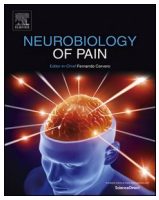
Neurobiology of Pain 16 (2024) 100161

Contents lists available at ScienceDirect

Neurobiology of Pain

journal homepage: www.sciencedirect.com/journal/neurobiology-of-pain



Original Research

The timing of the mouse hind paw incision does not influence postsurgical pain

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ARTICLE INFO

Keywords:
Circadian
Surgery
Hypersensitivity
Allodynia
Time course

ABSTRACT

Chronobiological approaches have emerged as tools to study pain and inflammation. Although time–of-day effects on the expression of pain after injury have been studied, it remains unaddressed whether the timing of the injury itself can alter subsequent pain behaviors. The aim of this study was to assess postsurgical pain behaviors in a mouse hind paw incision assay in a circadian-dependent manner. Incisions were made at one of four equally spaced time points over a 24-hour period, with evoked and spontaneous pain behaviors measured using the von Frey mechanical sensitivity test, Hargreaves' radiant heat paw-withdrawal test, and the Mouse Grimace Scale. Algesiometric testing was performed in C57BL/6 mice prior to and at multiple time points after incision injury, at the same time of day, until pain resolution. No statistically significant differences were observed between groups. This study adds to the literature on circadian rhythms and their influence on pain in the pursuit of more biologically informed pre- and postoperative care.

Introduction

Postsurgical pain is experienced by over 80 % of people undergoing surgeries (Mazda et al., 2021). Although most postsurgical pain is temporary, significant percentages of people develop chronic postsurgical pain after surgery (Kehlet et al., 2006). A strong predictor of chronic postsurgical pain is acute postsurgical pain intensity (Papadomanolakis-Pakis et al., 2021). Therefore, there is a need for better understanding the mechanisms underlying postsurgical pain and novel methods to mitigate it.

Chronotherapeutic approaches, which seek to optimize treatments through alignment with sleep/wake cycles or by taking advantage of circadian rhythms, are emerging methods to promote effective healing from illness or injury. There is evidence that the magnitude of pain sensitivity changes throughout the day (Knezevic et al., 2023). For example, neuropathic pain patients report increasing pain levels in the morning that peak in the evening, whereas patients with inflammatory pain tend to report higher pain in the morning (Gilron and Ghasemlou, 2014; Segal et al., 2017). A study of healthy human males found that pain sensitivity was greatest at night and dependent on endogenous rhythms and not sleep (Daguet et al., 2022). A recent review by Bumgarner and colleagues (2023) of circadian effects on pain in preclinical models also described variability in hamsters and mice, with some mouse strains showing peak pain behaviors in the dark (active) phase and others in the resting (light) phase.

Most of the literature describing chronotherapy of surgical interventions is focused on wound healing outcomes. A study from Montaigne et al. (2018) examined surgery time in human patients referred for isolated aortic valve replacements. The authors demonstrated that patients who had their surgery in the afternoon were less likely to experience adverse events than those who received surgery in the morning. Another group of researchers tested the effect of surgery time on wound healing from patients having hip replacement surgeries in the morning or afternoon, with the hypothesis that cortisol level would play a large role in postsurgical healing; again, afternoon surgery times yielded better outcomes (Kwon et al., 2019). In preclinical studies, it was observed that behaviorally rhythmic Siberian hamsters receiving cutaneous wounds in the morning healed faster than those receiving wounds in the evening (Cable et al., 2017). A study from Al-Waeli et al. (2020) evaluating the effect of surgery time on healing of tibial bone fracture in

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https://doi.org/10.1016/j.ynpai.2024.100161
Received 17 June 2024; Received in revised form 31 July 2024; Accepted 31 July 2024

C57BL/6 mice observed no differences between morning and evening surgeries. This study is the only extant one that included putative pain measurements (guarding and weight bearing), also showing no difference based on the time of day.

As limb guarding and weight bearing are uncommonly used (Sadler et al., 2022) and of unclear relevance as measures of neuropathic pain (Mogil et al., 2010), we wished to examine systematically whether the timing of a surgical incision injury—involving general anesthesia, wounding, and pain—affects subsequent pain behavior. The present study measured multiple pain behaviors in the mouse hind paw incision assay.

Materials and methods

Animals

C57BL/6NCrl mice were purchased from Charles River Laboratories (St. Constant, QC, Canada). Mice of both sexes were maintained on a 12:12 h light–dark cycle (lights on at 07:00 h) with same-sex littermates and had access to food (Harlan Teklad rodent diet #2920X; Envigo, Lachine, QC, Canada) and water *ad libitum*. Testing began no earlier than 6 weeks of age. All experiments were approved by the local animal care and use committee and confirmed to the guidelines of the Canadian Council on Animal Care.

Hind paw incisions

Zeitgeber time (ZT) is used to denote the timing of a procedure relative to light:dark cycles in a 12:12 h lights on/off facility, where ZT0 corresponds to lights-on (in our facility, at 07:00 h) and ZT12 corresponds to lights-off (at 19:00 h). Surgeries occurred in four different groups of mice at ZT2, ZT8, ZT14, and ZT20. These time points were chosen to avoid changes in the photoperiod during surgery.

The hind paw incisional wound protocol used was adapted from Pogatzki and Raja (2003), itself adapted from Brennan et al. (1996) in rats. Mice were anesthetized with 1.5–2.0 % isoflurane/oxygen through a nose cone. The toes of the left hind paw were secured with surgical tape. To ensure the mouse was fully anesthetized, a toe was pinched (either the exposed medial toe or on the right foot). Following an antiseptic swab of 2 % chlorhexidine gluconate in isopropyl alcohol solution (Baxedin 2 –70 % Solution, Omega Laboratories Ltd, Montreal, QC), a 2-mm incision was made with a #11 scalpel blade from the proximal edge of the heel down through the center of the hind paw. Using forceps, the skin was spread to expose the flexor digitorum brevis muscle, which was elevated with curved forceps underneath the medial side and pushed through. A second incision was made using the scalpel vertically through the flexor digitorum brevis muscle until it touched the forceps. The forceps were slowly pulled away, and the skin alone was sutured (Ethicon, 6–0, absorbable) at both the anterior and posterior ends of the wound. Finally, the tape was removed, the anesthesia discontinued, and the mice wakened slowly before being placed back into their home cage. For the dark phase surgeries, cages were covered with an opaque black sheet to minimize light leakage to the cage before and after the surgery.

Pain testing

For behavioral testing, due to potentially confounding circadian variation in pain sensitivity, all testing was performed at ZT8 (±1h). Testers were blinded to the ZT surgery group of animals during all behavioral testing. Post-surgery testing duration differed between measures (see below), and was based on time to resolution (i.e., return to baseline values). Timing of all testing is shown in Fig. 1.

von Frey mechanical sensitivity test

A series of nylon monofilaments were applied to the plantar surface of the hind paw to quantify changes in mechanical withdrawal thresholds. Mice ($n = 8$ mice/sex/ZT group) were placed in small cubicles (9 × 5 × 5 cm high) on a wire rack. The "up–down" method of Dixon (Chaplan et al., 1994) was used to determine 50 % withdrawal thresholds. Testing occurred two times on each foot one day before surgery, and on days 1, 3, 5, 7, 10, 14, 28, 35, 42 and 49 post–surgery. A replication experiment was conducted ($n = 8$ mice/sex/ZT group), with measurements were taken one day prior to surgery, and on days 2, 15, 30 and 50 post–surgery.

Hargreaves' radiant heat paw-withdrawal test

The Hargreaves' radiant heat paw-withdrawal test (Hargreaves et al., 1988) uses a localized beam of light to warm the plantar surface of the hind paw to measure changes in thermal (heat) thresholds. Mice ($n = 8$ mice/sex/ZT group; a separate group of mice from those being tested with von Frey fibers) were placed in small cubicles (9 × 5 × 5 cm high) atop a 0.5–inch-thick glass surface and the latency to paw withdrawal from the light beam recorded. Baseline measures were collected for each mouse 8 times on each paw on the day prior to surgery.

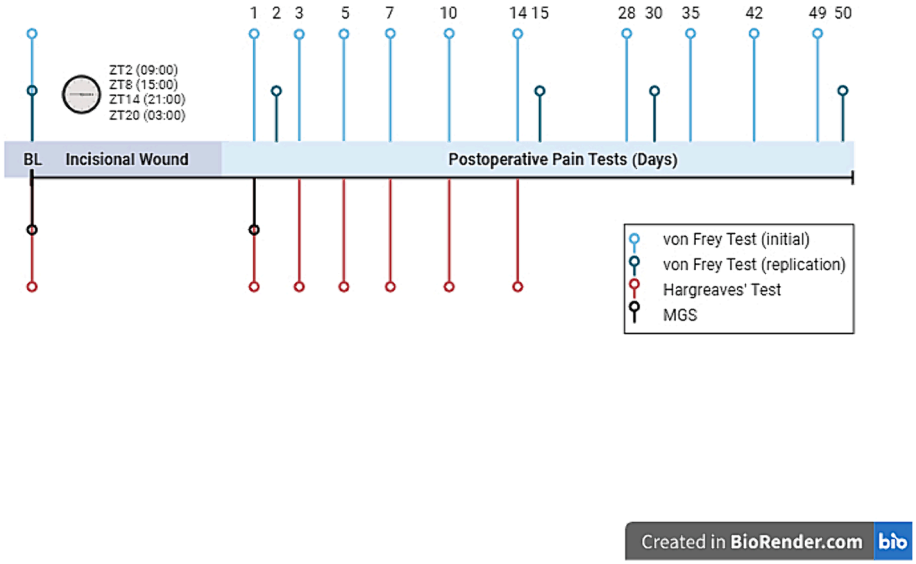

Fig. 1. Schedule of testing. Coloured lines and circles indicate baseline (BL) and postoperative pain testing days for all experiments.

Postoperative tests measured withdrawal responses using an average of 8 determinations on each paw 1, 3, 5, 7, 10 and 14 days post–surgery.

Mouse Grimace Scale

The Mouse Grimace Scale (MGS) is used to measure spontaneous pain via facial features (action units) such as orbital tightening, nose and cheek bulging, and changes in ear and whisker position (Langford et al.,

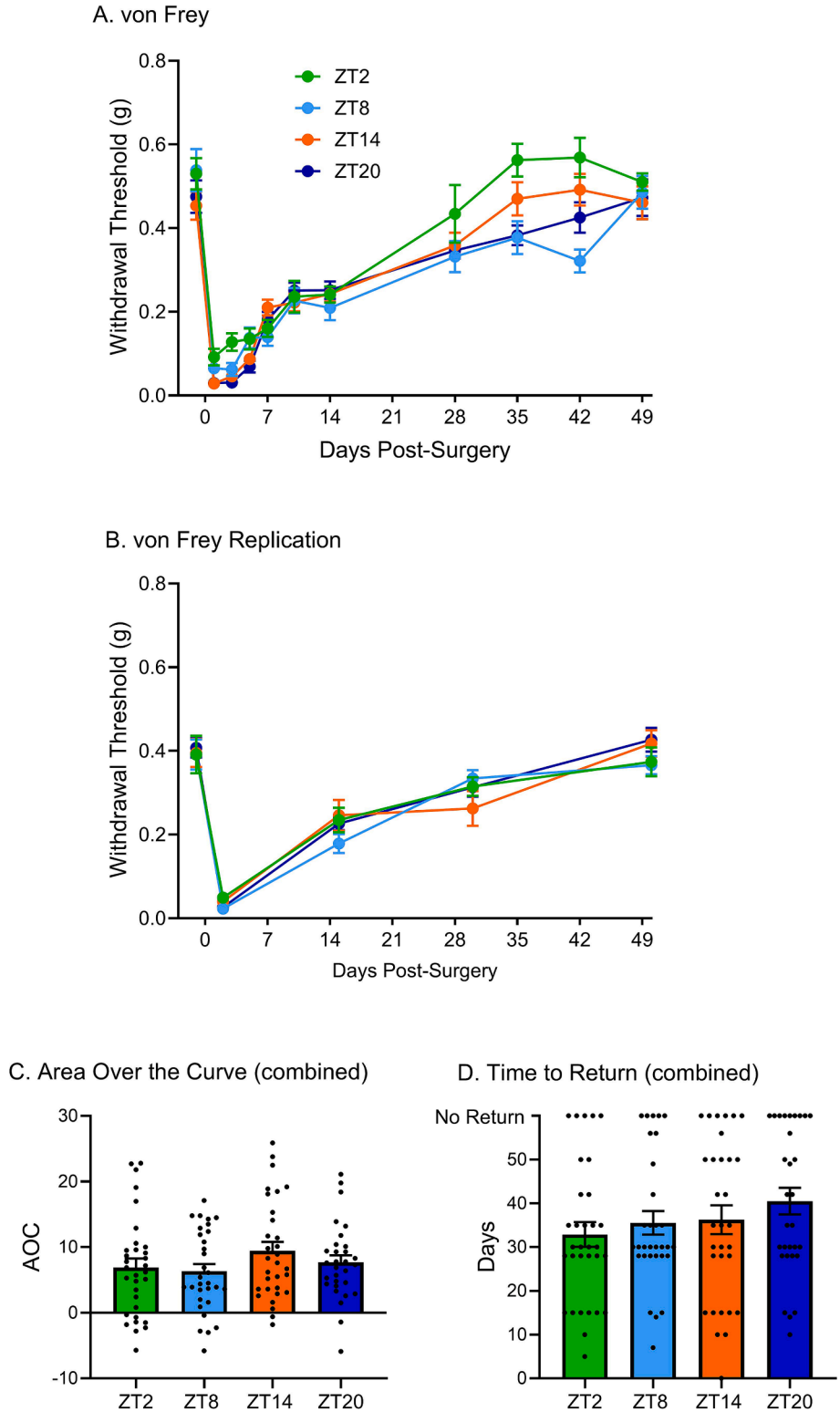

Fig. 2. Timing of surgery does not affect mechanical hypersensitivity after hind paw incision. A,B) Post-operative pain time course using von Frey filaments on the ipsilateral hind paw for the initial von Frey experiment (A), and the replication von Frey experiment (B). C) Area–over-the-curve (AOC) data for both runs combined. D) Time to return to baseline thresholds (see Methods) for both runs combined. Symbols and bars represent mean ± SEM for each surgery group and include data both males and females combined since no main effects of sex or interactions with sex were observed.

2010). The MGS has been used extensively as reliable indicator of postsurgical pain, especially within the first 24 to 48 h. Mice (n = 6–8 mice/sex/ZT group) were placed in cubicles and 30–min videos were captured one day prior to and one day after surgery. The time between surgery and the post-test videos varied by group to ensure the post-surgical tests all occur at ZT8 the following day: ZT2 is a 30-h difference, ZT8 is 24 h, and ZT14 is 18 h, and ZT20 is a 36-h difference. Video analyses were accomplished using VLC media player and Microsoft PowerPoint to procure still images of each mouse every 3 min (chosen semi-randomly, based on when the mouse's pose and optics allowed; see Langford et al., 2010) for both baseline and postoperative conditions (10 images per 30-min video). Still images were scored by an experimenter blinded to ZT surgery group.

Data analysis

Behavioral data collected individually from each subject were averaged into ZT group means and analyzed using analysis-of-variance (ANOVA). Area-over-the-curve (AOC) values, which summarize pain levels over the entire duration of the postsurgical pain episode, were calculated using the trapezoidal method. Time to return to baseline was quantified by inspection of data for each mouse, following procedures described previously (Parisien et al., 2022). Time to return consisted of the first of two consecutive days that a subject's withdrawal threshold or latency had returned to within 0.5 SD (via group means) of its baseline threshold. In cases where group significance was reached ($p < 0.05$), post-hoc analyses using Tukey's HSD were performed. Sex-disaggregated data were analyzed for all datasets. All analyses were carried out using GraphPad Prism v. 10. No data were excluded from analysis.

Results

Effect of surgery timing on mechanical hypersensitivity

Two separate von Frey runs were conducted, an initial and a replication experiment (shown in Fig. 2A,B). Results from both runs were combined by calculating the area over the threshold-time curves (AOCs), as a measure of the overall extent and duration of the post-operative pain episode (Fig. 2C). A two-way (ZT group and sex) ANOVA performed on AOC data revealed no main effects of ZT group ($F_{3,116}$ = 1.2, p = 0.32), sex ($F_{1,116}$ = 2.2, p = 0.14), or their interaction ($F_{3,116}$ = 0.3, p = 0.86). Furthermore, a two-between (ZT group and sex), one–within (using shared time points from both runs: day 2/3, day 14/15, day 28/30, and day 49/50) repeated measures ANOVA revealed a significant effect of repeated measure ($F_{4,364}$ = 205.8, $p < 0.001$) and sex ($F_{1,91}$ = 8.4, p = 0.005) but no other significant main effects or interactions. Finally, a two-way ANOVA (ZT group and sex) performed on Time to Return data (Fig. 2D) revealed no main effects of ZT group ($F_{3,116}$ = 1.2, p = 0.33), sex ($F_{1,116}$ = 1.3, p = 0.26), or their interaction

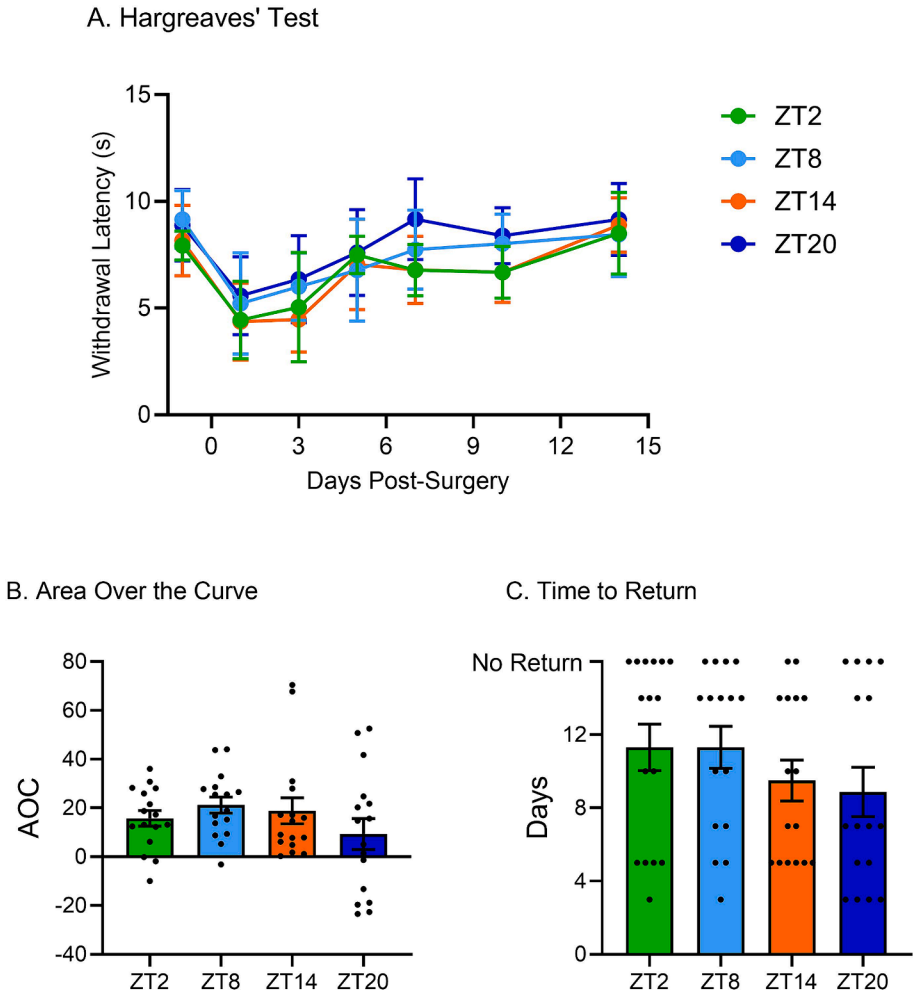

Fig. 3. Timing of surgery does not affect heat hypersensitivity after hind paw incision. A) Post–operative pain time course using Hargreaves' test of radiant heat paw-withdrawal on the ipsilateral hind paw. B) Area-over-the-curve (AOC) data. C) Time to return to baseline latencies (see Methods). Symbols and bars represent mean ± SEM for each surgery group and include data both males and females combined.

($F_{3,116} = 0.4$, $p = 0.76$).

Effect of surgery timing on heat hypersensitivity

Heat sensitivity before and at multiple time points after hind paw incision for the ipsilateral hind paw are presented in Fig. 3A. An analysis of the AOC data over the entire time course revealed no main effects of ZT group ($F_{3,56} = 1.1$, $p = 0.35$), sex ($F_{1,56} = 0.4$, $p = 0.51$), or ZT group x sex interaction ($F_{3,56} = 0.2$, $p = 0.90$) (Fig. 3B). Furthermore, a two–between (ZT group and sex), one–within repeated measures ANOVA revealed a significant effect of repeated measure ($F_{6,324} = 48.1$, $p < 0.001$) but no other significant main effects or interactions. Finally, a two-way ANOVA (ZT group and sex) performed on Time to Return data (Fig. 3C) revealed no main effects of ZT group ($F_{3,56} = 1.0$, $p = 0.39$), sex ($F_{1,56} = 0.1$, $p = 0.76$), or their interaction ($F_{3,56} = 0.6$, $p = 0.63$).

Effect of surgery timing on spontaneous pain

Spontaneous pain data as measured on the MGS are shown in Fig. 4. A repeated measures ANOVA performed on pre- versus post-incision MGS scores (Fig. 4A) revealed a highly significant effect ($F_{1,45} = 15.2$, $p < 0.001$) of repeated measures (i.e., a statistically significant increase in grimacing on day 1; 18–36 h post–surgery) but no ZT group × repeated measures interaction ($F_{3,45} = 0.05$, $p = 0.99$). As shown in Fig. 4B, no differences among ZT groups were observed in the average difference scores from pre- to post-surgery, as evidence by the lack of main effects of ZT group ($F_{3,41} = 0.08$, $p = 0.97$), sex ($F_{1,41} = 2.4$, $p = 0.13$), or ZT group × sex interaction ($F_{3,41} = 1.7$, $p = 0.17$).

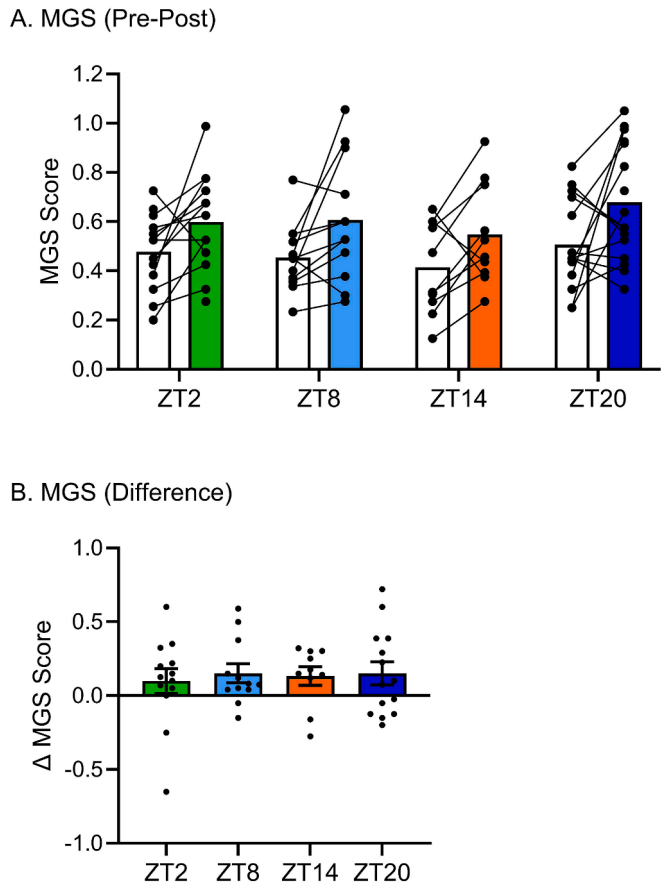

Fig. 4. Timing of surgery does not affect spontaneous pain after hind paw incision. A) Mouse Grimace Scale (MGS) scores pre- (open bars) and one day (18–36 h; colored bars) post–surgery in all ZT groups. B) Difference scores (post-surgery – pre-surgery MGS score) for all ZT groups. Symbols in both graphs represent individual mice and include data both males and females combined since no main effects of sex or interactions with sex were observed.

Discussion

To our knowledge, this is the first study to systematically examine postsurgical pain following surgeries performed at different times of day; the sparse literature on the topic has mainly focused on surgical wound healing. The only directly relevant study was that by Al-Waeli and colleagues (2020) described above, which measured bone fracture healing time in mice receiving the injury at one of two time points (ZT2 and ZT13), and assessed pain via weight bearing and limb guarding, which are not in common use in modern pain research (Sadler et al., 2022). Here, we used the most common mechanical (von Frey) and thermal (Hargreaves' test) assays (Zumbusch et al., 2024), and a validated measure of spontaneous pain (MGS) (Mogil et al., 2020). However, data from both their study and ours appear to suggest that there are no obvious differences in pain outcomes based on surgery timing.

Regardless of the paucity of preexisting data on surgical timing, wound healing, and pain, there was considerable *a priori* reason to believe that there might be differences in pain outcomes between ZT groups in this experiment. It is common for patients to have disrupted sleep and fatigue following surgical procedures (Luo et al., 2020). Gene expression of "clock genes" can change substantially when an individual is given an effective dose of anesthesia (Dispersyn et al., 2008), and there is growing preclinical evidence for an effect of anesthesia on clock genes in honeybees (Cheeseman et al., 2012), fruit flies (Li et al., 2020), and rodents (Ludin et al., 2021; Mizuno et al., 2022).

Anesthesia chronobiology has largely focused on the influence on melatonin rhythms, although many laboratory mouse strains, including the inbred C57BL/6 mice used here, are deficient in one or both enzymes needed for melatonin synthesis (Kennaway, 2019). Ludin and colleagues (2021) discussed an observation in C57BL/6 mice in which isoflurane treatment showed a delayed phase shift in *Per* gene expression, producing only a marginal change in wheel-running behavior. In other words, the shift in gene expression was stronger than the shift in the observable behavior. A similar situation may have occurred here, with surgery producing gene expression changes that did not result in pain behavior changes.

Various molecular cascades arise from the circadian clock based on the time of day. Clock–controlled genes, like those influencing neutrophil behavior (Ovadia et al., 2023), play a role in immune responses, and the immune system is increasingly thought to be critical to the development of pain (Kavelaars and Heijnen, 2021). It was our hypothesis that circadian variations in immune cell function would interact with the timing of the surgical wound. For example, neutrophils exhibit a circadian rhythm (Aroca-Crevillen et al., 2020), as do blood leukocytes (Kawate et al., 1981). Mast cells—in which degranulation can be triggered by surgical incision (Oliveira et al., 2011) to release substances like histamine, cytokines, and chemokines which can trigger nociceptor activation—show circadian variation in humans (Nakao et al., 2015) and rodents (Friedman and Walker, 1969). Finally, microglia, the resident immune cells of the central nervous system with a robust involvement in chronic pain (Chen et al., 2018), have been shown to be in a more activated state (with increased pro–inflammatory expression) during the light (rest) phase in rodents (Fonken et al., 2015). Cumulatively, considering the circadian variation in immune function, observing no difference in postsurgical pain after incisions between ZT surgery groups was perhaps an unexpected outcome.

Conclusions

The present study is a novel circadian examination of postsurgical pain in the mouse. We aimed to assess postsurgical pain behavior with commonly used, multi-modal pain assessments with incision injury time varied throughout the 24-h photoperiod. The results suggest there are no differences in postsurgical pain level or duration based on the timing of

the surgery. This should act to reassure researchers that they need not worry about surgical timing being a confound or a cause for variance in preclinical pain research. We note that absence of evidence is not evidence of absence, but this null conclusion is based on large sample sizes (n = 64–124 mice per modality), both sexes, and three separate modalities (mechanical hypersensitivity, heat hypersensitivity, and spontaneous pain).

It would be prudent for future work to compare hind paw incision to other types of surgery, and to consider circadian factors in research using other (sub)strains, ages, and species. More generally, measuring pain levels in conjunction with wound healing provides a more a complete picture of the recovery and is highly relevant clinically. If differences in pain levels or durations are shown to be caused by surgery timing, shifts in surgical scheduling might promote more optimal outcomes.

CRediT authorship contribution statement

Eleri L.F. McEachern: Writing – original draft, Visualization, Investigation, Formal analysis, Conceptualization. **Maria Zilic:** Investigation. **Susana G. Sotocinal:** Investigation. **Nader Ghasemlou:** Writing – review & editing, Conceptualization. **Jeffrey S. Mogil:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by the Canadian Institutes for Health Research and an unrestricted grant from the Louise and Alan Edwards Foundation (to J.S.M.).

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