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Effect of General Anaesthesia on Circadian Rhythms in Mice

Alma Orts-Sebastian
Department of Anaesthesiology.
University of Auckland

PhD Supervisor: Associate Professor Guy Warman
Co-supervisor: Dr James Cheeseman
Advisor: Dr Raewyn Poulsen
Abstract

After GA (general anaesthesia), patients often report symptoms of circadian misalignment that can last up to five days. Animal studies have now demonstrated that GA may be, at least in part, responsible for these effects, causing time-dependent shifts on circadian rhythms of behaviour, physiology, and cognitive parameters. However, due to the lack of consistency in the study design between experiments, the specific effect of GA on circadian rhythms in mammals, and the mechanism by which this occurs, is still unclear.

In this thesis, I sought to investigate the time-dependent effect of GA on behavioural rhythms in mice (C57BL/6VJU), with two of the most commonly used halogenated inhalational anaesthetic agents (isoflurane and sevoflurane), administered at different times of the day (circadian times, CTs), over a period of 24 hours. To do so, I collected data to construct a full phase response curve (PRC) for each anaesthetic agent. Animals received either a six-hour GA treatment or a 10-minutes GA treatment (control group) with isoflurane (1.5%) or sevoflurane (2.6%). 80 mice were treated with isoflurane for six hours and 78 mice were treated with isoflurane for 10 minutes. For the sevoflurane PRC, 75 animals were treated with sevoflurane for six hours and 24 mice were treated with sevoflurane for 10 minutes. The two anaesthetic agents produced (weak) type one PRCs. The results showed that after a six-hour treatment with isoflurane mice locomotor activity rhythms were delayed 1.11 hours (on average), when administered between CTs 8-12, with a maximum phase delay of 1.69 hours observed at CT 11.9. Six hours of sevoflurane administration phase delayed mice wheel running rhythms with 0.54 hours (on average) between CTs 6-11. A maximum phase delay after of 1.45 hours was observed at CT 8. No significant phase shifts were observed on mice circadian rhythms of locomotor activity after the control treatment (10 minutes of GA), at any time, with any of the anaesthetic agents studied. These results suggest that the effect of GA on the clock is time-dependent. No evidence of a change in the fundamental period of the mouse clock (tau) was found after anaesthesia, which suggests that the effect of GA on the clock is mediated by non-parametric phase shifting rather than parametric or continuous shifting.
I also investigated the combined effect of GA and light on mice circadian rhythms of locomotor activity. However, due to the time limitation of my PhD research, only two time points (CTs) were investigated for isoflurane and one for sevoflurane. Animals received either a light pulse (400 lux LED light source) for four hours or the same light while anaesthetised with either isoflurane (1.5%) or sevoflurane (2.6%).

Isoflurane administered with light between CTs 14-17 blocked the phase-shifting effect of light. The phase shift evoked by the concomitant treatment (light and isoflurane) between CTs 14-17 was 0.13±0.40 hours (n=5). However, when isoflurane and light were administered between CTs 7-11, the average of the induced phase delay in behavioural rhythms was 2.18±0.21 hours (n=12), showing that isoflurane was not blocking the effect of light on the clock. On the other hand, sevoflurane reduced the phase delay evoked by light when light and sevoflurane were administered together between CTs 14-17 but did not completely block it. The sevoflurane plus light-induced phase delay between CTs 14-17 was 0.61±0.3 hours (n=11).

These results suggest that GA does not simply pharmacologically block the effect of light on the clock at all circadian times as was previously thought, but more that the effect of GA and light on the clock depends on the circadian phase of the administration. Further studies need to be done to construct a full light and anaesthesia PRC for both anaesthetic agents in order to determine whether light could be used to reduce the anaesthesia-induced phase shift on circadian rhythms in mammals, as it does in invertebrates.
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<th>Description</th>
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<tr>
<td>Anaesthesia</td>
<td>Derived from the Greek “an” (without) and “aesthesia” (sensation).</td>
</tr>
<tr>
<td>Chi-squared (periodogram)</td>
<td>An implementation of the Enright periodogram (A mathematical procedure for the determination of periodicity in time series with equally-spaced data points) that utilises the chi-square distribution to determine the statistical significance of spectral power.</td>
</tr>
<tr>
<td>Circadian</td>
<td>Occurring or functioning in cycles of approximately 24 hours.</td>
</tr>
<tr>
<td>Circadian clock</td>
<td>An internal self–sustaining oscillator with a free–running period of approximately 24 hours which drives daily rhythms.</td>
</tr>
<tr>
<td>Circadian time</td>
<td>A standard time based on the free–running period of the rhythm (oscillation). The onset of activity by convention in diurnal animals is circadian time 0 (CT 0), whereas in nocturnal animals is time 12 (CT 12).</td>
</tr>
<tr>
<td>Circadian rhythms</td>
<td>Events that occur regularly every 24 hours.</td>
</tr>
<tr>
<td>Constant conditions</td>
<td>A research protocol intended to unmask the endogenous rhythmicity of the subject and where all time giving influences are removed.</td>
</tr>
</tbody>
</table>

**List of terms**
EC50  The concentration of a compound where 50% of its maximal effect is observed.

ED50  The effective dose for 50% of patients who are administered it.

Endogenous  Originating within an organism.

Endogenous rhythm  A periodically repeated sequence of events. It free–runs in the absence of temporal cues.

Entrainment  The synchronisation of the endogenous clock to an external cyclic geophysical cue (zeitgeber).

Exogenous  Originating from outside an organism.

Free–running period  The period of an endogenous oscillation (rhythm) revealed in constant conditions.

F6  1,2-dichlorohexafluorocyclobutane. An inhalational non–immobiliser that causes amnesia.

Isoflurane  2-chloro-2-(difluoromethoxy)-1,1,1-trifluoro-ethane. An inhalational anaesthetic that causes loss of consciousness.

Lipophilicity  The ability of a chemical compound to dissolve in fats, oils, lipids and non-polar solvents.

LD50  The median lethal dose required for death to occur in 50% of patients after a certain test duration.
| **Oscillator** | An entity capable of generating a periodic variation in the value of a physical or logical quantity, especially a regular variation above and below some mean value. |
| **Period** | The time to complete one full cycle of an oscillation. |
| **Phase** | Instantaneous state of an oscillation within a period. |
| **Phase Response Curve** | Graphical description of how the magnitude of phase shifts induced by single stimuli depends on the phase at which the stimuli are presented. |
| **Phase shift** | A discrete displacement of an oscillation along the time axis. |
| **Photoperiod** | Light-dark cycles that vary in the relative duration of the light. |
| **Process C** | Circadian sleep. Processes that are almost entirely independent of behavioural state. |
| **Process S** | Sleep homeostasis. Processes that are almost entirely dependent on the behavioural state, like the need for sleep. |
| **Process W** | Sleep inertia. Describes the time it takes after awakening to become fully alert. |
| **Rhythm** | Recurrence of an event at more or less regular intervals. |
| **Zeitgeber (time-giver)** | A stimulus capable of resetting the pacemaker or synchronising a self-sustaining oscillation. |
# List of abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>ANOVA</td>
<td>Analysis of variance</td>
</tr>
<tr>
<td>ARNTL</td>
<td>Aryl hydrocarbon receptor nuclear translocator-like protein</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BMAL1</td>
<td>Brain and muscle ARNT like-1</td>
</tr>
<tr>
<td>CaMK2</td>
<td>Calcium/calmodulin kinase 2</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CI</td>
<td>Confidence interval</td>
</tr>
<tr>
<td>CLOCK</td>
<td>Circadian locomotor output kaput</td>
</tr>
<tr>
<td>CT</td>
<td>Circadian time</td>
</tr>
<tr>
<td>CNS</td>
<td>Central nervous system</td>
</tr>
<tr>
<td>CREB</td>
<td>Component response element binding protein</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
</tr>
<tr>
<td>--------------</td>
<td>------------</td>
</tr>
<tr>
<td>CRTC1</td>
<td>CREB regulated transcription co-activator 1</td>
</tr>
<tr>
<td>Cry</td>
<td>Cryptochrome gene</td>
</tr>
<tr>
<td>CRY</td>
<td>Cryptochrome protein</td>
</tr>
<tr>
<td>DBP</td>
<td>D box binding protein</td>
</tr>
<tr>
<td>DD</td>
<td>Constant darkness</td>
</tr>
<tr>
<td>DMH</td>
<td>Dorsomedial hypothalamic nucleus</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EEG</td>
<td>Electroencephalogram</td>
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<tr>
<td>FEO</td>
<td>Food entrainable oscillator</td>
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<tr>
<td>FRP</td>
<td>Free running period</td>
</tr>
<tr>
<td>GA</td>
<td>General anaesthesia</td>
</tr>
<tr>
<td>GABA</td>
<td>γ (gamma) aminobutyric acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
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<tr>
<td>GHT</td>
<td>Geniculohypothalamic tract</td>
</tr>
<tr>
<td>GSK3β</td>
<td>Glycogen synthase kinase 3β</td>
</tr>
<tr>
<td>HCN</td>
<td>Hyperpolarization-activated cyclic-nucleotide-gated</td>
</tr>
<tr>
<td>HLF</td>
<td>Hepatic leukaemia factor</td>
</tr>
<tr>
<td>IGL</td>
<td>Intergeniculate leaflet</td>
</tr>
<tr>
<td>IR</td>
<td>Infrared</td>
</tr>
<tr>
<td>I.P.</td>
<td>Intraperitoneal</td>
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<tr>
<td>I.V.</td>
<td>Intravenous</td>
</tr>
<tr>
<td>LD</td>
<td>Light-dark</td>
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<tr>
<td>LED</td>
<td>Light emitting diode</td>
</tr>
<tr>
<td>LP</td>
<td>Light pulse</td>
</tr>
<tr>
<td>MAC</td>
<td>Minimum alveolar concentration</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Definition</td>
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<td>--------------</td>
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<tr>
<td>MASCO</td>
<td>Methamphetamine-sensitive circadian oscillator</td>
</tr>
<tr>
<td>MWM</td>
<td>Morris water maze</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NSAID</td>
<td>Non-steroidal anti-inflammatory drug</td>
</tr>
<tr>
<td>nACh</td>
<td>Nicotinic acetylcholine</td>
</tr>
<tr>
<td>NFIL3</td>
<td>Nuclear factor, interleukin 3 regulated</td>
</tr>
<tr>
<td>NMDA</td>
<td>N-Methyl-D-aspartic acid</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NPY</td>
<td>Neuropeptide Y</td>
</tr>
<tr>
<td>N-REM</td>
<td>Non-rapid eye movement</td>
</tr>
<tr>
<td>PACAP</td>
<td>Pituitary adenylate cyclase activating polypeptide</td>
</tr>
<tr>
<td>PAR-bZip</td>
<td>Proline and acidic amino acid-rich basic leucine zipper</td>
</tr>
</tbody>
</table>
PD  Pharmacodynamics

PER  Period protein

PICO  Palatable meal-inducible circadian oscillator

PK  Pharmacokinetics

P.O.  Post-operative

PRC  Phase response curve

Per  Period gene

PER  Period protein

REM  Rapid eye movement

RORα  Retinoic acid-related orphan receptor α

RNA  Ribonucleic acid

RW  Running wheel
<table>
<thead>
<tr>
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<th>Description</th>
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<tbody>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>SCN</td>
<td>Suprachiasmatic nucleus</td>
</tr>
<tr>
<td>SCN-X</td>
<td>Suprachiasmatic nucleus lesioned</td>
</tr>
<tr>
<td>SIK1</td>
<td>Salt inducible kinase 1</td>
</tr>
<tr>
<td>SWD</td>
<td>Shift work disorder</td>
</tr>
<tr>
<td>SWS</td>
<td>Slow wave sleep</td>
</tr>
<tr>
<td>TEF</td>
<td>Thyrotrophic embryonic factor</td>
</tr>
<tr>
<td>VLPO</td>
<td>Ventrolateral preoptic area</td>
</tr>
<tr>
<td>WICO</td>
<td>Wheel-inducible circadian oscillator</td>
</tr>
<tr>
<td>$\chi^2$</td>
<td>Chi squared</td>
</tr>
<tr>
<td>$\tau$</td>
<td>$\tau$. Fundamental period of the clock</td>
</tr>
<tr>
<td>$\Delta \tau$</td>
<td>$\tau$ increment ($\tau$ after-$\tau$ before)</td>
</tr>
<tr>
<td>5-HT</td>
<td>Serotonin</td>
</tr>
<tr>
<td>------------</td>
<td>----------------------------</td>
</tr>
<tr>
<td>6-OHMS</td>
<td>6-hydroxymelatonin sulphate</td>
</tr>
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Co-Authorship Form

This form is to accompany the submission of any PhD that contains published or unpublished co-authored work. Please include one copy of this form for each co-authored work. Completed forms should be included in all copies of your thesis submitted for examination and library deposit (including digital deposit), following your thesis Acknowledgements. Co-authored works may be included in a thesis if the candidate has written all or the majority of the text and had their contribution confirmed by all co-authors as not less than 65%.

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Chapter one, General introduction; Section 1.1; Page 20. Section 1.4.6.2 Effects of general anaesthesia on animal studies; Pages 47 to 55 including Table 1.5. Chapter 4: Section 4.6 Potential clinical implications and future directions; Page 123-127.

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<tr>
<td>First author. Undertook review of literature and prepared manuscript</td>
<td>75%</td>
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CO-AUTHORS

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<tr>
<th>Name</th>
<th>Nature of Contribution</th>
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<tr>
<td>Guy Warmen</td>
<td>Writing and editorial support</td>
</tr>
<tr>
<td>James Cheeseman</td>
<td>Writing and editorial support</td>
</tr>
<tr>
<td>Mathew Pawley</td>
<td>Writing and editorial support</td>
</tr>
<tr>
<td>Nicola Ludin</td>
<td>Writing and editorial support</td>
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Certification by Co-Authors

The undersigned hereby certify that:

- the above statement correctly reflects the nature and extent of the PhD candidate’s contribution to this work, and the nature of the contribution of each of the co-authors; and
- that the candidate wrote all or the majority of the text.

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<th>Name</th>
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<td></td>
<td>27/02/2019</td>
</tr>
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<td></td>
<td>1/03/2019</td>
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<tr>
<td>Nicola Ludin</td>
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I. Chapter one

1. General introduction to sleep, circadian rhythms, and GA

1.1 General introduction

The aim of the work described in this thesis was to isolate and examine the effect of GA on the circadian clock.

Despite GA being a widely used tool to enable surgery in humans and animals, its mode of action is still poorly understood. There is now convincing evidence that GA can cause profound and strongly time-dependent shifts on circadian rhythms of behaviour (sleep-wake cycles), physiology (core body temperature, blood pressure, heart rate and hormone release), and cognitive parameters (learning and memory) in a range of species. Nonetheless, our understanding of how circadian clocks interact with GA is still largely unknown.

This chapter provides a literature review of the relevant topics such as sleep, circadian rhythms, anaesthetic agents, and the effect of GA on the clock.

1.2 Description of sleep

Sleep is a vital characteristic in the animal kingdom. In general, animals spend at least half of their life asleep, and humans spend nearly a third of our lives sleeping (Hattar et al., 2006; Saper et al., 2010). However, very little is known about the sleep function. We know sleep is vital. Prolonged sleep deprivation produces metabolic, immune and thermal dysregulation, ultimately leading to death (Rechtschaffen et al., 1989). We also know sleep
is rhythmic. Some animals sleep at night, others sleep during the day; nevertheless, we all sleep.

Sleep is defined as a necessary reversible behavioural state of perceptual disengagement from, and unresponsiveness to, the environment (Carskadon et al., 2005). This means that sleep is not simply the absence of waking, but a special activity of the brain controlled by elaborate and precise mechanisms.

Before the discovery of rapid eye movement (REM) sleep in the 1950s, sleep was considered a passive, quiescent part of our daily lives. No real distinction was described between sleep and other states of quiescence such as coma, anaesthesia, or hibernation. However, we now know that different areas of our brains are active during sleep (Saper et al., 2005b), such as the nuclei of the hypothalamus and the brain stem or basal forebrain (Brown et al., 2010).

In mammals, there are two different types of sleep: rapid eye movement (REM) sleep and non-rapid eye movement (N-REM) sleep. N-REM sleep is further divided into four stages (from S1 to S4), each corresponding to an increasing depth of sleep. Sleep starts at S1 and deepens via S2 and S3 to S4, then proceeds to REM sleep. After the REM sleep period, the cycle starts from the beginning. The duration of one sleep cycle is about 90 minutes. Stage 2 of N-REM sleep is characterised by slow oscillation, sleep spindles, and high amplitude evoked potential (K-complex) waveforms. Low frequency (“synchronized”) brain waves dominate the deepest stages of sleep expressed in stages 3 and 4, which are characterised by delta waves. This is also called slow-wave-sleep (SWS) (Pace-Schott et al., 2002) (Figure 1.1)
Figure 1.1: Electroencephalogram tracings of the four stages of non–rapid eye movement sleep (figure adapted from (Carskadon et al., 2011)).

N-REM sleep is also characterised by minimal psychological activity (Carskadon et al., 2011) accompanied by waxing and waning muscle tone, low body temperature, and low heart rate (Brown et al., 2010).

On the other hand, brain activity is increased during REM sleep, which is associated with dreaming. This type of sleep is characterised by muscle atonia mediated by the inhibition of spinal motor neurons by brainstem mechanisms, and by cardiorespiratory irregularities. N-REM and REM sleep alternate in each of five cycles (stages I to IV plus REM) that occur over the course of the night of adult human sleep (four N-REM and one REM sleep). N-REM sleep timing is longer than REM sleep and mediates the onset of sleep (Carskadon et al., 2011). The sleep stages calculated across the night are often presented as a hypnogram, which describes the order and duration of each sleep stage (Figure 1.2).
1.2.1 Mechanism of sleep

Sleep has a profound effect on a number of physiological processes, such as body temperature, heart rate, cortisol, melatonin, and growth hormone levels. Humans require about six to seven hours of sleep per day to be well rested and functional (Dijk et al., 2005). Reducing nocturnal sleep increases physiological sleepiness the next day. If this reduction continues for successive nights, the need for sleep accumulates creating a sleep debt which is recouped only by sleeping (Dinges et al., 1997).

Different models have been developed to explain how sleep works. Such models distinguish between processes that are almost entirely dependent on the behavioural state, like the need for sleep, commonly called process S (sleep homeostasis); and processes that are almost entirely independent of the behavioural state, so-called circadian process C (the circadian clock) (Borbely, 1982). To explain the dynamics between these different processes, a third process was defined. Sleep inertia, or process W, which describes the time it takes after awakening to become fully alert (Akerstedt et al., 1995; Åkerstedt et al., 2004).

The most important interactions between these processes occur at the moments where the need for sleep reaches the upper threshold, transferring from waking to sleep, and the lower
threshold, changing from sleep to waking. Thus, the sleep homeostat can be explained as a sleep tank. The tank is drained with long periods of wakefulness, increasing the propensity to sleep (Dijk et al., 1994), and the circadian control of sleep is like an alarm clock that tells you when you can sleep and when you should be awake. Homeostasis maintains the duration and intensity of sleep, while the circadian rhythm determines the timing of the propensity for sleep (Deboer, 2018; Dement, 1999).

1.2.1.1 Mechanism of homeostatic control of sleep
A few theories have been proposed to explain the mechanisms underlying the homeostatic control of sleep. During long periods of wakefulness, more energy is consumed. At a molecular level, this corresponds to the breakdown of adenosine triphosphate (ATP) to adenosine. Adenosine has been proposed as a promoter of short-wave sleep. According to this theory, adenosine is the molecule that connects “energy balance to sleep regulation” (Benington et al., 1995). Another molecule, nitric oxide (NO), has also been related to energy metabolism and the homeostatic control of sleep. It appears that NO concentration increases after prolonged periods of wakefulness, promoting the increase in cellular adenosine concentration and contributing to homeostatic control (Porkka-Heiskanen et al., 2013). However it is unlikely that this explains the entire process of sleep as adenosine concentrations do not increase during prolonged wakefulness (6 hours) in regions important in behavioural state control (Strecker et al., 2000), and the homeostatic sleep drive persists even in the absence of adenosine receptors (Saper et al., 2010).

The ventrolateral preoptic area (VLPO) of the brain has also been directly linked with the promotion of sleep. The VLPO consists of two sub-regions that promote N-REM and REM sleep respectively. It also participates in the ascending and descending arousal systems that
produce wakefulness and sleep. This system is partially regulated by orexin/hypocretin secretion in the lateral hypothalamus (Pace-Schott et al., 2002). These neuropeptides were first discovered in 1998 and were identified as the cause of narcolepsy (Saper et al., 2005a), a disabling sleep disorder characterised by spontaneous daytime sleepiness and abnormal manifestations of REM sleep (Peyron et al., 2000). Orexin-sensitive neurons are mainly active during wakefulness. They have ascending projections to the cerebral cortex (ascendant arousal system), as well as descending projections to all the monoaminergic and cholinergic cell groups of the arousal systems. The VLPO and orexin/hypocretin system are critical mechanisms in regulating the wake-sleep system (Gvilia, 2010). Thus, multiple neuronal dynamics are likely to occur in the homeostatic regulation of sleep.

1.2.1.2 Mechanism of the circadian control of sleep

In humans and other mammals it is now well accepted that the circadian pacemaker is located in the suprachiasmatic nucleus (SCN) of the hypothalamus, and (in part) controls the sleep-wake cycle (Deboer, 2018). The SCN receives timing information from light-dark cycles by photoreceptors located exclusively within the retina of the eye. These photosensitive retinal ganglion cells, through the photopigment melanopsin, provide the primary light input to the SCN via the optic nerve and the retinohypothalamic tract (see section 1.3.3 Clocks throughout the body). Once the central pacemaker receives this input, it integrates the information and generates rhythms that will affect almost all cells in the body (Cuninkova et al., 2008; Jagannath et al., 2013). In this way, under normal conditions, the pacemaker is continuously adjusted to an external 24-hour daily rhythm.

Humans are a diurnal species; we sleep mostly at night and we do so at approximately 24-hour intervals. When we do not adhere to this pattern, for example when travelling across
time zones (jet-lag) or during shift work (Costa, 1996; DuPont, 2008), we experience a disruption of the circadian clock which manifests primarily as sleep disruption.

1.2.2 Consequences of disrupted sleep
Sleep as a rhythmic behaviour consists of an endogenous component driven by the internal clock and an exogenous component driven by lifestyle and environment. There is an intimate relationship between the circadian rhythm that controls an organism’s temporal programming and the sleep process. This means that environmental factors and lifestyle, stress, presence of other animals (in the animal kingdom), and health condition, all have their own influence on sleeping patterns (Beersma et al., 2007).

Sleep deprivation has been shown to have detrimental effects on coordination (Kahol et al., 2008), cognition, mood (Pilcher et al., 1996), health (Spiegel et al., 1999), and to alter the effects of drugs in an organism (Clement et al., 2008; Tufik et al., 1978). Performance during a period of sleep loss is directly dependent on the length of time awake and the circadian time, with longer periods of sleep loss resulting in worse consequences (Van Dongen et al., 2003). However, while the circadian and sleep loss effects on neurobehavioural performance are well established (Maire et al., 2018), less is known about how the systems interact and alter waking neurocognitive functions (Durmer et al., 2005). Given that the temporal regulation of sleep is governed by an interplay of homeostatic and circadian processes, the regulatory mechanisms of sleep could potentially serve to protect human neurobehavioural functions from degradation due to excessive wakefulness within and between circadian cycles (Van Dongen et al., 2003). It would be reasonable to think that there is a strong connection between sleep, circadian rhythms, and human health.
Sleep deprivation increases the risk of human-error related accidents (Doran et al., 2001). Published evidence suggests that long-term work shifts significantly increase fatigue and impair performance. Medical residents in hospitals traditionally work shifts of 24 to 30 consecutive hours that unquestionably increases the risk of serious medical errors and diagnostic mistakes, and have been shown in a national cohort study to increase the risk of harmful and fatal medical errors, besides detrimental effects to their own health (Lockley et al., 2007).

The overall prevalence of insufficient sleep in adults has been estimated at 20% (Durmer et al., 2005). Sleep deprivation has been shown to produce psychomotor impairments equivalent to those induced by alcohol consumption (Dawson et al., 1997), which are in turn aggravated by increased alcohol consumption (Roehrs et al., 2001). The key players in arousal regulation and motor response control under high sleep pressure conditions are located in subcortical structures, including the bilateral thalamus and the striatum. The effects of sleep loss on attention suggests that increased thalamic activity reflects a complex mutual interplay between the effects of sleep loss and the engagement in the assigned task. The role of the higher subcortical activity could be to provide a compensatory mechanism for the adverse circadian phase and high sleep pressure (Maire et al., 2018).

At a cognitive level, sleep deprivation produces involuntary microsleeps (impairment of cognitive performance due to a state of instability caused by competing sleep initiating and endogenous wake promoting factors (Arnal et al., 2015)). High attention performance is inconstant with spread errors of omission and commission. Response time slows, and both short-term recall and working memory performances decline (Durmer et al., 2005). Several studies have now shown that sleep deprivation can reduce spine density and attenuate...
synaptic efficacy in the hippocampus, which is critical for memory formation (Raven et al., 2018). Learning (acquisition) decreases and performance requiring divergent thinking deteriorates (Durmer et al., 2005).

Chronic loss of physiological sleep has been reported to adversely affect endocrine function, cardiovascular events (Van Dongen et al., 2003), and other health-related outcomes such as insulin resistance, diabetes, cancer, gastrointestinal disease, and pregnancy complications (Knutsson, 2003). Mood disorders, fatigue, and depression are also characteristic of sleep deprivation (Dinges et al., 1997).

1.3 Introduction to circadian rhythms and the circadian clock

1.3.1 An overview of circadian rhythms

Circadian rhythms orchestrate life. The term circadian was first introduced by Franz Halberg in the 1950s to describe an event that repeats itself with a period of approximately 24 hours. A rhythm is defined as the recurrence of an event at more or less regular intervals (Aschoff, 1981). Circadian rhythms (circa, about; dies, day) are therefore, events that occur regularly every 24 hours.

Circadian rhythms are endogenously generated and self-sustained (i.e. they free run) in the absence of environmental time cues (zeitgeber) over a period (τ) similar to, but seldom exactly, 24 hours (Schwartz et al., 1990b). They are synchronised to 24 hours on a daily basis by periodic environmental zeitgebers (Johnson et al., 1999) in a process known as entrainment.
1.3.2 The clock controlling circadian rhythms
Circadian rhythms are the overt manifestation of an endogenous circadian clock. The presence and anatomical location of circadian clocks has been established through a series of ablation/transplantation experiments, and more recently by molecular genetics studies (Takahashi, 2017).

1.3.2.1 The anatomical location of the clock
Clocks exist in almost all tissues in the body. The central circadian clock in mammals is located in the suprachiasmatic nuclei (SCN) in the anterior hypothalamus just above the optic chiasm (Mohawk et al., 2009). The SCN is comprised of a bilateral pair of small structures composed of around 20,000 neurons and is strategically positioned in order to receive inputs through both direct and indirect retina-SCN pathways (Reppert et al., 2001)(Figure 1.3).

![Figure 1.3: Location of the mouse suprachiasmatic nuclei (SCN) in the anterior hypothalamus, on top of the optic chiasm, beside the bottom of the third ventricle (black circles) (image provided by Dr Nicola Ludin).](image)

The role of the SCN in circadian time-keeping was first discovered by Moore and colleagues in 1972 when lesions in the area of the SCN (but not bilateral destruction of the primary optic tracts) in female rats resulted in loss of the oestrous response to light. However, at this time, little was known about the neural interactions underlying the
organisation of the mammalian circadian system or how circadian pacemakers could interacting with each other to generate circadian rhythms. In order to demonstrate the autonomy of the SCN as the central pacemaker it was necessary to prove that the SCN had the ability to maintain a rhythm dictating its phase and period. In 1979, Inouye and Kawamura used a Halsasz knife to isolate the portion of the hypothalamus that contained the SCN from previously entrained albino wistar rats. The electrical activity of rat brains were recorded after surgery by using bipolar electrodes inserted stereotaxically between the region of the SCN and the optic chiasm. The animals were also blinded by binocular enucleation. Following the hypothalamic surgery, the circadian rhythmicity in the whole animal was ablated, but rhythmicity was maintained in the section of cultured SCN tissues. The autonomy of the SCN in generating rhythms was demonstrated with this experiment (Inouye et al., 1979). A few years later, in an attempt to explain the biological basis of the generation of circadian rhythms in vertebrates, Pickard and colleagues (1982) studied the effect of unilateral ablation of the SCN on the dissociation or split of circadian activity patterns in golden hamsters maintained in constant light. Their results showed that unilateral ablation of the SCN had the same effect as the complete destruction of the SCN (loss of rhythmicity of locomotor activity). This suggested that the two structures of the SCN are reciprocally innervated and both are essential for the generation of circadian rhythms (Pickard et al., 1982). However, it was not until 1990, with the discovery of tau mutant hamsters that the SCN was confirmed to be the central driving oscillator of circadian rhythms (Ralph et al., 1990). The tau mutation results in a reduction in the period of the circadian rhythm from 24 hours to 22 hours in heterozygotes, and to about 20 hours in homozygotes. Ralph and colleagues first induced arrhythmicity in hamsters by ablating their SCN. Three weeks after ablation, a neuronal transplant of the SCN was performed.
Circadian rhythmicity was evaluated through the analysis of locomotor activity. Interestingly, the transplanted animals recovered their rhythmicity but with the period of the donor genotype. When wild type tissue was transplanted into the mutant, the period of the restored rhythm of the animals was always similar to 24 hours, whereas heterozygote donors exerted rhythms with a period close to 22 hours, and homozygote mutant tissue hosts showed rhythms of 20 hours. This experiment finally demonstrated that the SCN had the ability to restore a rhythm dictating its phase and period, and therefore demonstrated the autonomy of the SCN as the master pacemaker of circadian rhythms in mammals.

1.3.2.2 The molecular basis of the circadian clock

In 2017, Jeffrey C. Hall, Michael Rossbach and Michael W. Young, were awarded the Nobel Prize in Physiology or Medicine for their work on the molecular machinery that underlies all biological clocks and orchestrates circadian rhythms in living organisms. Using fruit flies as a model organism, they isolated a gene that controls normal daily biological rhythm. They showed that this gene encodes a protein that accumulates in the clock cells during the night and is degraded during the day. Subsequently, additional protein components of the circadian clock were identified, exposing the mechanism that governs the self-sustaining clockwork inside the cell. We now recognize that biological clocks function by the same principles in the cells of other multicellular organisms, including humans.

At a molecular level, the circadian clock in mammals is composed of the core clock genes *Clock, Bmal1, Per1, Per2, Per3, Cry1* and *Cry2*. Core clock genes are expressed not only in the SCN but also in most cells of the body. Both RNA and protein levels of the core clock genes oscillate over a period of approximately 24 hours as a result of cell-autonomous
transcriptional-translational autoregulatory feedback loops (Takahashi, 2017). Within the core molecular loop, CLOCK and BMAL1 proteins bind, creating a heterodimer. This heterodimer binds to regulatory elements containing E-box sequences in the promoters of Per1 and 2 and Cry 1 and 2, activating their expression. Together with the serine/threonine kinase casein kinase 1ε, (which phosphorylates PER1/2, CRY1/2 BMAL1), CLOCK:BMAL1 bind to regulatory elements in the Bmal1 promoter, inhibiting its transcription (Duguay et al., 2009). As BMAL1 levels fall, Per and Cry transcription declines, and PER and CRY protein levels decrease. Additional feedback loops involving other clock gene elements also regulate the expression of core clock genes. For instance, the CLOCK-BMAL1 complex regulates expression of the nuclear receptors (REV-ERBα and REV-ERBβ) and retinoic acid-related orphan receptor α (RORα), by acting on E-boxes present within their promoter (Evans et al., 2013). REV-ERBα and REV-ERBβ repress Bmal1 and Clock (Figure 1.4).
Finally, a third CLOCK–BMAL1 driven transcriptional loop involves the PAR-bZip (proline and acidic amino acid-rich basic leucine zipper) factors DBP (D box binding protein), TEF (thyrotrope embryonic factor) and HLF (hepatic leukaemia factor). These proteins interact at sites containing D boxes with the repressor NFIL3 (nuclear factor, interleukin 3 regulated; also known as E4BP4), which is driven by the REV-ERB–ROR loop. Together, these three interlocking transcriptional feedback loops generate cycles of transcription with various phases of expression (Takahashi, 2017).

1.3.3 Clocks throughout the body
Almost all physiological processes in mammalian organisms undergo daily oscillations. Although the SCN houses the central circadian pacemaker, robust circadian oscillations of
gene expression have also been observed in tissues like the spleen, cornea, liver, heart, kidneys, muscles, stomach, pancreas, thyroid gland, and adrenal gland (Yamazaki et al., 2009; Yoo et al., 2004). These clocks share the same molecular machinery that drives circadian rhythms in the SCN (although there are some differences in their regulation) and are called *peripheral clocks* (Schibler et al., 2016).

The SCN receives timing information from the light-dark cycles through the retinal ganglion cells, and in turn coordinates phasing of non-light-sensitive peripheral oscillators by both neuronal and humoral signals, body temperature rhythms and metabolites, or by synchronising local subsidiary oscillators in peripheral organs (Baron et al., 2014; Duguay et al., 2009; Kondratova et al., 2012; Schibler et al., 2016; Yamazaki et al., 2000). Peripheral clocks (and their communication with the SCN central clock) are essential to the regulation of circadian physiology in mammals (Cuninkova et al., 2008).

Circadian feedback loops participate in both SCN neurons and peripheral tissues. However, there are differences in how they work in these tissues. For instance, daily rest/activity cycles drive feeding-fasting rhythms, which generate daily oscillations in metabolism, entraining circadian oscillators in peripheral tissues such as in the liver (Schibler et al., 2016). Given that feeding-fasting rhythms have little impact on the phase of the SCN, they can uncouple peripheral from central clocks. This has been seen in mice, which, as nocturnal animals, consume most of their food during the night. When these animals’ feeding is restricted exclusively to light hours (their normal resting phase), it completely reverses the phase of circadian oscillators in peripheral cells such as in the liver but not in the SCN (Damiola et al., 2000). Additionally, many other tissues and brain regions have shown to contain circadian clocks with differing degrees of dependence on the SCN
(Cuninkova *et al.*, 2008). An example is the dorsomedial hypothalamic nucleus (DMH), reported to be important in the regulation of some circadian rhythms (independently from the SCN), such as rhythms of sleep and wakefulness, locomotor activity, corticosteroid secretion, and feeding. Cell-specific lesions of the DMH profoundly reduce all these rhythms (*Chou et al.*, 2003), demonstrating its role as circadian oscillator independent of the SCN.

### 1.3.4 The concept of entrainment

Entrainment is defined as the process by which an external cue or *zeitgeber* modifies an endogenous rhythm so that the period of the rhythm becomes equal to the *zeitgeber* that influences it. Circadian entrainment represents the daily adaptation of organisms to their environment (*Golombek et al.*, 2010), and it is therefore necessary to adjust the circadian phase of an internal rhythm to the environment (Aschoff, 1960). If an organism is isolated from external cues such as light, even if the endogenous period (τ) is very close to 24 hours, it will gradually differ from the period of entrainment and will become desynchronised from the day-night cycle. This means that the period of biological rhythms is a result of an interplay between an endogenous oscillator and the environmental cyclic variable (*Golombek et al.*, 2010). For instance, the C57bl/6 mouse has an average free running period (FRP) of 23.7 hours. Without entraining signals (in constant conditions), the rhythm will advance by 0.3 hours each day (Figure 1.5).
Figure 1.5: Double plot actogram created from the locomotor activity of one of the mice (C57bl/6VJU) used in this thesis. Light cues present during days 1–10 result in rhythm entrainment to the zeitgeber. Light. In the absence of the light cues (blue area) (days 11 to 21 in constant darkness), the rhythm is in a free-running state. That is, the circadian cycles persist with the period of the internal clock. In the example shown, the free running period (FRP) is slightly less than 24 hours: 23.7 hours.

Conversely, if the endogenous period is longer than 24 hours, the phase will be delayed with respect to the environmental time (Khalsa et al., 2003).

While circadian rhythms need to be entrained in order to maintain synchrony with the environment, sometimes a rhythm appears to be entrained but nonetheless is not. This process is called “masking. In this situation, the stimuli may have induced the expression of the overt behaviour but only temporarily, without affecting the phase of the rhythm and therefore without actually entraining the central pacemaker (Johnson et al., 2003). The main difference between masking and entrainment is that masking lasts only for the duration of the masking signal whereas entrainment lasts after the external stimuli ceases (Rietveld et al., 1993). A change in the sleep-wake cycle after a change in environmental conditions such as temperature, would be considered masking, which is different from entrainment (Mrosovsky, 1999). Entrainment modifies the endogenous rhythm. In addition to masking, other effects can be observed in locomotor activity rhythms during or after a period of
entrainment, and these are so called “transients” and “aftereffects”. During entrainment or right after releasing the animals into constant conditions, transient cycles can be observed in mice locomotor activity rhythms previous to stable entrainment to the zeitgeber or free run is established (Jud et al., 2005). These transient cycles have to be excluded from the determination of the internal period or phase shift. In turn, aftereffects can frequently be observed after a period of entrainment or after the exposure to a zeitgeber. Aftereffects are described as the effect of the circadian period length arising from a previous entrainment schedule or as a consequence of the exposure to a zeitgeber such as a light pulse (Beaulé et al., 2011).

Several stimuli are capable of entraining the central oscillator. One of the most extensively studied is light. The ability of light to exert different effects on the circadian clock makes it a great candidate for further study to better understand and investigate the fundamental basis of entrainment and to discriminate between masking and true entrainment.

1.3.4.1 Fundamental basis of entrainment: Mechanisms of photoentrainment
Colin Pittendrigh was one of the pioneers in the systematic study of photoentrainment. In order to understand the mechanisms by which light is capable of entraining the circadian clock, he studied the effects of discrete pulses of light on the free running rhythms of animals kept in constant darkness. He showed that light exposure delivered to a free running animal at different times in its circadian cycle has different phase-shifting effects on the animal’s free running period. While a discrete light pulse (of either 15 or 60 minutes) given to an animal during its active phase did not have much effect on the clock, when the same light pulse was given during the first half of the subjective night, at the time when the animal was meant to be asleep, the animal delayed its activity the next day. However, light
administered during the second half of the subjective night evoked phase advances in the animal’s circadian rhythms of behaviour (Pittendrigh et al., 1976a). This mechanism by which entrainment happens is called the discrete model or non-parametric model (phasic) (Pittendrigh et al., 1976b).

In contrast, Jürgen Aschoff focused his study of light as a zeitgeber through a continuous model, or parametric model (tonic), which he described in detail in 1960. This model states that longer durations (longer than 15-60 minutes) of light stimulation cause phase-specific accelerations or decelerations of the fundamental period of the oscillator (Aschoff, 1960), which allows the circadian pacemaker to continuously adjust the length of its cycle to the environmental light-dark (LD) cycle (Pendergast et al., 2010). Parametric entrainment is related to circadian photosensitivity to long-duration light stimuli.

Thus, in summary, light synchronises the mammalian circadian clock through phase shifts. These shifts are equal in magnitude (on average) to that of the entraining stimuli or zeitgeber, the difference between the periodicity of the LD cycles, and the period of the circadian rhythms (Sharma et al., 2005). However, the magnitude of these shifts is limited (Johnson et al., 2003). Molecular biological research into the understanding of the entrainment of circadian clocks since the times of Pittendrigh and Aschoff has contributed to the description of the mechanisms that underlie photic entrainment in mammals.

1.3.5 Molecular basis of photic entrainment in mammals
In mammals, several structures are involved in photic entrainment, including the retina and the retino-hypothalamic tract (RHT), the SCN, an indirect retinal projection to the SCN through the intergeniculate leaflet (IGL) of the lateral geniculate nucleus (Lowrey et al.,
2000), and a third major SCN afferent projection arriving from the median raphe nucleus (Morin et al., 2006).

The light entrainment signal is primarily perceived by specialised retinal ganglion cells containing the photopigment melanopsin (Hattar et al., 2006). The photic information is then processed through the retino-hypothalamic tract (RHT) resulting in induction of immediate early clock genes (Albrecht et al., 1997; Sharma et al., 2005).

In response to light, melanopsin-expressing retinal ganglion cells release glutamate (Glu) and pituitary adenylate cyclase activating polypeptide (PACAP) at the terminals of the RHT (Ding et al., 1997; Hannibal, 2002; Michel et al., 2006). The Glu released promotes the activation of N-methyl-D-aspartate (NMDA) receptors with an increase of $\text{Ca}^{2+}$ influx in SCN neurons, inducing a “phosphor-relay” signalling pathway that quickly promotes phosphorylation of cyclic adenosine monophosphate (cAMP) response element binding protein (CREB). Phosphorylated CREB (P-CREB) binds to cAMP response elements in the promoters of the genes Per1 and Per2, inducing their expression. Elevated PER1 and PER2 adjust the molecular feedback loop of the circadian oscillator to the light-dark cycle (Albrecht, 2012) (Figure 1.6). P-CREB has also been reported to promote the expression of a number of genes, including the immediate-early gene c-fos, which expression is specifically induced by night-light stimulation in the retina (Hannibal et al., 2001).
Entraining stimuli also promote the translocation of CREB regulated transcription co-activator 1 (CRTC1) to the nucleus. CRTC1 is a co-activator for CREB-driven transcription of *Per1*, as well as for transcription of salt inducible kinase 1 (*Sik1*). Light-induction peaks after 1 hour, then SIK1 inhibits further expression of *Per1* by phosphorylation and deactivation of CRTC1. Hence, SIK1 acts as a suppressor of the effect of light on the clock by blocking CREB-driven *Per1* transcription and therefore acts as a negative-feedback mechanism that effectively limits the magnitude of phase-shifting responses to light within the SCN (Jagannath *et al.*, 2013; Vitaterna *et al.*, 2006) (Figure 1.7). Entrainment is therefore a gradual process that requires repeatedly shifting stimuli over multiple days to adjust to an advanced or delayed LD cycle. The consequences of the SCN’s inability to make larger magnitude shifts are experienced as jet lag symptoms in humans, for example, when significant time zone displacement occurs.
This saturation of the response, or limit of entrainment, suggests that the regulation of Per1 gene transcription is likely to be only part of the mechanism involved. Other kinases, such as calcium/calmodulin kinase 2 (CaMK2) and glycogen synthase kinase 3β (GSK3β), seem to take part in regulating the intracellular localisation and stability of PER and BMAL1 proteins (Sahar et al., 2010). Further, the manipulation of the intensity (Sharma et al., 1999) and duration (Comas et al., 2006) of the administered photic zeitgeber can also modify the circadian period of a rhythm by acting on the photoreceptor pathway regulating the clock (Takahashi et al., 1984). One of the most powerful tools for dissecting and understanding entrainment and, in particular, the temporal gating for zeitgeber effects, is the construction of phase response curves (PRCs).

1.3.6 Phase Response Curves: The shifting effect of light
A phase response curve (PRC) is a plot of the phase shifts of a circadian rhythm in response to a stimulus (zeitgeber) given at different phases or circadian times (Aschoff, 1960). There are two types of PRC, classified regarding their strength in terms of the magnitude of the phase shift elicited by the stimulus or zeitgeber. Type 1 PRCs are elicited by weak stimuli.
and therefore display relatively small phase shifts (e.g. usually less than six hours). Type 1 PRCs show continuous transition between delays and advances. Contrarily, a strong stimulus yields Type 0 PRCs, which show maximal phase shifts of up to 12 hours, and transitions between advances and delays that are quite abrupt and discontinuous (Johnson, 1999; Johnson et al., 2003). When an external stimulus advances the phase of the rhythm studied, these shifts are plotted as positive values; phase delays are plotted as negative values; and the “dead zone” is the area in the subjective day in which the phase shift is zero. The abscissa represents the circadian time (CT), or time of the day when the stimulus is administered, from CT 0 to CT 24 (Figure 1.8).

Figure 1.8: Types of phase response curves. Advance phase shifts are plotted as positive values (blue) and phase delays are plotted as negative values (black). The “dead zone” is the area in the subjective day in which the phase shift is zero (red rectangle). (Adapted from (Johnson et al., 1999))

The shape and amplitude of a PRC shows the intrinsic property of the circadian oscillator and is characteristic for each species studied. Six different protocols were described by Aschoff (1960) for building a PRC; the two most famous are Aschoff type one and Aschoff type two. In the Aschoff type one protocol, a light pulse of a specific intensity is given whilst animals are in constant darkness. In that way, no other zeitgeber but light interferes
with the animal’s activity. In contrast, in the Aschoff type two protocol, the light pulse is given immediately after a period of entrainment in light-dark cycles. The disadvantage of this method is that the change into constant conditions might mask the effect of the pulse on the rhythm.

To determine the synergistic effect of two different zeitgeber on the clock machinery, “double-pulse” protocols are applied (Joy, 1992). Theoretically, when a phase-shifting stimulus is followed by a second stimulus, the latter stimulus will evoke its own supplementary phase shift, unless the stimulus is given during the dead zone (insensitive region, red rectangle, Figure 1.8) of the PRC, or the first stimulus makes the animal insensitive to the second stimulus by blocking the signal. The magnitude and phase angle of the phase shift evoked will be determined by the effect of the first stimulus on the clock. It is therefore possible to determine whether the stimulus has shifted the underlying pacemaker by establishing where in the PRC the second stimulus takes place.

1.3.7 Non-photic zeitgebers for the circadian clock
While the mechanisms underlying light entrainment are well established, those mediating non-photic entrainment are less well understood. Nevertheless, under natural conditions, organisms are known to be exposed to a wide range of periodic, non-photic zeitgebers (Mistlberger et al., 2011). Non-photic zeitgebers are non-light related cues that evoke behavioural arousal, affect the expression of clock genes, and therefore shift the phase of a rhythm (Ralph et al., 1992). These include, food availability, temperature cycles, social cycles, locomotor activity, and drugs or chemical agents (Johnson, 1999; Johnson et al., 2003; Sharma et al., 2005).
Two main signalling pathways have been identified that induce circadian gene expression in tissue cultured cells and provoke phase shifts in circadian rhythms independent from the light pathway: 1) a geniculohypothalamic tract (GHT) which originates in the thalamic intergeniculate leaflet (IGL) and uses neuropeptide Y (NPY), GABA, and endorphins as neurotransmitters (Harrington et al., 1988); and 2) a serotonergic median raphe nuclei projection to the SCN (Meyer-Bernstein et al., 1996; Wams et al., 2017).

These two signalling pathways suggest that non-photic stimuli have the ability to signal directly to the SCN clock and therefore entrain circadian rhythms independently from the light-dark cycle.

1.3.7.1 **Entrainment by temperature**

Daily cycles of ambient temperature act as a *zeitgeber* for circadian systems in heterothermic and in normothermic mammals (Van Someren et al., 2002). Pulses of temperature can evoke phase advances and delays in constant conditions in a phase-dependent manner (Johnson et al., 2003). However, while constant temperature cycles can have a strong and functional influence on activity profiles over the day in mammals, under a light-dark cycle, anti-phased temperature cycles cannot invert activity patterns. Circadian rhythms are temperature compensated (Ruby et al., 1999). Consequently, in mammals, temperature cycles are usually considered a weak *zeitgeber* in comparison to light (Wams et al., 2017). Nevertheless, they are directly related to rhythmic processes in the organism like running wheel activity rhythms (Refinetti, 2010).

1.3.7.2 **Entrainment by food**

One of the most dominant entrainment pathways after light input is feeding time (Schibler et al., 2003). Although feeding schedules often maintain a fixed relationship to the light
cycle under natural conditions, a change in this phase relationship can shift the food entrainable oscillator (FEO) that would normally act in synchrony. Feeding time has the ability to set the phase of the oscillators regulating rhythms in most peripheral tissues (Mistlberger et al., 2011). The suggested mechanism of the FEO is through the phasic release of serotonin (5-HT) in the hypothalamus, whose activity rhythm varies across the rest/activity cycle in rodents (Leibowitz et al., 1988) and is naturally secreted at the beginning of the natural feeding cycle (de Pontes et al., 2010). Peripheral organs related to the regulation of metabolic processes and digestion also exhibit circadian rhythms, which are synchronised with feeding time. This means that if food availability is restricted during the active period (day for diurnal animals, night for nocturnal), body temperature decreases, and the activities of these organs shift in order to adapt their rhythmicity to the new daily rhythm of food intake. The rhythmicity of clock gene expression in peripheral organs resets, whereas the phase of the rhythm in the SCN remains unchanged, as long as the LD cycle persists (Mistlberger et al., 2011; Schibler et al., 2003). Thus, under restricted feeding, the reduction of body temperature in animals might be a relevant factor to reset the clock. However, the mechanism by which temperature cycles might entrain peripheral clocks is still unknown (Mendoza, 2007).

1.3.7.3 **Entrainment by locomotor activity**

Although locomotor activity is an output from the clock, it can feed back into the clock. Restricting exercise can entrain the clock (Mrosovsky et al., 1989; Sharma et al., 2003), independently from the retinal input to the pacemaker (Marchant et al., 1996). In nocturnal animals (Challet, 2007; Flôres et al., 2016) as well as in humans (Buxton et al., 2003), exercise has shown to shift circadian clocks.
The entrainment of circadian rhythms by behavioural activity has been related to direct or indirect actions of serotonin (5-HT) and neuropeptide (NPY) on the SCN circadian pacemaker (Marchant et al., 1996) and on the reward system. Running wheel activity results in molecular adaptations within the mesolimbic dopamine pathway, including an increase in synaptic dopamine in the nucleus accumbens (Greenwood et al., 2011).

1.3.7.4 Entrainment by chemical agents

Drugs that interfere with any mechanism of the clock (chronobiotics) can induce phase shifts on circadian rhythms, resulting in chemical entrainment, therefore acting as zeitgebers (Challet et al., 2007; Chassard et al., 2004; Chassard et al., 2007). Indeed, there are PRCs described for a number of chemical agents in a variety of species. The term used to describe the study of time of day variation in drug pharmacodynamics (PD) and pharmacokinetics (PK) (Giraldez Davila, 1994) is chronopharmacology.

The mechanism by which a drug might be shifting the clock depends on the type of drug, dose, site, and mechanism of action (Perreau-Lenz et al., 2008), as well as time of day of the drug administration (Cheeseman et al., 2012), and the subject of study. Research looking at different drugs given at different times to different species highlights this fact. A role for GABA-active drugs in non-photic phase-shifting was first suggested by the finding that systemic injection of short-acting benzodiazepines (i.e., triazolam, midazolam, brotizolam), as well as the GABA_A agonist muscimol, produced phase shifts when the agonist is administered six hours after activity onset (Smith et al., 1989; Turek et al., 1986; Turek et al., 1989; Wee et al., 1989). In contrast, the GABA_A receptor antagonists bicuculline and picrotoxin increased GABA_A excitability when administered during both the subjective day and subjective night (Gribkoff et al., 2003).
Clinical general anaesthetics are predominantly GABAergic drugs (propofol, isoflurane, sevoflurane, desflurane). Results from molecular and behavioural studies show that anaesthetics can affect expression of the core clock components as well as behavioural rhythms, memory, and cognition (Orts-Sebastian et al., 2018; Poulsen et al., 2016). However, the mechanisms by which GA disrupts the clock are still largely unknown. The phase-shifting effect of anaesthetics could potentially result from these drugs causing a state of hyperactivation of the GABA receptor (Poulsen et al., 2016). Interestingly, at least in bees, light appears to counteract the phase-shifting effect of anaesthesia, suggesting that anaesthetics and light might be acting on the same pathway, and also providing a possible means of ‘treating’ anaesthetic-induced phase shifts (Cheeseman et al., 2012; Ludin et al., 2016; Poulsen et al., 2016).

As a main component of this thesis, the effect of different anaesthetic agents on the circadian clock will be described in detail in Section 1.4.6 Effects of GA on the circadian clock.

1.3.8 Other entrainable circadian oscillators
Other non-SCN-based circadian oscillators have also been described as possible entraining factors that operate independent of the SCN. The most studied circadian oscillator is the methamphetamine-sensitive circadian oscillator (MASCO).

Studies carried out in female rats with electrolytically lesioned SCN, and with their circadian locomotor rhythm completely abolished, showed that when methamphetamine was administered in the drinking water, robust rhythmicity in locomotor activity appeared in a dose-dependent period longer than 24 hours, suggesting that the MASCO does not need
the SCN to drive or restore circadian rhythms of locomotor behaviour (Honma et al., 1987). However, recent studies suggest that methamphetamine treatment by itself is not sufficient to re-establish rhythmicity. The presence of a running wheel (RW) to drive re-entrainment may be necessary, at least in SCN-lesioned (SCNX) C3H/HeN mice (Rawashdeh et al., 2017). It is therefore likely that the presence of the RW synergistically increases the dopamine release to a level that results in the lengthening of the period of the ultradian rhythm into circadian activity rhythms (Greenwood et al., 2011). A wheel-inducible circadian oscillator (WICO) as well as a palatable meal-inducible circadian oscillator (PICO) have been recently reported to be generated by non-canonical circadian clocks and have the power to entrain peripheral clocks independently of the SCN (Flôres et al., 2016).

In conclusion, many signalling pathways are expected to participate in the phase entrainment of circadian clocks (Dibner et al., 2010). Unfortunately, the synchronization pathways of different circadian oscillators by the SCN remain largely unknown; however, it is likely that these circadian oscillators work synergistically and need each other to actually drive, restore, or shift a rhythm (Rawashdeh et al., 2017). It is therefore vital to study the concomitant effect of different zeitgebers on the clock to clarify the bigger picture of how entrainment works.

1.3.9 Interaction of light and chemicals on the clock
Although several studies have examined the effect of light and drugs on the circadian system, few studies have focused on determining the interaction between photic and non-photic agents on clock functions and circadian rhythms.
As mentioned before, it is important to examine the interaction of different zeitgebers to better understand the way in which these stimuli act together. On this note, pharmacologically manipulating GABA receptors has been reported to cause phase shifts and can also modify light phase-shifting in mammals (Cardinali et al., 1998).

There are a number of studies showing that non-photic stimuli are able to inhibit light-induced phase shifts, and vice versa (Table 1.1). It is likely that multiple cellular components, signal transduction pathways, and transcriptional activations contribute to the shifting effects of light and drugs on the clock when administered together. It is reasonable to think that any drug that interferes with the light-shifting mechanism might modify the way both drugs and light affect the clock. However, as shown in Table 1.1, there is a lack of consistency in the interpretation of results, and therefore in the effect of the concomitant administration of light and drugs on the clock. This could be due not only to the use of different protocols, but to the time of administration, the intensity of light and dose of the drugs.

The phase-shifting effects of clinically used drugs are greatly relevant for human health and post-operative recovery, as drug-induced phase shifts can lead to the disruption of circadian rhythms, disrupted sleep timing and quality of life.
Table 1.1: Effect of the concomitant administration of drugs and light at a specific time phase on the clock

<table>
<thead>
<tr>
<th>Paper</th>
<th>Source of light</th>
<th>Animal model</th>
<th>Drug</th>
<th>Effect</th>
<th>Possible mechanism</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Ralph et al., 1985)</td>
<td>Monochromatic light</td>
<td>Male golden hamster</td>
<td>Bicuculline (selective antagonist of GABA)</td>
<td>Blocks light induced phase delay in a dose-dependent manner</td>
<td>Reduction of GABA sensitivity of GABAergic neurons</td>
</tr>
<tr>
<td>(Ralph et al., 1986)</td>
<td>Monochromatic light</td>
<td>Male golden hamster</td>
<td>Diazepam (GABA agonist)</td>
<td>Blocks light induced phase advance</td>
<td>Potentiation of GABA neurotransmission, adenosine neurotransmission, inhibition of calcium conductance and/or effects on cyclic nucleotide metabolism. Inhibition of C-Fos induction (Colwell et al., 1993). The effect on GABAB suggest that GABAB must also be related to light induced phase shift by different mechanisms, including inhibition of CAMP synthesis and voltage-dependent Ca+ conductance and reduction of neuronal excitability.</td>
</tr>
<tr>
<td>(Ralph et al., 1989)</td>
<td>Monochromatic light</td>
<td>Male golden hamster</td>
<td>Baclofen (GABAB agonist)</td>
<td>Blocks light induced phase advances and delays (dose-dependent)</td>
<td>All to block the phase-shifting effects of light Effects on calcium influx which appear to be responsible for many of the cellular events that underlie NMDA receptor activity</td>
</tr>
<tr>
<td>(Colwell et al., 1991; Colwell et al., 1993a; Colwell et al., 1992)</td>
<td>Monochromatic light</td>
<td>Male golden hamster</td>
<td>Dizocilpine (NMDA receptor antagonist) 3(2-carboxypiperazin-4-yi)-propyl-1-phosphonic acid (competitive NMDA receptor antagonist) Excitatory amino acid receptor antagonists—Nnepethyl-DaDaseapat (non-NMDA) antagonist 6,7-dinatriquinoxaline-2,3-dione</td>
<td>Blocks light induced phase advances and delays</td>
<td>Inhibitory action of sodium pentobarbital on C-Fos expression in the SCN. Enhance the chloride conductance regulated by the GABAA receptor Depression of the electrical excitability of SCN neurons. No inhibitory effect on C-Fos</td>
</tr>
<tr>
<td>Joy, 1992</td>
<td>White fluorescent light (600lux)</td>
<td>Male golden hamster</td>
<td>Triazolam (GABA agonist)</td>
<td>Blocks light induced phase advances and delays (dose-dependent)</td>
<td>Partially additive effect on the phase delay Injections and light pulses were given at saturating levels, which may have interfered with additivity. Its shifting effect is bound to locomotor activity</td>
</tr>
<tr>
<td>Gillespie et al., 1996</td>
<td>White light (120 lux)</td>
<td>Male golden hamster</td>
<td>Bicuculline (selective antagonist of GABA)</td>
<td>Increases the phase delay but not the phase advance</td>
<td>Reduces the size of the light induced phase delay Differs from Ralph and Menaker 1985 and 1989. It might be because of the administration of the drug in different areas of the brain. GABA can interact with other neurotransmitter systems within the SCN modifying the circadian response to light. Their mechanism of action is likely to be through activation of presynaptic 5HT1B receptors on retinal terminals, thereby decreasing light-induced neurotransmitter release.</td>
</tr>
<tr>
<td>Basu et al., 2015</td>
<td>15-min, 40-lux light pulse, Male Syrian hamsters</td>
<td>Sumatriptan (serotonin 5HT1B receptor agonists), Muscimol (GABAA agonist)</td>
<td></td>
<td>Attenuates both light-induced phase advances and phase delays</td>
<td>BMY7378 enhances photic responses is by changing the activity of the raphe nuclei to influence how the IGL responds to light, which influences the SCN</td>
</tr>
</tbody>
</table>
1.3.10 Circadian disruption of the clock

As explained previously, circadian rhythms control a variety of biological processes in all living organisms, from metabolism to physiology and behaviour. Therefore, the timing and alignment of circadian rhythms are essential to promote health and well-being in all organisms, including humans (Baron et al., 2014). This means that when circadian cycles are disrupted either by genetic or environmental factors, various physiological and behavioural processes might be adversely affected (Moore, 1997; Takahashi et al., 2008). Genetic mutations in clock gene expression can lead to physiological variation and have been associated with disease. For example, a mutation in Cry1 has been linked to familial delayed sleep phase disorder in humans (Patke et al., 2017). Modern societies are driven by social schedules more than the day-night cycle, resulting in perturbations of human circadian biology which can negatively impact both physiological function as well as mental well-being (Takahashi et al., 2008).

At a behavioural level, the most obvious circadian rhythm is the daily pattern of sleep and wakefulness (Merrow et al., 2005; Roenneberg et al., 2003), thus consequences of sleep disruption and circadian disruption are closely related (see 1.2.2 Consequences of disrupted sleep). Disruption of the circadian clock is manifested by sleep-wake cycle misalignment or disturbance, resulting in sleep disorders and altered circadian regulation of waking adaptive behaviour (Moore, 1997). Circadian dysregulation has also been linked to changes in feeding patterns, metabolic function, and mood disorders (Baron et al., 2014).
1.3.10.1 Causes and consequences of circadian disruption
Disruption of circadian rhythms may occur when the timing of the sleep-wake cycle is not well adjusted to the relative night, when feeding disorders are present, or even when the SCN is misaligned with the peripheral rhythms (Baron et al., 2014). Some of the health effects associated with circadian disruption include cognitive deficits (Gibson et al., 2010), breast cancer (Stevens, 2009), metabolic syndrome (Li et al., 2011), immune dysfunction (Edgar et al., 2016; Scheiermann et al., 2013), mood disorders (Lanfumey et al., 2013), and sleep disorders. (Baron et al., 2014; Dagan et al., 1999).

The causes of desynchronization can be external factors such as jet lag (Leigh Signal et al., 2008), shift work (Haus et al., 2013), or drugs such as general anaesthetic agents (Cheeseman et al., 2012). Alternatively, internal factors, such as aging (Touitou et al., 1983) or certain diseases (Lanfumey et al., 2013) can also perturb the circadian rhythm through metabolic and physiological pathways resulting in the loss of coordination of the circadian system (Takahashi et al., 2008).

In circadian misalignment or disruption, the synchronizers that drive the biological clock such as the light-dark cycle and sleep-wake cycle are dissociated from the biological clock. The invention and use of electric light a century ago, dramatically changed human behaviour, altering our night-time activity, and vastly increasing the prevalence of shift work (Touitou et al., 2010). In industrialised societies, shift work and social schedules which give rise to a discrepancy between work, social and biological time (termed social jet lag), are now a major cause of circadian disruption. According to Akerstedt and colleagues (2009), more than 20% of the population suffers from shift work disorder (SWD) (Åkerstedt et al., 2009). SWD is defined as a shift in schedule between workdays
and free days. Health problems result because of the conflict between displaced work hours and the output of the biological clock. Shift work, in particular night work, compels the worker to invert his/her normal ‘activity-rest’ cycle forcing him/her to adjust his/her body function to the night activity period (Costa, 1996). Such ‘adjustment’ entails a progressive phase shift of the body’s daily rhythmicity functions. It has also been reported that SWD interferes with the circadian and homeostatic regulation of sleep (Åkerstedt et al., 2009; Baron et al., 2014), promoting negative effects on human health such as subjective and physiological sleepiness, increased accident risk, and increased risk of cardiovascular and gastrointestinal diseases, and certain forms of cancer (Knutsson, 2003). Lifestyle and stress are also potential mediators of circadian and sleep disruption in shift workers (Knutsson, 2003).

Travelling across different time zones also disturbs the circadian clock and leads to health impairment and fatigue (Filipski et al., 2004; Leigh Signal et al., 2008). This circadian desynchronization is called jet-lag (Leigh Signal et al., 2008). Jet-lag happens due to the disharmonizing of the body's circadian structure and the day to night cycle of the destination. Because the human body's hypothalamic biological clock has an endogenous free-running period of longer than 24 hours, this symptomatology worsens when we travel East, where days start earlier. Like SWD, jet-lag has been reported to influence organism physiology (Lemmer et al., 2002) and the immune system (Sharma et al., 2016) with possible detriment of the physical performance until readjustment to the local time is achieved (Manfredini et al., 1998). The most common symptoms associated with jet-lag are exhaustion throughout the day, disturbances in sleep patterns, cognitive deficits, in particular memory impairment, weakness and irritability (Sharma et al., 2016). In addition,
chronic jet-lag has been reported to disrupt in the expression of clock genes in the central clock, liver, thymus, and peritoneal macrophages in a mice model (Castanon-Cervantes et al., 2010) and may even promote cancer (Filipski et al., 2004).

Interestingly, after surgery with GA, patients report similar symptoms to those those reported experiencing jet lag and shift work (Farr et al., 1988; Gögenur, 2010; Gögenur et al., 2009; Gögenur et al., 2007a). Since it is known that the circadian clock influences the pharmacologic sensitivity of many drugs (Chassard et al., 2004), it is possible that a two-way relationship may exist such that GA may at least in part be responsible for the post-operative circadian misalignment reported by these patients.

A recent study reported that during cardiac surgery with a cardiopulmonary bypass, the incidence of major adverse cardiac events was lower in the afternoon than in the morning. This was in line with ex vivo studies analysing human myocardium, which showed a morning-afternoon change in hypoxia-reoxygenation tolerance with morning transcriptional variations in circadian gene expression of Rev-Erbα (Montaigne et al., 2018). This could mean that not just afternoon surgery, but the anaesthesia treatment in the afternoon might provide perioperative myocardial protection that leads to improved patient outcomes, compared with morning surgery. However, the use of cardiac patients to study the impact of GA on circadian rhythms at a pre and/or post-operative level has significant limitations. Their pre-existent health condition and the effect of surgery itself might confound the role of GA in the post-operative circadian misalignment. Laboratory studies, however, allow us to completely isolate the effect of GA, and are vital to the understanding of the detrimental impact of GA on circadian rhythms and its significance for patients’ post-operative recovery.
Studies on humans (Madrid-Navarro et al., 2015) and bees (Ludin et al., 2016) have demonstrated the beneficial properties of light for post-operative circadian misalignment recovery after anaesthesia.

The effect of GA and different general anaesthetic agents in humans and in animal models will be discussed in depth in section 1.4.6 Effects of GA on the circadian clock).

1.4 General Anaesthesia

1.4.1 Definition of general anaesthesia
The term “anaesthesia” descends from the Greek “an” (without) and “aesthesia” (sensation). This term, suggested by Oliver Wendell Holmes in 1846, literally means a state without sensation, and includes the induction of a reversible loss of consciousness (Minert et al., 2016). However, the lack of sensation and consciousness is not enough to define anaesthesia; muscle atonia, analgesia, and amnesia must be included in the definition of the anaesthetic state (Minert et al., 2016). It is the purpose of an optimal anaesthetic to induce a state of sensory deprivation without motor response and with amnesia (Dispersyn et al., 2008).

1.4.2 Importance of general anaesthesia
GA is important for enabling human survival during and after surgery. It is estimated that 234 million major surgical interventions are performed every year around the world (Weiser et al., 2008). However, since the discovery of GA in 1942 by Crawford Williamson Long (Grasshoff et al., 2006), and the first successful public demonstration of ether anaesthesia in 1846 by William T. Morton (Rudolph et al., 2004), the study of, and search
for new compounds that produce loss of consciousness, muscle tone, and pain without side-effects has been ongoing. Compared to the first anaesthetics, (nitrous oxide, ethyl ether and chloroform), modern anaesthetics, such as isoflurane, sevoflurane and desflurane, are considerably safer (for the patient and the anaesthetist). They are not flammable and result in earlier recovery and improved patient welfare (Tonner, 2005). However, sleep disturbances, fatigue, and other unwanted side effects remain a problem even with modern anaesthetic agents (Kehlet et al., 2003).

1.4.3 Modern clinical general anaesthetics
Modern inhalational anaesthetics, such as sevoflurane and desflurane, were synthesized in 1960. However, concerns regarding the toxicity of earlier forms of halogenated anaesthetics delayed the standardisation of their use until the last decade of the 20th century (Young, 1995). Even today, there is still no single ideal anaesthetic agent. A combination of compounds with different properties (such as analgesia, amnesia, and diminution of autonomic reflexes) is used instead (Tonner, 2005). The co-administration of an analgesic and muscle relaxants allows lower doses of the hypnotic drug to be used, improving safety and survival. This is called balanced anaesthesia and was first introduced by S. Lundy in 1926 (Corssen, 1985). In 1996, Eriksson and colleagues stressed the importance of balanced anaesthesia as the “method of choice” and concluded that “no expensive new pharmacology or new technology is needed since old methods can be used more effectively” (Eriksson et al., 1996). Eriksson and colleagues combined lidocaine (local anaesthetic) with ketoprofen (NSAID) and fentanyl (analgesic) during anaesthesia. As a
result of this treatment, postoperative pain and analgesic requirements were successfully reduced, recovery times shortened, and nausea and vomiting episodes diminished.

1.4.4 Molecular basis of GA
For more than a century, the mechanism underlying the effects of general anaesthetic agents (anaesthesia, atonia, analgesia, and unconsciousness), has been a matter of debate. Meyer (1899) and Overton (1901) were the first researchers to propose a molecular theory for the mechanism of action of GA. They proposed the “lipid theory” which assumes that the anaesthetic agents act on the lipid bilayer of biological membranes by altering the function of ion channels by a non-specific mechanism (Grasshoff et al., 2006).

This theory was universally accepted for more than 60 years, but in the 1960s a new theory based on protein interaction was suggested (Franks et al., 1978). This theory was supported by the modulation of a water-soluble protein, the firefly luciferase, by general anaesthetic agents (Franks et al., 1984). Today, ion channels are considered the most important anaesthetic targets in the mammalian central nervous system (CNS) given their role in neuronal information processing (Hemmings Jr et al., 2005).

Currently, there are four inhalational and five intravenous anaesthetics used clinically to induce or maintain GA. The inhalational agents are isoflurane, sevoflurane, desflurane, and xenon - although the use of xenon is not as extensive as the other three inhalational anaesthetic agents due to its high price. The intravenous agents are propofol, etomidate, ketamine, methohexital, and thiopental. These nine general anaesthetic drugs are usually accompanied by a combination of compounds such as sedatives (e.g. benzodiazepines, midazolam, diazepam, and lorazepam). Most commonly used general anaesthetics, such
as, propofol, isoflurane, sevoflurane, and desflurane have been reported to enhance 
GABA\textsubscript{A}-induced chloride currents (Franks, 2008); whereas ketamine, nitrous oxide, and 
xenon seem to also inhibit NMDA synapses (Flohr, 2006). In addition, other possible 
molecular targets such as neuronal nicotinic acetylcholine (nACh) receptors (Raines et al., 
2002), cyclic-nucleotide-gated (HCN) channels, glycine receptors (Franks, 2008), 5-HT3 
receptors, glutamate receptors, and the two pore potassium channels (Garcia et al., 2010) 
have been described as mediators of the actions of volatile anaesthetics. While GABA\textsubscript{A} 
and NMDA receptors have been described as specific molecular targets of general 
aesthetic (Franks, 2008). The link between the molecular pathways and the anaesthetic-
induced loss of consciousness is still being investigated (Franks, 2008).

Through human imaging studies, it has been shown that the thalamus is deactivated during 
aesthetic (Franks, 2008). The thalamus manages peripheral information into the cortex 
(Alkire et al., 2000). Hence, it is possible that GA acts by blocking or disrupting the sensory 
information processed through the thalamus, and therefore the information transferred to 
the cortex (Hudetz, 2012). A correlation between the dose of propofol anaesthesia, the level 
of thalamic functioning, and consciousness in humans was established by Fiset and 
colleagues in 1999. In this study, propofol dose-dependently decreased cerebral blood flow 
in the areas linked to the loss of consciousness (thalamus, cuneus, precuneus, posterior 
cingulate, and orbito-frontal cortices), associative functions (like those related to visual 
inputs), and autonomic control (Fiset et al., 1999). Interestingly, similar variations in the 
thalamic cerebral blood flow were also observed in the progression between waking and 
slow-wave sleep (SWS) (Maquet et al., 1997). There is strong evidence indicating that
anaesthetics may induce loss of consciousness through sleep pathways and through pathways that maintain cortical activation and behavioural arousal (Franks, 2008).

1.4.5 Anaesthesia and sleep
As previously described, there is an intimate relationship between circadian rhythms and sleep. GA, as will be explained in the following section, disrupts both, therefore it is important to determine their similarities in order to understand their interaction and possible mechanism.

1.4.5.1 Similarities and differences between GA and sleep
The behavioural similarities between GA and naturally occurring sleep suggest that they might share a similar mechanistic basis (Table 1.2). For instance, the EEG patterns seen in stage two of the maintenance period of GA and stage three of N-REM sleep, are both characterised by SWS. At this stage, pain perception is decreased. In anaesthesia this means that the anaesthetic effect is sufficiently deep to perform surgery (Lavigne et al., 2015).

During SWS, induced by either GA or natural sleep, similar physical changes such as slow-wave sleep, waxing and waning muscle tone, decreased body temperature, and decreased heart rate are seen (Carskadon, 2011). Sleep deprivation can enhance anaesthetic potency (Tung et al., 2002). These data adds weight to the idea that sleep and anaesthesia share some neuronal pathways and have some common mechanisms (Franks, 2008). Despite these similarities, the onset, maintenance, offset, and effect on the circadian clock of sleep and anaesthesia are quite different (Tung et al., 2002) (Table 1.2). A study in rats showed that a 12-hour period of continuous sedation with propofol during the animals’ resting period, had restorative effects similar to that of naturally occurring sleep (Tung et al.,
However different anaesthetic agents might have different effects; Isoflurane administered to rat brain slices prevented electrical oscillatory behaviour in thalamo-cortical neurons that is necessary for generating wakefulness, and REM and N-REM sleep (Ries et al., 1993). This indicates that the mechanism of isoflurane-induced anaesthesia differs from sleep and suggests that isoflurane would not promote restorative effects like propofol and natural sleep.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Anaesthesia</th>
<th>Sleep</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Onset</strong></td>
<td>Drug-induced</td>
<td>Endogenously generated</td>
</tr>
<tr>
<td></td>
<td>No homeostatic control</td>
<td>Homeostatic and circadian regulation</td>
</tr>
<tr>
<td></td>
<td>Failure to initiate is non-existent</td>
<td>Failure to initiate is recognised as a pathology</td>
</tr>
<tr>
<td></td>
<td>Not altered by environmental factors</td>
<td>Modulated by environmental factors</td>
</tr>
<tr>
<td><strong>Mainte-</strong></td>
<td>Duration is dose-dependent</td>
<td>Duration is a function of homeostatic and circadian factors</td>
</tr>
<tr>
<td></td>
<td>Depth at a given anaesthetic dose is constant</td>
<td>Depth fluctuates rhythmically and spontaneously</td>
</tr>
<tr>
<td></td>
<td>Failure to maintain is non-existent</td>
<td>Failure to maintain is recognised as a pathology</td>
</tr>
<tr>
<td></td>
<td>Minimally affected by environmental factors</td>
<td>Altered by environmental factors</td>
</tr>
<tr>
<td><strong>Offset</strong></td>
<td>Return to normal wakefulness in hours to days</td>
<td>Return to normal wakefulness in minutes</td>
</tr>
<tr>
<td></td>
<td>Duration of anaesthesia and elimination of the agent governs the time to wakefulness</td>
<td>Timing of wakefulness governed by environment, sleep duration and circadian rhythm</td>
</tr>
</tbody>
</table>

Table 1.2: Comparison of the characteristics of anaesthesia and sleep onset, maintenance, and offset (Adapted from (Tung et al., 2004)).

Electroencephalography (EEG) recordings from 14 patients undergoing induction of GA with propofol showed reduced capacity for information integration in the brain (Lee et al., 2009). Other human studies have reported that patients often have no perception that time has passed whilst anaesthetised (Tung et al., 2004). In 176 cases of human surgical anaesthesia using volatile anaesthetics (desflurane, isoflurane, and sevoflurane), as well as propofol, etomidate, and barbiturates, it was shown that changes in the electrical
uncoupling of brain regions were similar between the different drugs (Mashour, 2004),
suggesting that this could be directly related to the loss of perceptual processing and
consciousness.

1.4.6  Effects of GA on the circadian clock
General anaesthetics disrupt the circadian clock. This disruption is associated with changes
in clock gene expression (Anzai et al., 2013), the sleep-wake cycle (Gögenur et al., 2009),
melatonin levels (Gögenur et al., 2007a), locomotor activity (Irifune et al., 1997; Kikuchi
et al., 2013a; Xia et al., 2016), and temperature (Åkerstedt et al., 1994; Dispersyn et al.,
2009; Gögenur, 2010; Gögenur et al., 2004; Gögenur et al., 2007a). But how this operates
in chronobiological terms is still largely unknown.

1.4.6.1  Previous data on clock effects of anaesthetics in humans
After GA, patients often report autonomic instability, hypothermia, cardiac dysrhythmias,
nausea, vomiting, and delirium (Campagna et al., 2003), in addition to fatigue and poor
sleep (Gögenur, 2010). These are symptoms often also experienced by shift workers and
trans-meridian travellers (Costa, 1996; DuPont, 2008) (see 1.3.10.1 Causes and
consequences of circadian disruption). It is therefore likely that these examples of circadian
misalignment share a common mechanism with GA by which they disrupt the circadian
clock, resulting in disruption of circadian rhythms that lead to the manifestation of these
symptoms.

To study the effect of GA on human circadian rhythms, melatonin and temperature are
usually used as circadian markers (Table 1.3 and Table 1.4). Melatonin and temperature
cyclically vary their rhythmicity in a circadian fashion (Dispersyn et al., 2008; Gögenur et
al., 2007a), therefore alterations in their rhythmicity can be used as indicators of desynchronization of the circadian time structure.

The timing of the decrease in temperature levels and increase in melatonin levels are important tools for the regulation of sleep onset (Gögenur et al., 2007a) which interestingly, has been reported to be disrupted after GA (Gögenur, 2010; Tung et al., 2004). For instance, melatonin feeds back onto the SCN clock, and this feedback is thought to play a determinant role in the functioning of the circadian system (Pevet et al., 2011). This suggests that the disturbances in the rhythm of melatonin secretion after surgery might be a cause of the sleep disturbance experienced in patients after GA (Gögenur et al., 2007b).

A study conducted by Kärkela and colleagues in 2002, showed that anaesthesia, in conjunction with surgery, acutely disrupted the normal circadian rhythm of melatonin by delaying the onset of nocturnal melatonin secretion (Kärkela et al., 2002). Nocturnal urine was analysed before and after minor knee operations for the melatonin urine metabolite 6-hydroxymelatonin sulphate (6-OHMS). Evening and morning saliva samples were also analysed radio-immunologically. The level of melatonin secretion during the postoperative evening was significantly decreased in comparison with the pre-operative evening. These patients also experienced postoperatively disrupted sleep (Kärkela et al., 2002).

Similarly, in a study carried out by Gögenur and colleagues in 2007, melatonin levels were taken from urine samples before and after minimally invasive surgery. 6-hydroxymelatonin sulphate was measured by radioimmunoassay and showed a significant phase delay in the timing of excretion in urine. In addition, sleep duration on the post-operative night was decreased. Although these disturbances could be due to pain and other disruptive post-operative factors associated with surgery (Chouchou et al., 2014), patients who
experienced low levels of post-operative pain still had clinically significant sleeping problems after surgery (Kain et al., 2003). These results may indicate that sleeping problems cannot be entirely attributed to the influence of post-operative pain. The disturbances observed in sleep and melatonin could be related, at least in part, to the administration of GA. Arai and colleagues (2004) obtained blood samples from 18 women undergoing gynaecological surgery. Samples were obtained five minutes before and after the administration of either 5% isoflurane or 7% sevoflurane anaesthesia. It was found that isoflurane increased blood melatonin concentrations after surgery, whereas sevoflurane decreased melatonin concentration (Arai et al., 2004). These differences might be explained through the different pharmacokinetic properties between both agents, which would correlate with the fact that patients anaesthetised with isoflurane emerge from GA more slowly than those anaesthetised with sevoflurane (Tonner, 2005). This may also indicate that isoflurane has a higher potential to increase circulating melatonin levels than sevoflurane (Arai et al., 2004). These findings are in line with results from a study carried out by Reber and colleagues in 1998. They found increased plasma levels of melatonin after gynaecological laparoscopic surgery with isoflurane but not with propofol.

Different anaesthetic agents may therefore have different mechanisms by which they disrupt the circadian clock. These differences in GA influence on melatonin rhythm may thus potentially explain the different phase misalignments observed with different anaesthetic agents between studies (Pevet et al., 2011), which, in addition, might as well be influenced by variations in pre-treatment therapies and differences between the operation protocols.
After GA with propofol, the recovery time had been reported to be faster than after isoflurane (Reber et al., 1998). In contrast, in another study, the concentration of melatonin was reported to decrease for two to three days after surgery with propofol (Guo et al., 2002). In Guo’s study all patients received a β-blocker treatment (cardioprotective) the morning before cardiopulmonary bypass surgery. β-blockers have been reported to inhibit melatonin secretion which might explain the depletion of melatonin blood levels after the recovery time of propofol anaesthesia when compared with Reber’s study (Reber et al., 1998) (Table 1.3).

<table>
<thead>
<tr>
<th>Author</th>
<th>Anaesthetic</th>
<th>Surgery</th>
<th>Effect on melatonin levels</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Kärkela et al., 2002)</td>
<td>Thiopental</td>
<td>Orthopaedic</td>
<td>Decreased 1 night after surgery and following morning</td>
</tr>
<tr>
<td>(Guo et al., 2002).</td>
<td>Propofol</td>
<td>Cardiac</td>
<td>Decreased 2 nights after surgery</td>
</tr>
<tr>
<td>(Gögenur et al., 2007a)</td>
<td>Propofol</td>
<td>Laparoscopy</td>
<td>Phase delay in aMT6s after surgery</td>
</tr>
<tr>
<td>(Arai et al., 2004).</td>
<td>Thiopental</td>
<td>Gynaecological</td>
<td>Increased melatonin blood concentration after surgery</td>
</tr>
<tr>
<td></td>
<td>Isoflurane</td>
<td>surgery</td>
<td>Decreased melatonin blood concentration after surgery</td>
</tr>
<tr>
<td></td>
<td>Sevoflurane</td>
<td></td>
<td>Increased melatonin plasma concentration after surgery</td>
</tr>
<tr>
<td>(Reber et al., 1998)</td>
<td>Isoflurane</td>
<td>Gynaecological</td>
<td>Increased melatonin plasma concentration after surgery</td>
</tr>
<tr>
<td></td>
<td>Propofol</td>
<td>surgery</td>
<td>Return to melatonin plasma baseline concentration after surgery</td>
</tr>
</tbody>
</table>

Table 1.3: Effect of GA on melatonin after surgery with different anaesthetic agents

In addition, it has been proposed that the sleep-inducing effect of melatonin in humans is mediated through its effect on core body temperature (Gögenur et al., 2007a). Core body temperature is regulated by the central circadian pacemaker and follows a well-defined circadian rhythm with a minimum in the early hours of the morning and a maximum in the late afternoon (Campbell, 2008). In the evening there is a reduction of light which
influences the SCN and induces an increase in melatonin production. Melatonin then increases peripheral temperature through vasodilation and the core body temperature drops, increasing sleep propensity (Gögenur, 2010). In a similar way, core body temperature falls during GA. During the induction of the anaesthetic state, the vasoconstrictor tone, which maintains the lower temperature of the peripheral zone, is inhibited. Vasodilatation occurs, heat is lost to the environment and the patient becomes relatively hypothermic. In major surgery, heat is also lost from the operation site, particularly by evaporation (Campbell, 2008). However, it remains unknown whether the disturbed post-operative circadian temperature regulation is a result of endocrine metabolic changes, or if it is a response to the stress caused by surgery. It has been published that in women who were given GA for laparoscopic surgery with propofol and thiopental, the phase relationship between melatonin and temperature was altered (Gögenur et al., 2007a). These researchers also found that temperature was increased in the evening after surgery, accompanied with the expected increase of melatonin (aMT6s). This persisted the following morning, contrary to what occurs in a normal physiological situation, creating a phase delay of the 24-hour pattern of temperature rhythm. Nevertheless, this reaction might be related to an acute inflammatory stress response to surgery (Gögenur et al., 2007a) rather than to the anaesthetic agent. This would explain the results obtained by this same group, in 2001, in which major surgery caused sleep disruption for a longer period of time than when compared to less invasive surgery (Gögenur et al., 2001). In this study, sleep architecture was disturbed for ≤ four weeks after major abdominal surgery but for only one week after laparoscopic cholecystectomy.
In addition, studies conducted with isoflurane anaesthesia alone (without any other concomitant or adjuvant medication), suggest that the internal circadian temperature cycle is independent of the clinical concentrations of isoflurane (Sessler et al., 1991). In this study the temperature cycle was significantly reduced on the day of anaesthesia but returned to normal on the subsequent day. Compared with the two days preceding isoflurane administration, there was no statistically significant change in any of the five male volunteers anaesthetised (Table 1.4).

<table>
<thead>
<tr>
<th>Author</th>
<th>Anaesthetic agent</th>
<th>Surgery</th>
<th>Effect in Core body temperature</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gögenur et al.,</td>
<td>Thiopental</td>
<td>Laparoscopic</td>
<td>Lack of decline in the evening.</td>
</tr>
<tr>
<td>2007; Gögenur et al., 2010</td>
<td>Propofol</td>
<td>Cholecystectomy</td>
<td>Increased in the evening after surgery and remained elevated on the post-operative morning</td>
</tr>
<tr>
<td>Sessler, 1991</td>
<td>Isoflurane</td>
<td>None</td>
<td>No significant changes two days post-surgery</td>
</tr>
</tbody>
</table>

Table 1.4: Summary of studies investigating the disruptive effect of GA on core body temperature, a marker of clock disruption

The human data suggest anaesthesia might have an effect on the clock because some circadian rhythms, such as melatonin and temperature, are generally disrupted following anaesthesia. However, these kinds of studies have large limitations. In humans, it is impossible to isolate the effect of GA itself, as it is unethical to administer GA to a subject who does not need it, and therefore it is not possible to study the effect of the anaesthetic agent on circadian rhythms in isolation from the effects of surgery and hospitalisation.

Thus, animals are our best choice for research into the effect of anaesthesia on circadian outputs at a molecular level, and in cognition and behaviour.
1.4.6.2 Effects of anaesthesia on the clock in animal studies
There is now a substantial body of evidence, with 29 published reports, to show that GA can have a profound shifting effect on the circadian clock at a molecular, cognitive, and behavioural level, in a range of vertebrate and invertebrate species (see Table 1.5). One proposed mechanism of this effect is via anaesthetic agents acting on the expression of core circadian clock genes (Poulsen et al., 2016). Phase shifts in the expression pattern of the genes controlling the clock result in shifts in physiological and behavioural rhythms at the whole animal level. This effect can be likened to chemically-induced jet-lag (Cheeseman et al., 2012).

In this section, evidence of the effect of different anaesthetic agents on experimental animals’ circadian clocks will be discussed in detail (see Table 1.5).

1.4.6.2.1 Evidence of the phase-shifting effect at a molecular level in experimental animals
Molecular studies have shown that there are effects of anaesthesia on clock genes. This suggests that GA could have an effect on circadian rhythms which would be translated into changes at a physiological/behavioural level.

At a molecular level, 2% isoflurane was shown to delay Per and Cry expression profiles in the honey bee brain (Cheeseman et al., 2012) and mouse SCN (Xia et al., 2015), when administered during the active period (day time for bees, night time for mice). This effect appears to be independent of light exposure during the anaesthesia treatment. In bees, isoflurane was administered in constant darkness (DD); whereas in mice, it was administered under light. Phase advances were reported in Bmal1 and Clock when 1.3% isoflurane was administered to mice during their active period for five hours (Song et al.,
Interestingly, another volatile halogenated anaesthetic agent, sevoflurane, has been reported to either suppress (Anzai et al., 2013; Kobayashi et al., 2007; Mori et al., 2014), or repress (Kadota et al., 2012), mPer2 and Per2 expression in brain and SCN respectively, when administered to rodents (rats and mice) in concentrations higher than 1 MAC (the minimum alveolar concentration of an inhalational anaesthetic that prevents movement in 50% of the subjects in response to a painful stimulus) during their active and rest phase (Kobayashi et al., 2007; Ohe et al., 2011; Sakamoto et al., 2005). However, when lower concentrations were administered during the animals’ resting phase there was no effect on mPer2 expression (Matsuo et al., 2016). These differences could be due to the different lighting conditions and animal models used in these studies. Propofol seemed to reduce Per1 and Per2 expression when administered either during the rat active phase (Yoshida et al., 2009) or rest phase (Ben-Hamouda et al., 2018). Other less commonly used anaesthetic agents, such as 2,2,2-tribromoetanol (240mg/kg), promote phase advances in Per2 expression in the liver when administered during the active phase of the mouse and phase delays when administered during the rest phase (Kubo et al., 2012) (Table 1.5).

Thus, it is likely that GA affects both central and peripheral clocks and these effects are dependent on the time of administration, the organ studied, the animal model, the drug administered, and the protocol applied (Nagamoto et al., 2016).
<table>
<thead>
<tr>
<th>Anaesthetic</th>
<th>Organism</th>
<th>Tissue</th>
<th>GA administration protocol</th>
<th>GA duration (hours)</th>
<th>GA during active phase</th>
<th>GA during rest phase</th>
<th>GA during active phase</th>
<th>GA during rest phase</th>
<th>GA during active phase</th>
<th>GA during rest phase</th>
<th>GA during active phase</th>
<th>GA during rest phase</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoflurane (2%)</td>
<td>Honey bee</td>
<td>Whole animal + brain</td>
<td>FRP</td>
<td>6</td>
<td>Delay</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Delay in Per and Cry expression profiles</td>
<td>None</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Isoflurane (1.4%)</td>
<td>Rat</td>
<td>Whole animal + hippocampus</td>
<td>LD cycles</td>
<td>4</td>
<td>Advance</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Kikuchi et al., 2013b)</td>
</tr>
<tr>
<td>Isoflurane (1%)</td>
<td>Mouse</td>
<td>Whole animal + SCN</td>
<td>LD cycles</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>Increase during subsequent rest period</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Isoflurane (2%)</td>
<td>Mouse</td>
<td>SCN + Peripheral mononuclear blood cells</td>
<td>LD cycles</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Delay in Cry1 and Per2 expression in the SCN</td>
<td>-</td>
<td>Expression of clock genes in peripheral mononuclear blood cells delayed</td>
<td>-</td>
</tr>
<tr>
<td>Isoflurane (1.3%)</td>
<td>Mouse</td>
<td>Whole animal + SCN</td>
<td>LD cycles</td>
<td>5</td>
<td>Delay</td>
<td></td>
<td>Delay in cry expression, advance in Bmal and Clock</td>
<td></td>
<td></td>
<td>Suppression of clock gene expression in brain and liver</td>
<td></td>
<td></td>
<td>(Gökmen et al., 2017)</td>
</tr>
<tr>
<td>Isoflurane (1.5%)</td>
<td>Rat</td>
<td>Whole animal</td>
<td>FRP</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td>Suppression of clock gene expression in brain and liver</td>
<td></td>
<td></td>
<td></td>
<td>(Kadota et al., 2012)</td>
<td></td>
</tr>
<tr>
<td>Sevoflurane (2.5%)</td>
<td>Mouse</td>
<td>Whole animal + SCN</td>
<td>FRP</td>
<td>4</td>
<td>Delay</td>
<td>Delay</td>
<td>-</td>
<td>-</td>
<td>mPer2 repression</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Anzai et al., 2013)</td>
</tr>
<tr>
<td>Sevoflurane (4%)</td>
<td>Rat</td>
<td>Whole animal + brain</td>
<td>FRP</td>
<td>8</td>
<td>-</td>
<td>None</td>
<td>-</td>
<td>Decrease</td>
<td>Per2 expression, phase delay</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Ohe et al., 2011)</td>
</tr>
<tr>
<td>Sevoflurane (2.5%)</td>
<td>Mouse</td>
<td>Whole animal + brain</td>
<td>FRP</td>
<td>4</td>
<td>-</td>
<td>Delay</td>
<td>-</td>
<td>Decrease</td>
<td>delay</td>
<td>Delay</td>
<td>-</td>
<td>-</td>
<td>Decrease in clock protein binding and acetylation of histone H4 of Per2 promoter</td>
</tr>
<tr>
<td>Sevoflurane (2.2%)</td>
<td>Rat</td>
<td>Brain</td>
<td>LD cycles</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Advances/delays in Per2 mRNA</td>
<td>No effect in Per2 mRNA</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sevoflurane (4.5%)</td>
<td>Rat</td>
<td>Multiple</td>
<td>LD cycles</td>
<td>0 - 6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Decrease in Per2 mRNA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sevoflurane (4%)</td>
<td>Rat</td>
<td>Brain</td>
<td>LD cycles</td>
<td>2 - 6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Suppression of circadian gene expression</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Kobayashi et al., 2007)</td>
</tr>
<tr>
<td>Drug</td>
<td>Duration of treatment</td>
<td>Animal</td>
<td>Main effects on the clock</td>
<td>Clock effects</td>
<td>Other effects</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>--------------------</td>
<td>-----------------------</td>
<td>-----------------</td>
<td>---------------------------</td>
<td>---------------</td>
<td>---------------</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Sevoflurane</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(4%) Mouse Cell lines (hypothalamic)</td>
<td>N/A</td>
<td>8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Suppression of Per2 expression</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Nagamoto et al., 2016)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(1.97%) (inhaled)</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Ocmen et al., 2016)</td>
<td></td>
</tr>
<tr>
<td>Desflurane (5.7%) (inhaled)</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Ocmen et al., 2016)</td>
<td></td>
</tr>
<tr>
<td>Pentobarbital (4 mg/100g) (i.v.)</td>
<td>Rat Whole animal</td>
<td>LD cycles</td>
<td>1</td>
<td>-</td>
<td>None</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Phase delay in melatonin rhythm</td>
<td>-</td>
</tr>
<tr>
<td>Pentobarbital (50mg/kg) (i.v.)</td>
<td>Mouse</td>
<td>Whole animal</td>
<td>FRP</td>
<td>N/A</td>
<td>Delay</td>
<td>Advance</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Ebihara et al., 1990)</td>
</tr>
<tr>
<td>Ether (20ml) (inhaled)</td>
<td>Rat Whole animal</td>
<td>LD cycles</td>
<td>1</td>
<td>-</td>
<td>None</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Prudian et al., 1997)</td>
</tr>
<tr>
<td><strong>Ketamine</strong> (100mg/kg) (i.p.)</td>
<td>Rat Whole animal</td>
<td>LD cycles</td>
<td>1</td>
<td>-</td>
<td>None</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>(Prudian et al., 1997)</td>
</tr>
<tr>
<td>(150mg/kg) (i.p.)</td>
<td>Rat Whole animal</td>
<td>LD cycles</td>
<td>4-4.5</td>
<td>Delay</td>
<td>Advance</td>
<td>Decrease</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Phase delay in melatonin rhythm</td>
<td>Phase advance in melatonin rhythm</td>
</tr>
<tr>
<td>Ketamine (10 mM +1 mM)</td>
<td>Mouse Fibroblasts</td>
<td>N/A</td>
<td>1</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Phase shift in Bmal1 and dbp expression + reduced amplitude of Cry1 and dbp</td>
<td>-</td>
</tr>
<tr>
<td><strong>Propofol</strong> (120mg/kg)</td>
<td>Rat SCN</td>
<td>FRP</td>
<td>1-3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Reduction in Per1 and Per2 expression (transient)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(120mg/kg) (i.p.)</td>
<td>Rat Whole animal</td>
<td>LD cycles</td>
<td>0.5</td>
<td>Advance</td>
<td>Advance</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Melatonin phase advance</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(120mg/kg) (i.p.)</td>
<td>Rat Whole animal</td>
<td>LD cycles</td>
<td>0.5</td>
<td>Advance</td>
<td>Advance</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Phase advance in temperature rhythms</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(60mg/kg) (i.p.)</td>
<td>Rat Whole animal</td>
<td>FRP</td>
<td>0.5</td>
<td>Advance</td>
<td>Advance</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(600 µg/kg/min)</td>
<td>Rat Brain</td>
<td>-</td>
<td>6</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Reduction in Per2 expression</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>(10 mg/min) (i.v.)</td>
<td>Rat Whole animal</td>
<td>LD cycles</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Period of rhythm of core body temperature lengthened</td>
<td>Period of rhythm of core body temperature shortened</td>
</tr>
<tr>
<td><strong>2,2,2-</strong> Tribromoethanol (240 mg/kg) (i.p.)</td>
<td>Mouse Whole animal + brain</td>
<td>FRP</td>
<td>1-4</td>
<td>None</td>
<td>None</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>Phase advance in Per2 expression</td>
<td>Phase delay in Per2 expression</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 1.5: Summary of the effect of general anaesthetic agents on circadian rhythms of behaviour in animals. Drug, duration of treatment, animal and main effects on the clock are detailed. ("GA" = General Anaesthesia, "FRP" = free running period, "-" not investigated or not part of the study).
1.4.6.2.2 Consequences of anaesthesia-induced “jet-lag” in cognition and memory in experimental animals

The potential effects of anaesthesia are not limited to those associated with rhythmic activity. There are other parameters that are intensely influenced by clock phase, such as learning and memory (Liu et al., 2014). For instance, we have previously demonstrated that the clock-shifting effect of isoflurane anaesthesia persists for at least three days in time-place learning in honeybees maintained in strong daily light cycles (Cheeseman et al., 2012). Acquisition and memory retention have also been reported to be impaired by the administration of different anaesthetic agents. In 18-month old male rats and 10-week old male mice, isoflurane administered at 1 MAC impaired learning skills in comparison with the controls (Cao et al., 2012). This effect was also observed in young (three-month old) and middle aged (12-month old) male rats. A recent study by Song and colleagues (2018), showed that five-hour exposure to 1.3% isoflurane during the active phase of mice, deteriorated memory and disrupted circadian rhythms. The duration of these effects was greatest in aged mice (18-month old). Isoflurane at 1 MAC administered for four hours impaired acquisition and memory retention while in the Morris Water Maze (MWM) a week after the treatment (Callaway et al., 2012), whereas sevoflurane did not impair acquisition learning and memory retention in young adult (8-10 week old) or aged rats (15-21 month old) (Callaway et al., 2012). It should be noted that in this study, experiments with young and aged rats were conducted at different circadian times, and therefore results are not directly comparable (Callaway et al., 2012). Sevoflurane administered for four hours in concentrations between 3% and 5% in seven-day old male rats was reported to affect spatial reference memory and locomotor activity performance. The performance of seven-day old male rats in the MWM temporarily deteriorated six, but not two weeks after sevoflurane anaesthesia treatment (Fang et al., 2012). In contrast, there
was no effect found on the performance of young adult male mice (nine-week old) in the MWM after a single four hour exposure to sevoflurane (and desflurane), nor after shorter (two hours) exposure during five consecutive days (Kilicaslan et al., 2013).

If GA is able to affect an animal’s memory and cognition to such magnitude, it is reasonable to think similar effects will be observed in the animal’s behaviour. Studies indicate that indeed they are.

1.4.6.2.3 Evidence of the phase-shifting effect of GA at a behavioural level in experimental animals

Studies on the effect of GA at a molecular level, and in relation to cognition and memory, indicate that GA affects the clock machinery via different molecular mechanisms. However, the specific mechanisms by which GA acts at different levels are still unclear. This is likely to be due to the variability and conflicting data published to date, probably because of the differences in the study design between experiments. If GA has the ability to disrupt the onset of mice locomotor activity by one to two hours, general anaesthetic agents must be acting as an important input on the central and possibly peripheral clocks, and therefore might have the ability to shift behavioural rhythms.

In experimental animals, the anaesthetic agents, isoflurane, sevoflurane, propofol, and ketamine, have all been shown to cause time-dependent shifts in daily behavioural rhythms. Ketamine, a NMDA receptor antagonist, has been shown to produce 60 to 150-minute phase advances in locomotor activity rhythms, measured by a passive infrared sensor, when administered to entrained rats during the resting phase; but 40 to 200-minute phase delays when administered during the active phase (Mihara et al., 2012). GABA agonists such as sevoflurane, propofol, and isoflurane seem to elicit various effects on circadian rhythms of behaviour. Sevoflurane anaesthesia
administered to mice during their free running period (FRP) was shown to cause a phase delay in the circadian rhythm of spontaneous locomotor activity, independent of the time of administration. Spontaneous locomotor activity was measured by using digital counters with passive infrared sensors (Kadota et al., 2012), however, looking at their phase response curve (PRC), it can be noticed that a higher phase delay was elicited when the anaesthetic agent was administered at circadian time point (CT 8). It is possible that the lack of significance regarding the time-dependent effect of the administration of sevoflurane was due to the number of animals used per CT. Only four animals per time point were tested by Kadota and colleagues (2012). It is important to design an experimental protocol that includes a sufficient number of mice so that there are enough data points to create a phase response curve that effectively describes the time-dependent effect of GA on circadian rhythms. The reason is that mice, when isolated in constant conditions (constant darkness) become active (start their active phase) at a different time of the day, Thus, even though this breed of mice have comparable FRP, the CT of treatment might vary between mice.

Sevoflurane anaesthesia administered to rats for eight hours in constant darkness at only one time point (CT 5) did not cause any phase shift in circadian rhythms of locomotor activity in the running wheel (Anzai et al., 2013). In contrast, studies with propofol administered to free running rats provoked one-hour phase advances in circadian rhythms of voluntary locomotor activity in the running wheel, when given during the transition from sleep to wakefulness (CT 10 to 12). However, there was no phase-shifting effect when propofol was administered at other times of the day. In this study, only 16 animals were used to build a six-time point PRC for propofol (Challet et al., 2007). It would be very interesting to see whether increasing the $n$ would show different results. In fact, with a slightly higher sample size, in entrained rats, propofol showed a phase
advance of 55 to 70 minutes of general locomotor activity measured by an implanted radio-telemetric recorder when administered at CT10 to CT16 (Dispersyn et al., 2009).

Isoflurane anaesthesia produced two-hour phase advances in spontaneous locomotor activity measured by a dielectric constant sensor with counters set beneath the cylindrical plastic cage, only when administered during the active phase in rats (Kikuchi et al., 2013b). However, general anaesthetic was only administered at CT 6 for the resting phase administration time, and at CT 18 for the active phase. Thus, it could be possible that general anaesthetic with isoflurane does shift circadian rhythms of locomotor activity during the resting phase, but at a different CT. Interestingly, when looking at a different animal species - such as the honey bee, which is a diurnal insect- isoflurane administered early in the morning (active phase) caused a profound phase delay, but no shift was observed when administered during the subjective night (rest phase) (Cheeseman et al., 2012; Ludin et al., 2016) (Table 1.5).

In conclusion, despite the amount of evidence published demonstrating that GA disrupts the circadian clock and circadian rhythms, at a molecular, cognitive and behavioural level, there is considerable disparity between studies on the type of effect. The mechanism by which this disruption happens is still unclear, as well as the time-dependency of this effect. It would be helpful to have a phase response curve for each anaesthetic agent used in theatre that described the anaesthesia-induced phase-shifting effect on circadian rhythms over a period of 24 hours. In order to do so, it is also necessary to develop a standardised protocol applicable to different anaesthetic agents and animal models, to make the results comparable. This would allow us to determine the way anaesthesia shifts circadian rhythms and disrupts the circadian clock.
It is also unclear what the combined effect of anaesthesia and light is on mammals. Again, due to the disparity between studies, it is not yet possible to determine whether light could be used as a therapeutic strategy to reduce the circadian disruption produced by GA, or whether GA is pharmacologically blocking the light induced phase shift or whether this effect is time-dependent. Further research is needed in order to increase our understanding of the clinical impact of light and GA on the clock.

1.5 Aim of the current study
Why anaesthesia causes post-operative sleep disruption is unclear. In this thesis it is hypothesised that this is partly due to the effect of anaesthesia on the circadian clock. It is clear from human and animal studies that GA disrupts circadian rhythms and therefore disrupts the circadian clock. However, due to confounding factors such as surgery and previous illness, as well as the lack of consistency in the study design between experiments, the specific effect of GA on circadian rhythms, and the mechanism by which this occurs, still remains largely unknown.

The effect of GA on circadian rhythms seems to be dependent on the time of the administration, the anaesthetic agent, protocol of administration, and the study subject. Thus, the purpose of this thesis is to examine and compare the effect on circadian rhythms of the two most widely used anaesthetic agents (isoflurane and sevoflurane) administered to mice at different times of the day in constant darkness.

Here, it is hypothesised that depending on the phase of the circadian oscillators at the time of the administration of the anaesthetic agent, mice locomotor activity rhythms will exhibit different shifts (if any), which may also differ in magnitude and direction.
In this thesis, the effect of light and anaesthesia administered concomitantly, on the circadian clock, were also studied.

Light is the *zeitgeber* that influences the most circadian rhythms (Berson *et al.*, 2002). Studies from our group on bees have demonstrated that light can decrease the shifting effect evoked by GA in bees’ locomotor activity rhythms (Ludin *et al.*, 2016). However, for small mammals, GA has been reported as a pharmacological blocker of the light-induced phase shift in rodent behaviour (Colwell *et al.*, 1993).

In this thesis, I hypothesise that, as in bees, light can reduce the GA- induced phase shift in mice circadian rhythms of behaviour, and therefore on the circadian clock.

Hence, the main questions that I plan to address and solve in my thesis are as follows:

1. Do general anaesthetic agents shift circadian rhythms of locomotor activity in mice?
2. Do general anaesthetics have different effects at different times of the day?
3. Do different anaesthetics have different effects?
4. Do light and GA administered together have different effects on the clock depending on the time of administration?

It is thus the target of my thesis to consider the role of chronobiology in mice anaesthesia-induced circadian disruption, and to further investigate the potential use of light to reproduce previous results observed in bees to restore or ameliorate the circadian function after an anaesthesia treatment, using the mouse as an animal model.
II. Chapter 2

2. Materials and Methods development

In this chapter the process of developing a protocol to administer two different volatile halogenated anaesthetic agents (isoflurane or sevoflurane), and the protocol development to record robust and steady locomotor activity rhythms to study the effect of GA on the mouse circadian clock, is explained. The protocol developed to study the interaction of light and anaesthesia with either isoflurane or sevoflurane on locomotor activity rhythms, is also explained in this chapter.

To test my two hypotheses, 1) GA evokes time-dependent phase shifts on circadian rhythms of locomotor activity in mice; and 2) light administered simultaneously with a volatile anaesthetic agent can influence the GA-induced phase-shifting effect on circadian rhythms of locomotor activity in the animal model studied - mice.

All my experimental protocols were based on Aschoff’s type one protocol (See Chapter 1, section 1.3.6 Phase Response Curves: The shifting effect of light).

2.1 Materials

2.1.1 Animals

The animals studied in this thesis are 8 to 12-week-old male C57BL/6 mice derived from the colony at the Vernon Jansen Unit, University of Auckland (C57BL/6VJU). Ethical approval for all studies described was obtained from the University of Auckland Animal Ethics Committee (approval numbers: 1654 and 1654/2).
The rationale for using the C57BL/6VJU strain was based on its robust and persistent circadian rhythms in wheel running behaviour, as is characteristic for this particular animal strain (Azzi et al., 2014; Chen et al., 2008; Duman et al., 2008; Novak et al., 2012; Ozburn et al., 2008; Pendergast et al., 2010; Sosunov et al., 2015; Yoo et al., 2004). This strain also has the same background as the transgenic Per2:LUC reporter line which our group is using in parallel experiments (reference number of the Ethics approval: 001128), enabling comparisons of gene expression and behavioural data.

Male mice were used in order to avoid the confounding effects on behavioural rhythms (Hildebrandt et al., 2008; 2012; Jud et al., 2005). Gender has significant effects on pharmacokinetics, metabolism, and other physiological parameters (Czerniak, 2001). This might be due to the differences in hepatic enzyme production between females and males, hormone production such as corticosterone, which impacts glucose levels, and especially sex hormones, which levels vary even between females from the same species depending on the stage of the oestrous cycle (Hildebrandt et al., 2008). This adds an additional rhythmic component that could interfere in the study of the impact of GA on circadian rhythms in mice. Animals under eight weeks of age were not used as they are unable to thermoregulate sufficiently when housed independently and they do not display robust wheel running rhythms (Jud et al., 2005; Valentinuzzi et al., 1997). Animals over 12 weeks were not used as older mice have shown to decrease the intensity of their performance on the running wheel (Valentinuzzi et al., 1997).

2.1.1.1 Housing

Animals were housed individually in standard transparent mouse cages (Dimensions: 8.4" x 14.25" x 5.6" H) fitted with a rodent running wheel (Figure 2.1a). Twelve cages were placed equidistant
from each other in four environmentally controlled chambers (Figure 2.1b). All standard institutional practices were followed for animal husbandry. A handful of bedding was placed in each cage (too much bedding prevents the running wheels from moving freely), and the cages were placed in position in the light-tight environmentally controlled chambers, and connected to the appropriate infrared (IR) silent micro switch (Figure 2.1a).

The light-tight chambers were fitted with a set of 14 LED light panels mounted above each shelf of the chamber (Figure 2.1b). This system could provide light intensities ranging from 100 to 1000 lux. Light cycles and light intensity in each environmentally controlled chamber could be independently controlled.

![Figure 2.1: a) Mouse cage with activity wheel and IR sensor for monitoring wheel revolutions. b) Circadian cabinet filled with 12 mouse cages each with running wheels. Mouse cages were placed equidistant from each other relative to the LED light.](image)

The cabinets were ventilated, sound attenuated, and temperature and humidity controlled. Temperature and humidity were monitored throughout the duration of the experiments (Coulbourn Instruments, Harvard Apparatus Company).
The mouse circadian system is extremely sensitive to even very low levels of illumination in the visible spectrum (Jud et al., 2005; Pendergast et al., 2010), therefore after entrainment animals were maintained under strict darkness (<0.1 lux). Extended periods of constant conditions are not detrimental to animal health or wellbeing (Jud et al., 2005). Any animal husbandry and the anaesthesia treatment were conducted in complete darkness and performed with the aid of infra-red nocturnal vision goggles (Figure 2.2).

![Night vision goggles](image)

Figure 2.2: Night vision goggles. Pulsar Edge 1x20

The cabinets were maintained in a room fitted with a dark room revolving door to prevent accidental light ingress while performing procedures in the dark.

### 2.2 Methods

To test my hypotheses, I designed an experimental protocol to record mice wheel-running locomotor activity rhythms, and to administer anaesthesia to multiple mice at the same time. This system allowed me to study changes on mice behavioural rhythms before and after the anaesthesia treatment.

The protocol designed for recording behavioural rhythms and for delivering the anaesthetic agents are described in the following sections.
2.2.1 Protocol development for recording locomotor activity rhythms

Voluntary wheel-running activity was chosen over monitoring general movement (e.g. with photosensors), as the circadian marker to evaluate the effect of GA on circadian rhythms. Because wheel-running activity only represents intended running of the animal, it therefore provides robust activity patterns that lack background activity (Albrecht et al., 2001; Jud et al., 2005).

Once animals were collected from the animal unit, they were placed in their activity cages (Figure 2.3a). The wheel-running locomotor activity was monitored using infra-red silent sensors connected to the running wheel of each cage (Figure 2.3b). The sensors detected the number of wheel revolutions in any given two-minute epoch. The data were binned in five-minute epochs and transformed into actograms (Figure 2.3c) (ClockLab, Goulburn instruments, USA).

Figure 2.3 a) Three environmentally controlled cabinets each containing 12 cages. b) Infrared silent sensor. c) Actogram of wheel-running activity of a single wild type mouse maintained in LD12:12 with LD transitions at 6am from day 2 to 6. Horizontal white lines represent lights on. Blue vertical lines represent the mouse locomotor activity on the running wheel.
Animals were acclimated to the environmental chambers in 24-hour light-dark cycles (LD 12:12) with lights on at 6am and lights off at 6pm. Lighting conditions were maintained similar to those from the animal unit where the animals were bred and habituated to the cage and circadian cabinets. Habituation lasted one to seven days before the recording and analysis of wheel-running activity commenced. After habituation, animals were entrained in LD 12:12 for 10 days, in order to ensure stable entrainment to the light cycle. Following entrainment, animals were transferred into constant conditions (constant darkness, and constant temperature and humidity) for a ‘baseline’ period of 14 days to determine their free running period. These 14 days were necessary to avoid the confounding effects of “transients” and the “after-effects of entrainment”, which could have masked the effects of anaesthesia on the rhythms (see Chapter 1, section 1.3.4 The concept of entrainment). Transients can persist for up to seven days following a light-dark (L-D) transition, thus 14 days are necessary to provide a minimum period of seven days for stable free run prior to the administration of anaesthesia (Azzi et al., 2014; Chabot et al., 2012; Chen et al., 2008; Jud et al., 2005; Yoo et al., 2004). On day 15, animals received the anaesthesia treatment. Following anaesthesia, locomotor activity was monitored in constant conditions for a further 14 days (Figure 2.4).
Figure 2.4: Experimental protocol design. Figure showing two double plotted actograms (A and B) from two individual mice. White spaces represent the light cycle, blue spaces represent dark cycles. The red rectangle represents the six hours of GA (A), the blue line represents the ten minutes treatment (B) on day 25. X axes represent the time of the day. The Y axes represents the days. GA = GA. FRP = free running period.

Data were collected continuously during the whole experiment via the proprietary ClockLab data collection software suite. Resulting activity records (or actograms) were analysed for changes in period and phase following exposure to GA.

Given the challenges posed by working in constant darkness with night vision goggles, and the novelty of the locomotor activity recording system, a pilot study with six animals was performed.

This first pilot study revealed problems with the locomotor activity recording.
2.2.1.1 Disrupted locomotor activity rhythms in mice. Animal behaviour and infrared sensors

Despite the fact that inter-individual variability in wheel-running activity (active versus resting phase) between animals was expected, the data collected during the pilot study showed that the animals were arrhythmic prior to anaesthesia (Figure 2.5).

As shown in Figure 2.5, mice locomotor activity in the running wheel was not as robust as expected. In actogram B, the sensor connected to the cage of one mouse detected abnormal activity over a period of 24 hours (red circles), and then no recording was obtained for more than 4 days (red square), despite running wheel activity from the animal observed daily during this period. In actogram D, peaks of locomotor activity in the running wheel (red circles) were detected by the IR sensor and continued to be observed in the resulting actogram after day 25 despite there being no mouse in the cage. Furthermore, when animals were switched to constant darkness prior to anaesthesia, a free running period could only be observed in one out of the six animals (Figure 2.5, actogram F).

As mentioned before, this animal model was especially chosen for its robust and persistent circadian rhythms in wheel running behaviour, therefore I suspected there was a problem with my recording system.
Figure 2.5: Double plotted actograms of six mice during the pilot study (A-F). The light-dark cycles of entrainment are scheduled from 3am to 3pm. White space represents light whereas blue space represents darkness. The purple rectangles represent the 6 hours treatment.
Furthermore, abnormal readings were observed from some of the IR sensors, which recorded running wheel activity in the cabinets that were not being used for the experimental procedure (Figure 2.6).

![Figure 2.6: Actogram from one of the sensors detecting aberrant signals in absence of activity. The first patterns shown correspond to 12 days of light-dark cycles (LD) (red right brace). The second patterns shown correspond to the period of 17 days in constant darkness (DD) (blue right brace).](image)

These two issues led me to further test my recording system and to investigate the environmental surroundings of my animals at the animal unit.

I discovered that construction work was being performed at the animal housing unit, which was found to be disturbing the animals and disrupting their activity patterns. Also, a technical issue with the IR sensors meant the beams from adjacent cages were being detected by incorrect sensors, resulting in cross talk between the infra-red sensors (see Figure 2.6). Interestingly, this effect was stronger during the dark period (Figure 2.6), suggesting that during the light cycles the bright light was attenuating the reflection of the infra-red signal from one sensor to the next one.
After consultation with the managers of the animal unit, an agreement was reached that I would be notified well in advance of any building work that was due to happen at the animal unit, so I could re-schedule my experiment if required to avoid disruption of the animals’ behaviour. To avoid the detection of infrared light emitted from adjacent silent sensors, I built light proof walls between cages. With these amendments, the cross talk between the infrared light beams of different cages was avoided. Defective sensors were replaced.

With these measures, a stable and reliable IR sensor recording of animals’ locomotor activity was achieved. However, the animal’s locomotor activity rhythms were still not as robust as expected. The actograms from the second pilot study (Figure 2.7) showed that most of the animals had increased locomotor activity more or less at the same time (between 8-11am). Interestingly this behaviour was mimicked in both cabinets used for this experiment. An example of this abnormal behaviour is highlighted with red circles in Figure 2.7. This was found to coincide with a new research group moving equipment into the room next to mine at the animal unit. Again, I suspected the noise was disrupting the animals’ circadian rhythms of locomotor activity.
Adjustment to periodic environmental changes is one of the key properties of the circadian system (Pendergast et al., 2010) (see Chapter 1, section 1.3.1 An overview of circadian rhythms). Circadian rhythms can be influenced by noise, vibrations, temperature, humidity, or pheromones. Due to this, it is important to perform any wheel-running experiment under defined environmental conditions (Jud et al., 2005).
The effects of noise on disrupting mouse behavioural rhythms can further be illustrated in Figure 2.8. The red circle highlights animal locomotor activity rhythms disruption during the entrainment and the purple circles during the free running period.

![Double plotted actogram of a mouse (mouse ID: 1.3.3) during its entrainment period maintained in LD cycles 12:12 with LD transition at 1pm for 10 days and its release into constant darkness for a period of 14 days. The red circle shows the disrupted behavioural activity during the entrainment. Purple circles highlight disruption due to noise during the free-running period.](image)

The disrupting effect of other research groups’ noise resulting in arrhythmic data on animals’ locomotor activity recordings was still an issue. After lengthy discussions, a new isolated location in the animal unit was allocated from which robust locomotor activity rhythms could be recorded.

A comparison of two double-plotted actograms from mice in LD cycles from the second pilot study - before and after relocation to a new room - is shown in Figure 2.9, demonstrating the difference in quality of data obtained between the two locations. The red circles show the disrupted
behavioural activity by the noise in the former room (Figure 2.9 a and b) which was completely absent during recordings in my new room (Figure 2.9 c and d).

![Figure 2.9](image)

Figure 2.9: Comparison between the actograms obtained during the Isoflurane phase response curve (PRC) experiment before (a and b) and after (c and d) changing rooms and getting a soundproof environment. Red circles mark the influence of the noise on mice locomotor activity before the move (a and b).

With all these measures, steady, reliable, and robust locomotor activity rhythm recordings were finally achieved.

### 2.2.2 Pilot protocol development for administering the GA treatment to multiple mice concurrently

An essential part of this thesis was to anaesthetise multiple animals concurrently for up to six hours, which logistically is very demanding.

The development of a protocol to enable concurrent anaesthetisation was more challenging than anticipated.
I first designed an experimental protocol for administering GA to six mice at the same time and purchased a commercially available Kent scientific “VetFlo” system. The system was composed of six inlets and six outlets, connected to a scavenger and six nose cones to allow for simultaneous yet independent anaesthetising of the mice (Figure 2.10a).

Each of the animals received the same concentration of isoflurane with the same flow rate, (2% isoflurane in 100% oxygen in a flow-rate of 1L/minute through a nose cone (Figure 2.10b)). This concentration was maintained during stage one (induction) and stage two (maintenance) of the anaesthesia treatment. Isoflurane, when administered at 2%, is an effective concentration to anaesthetise a large range of taxa, which is also very close, although slightly higher, to 1MAC in mammals. This concentration was chosen based on this statement and on previous findings from our group’s work with bees (Cheeseman et al., 2012) and flies (Li et al., unpublished), that showed that 2% of isoflurane shifts the circadian clock by evoking shifts on circadian rhythms of locomotor activity.

Body temperature was monitored and maintained using a “Right Temp” module and a heating pad (Kent Scientific). Oxygen saturations and heart rates were monitored on each mouse during the treatment, using Kent’s Physiosuite “Mouse Stat” system every 5-10 minutes. The Physiosuite “Mouse Stat” system is an electronic device designed for use on animals undergoing anaesthesia and consists of a rectal temperature probe and a paw clamp with a heart rate/oxygen saturation sensor. In order to maintain the constant conditions required for this type of chronobiological experiment, the other two parameters I constantly monitored during the whole experiment were environmental temperature and the humidity in the laboratory.
The use of inhaled general anaesthetic agents prevents the corneal blink reflex in mice (Gaertner et al., 2008), thus my mice kept their eyes open during GA administration. In order to prevent corneal drying and eye damage during the procedure, “Genteal Eye Gel” - a sterile, hypo-tonic, long acting lubricant gel (Gaertner et al., 2008; Gargiulo et al., 2012) was applied. Fluid balance was maintained by subcutaneous injections, given manually, at a rate of 10 ml/kg of body weight per hour, using 0.9% saline as suggested by the University veterinarian.

Figure 2.10: Figure showing a) The VetFlo system composed of tubes circuit with 6 inlets connected to one scavenger and 6 outlets connected to the vaporiser. b) Mouse under anaesthesia with a nose cone.

The pilot study was performed to acquire practice in the techniques required to anaesthetise animals and maintain animal care under limited visibility (night vision goggles, see Figure 2.2).

The first pilot study included six animals. These animals were divided in two groups, three control animals and three who received treatment. The control group received only 10 minutes of GA, and the treatment group received six hours of GA. Previous studies from our group on honey bees
showed that a 10-minute period of anaesthesia does not cause shifts in their circadian rhythms locomotor activity (Cheeseman et al., 2012). This period of anaesthesia was thus used as a control for potential effects of animal handling and anaesthetic gas exposure (Fueger et al., 2006; Gargiulo et al., 2012; Hildebrandt et al., 2008).

GA with isoflurane (2% in 100% oxygen) was administered in this pilot study at a single time point, at a flow rate of 1L/m to six mice. The protocol design is shown in Table 2.1.

<table>
<thead>
<tr>
<th>Study Stage</th>
<th>Treatment</th>
<th>Number of Days</th>
<th>Cumulative Days</th>
<th>Number of animals</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Light-dark cycles (LD:12:12)</td>
<td>10</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>Constant darkness (DD)</td>
<td>14</td>
<td>24</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>6 hours GA (isoflurane) or control (10 minutes GA)</td>
<td>1</td>
<td>25</td>
<td>3 mice GA for 6 hours or 3 controls 10 minutes GA</td>
</tr>
<tr>
<td>4</td>
<td>Constant darkness (DD)</td>
<td>14</td>
<td>39</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>Euthanasia</td>
<td>1</td>
<td>40</td>
<td>6</td>
</tr>
</tbody>
</table>

Table 2.1: Protocol of the pilot study to test the effect of GA with isoflurane in mice circadian rhythms of locomotor activity and number of animals involved. (GA = GA).

Although all procedures to maintain animal welfare were strictly adhered to during the pilot study, maintaining the animals under anaesthesia for six hours with a high survival rate was still a challenge.

2.2.2.1 Animal’s survival and welfare
Two animals out of three died during the 6-hour treatment, prompting the termination of the pilot study.
In order to rule out the possibility that the procedure I used to anaesthetise the animals was putting them at risk, I consulted the university veterinarian who conducted post-mortem examinations on the deceased animals. This revealed that the deceased animals had oedema and haemorrhages in all lobes of the lungs which would have severely affected gas exchange. Samples were sent to NZ Vet Pathology for histology. The results indicated that this was likely caused by hypersensitivity or an allergic reaction. Together with the veterinarian, I concluded that the death of the animals was due to pre-existing illness, rather than the method of anaesthesia, perhaps indicating underlying respiratory disease.

With the university veterinarian present, I performed the anaesthesia procedure on one of the surviving mice using the same treatment as that for the pilot study. The veterinarian found no fault with the procedure I was performing nor in the anaesthesia system. Thus, the theory of previous illness seemed valid as a cause of animal death in the pilot study.

As neither the post mortem examinations nor the consultation with the veterinarian revealed any failing in my anaesthesia protocol design that were putting animals at risk, I undertook a second study with a new set of animals whose health was pre-assured by the veterinarian before the experiment commenced.

The second study involved 24 animals. This number was chosen in order to optimise my working capacity (as 48 animals would be required for the actual experiments in this project).

Assistance from the University veterinarian was provided during the beginning of the six-hour anaesthesia procedure to assess animal welfare, this time in constant darkness using the night vision goggles.
Four mice died during the six-hour anaesthesia treatment in the second study. The animals were confirmed to be disease-free at the start of this study, thus this outcome led us to re-assess all of my experimental conditions. We discovered that the environmental temperature of the animal unit was $\leq 19 \pm 1^{\circ}$C. According to published literature, environmental temperature is a critical factor for mouse health, particularly when they are housed alone (as in my experiment). Normal room temperatures for mice should be between 21°- 23°C (Cesarovic et al., 2010; Comas et al., 2008; Comas et al., 2006; 2007). This could have potentially weakened the animals’ immune systems and compromised their health during the anaesthesia. Other researchers have reported experiencing similar outcomes to mine.

Following discussions with the managers of the animal unit, the temperature of our room, and the entire animal unit, was increased to improve animal welfare.

In addition, further discussion about methods of administering anaesthesia to multiple mice for six hours led us to the possibility that animals were rebreathing their own exhaled CO$_2$, which, together with the low temperature, could have been compromising survival.

I also sought advice overseas, initiating a collaboration with researchers at the Department of Anaesthesiology in the University of Nanjing, China, who had successfully conducted similar behavioural experiments. This led to the development of a new protocol for the administration of anaesthesia.

In the new anaesthesia protocol, the nose cones were replaced by a less sophisticated system, but one that allowed us to have more control over the animals’ reaction to the anaesthetic.
A specific anaesthesia chamber was designed and built at the workshop of the University of Auckland. The chamber was made from acrylic glass with a capacity of 9 litres. The box was sealed with an acrylic sliding lid. Two holes were made in parallel walls to install the inlet that connects the chamber with the anaesthesia system (vaporiser and oxygen cylinder), and an outlet that connects the box to a scavenger that absorbs excess anaesthetic from the circuit. An acrylic plate with drilling holes was placed two centimetres from the bottom so I could line the floor of the anaesthesia chamber with a colorimetric control layer of soda lime that absorbs excess CO2 produced as a result of mouse respiration. The separating walls also had equidistant drilling holes, so that the concentration of isoflurane stayed constant and identical everywhere in the chamber (Figure 2.11). A heating pad was placed underneath the anaesthesia chamber. The temperature of the platform where the animals were placed was constantly monitored with a testo data logger.

Figure 2.11: Sketch of the new system used to deliver the anaesthetic agent to six mice simultaneously. Instead of the previous nose cones, an anaesthesia chamber specifically designed and built by the University of Auckland workshop, was used for these experiments.
2.2.3 Final optimised protocol to study the effect isoflurane GA on circadian rhythms in mice

The major challenge to overcome was to deeply anaesthetise the animals for six hours without impeding animal health. It has previously been reported that isoflurane administration for more than 5 hours, in concentrations of 1 MAC or higher, in this breed of mice tends to cause perturbations of body temperature and a drop in respiratory rate (Wiersema et al., 1997), moderate acidosis (Szczęsny et al., 2004), and heart failure (Ewald et al., 2011).

In the first studies described, I had used Kent’s Physiosuite “Mouse Stat” system to measure oxygen saturation and heart rate every five minutes during the anaesthesia procedure as a means of monitoring animal health and welfare. However, despite continued monitoring using this system, the measures obtained provided no indication that the health of any of the mice was deteriorating. We therefore decided to adopt other means of monitoring in the final protocol.

This led to the development of the final protocol which successfully allowed us to study the effect of a general protocol for anaesthesia on circadian rhythms of mice locomotor activity rhythms.

The new anaesthesia protocol was divided into three stages: induction, maintenance, and emergence. The concentration for the induction stage was considerably increased from my previous protocol to achieve a faster induction of anaesthesia. Instead of isoflurane at 2%, the anaesthesia chamber was pre-filled with 5% of isoflurane in 100% oxygen (4L/minute flow) (Gargiulo et al., 2012; Valentim et al., 2008). The time required to fill the chamber with the desired concentrations was determined by using an anaesthesia gas monitor provided by Auckland City Hospital. A 0.2mm needle was attached and sealed into the anaesthesia chamber, and a connector was attached to the gas monitor to avoid any hazardous leaks of the anaesthetic agent into the room.
during the testing process. The time required to reach a concentration of 5% isoflurane in the anaesthesia chamber was five minutes.

Animals were again divided in two groups, one group receiving six hours of GA (treatment group) and the other receiving 10 minutes (control group). After pre-filling the anaesthesia chamber with isoflurane at 5% in 100% oxygen at a flow rate of 4L/minute, mice were introduced into the anaesthesia chamber and induced into an anaesthetic state. The animals were knocked down in a matter of seconds (30±5 seconds). The anaesthesia-induced stage was confirmed by the absence of movement plus the lack of response to a tail pinch. For the maintenance of anaesthesia (the second stage of the anaesthesia protocol), the concentration of isoflurane was reduced from 5% to 1.5% once the animals were fully knocked down. The different concentrations were regulated by using the vaporiser wheel regulator.

For the control comparison, the mice were induced into the anaesthetic stage with 5% of isoflurane in 100% at a flow-rate of 4L/minute. During ten minutes of anaesthesia maintenance, control group mice were constantly administered 2% isoflurane in 100% oxygen to mimic the anaesthesia conditions of the six-hour treatment. The flow rate remained the same for the whole treatment. The tail pinch reflex was used to monitor anaesthesia depth. In order to assess animal welfare, breaths per minute were counted every five minutes in each mouse. I used a stop watch and an anaesthesia monitoring sheet to record animal welfare during the anaesthesia treatment (Figure 2.12). If an animal suffered a decrease in its respiration rate under 25 breaths per minute, I placed this mouse in a separate sealed chamber with 100% oxygen until it reached the respiration rate of recovery (30 breaths per minute). Once the animal recovered to the target respiration rate it was then returned to the anaesthesia chamber with isoflurane. If more than a third of the animals were found
to have a lower respiration rate than the safe limit, the vaporiser was switched off and only oxygen was administered until the majority of animals reached the recovery respiration rate of 30 breaths per minute. Usually a pinch test (tail or foot pad) is used to monitor depth of anaesthesia, as this reflex is a much more sensitive indicator. However, this meant I had to open the lid of the anaesthesia chamber, exposing myself and my assistant to the anaesthetic gas. Thus, respiratory rate alongside tail movements and the loss of righting reflexes was used instead as a safer measure of the depth of the anaesthesia during the maintenance and emergence.

Following new advice from the university veterinarian, we no longer administered subcutaneous injections of 0.9% saline. This change was made due to post-mortem findings from one of the deceased animals in the previous study that the bladder was very full. As the animals were only under the effect of the anaesthesia and no surgical procedure was conducted, no loss of fluids was likely to be experienced, and hence saline administration was unnecessary.

With this protocol, the major goal was finally achieved: 98.8% of the animals anaesthetised either for 10 minutes or six hours survived the entire experiment.
The vaporiser was switched off 5.5 hours into the experiment. Due to the long offset of the anaesthetic, this was necessary to ensure the animals were only under the effect of the anaesthesia for six hours in total. After this time, animals were left in the anaesthesia chamber with oxygen until their recovery. Seventy-eight percent of the animals showed righting reflexes 30±15 minutes after switching off the vaporiser. Once animals exhibited the righting reflex, they were placed in new cages with new food, water, bedding, and nesting. All animals were weighed after the anaesthesia while still anaesthetised. The effect of the anaesthetic agent was considered finished.
when the animals woke up and stood up on their four legs. Animals were monitored until ambulatory movement was observed.

2.2.4 Protocol to study the effect of sevoflurane GA on circadian rhythms in mice

Using the final protocol I developed for isoflurane and with all that I learned in the process, I devised a protocol to enable the construction of a sevoflurane PRC. The survival rate with this protocol and set of experiments was 100%.

Using my isoflurane PRC protocol, I adjusted the anaesthesia concentrations based on the MAC for sevoflurane, so that my data could be directly comparable (Table 2.2).

<table>
<thead>
<tr>
<th>Study stage</th>
<th>Treatment</th>
<th>Number of days</th>
<th>Cumulative days</th>
<th>Number of mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Entrainment</td>
<td>10</td>
<td>10</td>
<td>105</td>
</tr>
<tr>
<td>2</td>
<td>FRP</td>
<td>14</td>
<td>24</td>
<td>105</td>
</tr>
<tr>
<td>3</td>
<td>GA (sevoflurane)</td>
<td>1</td>
<td>15</td>
<td>80/25</td>
</tr>
<tr>
<td></td>
<td>6h/10 minutes</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>FRP</td>
<td>14</td>
<td>39</td>
<td>105</td>
</tr>
<tr>
<td>5</td>
<td>Euthanasia</td>
<td>1</td>
<td>40</td>
<td>105</td>
</tr>
</tbody>
</table>

Table 2.2: Schedule of the sevoflurane PRC protocol. Treatment, number of days, cumulative numbers of days and numbers of mice are specified in five different study stages. (GA = General Anaesthesia).

Both isoflurane and sevoflurane are very similar regarding their adverse effects. Respiratory depression is the major adverse effect for both agents, and it is believed to be a result of inhibition of the respiratory control systems (e.g. feedback control central respiratory centres, various chemoreceptors, pulmonary reflexes, and neuronal input). Inhalational anaesthetics alter oxygen
supply and CO₂ elimination. Respiratory depression is often accompanied by acid-base imbalance (alterations in pH and HCO₃), and changes in the depth and frequency of respiration (Cesarovic et al., 2010).

With my target of preventing fatal outcomes (as previously experienced in my initial studies with isoflurane), I monitored respiratory rate during the whole treatment, following the same steps which led us to success in my experiment with isoflurane. The anaesthesia procedure was also carried out in my anaesthesia and oxygen chambers.

The onset of sevoflurane-induced anaesthesia is reported to be shorter than that described for isoflurane (Golembiewski, 2004). This made us think that theoretically, I would not need to use as high a dose of sevoflurane to achieve fast induction of anaesthesia as I did with isoflurane. However, in practice, I found that the onset of the anaesthetic state using sevoflurane was similar to what I observed with isoflurane. Consequently, as with isoflurane, a higher concentration was used for induction (5%). A concentration of 2.6% of sevoflurane in 100% oxygen at a flow rate of 4L/min was used for anaesthesia maintenance. The sevoflurane concentration of 2.6% corresponds to 1MAC for the animal model used (C57BL/6VJU) (Wiklund et al., 2008).

2.2.5 Calculation of the CTs of the intervention and generation of a phase response curve for each anaesthetic agent studied

The calculations of the CTs of the interventions were estimated through the LD scheduled on the animals’ 10-day period of entrainment. These calculations were based on the average FRP for C57BL/6 mice from the literature (23.7 hours) (Schwartz et al., 1990b). An FRP of 23.7 hours means animals wake up 18 minutes earlier each day. After 14 days in DD, the animals will wake
up 252 minutes before they did under LD conditions; 252 minutes = 4.2 hours. Using this estimate, the intervals at which lights were turned on and off in cabinets during the entrainment phase was calculated in order to schedule the time of anaesthesia to correspond with the desired CT for the animals and working hours at the animal unit.

For example:

For anaesthetic treatment administered at 9 am on day 25 of the experiment to correspond with approximately CT0 for mice, lights were turned on at 1pm and off at 1am. Therefore, during the entrainment period CT\(0 = 1\)pm, which is the beginning of the resting phase for mice since they are nocturnal animals. After 14 days in DD, CT\(0\) will be approximately 4.2h earlier than this (i.e. 9am); allowing the anaesthesia procedure to be performed from 9am to 3pm.

Standard PRC protocols involve the administration of a clock-shifting agent at multiple times of the day throughout the circadian cycle. Rodent light PRCs are sometimes restricted to the subjective night, with light pulses normally given only at cardinal wild-type PRC time points of the circadian cycle. However, I had no \textit{a priori} knowledge of when the phase-shifting times of anaesthetics were going to be. Thus, the GA-induced phase shift experiments were conducted at six different CTs: CT\(0\), CT\(4\), CT\(8\), CT\(12\), CT\(16\) and CT\(20\).

The number of animals required to build a phase response curve for six hours of treatment which significantly represented the animals’ behaviour was calculated to be between 80±5 animals. Power calculations showed that I only needed 3-7 animals per time point. However, in practice, due to greater inter-individual variability between active and resting phase in the six-hour treated
mice, at least seven animals were required for the intervals of circadian time (CT) where phase shifts were observed to be higher (CTs 4 to 12) (see 3.1.1. Isoflurane-induced time-dependent phase shift in circadian rhythms of locomotor activity rhythms in mice and 3.2.1 Effect of sevoflurane GA on the phase of circadian rhythms of behaviour). For the construction of the PRC, data were plotted as the average of the phase shift in a 4-hour bin ± SEM.

At the start of the entrainment period (10 days), the times at which lights were switched on and off were altered for each cabinet so that anaesthesia could be performed during normal working hours, and this would also correspond to different CTs for the animals in each cabinet. This enabled 1) animals to habituate to the environmental control chamber, 2) stable recording of their entrained rhythms to be obtained throughout the day, and 3) maximum success of experiments by ensuring anaesthesia was performed at a time of day when support was readily available.

### 2.2.6 Protocol for analysis of data using ClockLab

The following protocol was used to process all data (actograms) that was collected and analysed using ClockLab:

1. Download data. Either from the back up on the ClockLab folder in the U-drive of the Department of Anaesthesiology (data were saved and stored every two hours for the duration of the whole experiment, the backup was set to save to the server) or from the documents folder on the ClockLab computer at the animal unit.

2. Set template for analysis: this was an Excel spreadsheet labelled with the experiment and date, and columns organised to record the relevant information from each actogram.
3. Chi-squared ($\chi^2$) periodogram analysis was used to determine the tau ($\tau$) or fundamental period of each actogram before and after the intervention. The relevant days (seven days before treatment and seven days after treatment) were selected from the drop-down menu with chi-squared selected, and the $\tau$ is displayed onscreen.

4. Calculation of CT of intervention: this was calculated from what would have been the onset of mouse activity on the day of the intervention, extrapolated from the regression line of the onset of activity seven days prior to treatment. The CT was normalised for the different $\tau$ of the individuals using $\text{CT} \times \left(\frac{24}{\tau}\right)$.

Calculation of phase shifts: the onset of mice activity (CT 12) was the reference point used to determine change in phase and the CT of the intervention. Phase shifts were calculated on Days 1, 3 and 7 after the treatment, using extended regression lines from seven days before and after the intervention. Placing left and right cursors on the relevant day after the intervention revealed the direction and magnitude of the phase shift (Figure 2.13).

![Figure 2.13: Determination of a phase delay. This exemplar actogram shows the way in which ClockLab is used to determine the change in phase following anaesthesia (purple rectangle). The six-hour anaesthesia was administered on Day 9 on this actogram (Day 25 of the experimental protocol) at CT 8.6. Regression lines from seven days before the treatment (red) were compared to regression lines from seven days following the treatment. A phase delay of 2.42 hours (purple) was observed on Day 1 after anaesthesia. Phase delays of 2.26 hours (blue) and 2.03 hours (green) delay were observed on Days 3 and 7 respectively after treatment.](image-url)
On all except one occasion, the discrete phase shift was calculated by comparing the regression lines of the seven days before to the seven days after the intervention. For one of the experiments used to construct the isoflurane PRC, only six days of data were available after treatment was disrupted due to an unforeseen power cut (Figure 2.14).

![Figure 2.14: Double plot actogram from one single mouse. Light pulse due to the power cut (white line) on Day 17 is marked with a white circle. Control treatment of 10 minutes of GA is represented with a blue line and marked with a blue circle.](image)

### 2.2.7 Study of the concomitant effect of GA and light together using isoflurane or sevoflurane as the anaesthetic agents

To address my working hypothesis - “the phase-shifting effect of GA is mitigated by the concomitant administration of light during the anaesthesia procedure” - as seen in the honey bee (Ludin et al., 2016), I studied the effect of administering a light pulse at two different time points...
during anaesthesia, with either isoflurane or sevoflurane, on circadian rhythms of locomotor activity in mice.

The protocol for this experiment was based on previously published light PRC data in the same animal model and lighting system (Dallmann et al., 2011), so that results could be compared. However, since the magnitude of the interaction between light and anaesthesia on circadian rhythms in mice, over a period of 24 hours was unknown, and there is no light and anaesthesia PRC, due to the time limitation of my PhD research, only two time points were studied in these experiments for isoflurane and one for sevoflurane. The times for the concomitant treatment of light and anaesthesia together on the circadian rhythms of locomotor activity were selected as follows:

1. The time point (from the previously published light PRC work in C57BL/6 mice (Dallmann et al., 2011)) at which the light-induced phase-shift in locomotor activity rhythms in this breed of mice was maximal (from CT14 to CT17) following a light pulse of 400 lux for four hours.

The reason I chose this particular time point was due to the magnitude of the light-induced phase-shift at this CT Thus, any interaction between light and anaesthesia would be more relevant (at a chronobiological level) at this time point.

2. The time point from my own studies at which the anaesthetic agent tested induced the maximum phase-shift in mice circadian rhythms of locomotor activity.

Again, the reason to choose this CT was due to lack of knowledge regarding the interaction between these two zeitgebers and the concomitant effect on mice circadian rhythms of behaviour. Any effect at a chronobiological level would be more relevant at this time point.
For this experiment, I used two experimental groups per anaesthetic agent. One group received light by a light emitting diode (LED) module placed in the cabinet roof (Figure 2.15a) with a maximum intensity of 400 lux for four hours (control treatment). The other group received light and anaesthesia together (isoflurane or sevoflurane, depending on the anaesthetic agent being tested). The experiments were conducted at the same concentrations as the ones used in the previous GA PRCs protocols: 1.5% in 100% oxygen for isoflurane at a flow rate of 4L/m, and 2.6% in 100% oxygen at the same flow rate for sevoflurane.

The light intensity each animal received was double checked using an actiwatch (Figure 2.15b) and was equally distributed in the cabinets, so each animal received exactly the same light intensity (Figure 2.15a, Figure 2.15c). The range of light intensity each animal was exposed to at any one time varied from 395 to 400 lux depending on the position of the animal inside the cage. These values were measured with an Actiwatch monitoring system (Phillips Respitrronics) and were controlled and monitored with the ClockLab software I used to record and analyse locomotor activity data. The light intensity of the white LED lights of the circadian cabinets peaks at 450 and 550 nm.

![Figure 2.15: a) LED used during the light experiment, along with a light sensor which monitors the light intensity. b) the Actiwatch used to mimic the position of the animal inside each cage to measure the range of light intensity each animal may receive during the experiment. c) Equal distribution of the LEDs to ensure each animal received exactly the same light intensity.](image)
A new anaesthesia chamber that fitted inside the circadian cabinets was built (Figure 2.16). Thus, anaesthetised, and non-anaesthetised animals received the same light exposure.

Figure 2.16: Anaesthesia chamber specially designed to administer the anaesthetic agent inside of the circadian cabinets, so the treated group received exactly the same light as the control group.

The animals which received anaesthesia plus light on day 25 of the experimental protocol were first anaesthetised in constant darkness. Once the animals were under the anaesthesia stage (complete loss of consciousness measured by the loss of tail movements and righting reflex), the lights inside the cabinets were manually switched on for both animal groups (light, and light plus anaesthesia). Lights were only turned on for the length of the experiment, which was four hours. After this period, the animals were released into constant darkness where they recovered consciousness. Control animals were exposed to light without anaesthesia also for the four-hour period (Table 2.3). A warm pad was placed under the anaesthesia box inside of the circadian cabinet to keep the animals’ body temperature constant during the whole treatment, as was previously done during the anaesthesia PRC experiments. The temperature inside the chambers was monitored and recorded using a testo data logger.
<table>
<thead>
<tr>
<th>Study stage</th>
<th>Treatment</th>
<th>Number of days</th>
<th>Cumulative days</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Entrainment</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>Free running period</td>
<td>14</td>
<td>24</td>
</tr>
<tr>
<td>3</td>
<td>GA plus light 4h/ light 4h</td>
<td>1</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>no GA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Free running period</td>
<td>14</td>
<td>39</td>
</tr>
<tr>
<td>5</td>
<td>Euthanasia</td>
<td>1</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 2.3: Protocol for the study of the effect of light and general anaesthesia on circadian rhythms of locomotor activity when concomitantly administered. Two groups of mice were used. The control group received a light pulse of 400 lux for four hours and the treatment group received general anaesthesia (GA) using either isoflurane or sevoflurane plus light at 400 lux for four hours. Anaesthesia was administered under DD.

Eye drops were administered every 20 minutes to each mouse for the duration of the anaesthesia treatment to protect them from corneal damage during the experiment.

With these protocols, I built two PRC, one for isoflurane and one for sevoflurane, with its respective control treatments. This allowed the study of the time dependent effect of GA on the phase of the mice locomotor activity rhythm. I also studied the effect of GA on the fundamental period of the clock to further investigate the type of GA-induced phase shift on mice circadian rhythms (parametric or not parametric. See Chapter 1, section 1.3.4.1 Fundamental basis of entrainment: Mechanisms of photoentrainment), and compared the effect of light, and light and anaesthesia on the circadian function in mice.
2.2.8 Data analysis

The protocol used to analyse all data (actograms) collected using the ClockLab analysis software (Coulbourn Instruments, Pennsylvania, USA) was exactly the same as the protocol used for the isoflurane/sevoflurane PRC (see section 2.2.6 Protocol for analysis of data using ClockLab).

The data analysis protocol for the series of experiments looking at the concomitant treatment of GA plus light is identical to that described above in section 2.2.6 Protocol for analysis of data using ClockLab. A two-way analysis of variance (ANOVA) was used to determine whether the effect of the concomitant treatment (GA plus light) was significantly different from that of light only and/or GA only.
III. Chapter 3

3. Results

This chapter describes the different effects of two of the most commonly used general anaesthetic agents, isoflurane and sevoflurane, on the phase of mouse locomotor activity behavioural rhythms, the fundamental period of the mouse clock, and the combined effect of GA and light during the animals’ active and resting phases.

In order to investigate whether GA shifted behavioural rhythms in mice in a time-dependent fashion, I constructed a full phase response curve (PRC) for each anaesthetic agents studied. I also looked for changes in the fundamental period of the mouse clock \(\tau\) after the anaesthesia treatment to determine the type of shift (parametric or not parametric, see Chapter 1 1.3.4.1 Fundamental basis of entrainment: Mechanisms of photoentrainment) GA induces in behavioural rhythms. For that, I compared the \(\tau\) of each mouse before and after treatment and studied the time-dependency of this effect.

Finally, since previous studies from our department in bees showed that light and anaesthesia have opposing effects on the clock - with light reversing the GA-induced phase shift in bees’ behaviour.- I sought to investigate the effect of these two agents (light and GA), administered together at two different circadian times (CTs) (method and times of administration explained in section 2.2.7 Study of the concomitant effect of GA and light together using isoflurane or sevoflurane as the anaesthetic agents), on behavioural rhythms in mice.

My study was therefore based on two hypotheses:
1. Isoflurane and sevoflurane administered in constant conditions (DD), for six hours, phase shift circadian rhythms of locomotor activity in mice in a time-dependent manner.

2. Light administered simultaneously with a volatile anaesthetic agent, either isoflurane or sevoflurane, can influence the GA-induced phase-shifting effect on circadian rhythms of locomotor activity in the animal model studied - mice.

The data presented in this chapter involved the study of the mouse locomotor activity recording from 253 mice.

3.1 Effect of isoflurane GA on circadian rhythms of behaviour
In this section, I present the results describing the time-dependent phase-shifting effect of isoflurane GA on the phase of mice behavioural rhythms, as well as the effect of isoflurane on the fundamental period of the mouse clock.

3.1.1 Isoflurane-induced time-dependent phase shift in circadian rhythms of locomotor activity rhythms in mice
Mice locomotor activity was recorded and studied through actograms. An example of these actograms is presented below (Figure 3.1). These actograms show that isoflurane GA, when administered for six hours in DD, shifts mice circadian rhythms of locomotor activity in a time-dependent manner. Depending on the time at which isoflurane (red rectangle) was administered, it delayed (Figure 3.1 A), advanced (Figure 3.1 B), or had no effect (Figure 3.1 C) on the onset of mice wheel-running locomotor activity rhythms. The control treatment (10 minutes of isoflurane GA) (blue line highlighted with a circle), did not shift mice behaviour at any time (Figure 3.1 D, E, and F).
Figure 3.1: Exemplar of six actograms showing the effect of GA with isoflurane administered for either six hours (red rectangle) or ten minutes (blue line inside of a blue circle) on mice circadian rhythms of locomotor activity. Actograms A, B, and C show the phase-shifting effect evoked by six hours of GA. At CT 12 (A), where a phase delay of 0.89 hours on day one after treatment can be observed, at CT 20 (B), where there is a small advance on locomotor activity rhythms of 0.97 hours, and at CT 16 (C), where GA does not shift circadian rhythms of locomotor activity. Actograms D, E, and F show the absence of effect from a ten-minute isoflurane treatment on locomotor activity rhythms when administered at the same CTs as the six-hour treatment (D, CT12; E, CT20; F, CT16).
In order to study the time-dependency of the phase-shifting effect induced by six hours of GA on circadian rhythms of locomotor activity, the induced phase shift was measured on days one, three, and seven after treatment. As explained in Chapter 2, the phase shift evoked by GA was calculated for each animal by using extended regression lines from seven days before and after the intervention using the ClockLab data analysis system (see Chapter 2, 2.2.3 Protocol for analysis of data using ClockLab). With these data, a PRC describing the effect of GA on circadian rhythms over a period of 24 hours was plotted.

The figure presented is a weak type one PRC, characteristic of weak zeitgebers, displaying small phase shifts with continuous transition between delays and advances. The magnitude of the
isoflurane-induced phase shift on behavioural rhythms varies depending on the time of the administration of the anaesthesia treatment.

It can be observed in this mouse isoflurane PRC that the effect of a six-hour GA treatment with isoflurane on circadian rhythms of locomotor activity is different from the 10-minute control treatment. The confidence interval (CI 95%) of the difference between the mean of the induced phase shift after the six-hour treatment versus the control treatment, at each interval of time, was calculated. The CI 95% represents the accuracy or precision of an estimate. In a normal (gaussian) distribution (such as the distribution of the data describing the effect of GA on circadian rhythms), the CI is considered statistically significant when the 95%CI does not contain 0, the null hypothesis.

The CI of the difference between the mean of the induced phase shift of isoflurane administered for six hours versus 10 minutes does not contain 0 when isoflurane was administered between CTs 8-12, which means that the effect of the six-hour treatment was statistically significant and significantly differed from the control treatment at these CTs. Data are presented as the average of the phase shift ±SEM binned in four-hour epochs. After a six-hour treatment (n=80), the biggest phase delay in mice circadian rhythms of locomotor activity was observed when isoflurane was administered between CT 8-12. When isoflurane was administered between CT 20-24, small phase advances were observed in comparison to the control treatment. The value of the maximum phase delay, from a single mouse, was -1.69 hours and was observed at CT 11.9, whereas the maximum phase advance was 1.54 hours when isoflurane was administered at CT 23.9. No significant shifts were observed on mice circadian rhythms of locomotor activity after the control treatment of 10 minutes of GA (n=78), at any time. (Figure 3.2, Table 3.1).
Thus, as expected, when isoflurane was administered between CT8-12, the difference of the effect of the six-hour treatment was statistically significant and significantly differed from the control treatment (10 minutes).

<table>
<thead>
<tr>
<th>Circadian time (CT)</th>
<th>n</th>
<th>Six hours of isoflurane</th>
<th>n</th>
<th>10 minutes of isoflurane (control)</th>
<th>CI (95%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>8</td>
<td>-0.11 ±0.17</td>
<td>7</td>
<td>0.14±0.1</td>
<td>-0.25 (-0.768, 0.277)</td>
</tr>
<tr>
<td>4-8</td>
<td>10</td>
<td>-0.28±0.26</td>
<td>8</td>
<td>-0.17±0.2</td>
<td>-0.11 (-0.574, 0.353)</td>
</tr>
<tr>
<td>8-12</td>
<td>7</td>
<td>-1.11±0.27</td>
<td>6</td>
<td>0.28±0.23</td>
<td>-1.39 (-1.929, -0.841)</td>
</tr>
<tr>
<td>12-16</td>
<td>19</td>
<td>-0.3±0.11</td>
<td>20</td>
<td>-0.15±0.05</td>
<td>-0.16 (-0.472, 0.154)</td>
</tr>
<tr>
<td>16-20</td>
<td>15</td>
<td>0.09±0.16</td>
<td>23</td>
<td>-0.14±0.05</td>
<td>0.24 (-0.088, 0.560)</td>
</tr>
<tr>
<td>20-24</td>
<td>22</td>
<td>0.27±0.13</td>
<td>14</td>
<td>-0.06±0.06</td>
<td>0.32 (-0.012, 0.656)</td>
</tr>
</tbody>
</table>

Table 3.1: Table showing the confidence interval (CI95%) of the difference between the mean of the induced phase shift after the six-hour treatment, and the control treatment, at each interval of time of administration.

In addition, as explained in Chapter 1 (section 1.3.4 The concept of entrainment), the difference between masking and a phase shift is that a phase shift will persist for several days when the animals are under constant conditions. Thus, looking at the actograms in Figure 3.1 A and B, and Table 3.2, it can be seen that the phase shift evoked by six hours of isoflurane administered between CTs 8-12 persisted on day three after treatment and up to day seven.
Table 3.2: Data showing the phase-shifting effect of GA after six hours of isoflurane on mice circadian rhythms of mice locomotor activity calculated on day one, day three and day seven. Data are presented as the average of the phase shift evoked by the six-hour treatment and was binned in four-hour epochs ± SEM.

<table>
<thead>
<tr>
<th>Circadian time (CT)</th>
<th>n</th>
<th>Average phase shift on day 1</th>
<th>Average phase shift on day 3</th>
<th>Average phase shift on day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>7</td>
<td>-0.11 ±0.17</td>
<td>-0.26±0.22</td>
<td>-0.66±0.45</td>
</tr>
<tr>
<td>4-8</td>
<td>10</td>
<td>-0.28±0.26</td>
<td>-0.34±0.29</td>
<td>-0.46±0.41</td>
</tr>
<tr>
<td>8-12</td>
<td>7</td>
<td>-1.11±0.27</td>
<td>-1.04±0.32</td>
<td>-0.91±0.48</td>
</tr>
<tr>
<td>12-16</td>
<td>19</td>
<td>-0.3±0.11</td>
<td>-0.33±0.13</td>
<td>-0.41±0.18</td>
</tr>
<tr>
<td>16-20</td>
<td>15</td>
<td>0.09±0.16</td>
<td>-0.08±0.20</td>
<td>-0.19±0.30</td>
</tr>
<tr>
<td>20-24</td>
<td>22</td>
<td>0.27±0.13</td>
<td>0.20±0.14</td>
<td>-0.13±0.20</td>
</tr>
</tbody>
</table>

3.1.2 Effect of GA on the fundamental period of the mouse circadian clock (\(tau\)) before and after isoflurane GA

In order to further investigate the type of phase-shifting effect of GA on the circadian pacemaker, I looked for changes in the fundamental free running period (\(tau\)) of mice locomotor activity rhythms. In an animal model, in controlled conditions, it is possible to calculate the individual change in \(tau\). To do so, I first measured \(tau\) before and after each treatment (six hours or 10 minutes of isoflurane) for each individual mouse - from the last seven days before treatment of a total of 14 days in DD, as well as the seven days after treatment - and calculated the mean of the mice \(tau\) before and after GA. I then calculated the change in \(tau\) (\(\Delta tau = tau_{after GA} - tau_{before GA}\)) for each individual animal and averaged \(\Delta tau\) for the six-hour treatment and the 10 minutes treatment (Table 3.3).
Table 3.3: Table showing the mean of mice tau, the standard deviation (SD) and the standard error of the mean (SEM) before and after each treatment with isoflurane, as well as the change in tau (Δtau = tau after GA-tau before GA) from either the six hours or 10 minutes. n=sample size.

<table>
<thead>
<tr>
<th></th>
<th>Mean tau before 6 hours GA</th>
<th>Mean tau after 6 hours GA</th>
<th>Mean tau before 10 minutes GA</th>
<th>Mean tau after 10 minutes GA</th>
<th>Δtau 6 hours GA</th>
<th>Δtau 10 minutes GA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>23.72</td>
<td>23.75</td>
<td>23.78</td>
<td>23.74</td>
<td>0.025</td>
<td>-0.044</td>
</tr>
<tr>
<td>SD</td>
<td>0.21</td>
<td>0.21</td>
<td>0.19</td>
<td>0.21</td>
<td>0.117</td>
<td>0.124</td>
</tr>
<tr>
<td>SEM</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.02</td>
<td>0.013</td>
<td>0.014</td>
</tr>
<tr>
<td>n</td>
<td>81</td>
<td>81</td>
<td>80</td>
<td>80</td>
<td>81</td>
<td>80</td>
</tr>
</tbody>
</table>

The average of tau before any treatment for this breed of mice, C57bl/6VJU, was also calculated for all animals (261 mice) used in all the experiments (including free running animals before sevoflurane anaesthesia). The average of tau for n=261 is 23.7±0.18 hours. Data are represented as the mean of tau±SD (n=261). This means that in DD, mice free running period (FRP) is 0.3 hours (18 minutes) shorter than 24 hours. Data extracted from my experiments agree with previous publications (Schwartz et al., 1990a) for this breed of mice. The SD of mice tau (n=261) is 0.18 hours. This means that the normal FRP of this breed of mice varies, on average, 10.8 minutes.

The average of Δtau for the six-hour treatment was 0.025 hours, which is equal to 1.8 minutes, and the average of Δtau for the 10-minute treatment was 0.044 hours, which is equal to 2.6 minutes. Whilst it appears to be a statistically significant difference between treatments (p value <0.005), the magnitude of the tau increment for each treatment is smaller (1.8 and 2.6 minutes for the six-hour and 10-minute treatments, respectively) than the normal range of error (10.8 minutes). Thus, this means that the effect of isoflurane GA on the fundamental period of the mouse clock is not biologically relevant.

An overall change in tau considers all the values of the change in tau at all times, which is only indicative of the symmetry or asymmetry of the shift in tau. This means that if the effects of GA
on \( \tau \) are symmetrical at all times, the averaged \( \tau \) shift observed will be small. It is therefore logical to plot any change in \( \tau \) as a function of the CT of the administration. Thus, to see whether the effect of GA on \( \tau \) was time-dependent (similar to the phase of the rhythms), I built a \( \tau \) response curve. This graph shows the average of the difference on \( \tau \) (\( \tau \) after - \( \tau \) before) for each anaesthesia treatment administered for either six hours (red circles) or 10 minutes (blue circles) (Figure 3.3).

![Figure 3.3: Time-dependency effect on \( \tau \) after six hours isoflurane GA. Data are presented as the average of \( \tau \) binned in four-hour epochs ± SEM. The grey rectangle shows the normal range of error of \( \tau \) for this breed of animals (0.18 hours).](image)

Looking at the \( \tau \) response curve, even though it seems that six hours of GA has opposing effects on \( \tau \) in comparison to the 10-minute treatment between CTs 16-4, with a significant confidence interval which does not cross 0 between CTs 16-20 and 20-24 (Table 3.4), GA at any given CT interval has very little effect on \( \tau \) (see grey rectangle Figure 3.3). The average of the change in
tau is under the normal range of error for tau, (grey rectangle) independent of the duration of treatment and time of administration.

<table>
<thead>
<tr>
<th>Circadian time (CT)</th>
<th>n</th>
<th>Average [tau after GA-tau before 6 h isoflurane]</th>
<th>Average [tau after 6h GA-tau before 10 minutes GA]</th>
<th>Treatment (6 hours) – Control (10 minutes) (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>8</td>
<td>0.11±0.06</td>
<td>0.01±0.04</td>
<td>0.10(-0.023, 0.224)</td>
</tr>
<tr>
<td>4-8</td>
<td>10</td>
<td>0.05±0.05</td>
<td>-0.01±0.04</td>
<td>0.06(-0.049, 0.169)</td>
</tr>
<tr>
<td>8-12</td>
<td>7</td>
<td>-0.07±0.04</td>
<td>-0.04±0.07</td>
<td>-0.02(-0.154, 0.110)</td>
</tr>
<tr>
<td>12-16</td>
<td>19</td>
<td>0.01±0.02</td>
<td>-0.05±0.02</td>
<td>0.06(-0.011, 0.141)</td>
</tr>
<tr>
<td>16-20</td>
<td>15</td>
<td>0.03±0.02</td>
<td>-0.05±0.02</td>
<td>0.08(0.0001, 0.158)</td>
</tr>
<tr>
<td>20-24</td>
<td>22</td>
<td>0.02±0.03</td>
<td>-0.06±0.04</td>
<td>0.08(0.0003, 0.160)</td>
</tr>
</tbody>
</table>

Table 3.4: Data showing the time-dependency effect on tau after six hours of isoflurane GA. Data are presented as the average of tau binned in four-hour epochs ± SEM. The confidence interval of the difference between the mean of tau (six-hour treatment – 10-minute treatment) per CT is shown.

3.2 Effect of sevoflurane GA on circadian rhythms of behaviour

In this section I present the results describing the time-dependent effect of sevoflurane GA on the phase of the locomotor activity behavioural rhythm, as well as the effect of sevoflurane on the fundamental period of the mouse clock.

3.2.1 Effect of sevoflurane GA on the phase of circadian rhythms of behaviour

The effect of either six hours or 10 minutes of sevoflurane GA on circadian rhythms of locomotor activity was studied using actograms. In the following exemplar actograms (Figure 3.4), sevoflurane GA, like isoflurane, when administered for six hours in DD (n=75), also shifts circadian rhythms of locomotor activity in a time-dependent fashion. The six-hour treatment evoked phase delays (A), phase advances (B), or did not shift animals’ locomotor activity rhythms
at all (C) (Figure 3.4). The treatment with sevoflurane for 10 minutes (n=24) did not shift circadian rhythms of mice behavioural rhythms at any time (actograms D, E, and F).
Figure 3.4: Exemplar actograms showing the phase-shifting effect of GA with sevoflurane administered for either six hours (green rectangle) or ten minutes (blue line inside of a blue circle) on mice circadian locomotor activity rhythms. Actograms A, B, and C show the effect of sevoflurane after six hours of treatment. When administered at CT 8 (A) where a 0.71-hour phase delay is observed on day one after treatment, at CT 21 (B) where sevoflurane for six hours induces a 0.7-hour phase advance, and at CT 18 (C), where GA with sevoflurane does not shift circadian rhythms. Actograms D, E, and F show the absence of an effect of ten minutes of GA with sevoflurane at the same CTs as the six-hour treatment (D, CT8; E, CT 21; F, CT18).
In order to study the time-dependency of the phase-shifting effect induced by six hours of sevoflurane GA on circadian rhythms of locomotor activity, the induced phase shift was measured on days one, three, and seven after treatment. Data were analysed and plotted following the same protocol as for isoflurane. With the data obtained, a PRC describing the effect of sevoflurane GA on circadian rhythms over a period of 24 hours was plotted (Figure 3.5).

![Figure 3.5: Phase response curve describing the effect of GA with sevoflurane on mice circadian rhythms locomotor activity. Green circles are the mean of six hours of sevoflurane treatment (n=75). Blue circles represent the mean of 10 minutes of sevoflurane (control treatment) (n=24). Data are represented as the average of the phase shift binned in four-hour epochs ± SEM. Values from the time dependent GA-induced phase shift on mice behavioural rhythms were considered statistically and significantly different when the confidence interval of the difference between the mean of the induced phase shift after the treatments (six-hour GA versus 10 minutes GA) did not include the null hypothesis (did not include 0), as between CTs 4-8, and is represented by ⋆.](image)

When sevoflurane was administered between the middle and the end of the resting phase (CTs 6-11), the treatment delayed the onset of mice locomotor activity rhythms in the running wheel. The biggest phase delay from an individual mouse, 1.45 hours, was observed when GA was administered at CT 8. No significant phase shifts were detected when six-hour sevoflurane
treatment was delivered from CT 14 to CT3, in comparison to the control treatment. The maximum phase advance from a single mouse was observed when sevoflurane was administered at CT 23, where the six-hour treatment caused a 0.72 hours phase advance. The control treatment, 10 minutes of GA, did not shift circadian rhythms of locomotor activity in mice at any time (Figure 3.5, Table 3.5).

The confidence interval (CI 95%) of the difference between the mean of the induced phase shift after the six-hour treatment and the control treatment, at each interval of time, was calculated. The CI of the difference between the mean of the induced phase shift of sevoflurane administered for six hours versus 10 minutes did not contain 0, the null hypothesis, when sevoflurane was administered between CTs 4-8, which means that the effect of the six-hour treatment was statistically significant and statistically differed from the control treatment at these CTs (Table 3.5).

<table>
<thead>
<tr>
<th>Circadian time (CT)</th>
<th>n</th>
<th>Six hours of sevoflurane</th>
<th>n</th>
<th>10 minutes of sevoflurane (control)</th>
<th>Treatment -control (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>12</td>
<td>0.11±0.12</td>
<td>6</td>
<td>-0.015±0.14</td>
<td>0.13 (-0.271, 0.524)</td>
</tr>
<tr>
<td>4-8</td>
<td>9</td>
<td>-0.5±0.12</td>
<td>3</td>
<td>0.05±0.14</td>
<td>-0.55 (-1.083, -0.24)</td>
</tr>
<tr>
<td>8-12</td>
<td>9</td>
<td>-0.54±0.16</td>
<td>3</td>
<td>-0.11±0.08</td>
<td>-0.43 (-0.961, 0.099)</td>
</tr>
<tr>
<td>12-16</td>
<td>10</td>
<td>-0.03±0.19</td>
<td>3</td>
<td>-0.10±0.18</td>
<td>0.07 (-0.453, 0.593)</td>
</tr>
<tr>
<td>16-20</td>
<td>16</td>
<td>0.05±0.07</td>
<td>4</td>
<td>0.0±0.10</td>
<td>0.05 (-0.394, 0.495)</td>
</tr>
<tr>
<td>20-24</td>
<td>19</td>
<td>0.19±0.10</td>
<td>5</td>
<td>-0.032±0.12</td>
<td>0.22 (-0.179, 0.620)</td>
</tr>
</tbody>
</table>

Table 3.5: Data showing the phase-shifting effect of GA with sevoflurane on mice circadian rhythms of mice locomotor activity after six hours of treatment and 10 minutes (control). Data are presented as the average of the phase shift binned in four-hour epochs ± SEM. n represents the number of animals per data point. The confidence interval of the difference between the mean of the phase shift (six-hour treatment - 10-minute treatment) per CT is shown.
The phase-shifting-effect of sevoflurane was measured on days one, three, and seven after the treatment to estimate the duration of the phase shift. The phase-shifting effect evoked by sevoflurane when administered for six hours persisted up to day three when the treatment was administered between CTs 4-8 (Table 3.6).

<table>
<thead>
<tr>
<th>Circadian time (CT)</th>
<th>n</th>
<th>Average phase shift on day 1</th>
<th>Average phase shift on day 3</th>
<th>Average phase shift on day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-4</td>
<td>12</td>
<td>0.11±0.12</td>
<td>0.19±0.17</td>
<td>0.36±0.29</td>
</tr>
<tr>
<td>4-8</td>
<td>9</td>
<td>-0.5±0.12</td>
<td>-0.27±0.12</td>
<td>0.07±0.27</td>
</tr>
<tr>
<td>8-12</td>
<td>9</td>
<td>-0.58±0.16</td>
<td>-0.12±0.20</td>
<td>0.54±0.42</td>
</tr>
<tr>
<td>12-16</td>
<td>10</td>
<td>-0.03±0.19</td>
<td>-0.02±0.21</td>
<td>0.03±0.30</td>
</tr>
<tr>
<td>16-20</td>
<td>16</td>
<td>0.05±0.07</td>
<td>0.04±0.07</td>
<td>0.03±0.14</td>
</tr>
<tr>
<td>20-24</td>
<td>19</td>
<td>0.19±0.10</td>
<td>0.19±0.0.13</td>
<td>0.13±0.22</td>
</tr>
</tbody>
</table>

Table 3.6: Table showing the phase-shifting effect of GA with sevoflurane on mice circadian rhythms of mice locomotor activity calculated on day one, day three, and day seven after the anaesthesia treatment. Data are presented as the average of the phase shift binned in four-hour epochs ± SEM. n represents the number of animals per data point.

3.2.2 Effect of GA on the fundamental free running period of the mouse circadian clock (\( \tau \)) before and after sevoflurane GA

In order to further investigate the type of shifting effect sevoflurane GA evoked on the circadian rhythms, as with isoflurane, I sought for changes in the fundamental period of the mice clock. I did exactly the same analysis as I did for isoflurane, so data were directly comparable. I investigated whether sevoflurane GA altered mice \( \tau \) before and after each treatment, either six hours or 10 minutes. I measured \( \tau \) before and after the treatment for each individual mouse and calculated the mean to obtain the change in \( \tau \) (\( \Delta \tau \)) (Table 3.7).
Table 3.7: Table showing the mean of mice $\tau$, the standard deviation (SD) and the standard error of the mean (SEM) before and after each treatment with isoflurane, as well as the change in $\tau$ ($\Delta \tau = \tau_{after} - \tau_{before}$) from either the six hours or 10 minutes. n=sample size.

As with isoflurane, the magnitude of the change in $\tau$ is within the normal variance in $\tau$. The average of $\tau$ for n=261 was $23.7\pm0.18$ hours (data are represented as the mean of $\tau\pm$SD for n=261). Thus, since the magnitude of the average of the change in $\tau$ is $0.031$ (1.86 minutes) for the six-hour treatment, and $0.046$ hours (2.76 minutes) for the 10-minute treatment, the change in $\tau$ is within the normal biological margin ($0.18$ h = 10.8 minutes).

The CI calculated for the difference between the change in $\tau$ from the six-hour versus the 10-minute treatment included 0 for all the CTs (Table 3.8).

Not surprisingly, when change in $\tau$ before and after treatment was plotted in a $\tau$ response curve to see whether sevoflurane GA altered the mice free running period in a time-dependent manner, the average of $\tau$ plotted in four-hour epochs was within the normal error of the free running period for this breed of mice: 0.18 hours (Figure 3.6). Sevoflurane anaesthesia did not have a significant effect on the fundamental period of the mouse clock.
Figure 3.6: Time-dependency effect on $\tau$ after six hours sevoflurane GA. Data are presented as the average of $\tau$ binned in four-hour epochs ± SEM. The grey rectangle shows the normal range of error of $\tau$ for this breed of animals (0.18 hours).

Table 3.8: Data showing the time-dependency effect on $\tau$ after six hours of sevoflurane GA. Data are presented as the average of $\tau$ binned in four-hour epochs ± SEM. The confidence interval of the difference between the mean $\tau$ (six-hour treatment - 10-minute treatment) per CT is shown.
3.3 Effect of light on mice circadian rhythms of locomotor activity, and interaction of light and GA (with either isoflurane or sevoflurane) administered simultaneously during mice active and resting phase

As explained in Chapters 1 and 2, light is the external stimulus, or zeitgeber, that has the most profound shifting effect on the clock. The effect of light on circadian rhythms has been extensively studied in diurnal as well as in nocturnal animals. For diurnal species, the effect of light on the clock in the late subjective day or early subjective night (coinciding with dusk) results in phase delays; whereas light given in the late subjective night or early subjective day, (dawn) results in phase advances (Ludin et al., 2016). In nocturnal animals however, light evokes phase delays when administered at the beginning and in the middle of the subjective night (Figure 3.7), and results in phase advances when administered at the end of the subjective night. Light administered during the subjective day has no effect on behavioural rhythms in nocturnal animals (Dallmann et al., 2011; Pendergast et al., 2010). In order to have data which were comparable between all my studies, I reproduced a set of light experiments alongside light-and-anaesthesia experiments to study the interaction of these treatments and their effect on circadian rhythms of locomotor activity in mice.

3.3.1 Study of the effect of light on circadian rhythms of locomotor activity in mice

In these sets of experiments, I studied the phase-shifting effect of a four-hour light pulse period with an intensity of 400 lux, administered at the CT when light has shown to evoke phase delay behavioural rhythms in mice (between CT10-18) (Dallmann et al., 2011). These experiments were
designed according to the light PRC published by Dallmann and colleagues in 2011, so that data could be replicated and compared across studies.

As expected, light administered between CTs 10-18 phase delayed the onset of mice wheel running locomotor activity rhythms by almost three hours on average (Figure 3.7). Values are expressed as the average of the phase shift evoked by light on circadian rhythms of locomotor activity in mice, binned in four-hour epochs ± SEM (Table 3.9)

![Figure 3.7: Phase-shifting effect of light on circadian rhythms of mice locomotor activity. The open circles represent the average of the phase shift evoked by light on circadian rhythms of locomotor activity in mice binned in four-hour epochs ± SEM. The black line links the average of the phase-shifting effect of light in circadian rhythms. The grey circles represent the phase shift elicited by light in each individual mouse at the circadian time of administration.](image)

<table>
<thead>
<tr>
<th>Circadian time (CT)</th>
<th>n</th>
<th>Average phase shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-12</td>
<td>11</td>
<td>-1.5±0.29</td>
</tr>
<tr>
<td>12-16</td>
<td>13</td>
<td>-2.95±0.18</td>
</tr>
<tr>
<td>16-20</td>
<td>5</td>
<td>-1.65±0.42</td>
</tr>
</tbody>
</table>

Table 3.9: Table showing the data of the induced phase-shifting effect of light on circadian rhythms of mice locomotor activity. Data are shown as the average of the phase shift evoked by light on circadian rhythms of locomotor activity in mice binned in four-hour epochs ± SEM. n represents the number of animals per data point.
3.4 Effect of light and anaesthesia, with either isoflurane or sevoflurane, on circadian function

The aim of the following experiments was to investigate whether the effect of light on the circadian function, when administered during the anaesthesia treatment, was reproducible in small mammals, as was previously observed in bees, where light reversed the GA-induced phase shift in bees’ behaviour (Ludin et al., 2016).

3.4.1 Effect of light and GA with isoflurane on locomotor activity rhythms

Light was administered at an intensity of 400lux together with isoflurane at two different CTs, between CTs 14-17, where light had been reported to evoke the maximum phase delay (Colwell et al., 1993; Dallmann et al., 2011), and between CTs 7-11, where isoflurane has shown to evoke the maximum phase delay on circadian rhythms of locomotor activity from my experiments (see Figure 3.2). Both treatments, light and isoflurane together and light alone, were administered for four hours. The average of the CT at which the joint treatment was administered was CT 9.4±0.3 and CT 16.2±0.5, respectively.

In Figure 3.8, the isoflurane PRC and the average of the light induced phase shifts are represented in grey and open circles, respectively. The black circles represent the effect of isoflurane plus light administered simultaneously for four hours at CT9 (average of the CT of the time of the administration, between CT 7 and CT 11) and CT16 (average of the CT of the time of the administration, between CT 14 and CT 17).
Figure 3.8: Presentation of the effect of six hours of isoflurane GA on circadian rhythms of locomotor activity in mice (grey circles; n=80) (isoflurane PRC), and study of the shifting effect of light on circadian rhythms (open circles, n=29), in comparison to the shifting effect of light and anaesthesia administered together (black single circles) between CTs 7-11 (mice resting phase) (n=12) and CTs 14-17 (n=5) (mice active phase). Red dashed lines and red double arrow show the difference of the induced phase shift of light in comparison to the shifting effect evoked by light and GA (black circle) administered together during the mice resting phase. Blue dashed lines and blue double arrow show the difference of the induced phase shift of light in comparison to light and GA (black circle) administered together during the mice active phase. Data from the isoflurane PRC and light shifting effect are presented as the average of the phase shift binned in four-hour epochs ± SEM. The concomitant treatment of light and anaesthesia are represented by the average of the shifting effect binned in five and four-hour epochs. Error bars represent the SEM.

At CT16, isoflurane GA effectively blocked the phase-shifting effect of light. The light treatment given at this CT evoked almost a three-hour phase delay (2.64 h), however when administered simultaneously with isoflurane at this CT (interval of CTs between 14-17), the phase delay evoked by light was abolished and there was no significant phase shift on circadian rhythms of locomotor activity (0.13h) after the dual treatment, with a p value of <0.001. The significance of the effect of the dual treatment (light and isoflurane administered concomitantly) in comparison to light
individually (Figure 3.8, blue arrow), administered for four hours, on mice circadian rhythms of locomotor activity, was determined by a two-way ANOVA.

Interestingly, as-per Figure 3.8 and Table 3.10, when light and anaesthesia were given between CTs 7-11 a trend can be observed towards longer delays (Figure 3.8. red arrow) in the dual treatment in comparison to the shifting effect induced by light, however data are not statistically significant, with a p value of 0.59. At these CTs GA did not block the phase-shifting effect of light as it did between CTs 7-14.

<table>
<thead>
<tr>
<th>Treatment comparison</th>
<th>n</th>
<th>Average phase shift</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light phase shift CTs 7-11</td>
<td>11</td>
<td>-1.50±0.29</td>
<td>.059</td>
</tr>
<tr>
<td>Isoflurane +light phase shift CTs 7-11</td>
<td>12</td>
<td>-2.18±0.21</td>
<td></td>
</tr>
<tr>
<td>Light phase shift CTs 14-17</td>
<td>16</td>
<td>-2.64±0.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Isoflurane +light phase shift CTs 14-17</td>
<td>5</td>
<td>0.13±0.40</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.10: Table showing the phase shifting effect induced by light, and GA with isoflurane plus light, administered together for four hours at two different intervals of CT, on mice circadian rhythms of behaviour. n represents the number of animals per time point and treatment. Data was analysed by using a two-way ANOVA treatment versus time. P values are indicated for each group of treatments.

The effect of the concomitant treatment of light and anaesthesia appears to be, as it was for GA only, time-dependent.

The difference between the shifting effect of isoflurane administered independently was not directly compared to light or light plus GA due to the different duration of the treatments (see section 2.2.7 Study of the concomitant effect of GA and light together using isoflurane or sevoflurane as the anaesthetic agents).
3.4.2 Effect of light and GA with sevoflurane on locomotor activity rhythms

Following the study of isoflurane plus light, I investigated the effect of light and sevoflurane administered together for four hours at the time point (CT) where light had been reported to evoke the maximum phase delay (Colwell et al., 1993; Dallmann et al., 2011), between CTs 14-17. The average of the time of the administration of light and sevoflurane together was CT 15.49±0.26. The same protocol as with isoflurane and light was applied, so that data were comparable.

As can be seen in Figure 3.9, sevoflurane administered with light at CT 15 reduced the phase-shifting effect evoked by light on locomotor activity rhythms, however it did not completely block it. The induced phase delay of the concomitant treatment was significantly different from the effect of light administered independently. Sevoflurane administered at CT 15 did not shift circadian rhythms of locomotor activity, its effect on behavioural rhythms was not significantly different from the effect of the control treatment (10 minutes). Contrastingly, light given at CT 15 induces (on average) a 2.64 hours phase delay. The light and sevoflurane treatment significantly shifted circadian rhythms of locomotor activity in comparison to light, provoking (on average) a 0.61-hour delay and with a p value of <0.001 (Figure 3.9, green double arrow, Table 3.11).
Figure 3.9: Presentation of the effect of six hours of sevoflurane GA on circadian rhythms of locomotor activity in mice (grey circles, n=75) (sevoflurane PRC) and study of the shifting effect of light on circadian rhythms (open circles, n=29), and the shifting effect of light and anaesthesia administered together (black single circles, n=11) at CT 15 (average of the CT of the time of the administration of the light and sevoflurane treatment, between CTs 14-17). Green dashed lines and green double arrow show the difference of the induced phase shift of light in comparison to light and GA (black circle) administered together during the mice active phase. Data from the sevoflurane PRC and light shifting effect are presented as the average of the phase shift binned in four-hour epochs ± SEM. The treatment of light and anaesthesia are represented by the average of the shifting effect binned in four-hour epochs. Error bars represent the SEM.

<table>
<thead>
<tr>
<th>Treatment comparison</th>
<th>n</th>
<th>Average phase shift</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light phase shift CTs 14-17</td>
<td>16</td>
<td>-2.64±0.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Sevoflurane + light phase shift CTs 14-17</td>
<td>11</td>
<td>-0.61±0.30</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.11: Table showing the phase shifting effect induced by light, and GA with sevoflurane plus light administered, together for four hours, at a given interval of CTs (between 14-17), on mice circadian rhythms of behaviour. n represents the number of animals per time point and treatment. Data was analysed by using a two-way ANOVA treatment versus time. P value of the induced shifting effect of light versus light plus sevoflurane on mice circadian rhythms is shown.
3.4.3 Magnitude of the phase-shifting effect of GA with isoflurane and sevoflurane with light on the clock. Summary of the data.

Given the varying effects of different anaesthetic agents on circadian rhythms when administered together with light at different CTs, I analysed the magnitude of the induced phase shift of each treatment. I used a two-way ANOVA to study the statistical significance and plotted the average of the phase shift ± the SEM (Figure 3.10).

Figure 3.10: Plots showing the induced phase-shifting effect by a four-hour light pulse only during the animals’ resting phase between CTs 7-11, and during the animals’ active phase between CTs 14-17 (black circles). These are compared to the induced shifting effect of isoflurane and light administered simultaneously during the animals’ resting phase between CT 7-11, and during the animals’ active phase between CTs 14-17 (red circles), as well as the induced phase-shifting effect of sevoflurane plus light treatment only during the animals’ active phase between CTs 14-17 (green circle). Data are presented as the average of the phase-shifting effect. Error bars represent the SEM.

The effect of GA and light when administered together on circadian rhythms of locomotor activity, depended on the time of the administration and the anaesthetic agent used. Isoflurane administered with light at an intensity of 400lux for four hours in the middle of the mouse active phase (between CTs 14-17) blocked the phase-shifting effect of light. It is at these CTs that light evokes the maximum phase delay (almost 3 hours) in mice locomotor activity rhythms. The average of phase-shifting effect of light was reduced from -2.64 to 0.13 hours when light was administered with
isoflurane during the animals’ active phase, with a p value of <0.001 (Table 3.10). However, at the end of the resting phase, when isoflurane has shown to evoke, on average, its maximum phase delay on mice behaviour (-1.11 hours, n=7), the dual treatment did not block the light induced phase shift. At this time (CTs 7-11) a trend towards longer delays could be observed. The average of the phase delay induced in behavioural rhythms increased from -1.5 to -2.18 hours with a p value of 0.59 (Table 3.12). Sevoflurane, on the other hand, reduced the induced phase delay evoked by light between CTs 14-17, with a p value of <0.001 (Table 3.12), but did not completely block it as isoflurane did. The induced phase delay at these CTs decreased from -2.64 to -0.61 hours, whereas with isoflurane the light induced phase shift on mice circadian rhythms was reduced to 0.13 hours (Table 3.12).

<table>
<thead>
<tr>
<th>Treatment comparison</th>
<th>n</th>
<th>Average phase shift</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light phase shift CTs 7-11</td>
<td>11</td>
<td>-1.50±0.29</td>
<td>.059</td>
</tr>
<tr>
<td>Isoflurane +light phase shift CTs 7-11</td>
<td>12</td>
<td>-2.18±0.21</td>
<td></td>
</tr>
<tr>
<td>Light phase shift CTs 14-17</td>
<td>16</td>
<td>-2.64±0.17</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Isoflurane +light phase shift CTs 14-17</td>
<td>5</td>
<td>0.13±0.40</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Light phase shift CTs 14-17</td>
<td>16</td>
<td>-2.64±0.17</td>
<td></td>
</tr>
<tr>
<td>Sevoflurane +light phase shift CTs 14-17</td>
<td>11</td>
<td>-0.61±0.30</td>
<td></td>
</tr>
</tbody>
</table>

Table 3.12: Data are presented as the mean of the phase shift binned in five-hour epochs and four-hour epochs ± SEM. n represents the number of animals per data point. The p value for the light induced phase shift versus isoflurane and light induced phase shift at CTs 14-17 is <0.001 (4.6659E-8); and the p value for the light induced phase shift versus sevoflurane and light induced phase shift at CTs 14-17 is <0.001 (1.1963E-7).

An example of the effect of each treatment on locomotor activity rhythms is shown in Figure 3.11. These actograms show the phase shifts on the onset of mice running wheel activity rhythms after the light treatment at CT 15 (A and C) and CT 10 (B) (black opened rectangles), where the light induced phase delays were -2.84 hours (A and C) and -1.85 hours (B), respectively. The light and
isoflurane GA treatment (red opened rectangle, actogram D and E) at CT 16 and 10 evoked a phase advance of 0.14 hours and a phase delay of -1.40, respectively. Finally, the sevoflurane plus light treatment (green opened rectangle, actogram F) at CT 15 provoked a 0.65-hour phase delay.
Figure 3.11: Exemplar of actograms comparing the phase-shifting effect of light (black opened rectangle, actograms A, B and C), light and isoflurane GA (red opened rectangle, actograms D and E) and light and sevoflurane GA (green opened rectangle, actogram F), administered for four hours on circadian rhythms of locomotor activity. Actograms A, B and C show the phase-shifting effect on mice circadian rhythms of locomotor activity evoked by four hours of light from CTs 7-16. Actograms D and E show the concomitant shifting effect of isoflurane and light when administered for four hours at CTs 14-17 and CTs 7-11, respectively. Actogram F shows the shifting effect of sevoflurane and light when administered together for four hours between CTs 14-17.
IV. Chapter 4

4 General discussion and conclusions

The findings from this thesis strongly support the hypothesis that GA, with isoflurane and sevoflurane, shifts the mammalian circadian clock, and most importantly, that the effect of GA on the clock is time-dependent. The PRCs presented in this thesis are the first two comprehensive and closely controlled PRCs for the effects of anaesthetic agents on mammalian behavioural rhythms. Interestingly, light has also been previously shown to disrupt the circadian clock by causing profound and strong time-dependent shifts on circadian rhythms of behaviour in a range of species. In this thesis, I hypothesised that light had the ability to modify the GA-induced phase shift on mice behavioural rhythms as it does in bees, and therefore it could potentially be used to ameliorate or restore the GA-induced phase shift in mice circadian rhythms. However, from the results of this thesis, it appears that photic (light pulses of 400 lux from an LED light source) and non-photic (GA with either isoflurane (1.5%) or sevoflurane (2.6%)) stimuli, when administered concomitantly to mice in constant conditions, promote different magnitudes of disruption on mice circadian rhythms of behaviour, compared to when these agents were administered independently.

The findings from this thesis, at least for isoflurane and light, suggest that the effect of GA plus light on the clock is phase-dependent, which refuted previous research regarding the effect of light and GA on the clock (Colwell et al., 1993), however, further studies need to be conducted in order to confirm a similar/different effect for sevoflurane and light on mice circadian rhythms as well as to further understand how light and GA interact between each other and on the mice circadian
function at different CTs, and whether light could actually be used to restore the GA-induced phase shift.

Based on the similarities between these two anaesthetic agents, and how they interact with light and disrupt circadian rhythms, in this thesis, I hypothesise that similar time-dependent effects would be observed on mice behavioural rhythms after the administration of light and sevoflurane GA concomitantly, if more CTs were studied.

4.1 Comparison between my findings and previous work

Isoflurane and sevoflurane, when administered in constant darkness (DD) for six hours, both produced type one (weak) PRCs. Isoflurane administered for six hours at the end of the animals’ resting phase, between CTs 8-12, induced phase delays on circadian rhythms of locomotor activity. Sevoflurane evoked phase delays when administered in the middle of the animals’ resting phase, which was between CTs 4-8. The magnitude and amplitude of the sevoflurane-induced phase delays on behavioural rhythms (n=75) were, overall, smaller than the ones evoked by isoflurane (n=80). Isoflurane seemed to evoke a trend towards small phase advances when administered at the very end of the active phase, between CTs 20-24, and a dead zone was observed between CTs 0-6 and CTs 16-20, whereas sevoflurane showed a dead zone from CTs 13-2, and no phase advances.

When comparing the effects of both treatments (six hours versus 10 minutes), as well as treatment versus time, GA administered for ten minutes had no significant effect on circadian rhythms of locomotor activity. This validated that 10 minutes of GA was a reasonable control treatment for
all the conditions of the protocol designed to administer a volatile anaesthetic agent to multiple mice at the same time.

Out of the 29 studies published describing the effect of GA on the circadian clock, only one used PRCs to describe the time-dependent effect of isoflurane GA on bees’ locomotor activity rhythms (Ludin et al., 2012), and one used PRCs to describe the time-dependent effect of sevoflurane on gross locomotor activity rhythms in mice (Kadota et al., 2012). The magnitude of the phase shift elicited by six hours of isoflurane in bees was bigger than the one observed in this thesis in mice, and happened during the bees’ active phase. In the isoflurane PRC presented in this thesis, isoflurane delayed mice wheel running rhythms when administered during the animals’ resting phase. This difference could be due to GA having different effects on the clock genes driving the rest/activity rhythms in nocturnal and diurnal animals. Nocturnal animals are active when BMAL1/CLOCK levels in the SCN are high, whereas diurnal animals are active when PER/CRY in the SCN are high (Poulsen et al., 2016). The circadian system of diurnal and nocturnal animals is reported to be sensitive to different stimuli at different CTs (Mendoza et al., 2007). This theory (GA has different effects on the clock genes in nocturnal versus diurnal animals) is largely supported by experiments carried out with the GABA agonist muscimol. The injection of muscimol directly into the SCN causes phase advances in locomotor activity rhythms of nocturnal rodents, during the subjective day (Ehlen et al., 2006; Smith et al., 1989), whereas it causes phase delays in the circadian wheel-running rhythm in diurnal rodents (Novak et al., 2004). The differential responses between species with different activity patterns suggest that the underlying neural mechanisms in nocturnal animals is different to diurnal species.
Contrastively, Kadota and colleagues (2012) did not find any time-dependent effect of sevoflurane on mice locomotor activity rhythms, as shown in the PRC. However, they did find an overall phase delay, the magnitude of which was highest at the time point when sevoflurane evoked a maximum phase delay on locomotor activity rhythms in my experiments (between CTs 4-8). Interindividual variability between mice’s locomotor activity rhythms (beginning of the active phase versus beginning of resting phase) was not accounted for in Kadota and colleagues’ (2012) study. The SEM in their sample was too high to state that there was a time difference in the sevoflurane-induced phase shift. This is likely to be due to the small sample size per time point (n=4 per CT). In this study, mice were anaesthetised with sevoflurane (2.5%) for four hours on the 5th day after shifting them to DD, and then were returned to their original cages. However, lighting conditions during the anaesthesia treatment were not explained in this study. It could be a possibility that the time-dependent effect of sevoflurane on circadian rhythms was masked by light exposure during or after anaesthesia. The potential influence of light on the GA-induced-time dependent phase shift in mice behaviour was pointed out by their findings in their molecular experiments, where the mPer2 repression rate in the SCN of the DD group was higher than that of the LD group during the subjective day (lights on) after treatment, indicating that GA interferes with the clock gene expression patterns differently, depending on the lighting conditions. Given the exceptional influence of time, lighting conditions, and environmental noise on clock gene expression and on behaviour (Challet, 2007; Orts-Sebastian et al., 2018; Peirson et al., 2018), using strictly controlled protocols with a representative sample size has demonstrated, from the results in this thesis, to be essential to study the time-dependent effect of drugs on the circadian function.
Interestingly, although Kadota and colleagues’ (2012) protocol did not allow them to clearly determine the time-dependent effect of sevoflurane on behavioural rhythms of mice, their results regarding the CTs where the repression rate of mPer2 was observed in the DD group, matched the CTs of the sevoflurane-induced phase delay on behavioural rhythms presented in this thesis (CTs 4-8, subjective day). This suggests that the GA-induced phase shifting effect on mice behavioural rhythms might be, at least in part, due to GA altering the circadian expression of clock genes.

From the literature available, only 11 of the 29 studies published to date performed the administration of the anaesthesia treatment in constant conditions. Three did not specify the lighting conditions, and 15 were performed in light-dark cycles (see Table 1.5). In entrained rodents (animals exposed to LD cycles during the anaesthesia treatment), volatile anaesthetic agents seemed to disrupt behavioural rhythms only when the anaesthesia treatment was administered during the animals’ active phase (when the lights were off) and evoked phase advances (Kikuchi et al., 2013) and delays (Song et al., 2018) in rodents’ gross locomotor activity, depending on the study. However, sevoflurane administered in DD seemed to evoke only phase delays in both active and resting phases (Anzai et al., 2013; Kadota et al., 2012; Ohe et al., 2011). These effects of GA on animal behaviour have been related to the suppression (Anzai et al., 2013), repression (Kadota et al., 2012), or delay (Anzai et al., 2013; Ohe et al., 2011) in Per2 expression in the SCN and the brain.

Studies from our laboratory (Ludin, 2017) showed that isoflurane induced phase shifts in Per2 expression in mouse SCN in in vitro experiments, following a six-hour isoflurane treatment, at a concentration of 2% in air, and that the induced phase shifts were higher in magnitude (-6.9±1.33) than the behavioural phase shifts observed in my studies (-1.11±0.27). Interestingly, the timing of
the maximum phase delays in both molecular and behavioural studies were observed at the end of the mice resting phase, between CTs 8-12 (Figure 4.1)

![Diagram of mouse SCN isoflurane PRC and mouse isofoxane PRC from in vitro experiments compared to the mouse isofoxane PRC from in vivo studies.]

Figure 4.1: A) Mouse SCN isoflurane PRC showing the GA induced phase shift in Per2 expression in *in vitro* experiments, following a six-hour isoflurane treatment (provided by Dr Nicola Ludin from her PhD Thesis) compared to the B) mouse isofoxane PRC from my behavioural studies *in vivo*.

The smaller magnitude shifts at the whole animal level may have been the result of different phase shifts elicited in different circadian oscillators, probably located in different oscillatory tissues (Figure 4.2). The circadian system is composed of a hierarchy of circadian oscillators, where the SCN is the master pacemaker driving rhythmicity at different levels (see Chapter 1, section 1.3.2 The clock controlling circadian rhythms). The functioning of the circadian system is therefore likely to be a network of coupled and synchronised clocks throughout the body driven by a common cell-autonomous molecular mechanism. The existence of these additional circadian oscillators has been previously established in mice, where palatable meals (peanut butter), methamphetamine (methamphetamine sensitive circadian oscillator, MASCO), and the presence
of a running wheel, have shown to restore behavioural rhythmicity in arrhythmic mice (Flôres et al., 2016; Rawashdeh et al., 2017). However, the site of these oscillators is still unknown.

Figure 4.2: Diagram explaining the potential difference in magnitude of the phase shift induced by GA on circadian rhythms of behaviour versus SCN Per expression. I.e. If there are opposing phase shifts on different oscillatory tissues in the animal, it may explain why phase shifts in overall behaviour are much smaller than those on SCN clock gene expression. (MASCO= methamphetamine sensitive circadian oscillator; SCN= superchiasmatic nucleus; GA= general anaesthesia).

4.2 Effect of GA (and light) on the period of the oscillator
In order to further investigate the type of shift on circadian rhythms of behaviour evoked by GA, I looked at the effect of isoflurane and sevoflurane on the fundamental period of the mouse clock (tau). Conceptually, a phase shift can be separated into discrete (or non-parametric) and continuous (or parametric) (Aschoff, 1960; Pittendrigh et al., 1976). Discrete phase shifts occur by an immediate resetting of the phase of the oscillator, whereas continuous phase shifts result from a change in the fundamental period of the oscillator (See Chapter 1, Section 1.3.4.1 Fundamental basis of entrainment: Mechanisms of photoentrainment). The results in this thesis show that the
change in \( \tau \) after each GA treatment was within the normal margin of error for the FRP for this breed of mice, at all times. Therefore, there was no time-dependent shift attributable to the effect of GA on the period of the clock as there was for the phase of the rhythm. I found no evidence to infer that GA had a biologically significant effect on the fundamental period of the clock. In addition, according to the data presented in this thesis, isoflurane and sevoflurane are weak \textit{zeitgebers}, evoking shifts of small magnitudes on the phase of the locomotor activity rhythm. Altogether, this suggests that the effect of GA on the clock is mediated by non-parametric phase shift rather than parametric or continuous shifting.

I also looked at changes in \( \tau \) after the light treatment, and in agreement with previous research (Comas \textit{et al.}, 2006), \( \tau \) seemed to show a trend to lengthen in response to light at the circadian phase where the light-induced phase delays in behavioural rhythms were maximal (CTs 14.5-17.5), and seemed to show a trend to shorten at the circadian phase where the light-induced phase shifts were minimal (CTs 18-8). These data were not showed in this thesis due to the weakness of the observed trend and lack of statistical relevance of the change in \( \tau \) after the light treatment in my experiments. The average of the change in \( \tau \) was within the normal biological range for this breed of mice as well. This would suggest that both light and anaesthesia shift the circadian clock through non-parametric shifting mechanisms.

4.3 Potential mechanisms for the effect of GA on the clock
The general anaesthetic agents studied in this thesis are mainly gamma aminobutyric acid (GABA) agonists (activate the inhibitory GABAergic currents (Garcia \textit{et al.}, 2010)) and N-methyl-D-aspartate (NMDA) antagonist (block NMDA-stimulated currents (Martin \textit{et al.}, 1995)). They have
been reported to exert strong effects on the main neurotransmitter systems linked to the circadian control in the SCN, circadian entrainment, and the transduction of photic signals through the RHT (Poulsen et al., 2016).

Thus, it could be possible that one mechanism by which GA shifts the clock is due to GA interfering with the light-entrainment pathway of the circadian clock. Studies carried out by Colwell and colleagues in hamsters’ circadian rhythms found that GA interacts with the photic entrainment pathway by pharmacologically blocking the phase-shifting effect of light on rodents (Colwell et al., 1993). This was observed after administering halothane to hamsters for 18 minutes, three minutes before the start of the light pulse. The blocking effect of the light-induced phase shift was observed at CT 18, when light evoked phase advances in hamsters. Other intravenous anaesthetic agents also blocked the induced phase-shifting effect of light on behavioural rhythms at this CT (Colwell et al., 1993). This effect was reproduced in one of my experiments when light and isoflurane were administered together during the animals’ active phase, however, the opposite was observed when light and isoflurane were administered during the animals resting phase (see 3.4.1 Effect of light and GA with isoflurane on locomotor activity rhythms). The suggested mechanism was that anaesthetics interfered with the normal transmission of photic information to the circadian system, including the retina, the retinal projections to the SCN, and the SCN itself, through NMDA and GABA receptors blocking the phase-shifting effect of light (Colwell et al., 1993). However, studies in bees from our laboratory (Ludin et al., 2016), and the results presented in this thesis in mice, revealed that the mechanism by which GA acts on the circadian clock is far more complicated and still largely unknown.
Isoflurane and sevoflurane, both evoked type 1 “weak” PRCs, with mainly just phase delays during the animals’ resting phase. Despite sevoflurane, in comparison to isoflurane and other volatile anaesthetic agents, producing faster induction and recovery times at a clinical level (Tonner, 2005), at a molecular level they are very similar (see Figure 4.2). It has been reported that at 1MAC the anaesthesia depth for each agent was comparable (Cesarovic et al., 2010). Studies of evoked potentials and EEG in anesthetized patients, with either isoflurane or sevoflurane, also failed in proving any significant difference in their anaesthesia depth (Kameyama, 1994). Molecular studies do not provide enough evidence to clarify what the mechanistic difference between these two anaesthetic agents is.

Figure 4.3: molecular structure of the two volatile halogenated anaesthetic agents studied in this thesis, isoflurane and sevoflurane.

The anaesthetic stage induced by anaesthetic agents is characterised by the loss of consciousness, hypnosis, and immobility, and is processed through the thalamocortical network (Fukuda et al., 2011), and promoted by distinct subpopulations of GABA\(\alpha\) receptor subtypes (Drexler et al., 2011). In contrast, unlike other “true anaesthetics”, non-immobiliser agents such as F6, (1,2-dichlorohexafluorocyclobutane) do not produce immobility, do not enhance GABAergic currents and, importantly, do not shift activity rhythms in bees nor in rodents (Ludin, 2017; Raines et al., 2002), therefore it could be possible that the shifting effect evoked by GA on the clock is also
related to the anaesthetic properties mediated through GABAergic currents. Hence, if the GABAergic pathway is related to the photic entrainment and the GA-induced phase shift, different magnitudes of GABAergic activation would result in shifts of different magnitudes on the phase of biological rhythms, and thus, would interfere with the photic entrainment pathway at different levels. In addition, if the GABA regulation of the clock is phase-dependent, as has previously been stated (Ralph et al., 1989), it would make sense to see different time-dependent effects on circadian rhythms when GA, and light plus GA are administered at different circadian phases (active phase or resting phase) of the behavioural rhythm.

GA agents also affect the rhythmic expression of clock genes. The inhalational anaesthetic agents studied in this thesis, as previously mentioned, have been reported to suppress (Anzai et al., 2013), repress (Kadota et al., 2012), or delay (Anzai et al., 2013; Ohe et al., 2011) Per2 expression in the SCN and the brain in a time dependent manner. Interestingly, the peaks of expression of Per1 and Per2 in mice SCN coincide with the maximum phase delays that sevoflurane and isoflurane induced respectively, on circadian rhythms of locomotor activity in the results presented in this thesis, with mPer1 mRNA peaking early in the subjective day (CTs 4-8; sevoflurane maximum phase delay), and mPer2 mRNA peaking at the end of the subjective day (CTs 8-12; isoflurane maximum phase delay) (Yan et al., 1999). Therefore, it could also be likely that the phase-shifting effect of GA on circadian rhythms is related to the influence of GA on the expression patterns of clock genes. However, without molecular and behavioural studies performed with comparable and strictly controlled protocols, any conclusion is conjectural.

4.4 Does GA block the light signal to the clock?
Previous work in mammals proposed that anaesthesia acted only to block the light-induced phase shift to the clock. This is not the case in bees, as light can counteract the effect of GA (Ludin et al., 2016). But, what about in mammals?

If the light-induced phase shift was completely blocked by the anaesthesia treatment, it would not be possible to see any effect of light on locomotor activity rhythms when light and GA are administered at the same time. However, the results from this thesis showed otherwise. In Figure 4.3 a summary of my data and a comparison of previously published data in rodents is provided to further support the results and conclusion provided in this thesis.

The data provided in this thesis suggested that GA and light have different effects on the clock when administered together at different circadian times. This indicates that GA does not simply pharmacologically block the phase-shifting effect of light. Instead, this effect depends on the phase of the administration as well as the anaesthetic agent used and animal model (Figure 4.3).

Other GABAergic drugs such as diazepam have also shown phase-dependent effects on light-induced phase shifts (Ralph et al., 1986). Thus, it could be possible that, at least in part, the GABAergic activation promoted by GA on the clock interacts with the photic entrainment pathway, and that the different magnitudes of GABAergic activation, promoted by different anaesthetic agents, may influence magnitude of the light-induced phase shift in a phase-dependent manner.
Figure 4.4: Comparison of the effect of halothane (Colwell et al., 1993) at CT 18 (a), isoflurane (b) and sevoflurane (c) at CT 16 and CT 15 respectively, and isoflurane (d) at CT 9 when administered concomitantly with light on circadian rhythms of locomotor activity in mice. For bar graph (a), differences between treatment groups were evaluated by Colwell and colleagues using a Kruskal-Wallis one-way analysis of variance, followed by a Mann-Whitney U test. Values were considered significantly different if p < 0.05, which I have marked with a @. For the graphs, (b), (c) and (d), a two-way ANOVA on treatment versus time was applied. Values were considered significantly different if p < 0.05, marked with * or <0.001 marked with **.

This, together with the bee’s data, would suggest that different anaesthetic agents may promote different effects on the clock, and may interact at different levels with different entrainment pathways, including photic entrainment, which may also depend on the animal model studied.

### 4.5 Advantages and drawbacks

The results from this thesis provide strong evidence that GA, with isoflurane and sevoflurane, shifts circadian rhythms of behaviour in a time-dependent manner in mice. One of the advantages of this study is that the protocol designed has high enough resolution to determine the effects of GA on the clock. All the environmental conditions were strictly controlled, and the survival rate of the final protocol was 100%. The anaesthesia chamber for the anaesthesia delivery was exclusively designed in order to administer GA, or GA plus light, to multiple mice at the same
time with the same flow rate and concentration of the anaesthetic agent and allowed for the monitoring of mice welfare at all times.

Mice were only treated with isoflurane or sevoflurane, with no other treatment nor any surgical intervention that could have disrupted the clock. After a period of entrainment, animals were kept in strict constant darkness and constant environmental conditions, therefore the only external stimuli the animals received was the anaesthesia treatment. In this way, the effect of GA on circadian rhythms was isolated and studied in vivo. These results strongly indicated that GA itself shifts circadian rhythms of behaviour and the mouse circadian clock.

The same protocol was applied to the same breed of mice and in the same conditions (humidity, temperature, anaesthesia procedure, and animal welfare monitoring) in the light, and light and GA experiments, where only the treatment conditions were modified to study the effect of each treatment on circadian rhythms. Thus, results could be exclusively related to each treatment and could be compared and studied together avoiding any possible masking effect.

In addition, these experiments were performed on the same breed of mice as those used in a molecular study from our research group, on the effect of six hours of isoflurane in in vitro SCN slices taken from transgenic mPer2Luc mice. This offered an exceptional opportunity to study molecular and behavioural effects of GA in parallel. However, it should be noted here that whilst this approach may provide crucial information for understanding the mechanism of GA and its effects on the circadian clock in a mammalian model, culturing a tissue explant from an animal and directly stimulating it with a chemical, is different from administering that same chemical via respiratory inhalation. At a whole animal level, differences occur in the magnitude of the GA-induced phase shifts, and the resulting behavioural shifts are a combination of different
mechanisms involved in the generation of behavioural rhythms. Thus, while it is a very useful model, it is important to highlight that further studies must be done (see 4.6 Potential clinical implications and future directions).

Nevertheless, the data presented in this thesis suggests that volatile halogenated anaesthetic agents, as chronobiotics, could potentially be used to further investigate the mechanisms underlying the entrainment of the circadian clock, as well as the specific processes by which circadian rhythms are disrupted/shifted by different zeitgebers, such as light.

A limitation of this study includes the fact that mice are nocturnal mammals, and only males were used. Therefore, any possible human therapeutic applications must be carefully assumed. However, since constant condition protocols in humans have been related to a range of adverse effects including circadian misalignment (Kawasaki et al., 2018; Pagel et al., 2016), and it is unethical to anaesthetise a person who does not need anaesthesia, a mammalian animal model such as the mouse will continue to be useful to investigate the effects of GA on circadian rhythms.

I chose to use male mice exclusively in order to avoid the effect of the oestrous cycle on mice behavioural rhythms (see Chapter 2, Section 2.1.1 Animals) (Hildebrandt et al., 2008; 2012; Jud et al., 2005). Robust and steady locomotor activity recordings were essential in order to observe whether GA disrupted of rhythms of behaviour (the basis of my hypotheses).

4.6 Potential clinical implications and future directions
For a variable period of time after GA, sleep and other chronobiological, behavioural, and molecular disruptions have commonly been reported in patients (see Chapter 1, Table 1.3 and 1.4),
and in experimental animal models (see Chapter 1, Table 1.5). It is now evident that GA is, in part, responsible of these effects.

According to my results, the time of anaesthesia treatment is a determining factor for the disruption of the circadian clock. However, the lack of knowledge regarding chronotherapy in anaesthesia has often led researchers to confounding results - i.e. the administration of GA at different CTs between experiments, might have led to confounds in the interpretation of the results, due to the time-dependent effect of GA on circadian rhythms, and therefore in behavioural, cognition, and physiological parameters (Orts-Sebastian et al., 2018). In addition, the substantial lack of studies on anaesthetic agents with comparable protocols that distinguish the time-dependent effect of GA in each of the clock genes (see Chapter 1, Table 1.5) hampers our understanding of GA-induced circadian clock disruption.

In order to increase our understanding about how GA contributes to circadian clock disruption, and the disruption of behavioural rhythms, sleep, and post-operative recovery, it is vital to understand the specific chrono-pharmaco-physiological mechanisms by which GA operates at a central and peripheral level. To do so, future studies should investigate the molecular outputs of an anaesthesia treatment on the central circadian clock and peripheral clocks in parallel to behavioural studies, as well as the time-dependency effect of GA plus light at a molecular and behavioural level. A reasonable direction for future studies should include \textit{ex vivo} analysis of clock gene expression from SCN tissue and peripheral organs, harvested at different CTs post-anaesthesia using the same behavioural protocol developed in this thesis. This is called phase mapping and is used to observe the effects of \textit{in vivo} treatments, such as GA, on the circadian organisation. These set of experiments were originally planned; however, the lack of time and
funding hampered our progress. Results would provide evidence of the potential mechanisms by which anaesthesia induces molecular changes in the SCN clock and peripheral clocks, and how it translates into behaviour at a whole animal level.

Building full light and anaesthesia PRCs for different anaesthetic agents using the protocol developed in this thesis should be a priority. Results would provide evidence of how GA interacts with photic entrainment and would help to investigate whether light can be used to counteract the shifting effect of general anaesthesia in a mammalian model, as it does in bees.

In addition, it would also be interesting to include female mice in future behavioural experiments to extend our knowledge about the effect of GA on circadian rhythms in a broader and more representative sample.

Furthermore, a link between GA, post-operative recovery, and the disruption of circadian rhythms would be beneficial for a better control of co-variates such as the variability of symptoms associated with different operations, time of the operations in relation to the intensity and duration of the symptoms of circadian misalignment, previous illnesses, and gender.

### 4.7 Conclusions

In this thesis, I hypothesised that GA disrupts the circadian clock, being consequently responsible (at least in part) of the circadian misalignment patients experience after surgery. To investigate this hypothesis, an experimental protocol that allowed for continuous recording and monitoring of mice locomotor activity rhythms in time isolation was required.

The method development process of my study revealed that:
a) In order to anaesthetise multiple mice in DD for six hours with the exact same conditions and with a survival rate of 100%, a specific anaesthesia chamber with a continuous flow rate of 6L/min GA in 100% oxygen needs to be used so that animals can be constantly monitored, and anaesthesia can be effectively delivered.

b) Using strictly controlled protocols to isolate mice from any external stimuli such as noise, and keep animals in constant environmental conditions, is crucial to study the effect of GA on mice circadian rhythms and to get steady and reliable recordings.

In addition, the results from my thesis showed that GA disrupts circadian rhythms of behaviour in a time-dependent manner, causing phase shifts in behavioural rhythms (between 1.11 and 0.54 hours depending on the anaesthetic agent) when administered at the end (isoflurane GA, CTs 8-12) or in the middle (sevoflurane GA, CTs 4-8) of the animals’ resting phase.

The magnitude of this disruption depends on the anaesthetic drug, the duration of anaesthesia treatment, and the presence of light during the GA treatment. GA-induced phase shifts are statistically significant when GA is administered during the mice resting phase, evoking phase delays in locomotor activity rhythms.

It is likely that the anaesthesia-induced phase shift is related to the loss of consciousness through thalamo-cortical networks mediated by GABAergic currents and that this activation is phase-dependent. However, the molecular mechanism by which GA disrupts circadian rhythms of behaviour, and the range of the effects of GA on central and peripheral clocks, is unclear.

It is reasonable to think that GA interacts with photic entrainment through non-parametric mechanisms and that the influence of GA on the light pathway depends on the anaesthetic agent,
since the circadian responses of GA plus light on circadian rhythms are phase-dependent and differ between anaesthetic agents. This suggests that GA does not pharmacologically block the shifting effect of light, and therefore could open the possibility, after further study, of using light as a potential treatment to decrease or prevent the circadian disruption of the clock after surgery. The results from this thesis could not prove the potential usefulness of light as a therapeutic strategy to ameliorate the GA-induced phase shift in the circadian clock but establishes the line for future work in the field.

Thus, to fully understand how light and GA interact, and how they influence post-operative recovery, we need a full light and anaesthesia PRC for each anaesthetic agent, as well as molecular studies that allow us to compare molecular mechanisms with behavioural rhythms. This would reveal important information which, potentially, could be applied in therapeutic strategies to ameliorate and quicken patients’ recoveries. If successful, it could reveal a way to study how to reduce post-operative circadian and sleep disruption and potentially shorten hospital stays.

Finally, this thesis provides the first standardised and reproducible protocol to study the effect of GA, and GA plus light, on the clock in a mammalian model with a survival rate of 100%. Future studies implanting this experimental design would strongly benefit the field in terms of increased consistency between experiments, leading to informative comparisons of results and the ability to progress and expand the work.
Chapter 5

5 Appendices

5.1 Animal ethics approval

Protocol Number: 001654 & 001654/2

Project Title: The effect of GA on the behavioural rhythms of mice

5.2 Animal Ordering

In order to ensure animal supply and animal unit (Vernon Jansen Unit. VJU) stock availability, mice were ordered two months in advance, so that the animal unit had time to breed the number of adult animals required for each experiment. A VJU requisition form needed to be completed and sent to VJU orders (Figure 5.1).

![Requisition for Animals](image)

**Figure 5.1:** Vernon Jansen Unit (VJU) animal requisition form used to request animals
5.3 Animal collection

Once the animals were ready for collection from the VJU main warehouse, they were transported to the mice chronobiology lab in the VJU facility. They were weighed, identified, and marked by ear notches or holes produced by an ear punch device following the alternative numbering system (Figure 5.2). This system was used to create a unique identifier that allowed identification of animals in constant darkness (DD) and during the anaesthesia treatment.

![Alternative numbering system](image)

Figure 5.2: Alternative numbering system applied to create a unique identifier that allowed identification of animals in DD and during the anaesthesia treatment after collection

Animals were then placed in their individual autoclaved cages (Figure 5.3a) with bedding, nesting and food and water ad libitum. Each cage was identified with the mouse ID, cabinet and sensor number, date, AE approval number and date. The cages were connected to an IR silent sensor that counted the revolutions per minute of the running wheel (Figure 5.3b). The cages were placed inside the circadian cabinet (Figure 5.3c) for the adaptation period until the anaesthesia treatment.
Figure 5.3: Figure showing a) cages autoclaved and prepared with the bedding, nesting, and food and water (*ad libitum*). b) A mouse inside its cage after the ear punch and c) the IR silent sensors connected to the power panel in the circadian cabinets.
5.4 Circadian cabinet setup

5.4.1 Stacking cabinets

The cabinets were designed to be stacked up to four cabinets high. However, due to the dimensions of the ceiling space available in our room, the setup had to be re-organised with Cabinet 1 set apart from Cabinets 2, 3, and 4. Cabinet 2 was placed upon Cabinet 3, and 3 on top of 4. Cabinet 4 had little blocks, or “feet” on the underside, that fit into the steel frames. The frames and cabinets were marked according to the setup. The front part of the frame was identified by the locking wheels.

5.4.2 Computer standard setup.

There were two USB connections from the computer. One connected the computer to the black Clocklab interface (CL300). The other was connected to the blue RS 485 interface pictured below (Figure 5.4).

![Figure 5.4: Blue RS 485 interface.](image)

To connect the CL300 (Clocklab interface), the running wheel cables were connected to the satin cables on the cabinets. These were then connected to the white boxes on the side of the cabinets (Figure 5.5).
According to the commercial design of the cabinets, Cable 1 from the CL300 port connected to the top connector of the top cabinet; which connected to the second connector of the first cabinet, and so on down the side of the cabinet stack. However, as the distribution of the cabinets had to be re-arranged, these connections had to be lengthened and soldered together (Figure 5.6).

To connect the light controls from the CL300, a silver satin cable was used to connect from CL300 to ports 1-4 to the top RJ45 (Ethernet looking) connector of the light control, then daisy chain connections with the shorter silver satin cables were made from Cabinet 1 to Cabinet 2, onto Cabinet 3, then to Cabinet 4.
For the temperature/humidity/light sensor boards, the blue RS485 interface was plugged into a USB port. The connector cables were linked together from cabinet to cabinet in a daisy chain (Figure 5.7a). The connectors for the RS485 cables connected from the bottom and top of the black control boxes. Once the cabinets were connected to each other, temperature and humidity could be monitored from the computer (Figure 5.7b).

![Figure 5.7: a) Connectors for the RS485 cables connected together from cabinet to cabinet. b) Monitoring panel from the computer ClockLab software.](image)

The power for the cabinets was 12 volts. Plugs had to be modified for New Zealand connections since the equipment was American. There were three power sources for each cabinet, one for the black light control box, one for the wheel sensors, and one for the fans (Figure 5.8).

![Figure 5.8: Power sources for each cabinet highlighted by a blue circle.](image)
The wheel sensors were plugged into connectors inside the box (Figure 5.9a) and then clipped on to the side of the cage adjacent to the wheel (Figure 5.9b).

Figure 5.9: a) Wheel sensor plugged into the connectors inside the circadian cabinets and b) onto the mouse cage.

The computer and the Uninterruptible Power Supply (UPS) were placed in the adjacent room since complete darkness was required for the completion of the experiments. Cables were connected through a hole in the wall between the rooms.
5.4.3 Lights control and setup

Figure 5.10: Picture of the control panel located on the side of each of the cabinets.

- Manual light control to test the cabinets

To check that connections were correctly placed, a manual set up had to be performed since the company instructions had to be modified due to the size of the chronobiology laboratory.
First, the power had to be disconnected and re-connected. Once connected the green LED light (Figure 5.10) would start flashing. All the small levers on the binary control panel were positioned in the “up” position (this stopped the Chamber Control System from being able to control the lights in the cabinet and allowed the lights to be manually controlled). The dial underneath the control panel was turned on to adjust the lights.

- **Light control though Chamber Control System**

Each cabinet was numbered using the binary controls so that the Chamber Control system could identify each individual cabinet.

The Circadian Chamber Control Software had the ability to specify the light amplitude as well as the durations of the light cycle. This software worked in conjunction with the light control boards and sensor boards of the cabinet providing dawn and dusk lighting events. The Circadian Chamber Control Software also had the ability to monitor the temperature, humidity, and relative lux level within the cabinet with the addition of Temperature, Humidity, Light Sensor Board (P/N PT2-TH12). However, these conditions could not be changed, only monitored.

To set up the control of the light in the circadian cabinets via Chamber Control System, the jumper, indicated in the diagram, had to be removed and placed across both pins. Once the power supply was plugged in, the green LED, indicated in the diagram (Figure 5.10), started blinking. The light amplitude level could be set by setting all the smaller DIP switches (Figure 5.10, red rectangle) to the OFF position. Once the jumper was in place, the light level could be set while the green LED was blinking on the light control board. The light amplitude level had to be set within the first two
minutes after the board had received power. The power supply had to be disconnected and reconnected once again to be able to use the dial to adjust the lighting. Once this was done, and the LED was no longer blinking, the jumper had to be removed and placed over one of the pins as it was originally. When the jumper was placed on the single pin, the manual adjustment was inoperative, and the software/interface took control.

As long as the jumper stayed in this position, the cabinets would respond to Chamber Control rather than Clock Lab. The Chamber Control system was tested but not used due to its complexity, and because it did not provide any extra benefit over the ClockLab Light Control System.

- Light control via Clock Lab

Once the manual set up confirmed all the cabinets were correctly connected and operating, the ClockLab Light Control System was set up. With this system, lights could be controlled from the computer in the adjacent room without disturbing mice locomotor activity rhythms. In addition to its simplicity, this system was chosen over the Circadian Chamber Control Software because ClockLab Analysis Software was used for data analysis and this system had previously been used by my research group.

To set it up, the data cables and light control cables had to be interchanged and connected to the side of the black, light control box. Each box had two RJ45 connections. The cable going into the cabinet was plugged into the top RJ45 connector, and then daisy chained to the next box by cabling from the bottom connector of one cabinet to the top RJ45 connector on the next cabinet. All levers in the binary controls had to be on the “up” position.
The interface with the Clock Lab System was used to enable ClockLab to identify each cabinet.

For Cabinet 1, the interface jumper was attached to the first two pins in the Clock Lab interface.

For Cabinet 2, the interface jumper was attached to pins 3 and 4.

For Cabinet 3, the interface jumper was attached jumper to pins 5 and 6.

For Cabinet 4, the interface jumper was attached jumper to pins 7 and 8.

This system also allowed for the specification of light amplitude and duration parameters when ramping between lighting conditions. It worked in conjunction with the light control boards and sensor boards of the cabinet to provide dawn and dusk lighting events (although we did not use this option). The Clock Lab System also has the ability to monitor the temperature, humidity, and relative light intensity level (measured in lux) within the cabinet (Figure 5.11).

Figure 5.11: ClockLab light control screenshot from computer software.
5.5 Recording system set up

Data were collected continuously during the whole experiment via the proprietary ClockLab data collection software suite. The resulting activity records (actograms) were analysed for changes in period and phase following exposure to GA (Figure 5.12).

A sketch of how the locomotor activity rhythms recording system works can be seen in Figure 5.12.

Figure 5.12: ClockLab software configuration started when a mouse began running in the wheel. The IR light emitted by the silent switch is reflected by the steel bar of the running wheel and the reflection of the light beam is captured by the IR sensor of the switch. Each revolution per minute was transferred through the IR sensor to the ClockLab computer which recorded the revolutions per minute in actograms, where data were binned every two-minute epochs.
5.6 Animal welfare monitoring

5.6.1 Daily animal general monitoring

Daily general monitoring was applied to all experimental mice. Locomotor activity rhythm recording monitoring was performed twice per day (Figure 5.13).

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<th>male</th>
<th>Information for technicians:</th>
<th>Department</th>
<th>Anaesthesiology</th>
<th>Phone contacts</th>
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<td></td>
<td></td>
</tr>
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<td>Researcher</td>
<td>Alma Orts-Sebastian</td>
<td>2102214077</td>
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</tr>
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<td>IR</td>
<td>James Cheeseman</td>
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<td>Order number:</td>
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<td>#EAC</td>
<td>R001654</td>
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**State of the research:**
- **Numer of animals:** 24
- **Animal ID:** (cab.cage.ch/tg) from 1.1.01-C3/GA5 to 1.6.06-C3/GA5
- from 1.7.08-GA3/C5 to 1.12.15-GA3/C5
- from 3.25.21-C3/GA5 to 3.30.34-C3/GA5
- from 3.31.35-GA3/C5 to 3.36.40-GA3/C5

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</tbody>
</table>

Figure 5.13: Daily general monitoring sheet.

An action protocol was developed in case any abnormality was observed either from mice monitoring or from the locomotor activity recordings.
5.6.2 Animal welfare protocol (Adapted from Guidelines for monitoring of experimental mice, VJU)

Action protocol for animal welfare at the animal facilities (Figure 5.14).

![Diagram of animal welfare protocol]

- Abnormalities observed
- Check humane endpoints → AEC approval
  - 1. Weight loss of 20% or more plus one other clinical sign compared with control group
  - 2. Low activity performance for 24 hours plus one other clinical sign.
  - 3. No activity for more than 24 hours
- Yes, No, Not sure → Euthanasia, Increase monitor frequency, Investigate the cause → Solutions
- Advise VJU staff ← Advise AWO

Figure 5.14: Animal welfare monitor protocol (Adapted from Guidelines for monitoring of experimental mice, VJU) (AEC= Animal Ethics Committee) (VJU= Vernon Jansen Unit) (AWO= Animal Welfare Officer).

5.6.3 Animal welfare score sheet

If animals showed any sign of stress, pain, or disease it was recorded in the form below. Frequency of animal surveillance was increased, and human end points considered. Human end points are specific situations that denote the animal is in pain or distressed and therefore must be released from the experiment and euthanised (Figure 5.15).
Figure 5.15: Animal welfare score sheet.
5.6.4 Sick and death animal report and Animal Welfare Incident form

Regarding animal welfare, if anything unexpected occurred before, during or after manipulations, and this happened to affect animal welfare, it needed to be reported to the Animal Welfare Officer. Urgent welfare concerns had to be acted upon immediately by contacting a team leader or unit manager.

In all cases, the report of the incident had to be done by using the Sick and Dead Animal Report.

All deaths that occurred prior to the planned end of the experiment had to be reported to the Animal Welfare Officer as soon as possible so that a post mortem examination could be carried out if necessary. Sick animals had to be reported to the Animal Facility Manager as soon as possible, using a Clinical Incident Form (also known as a Sick and Dead Animal Report) which is available in all animal facilities (Figure 5.16).
Figure 5.16: Sick and Dead Animal Report.

Husbandry or experimental incidents that impacted animal welfare such as the power cut on the 26 September 2017, or the fire alarm on the 30 November 2017 had to be reported and the Animal Welfare Incident form had to be completed (Figure 5.17).
### Figure 5.17: Animal Welfare Incident form.

<table>
<thead>
<tr>
<th>Incident report ref no.</th>
<th>Date Received</th>
<th>AEC Protocol No.</th>
<th>Responsible investigator</th>
<th>Department</th>
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<table>
<thead>
<tr>
<th>Date of Incident</th>
<th>Time of Incident</th>
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<table>
<thead>
<tr>
<th>No. of animals affected</th>
<th>Description of Animal</th>
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<table>
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<tr>
<th>Incident identified by:</th>
<th>Incident witnessed by:</th>
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</table>

**Description of incident/observations:**

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<tr>
<th>Has immediate action been taken?</th>
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<tbody>
<tr>
<td>Specify details if known:</td>
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<table>
<thead>
<tr>
<th>Has the vet been notified?</th>
<th>Yes</th>
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</thead>
<tbody>
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<td>Specify details if known:</td>
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</table>

<table>
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<th>AEC protocol ref (if known):</th>
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<table>
<thead>
<tr>
<th>Researcher name (if known):</th>
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<table>
<thead>
<tr>
<th>Additional information:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tbody>
</table>

Submit completed form by email or via internal mail to:
- Animal Welfare Officer/University Vet
- Chair of AEC
- AEC Secretary

Any urgent animal welfare concerns must be actioned immediately, by contacting the vet, a team leader or unit manager.

**Created by:** FMRS  
**Version:** 1  
**Date approved:**
5.7 GA discrete phase shift and actograms from all the experiments

5.7.1 Isoflurane-induced phase shift (six-hour treatment)

The discrete phase shift induced by six hours of GA with isoflurane for each single mouse was calculated by comparing the regression lines of the seven days before to the seven days after the intervention as explained in sections 2.2.5 to 2.2.8 and plotted as per figure below:

Figure 5.18: Graph describing the discrete phase shift induced by six hours of isoflurane GA treatment on circadian rhythms of locomotor activity in each individual mouse per CT of administration (n=80).
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Table 5.1: Isoflurane induced phase shift per mouse per CT after six hours of treatment (n=80)
5.7.2 Isoflurane-induced phase shift (control treatment. 10 minutes GA)

The discrete phase shift induced by 10 minutes of GA with isoflurane for each single mouse was calculated by comparing the regression lines of the seven days before to the seven days after the intervention as explained in sections 2.2.5 to 2.2.8 and plotted as per figure below:

Figure 5.19: Graph describing the discrete phase shift induced by 10 minutes of isoflurane GA treatment on circadian rhythms of locomotor activity in each individual mouse per CT of administration (n=78).
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Table 5.2: Isoflurane induced phase shift per mouse per CT after 10 minutes treatment (n=78.)
5.7.3 Sevoflurane-induced phase shift (six hours treatment)

Plot of the discrete phase shifts induced by six hours of GA with sevoflurane for each single mouse at the CT of the administration.

![Graph showing sevoflurane discrete phase shift (6 hours treatment)]

Table 5.3: Graph describing the discrete phase shift induced by six hours of sevoflurane GA treatment on circadian rhythms of locomotor activity in each individual mouse per CT of administration (n=75).
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Table 5.4: Sevoflurane induced phase shift per mouse per CT after six hours treatment (n=75).
5.7.4  Sevoflurane-induced phase shift (10minutes GA)

The discrete phase shift induced by 10 minutes of GA with sevoflurane for each single mouse were plotted as per figure below:

Figure 5.20: Graph describing the discrete phase shift induced by 10 minutes of sevoflurane GA treatment on circadian rhythms of locomotor activity in each individual mouse per CT of administration (n=24).
### Table 5.5: Sevoflurane induced phase shift per mouse per CT after 10 minutes treatment (n=24)

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<td>4.48.55</td>
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<td>18.35</td>
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</tr>
<tr>
<td>1.8.9</td>
<td>23.99</td>
<td>-0.05</td>
</tr>
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</table>
5.7.4.1 Exemplar actograms showing the effect of noise and light on mice circadian rhythms

- Exemplar of the noise disruption due to a fire alarm at the FMHS. The fire alarm went off the day of the anaesthesia at 9pm. The animals were at the end of their active phase. The time of the fire alarm and the locomotor activity of the mouse at that time is highlighted with a black oval. The anaesthesia treatment is marked with a black rectangle. Locomotor activity data were excluded from the PRC due to possible interferences with the noise.

- Exemplar actograms of the circadian disruption due to the power cut at the FMHS. The power cut is highlighted with a black circle. The anaesthesia treatment is marked with a black rectangle. Locomotor activity data were excluded from the PRC due to the light disruption.
5.7.5 Discrete phase shift and actograms from isoflurane plus light and light experiments

**A)**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>CT</th>
<th>Light phase shift</th>
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<tr>
<td>4.38.44</td>
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<td>4.39.45</td>
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<td>10.4</td>
<td>-1.82</td>
</tr>
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<td>10.6</td>
<td>-2.13</td>
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<tr>
<td>4.42.48</td>
<td>10.8</td>
<td>-2.02</td>
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<td>4.43.50</td>
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<td>4.46.53</td>
<td>11</td>
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<td>-3.32</td>
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<td>2.16.18</td>
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<td>2.17.19</td>
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<td>2.23.26</td>
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<td>-2.63</td>
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<td>16.4</td>
<td>-2.57</td>
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</table>

**B)**

<table>
<thead>
<tr>
<th>Mouse</th>
<th>CT</th>
<th>Isoflurane plus light phase shift</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.26.30</td>
<td>10.0</td>
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<tr>
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</tr>
<tr>
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</tr>
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<td>7.8</td>
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</tr>
<tr>
<td>3.34.39</td>
<td>11.0</td>
<td>-2.37</td>
</tr>
<tr>
<td>3.35.40</td>
<td>9.2</td>
<td>-2.35</td>
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<tr>
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<tr>
<td>1.3.3</td>
<td>21.1</td>
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<tr>
<td>1.4.4</td>
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<tr>
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<td>16.9</td>
<td>0.81</td>
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</tbody>
</table>

Table 5.6: Table showing the A) light and B) isoflurane plus light induced phase shift per mouse per CT of administration (n=45.)
5.7.6 Actograms from sevoflurane plus light and light experiments

### A) Mouse ID  CT  Light phase shift
2.13.15 14.6 -3.61  
2.14.16 17.1 -2.25  
2.15.17 15.7 -2.99  
2.16.18 15.4 -2.78  
2.17.19 14.8 -3.01  
2.18.20 15.3 -3.36  
2.19.22 15 -3.21  
2.20.23 16.4 -2.02  
2.21.24 15.2 -2.63  
2.22.25 16.4 -2.57  
2.23.26 17 -1.15  
3.25.29 18.4 -0.24  
3.26.30 14.7 -1.6  
3.28.32 14.9 -2.84  
3.29.33 14.2 -3.32  
3.30.31 15.5 -2.82  

### B) Mouse ID  CT  Sevoflurane plus light phase shift
1.1.1 16.9 0.61  
1.2.2 15.3 -1.09  
1.3.3 15.3 -0.65  
1.4.4 15.9 -0.18  
1.5.5 14.0 -2.31  
1.6.6 15.9 -1.83  
1.7.8 15.1 -1.08  
1.8.9 14.1 -1.28  
1.9.10 16.0 0.36  
1.11.12 16.0 0.49  
1.12.13 15.9 0.25  

Table 5.7: Tables showing the A) light and B) sevoflurane plus light induced phase shift per mouse per CT of administration (n=27)
5.8 Summary of the data presented

Note: All the animals (but for the light and light and anaesthesia experiments) were in DD before, during and after treatment. The different background of the actograms is due to a feature of the ClockLab analysis software, which was merely used as a colour-code system to distinguish actograms from different experiments since data analysis sometimes took weeks to complete. The same system was used in the coloured bar that represent the GA treatment.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Number of actograms</th>
<th>Included in PRC</th>
<th>Reason</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isoflurane PRC (six-hour treatment)</td>
<td>80</td>
<td>80</td>
<td>-</td>
</tr>
<tr>
<td>Isoflurane PRC (10 minutes treatment)</td>
<td>81</td>
<td>78</td>
<td>No calculable phase shift day one after GA</td>
</tr>
<tr>
<td>Sevoflurane PRC (six-hour treatment)</td>
<td>75</td>
<td>75</td>
<td>-</td>
</tr>
<tr>
<td>Sevoflurane PRC (10 minutes)</td>
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<td>No calculable phase shift day one after GA</td>
</tr>
<tr>
<td>Isoflurane plus light and light (CTs 7-11 and 14-17)</td>
<td>47</td>
<td>45</td>
<td>No calculable phase shift day one after GA (2) CT out of the average stabilised (1)</td>
</tr>
<tr>
<td>Sevoflurane plus light and light (CTs 14-17)</td>
<td>29</td>
<td>27</td>
<td>No calculable phase shift day one after GA (1) No calculable phase shift day one after GA (1)</td>
</tr>
</tbody>
</table>

Table 5.8: Summary of the data presented in actograms in appendix 5.7. Number of actograms used per experiment.
References:


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Ehara taku toa, he takitahi, he toa takitini.

Kia ora, Aotearoa