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Spinal cord injury in rats disrupts the circadian system

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Title: Spinal cord injury in rats disrupts the circadian system

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Abstract

Spinal cord injury (SCI) perturbs many physiological systems. The circadian system helps maintain homeostasis throughout the body by synchronizing physiological and behavioural functions to predictable daily events. Whether disruption of these coordinated daily rhythms contributes to SCI-associated pathology remains under-studied. Here, we hypothesized that SCI in rats would dysregulate several prominent circadian outputs including glucocorticoids, core temperature, activity, neuroinflammation, and circadian gene networks. Female and male Sprague-Dawley rats were subjected to clinically relevant thoracic (T-) 9 moderate contusion SCI (or laminectomy sham surgery). Diurnal measures – including rhythms of plasma corticosterone (CORT), body temperature and activity (using small implanted transmitters), and intraspinal circadian and inflammatory gene expression – were studied prior to and post-surgery. SCI caused overall increases and disrupted rhythms of the major rodent glucocorticoid, CORT. Pre-surgery and sham rats displayed expected rhythms in body temperature and activity, whereas rats with SCI had blunted daily rhythms in body temperature and activity. In parallel, SCI disrupted intraspinal rhythms of circadian clock gene expression. Circadian clock genes can act as transcriptional regulators of inflammatory pathways. Indeed, SCI rats also showed dysregulated rhythms in inflammatory gene expression in both the epicenter and distal spinal cord. Our data show that moderate SCI in rats causes wide-ranging diurnal rhythm dysfunction, which is severe at acute time points, and gradually recovers over time. Normalizing post-SCI diurnal rhythms could enhance recovery of homeostasis and quality-of-life.

Significance Statement

Spinal cord injury (SCI) can cause physiologic dysfunction throughout the body. Internal physiologic function is typically synchronized with the environment through the circadian system. Despite the crucial roles of the spinal cord and the circadian system in optimizing whole-body function, it remains unclear whether SCI alters diurnal rhythms. Here, we hypothesized that SCI would disrupt key circadian output and feedback mechanisms. Moderate SCI in rats caused widespread disruption of diurnal measures – including glucocorticoids, body temperature, locomotor activity, and intraspinal clock and inflammatory gene expression. We identify the circadian system as a novel potential target for SCI therapies.

Introduction

Spinal cord injury (SCI) dysregulates a constellation of physiologic processes throughout the body. For instance, SCI suppresses peripheral immunity, which increases susceptibility to infection (Schwab et al., 2014). SCI predisposes to accumulating excess adipose tissue and to metabolic syndrome (Jones et al., 2003; Cragg et al., 2015; Sauerbeck et al., 2015). In addition, SCI causes sympathetic dysfunction, which contributes to cardiovascular issues such as autonomic dysreflexia and orthostatic hypotension (Alan et al., 2010; Inskip et al., 2012). Thus, SCI shifts prominent body systems away from a healthy equilibrium (i.e., physiologic homeostasis).

Physiological homeostasis is regulated by the circadian system, which synchronizes organisms to daily fluctuations in their external environment by temporally organizing internal systems (Fonken and Nelson, 2014). Circadian rhythms are endogenous free-running rhythms (persist even in constant darkness) lasting ~24 hours that are entrainable to external cues; when synchronized by light to day-night cycles, these rhythms are called “diurnal” rhythms. Initial circadian input occurs via light activation of retinal projections to the suprachiasmatic nucleus (SCN). SCN neurons entrain diurnal rhythms in other cells of the brain and body through neural (autonomic), humoral (e.g., glucocorticoids), and physiological cues (external cues that coordinate biological rhythms; e.g., feeding, activity, and body temperature) (Bedrosian et al., 2016). Circadian disruption can impair physiologic function, predispose to disease, and exacerbate post-surgery outcomes (Fonken et al., 2010; Li et al., 2011; Stevens et al., 2014). For example, disrupting the circadian clock with aberrantly timed light exposure following

traumatic brain injury in rats increased neuronal death and cortical lesion volume, resulting in worsened sensorimotor and cognitive deficits (Li et al., 2016).

Research in rodents and humans suggests that SCI likely disrupts the circadian system. In mice, SCI transiently increased and disrupted daily expression rhythms of the major rodent glucocorticoid, corticosterone (CORT) (Lucin et al., 2007). In rats, T3 spinal transection disrupted temperature rhythms for ~14 d post-SCI (West et al., 2015). Together, these results imply that SCI may disrupt diurnal rhythms; however, these studies omitted sham surgery groups, which are essential given that surgery itself can disrupt these systems. Further, these studies did not explore other key circadian outputs (activity, circadian clock genes, etc.). In humans, cervical SCI disrupted diurnal body temperature rhythms at chronic times (Thijssen et al., 2011), and individuals with SCI often experience sleep disturbances (Giannoccaro et al., 2013); yet diurnal rhythms in humans at acute-to-subacute post-SCI times remain under-studied. Moreover, the existence and post-SCI regulation of a molecular circadian clock in spinal cord remains unstudied. Therefore, there is a need to understand the timing and extent of SCI-elicited circadian disruption, and how this could relate to loss – and potential reacquisition – of physiologic homeostasis.

Here, we hypothesized that SCI in rats would disrupt physiological, behavioral, and molecular diurnal rhythms. Female and male rats with moderate-severity thoracic SCI displayed a strong increase in, and arrhythmia of, a major circadian entraining factor, CORT. In addition, SCI dampened daily rhythms in body temperature and activity. Moreover, spinal cords from

117 uninjured rats displayed robust diurnal regulation of clock genes; SCI dysregulated intraspinal
118 expression of clock and inflammatory genes, both in epicenter and in distal lumbar spinal cord.
119 We reveal that clinically relevant SCI in rats broadly disrupts circadian function, particularly
120 acutely post-injury.
121

Materials and Methods

Surgery and animal care

These experiments were approved by University of Colorado Boulder Institutional Animal Care and Use Committee and conformed with ARRIVE standards. In agreement with NIH guidelines, male and female rats were included in all experiments. Rats had standard chow and filtered tap water *ad libitum* and were maintained on a 12:12 light/dark cycle. All surgeries occurred between Zeitgeber time (ZT)-2 and -11. Sprague-Dawley sham/SCI rats (females: 200–250 g, males: 320-380 g; 2–3 months old; Envigo, Indianapolis, IN) received isoflurane anesthesia and T8 laminectomy; SCI rats additionally received moderate contusion injury (midline SCI; 150 kDyn, 1 s dwell; Infinite Horizon device, Precision Systems and Instrumentation, Fairfax Station, VA). Initial related studies involved pain testing, and inflammation was also studied, so analgesics were not given to any rats for consistency and limiting confounds (Detloff et al., 2008; Cho et al., 2009; Ellis et al., 2016; Grace et al., 2016). All rats received prophylactic and post-surgery intraperitoneal (i.p.) antibiotics (gentamicin sulfate), subcutaneous saline for 5 days post-injury (dpi), and post-SCI bladder voiding twice-daily (Gaudet et al., 2015; Gaudet et al., 2017).

Tissue processing, histology, and analysis

To evaluate lesion size and spared tissue area, tissue was collected for immunohistochemistry (n=7 male and female rats) at 7 dpi. Rats received i.p. pentobarbital overdose and were trans-cardially perfused with 0.9% saline, then 4% paraformaldehyde. Spinal cords were suspended in paraformaldehyde overnight, cyroprotected in 30% sucrose, and cryosectioned (16μm) (Gaudet

et al., 2015). For immunohistochemistry, slides were incubated with 10% normal donkey serum (one hour), then with primary antibodies (overnight; mouse anti-glial fibrillary acidic protein (GFAP) (1:100; 0869110, MP-Biomedicals, Santa Ana, CA) and rabbit anti-Iba1 (1:1000; 019-19741, Wako Chemicals, Richmond, VA), then with secondary antibodies (two hours; Alexa-488 donkey anti-mouse (A-21202) and Alexa-546 donkey anti-rabbit (A-10040); both 1:500; ThermoFisher) and DAPI (nuclear stain; D1306 ThermoFisher). Images were captured on an Olympus IX81 Microscope (Olympus, Waltham, MA), and analyzed using Fiji (Schindelin et al., 2012).

Locomotor testing

Locomotor recovery was assessed using the Basso-Beattie Bresnahan (BBB) scale (Basso et al., 1995) (female sham n=6, SCI=5; male sham n=7, SCI n=5) by two condition-blind observers prior to surgery; and at 1, 4, 7, 10, 14, 21, 28, 35, and 42 d post-surgery.

Collection and measurement of plasma corticosterone

Rats were pair-housed; cagemates had the same surgery (at start: female sham n=6, SCI=10; male sham n=6, SCI n=10; two female and one male SCI rat died). Rats were acclimated to handling for 5 days; then, blood samples were collected from immobilized unanesthetized rats via tail nick (<500μL/24h). Blood was collected pre-surgery, and 2, 7, and 14 d post-surgery at ZT0, 6, 12, and 18 (starting at ZT0 – CORT typically lowest). For dark phase collections, dim red light headlamps were used (Fonken and Nelson, 2014). Samples were centrifuged (10,000g,10 min) to isolate plasma. Plasma was used in a CORT ELISA (ADI-901-097; Enzo, Farmingdale, NY).

Several samples (various rats and timepoints) had insufficient plasma, so were excluded and samples that took longer than 3 min to collect were excluded (due to confounding stress-elicited CORT).

Body temperature and locomotor activity measurement

Individually housed rats were implanted i.p. with radiotelemetric transmitters (MiniMitter, Respironics; Bend, OR) (Fonken et al., 2012). After a one-week recovery, pre-laminectomy baseline activity/temperature was recorded for one week. Homecages on 12 TR-4000 receiver boards linked with DP-24 DataPorts (MiniMitter) continuously collected temperature/activity. Activity and body temperature were recorded for >6 weeks post-surgery.

Female and male experiments were completed separately due to a limited number of telemetry receivers. First, males (sham n=6; SCI n=5 – originally 6; one SCI rat transmitter did not transmit) were tested. Including shams was important: basic surgeries can disturb circadian rhythms (Farr et al., 1988). Sham/SCI rats received daily handling post-surgery (twice daily bladder emptying or handling for shams; at ~ZT2 and ZT10) and daily antibiotic/saline injections for 5 d post-surgery (~ZT2). Two weeks post-surgery, rats voided bladders independently and daily checks continued without handling. After the male experiment, experimental design was improved. For females (sham n=6; SCI n=6), pre-surgery handling (twice/day) acclimated the rats and reduced potential stress effects on post-surgery activity/temperature. Post-surgery antibiotic/saline injections (for 5 d post-surgery) occurred at afternoon (ZT10) animal care, to limit disruption of key early inactive phase rhythms.

188

189 **Diurnal regulation of gene expression**

190 For the uninjured PCR study, tissues were collected from uninjured female/male rats across the
191 day (ZT0, ZT6, ZT12, ZT18; n=6/sex/timepoint). Rats received i.p. pentobarbital overdose, then
192 were perfused with 0.9% saline. Tissues were flash frozen for PCR (L4-L5 was used for uninjured
193 spinal cord analyses; T8 (lesion) and L4-L5 were used for post-surgery analyses). L4-L5 was used
194 as a distal spinal cord site because it integrates hindpaw nociceptive information, which could
195 provide useful information for future studies. L4-L5 was used for the uninjured analyses, since
196 the T8 injury site was expected to have major shifts in gene expression (SCI vs. sham, given that
197 the lesion was present), whereas the lumbar spinal cord was predicted to have more minor SCI-
198 elicited changes. For the sham/SCI PCR study, female/male rats received sham/SCI surgery
199 (n=6/sex, except lumbar spinal cord, n=4/sex; 2 d post-surgery collection). Prior to surgery, rats
200 were handled to minimize post-surgery bladder care stress. SCI rats received post-surgery
201 bladder care and sham rats received similar handling (control for stress); the bladder care
202 immediately prior to tissue collection was omitted to limit circadian disruption. Tissue from
203 sham/SCI rats was collected at mid-light phase (ZT6; ~48 h post-surgery) and at mid-dark phase
204 (ZT18; ~60 h post-surgery) at 2 d post-surgery. Tissue collections were completed within 1-2
205 hours of the timepoint (e.g., ZT6 – tissue collected ZT5-7). Sub-groups of males and females
206 were collected on the same days to minimize between-day differences and to enable sex
207 comparisons.

208

Quantitative real-time PCR was completed as described (Fonken et al., 2018). Primers (Invitrogen) spanned exons (see Table 1 for sequences). Gene expression was assessed in duplicate and is presented relative to β-actin. There were no significant differences in β-actin expression between groups, and male, female, sham, and SCI rat samples were all run on the same plates to ensure consistency. PCR results were analyzed using $2^{-\Delta\Delta Ct}$ and normalized with female sham-ZT6 set to 1.

Statistics

Data were analyzed (SigmaPlot 13.0; Systat Software, San Jose, CA) using Student's *t*- or non-parametric Mann-Whitney U test; or ANOVAs (one- two- or three-way ANOVA, as appropriate). Holm-Sidak *post-hoc* tests were completed when appropriate for all tests involving more than two groups. Circadian data were analyzed using CircWave (https://www.euclock.org/results/item/circ-wave.html), which assesses independent circadian data (i.e., one individual contributes to a single data point) to identify waveforms and acrophases (rhythm peaks). Twice-daily post-surgery handling (voiding/injections) caused stress-elicited hyperthermia and hyperactivity in all groups and were excluded from CircWave analyses for clarity. Activity data were also analyzed using ClockLab 6.0 (Actimetrics, Wilmette, IL); actograms and wavelets were assessed for each rat and data from representative male sham and SCI individuals are presented. Actograms were scaled between 0 and 30. Researchers were blind to experimental group. Data were significant when $p<0.05$. Data plotted as mean±SEM.

Results

T8 spinal cord contusion (150 kDyn, 1 s dwell) causes tissue pathology and locomotor deficits

First, we examined tissue damage in male and female rats at 7 dpi in this SCI model (Gaudet et al., 2017) (Fig. 1). As expected, substantial tissue loss was observed at the T8 lesion epicenter (Fig. 1D); moving rostro-caudally away from the epicenter, there was progressively more tissue sparing (Fig. 1E). There were no significant differences in lesion size or tissue sparing in females vs. males (lesion volume: females, 8.15mm^3±0.70mm^3; males, 7.48mm^3±0.46mm^3; p>0.05).

Locomotor recovery after sham/SCI was assessed (Fig. 1F). At 1 dpi, average BBB scores indicated that SCI rats had movement of one hindlimb joint. By 42 dpi, SCI rats recovered frequent hindlimb stepping with no (or little) coordination (females, BBB score 10.6±0.6; males, BBB score 11.0±1.4).

Spinal cord injury causes a transient increase and arrhythmia in plasma CORT

CORT release is regulated in a circadian manner; this steroid hormone maintains metabolic homeostasis across the day and helps entrain extra-SCN circadian rhythms (Nicolaides et al., 2014). Here, we assessed whether SCI dysregulated plasma CORT levels and rhythms (Fig. 2). Prior to surgery, female and male rats showed peak-trough patterns in plasma CORT (Fig. 2A) (D'Agostino et al., 1982): CORT had cycle peak (acrophase) near early-mid active phase (around ZT12) (females ZT12: 1.4ng/mL±0.3ng/mL; males ZT12: 0.6ng/mL±0.1ng/mL), and was lowest at the start of the inactive phase (ZT0) (females: 0.6ng/mL±0.2ng/mL; males: 0.4ng/mL±0.1ng/mL). CORT levels were higher overall in pre-surgery females than males

(Cavigelli et al., 2005). In females, CORT was unusally high at ZT6, which could have been related to a handling-elicited CORT increase (Gartner et al., 1980). CORT was expressed rhythmically prior to injury in both females ($F_{2,37}$=3.465, p<0.05) and males ($F_{2,33}$=6.647, p<0.005).

SCI disrupted typical rhythms and significantly increased CORT levels at acute post-injury times (Females: main effect of injury: $F_{3,62}$ = 3.606, p < 0.05; males: main effect of injury: $F_{3,70}$ = 22.584, p < 0.001). Females with SCI had increased CORT at 7 dpi (vs. pre-surgery; at ZT0; p<0.05); males with SCI showed significant CORT increases at 2 dpi (vs. pre-surgery; at ZT0 and ZT6; p<0.001) and 7 dpi (at ZT0; p<0.05) (Fig. 2B). For average CORT concentration across the day, SCI in female and male rats caused significant increases in average CORT at 2 d post-surgery (females: vs. pre-surgery, p<0.05) (males: main effect of surgery; also at 2 dpi vs. both pre-surgery and sham, p<0.001) (Fig. 2C).

Spinal cord injury alters daily rhythms in body temperature

Body temperature and activity are two prominent outputs of the circadian system that also help entrain circadian rhythms in cells throughout the body (Reebs and Mrosovsky, 1989; Buhr et al., 2010). To measure these parameters, rats were implanted with a small transmitter and were studied prior to sham/SCI, and from acute-to-chronic times post-surgery. (The studies on male and female rats were completed consecutively, due to limited number of telemetry receivers – see Materials and Methods for details.)

Prior to surgery, female and male rats displayed the expected diurnal rhythms in body temperature: core temperature was higher during the active phase, and lower during the inactive phase (female-SCI pre-surgery acrophase ZT16.6) (male-SCI pre-surgery acrophase ZT17.3) (Fig. 3). Immediately after surgery (i.e., after replacing post-surgery rats on transmitter boards), SCI but not sham rats experienced hypothermia (lasted ~18 h) (ANOVA main effect: female: $F_{1,155}$=31.664, p<0.001; male: $F_{1,142}$=24.663, p<0.001; and significant differences at specific times; see figure).

After initial post-SCI hypothermia, female rats showed significant SCI-elicited temperature dysregulation. By 2 d post-surgery, female sham and SCI rats both showed approximately typical rhythms in core temperature (Circwave, all showed rhythmicity). Compared to sham rats, female SCI rats had shifted (advanced) acrophases between 2-6 d post-surgery and increased inactive phase temperatures between 2-5 d post-surgery (2-6 d sham acrophases: ZT15.5, 15.7, 14.9, 15.9, respectively; 2-6 dpi SCI acrophases: ZT12.0, 12.9, 13.0, 14.5, respectively) (ANOVA main effect of treatment and injury-time interaction, 2-3 d post-surgery: $F_{1,203}$=6.678, p<0.05; sham v. SCI: 2 d post-surgery: ZT3,6,9 and 3 d post-surgery: ZT3,6,9 – p<0.05; 4-5 d post-surgery: $F_{1,203}$=12.207, p<0.01; sham v. SCI: 6 d post-surgery: ZT18 – p<0.05) (inactive phase temperature: SCI>sham, 1-5 d post-surgery, p<0.05). SCI rats displayed no significant differences vs. sham at 7, 13, or 14 d post-surgery (7 dpi: ANOVA, no main effect of treatment: $F_{1,203}$=0.982; 13-14 dpi: ANOVA, no main effect of treatment: $F_{1,203}$=1.147, p>0.05).

296 Male rats showed a similar pattern to females of SCI-elicited temperature dysregulation, but
297 with somewhat delayed recovery of rhythms. Sham rats at 2-3 dpi showed approximately
298 typical rhythms in core temperature (2 and 3 d post-surgery sham acrophases: ZT13.3 and
299 ZT16.2; all showed rhythmicity); conversely, male SCI rats at 2-3 dpi did not have significant
300 core temperature rhythms (ANOVA main effect of treatment: $F_{1,186}$=0.688, p>0.05). Male SCI
301 rats displayed shifted acrophases and increased inactive phase temperatures compared to
302 sham rats between 4-5 d post-surgery (acrophases: sham-4 d post-surgery: 16.4, SCI-4 dpi: 13.7,
303 sham-5 d post-surgery: 16.4, SCI-5 dpi: 12.5) (ANOVA main effect of treatment: $F_{1,186}$=0.657,
304 p>0.05) (inactive phase temperature: SCI>sham, 4-5 d post-surgery, p<0.05). There were no
305 significant differences caused by SCI in core temperature at 6-7 d post-surgery (ANOVA, no
306 main effect of treatment: $F_{1,186}$=0.400, p>0.05) nor at 13-14 d post-surgery (ANOVA, no main
307 effect of treatment: $F_{1,186}$=2.794, p>0.05).
308
309 To understand more broadly whether SCI influenced temperature rhythms, daily temperature
310 was processed in ClockLab in grouped multi-day "bins" (Fig. 4). Prior to surgery, peak daily
311 temperature phase occurred in females at an angle of 263.5^{o} (or ZT17.8) and in males at 275.2^{o}
312 (or ZT18.3). After surgery, females with SCI showed no significant difference in phase from
313 shams (p > 0.05; two-way RM ANOVA with Holm-Sidak *post-hoc*). In contrast, males with SCI
314 displayed significantly advanced temperature phases: SCI males had earlier temperature
315 acrophase starting at 2-5 d post-surgery (ANOVA main effect of treatment: $F_{1,81}$=43.299, p <
316 0.001) (2-5 d post-surgery: male-sham, phase angle 261.9^{o} ± 3.4^{o} (=ZT17.5); male-SCI, 208.3^{o} ±
317 15.3^{o} (=ZT13.9), p < 0.05) and these SCI-advanced rhythms continued through all 5 d bins

examined through 26-30 d post-surgery (26-30 d post-surgery: male-sham, 268.7° ± 4.9° (=ZT17.9); male-SCI, 249.9° ± 4.1° (=ZT16.7), $p < 0.05$). At 31-35 d post-surgery and beyond, male SCI rat temperature acrophases were not significantly different from male sham rats (e.g., 31-35 d post-surgery: male-sham, 269.5° ± 3.5° (=ZT18.0); male-SCI, 257.2° ± 4.° (=ZT17.1), $p > 0.05$).

Overall, SCI altered average core temperature at early post-injury times – particularly due to increased temperature during the inactive phase – and showed substantial recovery at subacute times. SCI males (vs. females) displayed significant and longer-lasting shifts in temperature acrophase.

Spinal cord injury disrupts amount and diurnal rhythms of activity

Activity patterns are a strong index of integrity of the circadian system. Although SCI clearly reduces activity, it is also possible that it disrupts activity rhythms. Rhythmic activity is important for entraining peripheral circadian rhythms (Reebs and Mrosovsky, 1989; Tahara et al., 2017). Prior to surgery, rats showed typical diurnal patterns of activity, with more activity during the active phase (female-SCI pre-surgery acrophase ZT15.1) (male-SCI pre-surgery acrophase ZT17.9) (percent of counts in active phase – females: 65%±2%; males: 67%±2%).

After surgery, differences in activity between sham and SCI rats were mainly during the active phase (Fig. 5). Sham rats showed relatively typical activity levels and diurnal activity rhythms by 2-3 d post-surgery (2 and 3 d post-surgery sham acrophases: female, ZT15.2 and ZT16.2; male,

ZT15.8 and ZT16.8). In contrast, female and male SCI rats had strongly reduced activity throughout the day at 2 dpi (total counts – female-sham: 13000±2000, female-SCI: 7000±2000, $p<0.05$; male-sham: 13200±800, male-SCI: 3200±800, $p<0.001$). Although this pattern of post-SCI reduced activity continued for days, there was some recovery of diurnal activity rhythms over the first 5 dpi. At 6-7 dpi, female and male rats with SCI showed further recovery of activity, but not 6 dpi, though their overall activity remained significantly lower than sham rats. Patterns of activity at 6 d post-surgery and beyond were not significantly different between sham and SCI rats in both females and males (percent counts in active phase at 6 d post-surgery – female-sham: 70%±2%, female-SCI: 63%±3%, $p>0.05$; male-sham: 70%±2%, male-SCI: 62%±3%, $p>0.05$).

At 13-14 d post-surgery, female and male SCI rats had more similar activity patterns to shams (acrophases: female-sham: 13.9, female-SCI: 13.3, male-sham: 16.2, male-SCI: 15.8) (total counts in active phase at 14 dpi – female-sham: 9000±900, female-SCI: 7000±1000, $p>0.05$; male-sham: 8500±500, male-SCI: 6600±500, $p<0.05$). These patterns were even more similar at a chronic post-SCI timepoint (acrophases: female-sham: 15.8, female-SCI: 15.7, male-sham: 16.1, male-SCI: 15.5). Therefore, SCI transiently dampens activity levels, and the timing of post-SCI recovery of daily activity counts and rhythms parallels recovery of other circadian measures.

Spinal cord injury delays recovery of 24-hour activity rhythms

To establish post-surgery latency to recovery of 24 h activity rhythms, actogram and wavelet analyses were performed. These qualitative assessments help visualize diurnal rhythms. Data

362 from one male sham rat and one male SCI rat (both representative) are presented. Prior to
363 surgery, the rats displayed expected rhythms in activity (Fig. 6A). After sham surgery, rats
364 recovered relatively typical rhythms within ~1 d post-surgery. In contrast, after SCI, rats
365 recovered 24 h activity rhythms around 6-7 d post-surgery (Fig. 6B,C; see yellow arrows).
366 Therefore, SCI substantially delayed recovery of diurnal activity rhythms.
367
368 **Spinal cord injury disrupts clock gene expression in epicenter and lumbar spinal cord**
369 Rhythms of circadian "clock" gene expression exist throughout the body; however, clock gene
370 expression has not been systematically characterized in the spinal cord. Thus, first we sought to
371 establish how clock genes are regulated by time-of-day in uninjured rat spinal cord (Fig. 7A).
372 Our data reveal that clock genes are expressed rhythmically in the L4-L5 spinal cord. The "core
373 clock" components *Per2*, *Cry1*, and *Bmal1* were all regulated in spinal cord by time and sex
374 (*Cry1*, *Bmal1*: main effect of time (p<0.001) and sex (females>males, p<0.001); *Per2*: significant
375 time-sex interaction, $F_{3,44}$=2.953, p<0.05) and were expressed rhythmically across the day (all in
376 both females and males) (acrophases: *Per2* female ZT15.2±1.2, male ZT12.2±1.2; *Cry1* female
377 ZT16.3±1.4, male ZT16.8±1.5; *Bmal1* female ZT23.0±1.4, male ZT0.8±1.3). Similarly, *Rev-erbα* –
378 a transcription factor in a secondary circadian feedback loop – was regulated in spinal cord by
379 time-of-day (interaction with sex; $F_{3,44}$=3.953, p<0.05) and was rhythmically expressed (in
380 females and males) (*Rev-erbα* acrophases: female ZT6.0±1.4, male ZT6.6±1.1).
381
382 To establish whether SCI alters molecular circadian rhythms, rats were subjected to T8 sham
383 (laminectomy) or SCI surgery, and tissue was collected at 2 d post-surgery in the light (ZT6) or

dark (ZT18) phase. Clock gene expression was assessed in T8 epicenter and lumbar spinal cord (Fig. 7B,C). In T8 epicenter, SCI (vs. sham surgery) substantially downregulated key clock genes *Per2*, *Cry1*, *Bmal1*, and *Rev-erbα* ($F_{1,45}$=45.7, 113.2, 82.1, 135.6, respectively; all p<0.001; main effect at ZT6 and ZT18; Fig. 7B). In addition, there were time-of-day differences in sham rats that were abolished by SCI in several genes (*Per2*, *Cry1*, and *Rev-erbα*; post-hoc), and there were sex differences in *Cry1* and *Rev-erbα* expression (main effect; males higher).

Dysregulation of circadian genes by SCI extended beyond the lesion site. Lumbar spinal cord from SCI (vs. sham) rats displayed significantly reduced expression of *Per2*, *Cry1*, *Bmal1*, and *Rev-erbα* ($F_{1,30}$=40.7, 48.4, 62.2, 23.8, respectively; all p<0.001, all main effects; SCI also downregulated *Per2* at ZT6 and ZT18) (Fig. 7C). In addition, there were main effects on clock gene expression of time (*Cry1*, *Bmal1*, *Rev-erbα*) and sex (*Per2*, *Cry1*, *Bmal1*). Additional clock genes dysregulated by SCI included *Per1* and *Clock* (Table 2).

Spinal cord injury disrupts inflammatory gene expression in epicenter and lumbar spinal cord

Inflammatory genes in the CNS can also be regulated by time-of-day, and this likely confers differential immunocompetence across the day (Fonken et al., 2015). In the healthy adult rat spinal cord, several inflammatory genes were expressed differentially across the day (Fig. 8A): the pro-inflammatory cytokines *IL-1b* (main effect of time, $F_{3,43}$=3.54, p<0.05; females had significant daily rhythm, p<0.05) and *Tnfa* (main effect of time, $F_{3,44}$=5.57, p<0.005; males had significant daily rhythm, p<0.01); the inflammatory cytokine *IL-6* (main effect of time, $F_{1,44}$=6.99, p<0.001; males had significant daily rhythm, p<0.005); and the microglial/macrophage

406 activation marker *Cd68* (significant interaction of time and sex, $F_{3,44}$=5.51, p<0.005; males had
407 significant daily rhythm, p<0.005).
408
409 SCI significantly dysregulated inflammatory gene expression in epicenter and lumbar spinal cord
410 (main effects; Fig. 8B,C). *IL-1b* was significantly upregulated in SCI (vs. sham) rats in the T8
411 lesion epicenter, but was downregulated in the lumbar spinal cord ($F_{1,45}$=11.9, $F_{1,30}$=144.7,
412 respectively; both p<0.001; main effects). *Tnfa* was upregulated by SCI in both the epicenter
413 and the lumbar spinal cord ($F_{1,45}$=12.9, $F_{1,29}$=7.9, respectively; both p<0.001; main effects) (also
414 main effect of time: lumbar). *IL-6* was increased in SCI rats in the epicenter, but was reduced in
415 the lumbar spinal cord ($F_{1,44}$=43.6, p<0.001, $F_{1,29}$=4.5, p<0.05, respectively; main effects; also
416 main effect of sex in both tissues). *Cd68* was increased by SCI in the epicenter, but was
417 downregulated in the lumbar spinal cord ($F_{1,44}$=32.6, $F_{1,29}$=31.2, respectively; both p<0.001;
418 main effects) (also main effect of time (epicenter and lumbar) and sex (epicenter)). (SCI also
419 altered intraspinal expression of *Iba1*, an RNA expressed by microglia/macrophages, and *Mhc II*,
420 an antigen-presenting molecule expressed by microglia/macrophages; Table 2). Thus, SCI
421 robustly dysregulated intraspinal clock and inflammatory gene expression.
422

Discussion

This study used female and male rats to determine whether SCI dysregulated acute-to-chronic physiological and behavioral rhythms and related molecular outputs. Moderate T8 spinal cord contusion dysregulated plasma CORT – a key humoral output of, and feedback signal for the circadian system – particularly at 2 and 7 d post-surgery. In addition, SCI dampened diurnal rhythms in locomotor activity and body temperature. SCI strongly decreased expression of several clock genes in the epicenter and lumbar spinal cord, and also altered expression of intraspinal inflammatory genes at two times of day. Therefore, moderate SCI in a rat model severely disrupts daily rhythms in CORT, activity, body temperature, and intraspinal gene expression. The physiological parameters studied gradually reacquired more typical daily rhythms over time. Our results suggest that moderate contusion SCI causes widespread, transient disruption of physiologic homeostasis (Fig. 9).

Locomotor recovery after SCI

As expected, rats with SCI showed substantial deficit in hindlimb movement at 1 dpi, and the rats recovered frequent stepping by the chronic 42 dpi timepoint. Locomotor recovery had not previously been assessed in this model (a T8 150 kDyn contusion with 1 s dwell) (Putatunda et al., 2014; Gaudet et al., 2017; Hook et al., 2017). Interestingly, post-SCI locomotor recovery presented here complements others' findings: our average BBB score of 10.6 in female 42 dpi rats supports that this injury severity is between the severities reported previously (vs. 150 and 200 kDyn with 0 s dwell (Scheff et al., 2003)).

SCI disrupts diurnal rhythms and physiologic homeostasis

Diurnal rhythms help optimize organism function across the day; thus, disrupting diurnal rhythms likely shifts an animal's physiology away from homeostasis. Prolonged diurnal disruptions have detrimental effects for the individual. Here, we identify SCI as a disruptor of several prominent physiological rhythms – CORT, core temperature, and activity.

Glucocorticoids are steroid hormones that help synchronize extra-SCN circadian rhythms (Balsalobre et al., 2000). This is possible because SCN-derived signals entrain CORT rhythms to light and SCN cells lack CORT receptors, whereas most non-SCN cells express CORT receptors. In this manner, CORT can modulate peripheral circadian clocks directly (e.g., CORT-receptor complex drives *Per1* and reduces *Rev-erb*α transcription) (Oster et al., 2006; Dickmeis, 2009; Surjit et al., 2011). CORT is potently upregulated by immune activation and helps resolve inflammatory responses; however, CORT is also critically involved in stress responses (Frank et al., 2013) and basic homeostatic functions (Nicolaides et al., 2014). Given these roles of CORT in health and pathology, we hypothesized that SCI would dysregulate CORT rhythms. Indeed, male and female rats with acute SCI had increased CORT levels and disrupted CORT rhythms. Previous work supports our findings: female mice with T9 contusion SCI had acute, transient dysregulation of glucocorticoid rhythms (Lucin et al., 2007), although no sham mice were included. A recent study by Pruss *et al.* found that SCI in mice increased serum CORT (T1 SCI>T9 SCI; 3 dpi CORT with no time-of-day specified); similarly, humans within 96h post-SCI showed increased serum cortisol (Pruss et al., 2017). SCI in mice and humans substantially increases susceptibility to peripheral infection (pneumonia). In mice, SCI-elicited CORT increases could be

467 quenched by removing the major source of systemic CORT, the adrenal glands, but
468 adrenalectomy did not reduce frequency of pneumonia (Pruss et al., 2017). Interestingly,
469 transplanting denervated adrenal glands into adrenalectomized SCI mice normalized CORT
470 levels and limited susceptibility to pneumonia (Pruss et al., 2017); also see (Oster et al., 2006).
471 Thus, accelerating recovery of CORT rhythms could help regain homeostasis, including diurnal
472 rhythms.
473
474 We also revealed that daily rhythms in body temperature were significantly altered at acute
475 post-SCI times and gradually recovered. Immediate post-SCI hypothermia lasted ~24 h
476 (hypothermia not observed in shams). Body temperature acrophase was particularly disrupted
477 in male rats with SCI, which had altered temperature acrophase that persisted through 30 dpi.
478 Others also observed SCI-elicited temperature arrhythmia in rats (West et al., 2015). Similarly,
479 humans with acute trauma (including SCI) commonly experience hypothermia, which can co-
480 present with other pathologies and lead to death (Kirkpatrick et al., 1999). In humans with
481 chronic SCI, diurnal regulation of core body temperature was disrupted after cervical, but not
482 thoracic SCI (though acute post-SCI rhythms were not assessed) (Thijssen et al., 2011). Body
483 temperature rhythms help coordinate peripheral circadian rhythms (Buhr et al., 2010); thus,
484 optimizing post-SCI recovery of body temperature rhythms could accelerate recovery of
485 homeostasis.
486
487 Activity rhythms were transiently dampened by SCI, which could have more widespread
488 implications for the circadian system. For example, timed activity can be used to help

strengthen diurnal rhythms: in mice with a mutation in core circadian clock machinery, scheduled (late night) wheel running strengthened molecular and physiological rhythms (Schroeder et al., 2012). Behavioral activity feeds back to the SCN: exercise in mice suppresses SCN neuron activity, and exercising at the proper time of the cycle increases SCN diurnal rhythm amplitude (van Oosterhout et al., 2012). In humans, exercising at abnormal times (during the night) phase shifts diurnal rhythms (Youngstedt et al., 2016). Therefore, post-SCI diurnal rhythms may be strengthened by optimizing the time-of-day of key activities, including rehabilitation, social activity, and feeding.

Glucocorticoids, body temperature, and activity modulate circadian oscillators throughout the body, so SCI-elicited dysregulation could alter daily rhythms more broadly. Indeed, typical diurnal rhythms in body function can be disrupted in humans by SCI (e.g., sleep (Giannoccaro et al., 2013)) and by related medical interventions (Li et al., 2011), so expediting recovery of diurnal rhythms after SCI could improve neuroprotection and functional outcomes.

It is important to note that we had limited telemetry receivers, so we completed the body temperature and activity transmitter study separately in males and females. In addition, limitations were noted after completing the male study and methods were improved for the female portion (e.g., rats were acclimated to twice daily handling prior to surgery), which could have affected the consistency of the results. Despite this, the results were remarkably similar between males and females – both for sham and SCI rats – and differences existed between sham and SCI groups. Our experience and data also highlight that typical post-surgery rodent

511 care and behavioral testing in many research labs (e.g., twice-daily rodent care during the
512 light/inactive phase) likely further disrupts daily rhythms (as well as sleep), and could be a
513 confound to the factor under study (e.g., (McEwen, 2006). Future SCI studies could improve
514 design by having the daily injections and major care immediately before the active phase, and
515 by having a second potential daily care time during the dark or at another minimally disruptive
516 time (using shifted light schedules in animal housing, if helpful). In addition, given that intense
517 rodent care during the beginning of the inactive phase is likely a strong disruptor of rhythms,
518 future studies could compare whether this type of disruption affects anatomical and neurologic
519 outcomes compared to a "circadian-optimized" care schedule. These findings would have
520 translational relevance, since early post-SCI patients in hospitals likely experience excess
521 circadian disruptions (that could be improved with relatively simple interventions).
522
523 Another distinction between studies described herein is that the telemetry study was
524 completed with individually-housed rats (to enable measuring a single rat's temperature and
525 activity), whereas all other studies were completed with rats housed in pairs. This is important
526 to note, given that housing rats individually is a stressor (Von Frijtag et al., 2000; Westenbroek
527 et al., 2005) and can impair recovery after CNS injury (Craft et al., 2005; Karelina et al., 2009;
528 Gaudier-Diaz et al., 2017). Furthermore, single housing may reduce relative activity (vs. having a
529 cagemate) and therefore likely worsens other aspects of recovery (e.g., locomotor recovery)
530 (Van Meeteren et al., 2003).
531
532 **SCI alters intraspinal expression and diurnal regulation of clock and inflammatory genes**

533 At 2 d post-surgery, SCI remarkably reduced expression of clock genes was observed in the
534 lesion epicenter, and a more modest (but significant) decrease also occurred in the lumbar
535 spinal cord. In the lesion epicenter, this is likely affected by altered cell composition: sham T8
536 spinal cords contain typical CNS cellular constituents, whereas SCI T8 epicenters include various
537 activated immune, glial, and other cells (Gaudet and Fonken, 2018). Epicenter-localized cells
538 could have different clock gene levels that cause the massive decrease observed. Another
539 possibility is that SCI dysregulates a finely-tuned circadian and inflammatory gene expression
540 network: immune cell-specific circadian gene dysregulation can provoke inflammatory
541 responses (Nguyen et al., 2013; Fonken et al., 2016; Segal et al., 2018); conversely, induction of
542 cytokines such as TNF-α and IL-1β can suppress expression and/or function of circadian genes
543 (Cavadini et al., 2007). Indeed, intraspinal *Tnfa* and *IL-1b* diurnal regulation inversely correlated
544 with *Per2* expression, both in uninjured spinal cord and in the SCI epicenter. Similarly, Rev-erbα
545 reduces IL-6 expression (Gibbs et al., 2012); here, diurnal variation in *Rev-erbα* and *IL-6* levels
546 were inversely related in uninjured spinal cord, and the SCI-elicited decrease in epicenter *Rev-*
547 *erbα* paralleled an increase in *IL-6*. These findings underscore the potential relevance of clock
548 and inflammatory gene cross-talk. In addition, SCI downregulated clock genes even distal to
549 lesion in lumbar spinal cord, where cell infiltration likely has limited influence. Overall,
550 decreased and arrhythmic clock gene expression by SCI could broadly regulate immune and
551 homeostatic function in the CNS.
552
553 Inflammatory gene expression was differentially affected by SCI in epicenter and lumbar spinal
554 cord. In the epicenter, most genes assessed were upregulated; conversely, most genes assessed

555 in the lumbar spinal cord were downregulated. L4-L5 spinal cord was chosen as a distal spinal
556 cord site, because it integrates hindpaw nociceptive information. At later times post-SCI, excess
557 below-level intraspinal inflammation likely contributes to SCI-elicited neuropathic pain (Hains
558 and Waxman, 2006; Detloff et al., 2008), and we found that this contusion model causes
559 neuropathic pain symptoms by 14 dpi in female and male rats (Gaudet et al., 2017). The
560 reduced expression of inflammatory markers at 2 dpi may represent an initial remote
561 intraspinal dampening of the immune response, which may precede chronic
562 neuroinflammation that exacerbates neuropathic pain and pathology.
563
564 Here, two timepoints (middle of the light (ZT6) and dark (ZT18) phase) were used to assess
565 clock and inflammatory gene expression at 2 d post-SCI. This is a limitation of the study, since
566 with two timepoints it is not possible to make more broad conclusions about the effects of SCI
567 on circadian phase, amplitude, and average expression. Future studies could examine
568 expression at additional times of day. Further, disruption of circadian outputs persisted beyond
569 2 dpi, including rhythms of serum CORT, body temperature, and activity (all were disrupted
570 until at least 7 dpi) - yet our study only assessed gene expression changes at 2 d post-surgery.
571 Gene expression was assessed at 2 d post-surgery because robust changes in the circadian
572 system were seen at this time, and because early changes in lesion pathology and inflammation
573 likely have strong effects on recovery trajectory (Noble et al., 2002; Kigerl et al., 2009; Gaudet
574 et al., 2018; McCreedy et al., 2018). It is also important, however, to understand how clock and
575 inflammatory gene patterns shift through sub-acute and chronic times post-surgery. In the

future, studying circadian gene expression changes at additional times post-SCI would provide more information about the robustness and duration of SCI's effects on the circadian system.

Future directions

Our study highlights that SCI disrupts whole-body functions, yet unresolved questions remain about post-SCI circadian disruption and related potential therapies. As shown in Fig. 9, SCI alters regulation of several circadian outputs. Daily rhythms are entrained by light input to the retina influencing the SCN, which is likely protected from changes caused by SCI (e.g., SCN neurons lack CORT receptors (Balsalobre et al., 2000; Woodruff et al., 2016), unlike most cells of the body). However, peripheral rhythms are synchronized and entrained by other circadian oscillators – including CORT, body temperature, activity, and autonomic circuits (Buijs et al., 2016) – that are perturbed by SCI. Therefore, the disruption of circadian outputs could contribute to more widespread circadian disruption throughout the body. Here, a moderate T8 spinal cord contusion was used; future studies could establish whether more severe injuries or injuries at other spinal levels (e.g., cervical or high thoracic, which would more strongly perturb autonomic function (Inskip et al., 2009)) differentially alter post-injury circadian dynamics. In addition, our study did not address whether circadian disruption was simply a result of injury, or whether disruption itself further exacerbated outcomes. Future studies could determine whether amplifying post-SCI rhythms using timed exercise, feeding, light-dark schedule manipulations, injections of circadian-related drugs, etc. could improve recovery measures (discussed more below). Strengthening of post-SCI rhythms could also influence other long-term recovery measures, such as survival, neuroprotection, chronic pain, autonomic function,

598 and general health. Further, since altering circadian rhythms affects other aspects of
599 metabolism, metabolic cage telemetry and calorimetry (Gaudet et al., 2016) could reveal
600 whether boosting post-SCI rhythms improves short- and long-term metabolic health. Finally, to
601 better understand the effect of SCI on circadian rhythms, SCI researchers could adopt other
602 models and strategies frequently used in circadian research – such as the *PER::luciferase*
603 transgenic rodent, which uses the clock gene promoter from *Per* to express the bioluminescent
604 *luciferase* gene and enables studying rhythm synchrony/dysregulation *in vitro* (Yamazaki et al.,
605 2000) and *in vivo* (Tahara et al., 2012).
606
607 If SCI-elicited circadian disruption worsens recovery and metabolism, amplifying diurnal
608 rhythms using entraining strategies soon after injury could expedite recovery (Roenneberg and
609 Merrow, 2016). For instance, improving darkness of rooms during nights (minimize brightness
610 of lights/monitors; use red lights and filters) and incorporating bright morning light (e.g., having
611 an outdoor-facing window in the room) (Engwall et al., 2014), feeding during the day (Stokkan
612 et al., 2001; Fonken et al., 2010), and optimizing time-of-day of rehabilitation/activity
613 (Schroeder et al., 2012) could improve sleep-wake cycles and re-entrain diurnal rhythms. Using
614 these and other strategies to accelerate post-SCI recovery of homeostasis could boost key early
615 recovery processes and overall post-injury outcomes.
616
617 **Conclusions**
618 In conclusion, we used a clinically-relevant rat spinal contusion model to assess how SCI affects
619 circadian dynamics. We established that moderate thoracic SCI has broad effects on diurnal

620 rhythms, including disrupted rhythms of plasma CORT levels, activity, body temperature, and
621 intraspinal gene expression. SCI caused robust diurnal rhythm disruption at acute post-SCI
622 times (2 and 7 dpi; across various circadian measures); SCI rats recovered more typical diurnal
623 rhythmicity by subacute-to-chronic times (14-42 dpi). SCI associated disruption of these key
624 regulators of physiologic homeostasis may feed back to impede SCI recovery. Future discovery
625 and clinical SCI studies could incorporate measures of circadian function, which may reveal
626 post-SCI “chronotherapies” that help regain homeostasis and improve recovery.

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628
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852

Figure Legends

Figure 1. T9 contusion SCI (150 kDyn, 1 s dwell) causes extensive tissue loss at/near epicenter with associated locomotor impairment. (A-B) Spinal cords from female (A) and male (B) rats show substantial pathology and cavitation at 7 d post-SCI. Glial fibrillary acidic protein (GFAP) immunoreactivity was used to visualize and assess tissue pathology; dotted lines outline approximate lesion border in each section. (C) Example of GFAP (astrocytes, green) and Iba1 (microglia/macrophages, red) immunoreactivity in the 7 dpi lesion site (blue = nuclei; DAPI). $GFAP^+$ astrocytes form the glial scar; $Iba1^+$ macrophages/microglia exist in the epicenter and $Iba1^+$ microglia are present in the lesion penumbra. (D,E) Analysis of lesion area and volume (D) and percent spared tissue (E) at 7 d post-SCI. There were no significant sex differences in lesion size or percent spared tissue. (F) Moderate T9 SCI caused substantial immediate locomotor deficits in female and male rats that recovered over time, as assessed in an open-field using the BBB scale. There were no significant differences in BBB scores between female and male SCI rats. Scale bar = 1 mm.

Figure 2. SCI in female and male rats disrupts diurnal rhythms in corticosterone (CORT). (A) Prior to surgery, female and male rats exhibited diurnal rhythms in CORT, with nadir levels at the beginning of the inactive (light) phase and peak levels towards the beginning of the active (dark) phase. (B) SCI increased CORT levels and altered CORT rhythms at 2 and 7 dpi; CORT rhythms normalized by 14 dpi. (C) Average CORT across the day in female and male SCI rats was increased at 2 dpi. * indicates ZT differences or SCI vs. pre-surgery, ANOVA with Holm-Sidak *post-hoc*, $p < 0.05$; † indicates SCI vs. sham at that timepoint, $p < 0.05$.

Figure 3. SCI in female and male rats disrupted diurnal rhythms of core body temperature. Prior to surgery, female and male rats exhibited typical diurnal core temperature rhythms. Immediately after

877 surgery, SCI rats displayed hypothermia. SCI disrupted diurnal rhythms in core temperature between 2-5
878 d post-surgery. Diurnal temperature regulation was similar between sham and SCI rats by 13-14 d
879 post-surgery. * indicates $p < 0.05$ for sham vs. SCI at that timepoint, ANOVA with Holm-Sidak *post-hoc*.
880
881 **Figure 4.** SCI significantly advanced temperature acrophase in males, but not females. Temperature
882 phase angles (in bins of 4-5 d) were studied in female and male rats prior to surgery, then up to 50 d
883 post-surgery. (A) Compared to shams, females with SCI showed no significant difference in phase angle
884 after surgery. (B) SCI males had significantly advanced temperature acrophases from 2-5 d through 26-
885 30 d post-surgery. (C) Phase plot showing how SCI affected phase angle over time post-surgery (up to 25
886 d for clarity). Angle indicates time of acrophase; radial distance depicts time post-surgery. * indicates $p <$
887 0.05.
888
889 **Figure 5.** SCI in female and male rats disrupted diurnal rhythms in activity. Data show cumulative activity
890 counts per 3 hour time period. Prior to surgery, female and male rats exhibited typical diurnal rhythms
891 in activity, with rats more active in the dark phase. As expected, SCI rats were less active at acute times
892 post-injury (up to 7 d post-surgery). Interestingly at sub-acute (14 d post-surgery) to chronic times, SCI
893 rats regained activity levels and diurnal rhythms in activity that were similar to sham rats. * indicates $p <$
894 0.05 for sham vs. SCI at that timepoint, ANOVA with Holm-Sidak *post-hoc*.
895
896 **Figure 6.** Rats with SCI show delayed recovery of diurnal activity rhythms. These representative data are
897 from one male sham and one male SCI rat. (A,B) Actograms display continuous measures of activity
898 across the day (ZT; x-axis) and over time post-surgery (y-axis). (A) Prior to surgery, these rats in the sham
899 and SCI groups had expected diurnal patterns of activity (i.e., increased activity between ZT12-24; higher
900 black bars). (B) After surgery, sham rats quickly recover more typical activity rhythms, whereas SCI rats

show delayed post-surgery recovery of rhythms. Yellow arrows highlight approximate latency to recovery of 24 h rhythms; also shown in C. (C) Wavelet analysis shows that pre-surgery rats display strong 24 h rhythms, and that sham and SCI rats have different post-surgery latencies to recover 24 h activity rhythms (yellow arrows). The sham rat recovered ~24 h rhythms within 1 d post-surgery, whereas the SCI rat recovered ~24 h rhythms around 6 d post-surgery. Intensity of rhythm across days is represented by the colour continuum: purple (minimal rhythm) through blue and green to red (intense rhythm).

Figure 7. Diurnal regulation of clock genes in spinal cord from uninjured rats, and from sham/SCI rats at 2 d post-surgery. (A) Female and male rat spinal cords express clock genes, and clock gene expression varies through the day. *Per2*, *Cry1*, *Bmal1*, and *Rev-erba* displayed rhythmic expression in the spinal cord. Females generally expressed higher levels of clock genes. (B) SCI disrupts clock gene expression at the lesion epicenter. Clock gene expression at ZT6 and ZT18 was assessed. Sham spinal cords showed clock gene variation between the two timepoints (e.g., *Per2*, *Cry1*, and *Rev-erba*). After SCI, injury epicenters showed strongly reduced expression and ablated diurnal variation of these four clock genes, suggesting circadian disruption. (C) SCI disrupts clock gene expression in spinal cord distal to injury. The lumbar spinal cord, which was not directly injured by contusion, showed decreased expression of the four clock genes examined in SCI versus sham rats. There were also time-of-day differences for all genes presented, and a significant main effect of sex for *Per2*, *Cry1*, and *Bmal1*. Black ~ indicates that females or males for that gene show significant rhythm; red **+** indicates injury difference (sham v. SCI), $p < .05$; yellow hourglass indicates time difference, $p < .05$; blue gender symbol indicates sex difference, $p < .05$. Symbols at top of each graph indicates significant main effect; symbols above/below data indicate significant interactions. ZT = Zeitgeber time (hours since lights-on).

Figure 8. Diurnal regulation of inflammatory genes in spinal cord from uninjured rats, and from sham/SCI rats at 2 d post-surgery. (A) Female and male rat spinal cord expression of inflammatory genes varies through the day. *IL-1b*, *Tnfa*, and *Cd68* displayed time-of-day expression differences in the spinal cord. Rhythmic expression was observed in *IL-1b* (females), *Tnfa* (males), *IL-6* (males), and *Cd68* (males). Sex affected intraspinal *IL-6* and *Cd68* gene expression. (B) SCI alters inflammatory gene expression at the lesion epicenter. SCI increased expression of *IL-1b*, *Tnfa*, *IL-6*, and *Cd68* expression. Epicentre *IL-6* was also regulated by sex, and *Cd68* was also regulated by ZT and by sex. (C) SCI modulates inflammatory gene expression in spinal cord distal to injury. SCI reduced lumbar spinal cord expression of *IL1b*, *IL-6*, and *Cd68*, and increased expression of *Tnfa*. There were also significant main effects of ZT (*Tnfa*, *Cd68*; both lower at ZT18) and sex (*IL-6*; females higher than males). Black ~ indicates that females or males for that gene show significant rhythm; red **+** indicates injury difference (sham v. SCI), $p < .05$; yellow hourglass indicates time difference, $p < .05$; blue gender symbol indicates sex difference, $p < .05$. Symbols at top of each graph indicates significant main effect; symbols above/below data indicate significant interactions.

Figure 9. SCI disrupts diurnal rhythms. (A) Diurnal rhythm control under homeostatic conditions. Initial circadian input occurs via light activating specialized retinal ganglion cells that project directly to the suprachiasmatic nucleus (SCN). The SCN is the master circadian oscillator; in turn, this regulates extra-SCN ("peripheral") rhythms via direct and indirect routes. The SCN controls peripheral clocks directly via autonomic control (sympathetic and parasympathetic innervation), whereas the SCN controls peripheral clocks indirectly through regulation of physiologic and humoral factors. Appropriately entrained clocks throughout the body (likely every cell) optimize organismal performance for time-of-day. (B) Diurnal rhythm control is disrupted by SCI. Our data suggest that SCI disrupts rhythms of key zeitgebers – including body temperature, activity, and CORT (yellow bolts) – which could disrupt peripheral clock

949 entrainment. In addition, data from other groups suggest that SCI also disrupts additional entraining
950 factors (e.g., feeding, melatonin, and autonomic input; grey bolts). Ultimately, prolonged SCI-elicited
951 disruption of these entraining factors could contribute to loss of homeostasis and suboptimal repair.
952
953 **Table 1.** Primer sequences and related function of assessed RNAs.
954
955 **Table 2.** Expression of additional clock (*Per1, Clock*) and inflammatory (*Iba1*, *Mhc II*) genes at 2 d post-
956 surgery in the lesion epicenter and lumbar spinal cord (data only for genes not shown in figures). For
957 statistical comparisons (far right column): “SCI” = significant main effect of SCI vs. sham; “ZT” =
958 significant main effect of time (ZT6 vs. ZT18); “sex” = significant main effect of sex; “Sham x ZT” =
959 significant interaction between sham and ZT; “N.S.” = no significant difference.
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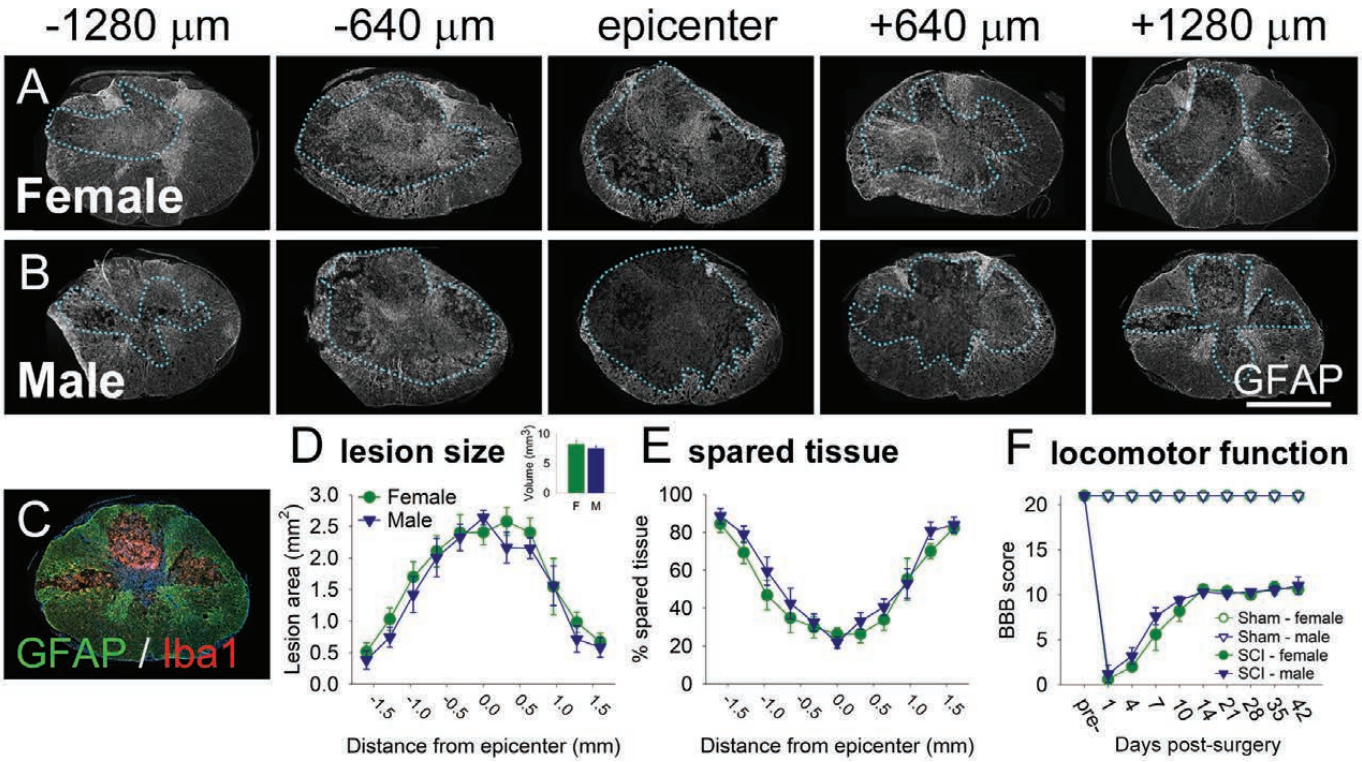
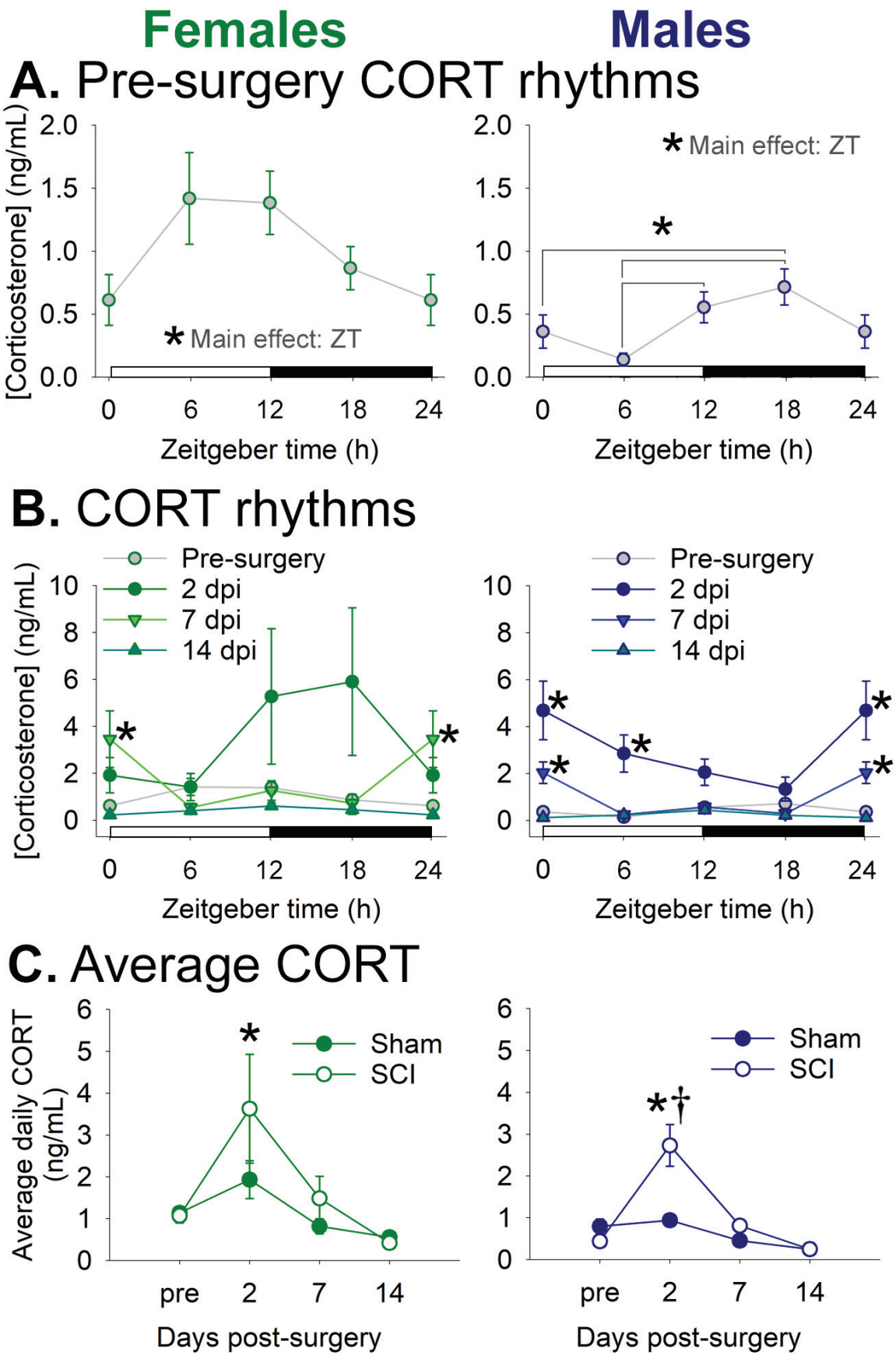
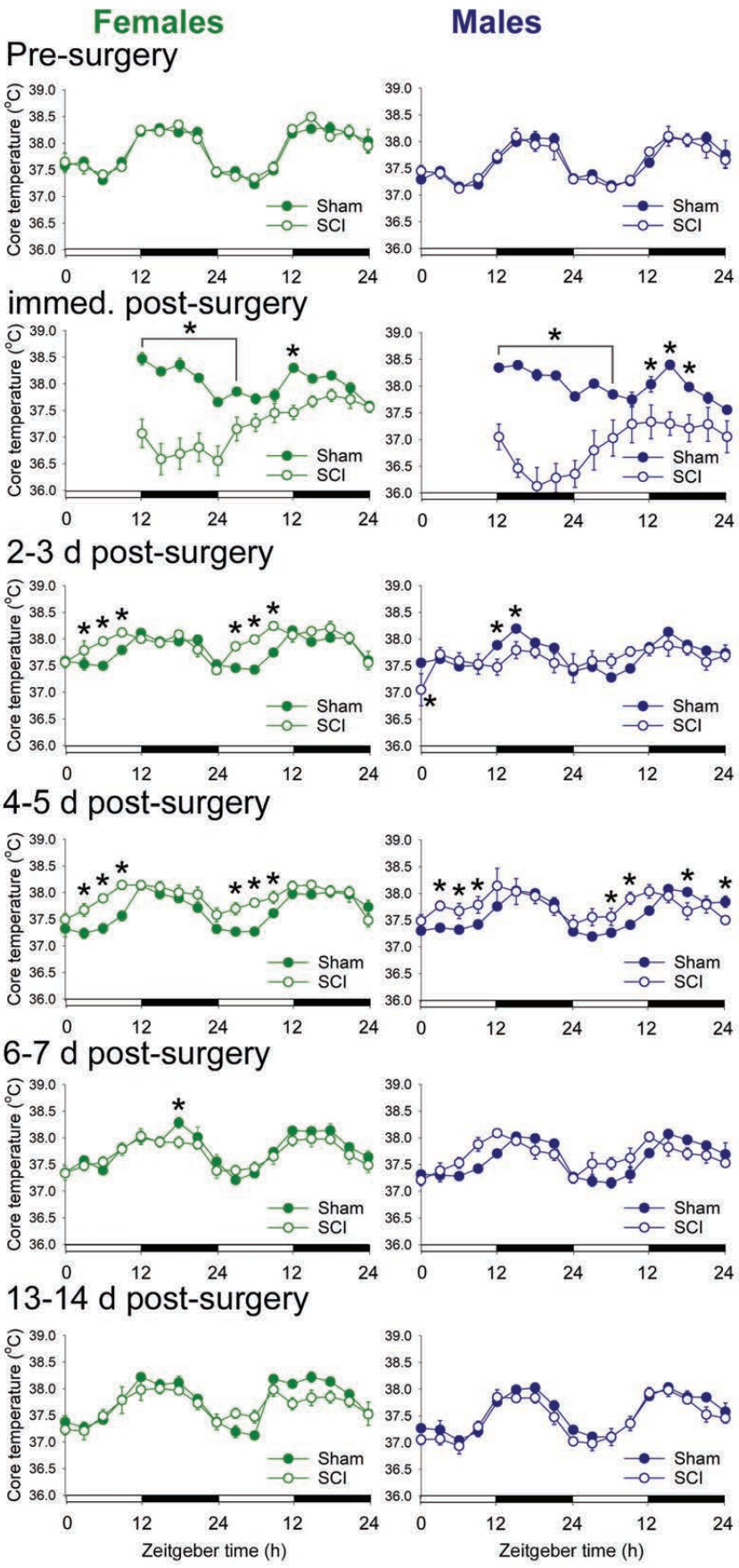
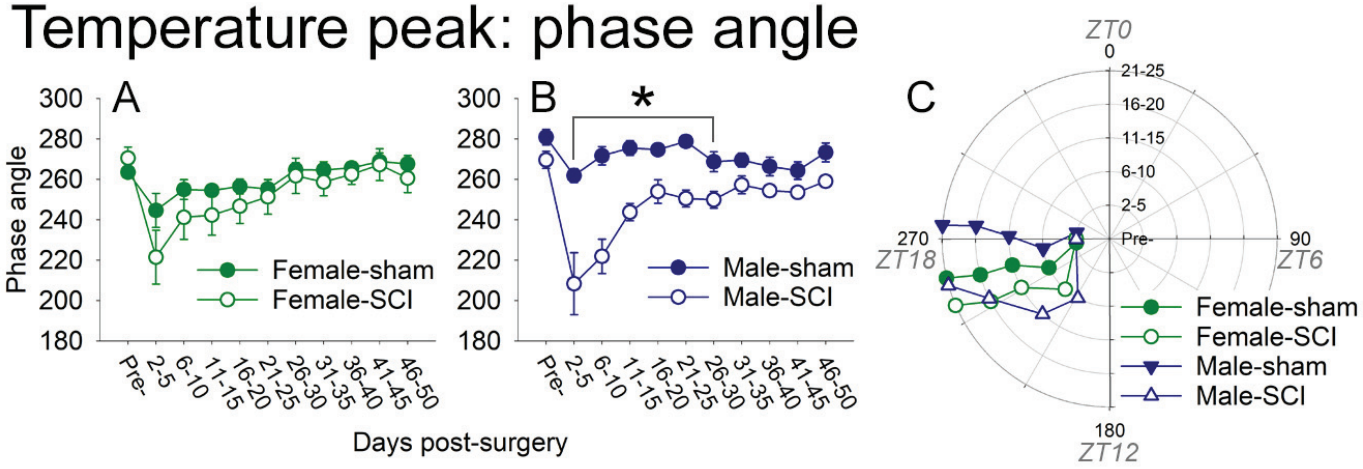
961
962

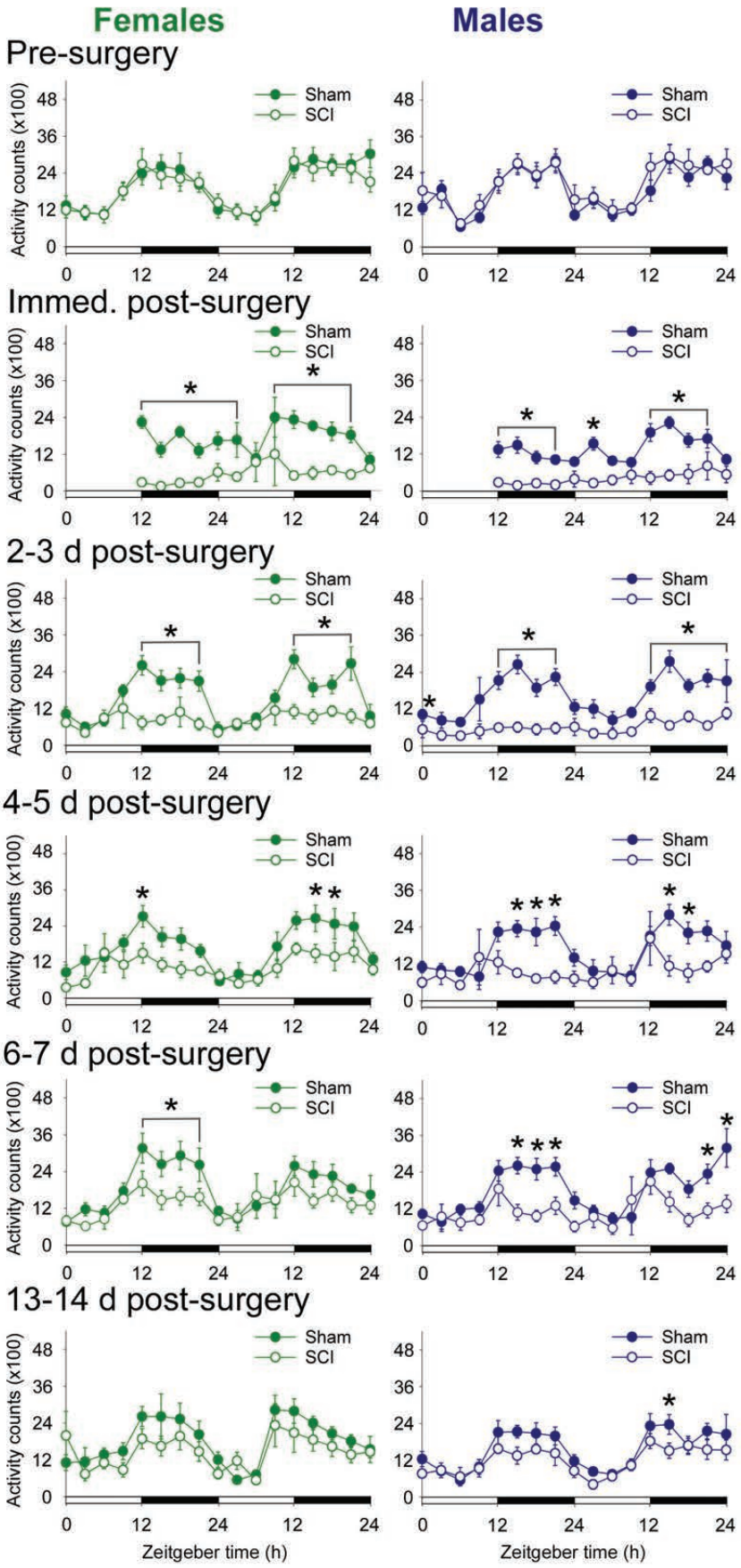


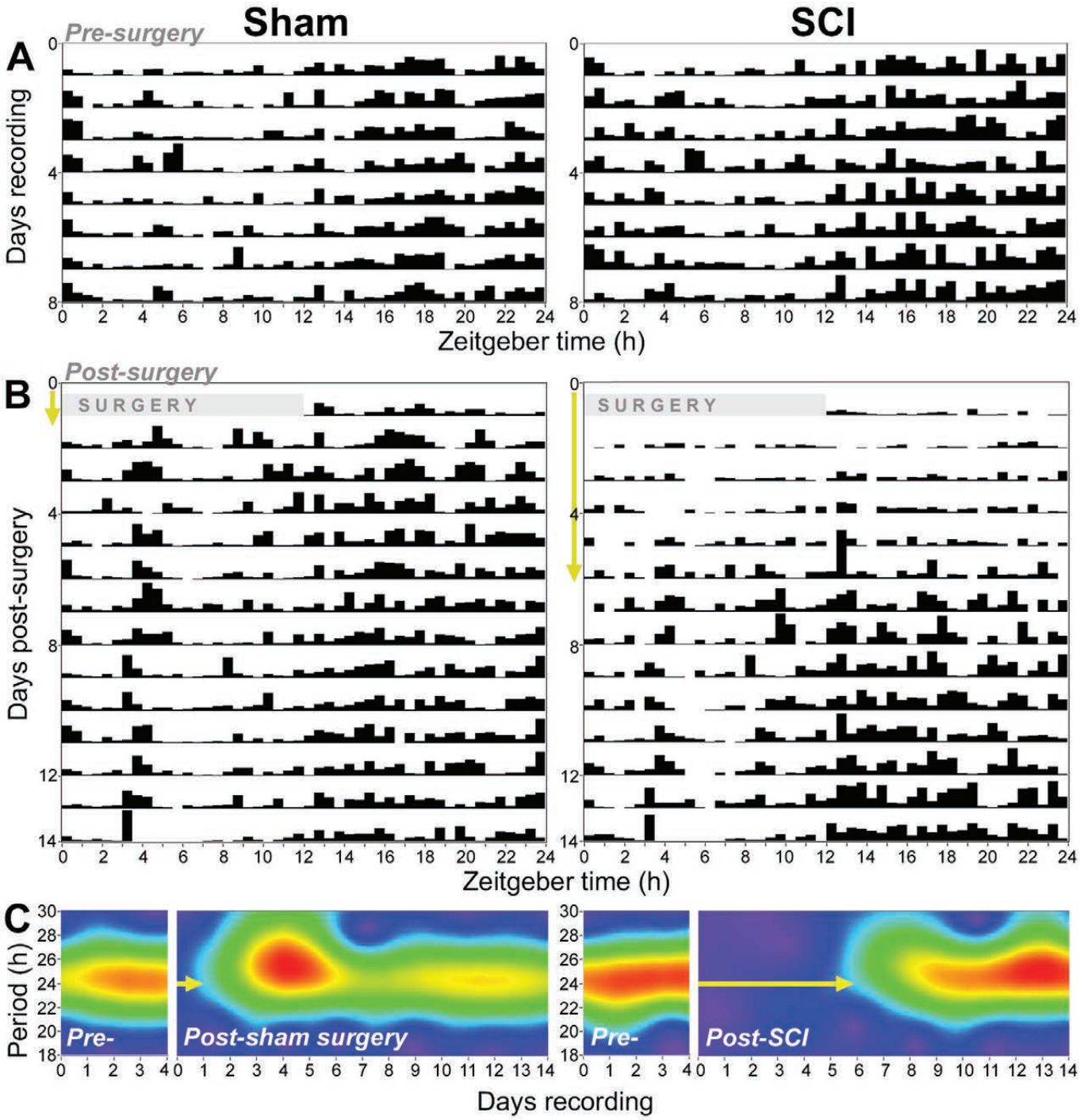


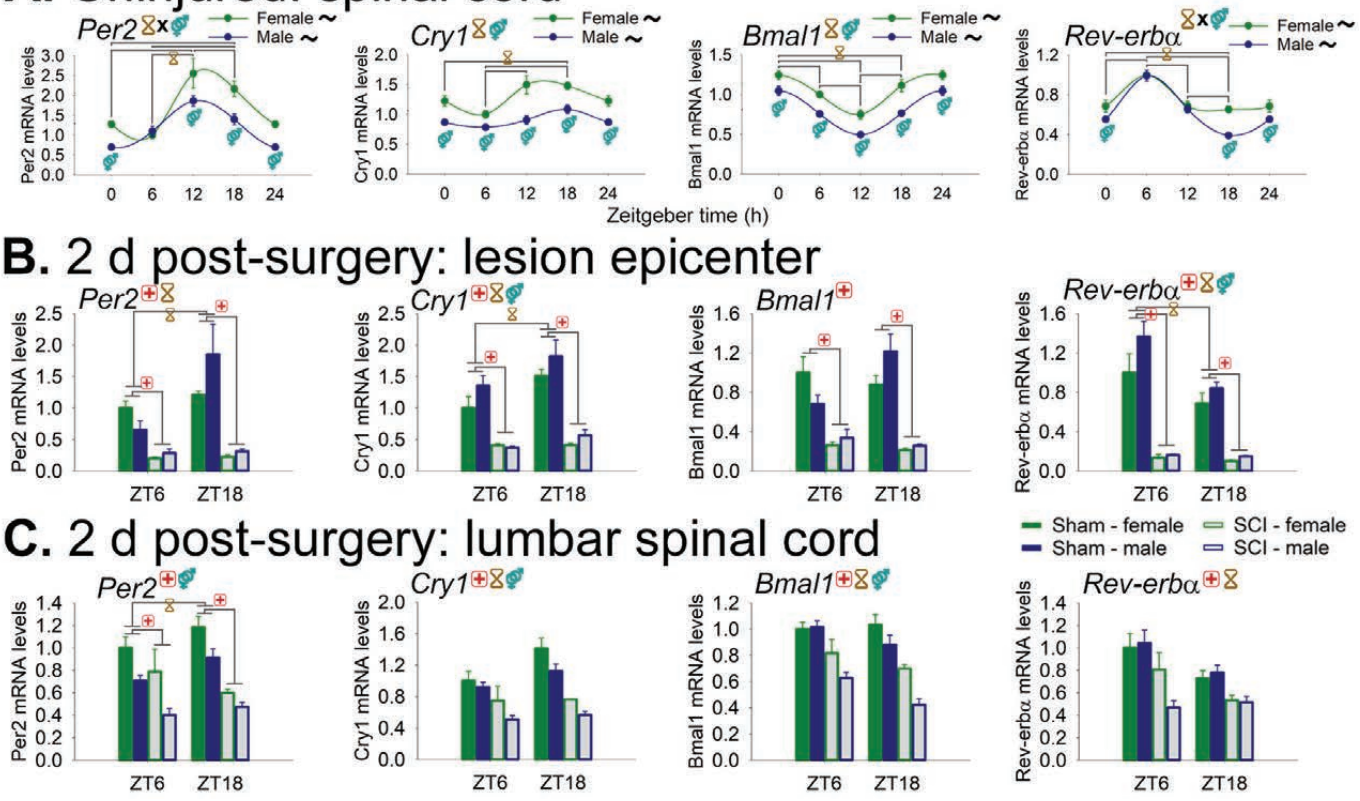


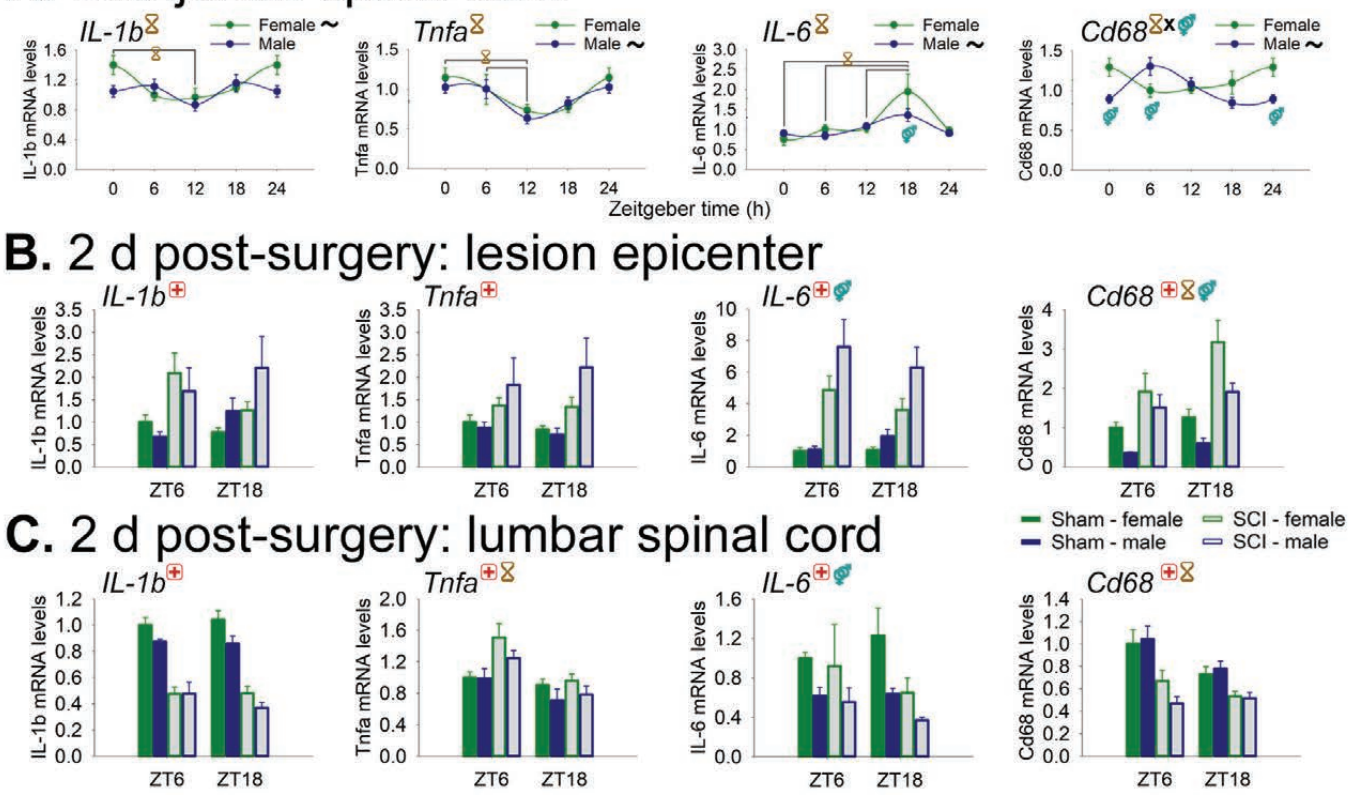
Temperature peak: phase angle



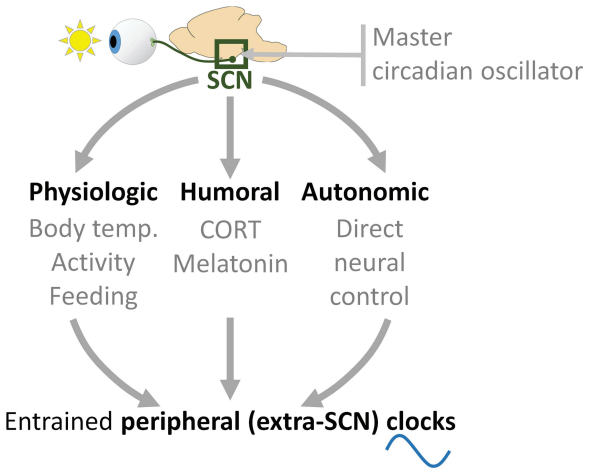




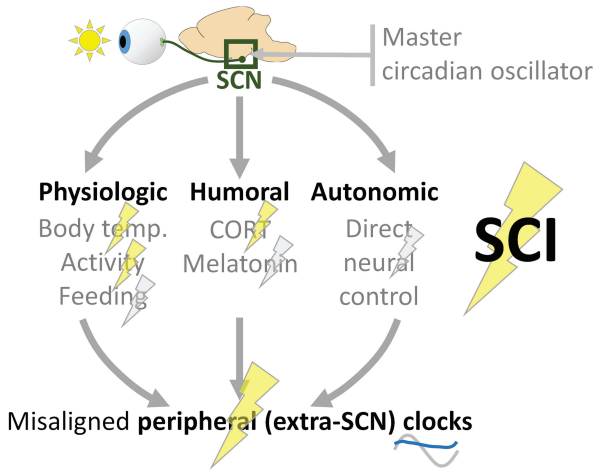




A. Healthy: circadian system in homeostasis



B. Post-SCI: Disrupted circadian system



Gene	Protein function	Primer sequences (5'-3')
b-actin	Housekeeping control	F: TTCCTTCCTGGGTATGGAAT R: GAGGAGCAATGATCTTGATC
Bmal1	Circadian clock gene – with Clock, activates *Per* and *Cry* transcription	F: AAAATGCAAGGGAGGCCCAC R: TCTAACTTCCGGGACATCGC
Clock	Circadian clock gene – with Bmal1, activates *Per* and *Cry* transcription	F: CTGCTGACAAAAGCCAAGAT R: GACTTTCTTGAGCTTCTGGA
Cry1	Circadian clock gene – with Per, represses *Bmal* and *Clock* transcription	F: GTGGTGGCGGAAACTGCTCTC R: ACTCTGTGCGTCCTCTTCCTGA
Per1	Circadian clock gene – with Cry, represses *Bmal* & *Clock* transcription	F: GTGCAGGCTAACCAGGAATA R: GCGGAGAGTGTATTCAGATG
Per2	Circadian clock gene – with Cry, represses *Bmal* & *Clock* transcription	F: ACAAGCGGCTGCAGTAGTGA R: TTCAAGGTTGCCAGCGTGCT
Rev-erba (*Nr1d1*)	Circadian clock gene – represses expression of core clock proteins	F: AGACGCTGTGCGTTTTGGAC R: TGTGGGAACTGAGAGAAGCC
Cd68	Inflammation – expressed by microglia/macrophages; activation marker; cell homing and adhesion	F: CAAGCAGCACAGTGGACATTC R: CAAGAGAAGCATGGCCCGAA
Iba1 (*AIF1*)	Inflammation – expressed by all microglia/macrophages; increased with activation; binds calcium & actin	F: GGCAATGGAGATATCGATAT R: AGAATCATTCTCAAGATGGC
IL-1b	Pro-inflammatory cytokine; secreted mainly by microglia/macrophages	F: CCTTGTGCAAGTGTCTGAAG R: GGGCTTGGAAGCAATCCTTA
IL-6	Cytokine with mixed pro- and anti-inflammatory roles	F: AGAAAAGAGTTGTGCAATGGCA R: GGCAAATTTCCTGGTTATATCC
Mhc II	Membrane-bound receptor; expressed by antigen-presenting cells (including microglia and macrophages); presents antigens	F: AGCACTGGGAGTTTGAAGAG R: AAGCCATCACCTCCTGGTAT
Tnfa	Pro-inflammatory cytokine; secreted mainly by microglia/macrophages	F: CAAGGAGGAGAAGTTCCCA R: TTGGTGGTTTGCTACGACG

Table 1. Primer sequences and related function of assessed RNAs.

	Female				Male					
	ZT6		ZT18		ZT6		ZT18		Significant differences	
Gene	**Sham**	**SCI**	**Sham**	**SCI**	**Sham**	**SCI**	**Sham**	**SCI**	**Main effect**	**Interactions**
Per1	1.00±.10	0.39±.06	1.19±.07	0.23±.03	1.01±.17	0.35±.06	2.01±.57	0.34±.01	SCI: ZT6+18 SCI, sex	Sham x ZT N.S.
- Lesion	1.00±.05	1.04±.29	0.97±.09	0.70±.03	0.78±.08	0.57±.07	0.84±.12	0.50±.02		
- Lumbar										
Clock	1.00±.16	0.32±.03	1.21±.18	0.27±.04	0.90±.18	0.36±.09	1.56±.33	0.29±.03	SCI	Sham x ZT
- Lesion	1.00±.03	0.71±.10	1.07±.06	0.64±.04	0.94±.07	0.53±.07	0.83±.08	0.45±.04	SCI	Sham x ZT
- Lumbar										
Iba1	1.00±.21	0.75±.03	0.86±.08	0.93±.09	0.69±.09	0.41±.07	0.59±.04	0.78±.14	Sex	SCI x ZT;
-Lesion	1.00±.07	1.36±.04	0.96±.08	1.38±.13	1.32±.05	2.06±.45	1.23±.12	1.54±.18	N.S.	ZT6 x inj.
-Lumbar										N.S.
Mhc II	1.00±.17	0.21±.05	0.82±.09	0.21±.03	0.65±.14	0.23±.05	0.79±.14	0.22±.05	SCI	N.S.
- Lesion	1.00±.19	0.50±.05	0.54±.21	0.54±.18	1.59±.38	0.86±.14	1.09±.30	0.54±.11	SCI, sex	N.S.
- Lumbar										

Table 2. Expression of additional clock (*Per1, Clock*) and inflammatory (*Iba1*, *Mhc II*) genes at 2 dpi in the lesion epicenter and lumbar spinal cord (data only for genes not shown in figures). For statistical comparisons (far right column): "SCI" = significant main effect of SCI vs. sham; "ZT" = significant main effect of time (ZT6 vs. ZT18); "sex" = significant main effect of sex; "Sham x ZT" = significant interaction between sham and ZT; "N.S." = no significant difference.