5D).

Identical regression analyses of activity on healing were performed using the 100% healing criterion as the dependent variable, but no significant relations were evident ($R^2<0.07$, $df=9-15$, $p>0.30$, all comparisons; data not illustrated).

3.9 Relations between CR power and healing rates

Lastly, to take advantage of the broad range of CR power across all animals in these experiments, an exploratory analysis was performed in which data from all rounds were subjected to a regression analysis, with circadian power (Qp values from the χ^2 periodogram) as the predictor variable and 50% healing rate as the dependent variable. Circadian power was a significant negative predictor of 50% wound healing values ($R^2=0.21$, $df=39$, $p<0.005$; Fig. 6A), increased circadian power measured prior to wounding significantly predicted delayed healing to 50%. To assess whether these effects were being driven solely by the relatively lower Qp values in ARR hamsters, supplementary regression analyses were performed separately on ARR and RHYTH data. Circadian power (Qp) significantly positively predicted 50% wound healing rate among RHYTH hamsters ($R^2=0.16$, $df=25$, $p<0.05$; Fig. 6B). No significant relation was evident between Qp values and 50% healing rate among ARR hamsters ($R^2<0.01$, $df=13$, $p>0.90$; Fig. 6C).

4. Discussion

Here we characterize for the first time multiple effects of circadian organization on cutaneous wound healing. Healing rates were influenced by the circadian time of the injury and by circadian integrity (RHYTH vs. ARR). RHYTH hamsters healed slower when wounds were administered in the early active period (ZT18) as compared to the early inactive period (ZT03). In contrast, ARR hamsters exhibited no such circadian modulation of wound healing. Irrespective of wounding time, ARR hamsters healed slower than RHYTH hamsters wounded at ZT03, an effect not present in hamsters wounded at ZT18. Among RHYTH hamsters, the strength of circadian entrainment to the LD cycle was a significant predictor of wound healing rate, as was a prospective measure of locomotor activity during the interval immediately following wounding. The present work is consistent with recent studies of molecular circadian influences on wound healing (Kowalska et al., 2013) and suggests that a functional circadian system is essential for optimal healing following a cutaneous wound.

Among RHYTH hamsters, ZT18 wounds exhibited larger wound sizes during what is classically considered to be the “inflammatory stage” (3–5 days post-wounding) and slower overall wound healing rates (Fig. 1). The phases of wound healing are defined by the recruitment of specific cells to the site of injury. Coordination of the immune response following tissue damage is essential to maintaining the healing process. The proliferative phase of tissue regeneration and repair initiates after the wound has been cleared of foreign bacteria, and an extracellular matrix has begun formation for scaffolding skin repair (Velnar et al., 2009). In mice, the magnitude of cytokine production and of neutrophil and macrophage trafficking to the site of tissue damage exhibits a clear CR (Gibbs et al., 2014). Several groups have documented a clear progression of leukocyte migration to the cutaneous wound site, using sponge-based capture of infiltrating cells over time, followed by flow cytometric analyses of leukocyte subtypes (Viswanathan & Dhabhar, 2005; Neeman et al., 2012). Indeed in the DPS-ARR hamster model we have previously documented disrupted rhythms in antigen presenting cells (dendritic cells) in the skin of non-wounded hamsters (Prendergast et al., 2013). In future studies, flow-cytometric based approaches could be

fruitfully deployed to determine if circadian disruption impairs the orderly sequence of immune cell migration to the wound site. In addition, an endogenous circadian clock in macrophages has been shown to drive a circadian rhythm in the magnitude of the inflammatory response to immune challenge (Keller et al., 2009). Circadian oscillations in skin cells, which are abolished in SCN lesioned mice, may affect the efficiency of migration, tissue growth and regeneration, all of which contribute to wound healing (Plikus et al., 2015; Tanikoa et al., 2009). If coordination of cellular recruitment to wound site depends, in part or in whole, on endogenous CRs either in skin or immune cells, or in the CNS, and is communicated to immune cells, then CRs in the response to tissue damage would be expected. Indeed, the present work suggests a quantitative relation between the amplitude of the pacemaker in the CNS (which drives CRs in locomotor activity) and rates of wound healing.

Conversely, hamsters designated as ARR not only failed to exhibit CRs in healing, but also were slower to heal wounds, again underscoring the importance of circadian coordination of events for optimal wound healing. Regardless of time of day, ARR-ZT03 and ARR-ZT18 hamsters exhibited comparable magnitudes of post-biopsy inflammatory responses and comparable intervals required reach 50% and 100% healing metrics. ARR hamsters lack a detectable circadian rhythm in multiple aspects of physiology and behavior (Grone et al., 2011; Prendergast et al., 2015; Ruby et al., 1996; Ruby et al., 2004). While it is possible that prolonged post-DPS circadian disruption (10–11 months) may have affected healing rates or immune function, there are no data presently available to speak to such an interaction.

Enhanced healing rates in RHYTH relative to ARR hamsters suggest a marked influence of the circadian system on wound healing processes. The presence of CRs may coordinate cellular recruitment to the site of the wound over multiple stages of the healing process. Absent robust CRs, stages of wound healing and tissue repair evidently require greater intervals of time, which may leave the individual more susceptible to infection (Rojas et al., 2002).

A sudden increase in wound size occurred in Round 2, ZT18 wound sizes between Days 9 and 10; this occurred in both RHYTH and ARR groups. We have no clear explanation for this transient increase in wound size, and it is possible, if not likely, that this increase had a quantitative effect on wound size comparisons on Day 10, and for all the days that followed. This may have contributed to the strong (though non-significant) trend towards a difference between Round 2 ZT-18 ARR and Round 1 ZT-18 RHYTH hamsters. Absent this unexpected size increase, the wound healing patterns may have been indistinguishable from one another between chronotypes. In addition, the number of days required to achieve 50% and 100% healing may have been affected in Round 2 ZT18 hamsters by this anomalous increase in wound size. Importantly, no hamster achieved criteria for 50% or 100% healing, only to not qualify on the following day because of this size increase; however, some hamsters, on Day 9, may have been poised to achieve 50% healing on the following day, only to fail to achieve the criterion (on Day 10) because of this anomalous size increase.

In both experimental Rounds, immediately after biopsy, wound sizes in RHYTH-ZT18 were noticeably smaller than those of several other groups (ARR-ZT03, ARR-ZT18, RHYTH-

ZT03, [Round 2 only]). Such differences were not evident in RHYTH-ZT03 hamsters compared to any other groups. This outcome is unlikely to be an artifact of photography in the dark, as validation studies were performed to ensure that ambient lighting did not affect RWS measures of wound size. Experimenters could not be blind to wounding time, but were blind to circadian chronotype at the time of biopsy and photography, and wound photography was completed in <30 seconds post-biopsy. Although this outcome indicates that a functioning circadian system is required for this CR in the immediate response to cutaneous trauma, the mechanism by which this effect manifests is not clear. CRs exist in the contractile properties of skin, and in vasoconstriction and dilation (Aoki et al., 2001; see Reeve, 1975 for review; Brown et al., 2014), processes which participate in immediate response to cutaneous damage. However, we note that rapid contraction (i.e., smaller initial measures of absolute wound size) of skin in the ZT18 hamsters did not translate to an accelerated healing process, indeed, the opposite was the case.

Among RHYTH hamsters, healing was faster in RHYTH-ZT03 relative to RHYTH-ZT18 hamsters. Locomotor activity (LMA) data collected in the present study permitted limited insights into the mechanism(s) that may mediate such CRs in wound healing. Greater amounts of locomotor activity in the 8 h window following wounding strongly predicted slower healing to 50% in both populations of RHYTH hamsters, but not in ARR animals. Because site immobilization has been shown to markedly accelerate wound healing rates (Järvinen & Lehto, 1993; Johnson, Ratner and Nelson, 1992), we predicted that LMA typical of that which occurs during the interval immediately following a ZT18 wound (elevated LMA in the early active phase) may agitate the wounded tissue and thereby negatively impact healing (cf. ZT03-wounded hamsters, which are in the rest phase). Consistent with this conjecture, analyses performed here indicate that LMA was a significant negative predictor of wound healing rate in both groups of RHYTH hamsters. Although the data do not establish causality, they point to behavioral output of the circadian clock in CNS (circadian gating of locomotor activity) as a potential mechanism for modulating innate immune function. However, if LMA during the immediate post-wounding interval were sufficient to explain healing rates then one would expect it to predict healing rates among ARR hamsters as well.

Power of the behavioral circadian waveform (Qp) was also associated with healing rate (Fig. 6): in both groups of RHYTH hamsters, higher CR power predicted faster healing, strongly suggesting that CR robustness conveys immunological advantages. This may be mediated via coordination of immunological events integral to the inflammation / repair / remodeling process, the gating of LMA, or both. It should be noted, however, that neither LMA nor circadian power alone fully explains the effect of circadian *chronotype* on wound healing. ARR animals exhibited markedly reduced LMA at both times of day relative to RHYTH-ZT18 hamsters, yet ARR hamsters healed at the same rate or slower than RHYTH-ZT18 wounded hamsters. Locomotor activity, and circadian amplitude may only predict healing rate in RHYTH animals, and are not sufficient to explain the differences in healing rates between chronotypes, suggesting that unspecified, but activity-independent, possibly circadian, mechanisms participate in delayed wound healing in ARR hamsters.

Taken together, the present data indicate that circadian time and integrity impact skin wound healing, modulating innate immune processes that unfold over the course of ~2 weeks. The data emphasize the importance of a robust circadian system for optimal wound healing. The mechanisms by which the circadian system impacts the multiple stages of wound healing remain to be fully understood, however, clock-driven variance in locomotor activity levels and omnibus CR robustness predicted wound healing rates among RHYTH hamsters. Specification of the mechanisms by which CRs impact wound healing has practical implications, as injuries sustained by individuals with disrupted CRs (e.g., shift-work schedules, following transmeridian travel) may heal at a reduced rate, increasing risk of infection or prolonged disability. Even in individuals with relatively stably-entrained CRs, the timing of cutaneous wounds is predictive of recovery rates and may impact health outcomes.

Acknowledgments

The authors thank Dr. Betty Theriault for expert veterinary care and technical assistance. This work was supported by NIH Grant AI-67406 from the National Institute of Allergy and Infectious Diseases.

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Highlights

Circadian rhythms (CRs) in healing rates were documented in female Siberian hamsters

In nocturnal hamsters, skin wounds heal faster if they occur prior to the rest phase

Behaviorally circadian arrhythmic hamsters heal wounds slower, with no CRs in healing

Figure 1.

(A) Mean ($\pm$ SEM) Relative Wound Size (RWS) during cutaneous wound healing of RHYTH hamsters that received punch biopsies at ZT03 (open circles; $n=8$) or ZT18 (filled circles; $n=8$) in Round 1. (B, C) Mean number of days ($\pm$ SEM) required for wounds depicted in Panel A to reach criteria for 50% and 100% healed. (D) Mean $\pm$ SEM RWS of RHYTH hamsters wounded at ZT03 (open circles; $n=5$) or ZT18 (filled circles; $n=5$) in Round 2. (E, F) Mean number of days ($\pm$ SEM) required for wounds depicted in Panel D to reach criteria for 50% and 100% healing. (G) Mean ($\pm$ SEM) RWS ARR hamsters wounded at ZT03 (open circles; $n=8$), or ZT18 (filled circles; $n=6$). (H, I) Mean number of days ($\pm$

SEM) required for wounds depicted in Panel G to reach criteria for 50% and 100% healing. In all experiments biopsies / wounds were performed on Day 0 and healing was monitored for 17 days. # $p < 0.10$, * $p < 0.05$, vs. corresponding ZT03 value.

Figure 2.

(A, B) Mean ($\pm$ SEM) RWS of robustly-entrained RHYTH hamsters wounded in Round 1 (open circles; $n=16$) and entrained RHYTH hamsters in Round 2 (filled circles $n=10$) wounded at ZT03 or ZT18. (C, D) Mean ($\pm$ SEM) number of days required for wounds to reach 50% healed criteria among Round 1 RHYTH (R1, white bars) and Round 2 RHYTH hamsters (R2, black bars) wounded at ZT03 (Panel C) or ZT18 (Panel D). [#] $p<0.10$, ^{*} $p<0.05$ vs. corresponding R1 RHYTH value.

Figure 3.

(A, D) Mean ($\pm$ SEM) RWS during cutaneous wound healing of Round 1 RHYTH (open circles) and Round 2 ARR hamsters (filled circles) wounded at ZT03 (Panel A) or ZT18 (Panel D). (B, C) Mean number of days ($\pm$ SEM) required for wounds to reach criteria for 50% and 100% healed in Round 1 RHYTH (white bars) and ARR (black bars) hamsters wounded at ZT03. (E, F) Mean number of days ($\pm$ SEM) required for wounds to reach criteria for 50% and 100% healed in Round 1 RHYTH (white bars) and ARR (black bars) hamsters wounded at ZT18. * $p < 0.05$, ** $p < 0.01$ vs. corresponding RHYTH value.

Figure 4.

(A, B) Mean ($\pm$ SEM) RWS during cutaneous wound healing of Round 2 RHYTH (open circles) and ARR hamsters (filled circles) wounded at ZT03 (Panel A) or ZT18 (Panel B). (C, D) Mean number of days ($\pm$ SEM) required for wounds to reach criteria for 100% healed in Round 2 RHYTH (white bars) and ARR (black bars) hamsters wounded at ZT03 (Panel C) or ZT18 (Panel D). * $p < 0.05$ vs. corresponding RHYTH value.

Figure 5.

(A) Mean ($\pm$ SEM) locomotor activity during the 8 h epochs (ZT03-ZT11, white bars; ZT18-ZT02, black bars) immediately following the timing of the punch biopsy for Round 1 RHYTH, Round 2 RHYTH, and ARR hamsters wounded at ZT03 (white bars) or ZT18 (black bars; note: all activity data collected in the days prior to wounding). (B, C, D) Linear correlation analyses of locomotor activity as a predictor of wound healing rate (time to 50% healed) in Round 1 RHYTH (Panel B), Round 2 RHYTH (Panel C), and ARR (Panel D) hamsters. In Panel A: ** $p<0.01$ vs. corresponding ZT03 value, *** $p<0.001$ vs. corresponding ZT03 value.

Figure 6.

(A–C) Linear correlation analyses of a measure of circadian power (Qp value in the χ^2 periodogram) as a predictor of wound healing rate (time to 50% healed) in all experimental hamsters (Panel A), all RHYTH hamsters (Panel B), and all ARR hamsters (Panel C).