2,2,2-Tribromoethanol Phase-Shifts the Circadian Rhythm of the Liver Clock in Per2::Luciferase Knockin Mice: Lack of Dependence on Anesthetic Activity

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ABSTRACT

Comprehensive gene expression profiling in mice in response to the inhalation of sevoflurane has revealed that circadian clock gene expression is affected strongly in the liver, heart, lung, and kidney, in this order, but moderately in the spleen and slightly in the brain. Therefore, we examined whether the administration of general anesthetics at different times of the day induces phase shifts of the liver clock in Per2::Luciferase knockin mice. One to 4 days of intraperitoneal injection of 2,2,2-tribromoethanol (240 mg/kg, anesthetic time 60 min) or 2,2,2-trichloroethanol (240 mg/kg, 60 min), common anesthetics in veterinary surgery, caused phase delays when injected during the daytime and phase advances when injected during the nighttime. Inhalation administration of isoflurane for 30 or 60 min during the daytime did not induce a phase delay. Injection of propofol (300 mg/kg, 17 min) during the daytime induced an insignificant phase delay of the Per2 bioluminescence rhythm. Injection of 2,2,2-tribromoethanol did not induce a phase shift in the suprachiasmatic nucleus, the main oscillator, or in behavioral locomotor rhythms, suggesting that 2,2,2-tribromoethanol induced phase shifts of the liver clock independent of the main suprachiasmatic clock. The expression of clock genes, such as Bmal1 and Clock, in mouse liver was decreased strongly 1 and 4 h after a single injection of 2,2,2-tribromoethanol. These results demonstrate that 2,2,2-tribromoethanol or 2,2,2-trichloroethanol produce phase shifts of the peripheral clock, independent of anesthetic activity. These anesthetics may cause circadian rhythm disorders in peripheral organs when administered as general anesthetics several times during the day.

Introduction

General anesthesia can be described as a pharmacologic state involving amnesia, immobility, unconsciousness, and analgesia (Naguib et al., 2007). Two kinds of general anesthetics are used commonly in human and animal surgery: intravenous/intraperitoneal agents (propofol, ketamine, 2,2,2-tribromoethanol, and thiopental) and inhaled gases (sevoflurane, isoflurane, and halothane). In the days after general anesthesia, patients often complain of sleep and mood disorders and altered vigilance. The disruptive effects of general anesthesia on circadian time structure might have considerable consequences in humans and sustain postoperative wake-sleep disorders (Bass and Takahashi, 2010).

Circadian clock genes are involved in the generation and resetting of circadian rhythms. Clock genes are expressed robustly in the hypothalamic suprachiasmatic nucleus (SCN) as well as other brain areas and peripheral tissues, such as liver, lung, heart, kidney, and skeletal muscle (Dibner et al., 2010). The mutation of circadian clock genes, such as Bmal1, Per1/Per2, Cry1/Cry2, and Clock, has reported effects on normal circadian locomotor activity rhythms and various physiologic functions (Ko and Takahashi, 2006; Siepka et al., 2007). Therefore, general anesthetics may induce deficits of clock gene expression, resulting in disturbed wake-sleep rhythms. Sevoflurane suppresses Per2 gene expression in the rat brain over a long time period (Kobayashi et al., 2007). This anesthetic suppresses not only circadian expression but also light-induced expression of the Per2 gene in the SCN (Ohe et al., 2011). Continuous intravenous injection of propofol also suppresses Per2 gene expression in rat brain (Yoshida et al., 2009). Circadian core temperature and rest-activity rhythms have been reported to be disrupted by propofol injection during late rest periods and early active periods in rats. Propofol induces significant 60- to 80-min
phase advances in both rest-activity and body temperature rhythms (Dispersyn et al., 2009). However, sevoflurane inhalation during the early rest period causes a phase delay of the locomotor activity rhythms in mice under constant dark (DD) conditions (Ohe et al., 2011). Taken together, these results suggest that anesthetics may affect circadian rhythms by regulating Per2 gene expression in the brain.

Sakamoto et al. (2005) performed comprehensive gene expression profiling in mice in response to the inhalation of sevoflurane and found that the expression of circadian clock genes, such as Per2, Cry2, Per3, Bmal1, Clock, Rev-erb-a, Dec1, Dbp, and E4bp4, was affected strongly in the liver, heart, lung, and kidney, in that order, but modestly in the spleen and slightly in the brain. This indicates that general anesthetics may affect peripheral circadian clock systems and disturb many circadian functions, such as circulation, energy metabolism, and digestion, because these physiologic functions are reported to be under circadian control (Garaulet and Madrid, 2010; Shibata et al., 2010; Takeda and Maemura, 2010). Although a central clock such as the SCN and/or a brain clock such as the cerebral cortex are known now to be affected by general anesthesia, no one knows whether general anesthesia can affect peripheral clock systems. Thus, this experiment is important to know some aspects of side effects of general anesthesia. In the present study, we examined whether the inhalation of isoflurane or the intraperitoneal injection of 2,2,2-tribromoethanol phase-shifts the circadian Per2 gene expression rhythm in the livers of Per2::Luciferase (Per2::Luc) knockin mice and causes acute changes in clock gene expression in the liver. In addition, we evaluated compounds structurally related to 2,2,2-tribromoethanol to determine whether the phase resetting effect is specific to the structure of 2,2,2-tribromoethanol or to its anesthetic activity.

Materials and Methods

General Conditions of Animals and Housing. Per2::Luc knockin mice (founders courtesy of Dr. Joseph Takahashi, Northwestern University, Chicago, IL) (Yoo et al., 2004) were bred in-house. We prepared Per2::Luc heterozygous male mice for mating with female mice from the ICR strain (Tokyo Experimental Animals Co., Tokyo, Japan). From this crossing, we obtained F1 hybrid Per2::Luc heterozygous males that weighed 25 to 35 g at the start of the experiment. Male ICR mice (25–35 g) were used for the experiments on anesthetic activity, locomotor activity, and acute change in clock gene expression. We observed no differences in the peak times of bioluminescence in livers from the original male or F1 hybrid mice. We observed no differences in the peak times of bioluminescence in livers from the original male or F1 hybrid mice.

Assessment of Anesthetic Activity In Vivo. Male ICR mice (25–35 g) were used for this experiment. Assessment of general anesthetics was evaluated by measuring the time (minutes) until the recovery of the righting reflex response after the intraperitoneal injection of each agent except isoflurane. Some mice were anesthetized with inhalant anesthesia (2.5% isoflurane in 100% oxygen) for 30 or 60 min with the use of an inhalant anesthesia apparatus (XGI-8 Gas Anesthesia System; Caliper Life Sciences, Hopkinton, MA).

Phase Shifting Effects on Locomotor Activity Rhythms. Male ICR mice (25–35 g) were housed individually, and general locomotor activity was recorded with an infrared radiation sensor (FSB; Omron, Tokyo, Japan) and analyzed with ClockLab software (Actimetrics, Wilmette, IL). Analysis of the activity was measured as the number of sensor counts per 6 min. Vehicle or 2,2,2-tribromoethanol (240 mg/kg) was injected for 4 days under LD cycles, and then mice were placed in the DD condition for 10 days or more. The phase of activity onset was measured before DD and after DD, and the phase difference (minutes) was calculated.

Preparation and Measurement of Bioluminescence from Per2::Luc Mice. After daily injections of agents for 1 to 4 days, Per2::Luc mice were killed at ZT3 for the assessment of the bioluminescence rhythm in the liver or SCN. Liver blocks were removed rapidly and placed in ice-cold Hanks’ balanced salt solution (pH 7.2; Sigma-Aldrich, St. Louis, MO). Four pieces were taken randomly from the four lobes of the liver from one mouse. For SCN experiments, 300-μm-thick SCN slices were prepared with a microtome (Dohan Co., Osaka, Japan). Each tissue explant was placed into a 35-mm Petri dish (Iwaki, Tokyo, Japan) sealed with parafilm (Sigma-Aldrich), and the tissues were cultured in 1.3 ml of Dulbecco’s modified Eagle’s medium (Invitro, Carlsbad, CA) supplemented with 2.7 mM NaHCO3, 10 mM HEPES, 20 mg/l kanamycin (Sigma-Aldrich), 5 μg/ml insulin (Sigma-Aldrich), 100 μM putrescine (Sigma-Aldrich), 100 μg/ml human transferrin (Sigma-Aldrich), 20 nM progesterone (Sigma-Aldrich), 30 nM sodium selenite (Sigma-Aldrich), and 0.1 M d-luciferin sodium salt (Invitro). The cultures were incubated at 37°C, and bioluminescence was monitored each minute over 10-min intervals with a dish-type luminometer (LumiCycle; Actimetrics).

Assessment of the Circadian Phases of Liver and SCN Clocks. Original data (1-min bins) were smoothed by an adjusting-averaging method with 2-h running means, as described previously (Meerlo et al., 1997). The datasets then were detrended by subtracting the 24-h running average from the raw data with the use of R software (R Development Core Team; http://www.r-project.org/). The program was created by Tsuyoshi Yaita, Naoki Furutani, and Dr. Shigenobu Shibata (Waseda University, Tokyo, Japan). Peaks were defined as points where the bioluminescence was higher than that of both sides and were confirmed by waveform. In general, the peak phase time (pZT) is evaluated by two peaks, because the first peak is affected occasionally by the movement of the culture dish (Hayasaka et al., 2007; Ohta et al., 2008). The averaged peak phase (pZT) was calculated from four liver pieces from one mouse. The period of Per2::Luc activity (recorded from 24 to 72 h in vitro) was assessed for each liver culture and calculated by averaging the period between the first and the second peaks and the period between the second and the third peaks.

Total RNA Isolation and Real-Time Reverse-Transcription Polymerase Chain Reactions. Tissue mRNA was measured by real-time reverse-transcription polymerase chain reaction (RT-PCR), as described previously (Kudo et al., 2007, 2008). Mice (n = 4 per time point) were anesthetized deeply with ether, and livers were isolated rapidly. The total RNA was extracted with ISOGEN reagent (Nippon Gene Co., Ltd., Tokyo, Japan), and the concentration was determined with a SmartSpec 3000 (Bio-Rad Laboratories, Hercules, CA). The DNA digestion step was performed with RQ1 RNase-free DNase (Promega, Madison, WI) after an incubation step at 37°C for 30 min. The enzyme was inactivated by RQ1 DNase Stop Solution (Promega). Fifty nanograms of total RNA was reverse-transcribed and amplified with a One-Step SYBR RT-PCR kit (Takara, Otsu, Japan) in an iCycler PCR machine (Bio-Rad Laboratories). Specific primer pairs were designed based on published data for the β-actin, Per2, Dec1, Ror, Rev-erba, Bmal1, and Clock genes. The β-actin, Per2, Dec1, and Bmal1 primers were designed to cross exon-intron boundaries.
The primer sequences were as follows: mouse β-actin, 5'-TGACAGGAT-GCAGAAGGAGA-3', 5'-GCTGGAAAGGTGGAACATGGA-3' [these forward (5'-3') and reverse (3'-5') are here and below]; mouse Per2, 5'-TGTTGTGCTTACACGGGTGTCTCA-3', 5'-ACGTTTGGTTGGCCATGAA-3'; mouse Dec1, 5'-CCAGCGGGAAATAAAAC-3', 5'-TTCACTGGGCAAATGACTC-3'; mouse Rev-erba, 5'-CGTCTCAG-CTTCTTACGAC-3', 5'-GCAGCTGGAGAACAAGG-3', 5'-AGGGATATCTCAATGATGAC-3'; mouse Bmal1, 5'-CCACCTCAAGCCATATAACA-3', 5'-GACGAGTTTGAATTCACACCT-3'; mouse Clock, 5'-AAGATCTCGGTCGTGACAATC-3', 5'-TTCCTCTGTGACTGAGCT-3'. Real-time RT-PCR was performed under the following conditions: cDNA synthesis at 42°C for 15 min followed by 95°C for 2 min, PCR amplification for 40 cycles with denaturation at 95°C for 5 s, and annealing and extension at 60°C for 20 s. The relative levels of expression were normalized to that of 18S rRNA.

**Statistical Analysis.** All of the values are expressed as mean ± S.E.M. For statistical analyses, one- or two-way analysis of variance (ANOVA) was applied using StatView software (SAS Institute, Cary, NC). Tukey-Kramer’s test was used to compare the groups. A significant phase delay of the liver clock was observed, with a dependence on the injection time (F = 24.5, P < 0.01 for ANOVA; P < 0.01 versus control, Tukey-Kramer’s test; Fig. 1, C and D). Figure 1 shows representative data for raw (A) and detrended (B) liver bioluminescence rhythms. In this study, normalized PCR products from vehicle-treated mice were set at 100%. Data were analyzed by the 5-s Ct method. A melt-curve analysis then was performed to ensure no amplification of nonspecific products.

**Drugs and Agents.** 2,2,2-Tribromoethanol and 2-methyl-2-butanol, a vehicle for 2,2,2-tribromoethanol, were purchased from Sigma-Aldrich. Ethanol and 2,2,2-trimethoxacetic acid were purchased from Wako Pure Chemicals (Osaka, Japan). 2,2,2-Trichloroethanol and 2-methyl-2-butanol, a vehicle for 2,2,2-tribromoethanol, were purchased from Sigma-Aldrich. Isoflurane was inhaled at 2.5% (Abbott Laboratories, Abbott Park, IL). Propofol was obtained from Wako Pure Chemicals and dissolved in soybean oil.

**Statistical Analysis.** All of the values are expressed as mean ± S.E.M. For statistical analyses, one- or two-way analysis of variance (ANOVA) was applied using StatView software (SAS Institute, Cary, NC), and post hoc analysis was conducted with Tukey-Kramer’s test.

## Results

**Phase Delay of the Liver Bioluminescence Rhythm by 2,2,2-Tribromoethanol.** When we injected 2,2,2-tribromoethanol into Per2::Luc knockin mice for sham surgery at midday (around at ZT6), we incidentally found a phase delay of the liver clock by monitoring with a continuous bioluminescence recording system, a dish-type luminometer (Lumisphere). Multiple rounds of entraining stimulation can cause a strong reset of the circadian rhythm. For example, presentation of restricted feeding stimuli for 1 to 4 days during the daytime produces a phase advance of the liver clock, with a dependence on the restricted feeding days (Hirao et al., 2010). Therefore, we injected 2,2,2-tribromoethanol for 1, 2, or 4 days at ZT6. A significant phase delay of the liver clock was observed, with a dependence on the injection time (F = 19, P < 0.01 for ANOVA; P < 0.01 versus control, Tukey-Kramer’s test; Fig. 1, C and D). Figure 1 shows representative data for raw (A) and detrended (B) liver bioluminescence rhythms for a mouse injected with 2,2,2-tribromoethanol for 4 days. Injection of 2,2,2-tribromoethanol clearly induced a phase delay of the liver clock without affecting the free-running rhythm (20.43 ± 0.51 h [n = 4] for vehicle control; 20.54 ± 0.29 h [n = 5] for 2,2,2-tribromoethanol) (Table 1).

**Dose-Dependent and Phase-Dependent Phase Shifts of the Liver Bioluminescence Rhythm by 2,2,2-Tribromoethanol.** To elucidate the relation between the amplitude of the phase delay and the strength of anesthetic activity, we examined dose dependence. Both amplitudes of the phase delay (F = 24.5, P < 0.01 for ANOVA; P < 0.01 versus control, Tukey-Kramer’s test; Fig. 2A) and the anesthetic activity (F = 37.6, P < 0.01 for ANOVA; P < 0.01 versus control, Tukey-Kramer’s test; Fig. 2B) were significantly and dose dependently increased with an increasing dose of 2,2,2-tribromoethanol. In the next experiment, 240 mg/kg 2,2,2-tribromoethanol was injected at ZT2, ZT6, ZT10, ZT14, ZT18, or ZT22 for 2 days. Injections at ZT2, ZT6, or ZT10 caused a significant phase delay of the liver bioluminescence rhythm, and the injection at ZT14 had no effect. Whereas the injection at ZT18 or ZT22 produced a significant phase advance (Fig. 2, C and D).

**Structure-Specific or Anesthetic-Specific Effects on the Phase Delay of the Liver Bioluminescence Rhythm.** To understand whether phase shifts of the liver clock by 2,2,2-tribromoethanol are agent-specific, structure-specific, or anesthetic activity-specific, we prepared other chemicals to examine their activities with respect to the phase delay and...
injected these at ZT6 for 4 days (Fig. 3). Injection of 2,2,2-trichloroethanol (240 mg/kg) produced an anesthetic activity similar to that of 2,2,2-tribromoethanol (Table 1), and it caused a significant, but moderate, phase delay compared with that of 2,2,2-tribromoethanol ($P < 0.01$ versus control, Tukey-Kramer’s test). Chemicals with structures similar to that of 2,2,2-tribromoethanol (ethanol, 2-bromoethanol, and trichloroethanol 240 mg/kg) produced an anesthetic activity in the present ex vivo experiments, we ruled out the possibility that the phase delay of the liver clock (Fig. 3). In this experiment, we prepared two different vehicles (2-methyl-2-butanol and soybean oil) and did not observe any anesthetic activity or phase shifting effects (Fig. 3; Table 1).

Phase Shift of the SCN Bioluminescence Rhythm by 2,2,2-Tribromoethanol. In the present ex vivo experiments, we ruled out the possibility that the phase delay of liver Per2 expression rhythm by 2,2,2-tribromoethanol injection was caused indirectly by the phase delay of the SCN rhythm. We examined the phase of the SCN bioluminescence

### Table 1

**Effects of various compounds structurally or functionally related to 2,2,2-tribromoethanol on the free-running period of the bioluminescence rhythm in the liver and anesthetic activity**

<table>
<thead>
<tr>
<th>Dose</th>
<th>Free-Running Period (hour)</th>
<th>Loss of Righting Reflex (min)</th>
<th>p Value</th>
<th>$P$ Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>20.4 ± 0.52 (4)</td>
<td>0.01 ± 0.002 (6)</td>
<td>N.S.</td>
<td>**</td>
</tr>
<tr>
<td>2,2,2-Tribromoethanol</td>
<td>20.5 ± 0.29 (5)</td>
<td>58.5 ± 7.9 (5)</td>
<td>N.S.</td>
<td>**</td>
</tr>
<tr>
<td>2,2,2-Trichloroethanol</td>
<td>20.9 ± 0.35 (4)</td>
<td>66.3 ± 14.0 (5)</td>
<td>N.S.</td>
<td>**</td>
</tr>
<tr>
<td>2-Bromoethanol</td>
<td>20.1 ± 0.29 (4)</td>
<td>9.3 ± 0.6 (5)</td>
<td>N.S.</td>
<td>**</td>
</tr>
<tr>
<td>2,2,2-Tribromoacetic acid</td>
<td>20.6 ± 0.31 (4)</td>
<td>18.6 ± 2.0 (5)</td>
<td>N.S.</td>
<td>**</td>
</tr>
<tr>
<td>Ethanol</td>
<td>20.2 ± 0.24 (4)</td>
<td>0.01 ± 0.001 (5)</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Propofol</td>
<td>21.2 ± 0.10 (4)</td>
<td>17.3 ± 3.9 (5)</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Isoflurane (30 min)</td>
<td>2.5%</td>
<td>30 (4)</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Isoflurane (60 min)</td>
<td>2.5%</td>
<td>60 (4)</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>2-Methyl-2-butanol</td>
<td>0.4 mg/kg</td>
<td>1.9 ± 1.1 (5)</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
<tr>
<td>Soybean oil</td>
<td>1.7 mg/kg</td>
<td>0.01 ± 0.001 (5)</td>
<td>N.S.</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

N.S., not significant.

**P** $< 0.01$ versus control (Tukey-Kramer’s test).

![Graph A](image1)

**Graph A.** Control ○ 2,2,2-Tribromoethanol

![Graph B](image2)

**Graph B.** Loss of righting reflex (min) vs. dose (mg/kg).

![Graph C](image3)

**Graph C.** Effects of various compounds structurally or functionally related to 2,2,2-tribromoethanol on the free-running period of the bioluminescence rhythm in the liver and anesthetic activity.

![Graph D](image4)

**Graph D.** Dose-response effect (A and B) and ZT effect (C and D) on the 2,2,2-tribromoethanol-induced entrainment of the liver bioluminescence rhythms in mice. A. Per2::Luc knockin mice were injected with 2,2,2-tribromoethanol (24–240 mg/kg i.p.) at ZT6 for 4 days and then killed to obtain recordings of the liver bioluminescence rhythms. The amplitude of the phase delay was dose dependent. B, evaluation of the anesthetic activity of a single injection of 2,2,2-tribromoethanol (24–240 mg/kg i.p.) at ZT6. Time to recovery of the righting reflex (min) was measured after the injection. C, protocol for drug treatment. Per2::Luc knockin mice were injected with 2,2,2-tribromoethanol at each ZT for 2 days and then killed to obtain recordings of the liver bioluminescence rhythms. Horizontal white and black bars indicate the light period. ZTs of ZT0 and ZT12 were used as the lights-on and lights-off times, respectively, and pZT was used in the ex vivo experiments. Each arrow indicates each injection time. D, averaged phase shifts by 2,2,2-tribromoethanol injected at each ZT for 2 days. Open circles and closed circles denote control (saline) and 2,2,2-tribromoethanol treatments, respectively. The numbers in parentheses refer to the number of mice. *, $P < 0.05$; **, $P < 0.01$ versus control (Tukey-Kramer’s test).
rhythm after 2,2,2-tribromoethanol injection at ZT6 for 4 days (Fig. 4A). The phase was unaffected by 2,2,2-tribromoethanol injection, and there were no significant differences in the phase of the SCN rhythm between the saline injection group and the 2,2,2-tribromoethanol injection group ($P < 0.05$ versus control, Tukey-Kramer's test; Fig. 4B). In Fig. 4C, summary data are shown.

No Phase Shift of the Locomotor Activity in Mice Treated with 2,2,2-Tribromoethanol. The locomotor activity rhythm is controlled by the SCN-dependent circadian rhythm. Similar to our finding that 2,2,2-tribromoethanol failed to cause a phase delay of the SCN rhythm, we confirmed that 4 days of injection of 2,2,2-tribromoethanol did not induce a phase delay of the locomotor activity rhythms. In Fig. 5, A and B, 240 mg/kg 2,2,2-tribromoethanol was injected at ZT6 for 4 days under LD conditions, and then mice were placed into the DD condition to examine the phase angle of the initiation of locomotor activity. Injection of vehicle or 2,2,2-tribromoethanol exerted no change on the phase angle of locomotor activity ($P < 0.05$ versus control, Tukey-Kramer's test; Fig. 5C).

Acute Change of Clock Gene Expression in the Livers of Mice Treated with a Single Injection of 2,2,2-Tribromoethanol. Although the anesthetic activities of 2,2,2-tribromoethanol and isoflurane were similar (Table 1), the phase delay effect differed between them. Responses of
clock gene expression to 2,2,2-tribromoethanol or isoflurane may differ between them. Clock and Bmal1 gene expression was decreased significantly 1 and 4 h after the injection of 2,2,2-tribromoethanol (240 mg/kg) \(P < 0.01 \text{ versus control, Tukey-Kramer's test; Fig. 6, but not by isoflurane (60 min), compared with that of the control (saline injection). Rev-erba gene expression also was attenuated strongly by 2,2,2-tribromoethanol \(P < 0.01 \text{ versus control for 1 h, } P < 0.05 \text{ versus control for 4 h, Tukey-Kramer's test) and moderately by isoflurane. Expression of Per2 and Dec1 was basically unaffected by 2,2,2-tribromoethanol or isoflurane.}

**Discussion**

In the present study, we found that the intraperitoneal injection of 2,2,2-tribromoethanol at ZT2–ZT10 or at ZT18–ZT22 induced a phase delay or a phase advance, respectively, of the Per2 gene expression rhythm. A 2,2,2-tribromoethanol-induced phase delay of the Per2 gene expression rhythm exhibited dose dependence and injection time dependence. Ohe et al. (2011) recently demonstrated that the inhalation of sevoflurane at ZT3 phase-delayed the mouse activity rhythm. Thus, general anesthetics may induce a phase delay of circadian rhythms when these agents are administered during the daytime. However, Dispersyn et al. (2009) reported that the administration of propofol (120 mg/kg i.p.) at ZT6, ZT10, or ZT16 phase-advances the core body temperature rhythm and the rest-activity rhythm in rats. The duration of anesthesia was between 25 and 30 min. In the present study, we demonstrated that the administration of propofol (300 mg/kg i.p.) at ZT6 did not induce phase shifts of the Per2 gene expression rhythm, but the anesthesia duration was compatible \(17.3 \pm 3.9, n = 4\). The discrepancy between the present results and those of Dispersyn et al. (2009) might be explained by species differences.

Propofol, GABA agonist, exerts an inhibitory effect on Per1 and Per2 gene expression in the SCN and advances the circadian rhythm (Ehlen et al., 2006; Legan et al., 2009). The benzodiazepine midazolam has been used as an intravenous anesthetic, and the mechanisms of action of benzodiazepines and general anesthetics may be similar with respect to circadian rhythm. We reported previously that brotizolam induces a phase advance of the activity rhythms in hamsters when injected during the daytime and that it decreased the circadian rhythm. We reported previously that brotizolam induces a phase advance in the previous study, and Per2 expression was decreased by brotizolam but was unaffected by 2,2,2-tribromoethanol. Thus, the activation of benzodiazepine receptors does not participate in the mechanism of 2,2,2-tribromoethanol. It has been reported that ethanol suppresses photic and serotonergic circadian clock phase resetting in mice (Ruby et al., 2009; Brager et al., 2011). However, the phase delay of the liver Per2 gene rhythm by 2,2,2-tribromoethanol is not caused by the suppression of photic exposure at ZT2–ZT10, because the disruption of photic signals during the daytime produces a phase advance but not a phase delay (Shibata et al., 2010). Phase resetting of the liver Per2 gene by 2,2,2-tribromoethanol is a specific phenomenon and is not associated with benzodiazepines or ethanol.

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**Fig. 5.** No phase shift of the mouse locomotor activity rhythm induced by daily injections of 2,2,2-tribromoethanol for 4 days. Per2::Luc knockin mice were injected with saline (control) or 2,2,2-tribromoethanol (240 mg/kg i.p.) for 4 days under LD conditions, and then these mice were placed into the DD condition for 10 days or more. A and B, representative examples of double-plotted actograms for the saline group or drug group, respectively. Horizontal white and black bars indicate the light period. ZT0 indicates lights-on, and ZT12 indicates lights-off. Arrows indicate the injection time (ZT6) for 4 days under the LD cycle. Two straight solid lines in each actogram indicate the onset of locomotor activity under LD and DD conditions, respectively. C, averaged phase angles (h) between the onset of locomotor activity under LD and DD conditions. Numbers in parentheses refer to the number of mice. No significant difference was observed between the two groups (Tukey-Kramer’s test).
2,2,2-Trichloroethanol induced an anesthetic effect similar to that of 2,2,2-tribromoethanol; however, 2,2,2-trichloroethanol produced a relatively weak but significant phase delay compared with that of 2,2,2-tribromoethanol. It is known that 2,2,2-trichloroethanol is a major metabolite of chloral hydrate, which is used as a general anesthetic. A dose of 120 mg/kg 2,2,2-tribromoethanol resulted in a weak anesthetic effect but a significant phase delay of the liver rhythm. Taken together, these data suggest that the phase shifting effect of 2,2,2-tribromoethanol or 2,2,2-trichloroethanol is independent of the anesthetic effect of these chemicals.

When we further examined the effects of structurally similar compounds, such as 2-bromoethanol, 2,2,2-tribromoacetic acid, and ethanol, on the circadian rhythm of the liver clock, none of these agents affected the phase of the Per2 rhythm. Although the detailed mechanism is not known, the structural specificities of 2,2,2-tribromoethanol and 2,2,2-trichloroethanol may be involved in the phase shifts of the liver clock. The tribromide structure appears to be important for phase shifting compared with the trichloride structure. Tribromide and/or trichloride structures are also important for phase shifting compared with the monobromide structure.

In the present study, the injection of 2,2,2-tribromoethanol induced a small but significant phase delay when administered as a single injection. However, 2- or 4-day injections dramatically phase-delayed the Per2 bioluminescence rhythm. Therefore, the administration of 2,2,2-tribromoethanol several times at fixed times, rather than as a single dose, may disturb the peripheral circadian rhythm.

Although the inhalation of isoflurane (2.5%) maintained an anesthetic effect during the inhalation period (30 or 60 min), isoflurane slightly phase-delayed the liver Per2 rhythm in the present study. Sakamoto et al. (2005) performed comprehensive gene expression profiling in mice in response to the inhalation of sevoflurane and found that circadian clock gene expression, such as that of Per2, Cry2, Per3, Bmal1, Clock, Rev-erba, Dec1, Dbp, and E4bp4, was affected strongly in the liver, heart, lung, and kidney but was moderately affected in the spleen and only slightly affected in the brain. It is believed that general anesthetic-induced decreases in Per2 gene expression in the brain, including the SCN, may cause phase shifts in behavioral rhythms and body temperature rhythms, because these circadian rhythms are controlled by the central nervous system. Gene expression levels of Bmal1 and Rev-erba are strongly down- and up-regulated in the liver by sevoflurane inhalation, respectively (Sakamoto et al., 2005). Thus, Sakamoto et al. (2005) suggest that not only the brain and SCN clocks but also peripheral clocks are pharmacologic targets for general anesthetics. We demonstrated recently that an acute increase in Per2 gene expression in the liver may be related to refeeding- and insulin-induced phase shifts (Hirao et al., 2010; Tahara et al., 2011). In the present study, Rev-erba, Bmal1, and Clock, but not Per2, gene expression was decreased strongly by 2,2,2-tribromoethanol.

![Fig. 6. Effect of a single administration of 2,2,2-tribromoethanol or isoflurane on clock gene expression in the liver. Mice were injected with 2,2,2-tribromoethanol (240 mg/kg) or inhaled isoflurane (2.5%, 60 min) at ZT6 and were killed 1 or 4 h after the administration. Expression of clock genes then was measured. Relative levels of each target gene PCR product were normalized to that of β-actin, and gene expression level in control mice 1 h after administration was set at 100%. * P < 0.05; ** P < 0.01 versus vehicle-treated mice (control, saline injection) (Tukey-Kramer’s test).](https://jpet.aspetjournals.org/content/10/10/704/F6)
Administration of sevoflurane does not affect Per2 gene expression in the liver (Sakamoto et al., 2005). Thus, phase shifts of the liver clock by 2,2,2-tribromoethanol may be explained by acute changes in the expression levels of Rev-erba, Bmal1, and Clock, but not the Per2 gene. Further experiments are necessary to elucidate the mechanisms of up- and down-regulation of each clock gene.

In contrast to the liver clock, the phase of the Per2 gene expression rhythm in the SCN was unaffected by 4-day treatment with 2,2,2-tribromoethanol. In the present locomotor rhythm experiments, intraperitoneal injection of 2,2,2-tribromoethanol for 4 days, followed by the placement of the mice into the DD condition, did not affect the phase of the locomotor activity rhythm. These data suggest that 2,2,2-tribromoethanol may not affect SCN-dependent circadian rhythms; a phase shift of the liver clock by 2,2,2-tribromoethanol is independent of the SCN clock.

In the present study, we can divide general anesthetics into two categories, one being phase-resetting and the other being non-phase-resetting. The former group includes 2,2,2-tribromoethanol and 2,2,2-trichloroethanol, and the latter includes propofol and isoflurane. Although 2,2,2-tribromoethanol is a popular anesthetic in veterinary medicine and for animal experiments, it may be better to select other general anesthetics to avoid side effects such as circadian rhythm disorders of peripheral clocks. Propofol and isoflurane are popular anesthetics in human medicine, and effects on peripheral clocks may not occur.

Authorship Contributions

Participated in research design: Shibata.
Conducted experiments: Kubo and Hiroa.
Contributed new reagents or analytic tools: Kubo and Hiroa.
Performed data analysis: Kubo and Hiroa.
Wrote or contributed to the writing of the manuscript: Shibata.

References


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