Biochemistry and Biophysics Reports 30 (2022) 101258

Contents lists available at ScienceDirect

Biochemistry and Biophysics Reports

journal homepage: www.elsevier.com/locate/bbrep

Differences in recovery processes of circadian oscillators in various tissues after sevoflurane treatment *in vivo*

Takeshi Yamaguchi [a,1], Toshiyuki Hamada [b], Norio Iijima [a,*,1]

[a] Center for Basic Medical Research, International University of Health and Welfare, Ohtawara, Tochigi, Japan
[b] Department of Pharmacology, International University of Health and Welfare, Ohtawara, Tochigi, Japan

ARTICLE INFO

Keywords:
Circadian rhythm
Suprachiasmatic nucleus
Lateral ventricle choroid plexus
Fourth ventricle choroid plexus
Pineal gland
Sevoflurane

ABSTRACT

The inhalation anesthetic sevoflurane reversibly suppresses *Period2* (*Per2)* mRNA expression in the suprachiasmatic nucleus (SCN). However, a discrepancy exists in phase shifting of the *Per2* expression rhythm between sevoflurane application in rats (*in vivo* application) and explants (*ex vivo* application). This investigation aimed to resolve this issue. First, tissues from the SCN, choroid plexus in the lateral ventricle (CP-LV), and choroid plexus in the fourth ventricle (CP–4V), which are robust circadian oscillators, and pineal gland (PG) tissue, which is a circadian influencer, were prepared from *Per2::dLuc* transgenic rats. Significant phase responses of bioluminescence rhythms for different preparation times were monitored in the four tissue explant types. Second, tissue explants were prepared from anesthetized rats immediately after sevoflurane treatment, and bioluminescence rhythms were compared with those from non-anesthetized rats at various preparation times. Regarding bioluminescence rhythm phases, *in vivo* application of sevoflurane induced phase shifts in CP-LV, CP-4V, and PG explants according to the times that rats were administered anesthesia and the explants were prepared. Phase shifts in these peripheral explants were withdrawn due to the recovery period after the anesthetic treatment, which suggests that peripheral tissues require the assistance of related tissues or organs to correct phase shifts. In contrast, no phase shifts were observed in SCN explants. These results indicated that SCN explants can independently correct bioluminescence rhythm phase. The bioluminescence intensity of explants was also decreased after *in vivo* sevoflurane application. The suppressive effects on SCN explants were withdrawn due to a recovery day after the anesthetic treatment. In contrast, the suppressive effects on the bioluminescence intensities of CP-LV, CP-4V, and PG explants remained at 30 days after anesthesia administration. These results suggest that anesthetic suppression is imprinted within the peripheral tissues.

1. Introduction

General anesthesia, which has been used for over 175 years, is given to patients for analgesia and sedation during surgical procedures. Enhancement of its safety has brought significant advances in surgical procedures [1–3]. Despite the long history of general anesthesia use, its molecular mechanism is still not well understood. Comprehensive microarray analyses of gene expression related to anesthetics have been performed, and the results revealed that sevoflurane, the most widely used inhalation anesthetic, affected the expression of 1.5% of 10,000 genes in various rat organs, including clock genes [4]. Circadian clock is obviously a key biological function in human. If it is perturbated due to unhealthy life styles that pose circadian rhythm disturbances, the risks of serious illness will increase [5]. Although numerous studies have examined the effects of anesthetics on circadian rhythm, a common understanding has not been obtained [6,7]. Recently, desflurane which is new type of anesthetics used in the clinical practices is also reported to affect the rest/activity rhythm in mice [8].

We have been focusing on *Period2* (*Per2*), a component of the ‘‘core loop’’ of the circadian clock [9,10]. *Per2* expression in the suprachiasmatic nucleus (SCN), the master clock of the body, is reversibly suppressed by sevoflurane treatment (*in vivo* application) [11–13]. Although the *Per2* expression level rapidly recovers from suppression after anesthetic treatment, phase shifts of the *Per2* expression rhythm were not clearly observed. Furthermore, *in vivo* sevoflurane application does not affect the rest/activity rhythm clearly [12,13]. In contrast,

* Corresponding author.
E-mail address: niijima@iuhw.ac.jp (N. Iijima).
[1] These authors contributed equally to this study.

https://doi.org/10.1016/j.bbrep.2022.101258
Received 12 January 2022; Received in revised form 23 March 2022; Accepted 1 April 2022

sevoflurane application to SCN explants from *Per2::dLuc* transgenic rats (*ex vivo* application) showed suppressive effects on bioluminescence intensities in the same manner as *in vivo* application; in addition, obvious phase shifts of the bioluminescence rhythm were observed after anesthetic treatment [13,14]. Therefore, considering the recovery of *Per2* mRNA expression/bioluminescence after anesthetic suppression, a discrepancy exists between *in vivo* and *ex vivo* application in bioluminescence phase shifts. To resolve this discrepancy, the bioluminescence rhythm phases of explants prepared from both anesthetized and non-anesthetized rats were compared in this study because explants are unaffected by the phase correction phenomenon that occurs in related brain areas. If the bioluminescence phases differ between conditions, the circadian clock in the SCN may be capable of *in vivo* regulation of the circadian time via related brain areas or peripheral tissues. Conversely, a lack of phase differences would suggest that the explants contain machinery to return the perturbed circadian clock to its original phase.

According to recent reports, the choroid plexus in the lateral ventricle (CP-LV) and the choroid plexus in the fourth ventricle (CP–4V) are robust circadian oscillators [15–17]. The CP-LV and CP-4V are sources of melatonin in the brain in addition to the pineal gland (PG), which affects the circadian phases in the SCN via feed-back information [18,19]. In this study, effects of i*n vivo* sevoflurane application on explants from the CP-LV, CP-4V, and PG, in addition to SCN explants, were examined.

Our experimental design was as follows. Four types of explants were prepared at various times of the day, and the bioluminescence phase responses of each tissue according to the explant preparation time were examined. On the basis of the results, the bioluminescence from explants of anesthetized and non-anesthetized rats was compared to examine the effects of anesthetics on the phase and intensity of bioluminescence. Finally, bioluminescence from the explants was examined to investigate whether the anesthetic effects were withdrawn due to the recovery period after the anesthetic treatment.

2. Materials and methods

2.1. Animals

Per2::dLuc transgenic male rats (8–14 weeks old) were used to monitor the bioluminescence of explants [13,14,20]. Rats were kept on a 12 h light/12 h dark cycle at 21 °C with unrestricted access to food (Lab MR stock, NOSAN, Kanagawa, JAPAN) and water. The Committee of Animal Research of the International University of Health and Welfare approved this study (No. 17006).

2.2. Anesthetic treatment of individual rats (in vivo application of sevoflurane)

Rats were placed in a chamber (44 x 32 x 16 cm) and exposed to a gas mixture containing 4% sevoflurane (NIKKO Pharmaceutical Co., Gifu, Japan) and 60% O_2 at a flow rate of 1 L/min via a vaporizer (NARCOBIT-E(II), Natsume Seisakusho Co., Ltd., Tokyo, JAPAN). Rats were anesthetized for 6 h while the temperature in the chamber was maintained between 26 °C and 29 °C by a heating pad [13].

2.3. Experiment 1: detection of the bioluminescence phase response to time for preparing explants

Transgenic rats (n = 4–8) were sacrificed by decapitation at Zeitgeber Time (ZT) 2, 8, 11, 14, and 20. Immediately after decapitation, explants were prepared from the SCN, CP-4V, CP-LV, and PG. Explant preparation and bioluminescence monitoring were conducted in the same manner as in our previous report [17]. The ZT of each peak time point of the bioluminescence rhythm in the explants was determined for the four types of tissue, which were prepared at various times during the day.

The methods used to examine the degree of phase shift are described in the supplemental experimental procedures.

2.4. Experiment 2: effects of in vivo sevoflurane application to the four explant types on the phase and intensity of the bioluminescence rhythm

Four types of tissue explants were prepared from anesthetized rats at ZT2, 8, 14, and 20, and the bioluminescence rhythm was monitored. The peaks in anesthetized rats, which appeared 12 h before and after the peaks in non-anesthetized rats, were identified. To predict the effects of anesthetic administration on bioluminescence rhythm phases in explants, the ZTs of bioluminescence peaks in anesthetized rats were compared with the corresponding peaks of explants from non-anesthetized rats that were prepared at the same ZT.

To examine suppressive effects of sevoflurane on the explants, the intensities of each peak (peak intensity of bioluminescence: PIB) in the explants from anesthetized rats were compared with the corresponding PIBs of non-anesthetized rats. The dataset obtained in *Experiment 1* was used in *Experiment 2* because the experimental conditions of non-anesthetized rats were identical.

2.5. Experiment 3: influence of recovery period after the anesthesia

After anesthetic treatment (ZT14–20), the rats were housed in cages in the same manner as before anesthesia. The explants were prepared at ZT20 at 24 h, 7 days, and 30 days after anesthetic administration. The PIB and peak ZT of the bioluminescence rhythm were compared with those of non-anesthetized rats that were prepared at ZT20. The datasets obtained from non-anesthetized rats in *Experiment 1* and the datasets from *Experiment 2* in which explant-preparation was performed immediately after wearing off anesthesia, were used in *Experiment 3*.

2.6. Statistical analysis

Statistical analysis of the phase response curve was performed using one-way analysis of variance with a Bonferroni post-hoc test. The bioluminescence phase and intensity in the two groups were compared using Welch's *t*-test or Student's t-test.

3. Results

3.1. Phase response of the bioluminescence rhythm to the explant preparation time

The peak ZT of each type of explant fluctuated depending on the explant preparation ZT (Supplemental Fig. 1). For the first peak, the time lags between the ZT of the earliest and latest peaks were 3.4 h in the SCN, 5.8 h in the CP-LV, 5.9 h in the CP-4V, and 5.5 h in the PG (Supplemental Fig. 2). Cosine curve fitting of the bioluminescence intensity at 1 h after explant preparation (Supplemental Fig. 3) showed that the *in vivo* peak times were ZT12 in the SCN, ZT12 in the CP-LV, ZT 15 in the CP-4V, and ZT 21 in the PG (Supplemental Fig. 4). As shown in Fig. 1, which is based on Supplemental Fig. 2 and Supplemental Fig. 4, the phase response curve indicated that the most significant phase delay in the SCN occurred at ZT11. However, in the CP-LV, CP-4V, and PG, the most significant delay occurred at ZT14.

3.2. Effects of and recovery from in vivo sevoflurane application with regard to the bioluminescence rhythm of SCN explants

Regardless of the ZT when the explants were prepared, no difference was observed between anesthetized and non-anesthetized rats regarding the appearance of the first peak of the bioluminescence rhythm in the SCN. These results indicated that i*n vivo* application of sevoflurane did not cause a phase shift in SCN explants (Fig. 2A).

The most common pattern of bioluminescence in explants from the

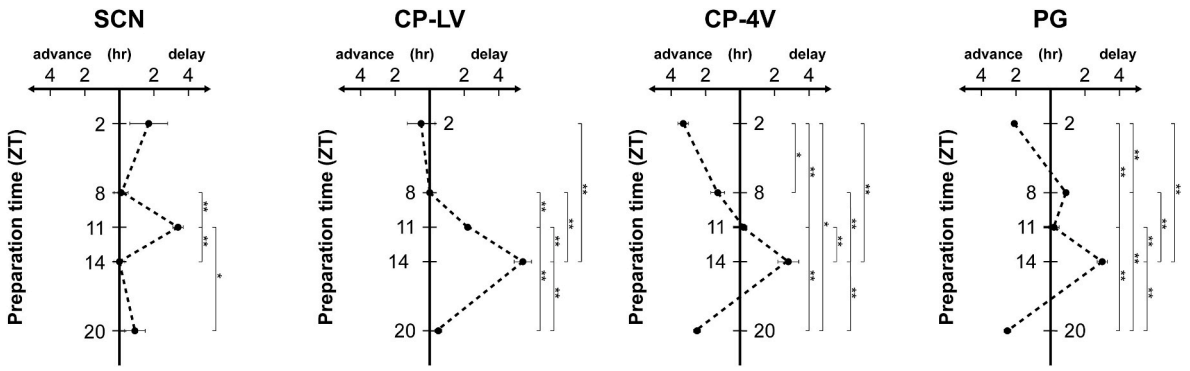


Fig. 1. Phase response curves of bioluminescence rhythms according to explant preparation times for different types of tissue. The phase shift compared with the *in vivo* peak time (Supplemental Fig. 4) is presented as the mean time difference ±standard error. Significant differences between two values are indicated by * $P < 0.05$ and ** $P < 0.01$.

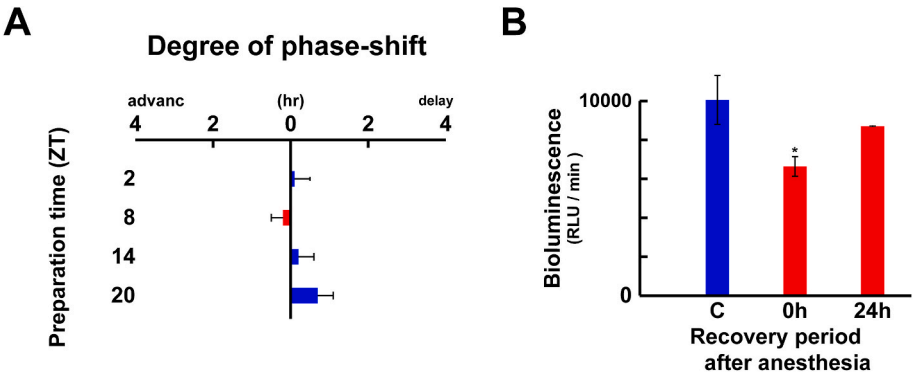


Fig. 2. Effects of *in vivo* sevoflurane application on bioluminescence rhythm in SCN explants. A: Time lag between first peak ZT of explants from anesthetized rats and those from non-anesthetized rats are shown for each explant preparation ZT. Phase advances (shown in red) and phase delays (shown in blue) are presented as the mean time difference ± standard error. No difference was observed between groups. B: Recovery of bioluminescence intensity of SCN explants after *in vivo* sevoflurane application in rats. The bioluminescence intensities of first peaks were recorded in rats of 0 h, 24 h after anesthetic treatment (ZT14–20, red) and compared with those of non-anesthetized rats (blue) (*$P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

SCN was a lower intensity in anesthetized rats than in non-anesthetized rats (Supplemental Fig. 5). In particular, the first, second, and third PIBs of SCN explants from anesthetized rats that were prepared at ZT2 and ZT20 were significantly lower than those from non-anesthetized rats prepared at the same ZT (Supplemental Fig. 6).

Regarding the recovery of PIB, when SCN explants were prepared 24 h after anesthesia, the PIBs did not show a significant decline compared with those of non-anesthetized rats (Fig. 2B). These results indicated that the SCN required a day to get recovered from the perturbation due to sevoflurane.

3.3. *Effects of and recovery from in vivo application of sevoflurane with regard to bioluminescence rhythms of peripheral explants*

Regarding the phase shift, CP-LV explants that were prepared at ZT20 showed significant delays in the first peak. CL-4V and PG explants also showed phase shifts depending on the preparation time (Fig. 3A). Regarding recovery to the original phase, significant phase delays were observed in CP-LV and CL-4V explants that were prepared at ZT20 at 24 h after anesthesia administration. In contrast, explants from rats that completed 7 days of recovery period after anesthesia showed no significant phase shifts (Fig. 3B). These results indicated that CP-LV and CL-4V explants require the recovery period of several days to withdraw the effects of anesthetics on phase shifts.

Regarding the bioluminescence intensity, comparable suppressive patterns were observed in peripheral explants and the SCN (Supplemental Fig. 5). The PIBs of CP-LV, CP-4V, and PG explants from anesthetized rats appeared to exhibit significantly lower intensity than those of corresponding explants from non-anesthetized rats, depending on the time of explant preparation (Supplemental Fig. 6). These results indicated that the suppressive effects of *in vivo* sevoflurane application persisted in peripheral explants even after wearing off anesthesia.

Regarding the recovery of bioluminescence intensity, the CP-LV, CL-4V, and PG explants prepared at ZT20 showed significantly lower PIBs, even at 30 days after anesthesia administration (Fig. 3C), indicating that the anesthetic-induced suppression of bioluminescence intensity is imprinted within the peripheral tissues.

4. Discussion

The present study revealed that explant preparation acted as a Zeitgeber on the bioluminescence rhythm of four types of tissue explants due to *Per2* transcription machinery. In contrast, vanderLeest et al. reported that the phase of the electrical activity rhythm in SCN explants was not altered by time when the explants were prepared within one day [21]. The electrical activity of the SCN has a significant impact on the *Per1* expression machinery [22]. *Per2* mRNA expression is driven by the same transcription mechanism as *Per1* expression. In the SCN, conflicting results were obtained between our study and the report by vanderLeest et al.. Therefore, the circadian rhythm of transcriptional activity demonstrated in our study was divergent from the electrical activity rhythm. Furthermore, the time lag between the earliest and latest peaks of bioluminescence rhythm in the four types of tissue, as indicated by the phase-response curve, were shorter in SCN explants than those in CL-LV, CL-4V, and PG explants. This may have occurred because of the higher resilience of SCN explants to stress exerted by explant preparation than that of other peripheral explants. The bioluminescence rhythm phases observed in the four types of explants from non-anesthetized rats were set to the "original phase".

The phase shifts in SCN explants from anesthetized rats were not notable in this study. This result indicated that SCN explants could independently restore the perturbed circadian phases to those observed

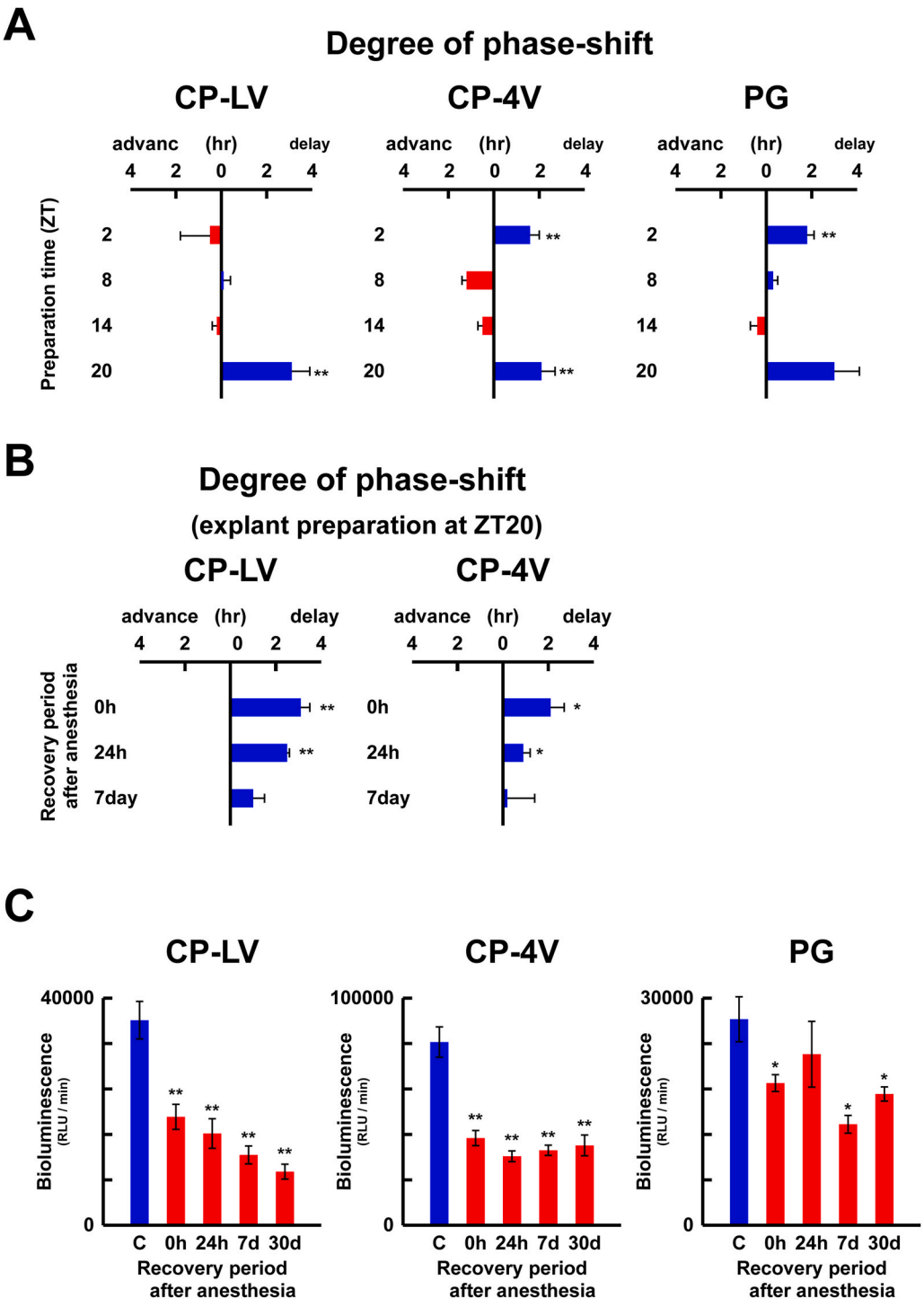


Fig. 3. A: Effects of *in vivo* sevoflurane application on the bioluminescence rhythm in peripheral explants. The time lag between the first peak ZT of explants from anesthetized rats and those from non-anesthetized rats is shown for each explant-preparation ZT. Phase advances (shown in red) and phase delays (shown in blue) are presented as the mean time difference ± standard error (*P < 0.05, **P < 0.01). B: Recovery of bioluminescence phase of explants after *in vivo* sevoflurane application to rats. ZTs of the first peaks were recorded in rats of 0 h, 24 h, and 7 days recovery after anesthetic treatment (ZT14–20) and compared with those of non-anesthetized rats. C: The bioluminescence intensities of first peaks were recorded in rats of 0 h, 24 h, 7 days, and 30 days recovery after anesthetic treatment (ZT14–20, red) and compared with those of non-anesthetized rats (blue) (*P < 0.05, **P < 0.01). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

before anesthesia. According to a previous study, suppression of *Per2* mRNA expression in the brain restored the normal expression pattern, while no obvious phase shift was exhibited after anesthetic treatment [11–13]. Extrapolating from this *ex vivo* experimental result, the SCN in the brain can induce phase correction under *in vivo* conditions by itself in the same manner as that observed under *ex vivo* conditions.

Mori et al. reported that *in vivo* application of sevoflurane inhibited binding of the histone acetyltransferase CLOCK to the *Per2* promoter region in the SCN, leading to suppression of *Per2* mRNA expression [23]. Immediately after wearing off anesthesia, *Per2* expression in the brain or bioluminescence in SCN explants rapidly restored the original phases. Even during the effects of anesthesia, CLOCK/BMAL in the SCN showed enhanced ability to transcribe *Per2*/luciferase mRNA. Further investigation into the mechanism of this phenomenon involving CLOCK/BMAL is necessary.

Phase shifts in the bioluminescence rhythm appeared after *ex vivo* sevoflurane application, while the phases showed an increasing trend, which is closely associated with gamma-aminobutyric acid (GABA) receptors [14]. GABA receptors are expressed in the SCN [24], and $GABA_A$ receptors have a particular protein region that responds to sevoflurane [25]. $GABA_A$ receptor activation suppresses *Per1* and *Per2* mRNA in the SCN during the middle of the subjective day [26]. These reports suggest that the SCN itself has a mechanism to sense sevoflurane anesthetics. However, the same machinery may not function in the brain when the

anesthetic is applied under *in vivo* conditions. In a previous report, sevoflurane was intermittently administered to a small glass chamber containing explants, and the same concentration used under *in vivo* conditions was maintained. Additionally, the explants were exposed to air movement [13,14]. However, comparable circumstances such as air movement or high anesthetic concentrations cannot be expected inside the brain in practice. The mechanism responsible for the suppressive effects of *Per2* mRNA expression under *in vivo* conditions may differ from the phase shift mechanism of *ex vivo* anesthetic application. Regarding suppressing *Per2* mRNA expression or bioluminescence, this could be explained if anesthetics act on neurons outside the SCN as follows. The SCN is innervated mainly by the lateral geniculate body and raphe nucleus. Neuropeptide Y and serotonin, which are produced in the lateral geniculate body and raphe nucleus, respectively, have been reported to affect *Per2* mRNA expression in the SCN [27–32]. In addition, orexin, which is related to sleep, is produced in the lateral hypothalamic area and has also been reported to affect *Per2* mRNA expression [33].

The present study showed that *in vivo* application of sevoflurane during late night induces phase shifts in CP-LV, CP-4V, and PG explants. These three types of tissues are known to be innervated via the autonomic nervous system [34–36]. The electrical activity of the SCN is decreased at night. Therefore, even if suppressive effects occur in CP-LV, CP-4V, and PG explants, the SCN is not a candidate area for sevoflurane action. For example, autonomic nervous system nuclei could be affected by sevoflurane.

This investigation also revealed that anesthetic treatment suppressed PIBs of four types of tissues. The results suggest that anesthetic treatment suppresses *Per2* expression/the bioluminescence rhythm of four types of explants during consecutive hours. Suppression of PIBs in SCN explants was restored after one day of recovery. According to the results of similar reports, *Per2* mRNA expression was decreased on the day after anesthesia was applied in mice under *in vivo* conditions compared with mice without anesthetic treatment [11,12]. After wearing off anesthesia, an *in vivo* intervention using related tissues is necessary for the SCN to restore the normal bioluminescence intensity level. These related tissues may include the three tissues other than SCN in the brain examined in this study. In contrast, the PIBs of peripheral explants did not recover from suppression after anesthesia even if the rats were bred for 30 days before being sacrificed. The results may suggest that certain epigenetic events such as DNA or histone methylation may contribute to the effects on bioluminescence intensity.

5. Conclusion

The resilience of the circadian oscillator in the SCN to the effects of sevoflurane was predominant over that of other tissues. In contrast, the bioluminescence intensity, which indicated gene expression in peripheral tissues was prone to remain low after sevoflurane treatment.

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

No data was used for the research described in the article.

Acknowledgments

We are grateful to Prof. Yasufumi Shigeyoshi (Department of Anatomy and Neurobiology, Kinki University School of Medicine, Japan) for providing *Per2::dLuc* transgenic rats. We are also grateful to Prof. Naoki Shimada (Center for Basic Medical Science, International University of Health and Welfare) for consultation regarding the statistical analysis. This work was supported by Grants-in-Aid from the Japan Society for the Promotion of Science (JSPS)-KAKENHI, Japan [grant No. 19K09335 to N.I., 20K16607 to T. Y.]. We thank Ms Kaoru Iijima for language editing of the manuscript. We thank Edanz (www.edanzediting.com/ac) for editing a draft of this manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2022.101258.

References

[1] B.R. Brown, E.J. Frink, The safety of sevoflurane in humans, Anesthesiology 79 (1993) 201–202.
[2] J.B. Forrest, K. Rehder, C.H. Goldsmith, M.K. Cahalan, W.J. Levy, L. Strunin, W. Bota, C.D. Boucek, R.F. Cucchiara, S. Dhamee, Multicenter study of general anesthesia. I. Design and patient demography, Anesthesiology 72 (1990) 252–261.
[3] W.J. Levy, Clinical anaesthesia with isoflurane. A review of the multicentre study, Br. J. Anaesth. 56 (Suppl. 1) (1984), 101S–112S.
[4] A. Sakamoto, J. Imai, A. Nishikawa, R. Honma, E. Ito, M. Kawamura, R. Ogawa, S. Watanabe, Influence of inhalation anesthesia assessed by comprehensive gene expression profiling, Gene 356 (2005) 39–48.
[5] W. Ruan, X. Yuan, H.K. Eltzschig, Circadian rhythm as a therapeutic target, Nat. Rev. Drug Discov. 20 (2021) 287–307.
[6] A. Wirz-Justice, M. Frank, Does anaesthesia stop the clock? Sleep Med. Rev. 37 (2018) 3.
[7] R.C. Poulsen, G.R. Warman, J. Sleigh, N.M. Ludin, J.F. Cheeseman, How does general anesthesia affect the circadian clock? Sleep Med. Rev. 37 (2018) 35–44.
[8] R. Imai, H. Makino, T. Katoh, T. Kimura, T. Kurita, K. Hokamura, K. Umemura, Y. Nakajima, Desflurane anesthesia shifts the circadian rhythm phase depending on the time of day of anesthesia, Sci. Rep. 10 (2020) 18273.
[9] L.P. Shearman, S. Sriram, D.R. Weaver, E.S. Maywood, I. Chaves, B. Zheng, K. Kume, C.C. Lee, G.T. van der Horst, M.H. Hastings, S.M. Reppert, Interacting molecular loops in the mammalian circadian clock, Science 288 (2000) 1013–1019.
[10] D.P. King, J.S. Takahashi, Molecular genetics of circadian rhythms in mammals, Annu. Rev. Neurosci. 23 (2000) 713–742.
[11] Y. Ohe, N. Iijima, K. Kadota, A. Sakamoto, H. Ozawa, The general anesthetic sevoflurane affects the expression of clock gene *mPer2* accompanying the change of NAD^+ level in the suprachiasmatic nucleus of mice, Neurosci. Lett. 490 (2011) 231–236.
[12] K. Kadota, N. Iijima, Y. Ohe-Hayashi, K. Takumi, S. Higo, A. Sakamoto, H. Ozawa, Time-dependent repression of *mPer2* expression in the suprachiasmatic nucleus by inhalation anesthesia with sevoflurane, Neurosci. Lett. 528 (2012) 153–158.
[13] M. Anzai, N. Iijima, S. Higo, K. Takumi, I. Matsuo, K. Mori, Y. Ohe, K. Kadota, T. Akimoto, A. Sakamoto, H. Ozawa, Direct and specific effect of sevoflurane anesthesia on *rat Per2* expression in the suprachiasmatic nucleus, PLoS One 8 (2013), e59454.
[14] I. Matsuo, N. Iijima, K. Takumi, S. Higo, S. Aikawa, M. Anzai, H. Ishii, A. Sakamoto, H. Ozawa, Characterization of sevoflurane effects on *Per2* expression using *ex vivo* bioluminescence imaging of the suprachiasmatic nucleus in transgenic rats, Neurosci. Res. 107 (2016) 30–37.
[15] J. Myung, C. Schmal, S. Hong, Y. Tsukizawa, P. Rose, Y. Zhang, M.J. Holtzman, E. De Schutter, H. Herzel, G. Bordyugov, T. Takumi, The choroid plexus is an important circadian clock component, Nat. Commun. 9 (2018) 1062.
[16] T. Quintela, C. Sousa, F.M. Patriarca, I. Gonçalves, C.R. Santos, Gender associated circadian oscillations of the clock genes in rat choroid plexus, Brain Struct. Funct. 220 (2015) 1251–1262.
[17] T. Yamaguchi, T. Hamada, T. Matsuzaki, N. Iijima, Characterization of the circadian oscillator in the choroid plexus of rats, Biochem. Biophys. Res. Commun. 524 (2020) 497–501.
[18] P. Pevet, E. Challet, Melatonin: both master clock output and internal time-giver in the circadian clocks network, J. Physiol. Paris 105 (2011) 170–182.
[19] T. Quintela, I. Gonçalves, M. Silva, A.C. Duarte, P. Guedes, K. Andrade, F. Freitas, D. Talhada, T. Albuquerque, S. Tavares, L.A. Passarinha, J. Cipolla-Neto, C.R. A. Santos, Choroid plexus is an additional source of melatonin in the brain, J. Pineal Res. 65 (2018), e12528.
[20] P.J. He, M. Hirata, N. Yamauchi, S. Hashimoto, M.A. Hattori, The disruption of circadian clockwork in differentiating cells from rat reproductive tissues as identified by in vitro real-time monitoring system, J. Endocrinol. 193 (2007) 413–420.
[21] H.T. vanderLeest, M.J. Vansteensel, H. Duindam, S. Michel, J.H. Meijer, Phase of the electrical activity rhythm in the SCN in vitro not influenced by preparation time, Chronobiol. Int. 26 (2009) 1075–1089.
[22] S. Yamaguchi, H. Isejima, T. Matsuo, R. Okura, K. Yagita, M. Kobayashi, H. Okamura, Synchronization of cellular clocks in the suprachiasmatic nucleus, Science 302 (2003) 1408–1412.
[23] K. Mori, N. Iijima, S. Higo, S. Aikawa, I. Matsuo, K. Takumi, A. Sakamoto, H. Ozawa, Epigenetic suppression of mouse *Per2* expression in the suprachiasmatic nucleus by the inhalational anesthetic, sevoflurane, PLoS One 9 (2014), e87319.

[24] B.F. O'Hara, R. Andretic, H.C. Heller, D.B. Carter, T.S. Kilduff, $GABA_A$, $GABA_C$, and NMDA receptor subunit expression in the suprachiasmatic nucleus and other brain regions, Brain Res Mol Brain Res 28 (1995) 239–250.
[25] K. Nishikawa, N.L. Harrison, The actions of sevoflurane and desflurane on the gamma-aminobutyric acid receptor type A: effects of TM2 mutations in the alpha and beta subunits, Anesthesiology 99 (2003) 678–684.
[26] C. Ehlen, C.M. Novak, M.C. Karom, K.L. Gamble, K.N. Paul, H.E. Albers, $GABA_A$ receptor activation suppresses Period 1 mRNA and Period 2 mRNA in the suprachiasmatic nucleus during the mid-subjective day, Eur. J. Neurosci. 23 (2006) 3328–3336.
[27] C. Fukuhara, J.M. Brewer, J.C. Dirden, E.L. Bittman, G. Tosini, M.E. Harrington, Neuropeptide Y rapidly reduces *Period* 1 and *Period* 2 mRNA levels in the hamster suprachiasmatic nucleus, Neurosci. Lett. 314 (2001) 119–122.
[28] J.M. Brewer, P.C. Yannielli, M.E. Harrington, Neuropeptide Y differentially suppresses *per1* and *per2* mRNA induced by light in the suprachiasmatic nuclei of the golden hamster, J. Biol. Rhythm. 17 (2002) 28–39.
[29] R.C. Besing, L.M. Hablitz, J.R. Paul, R.L. Johnson, R.A. Prosser, K.L. Gamble, NPY-induced phase shifts of PER2::LUC rhythms are mediated by long-term suppression of neuronal excitability in a phase-specific manner, Chronobiol. Int. 29 (2012) 91–102.
[30] K. Horikawa, S. Yokota, K. Fuji, M. Akiyama, T. Moriya, H. Okamura, S. Shibata, Nonphotic entrainment by $5\text{-}HT_{1A/7}$ receptor agonists accompanied by reduced Per1 and Per2 mRNA levels in the suprachiasmatic nuclei, J. Neurosci. 20 (2000) 5867–5873.
[31] T.J. Varcoe, D.J. Kennaway, A. Voultsios, Activation of $5\text{-}HT_{2C}$ receptors acutely induces Per gene expression in the rat suprachiasmatic nucleus at night, Brain Res Mol Brain Res 119 (2003) 192–200.
[32] T.J. Varcoe, D.J. Kennaway, Research centre activation of $5\text{-}HT_{2C}$ receptors acutely induces Per1 gene expression in the rat SCN in vitro, Brain Res. 1209 (2008) 19–28.
[33] M.D.C. Belle, A.T.L. Hughes, D.A. Bechtold, P. Cunningham, M. Pierucci, D. Burdakov, H.D. Piggins, Acute suppressive and long-term phase modulation actions of orexin on the mammalian circadian clock, J. Neurosci. 34 (2014) 3607–3621.
[34] M. Lindvall, L. Edvinsson, C. Owman, Histochemical, ultrastructural, and functional evidence for a neurogenic control of CSF production from the choroid plexus, Adv. Neurol. 20 (1978) 111–120.
[35] M. Lindvall, C. Owman, Autonomic nerves in the mammalian choroid plexus and their influence on the formation of cerebrospinal fluid, J. Cerebr. Blood Flow Metabol. 1 (1981) 245–266.
[36] J.A. Nathanson, Beta-adrenergic-sensitive adenylate cyclase in secretory cells of choroid plexus, Science 204 (1979) 843–844.

Supplemental experimental procedures

Examination of the phase shift degree

Bioluminescence intensities detected from the explants 1 h after explant preparation (initial bioluminescence) were considered to reflect the relative amount of luciferase contained in each tissue at the time of sacrifice (Supplemental Fig. 3). Initial bioluminescence values were plotted against the preparation time (Supplemental Fig. 4). Cosine curve fitting was performed on bioluminescence intensities from four types of tissues using Acrophase software (https://www.circadian.org/softwar.html) to define the putative peak times of luciferase expression in the brain (*in vivo* peak time). Phase shifts caused by explant preparation were calculated by subtracting the *in vivo* peak time from ZT of the first bioluminescence peak for the four types of explants.

Supplemental Fig. 1 The most common data patterns of bioluminescence in explants prepared at various times during the day are superimposed for each type of tissue. The bioluminescence intensities of the first peaks for each type of tissue were adjusted to 100. The bioluminescence rhythms observed in the superchiasmatic nucleus, choroid plexus in the lateral ventricle, and choroid plexus in the fourth ventricle during 5 days and the pineal gland during 4 days are shown. The first peaks that appeared in the four types of

tissue are displayed in enlarged views on the top left of each graph.

Supplemental Fig. 2 Changes in bioluminescence phases in response to the explant preparation time of day. The peak ZTs are presented as the mean ± standard error. The peak ZTs in each phase (first to fifth phases) are plotted and connected with different colored dotted lines for each tissue.

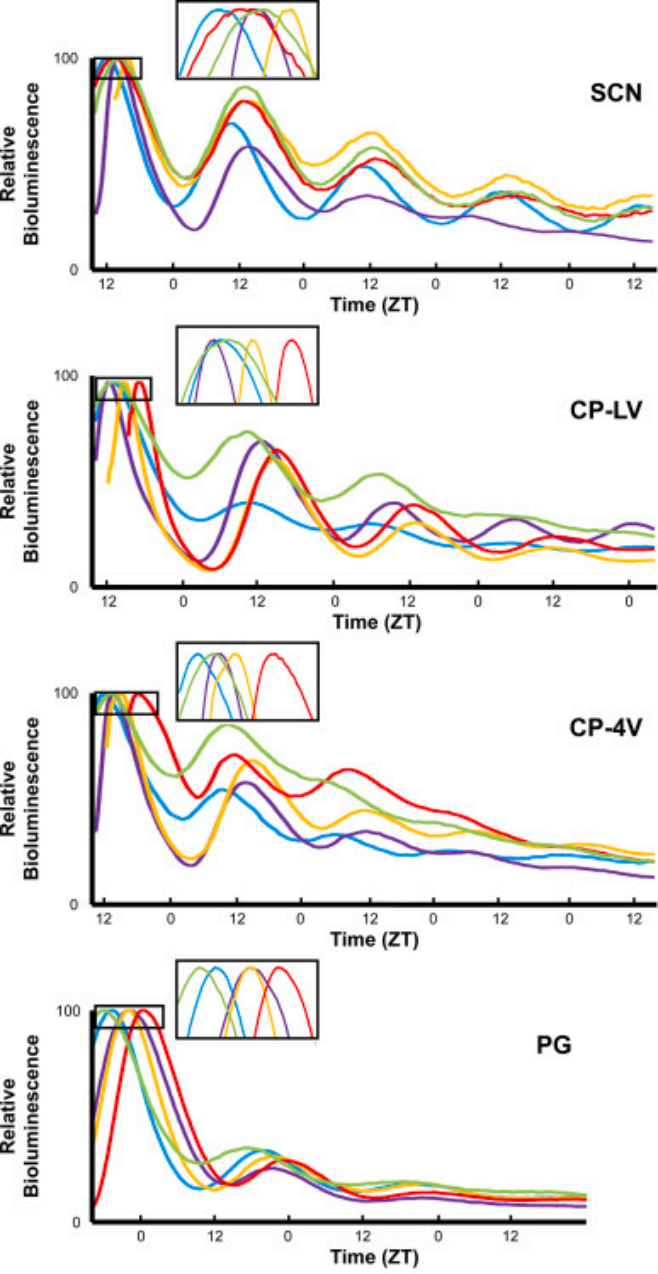
Supplemental Fig. 3 Various bioluminescence rhythms over one day are superimposed. The horizontal axis indicates time after explant preparation. Bioluminescence intensities at 1 h after explant preparation (initial bioluminescence) indicate the relative amount of luciferase in each tissue at the time of sacrifice.

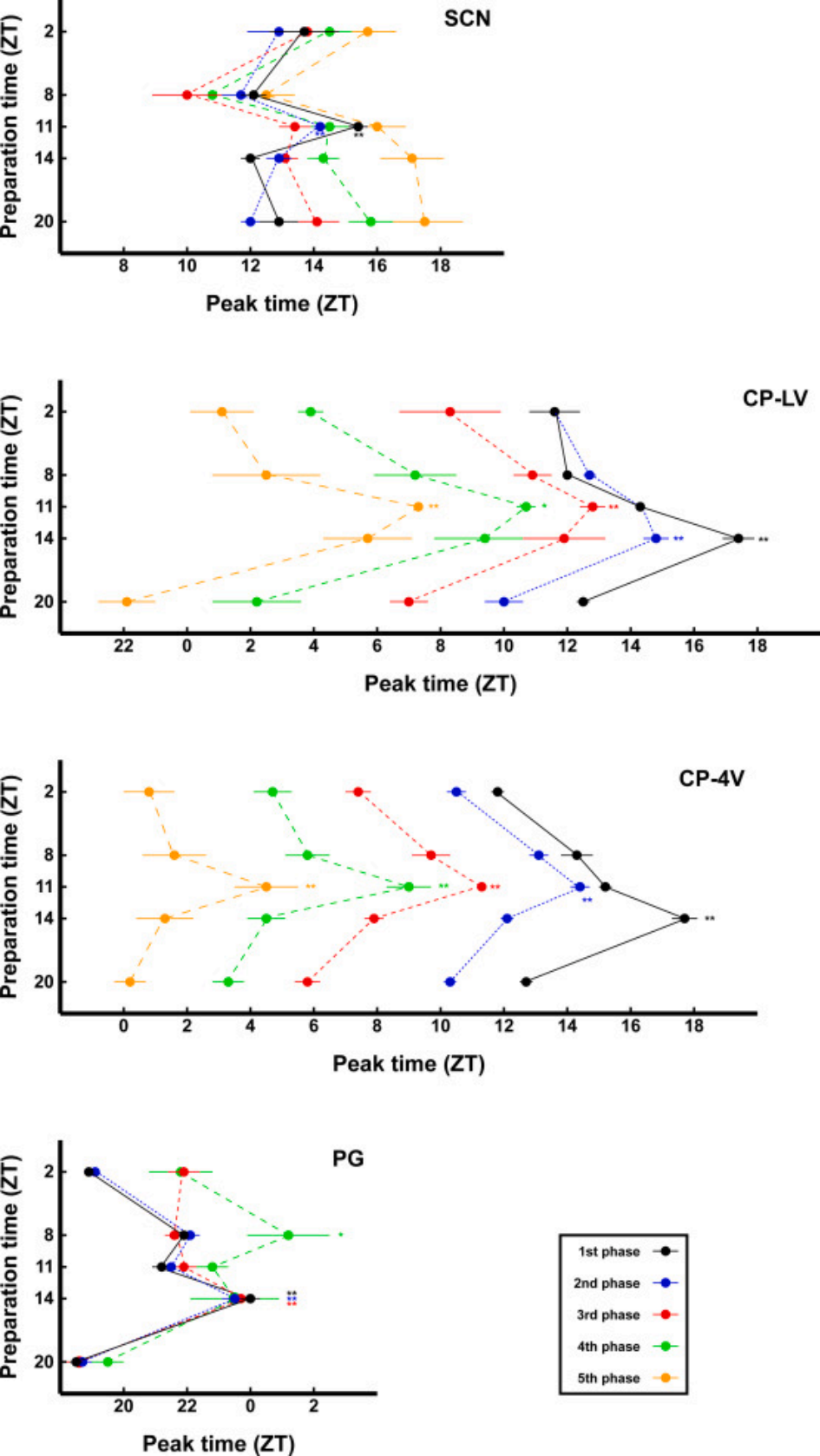
Supplemental Fig. 4 Initial bioluminescence intensities are plotted against the explant-preparation Zeitgeber time (ZT). ZTs of the *in vivo* peaks (*in vivo* peak time) in the tissues were determined by cosine curve fitting.

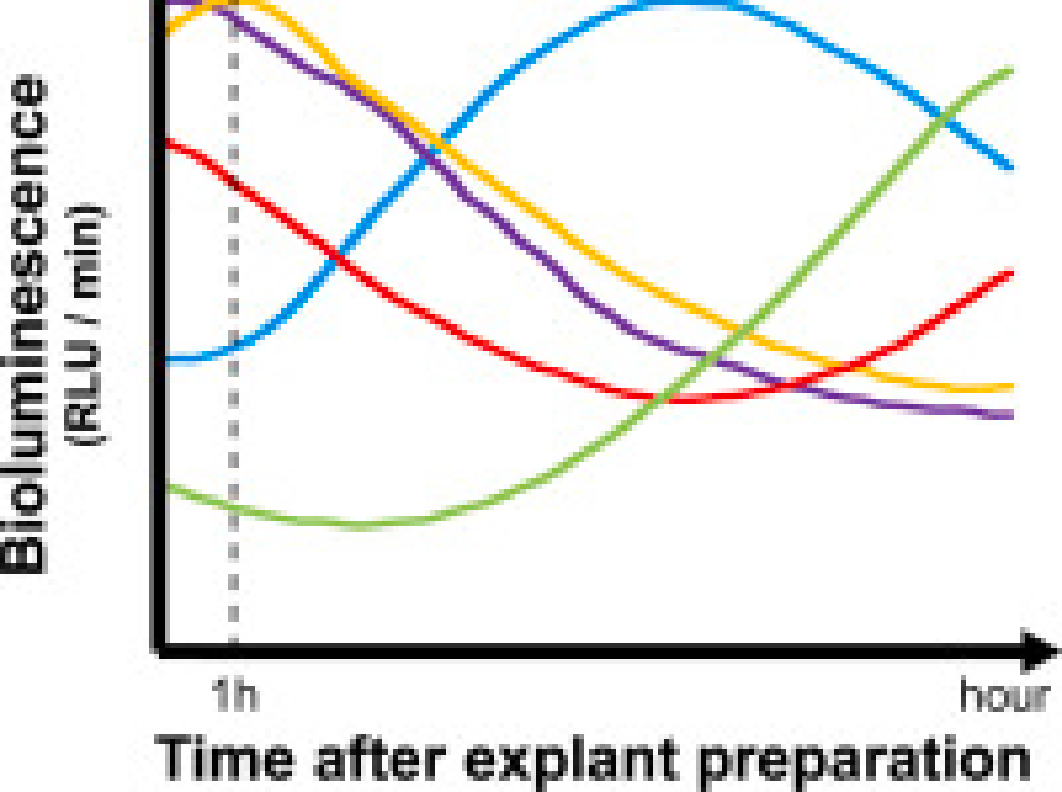
Supplemental Fig. 5 The most common bioluminescence patterns from explants of anesthetized rats (red) and non-anesthetized rats (blue) are superimposed for each type of

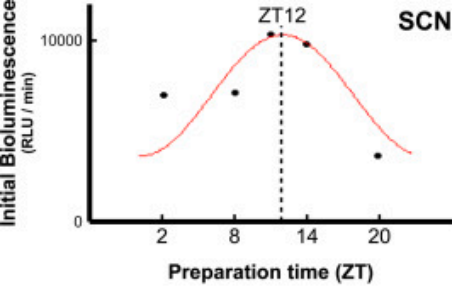
tissue.

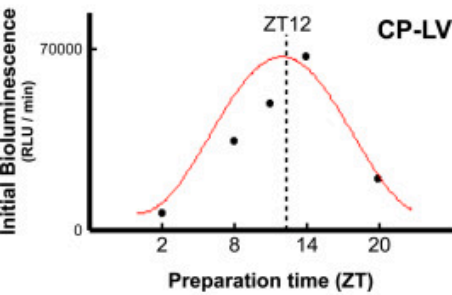
Supplemental Fig. 6 The suppressive effects of *in vivo* sevoflurane application on the bioluminescence peak intensities of explants are indicated. The intensities detected at the peak time points in explants from both anesthetized (red) and non-anesthetized rats (blue) were compared for each explant preparation time. Significant differences between two values are indicated by * $P < 0.05$ and ** $P < 0.01$.

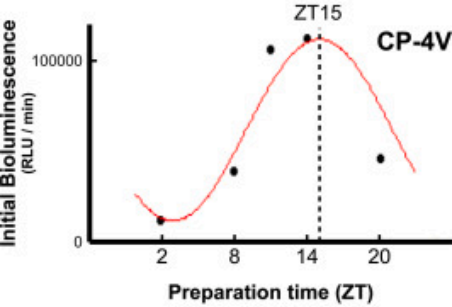

SCN
CP-LV
CP-4V
PG
Relative Bioluminescence
100
0
12
0
Time (ZT)
preparation time
ZT2
ZT8
ZT11
ZT14
ZT20

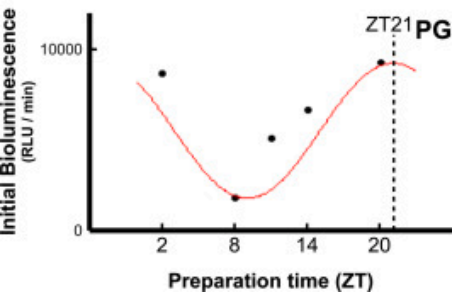

SCN
Preparation time (ZT)
2
8
11
14
20
8
10
12
14
16
18
Peak time (ZT)
CP-LV
Preparation time (ZT)
2
8
11
14
20
22
0
2
4
6
8
10
12
14
16
18
Peak time (ZT)
CP-4V
Preparation time (ZT)
2
8
11
14
20
0
2
4
6
8
10
12
14
16
18
Peak time (ZT)
PG
Preparation time (ZT)
2
8
11
14
20
20
22
0
2
Peak time (ZT)
1st phase
2nd phase
3rd phase
4th phase
5th phase

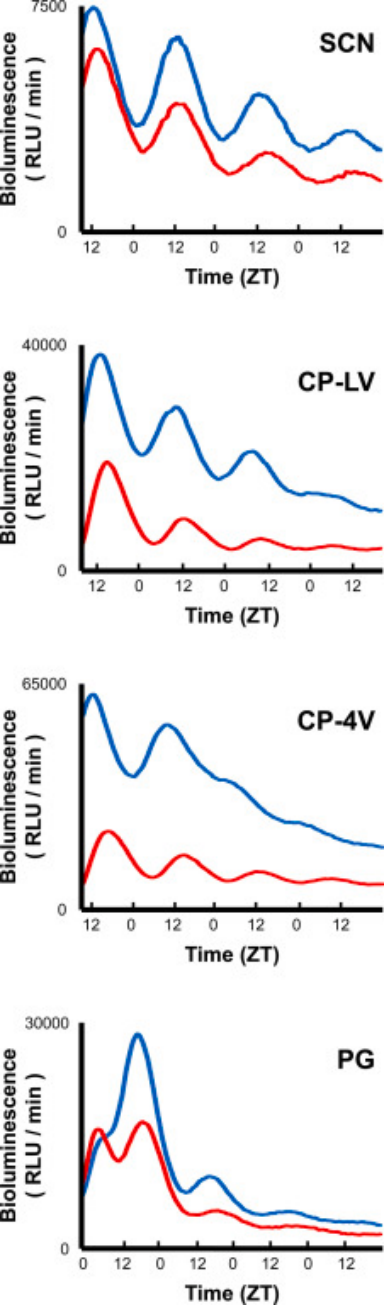

Bioluminescence
(RLU / min)
1h
hour
Time after explant preparation

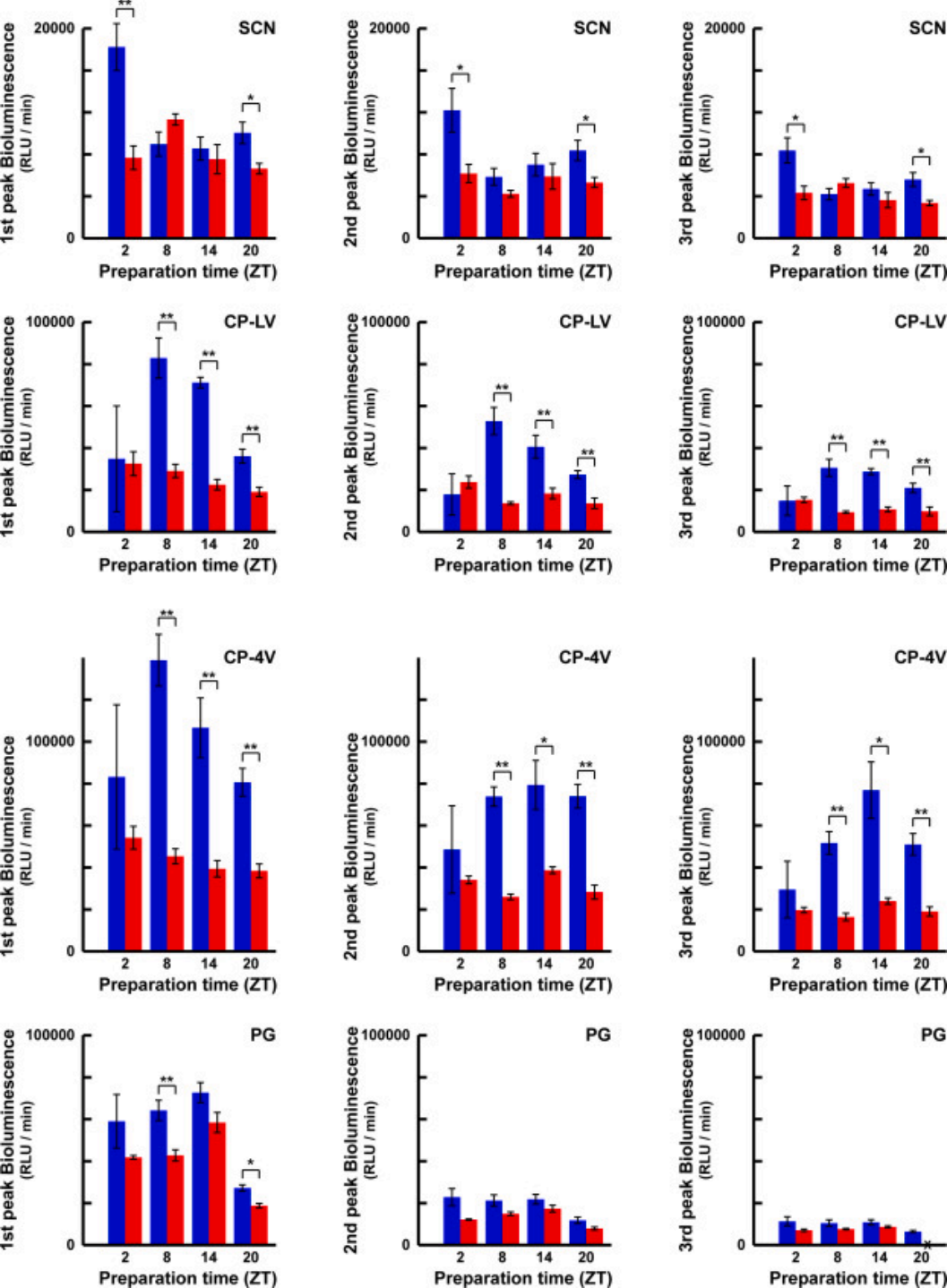

ZT12
SCN
10000
0
Initial Bioluminescence
(RLU / min)
2
8
14
20
Preparation time (ZT)


ZT12
CP-LV
70000
0
Initial Bioluminescence
(RLU / min)
2
8
14
20
Preparation time (ZT)


ZT15
CP-4V
100000
0
Initial Bioluminescence
(RLU / min)
2
8
14
20
Preparation time (ZT)


ZT21
PG
10000
0
Initial Bioluminescence
(RLU / min)
2
8
14
20
Preparation time (ZT)


7500
Bioluminescence
(RLU / min)
SCN
0
12 0 12 0 12 0 12
Time (ZT)
40000
Bioluminescence
(RLU / min)
CP-LV
0
12 0 12 0 12 0 12
Time (ZT)
65000
Bioluminescence
(RLU / min)
CP-4V
0
12 0 12 0 12 0 12
Time (ZT)
30000
Bioluminescence
(RLU / min)
PG
0
0 12 0 12 0 12 0 12
Time (ZT)


SCN
1st peak Bioluminescence (RLU / min)
2nd peak Bioluminescence (RLU / min)
3rd peak Bioluminescence (RLU / min)
20000
0
2
8
14
20
Preparation time (ZT)
CP-LV
100000
CP-4V
PG