Photoperiodic properties of circadian rhythm in rat

by

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To my loving grandparents, YaoXiang Zhang and AnNa Yu

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Abstract

Animals have intrinsic circadian periods of around 24 hours. In order to synchronize to the 24-hour day, circadian rhythms entrain to photic time cues and establish a consistent daily rhythm. The maintenance of a stable phase relationship between circadian rhythms and the light-dark (LD) cycle is necessary for entrainment to occur. It is commonly thought that there is a fixed relationship between the phase angle of entrainment and the intrinsic free-running period, where a smaller phase angle reflects a shorter period. Furthermore, it was thought that individuals with smaller phase angles would adjust faster to advance shift of the LD cycle, and slower to delay shift of the LD cycle. This dissertation reevaluated these relationships in rats using long-term *in vivo* pineal microdialysis to carefully monitor melatonin secretion rhythm. The studies presented in this dissertation examined both the interindividual differences between outbred rats, as well as differences in melatonin rhythms of inbred rats entrained to different photoperiods. Under long photoperiod (short night), melatonin rhythm has smaller phase angles and shorter durations; the opposite is true for short photoperiods. The phase angle variation, natural as well as manipulated, did not correlate with the free-running period variation in rats. Furthermore, individuals with smaller phase angles reentrained faster in both the advance and delay direction. To gain a greater understanding of the role of photoperiod in melatonin phase angle, the adjustment of the melatonin rhythm to an expansion or compression of dark period was studied by altering the dark period symmetrically or unidirectionally. While reentrainment to an 8h night

resulted in very similar melatonin profiles regardless of how the dark period was altered, reentrainment to a 16h night depend significantly on whether the dark period was expanded symmetrically or by morning or evening expansion. These findings demonstrate that the relationship between period, phase, and pattern of reentrainment are quite different than is currently believed, and new analysis may be necessary to understand the underlying biology of the circadian timing system.

Chapter 1

Introduction

1.1 The circadian system

Nearly all organisms and biological processes exhibit a daily circadian rhythm. For example, individuals keep a regular sleep-wake schedule, and physiological factors such as heart rate, body temperature, and melatonin production all cycle each day with regularity. Such circadian rhythms evolved in response to the 24 hour (h) light-dark (LD) cycle produced by the Earth's rotation around the Sun. Under a consistent schedule, biological circadian rhythms maintain a set temporal phase relationship with the environmental LD cycle. In the absence of external time cues under constant darkness (DD), circadian rhythms persist with a close to 24h "free-running" period that's specific to each species and unique to each individual (Figure 1-1).

An internal biological time keeping system drives the persistence of endogenous circadian rhythms. In mammals, the central biological clock is located in the suprachiasmatic nuclei (SCN) of the anterior hypothalamus, which is often referred to as the "central pacemaker" (Klein et al., 1991). The SCN coordinates biological processes to provide temporal organization within the organism, and uses the presence of light to synchronize the internal clock to the external time. This process, called "entrainment", orchestrates the behavioral, physiological and biochemical processes to ensure optimum function in coordination with time of day.

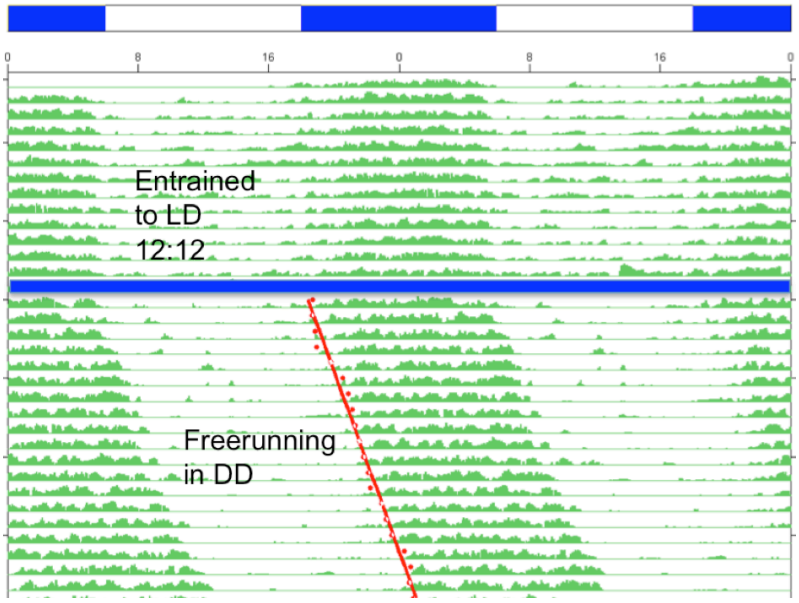

Figure 1-1 Entrained and free-running core body temperature rhythm of a rat

The actogram displays the rise and fall in core body temperature of a rat (in green). Each horizontal line represents 48h, and consecutive 24h days are plotted on top of each other. Blue bars mark the dark period each day. The rat was at first entrained to a 12h light 12h dark schedule (LD12:12h), then allowed to free-run in complete darkness (DD). Under entrained conditions, the temperature rhythm is synchronized to the 24h day. In the absence of light cues, the temperature rhythm begins to free-run. The red line marks the temperature onset each day under DD, and shows that the free-running rhythm has a greater than 24h period. (Zhang et al., unpublished data).

1.2 Importance of entrainment

As the behavioral, physiological and biochemical processes required for optimum fitness change from morning to night, it is imperative for the internal circadian rhythm to be entrained in anticipation of the environmental change each day. In nocturnal rodents for example, entrainment of behavioral rhythm is a matter of life and death: a rat that ventures outside of the burrow in broad daylight falls easy prey to many predators in the wild. In humans, the importance of entrainment is commonly felt when transmeridian travel, daylight saving time, or other acute changes to our schedule disrupt the circadian system. Inconsistencies between biological processes and desynchronization with the external schedule have wide-ranged health consequences. Before the circadian system can readjust or "reentrain" to the environmental time, symptoms of sleep disturbance, gastrointestinal distress, and decreased cognitive performance occur along with general feelings of ailment in what is commonly referred to as "jetlag" (Wright et al., 1983; Winget et al., 1984; Cho et al., 2000). Furthermore, chronic circadian disruptions result in higher incidents of cancer (Filipski et al., 2004), sleep disorders (Drake et al., 2004), cardiovascular disease (Boggild and Knutsson, 1999), digestive diseases (Caruso et al., 2004), as well as other health issues (Rajaratnam and Arendt, 2001; Costa, 2003). Reentrainment of the circadian clock is a slow process that takes many days. To minimize the health issues associated with circadian disruption, it is imperative to gain a better understanding of the properties of circadian entrainment and reentrainment.

Beyond attaining optimum fitness on a daily basis, circadian entrainment also has the vital function of regulating seasonal physiology in photoperiodic animals (i.e. animals with seasonal phenotypes). In temperate and arctic latitudes, the duration of daylight (i.e.

"photoperiod") within a 24h day lengthens in the summer and shortens in the winter. Entrainment to a particular photoperiod changes the nightly duration of melatonin production, which triggers biological responses that allow animals to cope with seasonal changes in the wild (Goldman, 2001; Malpaux et al., 2001). These responses include annual cycles in reproduction, activity, body weight, molting, and thermoregulation. Even though humans and rats are not considered photoperiodic, their circadian rhythms still display differences under different photoperiods. For instance, the duration of locomotor activity for nocturnal rodents is shorter in the summer (Puchalski and Lynch, 1991; Elliott and Tamarkin, 1994), and in humans, decreased exposure to light in the winter months often results in fatigue and seasonal affective disorder (Terman et al., 1989). Study of circadian entrainment to different photoperiods not only reveals information on control of seasonal phenotypes, but also provides a better grasp of the components in the circadian system.

1.3 Mechanisms of mammalian photic entrainment

Apart from light, other time cues (known as "zeitgebers") exist that entrain the circadian clock; these include regular cycles in temperature (Rensing and Ruoff, 2002), food availability (Stephan, 2002), exercise (Yamanaka et al., 2006), and intake of pharmacological agents such as melatonin (Lewy et al., 2005). The presence of light is by far the strongest zeitgeber, and this dissertation focuses on the photic entrainment of mammals.

When light hits the retina, it activates melanopsin-containing retinal ganglion cells that transmit the photic information via the retinohypothalamic tract (RHT) directly to the ventrolateral region of the SCN (vlSCN) (Figure 1-2; Foster and Hankins, 2002; Hattar et

al., 2002; Moore et al., 2002). The vlSCN imparts the photic timing signal to the dorsomedial region of the SCN (dmSCN), whose role is to maintain an endogenous oscillation that synchronizes the biological rhythms of the organism (Moore et al., 2002; Nagano et al., 2003). The excitatory neurotransmitter glutamate communicates a change in external schedule by activating N-methyl-D-aspartate (NMDA) receptors in the SCN, which induces an influx of calcium (Ca^{2+}) that leads to the production of nitric oxide (NO) (Ding et al., 1994; Mintz and Albers, 1997; Mintz et al., 1999). Induction early in the night results in a phase shift in the delay direction as NO causes nuclear Ca^{2+} influx by activating ryanodine receptors (Ding et al., 1998). Induction late in the night causes phase advance as NO activates guanylate cyclase to produce cGMP, which in turn activates the MAPK-CREB signaling pathway (Golombek et al., 2004). The RHT also releases the neurotransmitter pituitary adenylate cyclase activating peptide (PACAP) at the SCN (Hannibal et al., 1997). Other SCN inputs that contain photic information include those from the thalamic intergeniculate leaflet (IGL), and the raphe nucleus (Krout et al., 2002).

The timing of circadian oscillations is maintained at the cellular level through a molecular feedback loop of clock genes (Cermakian and Sassone-Corsi, 2000; Reppert and Weaver, 2001; Okamura et al., 2002). Inside the nucleus, CLOCK and BMAL1 proteins form a heterodimer that activates the transcription of *period* 1 and 2 genes (*Per1* and *Per2*). The PER proteins translated in the cytoplasm are phosphorylated by casein kinase 1 epsilon (CK1ε) and rapidly degraded. Over time, more PER proteins accumulate in the cytoplasm and are shuttled into the nucleus after forming dimers with *cryptochrome* (CRY) or other PER proteins. This protein complex acts on the

CLOCK/BMAL1 heterodimer to inhibit the transcriptional activation of *Per* genes. As the protein complex destabilizes through the release of PER proteins back into the cytoplasm, the molecular cycle starts again. During phase shifts, light promotes the transcription of *Per* genes, with *Per1* playing an essential role for phase advance and *Per2* for phase delay (Albrecht et al., 2001). Almost all cells in the body express clock genes, and their synchronization depends on output signals from the SCN.

1.4 SCN outputs

The SCN projects directly to a number of regions, and communicates timing information via rhythmic release of arginine-vasopressin (AVP), vasoactive intestinal peptide (VIP), γ-Aminobutyric acid (GABA), and glutamate (Dibner et al., 2010). The direct targets of the SCN include the paraventricular nucleus (PVN), dorsomedial hypothalamus (DMH), medial preoptic area (MPOA), and lateral hypothalamus (LH). These regions influence the endocrine and autonomic nervous system, allowing the SCN to control timing of sleep, activity, heart rate level, body temperature, hunger, thirst, metabolism, and other biologic processes (Morin, 2007; Dibner et al., 2010).

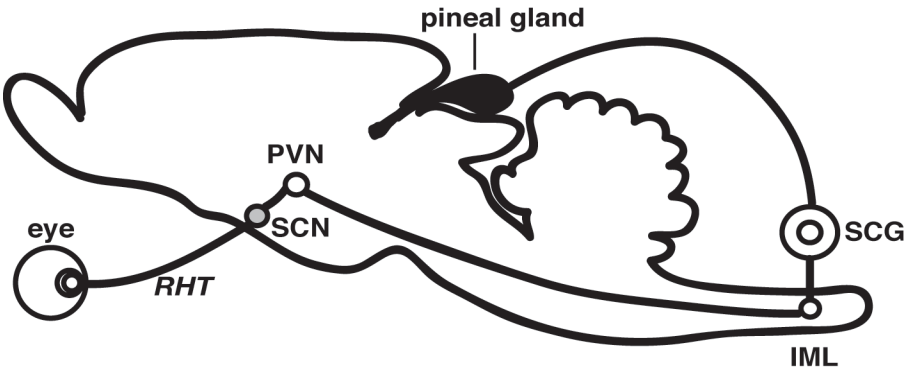

Figure 1-2 Melatonin synthesis pathway in the rat brain

To entrain the circadian clock, light signals received by the eyes transmit to the SCN via the retinohypothalamic tract (RHT). The SCN subsequently controls melatonin production in the pineal gland via a multi-synaptic pathway. Signals from the SCN transmit to the paraventricular nucleus of the hypothalamus (PVN), the intermediolateral cell column of the spinal cord (IML), and the superior cervical ganglion (SCG), before reaching the pineal gland. (Adapted from Borjigin, J., & Liu, T. 2008). Application of long-term microdialysis in circadian rhythm research. Pharmacol Biochem Behav, 90(2), 148–155.)

One of the most studied projections from the SCN is part of the retino-hypothalamo-pineal pathway that controls the synthesis and secretion of melatonin at the pineal gland (Figure 1-2). The circadian information sent out from the SCN is accurately portrayed by the melatonin profile, and it in turn plays a major role in regulating various biological processes (discussed in section 1.6 below). In addition to direct circadian control of melatonin synthesis, the retino-hypothalamo-pineal pathway also causes the immediate suppression of melatonin synthesis in the presence of light at night (Lewy et al., 2001). The extent of acute suppression depends on the wavelength, intensity, and duration of the light pulse. All vertebrate organisms produce melatonin at night, regardless of whether they are diurnal or nocturnal. In rats entrained to a steady light and dark (LD) cycle, melatonin concentration rises only after at least 1h following the onset of darkness (Borjigin et al. unpublished data), reaches maximum levels in the middle of the night, and drops off right before the light turns on. The time lag preceding melatonin onset is indicative of the minimum amount of time necessary for the biochemical processes involved in melatonin synthesis to occur; these processes are briefly discussed below.

1.5 Pineal melatonin synthesis pathway

The SCN connects to the pineal gland via the PVN (Moore and Klein, 1974; Teclemariam-Mesbah et al., 1999). There are other inputs to the pineal gland, and they are reviewed in (Simonneaux and Ribelayga, 2003). At the PVN, SCN projections release GABA during the day to inhibit melatonin synthesis, and glutamate at night to stimulate melatonin synthesis (Perreau-Lenz et al., 2004). At the pineal gland, sympathetic neurons from the SCG receive these regulatory inputs, and release noradrenalin to simulate melatonin synthesis (Arendt, 1995). Melatonin synthesis takes

place within the major cells of the pineal gland called pinealocytes. The adrenergic regulation of melatonin synthesis is outlined in (Takahashi, 1994). Briefly, α1 and β-adrenergic receptor activities result in elevation of intracellular cyclic adenosine monophosphate (cAMP), which activates the production of serotonin N-acetyltransferase (AANAT) (Klein, 1985; Krause and Dubocovich, 1990). AANAT plays a major role in the enzymatic synthesis of melatonin.

Figure 1-3 shows the four enzymatic steps involved in melatonin synthesis (Arendt, 1995; Simonneaux and Ribelayga, 2003; Borjigin and Liu, 2008). Since the pineal gland is outside of the blood brain barrier, pinealocytes are able to take up tryptophan from the blood, which tryptophan-hydroxylase 1 (TPH1) converts to 5-hydroxytryptophan. Aromatic amino acid decarboxylase (AADC) converts the 5-hydroxytryptophan to 5-hydroxytryptamine, (a.k.a. serotonin; 5HT). AANAT converts 5HT to N-acetylserotonin (NAS), which is then methylated by Hydroxyindole-O-methyl transferase (HIOMT) to become melatonin (Arendt, 1995; Borjigin et al, 1999; Simonneaux and Ribelayga, 2003). The transcriptional regulation of these melatonin synthesis enzymes follows a day/night cycle, and is reviewed in Li et al. (1998) and Bernard et al. (1999). The synthesized melatonin is released into the bloodstream, where it goes on to play crucial roles in the regulation of circadian rhythms, seasonal behaviors, sexual development, and metabolism.

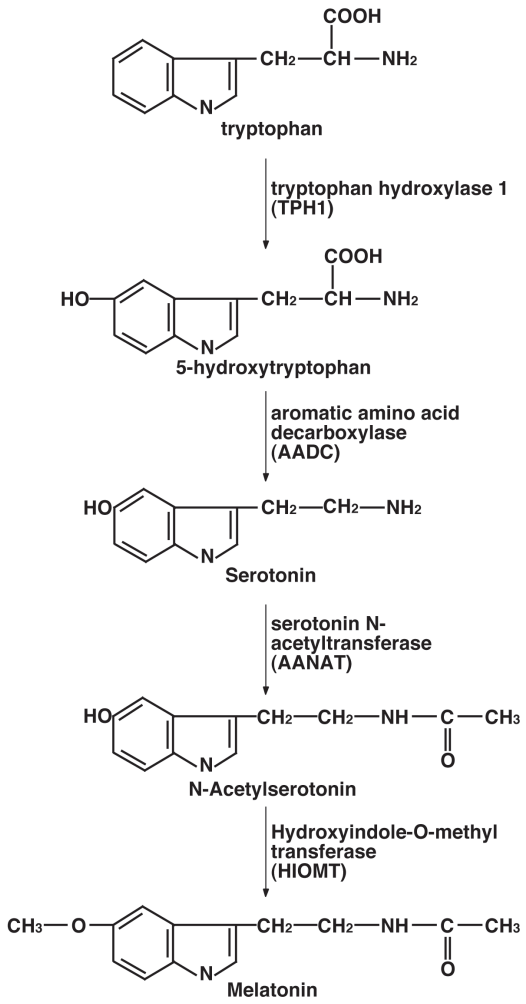

Figure 1-3 Biochemical synthesis of melatonin

Melatonin is synthesized from tryptophan by four enzymes: tryptophan hydroxylase 1 (TPH1), aromatic amino acid decarboxylase (AADC), arylalkylamine N-acetyltransferase (AANAT), and hydroxyindole-O-methyltransferase (HIOMT). In our studies, we routinely collect data for serotonin, N-acetylserotonin, and melatonin in a single HPLC chromatogram. (Adapted from Borjigin, J., & Liu, T. (2008). Application of long-term microdialysis in circadian rhythm research. Pharmacol Biochem Behav, 90(2), 148–155.)

1.6 Role of melatonin

Melatonin has a wide variety of effects in the body, and modulates cells and tissues in an assortment of mechanisms that are reviewed in (Reiter et al., 2010). The major melatonin membrane receptors include G protein coupled receptors called MT1 and MT2 (Hardeland, 2009), and major nuclear binding sites include retinoic acid receptors such as RORα1, RORα2, RZRα, and RZRβ (Carlberg, 2000). Beyond activating membrane receptors and nuclear binding sites, melatonin also acts as an antioxidant and protects lipids, proteins, and DNA from oxidative stress by directly detoxifying free radicals and related oxygen derivatives (Allegra et al., 2003). Melatonin's effects on sexual development, metabolism, and inhibition of cancer have been well documented (Macchi and Bruce, 2004; Reiter et al., 2007; Reiter et al., 2010; Tan et al., 2010). Its other major roles are discussed in greater detail below.

1.6.1 Circadian regulation

The consistent nightly melatonin secretion acts as a messenger of the central pacemaker, and synchronizes the circadian rhythms of several biological outputs. Although melatonin is secreted at night in all animals, it has different effects on diurnal and nocturnal animals. For example, melatonin promotes sleep, lowers body temperature, reduces blood pressure, and decreases mental performance in diurnal humans (Cagnacci et al., 1992; Skene and Arendt, 2006; Simko and Pechanova, 2009), but has the opposite effects in nocturnal rats.

While the SCN controls melatonin synthesis rhythm, melatonin also acts on the MT1 and MT2 receptors at the SCN to entrain the circadian clock (Rivera-Bermudez et al., 2004). Administration of exogenous melatonin results in phase shift of body

temperature, sleep timing, and endogenous melatonin rhythms in mammals, with evening administration of melatonin leading to phase advance, and morning administration leading to phase delay (Arendt and Skene, 2005). The chronobiotic functions of exogenously administered melatonin have been used in humans to minimize circadian disruption during jet lag, and to promote sleep (Lavie, 1997; Arendt, 2009).

1.6.2 Seasonal regulation

Just as daily melatonin rhythm imparts circadian timing information, melatonin duration serves as an internal indicator of the seasons (Reiter, 1993). Since melatonin is only produced at night, the duration of melatonin is shorter in the short summer nights, and longer in the long winter nights (Illnerova and Vanecek, 1980; Goldman, 2001). The duration of melatonin triggers changes such as molting, pelage growth and color change, hibernation, migration, metabolism, and seasonal reproduction (Deboer and Tobler, 1996; Anderson et al., 2003; Lincoln et al., 2006; Atgie et al., 2009; Masumoto et al., 2010). Studies suggest that the pars tuberalis (PT) of the pituitary plays an important role in inducing seasonal phenotype. Melatonin released into the bloodstream binds to MT1 receptors on the pars tuberalis (PT), and the duration of nightly melatonin secretion initiate molecular pathways that leads to physiological changes (Hut, 2011).

1.6.3 Melatonin as a circadian marker

For the research purposes of this dissertation, the most important aspect of melatonin is its function as the best available in vivo marker for analysis of the circadian clock (Illnerova et al., 1989; Honma et al., 1997; Klerman et al., 2002; Benloucif et al., 2008; Borjigin and Liu, 2008). Melatonin synthesis and secretion at the pineal gland follows a precise rhythm that accurately represents the circadian clock activities at the SCN. In

comparison to other circadian markers traditionally used in animal studies (e.g. locomotor activity, body temperature, heart rate), melatonin is superior in the following ways: 1. Traditional circadian markers tend to produce noisy data. While activity, heart rate, and body temperature rhythms maintain a constant phase with the LD cycle, it is sometimes difficult to identify the daily onset of the rhythm, and almost impossible to decipher the daily offset. In comparison, melatonin measurements produce very clear results with little noise, and show a dramatic nightly rise and fall in concentration that allows for the confident identification of both onset and offset (Liu and Borjigin, 2006). 2. Melatonin rhythm is precise and highly reproducible under entrained conditions. In fact, an entrained individual has the exact same melatonin onset and offset each day (Figure 1-4). A circadian disruption is thus made obvious by daily variation in melatonin profile, and reentrainment can be clearly identified by noting when both the melatonin onset and offset re-stabilize to remain the same each day (Liu and Borjigin, 2005a). 3. Following a circadian disruption, the precise identification of melatonin onset and offset allows for analysis of the dynamics of reentrainment. 4. Melatonin rhythms are not affected by stress such as surgery, sickness, hunger, and fear. In comparison, locomotor activity, heart rate, and temperature rhythms are disrupted for over a week following surgery, and can be influenced by other non-circadian stressors (Drijfhout et al., 1995; Liu and Borjigin, 2005c).

Melatonin is the preferred marker for circadian analysis in human studies, but despite its benefits, melatonin is rarely used in animal studies. In human subjects, a profile of melatonin synthesis rhythm can be constructed from repeated sampling of melatonin measurements from the blood plasma, saliva, or urine of a single individual (Benloucif et

al., 2008). Individual laboratory rodents on the other hand have limited saliva, blood, and urine volume, making it impossible to take frequent samples over long periods of time. By sacrificing multiple animals around the clock, a melatonin profile can be obtained; however, inter-individual differences cannot be discerned in such studies, and a heroic effort is required on the part of the researcher. In comparison to such efforts, continuous measurements of activity, heart rate, and temperature rhythms are easier to obtain, and are frequently used in circadian studies of rodents.

1.7 Pineal microdialysis approach in circadian research

To fully utilize this marker in rodents, the Borjigin lab developed a long-term automated pineal microdialysis system that overcomes the acquisition issues mentioned above (Sun et al., 2003; Borjigin and Liu, 2008; Zhang et al., 2011). Using this system, we are able to obtain accurate melatonin secretion profiles directly from the pineal glands of freely moving rats (described in detail in Chapter 2).

The system offers a number of advantages in deciphering the circadian clock. Firstly, the automation of this process by coupling microdialysis with on-line high-pressure liquid chromatography (HPLC) bypasses the labor-intensive and error-prone manual handling of samples, and increases accuracy of the results. Secondly, continual measurements can be made for around a month, allowing long-term perturbations of the clock to be followed over time in the same animals (Figure 1-5; Sun et al., 2003). Thirdly, melatonin concentration is recorded every 20min in an individual; this temporal resolution together with the precision of melatonin rhythm allows for the detection of small changes in circadian phase (Figure 1-4 and Figure 1-5). Lastly, past melatonin studies relied on results averaged between a group of animals, in experiments that lasted

for only 1-4 days (Azekawa et al., 1990; Azekawa et al., 1991; Drijfhout et al., 1993; Perreau-Lenz et al., 2005). By using the long-term pineal microdialysis system, inter-individual variations of melatonin rhythm can be verified over time, allowing for the differentiation of chronotypes and reentrainment kinetics in laboratory animals (Liu and Borjigin, 2006).

One shortcoming of the pineal microdialysis system is the inaccurate representation of melatonin amplitude. Because the amplitude of melatonin depends on the surgical position of the trans-pineal microdialysis probe, we cannot compare melatonin amplitude between individuals. Individuals with probes through the central region of the pineal gland would provide a higher amplitude data than those with probes on the peripheral regions. We also cannot compare melatonin amplitude within the same individual. As shown in Figure 1-5, due to clogging of the probe, growth of the rat skull, and other technical issues, the amplitude of melatonin will artificially decrease over time. Thus, throughout our experiments, melatonin synthesis rhythms are examined in terms of percent of daily maximum values (Figure 1-4).

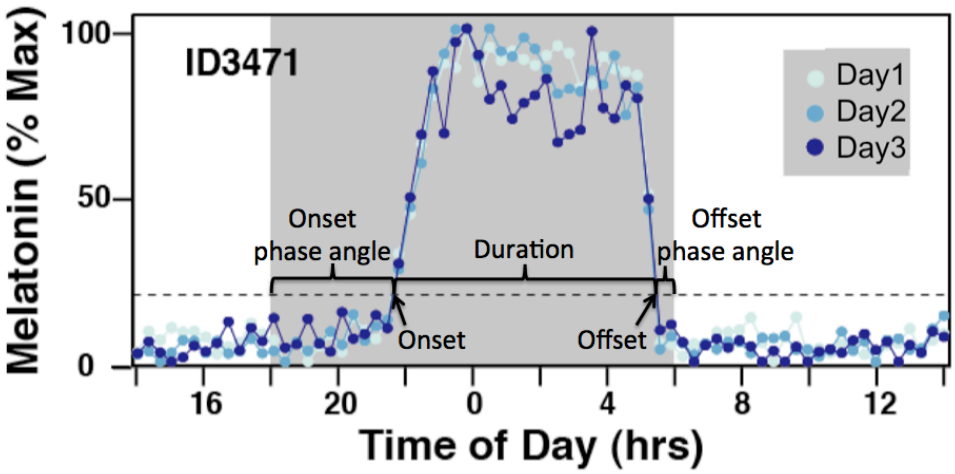

Figure 1-4 Daily melatonin secretion profiles from a single rat

The daily melatonin profile of a single rat entrained to LD12:12h is shown for three consecutive days. The gray shaded area represents time of darkness. The y-axis displays percentage of the daily maximum value. The dotted line represents 20% of the daily maximum value. Melatonin onset and offset timings are defined by when melatonin concentration rises beyond and falls below 20% of the daily max. Melatonin duration is defined by the time difference between onset and offset. Melatonin onset phase angle is the time difference between light-off and melatonin onset, and offset phase angle is the time difference between melatonin offset and light-on. (Adapted from **Zhang, L. S**., Liu, T., & Borjigin, J. (2011). Long-term Automated HPLC Analysis of Microdialysis Samples from Multiple Freely Moving Animals. LC World Talk, (1), 10–13.)

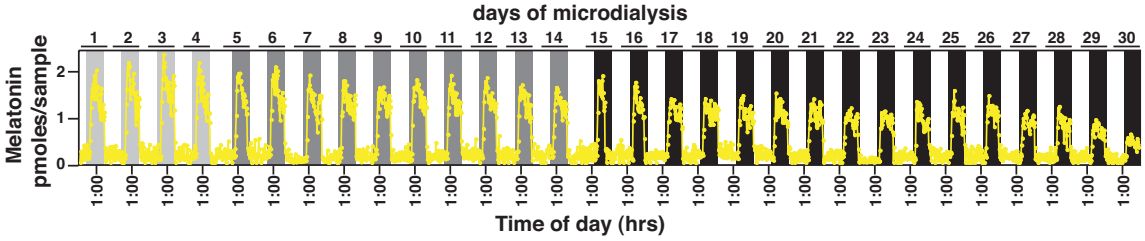

Figure 1-5 Long-term pineal microdialysis of a single rat

The rat was entrained in light:dark (LD) 12:12h (lights-off at 18:00h) for more than 2 weeks prior to the pineal microdialysis. Melatonin profiles were shown for the last 4 days of entrainment with lights-off at 18:00 h (days 1-4; lighter gray shades), following an LD delay of 3 hrs (days 5-14; darker gray shades), and following an LD delay of 6 hrs (days 15-30; black shades). Detailed profiles of each shift are published in (Liu and Borjigin, 2005c). (Adapted from Borjigin, J., & Liu, T. (2008). Application of long-term microdialysis in circadian rhythm research. Pharmacol Biochem Behav, 90(2), 148–155.)

When a rat is entrained to a LD cycle, its melatonin profiles expressed in daily percentage values are so consistent that they look almost identical each day (Figure 1-4). We can identify several circadian factors from the melatonin profiles: melatonin onset and offset timing determined at 20% of the daily maximum value, melatonin duration determined from time difference between onset and offset, and the entrained phase difference between melatonin and the LD cycle (i.e. "phase angle") (Figure 1-4). Since melatonin is only synthesized in the dark, the phase angles are expressed in positive values of the time difference between light-off and onset, and between offset and light-on.

1.8 Goals and Significance

This dissertation relies extensively on the use of rat pineal microdialysis for longitudinal investigation of the circadian clock response to light. Chapter 2 describes the pineal microdialysis system in detail, as well as provides information on the use of telemetry implants for recording activity, heart rate, and body temperature rhythms. The ability to distinctly and clearly identify both onset and offset of the melatonin rhythm for long periods of time within the same individuals offers many new insights into the circadian system. Throughout the dissertation, rats were entrained to three different photoperiods: a middle photoperiod of 12h light and 12h dark (LD12:12h), a long photoperiod of 16h light and 8h dark (LD16:8h), and a short photoperiod of 8h light and 16h dark (LD8:16h). The longer the photoperiod, the shorter the melatonin duration and phase angles. By using photoperiod to manipulate the entrained melatonin profile, the studies described in this dissertation aim to shed light on the relationships between the

free-running period, the entrained phase angle, and the speed of re-entrainment following a shift of the LD cycle.

In Chapter 3, I test the hypothesis that there is not a strong relationship between the phase angle of entrainment and the steady state free-running period. The experiments include the examination of inter-individual variation in phase angle and period in outbred rats, as well as photoperiod manipulation of phase angle in inbred rats. The results of this study contradict the circadian theory that a smaller phase angle is correlated to a shorter free-running period.

Chapter 4 tests the hypothesis that a smaller melatonin onset phase angle correlates with faster reentrainment to a new LD schedule. Both inter-individual variations in outbred rats, as well as photoperiod manipulation of phase angle in inbred rats were analyzed. Finding that a smaller melatonin onset phase angle result in faster reentrainment provides important insight into the reducing the duration of jetlag and other circadian disruptions.

Chapter 5 focuses on the reentrainment of melatonin rhythm from LD12:12h to LD8:16h or LD16:8h. Depending on whether the photoperiod was compressed or expanded symmetrically or unidirectionally, the final entrained profiles as well as the dynamics of entrainment differed significantly between treatments. These findings have notable implications on the properties of circadian reentrainment, as well as on photoperiod regulation of seasonal phenotypes.

Chapter 2

Experimental methods

This chapter covers the common experimental methods used throughout this dissertation. All experimental and surgical procedures were performed in accordance with the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan, and the NIH handbook.

2.1 Long-term pineal microdialysis of freely moving rats

2.1.1 System overview

Online microdialysis integrated with real-time high-pressure liquid chromatography (HPLC) is a powerful tool for providing information on the circadian pacemaker. Designed by the Borjigin lab, this procedure provides a method for long-term *in vivo* measurement of pineal secretions in freely acting animals (Sun et al., 2003; Borjigin and Liu, 2008).

The HPLC system consists of one Shimadzu SCL-10A VP controller, two Shimadzu LC-20AD isocratic pumps, a CTO-20AC column oven containing 2 Supelco C18 reversed phase columns, two RF-10AXL detectors, two VICI Cheminert® sample injectors (2-position/10-port actuator), and a VICI digital sequence programmer (Figure 2-1). Each system is designed to analyze pineal dialysates from four rats, with two rats to each detector. The Borjigin lab currently operates 5 systems, for a maximum capacity of 20 rats at a time. As shown in Figure 2-2, detectors A and B analyze dialysates from rats A1 and B1 simultaneously for 10 minutes, then switches to rats A2 and B2. Each rat is

thus analyzed every 20 minutes, and consecutive samples can generally be measured for up to one month.

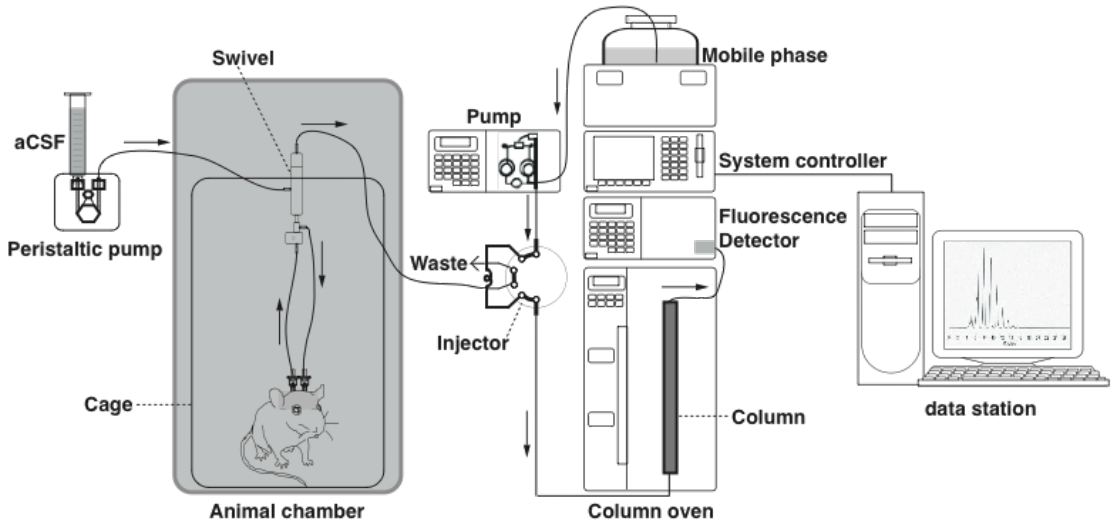

Figure 2-1 Overview of the microdialysis-HPLC system

Components and connections for the analysis of one rat are shown. In a full setup, with the exception of the system controller, all other components are doubled to allow the analysis of dialysates from four rats under one system. Artificial cerebral spinal fluid is pumped through the pineal microdialysis implants. The dialysate from the pineal gland is collected in the sample injector, which is then injected into the HPLC column. For simplicity, a 1-position/6-port valve is shown instead of the 2-position/10-port valve used in the actual sample injector. Excess dialysate and mobile phase are delivered into waste lines. (Adapted from Borjigin, J., & Liu, T. (2008). Application of long-term microdialysis in circadian rhythm research. Pharmacol Biochem Behav, 90(2), 148–155.)

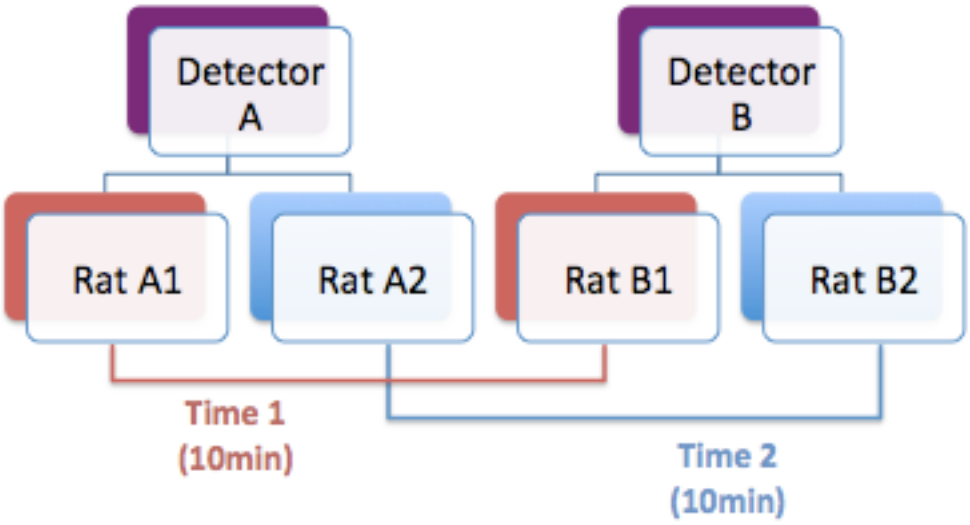

Figure 2-2 Data processing sequence

VICI digital sequence programmer controls the 2-position/10-port actuator. Detector A analyzes dialysate from rat A1 while detector B simultaneously analyzes rat B1. Dialysates from rats A2 and B2 are then subsequently analyzed. The analysis switches back and forth between rat 1 and rat 2 every 10 minutes. (Adapted from **Zhang, L. S**., Liu, T., & Borjigin, J. (2011). Long-term Automated HPLC Analysis of Microdialysis Samples from Multiple Freely Moving Animals. LC World Talk, (1), 10–13.)

2.1.2 Trans-pineal microdialysis probe implantation

2.1.2.1 Microdialysis probe construction

Each microdialysis probe is hand crafted from 21-gauge and 25-gauge blunt needle tips. The 25-gauge blunt tip needle shaft (length of 1 inch) forms the inner shaft in direct contact with dialysis samples. For structural support, the 25-guage needle shaft is inserted into a 21-gauge needle with a shaft length of 0.5 inches (Figure 2-3A). The needles are held together by epoxy glue, and then bent to shape using a needle nose plier as shown in Figure 2-3B to make TC4-left probe. For TC4-right probe, a die grinder sharpened tungsten rod is attached to serve as the guide wire during surgical implantation. The 1.5 to 2 inch long tungsten rod is threaded though a 1 to 1.5 inch semipermeable microdialysis hollow fiber (molecular weight cut off of 13 kd). Both the rod and hollow fiber are inserted into the end of a TC4-left to make TC4-right probe (Figure 2-3C). Epoxy is used to secure the hollow fiber to the 25-gauge tip and to the tungsten rod. Only tiny amount of epoxy is used at the junction of tungsten rod and microdialysis fiber to minimize damage to the brain during insertion. The complete dialysis probes are allowed to dry for at least 16h before use.

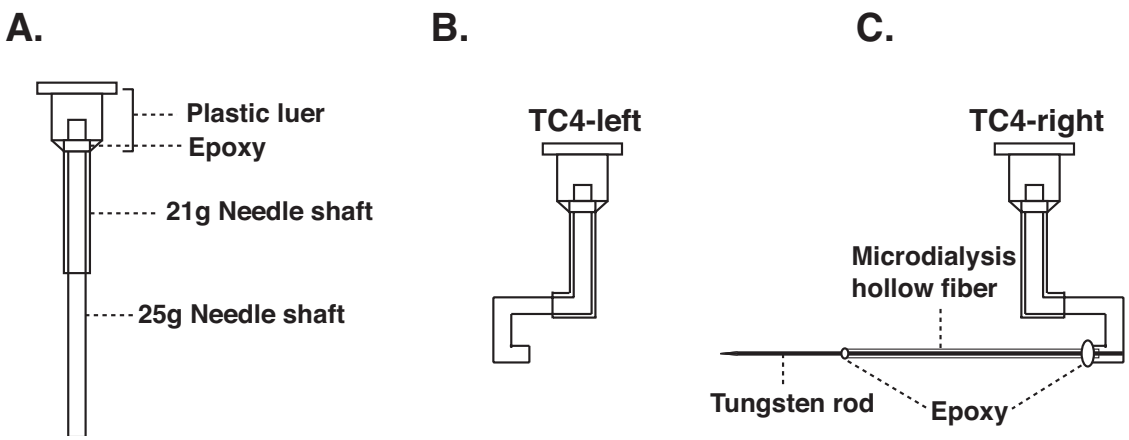

Figure 2-3 Microdialysis probe construction

Dr. T.C. Liu designed the current model of the microdialysis probe. The probe consists of a left and right part (TC4-left and TC4-right). **(A)** The base of the probes is constructed from 21 and 25-gauge blunt tip needles held together by epoxy. **(B)** The needle shafts are bent to shape as shown to construct TC4-left. **(C)** TC4-right is constructed by attaching a sharpened tungsten rod threaded through a microdialysis hollow fiber to a TC4-left. During surgery, TC4-right is threaded through the pineal gland. The tungsten rod is then removed, and the hollow fiber is attached to TC4-left by epoxy. (Adapted from Borjigin, J., & Liu, T. (2008). Application of long-term microdialysis in circadian rhythm research. Pharmacol Biochem Behav, 90(2), 148–155.)

2.1.2.2 Dialysis probe implantation

Rats are anesthetized by inhalation of isoflurane. The head is shaved and positioned flat in a stereotaxic instrument. We expose the skull by a 2cm coronal incision between the two ears along the interaural line, and score the position of the stabilizing screws and holes for the probe to pass through with a cauterizer as shown on Figure 2-2A. The pineal gland is located beneath the lambda (the intersection between lambdoidal and saggital sutures). The position of holes for the probe to pass through will depend on the species, strain, and age of the animal, and needs to be evaluated for each type of animal. For 8-week old male Sprague Dawley rats for example, the position should be AP of +0.8 mm and V of -2.5 mm. We use a small hand drill to make 1mm diameter openings for 3 stainless steel stabilizing screws. The holes for the probe are 0.5mm diameter on the right and 1.0mm diameter on the left. The smaller right side hole prevents the tip of the 25-gauge needle from penetrating the skull. The larger left side hole allows the probe to easily exit the brain during implantation. TC4-right probe is carefully inserted from the right side of the skull out the left side (Figure 2-2B). The tungsten wire is removed by melting the epoxy on the left side. We then cut off excess dialysis membrane and insert the end into the TC4-left probe under the microscope using a micromanipulator mounted to the steriotaxic instrument (Figure 2-2C). The membrane is carefully secured to the probe with epoxy. The left and right probes are secured to the skull and anchor screws with dental cement. Lastly, we suture the skin, and house the rat individually in light-controlled chambers.

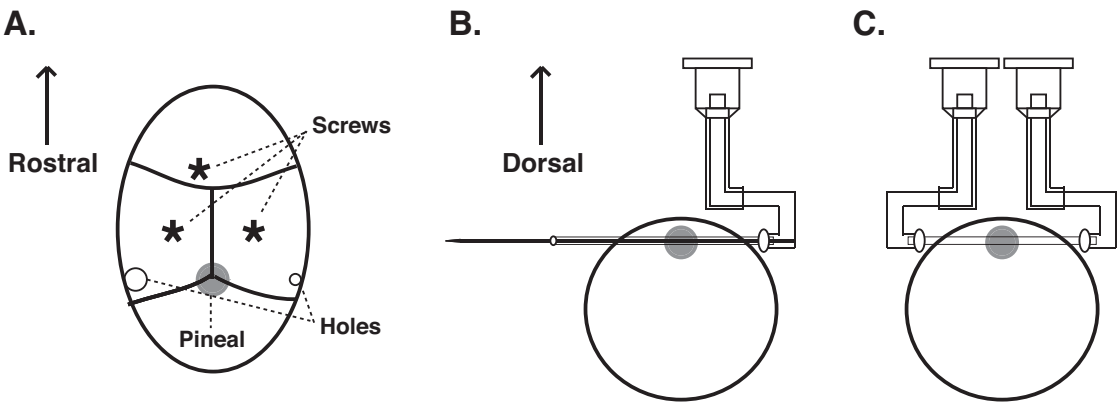

Figure 2-4 Pineal microdialysis implant surgery

(A) Diagram of a rat's skull viewed from the top. The positions of stabilizing screws are noted by the * symbol. The pineal gland is generally located beneath the lambdoidal and saggital sutures of the skull. Two holes are drilled on either side of the skull: a small hole on the right for inserting the tungsten guide wire and microdialysis membrane, and a larger hole on the left for the wire and membrane to exit the skull. **(B)** Diagram of the rat's skull looking from the back (up is dorsal). TC4-right probe is inserted from the hole on the right, through the pineal gland and out the hole on the left. **(C)** Once through the skull, we removed the tungsten wire, and cut the membrane to about 5mm out side of the skull. The membrane is then sealed to TC4-left probe with epoxy. Lastly, the whole structure is secured with dental cement, and the animal is sutured and allowed to rest for 1-2 days. (Adapted from Borjigin, J., & Liu, T. (2008). Application of long-term microdialysis in circadian rhythm research. Pharmacol Biochem Behav, 90(2), 148–155.)

2.1.3 Sample acquisition

After pineal microdialysis probe implantation, rats are allowed to rest for one to two days before they are connected to the microdialysis system as shown in Figure 2-1. The 21-gauge needle base of the probe can plug directly into PEEK tubing sealed against a syringe tip. Artificial cerebral spinal fluid is delivered to each implant via Instech peristaltic pumps, with two rats per pump at 2μl per minute. Each rat is linked to the peristaltic pump through a series of PEEK tubing connected through an Instech duel channel swivel. The swivel is mounted on a counterbalance arm providing both vertical and horizontal mobility. Freely moving rats tethered to the swivel are housed individually in cages situated in light controlled chambers. Dialysates are collected and delivered to the HPLC system through the sample injector (Figure 2-1). Two rats are connected to each sample injector. Staggered sample collection and analysis doubles the output of this system; while the dialysate from one rat is analyzed, the dialysate of the other rat is collected in the 20μL loop of the sample injector, with the excess running off into the waste. Every 10 minutes, the sequence programmer gives a signal to the fast microelectric actuator, and the previously collected dialysate is injected from the 2-position/10-port valve (Figure 2-2). The acquisition time window for each sample is 8 minutes followed by 2 minutes of system equilibration, for a combined time of 10 minutes for each run.

For purpose of circadian rhythm studies, the peaks of interest on a chromatograph are melatonin (MT) and its precursors, serotonin (5-HT) and N-acetylserotonin (NAS). These indoles are naturally fluorescent (Chin, 1990). Separation of each sample is conducted by reversed phase C18 column, maintained at 45°C. The mobile phase is

pumped at 1.5mL per minute, and consists of 34% methanol with about 10 mM sodium acetate (pH 4.5). Due to slight differences in each system, the exact concentration of sodium acetate must be adjusted for each detector so that the NAS, 5-HT and melatonin peaks are present and distinct during each run. The final adjusted retention times of the three peaks from nighttime pineal dialysates are shown in Figure 2-5. Note that dialysates are directly analyzed without any purification process, and it is of importance to ensure additional peaks do not interfere with the peaks of interest.

2.1.4 Data analysis

Data collection and sequence processing is performed on ClassVP firmware from Shimadzu. The data sequence is processed daily, and the resulting report is pasted into a preformatted Microsoft Excel worksheet for each rat, with data obtained every 20 minutes forming a profile of pineal NAS, 5-HT and melatonin concentrations through time. Figure 2-6 shows two consecutive days of data for a rat housed in cycles of 12 hours of light (6am to 6pm) and 12 hours of darkness (6pm to 6am). The concentration of 5-HT remains relatively constant, spiking only right before onset of NAS and MT. Both NAS and MT are detectable only at night; their concentrations rise dramatically several hours into the night, and falls to basal levels before the light comes on each day.

This dissertation focuses specifically on melatonin as a circadian marker. To ensure accurate representation of melatonin concentrations, the nighttime melatonin values are checked in ClassVP firmware by manually adjusting the automatically generated baseline (as depicted by the red lines in Figure 2-5). This manual inspection focuses on time points where the daily maximum concentration, onset, and offset occur. Graphing continuous time points generates an accurate representation of nightly melatonin profile.

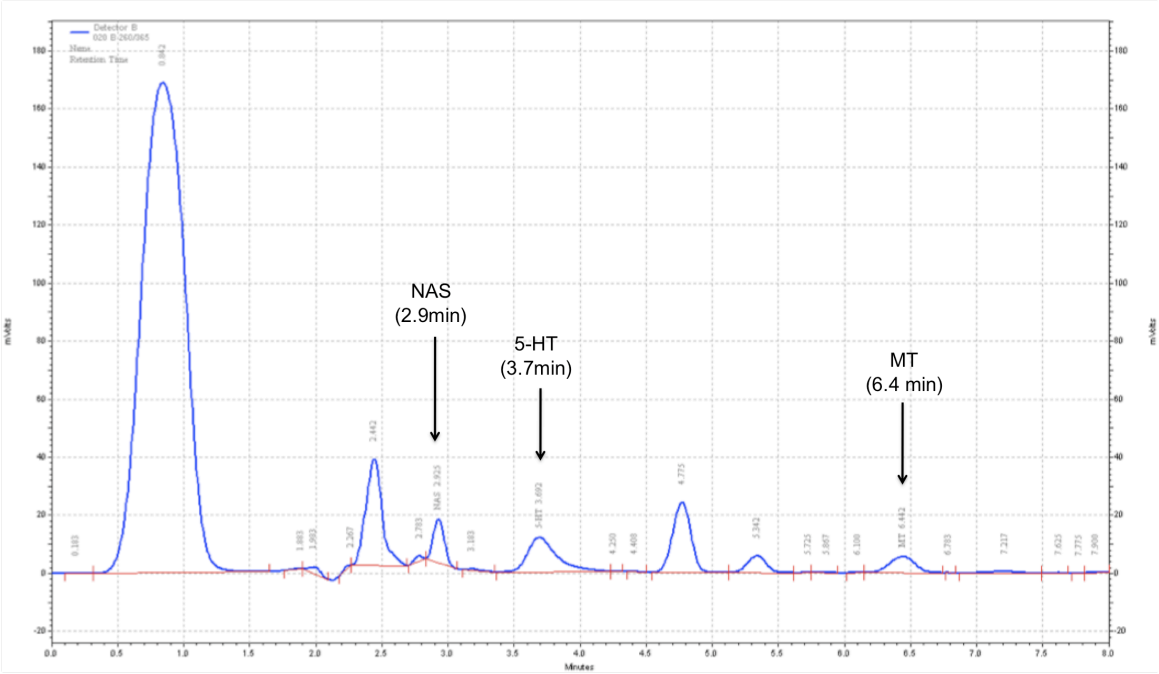

Figure 2-5 Typical nighttime trace of a single pineal dialysate sample

A single sample from an individual was collected in the sample loop then injected into the detector. The generated trace shows the peaks for N-acetylserotonin (NAS), serotonin (5-HT) and melatonin (MT) at their ideal retention times. The acquisition time window is 8min for each sample, followed by a 2min of system equilibration before acquisition of the next sample. The automatically generated base line shown in red is subsequently adjusted manually to ensure accurate representation of melatonin concentration, which is the area under the curve. (Adapted from **Zhang, L. S**., Liu, T., & Borjigin, J. (2011). Long-term Automated HPLC Analysis of Microdialysis Samples from Multiple Freely Moving Animals. LC World Talk, (1), 10–13.)

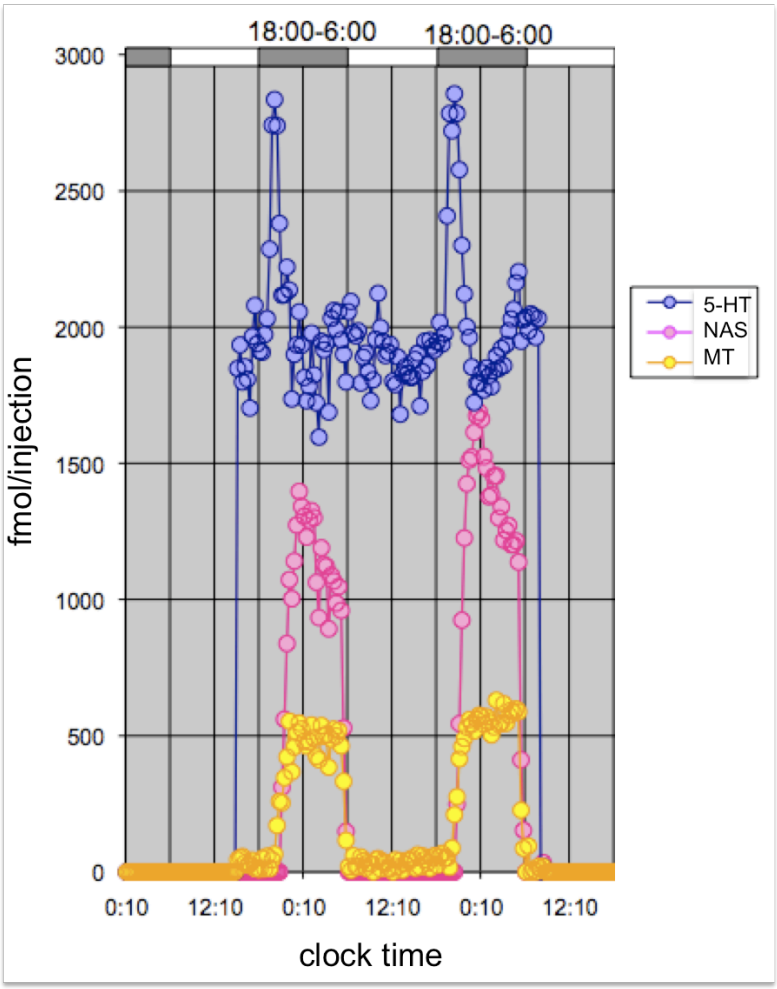

Figure 2-6 Microdialysis profile of 5-HT, NAS, and MT

Two consecutive days of data from a single rat are displayed, showing the concentrations of 5-HT, NAS and MT produced in the pineal gland over time. The shaded bars above the graph indicate periods of darkness. (Adapted from **Zhang, L. S**., Liu, T., & Borjigin, J. (2011). Long-term Automated HPLC Analysis of Microdialysis Samples from Multiple Freely Moving Animals. LC World Talk, (1), 10–13.)

2.1.5 Experiments

In this dissertation, the melatonin profiles of rats are observed under 24-hour cycles of light and darkness in one of three photoperiods (LD16:8h, LD12:12h, and LD8:16h). In Chapter 3, I also present the melatonin profiles of rats free-running under constant dark conditions (DD). The precision and frequency in which the data are obtained through the automated HPLC system allows for an unambiguous determination of MT onset, offset, duration, and phase angles (Figure 1-4).

2.2 Telemetry measurements

We record the locomotor activity, heart rate, and core body temperature rhythm of rats by using PDT 4000 E-mitters (Mini Mitter, Sunriver, OR). E-mitter communicates via radiofrequency field produced by an ER-4000 energizer/receiver placed below rat cages. Data output from the receivers are managed by Vital View™ (Mini Mitter) software.

2.2.1 Surgical Procedures

Rats are anesthetized by isoflurane inhalation, and a 2cm incision is made in the abdominal wall under sterile conditions. The E-mitter transmitter is placed inside the abdominal cavity, and the muscle wall and skin sutured. Because surgery affects the output of these rhythms, animals are allowed to recover for at least one week before start of data collection.

2.2.2 Data Collection and Analysis

We use Clocklab software (Actimetrics, Evanston, IL) to analyze the telemetry data. Actographs are generated with the built-in software features, and the free-running periods are calculated using Chi-squared Periodogram analysis (Sokolove and Bushell, 1978).

Chapter 3

Dissociation between phase angles of entrainment and free-running period

3.1 Introduction

The central circadian pacemaker at the suprachiasmatic nuclei shapes a variety of body rhythms such as locomotor activity, core body temperature, heartbeat frequency, blood pressure, and synthesis and secretion of several hormones including melatonin (Rusak and Zucker, 1975; Hastings et al., 2007). When individuals are situated under complete darkness (DD) for long periods of time without external time cues, these rhythms continue with a free-running period that deviates from the 24h solar cycle. In order to adapt to the 24h day, an individual must entrain their endogenous circadian rhythm to external time cues. The most potent time cue is that of the LD cycle (Stephan and Zucker, 1972; Ibuka and Kawamura, 1975; Duffy and Wright, 2005). When stably entrained, the circadian rhythm and the external time cue exhibit steady phase differences known as the phase angle of entrainment.

3.1.1 Accepted relationship between entrained phase angle and free-running period

Interindividual variations exist for both the phase angle and free-running period of animal and human subjects. More than three decades ago, several original studies established a qualitative relationship between phase angle and period, where a smaller phase angle corresponds to a shorter period in birds (Aschoff and Wever, 1962, 1966), lizards (Hoffmann, 1963), rodents (Pittendrigh and Daan, 1976a), and humans (Aschoff

et al., 1969). Based on these findings, the relationship between circadian phase and period has since become an established theory that is used to predict behavior. More recently, human studies using melatonin rhythm as a marker also confirm the notion of a linear positive correlation between phase angle and free-running period (Gronfier et al., 2007).

3.1.2 Published findings that contradict the relationship

Despite these corroborating findings, the relationship between phase angle and free-running period is not always so clear. In a study using human fibroblast cells (Brown et al., 2008), individuals with phase angle and period within the normal range did not show a clear positive correlation between the two factors. Only in individuals with more extreme chronotype did the correlation between phase angle and period become apparent (Brown et al., 2008). Furthermore, data from circadian mutants also suggest that phase angle and free-running period are independent factors. The per2 -/- mutants, for instance, possess very short periods and yet have wildtype-like phase angles (Zheng et al., 1999; Zheng et al., 2001). Similarly, cry2 -/- mutants display abnormally long periods and still have normal phase angles (Thresher et al., 1998; Van der Horst et al., 1999; Vitaterna et al., 1999). Additionally, published results from our own lab suggest that rats with earlier melatonin onsets do not necessarily have shorter free-running periods (Liu and Borjigin, 2005a). Together, these data indicate that phase angle of entrainment and free-running period may not in fact be directly related.

3.1.3 What this chapter offers

In this chapter, I reinvestigate the phase angle and period relationship in rats using multiple rhythm markers, especially melatonin. I examined both the onset and offset

phase angles of entrained melatonin profiles. Onset phase angle is defined as the time difference between light-off and melatonin onset, whereas the offset phase angle is defined as the time difference between melatonin offset and light-on. The free-running period in its stable state was obtained from locomotor activity, heart rate, and core body temperature rhythms of the same rats recorded for up to three months.

We hypothesize that there is not a strong relationship between the phase angle of entrainment and the free-running period. The investigation of the phase angle and free-running period relationship takes place in two major sections in this chapter. In section 3.3.1, I focus on the interindividual differences in phase angle and period of outbred rats, and report the correlation between melatonin phase angles from microdialysis measurements and free-running period obtained from telemetry recordings. In section 3.3.2, melatonin onset and offset phase angles were manipulated by entraining inbred Fischer rats to long or short day-lengths (called "photoperiods"). The differences in the manipulated phase angles were compared to the intrinsic period when the rats were stably free-running under DD.

3.2 Methods

3.2.1 Animals

Rats were housed individually in light-tight chambers at controlled temperature (20-25°C). Food and water were freely available. Illumination was supplied by white fluorescent lamps (~400 lux at cage level). Measurement of melatonin phase angles via pineal microdialysis and measurements of activity, heart rate, and body temperature via telemetry were as described in Chapter 2.

3.2.1.1 Individual differences in outbred rats

Male Wistar (WI) and Sprague-Dawley (SD) rats ordered from Harlan (Indianapolis, IN) were maintained under LD12:12h (lights on at 6:00AM) for a minimum of 2 weeks prior to microdialysis experiments. Their melatonin synthesis rhythms were recorded for at least 5 days under steady state entrainment to determine onset and offset phase angles. The animals were then placed under DD, and their free-running periods were determined from telemetry measurements of activity, heart rate, and body temperature after around 30 days in DD.

3.2.1.2 Photoperiod manipulation of phase angle in inbred rats

Adult Fischer 344 (F344) rats ordered from Harlan were housed under LD8:16h or LD16:8h for 2 months to entrain the animals to their respective photoperiod. Entrained melatonin synthesis rhythm was recorded from 4 rats in each photoperiod, for at least 5 days. The activity, heart rate, and temperature rhythms of a separate group of F344 rats were recorded via telemetry for 47 days while entrained under LD8:16h or LD16:8h, then for more than 90 days in DD.

3.2.2 Free-running period measurement

Under constant conditions, the previous entrainment history has an effect on the circadian rhythm, which is referred to as "after-effects" (Pittendrigh and Daan, 1976a; Liu and Borjigin, 2005a). It may take many weeks for the period to stabilize after introduction of DD (Jilge, 1980). Unfortunately, pineal microdialysis experiments can generally only last up to one month, which makes it technically unfeasible to obtain both phase angle and steady state free-running period from the melatonin rhythms of the same

individual. Thus, the free-running period was obtained from telemetry measurements, to be compared to the phase angles of entrained melatonin profiles in the same individuals.

3.2.2.1 Telemetry recording

Rats were housed individually with food and water freely available under LD cycle, then under DD for 3 months. Their locomotor activity, heart rate, and core body temperature were monitored via telemetry (Chapter 2.2). The period of the rhythm markers were then calculated using ClockLab software's Chi-squared Periodogram analysis (Sokolove and Bushell, 1978).

3.2.2.2 Comparison of period obtained from melatonin versus telemetry measurements

To test the validity of comparing activity, heart rate, and body temperature against melatonin rhythms, a separate cohort of rats was housed for 2 months under DD before implanted with microdialysis probe. After measuring melatonin for a few weeks, we implanted the same rats with telemetry transmitters, allowed them to rest for one week, then recorded their activity, heart rate, and temperature rhythms. The presence of after-effect under constant conditions can be detected by changes in melatonin duration (Liu and Borjigin, 2005a). The melatonin profile of these free-running rats maintained a steady shift each day at a constant period (Figure 3-1A), with onset and offset running parallel to each other (Figure 3-1B). From this melatonin data, we can confidently say that this cohort of animals has reached steady free-running state. Compared to melatonin rhythm, activity, heart rate, and temperature rhythms are harder to discern (Figure 3-1C). However, the individual free-running periods of melatonin and temperature rhythms are highly correlated with each other (Figure 3-1D; r^2=0.825 p=0.01). Similar results were

seen for activity and heart rate as well, thus indicating that it is reasonable to compare entrained melatonin rhythm to free-running telemetry rhythms.

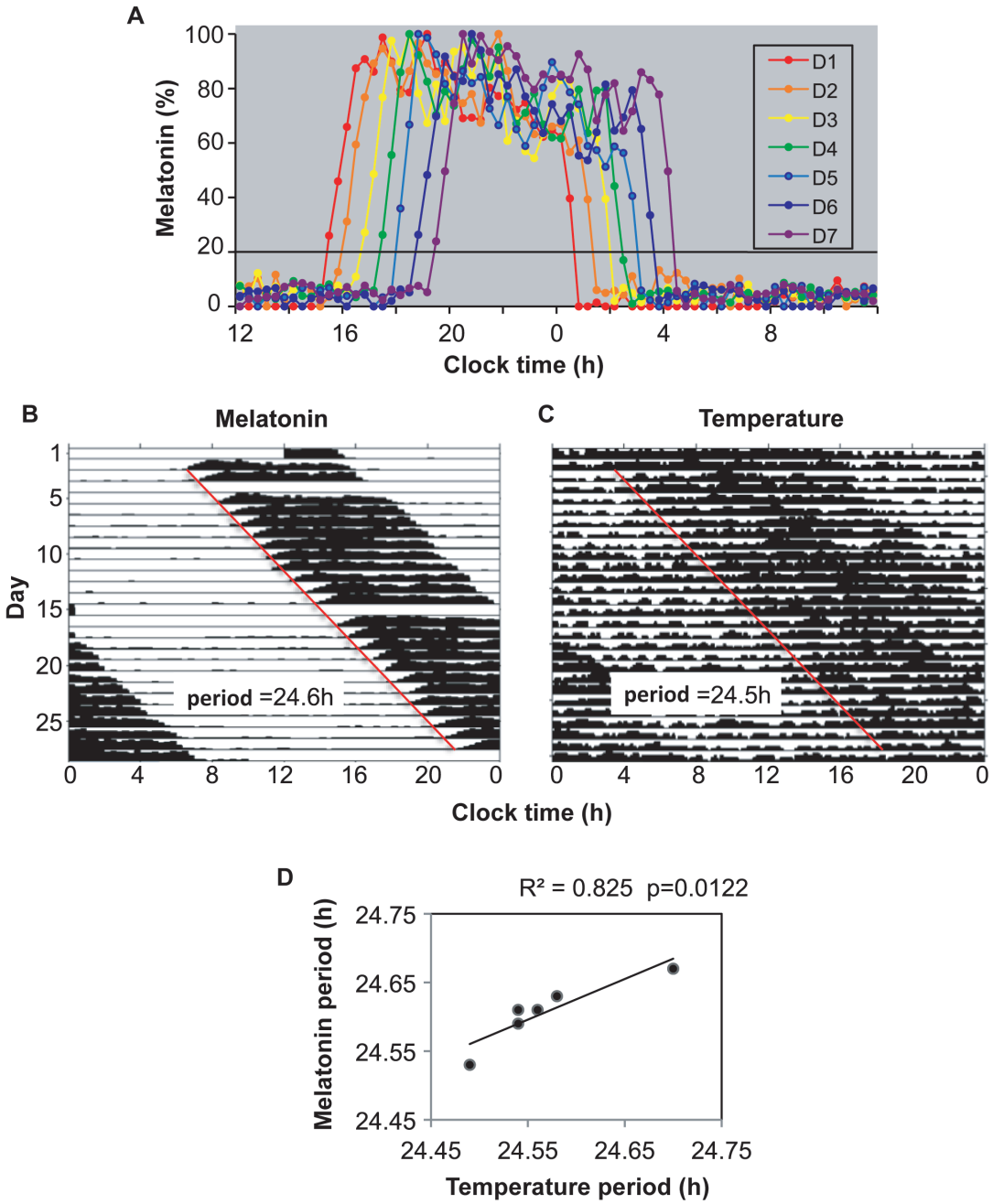

Figure 3-1: Comparison of melatonin and temperature free-running periods

(A) Free-running melatonin profile of a rat after two months under DD. **(B)** Melatonin and temperature actograms of the same rat. **(C)** Correlation between free-running periods obtained from melatonin and temperature rhythms. Each data point represents one rat. (Zhang et al., manuscript in preparation).

3.3 Results

3.3.1 Interindividual differences in phase angle and free-running period of outbred rats

3.3.1.1 Variations in entrained melatonin profiles

The melatonin profiles of WI and SD rats examined in this study varied greatly between individuals (n = 13; Figure 3-2A). Melatonin onset phase angle ranged between 108min and 285min, for a variation of 177min among individuals. Melatonin offset phase angle had a smaller range of 62 min, and varied between 58 min before and 4 min after light-on (+58min and -4min respectively). Melatonin duration was between 418min and 601min for these individuals, for a range of 183min.

A strong negative correlation existed between the duration of melatonin secretion and melatonin onset phase angle (Figure 3-2B; $R^2 = 0.862$, $p < 0.0001$), but there did not seem to be a correlation between duration and offset phase angle (Figure 3-2C; $R^2 = 0.116$, $p = 0.255$). Such results indicate that the difference in melatonin duration was mainly attributed to difference in melatonin onset timing. There was also no relationship between the melatonin onset and offset (Figure 3-2D; $R^2 = 1.11E\text{-}03$, $p = 0.914$), indicating that it is worthwhile to compare both the onset and offset phase angle against the free-running period.

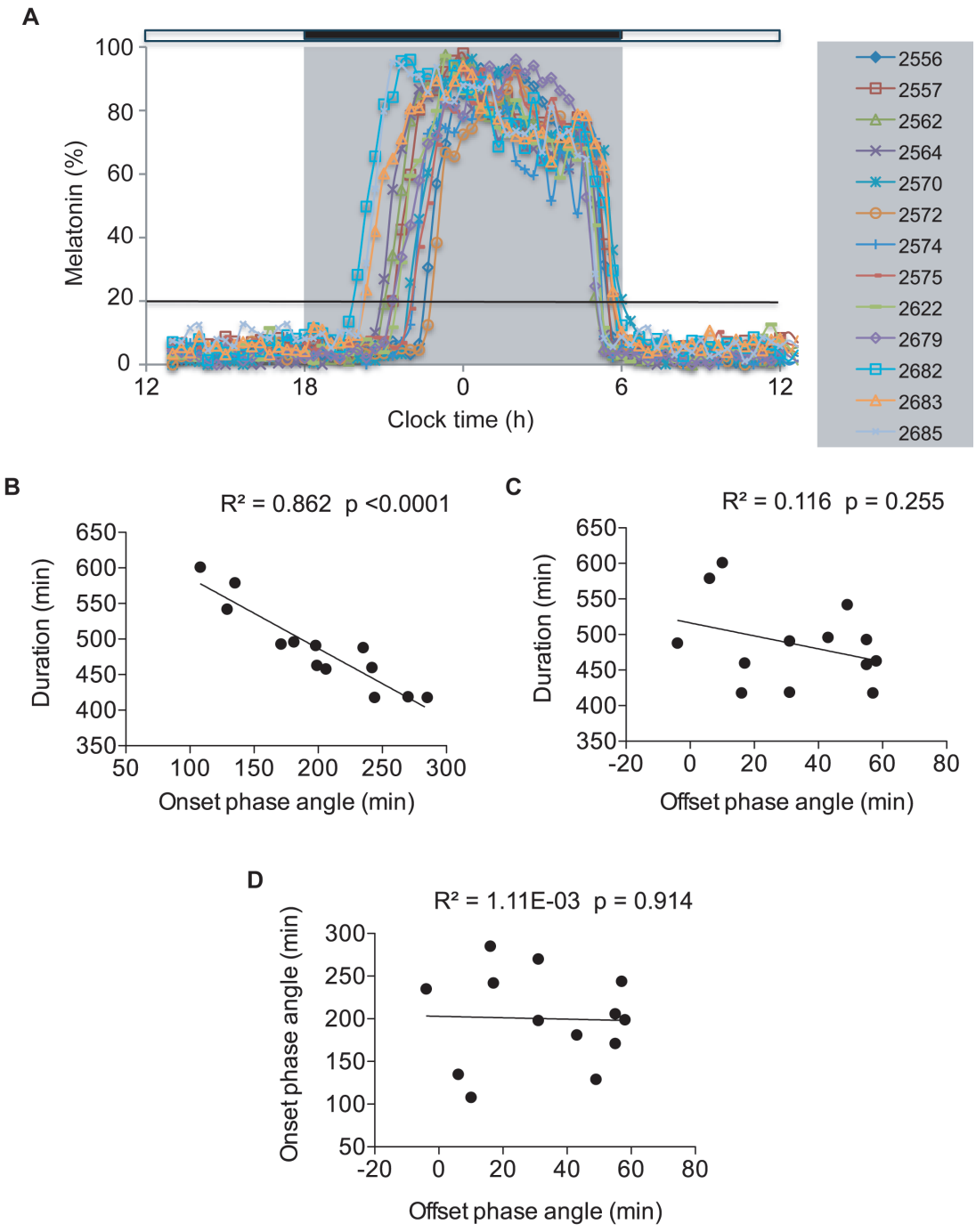

Figure 3-2 Interindividual differences of melatonin profile.

(A) Melatonin profiles of nine rats showing large onset variation (177min), small offset variation (62min), and duration variation of 183min. Melatonin duration is **(B)** highly correlated with onset phase angle, **(C)** but not with offset phase angle. **(D)** There is no correlation between melatonin onset and offset phase angles. (Zhang et al., manuscript in preparation).

3.3.1.2 Lack of relationship between entrained phase angle and free-running period

The free-running period of WI and SD rats used in this experiment ranged between 23.88h and 24.48h, for a difference of 36min between individuals (Figure 3-3). The period obtained from temperature rhythms did not show any correlation with melatonin onset phase angle (Figure 3-3A; $R^2 = 0.014$, $p = 0.715$), or offset phase angle (Figure 3-3B; $R^2 = 0.196$, $p = 0.149$). There was also no correlation when period was obtained from activity or heart rate rhythms (data not shown).

3.3.2 Photoperiod manipulation of phase angles of inbred F344 rats

3.3.2.1 Photoperiodic manipulation of phase angle and duration

The melatonin profiles of inbred F344 rats were very similar under identical conditions. By entraining these rats to different photoperiods, their melatonin onset and offset phase angles were dramatically altered (Figure 3-4). Rats entrained under LD16:8h had a mean onset phase angle of 104.6min (SEM = 4.9). Rats entrained under LD8:16h had a much larger onset phase angle with a mean of 214.7min (SEM = 9.121min). The offset phase angle was also shorter under LD16:8h than in LD8:16h, with a mean of -2.33min (SEM = 7.253min) and 118min (SEM = 4.863min) respectively.

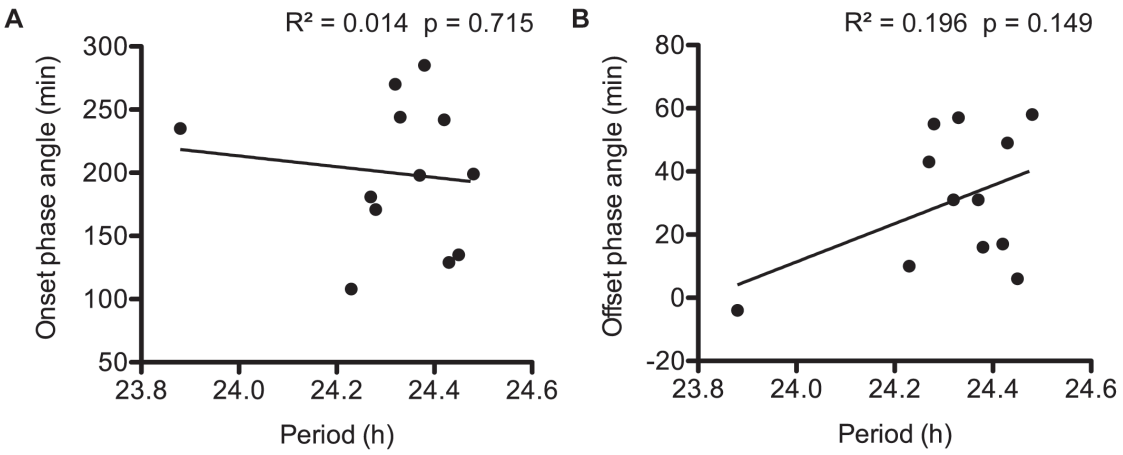

Figure 3-3 Lack of correlation between phase angle and free-running period.

No correlation was found between free-running period and **(A)** melatonin onset phase angle or **(B)** melatonin offset phase angle. (Zhang et al., manuscript in preparation).

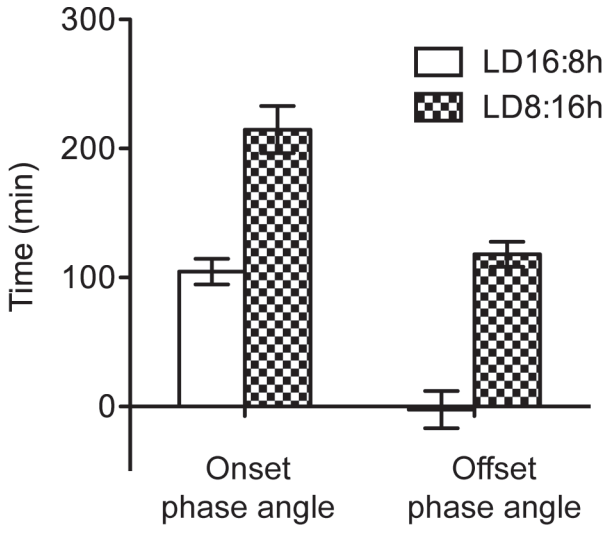

Figure 3-4 Entrained phase angles under long and short photoperiods

F344 inbred rats were entrained under LD16:8h (n=4) or LD8:16h (n=4). The entrained melatonin onset and offset phase angles are significantly different under long and short photoperiods. (Zhang et al., manuscript in preparation).

3.3.2.2 Identical steady state free-running period regardless of previous phase angle and duration

The locomotor activity, heart rate, and temperature rhythms of a separate group of F344 rats were monitored while entrained under LD16:8h and LD8:16h, then for three months after placement under DD. Representative temperature recordings are shown in Figure 3-5. For several days after the change to DD, rats entrained under LD16:8h experienced bidirectional expansion of nighttime rhythm duration (Figure 3-5A), while rats entrained under LD8:16h experienced slight compression of nighttime rhythm duration (Figure 3-5B).

The period of these rats were measured in 30-day increments for three months under DD (Figure 3-6). In most cases, an individual's activity, heart rate and temperature rhythms yielded the same period under DD. The exception was in the first 30 days after the change to DD, where two rats entrained under LD16:8 (ID4684, ID4763) had activity and heart rate period of 24h, while temperature rhythm had a period of 24.17h. Out of the rats entrained under LD16:8h, only rat ID4693 had a period of 24.17h in all three rhythm markers during the first 30 days; the rest of the rats exhibited rhythms with a 24h period with all three markers (ID4764), or just from activity and heart rate. In contrast, most of the rats entrained under LD8:16 attained period of 24.17h in the first 30 days, with the exception of rat ID4710 with a period of 24.33h. At 60 days, all rats had a period of 24.17h, regardless of the vastly different phase angles when entrained under long and short photoperiods. All rats maintained the same free-running period even at 90 days (Figure 3-6).

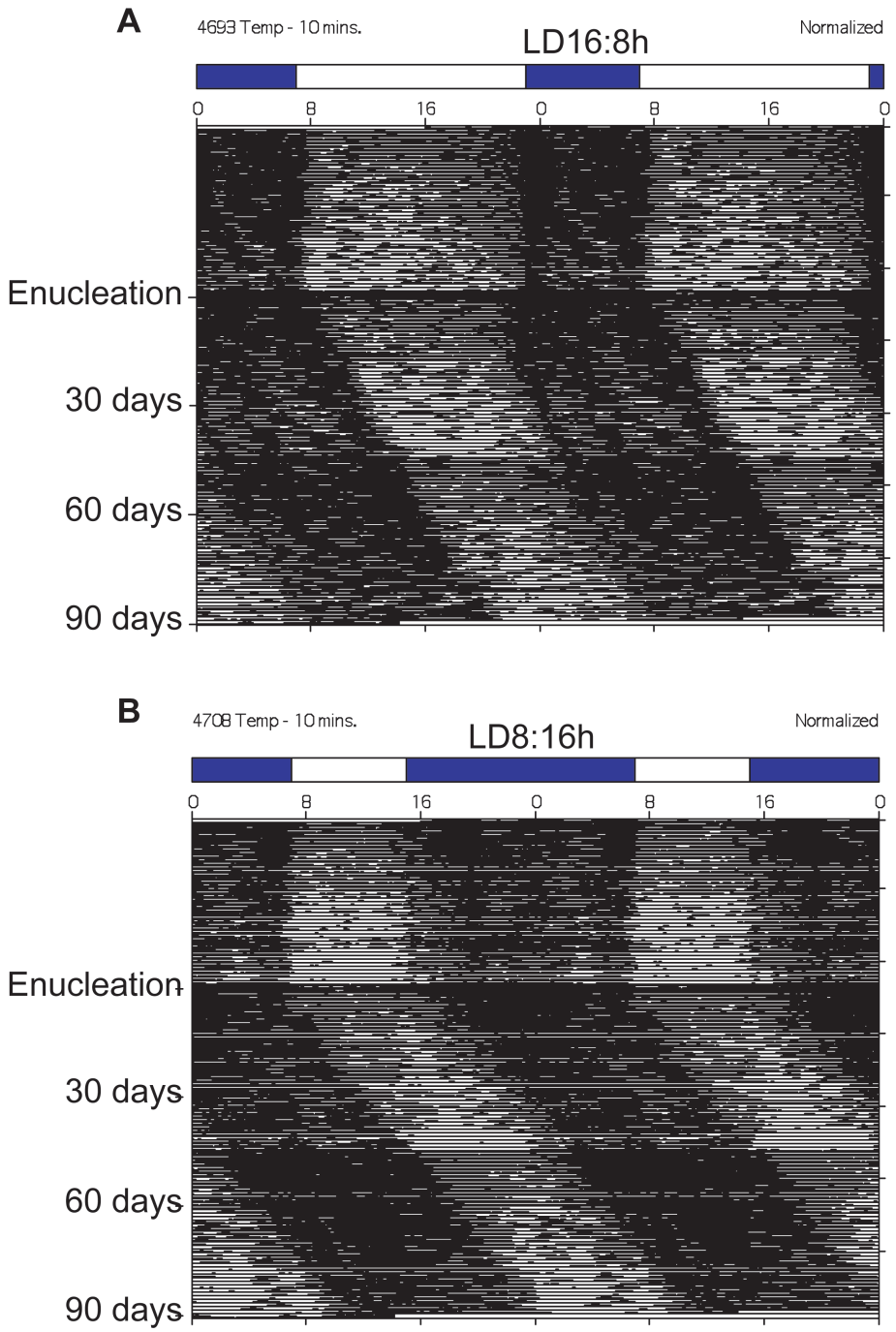

Figure 3-5 Temperature actogram during photoperiod entrainment and free-run

Example of temperature rhythms recorded from a F344 rat entrained to **(A)** LD16:8h (ID4693), or **(B)** LD8:16h (ID4708). After recording almost two months of entrained rhythm, the rats were allowed to free-run under constant conditions. Their free-running periods were analyzed after 30, 60, and 90 days under DD. (Zhang et al., manuscript in preparation).

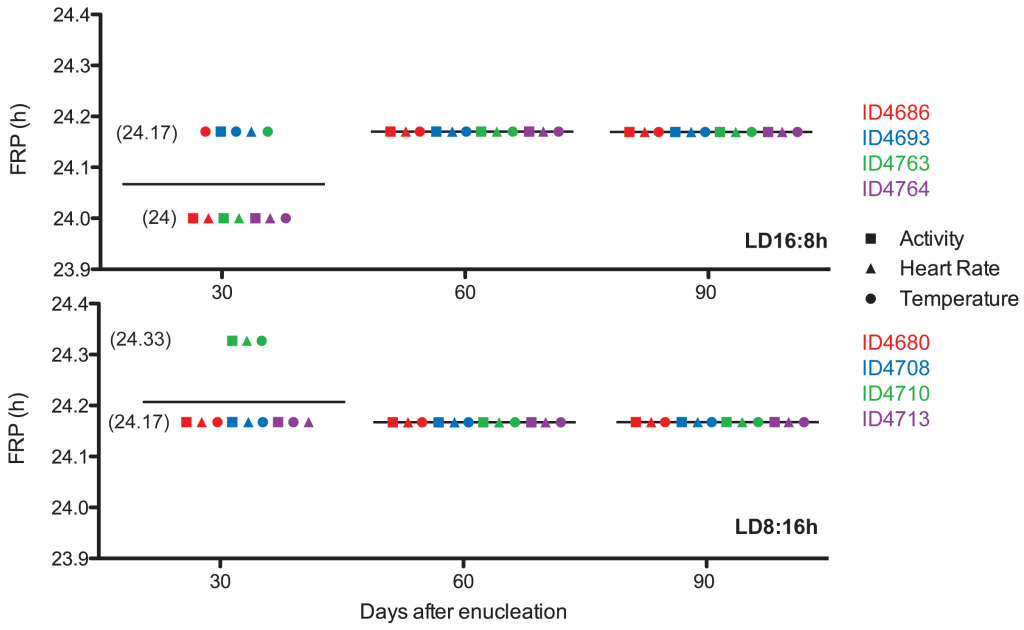

Figure 3-6 Free-running period of rats that were entrained to LD16:8h or LD8:16h

Periods from activity, heart rate, and temperature rhythms of inbred F344 rats plotted for 30, 60, or 90 days under DD. Periods were determined in 30-day bins. Individual rats are colored differently, and each circadian marker is represented by a symbol. In the first 30 days in DD, the period showed individual variations – rats entrained under LD16:8h tended to have periods of either 24h or 24.17h, while rats entrained under LD8:16h had periods of 24.17h or 24.33h. In the second and third month, all rats had a free-running period of 24.17h regardless of previous entrainment. (Zhang et al., manuscript in preparation).

3.4 Discussion

In examining interindividual differences in outbred rats as well as manipulating melatonin phase angle in inbred rats, the data in this chapter suggest that the melatonin phase angles of entrainment do not directly correspond to the free-running period. This finding contradicts previous animal and human studies where the phase angle difference was strongly related to the circadian period (Aschoff and Wever, 1966; Pittendrigh and Daan, 1976a; Gronfier et al., 2007).

It is important to note that the free-running period discussed in this chapter is specifically the steady state free-running period, and not just rhythmic period recorded when the animal is in the initial days of free-run under constant conditions. Furthermore, the phase angle is defined by the phase difference between driving oscillation (LD cycle) and driven oscillation (melatonin synthesis rhythm). Specifically, the time difference between light-off and melatonin onset (onset phase angle), as well as melatonin offset and light-on (offset phase angle), measured with melatonin under steady state entrainment to a LD cycle.

3.4.1 Previous establishment of relationship between phase angle and period

3.4.1.1 Varied definitions of phase angle and period

The early studies established the relationship between phase angle and free-running period by using behavioral markers to identify circadian components (Aschoff and Wever, 1966; Pittendrigh and Daan, 1976a). In many cases, the phase angle used was not the entrained phase angle, and the free-running period may not have reached a steady state. In Pittendrigh's study for example, the phase angle of white-footed mice was not obtained under LD entrainment, but was instead measured from an entraining light pulse,

administered after the animal was free-running in DD (Pittendrigh and Daan, 1976a). The difference in phase angle from an entraining light pulse versus that of a LD regimen will be of interest to explore further using melatonin as a very precise circadian marker.

In a more recent study of human circadian rhythm using melatonin as a marker, Gronfier et al. (2007) found a closely fitted linear relationship between phase angle and period in their subjects. However in this case, the phase angle does not involve the onset of a time cue such as light, but is instead the time difference between habitual bedtime and melatonin onset. This phase difference between two circadian outputs cannot be said to be a phase angle of entrainment, which must involve an external time cue. Furthermore, the period in this case is determined via a forced desynchrony protocol (Carskadon et al., 1999), and may not reflect the free-running period with the same accuracy as under constant conditions.

3.4.1.2 After-effects

The free-running period measurements are further complicated by after-effects. After-effects take place during the period of time under constant conditions before the free-running period reaches its final stable state (Pittendrigh and Daan, 1976a; Liu and Borjigin, 2005a). In some cases, after-effects may last for a very long time (Jilge, 1980), are hard to detect, and may thus be unknowingly included in reports of free-running periods.

In this study, after-effects of animals entrained under LD16:8h and LD8:16h are clearly evident following transition to DD. For both photoperiod groups, interindividual variation was evident only in the first 30 days after DD; at 60 and 90 days, all F344 rats had the exact same period signifying that after-effects influence the period in the first 30

days (Figure 3-6). Exactly when the steady state occurs for each photoperiod requires further analysis with larger number of rats. Under the influence of after-effect, it did indeed seem that a relationship exists between entrained phase angle and free-running period. For the rats that had smaller phase angles by being entrained under LD16:8h (Figure 3-4), most of them exhibited a shorter period of 24h at first, especially in activity and heart rate rhythms (Figure 3-6). Likewise for LD8:16h entrained rats with large phase angles, one of them had a period of 24.33h in the first 30 days after DD (Figure 3-6). Thus, after-effects may create a seemingly corresponding relationship between phase and period.

3.4.2 Supporting evidence for a lack of direct correlation between phase angle and period.

Various published studies have indirectly corroborated our results. For instance, in a human study that used forced desynchrony to identify the circadian period, Duffy and Czeisler (2002) examined the relationship between period and phase angle from core body temperature of young and old individuals. They found that the correlation between phase and period did not exist in old people. Furthermore, they found that although old people had significantly earlier wake times (which can be interpreted as smaller phase angle for activity onset), the circadian period was not significantly different between young and old people. Like with the F344 rats, this study showed that phase angle seemed to be more flexible over time, while the intrinsic free-running period did not seem to change significantly with age in humans.

In another study of circadian free-running period length of early and late human chronotypes (Brown et al., 2008), data from dermal fibroblast cells showed that a relationship between phase angle and period was only evident for extreme chronotypes

with periods short as 23.7h and as long as 25.7h. Within the normal range of periods between 24h and 25.5h, the relationship was ambiguous, which further suggests the lack of a direct correlation between phase angle and period. In our study of outbred rats, with the exception of one rat having a period of 23.88h, the period of all other rats ranged between 24.23h and 24.48h for a difference of only 15min. For the range of periods examined, we did not see any correlation between phase angle and period. It would be of interest to verify this lack of correlation in animals with extreme periods as well, especially those with periods in the 25h range.

When considering the extreme differences in free-running period of circadian mutants, further doubts are cast upon the established phase angle and period relationship. Figure 3-7 summarizes the published results on various circadian mutants (Vitaterna et al., 1994; Shearman et al., 1997; Thresher et al., 1998; Van der Horst et al., 1999; Zheng et al., 1999; Bunger et al., 2000; Bae et al., 2001; Cermakian et al., 2001; Zheng et al., 2001). In all cases, the phase angle was measured under LD12:12h entrained conditions, and wheel running activity was used as a marker. These mutants displayed a large range in their free-running period, spanning from 22.1h to 24.6h (difference of 2h30min); however, their phase angle estimates were all within 22.6min of each other. Additionally, there is no clear linear relationship, especially not one that shows a positive correlation between the period and phase angle measured in these mutants.

The fact that running wheel was used to measure circadian rhythm in these mutants may have influenced the results – the presence of a running wheel actually serves as a motivating reward, and can alter behavioral rhythms (Stewart et al., 1985; Edgar and Dement, 1991; Ruiz de Elvira et al., 1992; Sherwin, 1998). For example, it has been

found in ennucleated rats that the free-running period obtained from wheel running activity is shorter than that of locomotor activity (Yamada et al., 1990). In this study, telemetry measurements of locomotor activity, heart rate, and core body temperature were measured at the same time to discern an accurate representation of the free-running period, free from external factors. Interestingly, after the first 30 days following placement in DD, the temperature rhythm of two of the rats entrained under LD16:8h had a longer period (24.17h) than the activity and heart rate rhythms within the same individual (24h). This result further highlights the importance of using multiple markers to accurately determine the free-running period.

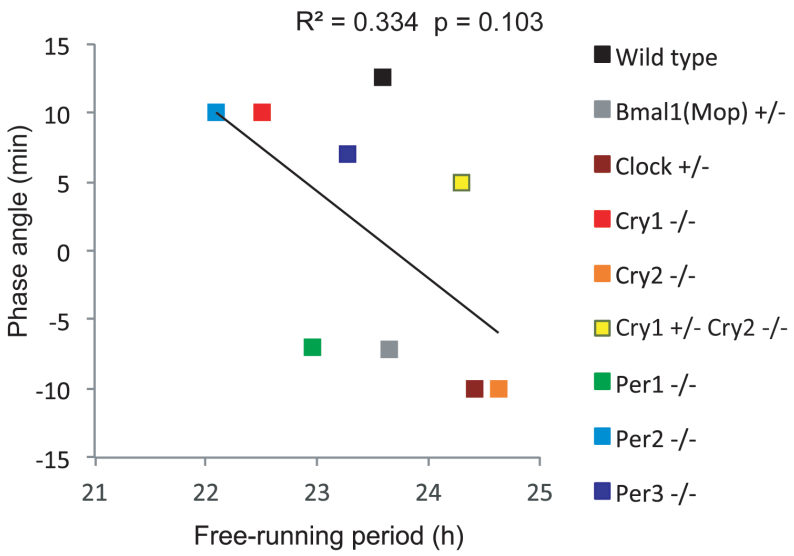

Figure 3-7 Phase angle and period of circadian mutants

Previously published results on the phase angle and free-running period of mutant mice measured from wheel running activity. The genotypes of the mice are wild type C57BL/6J (B6) (Vitaterna et al., 1994; Van der Horst et al., 1999; Bunger et al., 2000), Bmal1(Mop)+/- (Bunger et al., 2000), Clock+/- (Vitaterna et al., 1994), Cry1-/- (Vitaterna et al., 1994; Van der Horst et al., 1999), Cry2-/- (Vitaterna et al., 1994; Thresher et al., 1998; Van der Horst et al., 1999), Cry1+/- Cry2-/- (Van der Horst et al., 1999), Per1-/- (Cermakian et al., 2001; Zheng et al., 2001), Per2-/- (Zheng et al., 1999; Zheng et al., 2001), and Per3-/- (Shearman et al., 1997; Bae et al., 2001). (Zhang et al., manuscript in preparation).

3.5 Conclusion

The major finding of this study is that there is no direct relationship between phase angles and period when phase angles are defined by melatonin synthesis rhythm entrained to a LD cycle, and period is defined under steady state free-running conditions using multiple markers. The lack of relationship between phase angle and period holds true both when examining interindividual differences, as well as through photoperiodic manipulation of phase angle.

In considering interindividual differences in entrained melatonin profiles, this chapter shows melatonin onset phase angle bears no relationship with the offset phase angle. However, a linear relationship does exist between melatonin onset phase angle and duration, where earlier onset indicates longer melatonin secretion. When entrained to different photoperiods, longer photoperiod begets shorter onset and offset phase angles.

In conclusion, while conflicting evidence exist for a positive correlation between phase angle and period, our results clearly suggest the absence of a strong linear relationship between phase angle and period. The discrepancy between our findings and others can be accredited to the sensitivity of melatonin as a circadian marker, the definition of entrainment, and the influence of after-affect when animal is released into DD.

3.6 Future directions

3.6.1 Wider range of phase angle and periods

Brown's study (2008) suggests that the correlation between phase angle and free-running period may indeed exist at more extreme phase angle and period values. To determine the range outside which a correlation appears between these two factors,

animals with large differences in phase angles and periods should be measured with the same methods as this study.

In addition to finding rats with large interindividual differences, the phase angle and period can be further manipulated. In this study we manipulated the phase angle of inbred rats by entraining them to different photoperiods. In future studies, the period of inbred rats can be manipulated pharmacologically to examine its effect on the phase angle. Certain chemical substances have been found to lengthen the free-running period; these include deuterium, lithium, and monoamine oxidase inhibitors (Bruce and Pittendrigh, 1960; McDaniel et al., 1974; Daan and Pittendrigh, 1976c; Richter, 1977; Kripke and Wyborney, 1980; Wirz-Justice and Campbell, 1982). The period can also be altered with changes in endocrine hormones via methods such as ovariectomy or castration (Daan et al., 1975; Morin et al., 1977; Takahashi and Menaker, 1980; Morin and Cummings, 1981). In most of these cases, the phase angle is altered along with the free-running period, and it would be of interest to see if there are correlations in these changes using melatonin as a clear circadian marker.

3.6.2 After-effects

In our previous studies, we have shown that melatonin onset and offset free-run at different rates during the first few days in DD (Liu and Borjigin, 2005a). While it is clear that the asymmetric free-running of melatonin onset and offset and consequent change in melatonin duration is a transient phenomenon, it is not clear how long after-effects generally last. In this chapter, free-running melatonin rhythms measured two months following placement under DD showed consistent duration in all animals examined (Figure 3-1). This suggests that melatonin after-effect ends within 2 months for most

rats. Further studies of melatonin rhythm under DD should be performed to identify the duration of after-effect, and to determine the temporal dynamics of melatonin onset and offset in DD. Specifically, it would be of interest to compare the after-effects in melatonin rhythm following entrainment to long and short photoperiods. The data presented in this study suggests that melatonin duration increases in DD after entrainment to LD16:8h, and decreases in DD after entrainment to LD8:16h. It would be interesting to investigate whether animals subjected to frequent photoperiod changes alternating between 8h and 16h would exhibit shorter after effects. Furthermore, the differences in after-effects between circadian markers can be investigated, especially in terms of the dissociation of periods measured from different circadian markers within the same individual for the first few days in DD (Figure 3-6).

Chapter 4

Shorter melatonin onset phase angle predicts faster reentrainment rate

Chapter 3 concludes that there is no direct relationship between phase angle and period. Part of the theory that assumes a relationship between phase angle and period also states that speed of reentrainment depends on length of free-running period, where individuals with larger onset phase angle (and thus longer free-running period) would reentrain faster to a delay shift in schedule (Aschoff et al., 1975). In this chapter we explore the relationship between phase angle and reentrainment speed to verify predictive circadian elements.

4.1 Introduction

When an individual's internal circadian rhythms are entrained to the environment, their behavioral, physiological, and biochemical processes are homeostatically regulated to ensure optimum coordination with time of day. When the environmental schedule is shifted as occurs when humans travel across time zones or experience daylight savings time, the circadian system must readjust to the new time schedule. During the period of reentrainment, the disrupted circadian system results in symptoms of jet lag in humans, which include decreased cognitive performance, sleep, cardiovascular, and gastrointestinal distress, along with general feelings of ailment (Rajaratnam and Arendt, 2001; Herxheimer and Waterhouse, 2003). The duration of jet lag symptoms is associated to the duration of circadian reentrainment. Individuals reentrain to the new

external schedule at different rates, and identification of factors that accelerate the reentrainment rate would help to reduce the duration of jet lag.

The rate of reentrainment has been attributed to several factors. One such factor is the magnitude of the shift: it takes more days to reentrain to a larger shift in schedule (Aschoff and Wever, 1963). Light intensity also plays a role where brighter light leads to faster reentrainment (Aschoff et al., 1975). Another crucial factor is the direction of the phase shift. For example, diurnal birds (Aschoff and Wever, 1963) and lizards (Fischer, 1961) will reentrain faster to an advance shift than a delay shift, while the opposite has been shown for nocturnal rats (Halberg et al., 1971) and flour beetle (Chiba et al., 1973). The preference for advance or delay is not attributed to diurnality or nocturnality, but to the free-running period being longer or shorter than 24h.

Previous publications suggest that an individual's free-running period plays a role in determining the course of reentrainment. For example, with a 12h shift of the LD cycle, lizards with a free-running period shorter than 24h shift in the advance direction, while lizards with a period greater than 24h shift in the delay direction (Hoffmann, 1969). The propensity for animals with shorter free-running periods to more readily shift in the advance direction was thought to drive the difference in reentrainment rate to advance and delay shifts (Aschoff et al., 1975). In other words, animals with short periods would shift faster in the advance direction, while animals with longer than 24h periods would shift faster in the delay direction.

Under the assumption that the phase angle depends on the free-running period, an individual with an smaller phase angle (which assumes a shorter period) would reentrain faster to an advance shift, while individuals with larger phase angle would reentrain faster

to a delay shift. Indeed, it has been found that chaffinches with later activity onset reentrained faster to delay shifts (Aschoff and Wever, 1963).

Exceptions to the phase angle prediction of reentrainment rate have been found. In response to a 12h shift, chaffinches and bullfinches with earlier activity onset actually reentrained faster in the delay direction, even thought the opposite was true in the same birds following a 6h delay shift (Pohl, 1978). Also, squirrel monkeys have free-running periods greater than 24h (Ferraro and Sulzman, 1988), but they reentrained faster to an 8h advance than an 8h delay shift (Boulos et al., 1996a). Preliminary studies in our own lab of 1h, 3h, 4h, and 6h delay shifts also suggest that larger phase angles do not always reentrain faster than shorter phase angles, particularly with larger delay shifts. We hypothesize that individuals with smaller phase angle (earlier onset) will reentrain faster to both advance and delay shifts in schedule. To precisely examine the relationship of phase angle and reentrainment rate, this chapter considers both interindividual differences in reentrainment, as well as the effect of photoperiod manipulation of phase angle on rate of reentrainment.

4.2 Method

4.2.1 Animals

Rats were housed individually in light-tight chambers at controlled temperature (20-25°C). Food and water were freely available. Illumination was supplied by white fluorescent lamps (~400 lux at cage level). Measurement of melatonin phase angles via pineal microdialysis is as described in Chapter 2.1.

4.2.1.1 Individual differences in outbred rats

In a preliminary study, male Sprague-Dawley (SD) rats ordered from Harlan (Indianapolis, IN) were maintained under LD12:12h (lights on from 6:00 – 18:00) for a minimum of 2 weeks prior to microdialysis experiments. Their melatonin synthesis rhythms were recorded for at least 4 days to determine the original entrained melatonin profile prior to a 1h delay shift of the LD cycle.

4.2.1.2 Photoperiod manipulation of phase angle of inbred rats

Adult Fischer 344 (F344) rats ordered from Harlan were housed under LD16:8h or LD8:16h for 2 months to entrain the animals to the long or short photoperiods. F344 rats maintained in LD12:12 were also housed on site for at least 2 weeks prior to microdiaysis experiments. Entrained melatonin synthesis rhythm was recorded for at least 4 days in each photoperiod before a 2h phase shift (Figure 4-1).

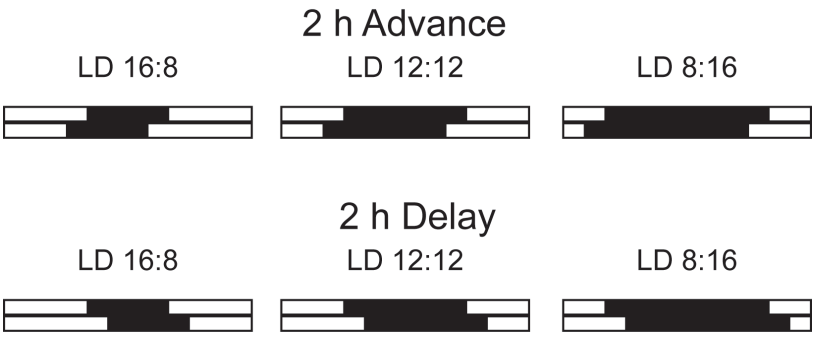

Figure 4-1 LD cycle shift paradigm

Inbred F344 rats were entrained under three different photoperiods, then subjected to an acute advance or delay shift of 2h. (Zhang et al., manuscript in preparation).

4.2.2 Definition of reentrainment

Since melatonin profiles entrained under LD12:12h and LD8:16h have identical onset and offset each day, reentrainment under these photoperiods was defined as when the onset and offset remained within the same 20min bins for at least 3 days following phase shift in LD cycle. For rats entrained under LD16:8h however, there were further complications. Throughout the photoperiod studies, we noticed that entrainment was less stable in animals exposed to the extremely long photoperiod (LD16:8h) compared to those in shorter photoperiods (LD12:12h and LD8:16h). We exposed three separate cohorts of rats to photoperiods of LD16:8h, LD12:12h, and LD8:16h for 1 to 2 months and tested their entrained melatonin profiles using pineal microdialysis. As shown in Figure 4-2, melatonin profiles of rats entrained under LD8:16h and LD12:12h were very consistent from day to day, with almost identical nightly onset and offset (Figure 4-2A and B). The exception was rat ID4681, whose offset deviated from the rest of the data on day 1 (Figure 4-2Ac). However, a slight variation during the first night is commonly seen when online dialysis occurs within 2 days of surgery (Liu and Borjigin, 2006). In comparison to the rats entrained under LD8:16h and LD12:12h, rats entrained under LD16:8h had very inconsistent melatonin profiles that shifted daily (Figure 4-2C). A closer examination of the daily onset and offset of rats entrained under LD16:8h showed that their values varied as much as 60min for a single individual (Figure 4-2Cb and Cc). The daily variations were taken into consideration when determining reentrainment for rats under LD16:8h. Unlike for rats under LD8:16h and LD12:12h, rats entrained under LD16:8h were considered reentrained when their melatonin profile shifted to the expected position, and maintained the same amount of variance as before the shift.

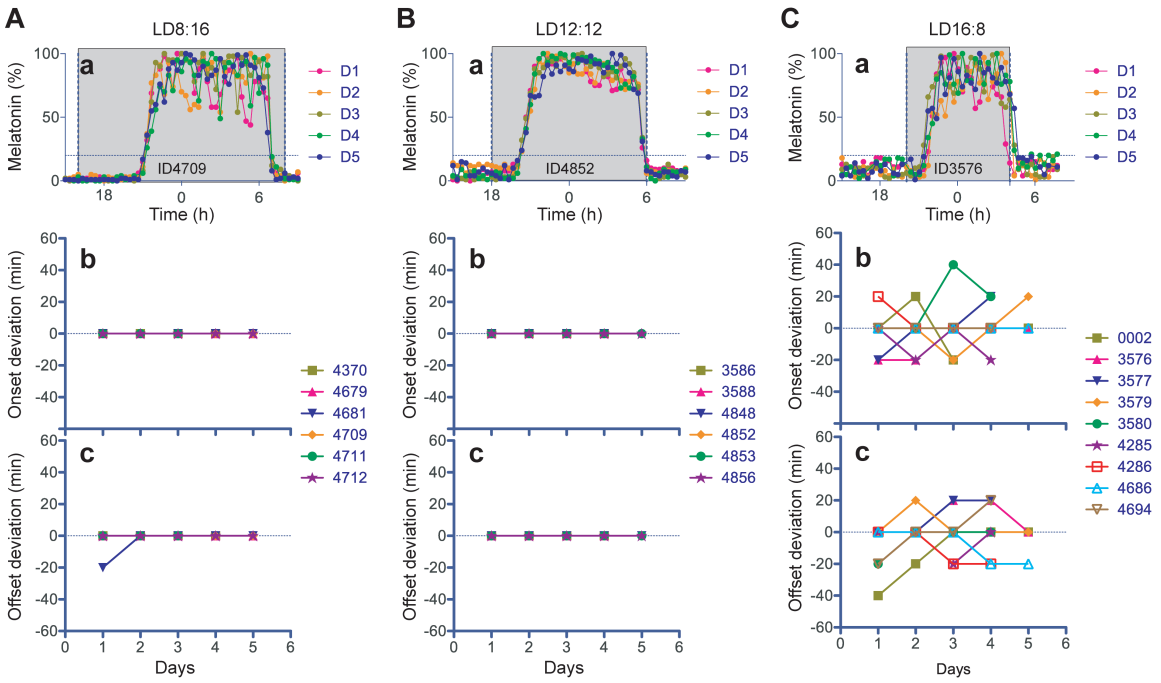

Figure 4-2 Stability of melatonin rhythms under different photoperiods

Inbred F344 adult male rats born and raised in LD12:12h, were exposed to **(A)**LD8:16h (n=6), **(B)** LD12:12h (n=6), and **(C)** LD16:8h (n=9) for one (ID4285-4856) or two (ID002-3588) months before melatonin was recorded via pineal microdialysis. The top (a) panels represents 5 consecutive days of data from an individual rat. The bottom panels show the deviation from the means of melatonin onset (b) and offset (c) for 4 or 5 consecutive days. A value of 0 means there is no change. The melatonin onset and offset for rats in LD8:16h and LD12:12h did not change from day to day. The only exception was ID4681 who's offset on day 1 was different from the subsequent days. In contrast, the melatonin onset and offset of rats in LD16:8h, changed almost daily, displaying range of deviation up to 60min. (Zhang et al., manuscript in preparation).

4.3 Results

4.3.1 Reentrainment is a slow process

Figure 4-3 provides an example of a single individual's response to a 3h LD shift in the delay direction. Under entrained conditions, the melatonin profile looks almost identical each night (D-2, -1, 0 of Figure 4-3). By graphically delaying the entrained profile by 3h, we obtain what the melatonin profile is expected to look like after reentraining to a 3h LD delay (D0# of Figure 4-3). In the first night under the new LD cycle (D1), melatonin onset immediately shifted to almost the expected position; the offset shifted only slightly in the delay direction. By D5, offset reached the expected position, but it wasn't until D8 that the onset also arrived at its expected position. Even then, the melatonin profile continued to make small adjustments, as is most apparent on D16 when both the onset and offset overshot the expected position. It took many days before the melatonin profile regains its original stability. In summary, in response to a LD phase shift, melatonin profile first reaches the expected position, and then fine-tunes itself to stably entrain to the new LD cycle.

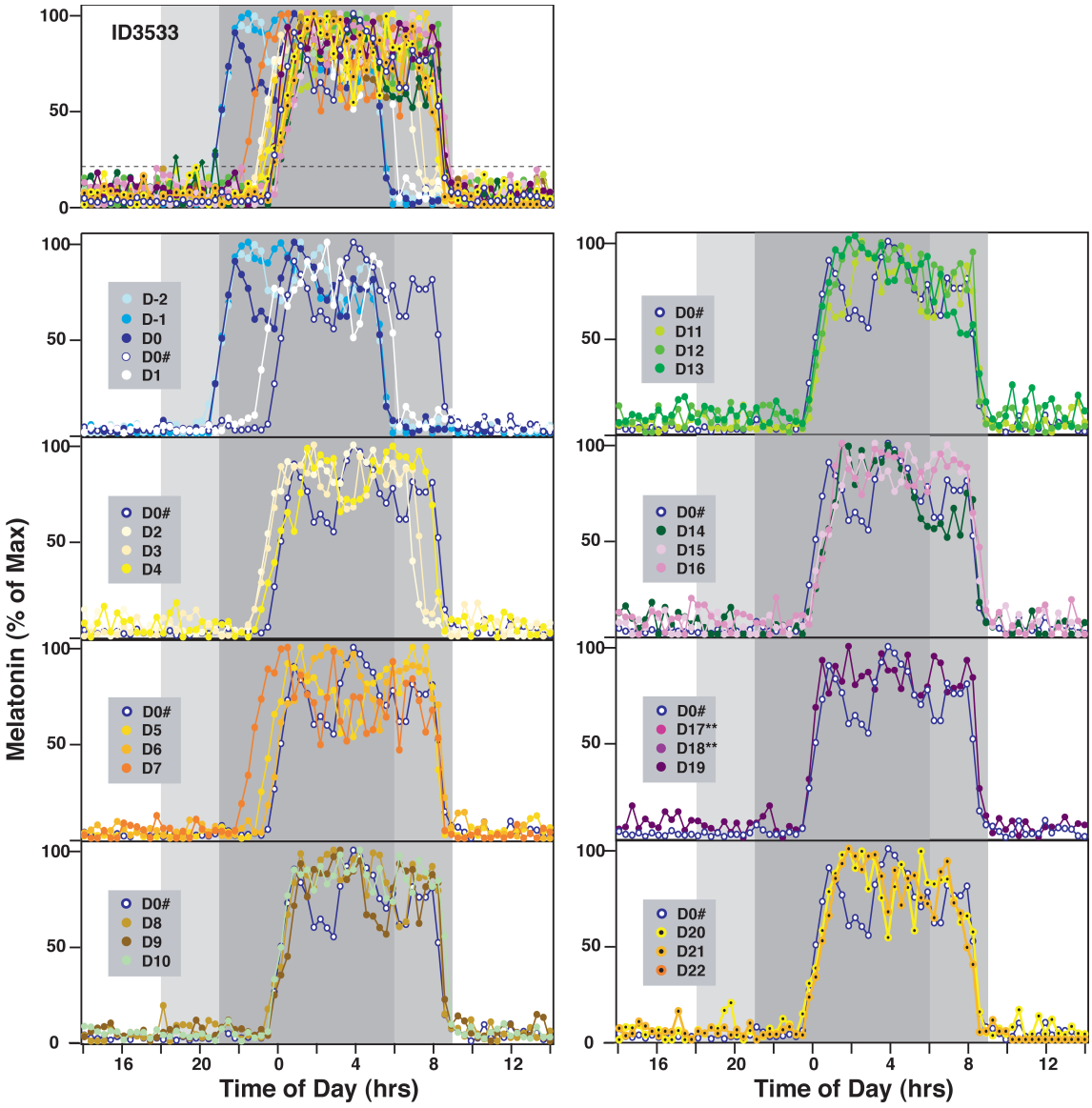

Figure 4-3 Melatonin profiles in response to a 3 hour delay shift of the LD cycle

An SD rat entrained under LD12:12h experienced an acute 3h delay in LD cycle. The original dark period is shaded in light gray (from 18:00 to 6:00), and over laps with the new dark period shaded in dark gray (from 21:00 to 9:00). The top-left panel summarizes melatonin profiles before and after the shift, and the bottom panels break the reentrainment process down into days. Three consecutive days of melatonin entrained under the original LD cycle are shown labeled as D-2, D-1, and D0. A copy of the entrained profile is graphically shifted to show the expected position of melatonin profile after reentrainment to a 3h delay shift (dark blue open circles; D0#). The melatonin profile on the night of the shift is shown in white (D1), followed by 21 subsequent nights of melatonin profiles labeled D2 to D22. Data for D16** and D17** are missing due to technical issues. (Zhang et al., manuscript in preparation).

4.3.2 Interindividual differences in speed of reentrainment

Preliminary data from SD rats showed that there were interindividual differences in the speed of reentrainment. In response to an 1h delay in the LD cycle, some rats reentrained much faster than others. In the example shown in Figure 4-4, both the melatonin onset and offset of rat ID2221 reached the expected position by day 2 (D2) and became stably entrained to the new LD cycle. Rat ID2241 on the other hand did not reach the expected onset timing until day 8 (D8), and did not stably reentrain until D12. These two rats were part of a group of 7 rats that underwent a 1h delay shift; the onset phase angle of these rats ranged between 60min and 190min, and their melatonin duration ranged between 480min and 620min (Figure 4-5). As discussed in Chapter 3, rats with earlier melatonin onsets tends to have longer melatonin durations, and the same held true for the 7 rats used in this experiment. When the number of days it took the animal to reentrain was plotted against melatonin onset phase angle, we saw a tight positive correlation where individuals with smaller phase angle reentrained faster (R^2=0.96 Figure 4-5B). As expected, a tight correlation was also found between melatonin duration and reentrainment rate, where individuals with longer durations reentrained faster (R^2=0.93 Figure 4-5C).

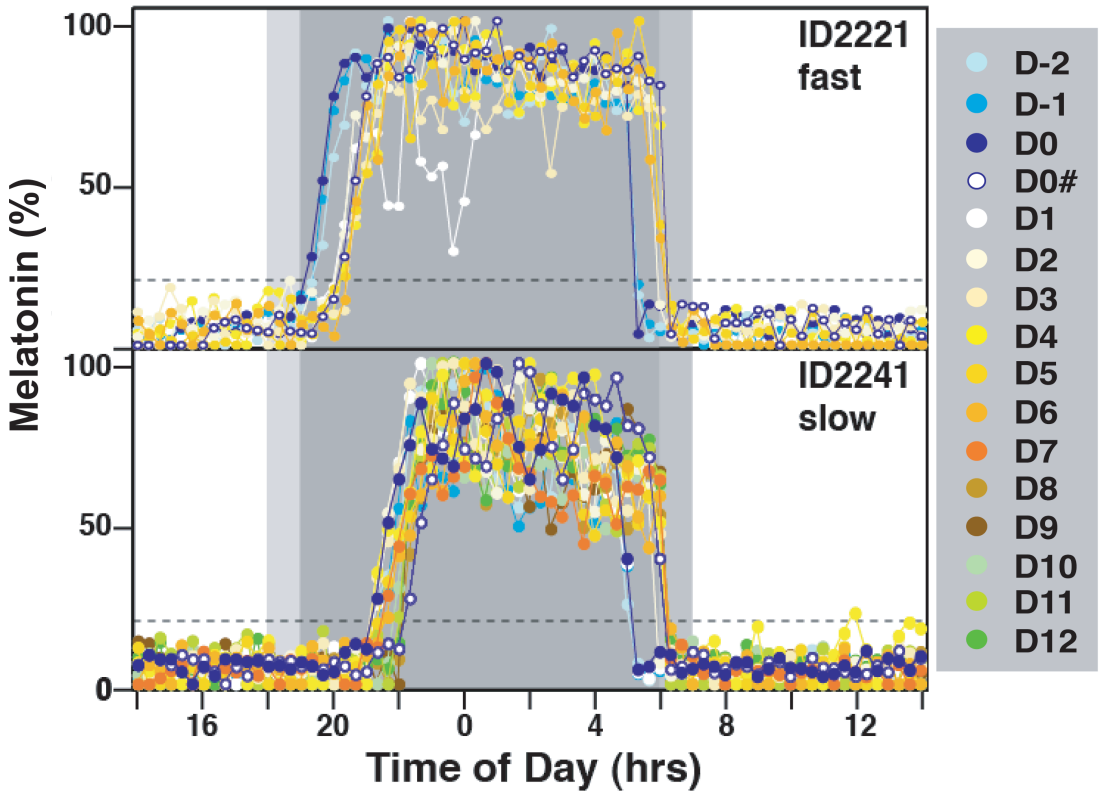

Figure 4-4 Differences in speed of reentrainment between individuals.

Individuals reentrained to a 1h delay shift of the LD cycle at different speeds. For rat ID2221, both the melatonin onset and offset reached the expected position by day 2 (D2) and became stably entrained to the new LD cycle. Rat ID2241 did not reach the expected onset timing until day 8 (D8), and did not stably reentrain until D12. (Zhang et al., manuscript in preparation).

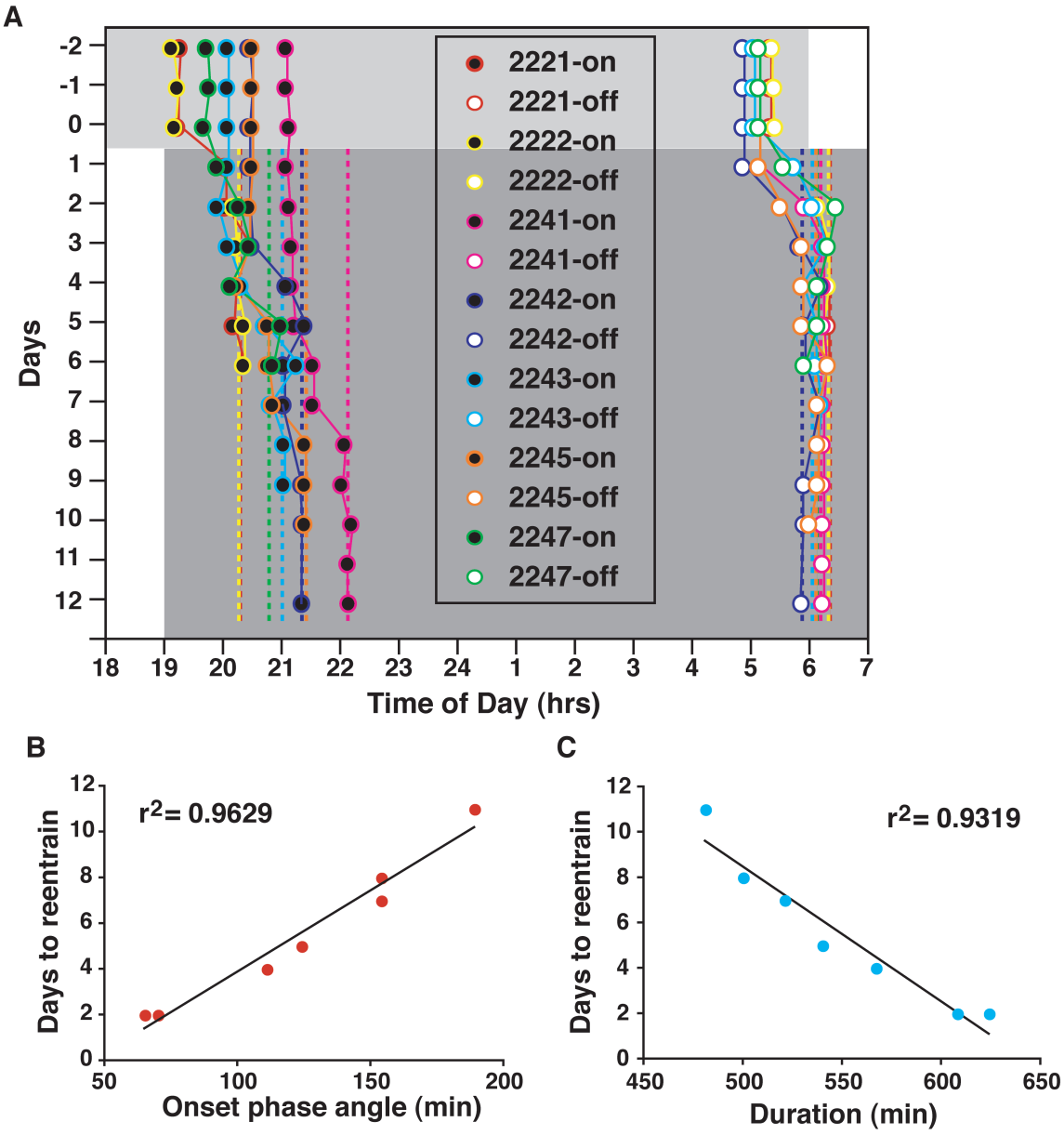

Figure 4-5 Reentrainment to an 1h delay shift of the LD cycle

(A) The daily melatonin onset and offset of 7 rats are shown as they reentrained to an 1h delay shift of the LD cycle. Melatonin onset is labeled with closed circles, and offset with open circles. The gray boxes represent period of darkness both before the shift (light gray) and after the shift (dark gray). The expected position of melatonin onset and offset after the shift is represented by the dashed line color coded for each individual rat. The legend in the center shows the rats' ID number and color code. The number of days it took for rats to reentrain to the 1h delay shift was closely correlated to both the **(B)** melatonin onset phase angle, and the **(C)** melatonin duration of the individuals. (Borjigin et al., manuscript in preparation).

4.3.3 Photoperiod manipulation

4.3.3.1 Effect of photoperiod on phase angle and duration

Inbred F344 rats have very similar melatonin profiles under the same entrained conditions. For rats entrained under LD16:8h, LD12:12h and LD8:16h, the shorter the dark period, the smaller the phase angle and shorter the duration of nightly melatonin synthesis (Figure 4-6). The difference in phase angle and duration was significant for each photoperiod (Figure 4-6B). The mean phase angle was 1.83h for LD16:8h (SEM = 0.0980h), 2.41h for LD12:12h (SEM = 0.127h), and 4.33h for LD8:16h (SEM = 0.305h). The mean durations were 6.30h (SEM = 0.186h), 9.16h (SEM = 0.162h), and 10.5 (SEM = 0.215h) for LD16:8h, LD12:12h, and LD8:16h respectively.

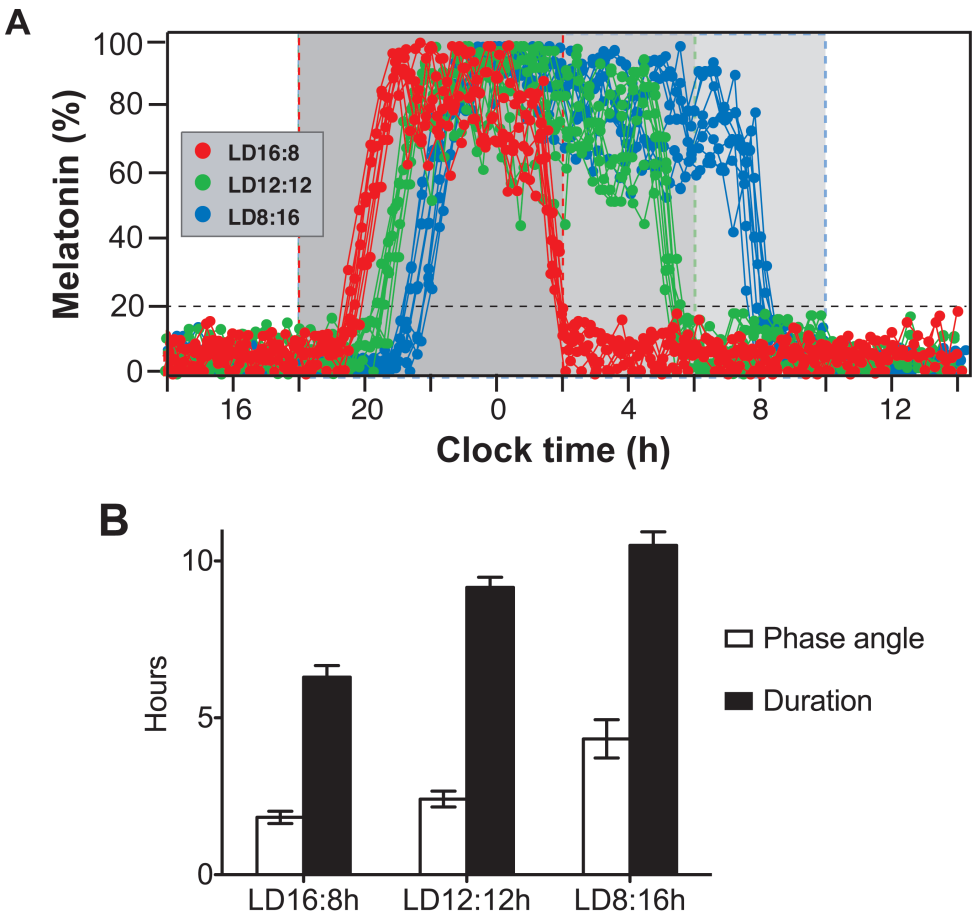

Figure 4-6 Entrained melatonin profiles under different photoperiods

(A) Inbred F344 rats were entrained under LD16:8h (red), LD12:12h (green), and LD8:16h (blue). The gray box outlined in dashed lines represents the duration of darkness for each photoperiod. **(B)** The bar graph depicts the entrained melatonin onset phase angle and duration under LD16:8h (n=9), LD12:12h (n=9), and LD8:16h (n=9). (Zhang et al., manuscript in preparation).

4.3.4 Reentrainment to 2h advance or delay shift under different photoperiods

Regardless of the direction of shift, rats under longer photoperiods with smaller phase angles reentrained faster. A one-way analysis of variance (ANOVA) test shows significant difference between the photoperiod groups (2h advance: $F=18.9$, $p<0.0001$; 2h delay: $F=41.65$, $p<0.0001$). In response to a 2h advance shift, the mean number of days to reentrain was 4.75 days (SEM = 0.250), 8.22 days (SEM = 0.521), and 10.2 days (SEM = 0.547) for LD16:8h, LD12:12h, and LD8:16h respectively. For 2h delay, the mean number of days to reentrain was 4.00 days (SEM = 0.365), 5.75 days (SEM = 0.479), and 9.80 days (SEM = 0.583) for LD16:8h, LD12:12h, and LD8:16h respectively.

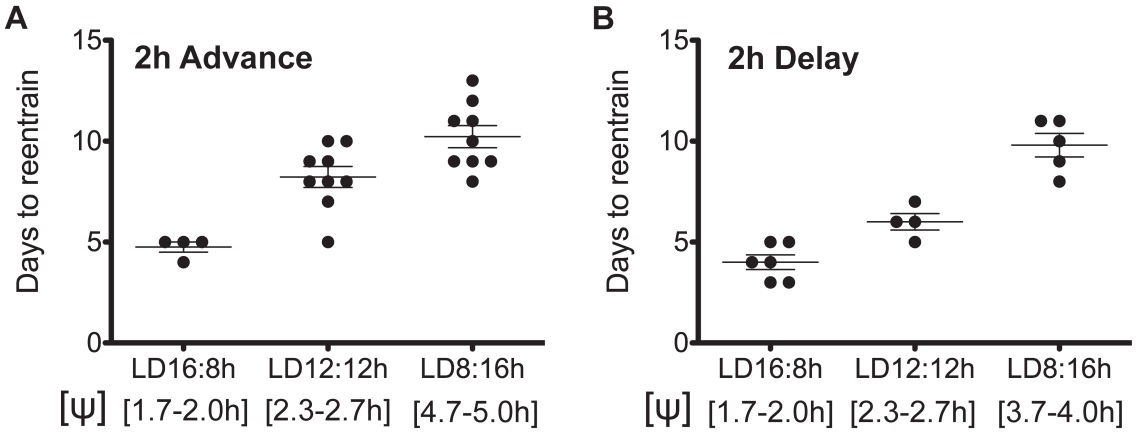

Figure 4-7 Speed of reentrainment under different photoperiods

The number of days it took rats to reentrain to a **(A)** 2h advance shift, and **(B)** 2h delay shift are plotted for each photoperiod. The original phase angles (ψ) before the shift for each photoperiod is displayed in brackets. (Zhang et al., manuscript in preparation).

4.3.5 Change in phase angle after phase shift

After reentrainment, the onset and offset phase angles did not always remain the same (Figure 4-8). At least one animal from each photoperiod and shift group had an onset and/or offset phase angle that differed from its original phase angle. The difference between photoperiods was significant only for onset phase angle of rats under LD8:16h after a 2h advance shift, where the majority of rats had a phase angle that's 40min larger than before the shift (Figure 4-8A; one-way ANOVA: $F=23.38$, $p<0.0001$). Although not statistically different from the other photoperiods, the offset phase angle in majority of rats under LD12:12h also increased by 20min after a 2h advance shift (Figure 4-8B).

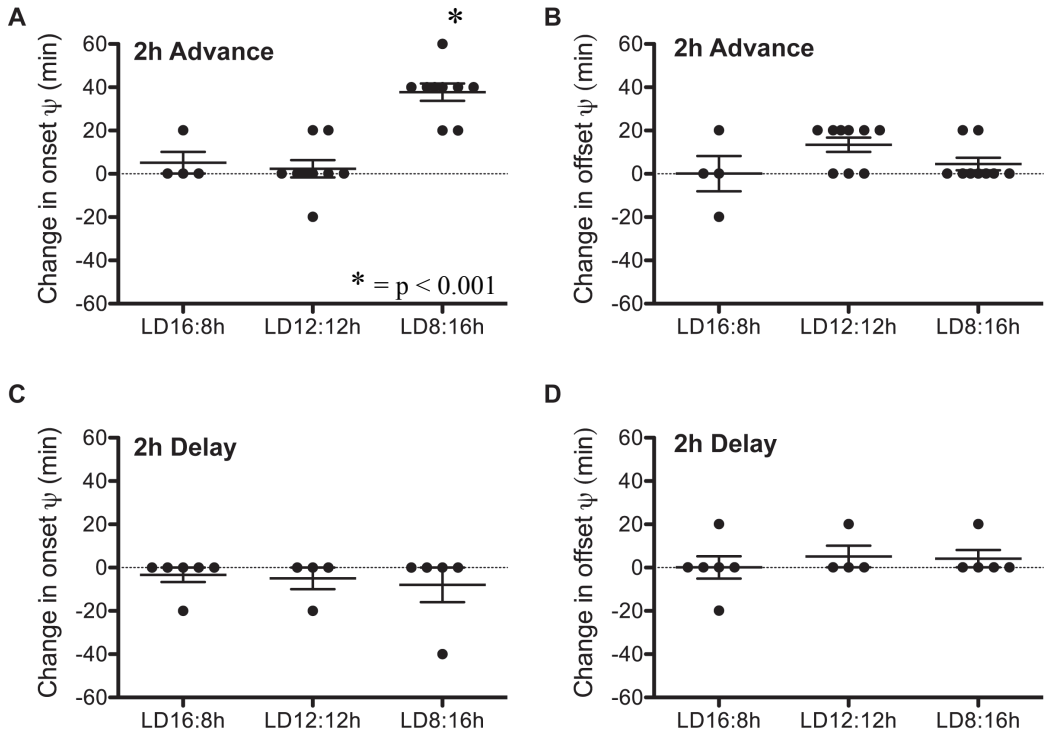

Figure 4-8 Change in phase angle after LD shift

The change in **(A)** onset and **(B)** offset phase angles (ψ) following 2h advance shift of the LD cycle are plotted for the three photoperiods. The change in **(C)** onset and **(D)** offset phase angles following 2h delay shift are also plotted. For onset phase angle change, positive numbers mean the time between light-off and melatonin onset increased after the shift (onset later than expected). For offset phase angles, positive numbers mean the time between melatonin offset and light-on increased after the shift (offset earlier than expected). (Zhang et al., manuscript in preparation).

4.4 Discussion

4.4.1 Preliminary data on interindividual differences of reentrainment

SD rats that underwent a 1h delay shift displayed distinct differences in their rate of reentrainment. That difference in speed was highly correlated to both melatonin onset phase angle, as well as melatonin duration. Individuals with earlier melatonin onset and longer melatonin duration reentrained faster. To determine whether melatonin duration alone has a consistent correlation with reentrainment rate, we needed a way to dissociate the positive relationship between phase angle and duration. Photoperiod entrainment offered the perfect tool for this experiment. Under the same photoperiod, outbred animals differed from each other in that individuals with earlier melatonin onset also have longer melatonin duration. By entraining inbred animals with little interindividual variation to different photoperiods, we get rats with earlier onset and shorter duration in shorter nights. If rats under short nights reentrained faster, then earlier melatonin onset is indicative of a faster reentrainment rate. If rats under long nights reentrained faster, then longer melatonin duration is indicative of a faster reentrainment rate. The experimental results show that rats reentrained faster under shorter nights, which means that smaller phase angle is truly indicative of reentrainment speed, and longer melatonin duration is not associated with faster reentrainment speed.

4.4.2 Phase angle and period theory of reentrainment

Previous studies suggest that individuals with shorter free-running period would reentrain faster to an advance phase shift and slower to a delay phase shift (Aschoff et al., 1975). Assuming that the theory of a direct correspondence between phase angle and period holds true, then individuals with smaller phase angles would reentrain faster to

advance and slower to delay shifts. This was certainly not the case for the rats in this study. Regardless of whether we consider interindividual differences in phase angle, or photoperiod manipulation of phase angle, individuals with smaller phase angles reentrained faster in both the advance and delay direction. This finding provides further proof of the dissociation between phase angle and free-running period (cf. Chapter 3). In regards to rat melatonin rhythm, we find that earlier melatonin onset is very much indicative of a faster reentrainment speed regardless of direction of shift. Considering that F344 rats have identical free-running periods (Figure 3-6), and yet very different reentrainment rates under different photoperiods, the extent to which period controls reentrainment rate requires further investigation.

4.4.3 Magnitude of phase shift

One factor that affects reentrainment speed is the magnitude of the phase shift. In this chapter, I presented data for both 1h and 2h delay shift in LD12:12h. The outbred SD rats reentrained to the 1h delay shift in 2 to 11 days. In comparison, the inbred F344 rats reentrained to the 2h delay shift in 5 to 7 days. The larger spread of reentrainment rates for outbred rats compared to F344 rats was expected, however, their means also did not significantly differ from each other. For SD rats the mean reentrainment rate to a 1h delay was 5.57 days (SEM=1.25), and for F344 rats a 2h delay took on average 5.75 days (SEM = 0.479). From this data, it does seem that variation in phase angle plays a larger role on reentrainment rate than does the magnitude of phase shift. However, number of hours shifted still does impact reentrainment rate. In our preliminary delay shift studies, while there were no significant differences between 3h and 4h delay shifts, a 6h delay shift definitely took longer to reentrain to than a 1h shift. By the end of a month long

recording of melatonin data, none of the rats completed reentrainment to the 6h shift (data not shown).

4.4.4 Change in phase angle following phase shift.

It is often assumed that circadian rhythms will return to the same phase angle with the light cue after reentraining to a phase shift; however, that is not always the case as shown in this chapter. In each experimental group, at least one animal did not return to its original phase angle after the shift. The change is especially prominent following a 2h advance shift for the onset phase angles of rats under LD8:16h, and less so for offset phase angle of rats under LD12:12h. In previous publications, a change between pre- and post-shift phase angle have been noted following a 12h shift in chaffinch and dormouse (Pohl, 1978). Aschoff et al. (1975) postulated that a new LD photic cue would enforce a compression of phase angle values towards a species specific preferred value. However, this explanation does not account for why under LD8:16h the onset phase angle became on average 40min larger following 2h advance shift but not following 2h delay shift (Figure 4-8). The change in phase angles will be interesting to explore in future studies.

4.4.5 Photoperiod entrainment

The F344 rats under LD8:16h had significantly different phase angles between the group used for 2h advance shift (phase angles in the range of 4.7-5.0h), and the group used for 2h delay shift (phase angles in the range of 3.7-4.0h). The cause for this difference was the inadvertent use of different paradigms to entrain the rats to LD8:16h from LD12:12h. For advance shift rats, the dark period was expanded symmetrically; for delay shift rats, the dark period was expanded into the morning hours. The effect of

photoperiod entrainment paradigm on the melatonin phase angle is the focus of Chapter 5.

With regards to rate of reentrainment, rats with free-running periods of greater than 24h are expected to reentrain faster to delay shift than advance shift based on the established theory. Since the LD8:16h rats used for 2h delay had smaller phase angle than those for 2h advance, we should expect them to reentrain even faster to a delay shift than advance shift. However, our data show no significant differences in rate of reentrainment between advance and delay shift. In general, the mean speed of reentrainment for each photoperiod was faster for delay than advance shift; however, that difference is only significant under LD12:12h (advance: 8.22 days, SEM = 0.521; delay: 5.75 days, SEM = 0.479). This seemingly photoperiod dependent difference in reentrainment rate to advance versus delay shifts may require further examination.

4.5 Conclusion

From our data we can conclude that rats with earlier melatonin onset will generally reentrain faster to a phase shift, regardless of the direction of shift. This is true for both natural individual variation of melatonin rhythm, as well as for photoperiod manipulation of melatonin rhythm.

4.6 Future directions

4.6.1 Individual differences in advance shift

To insure that the speed of reentrainment is truly dependent on melatonin onset phase angle and not on reentrainment property of different photoperiods, it is important to establish the same results in outbred rats with different phase angles for both the advance and delay shifts. While delay shifts of varying magnitudes have been performed for SD

and WI rats, not many successful advance shift experiments have taken place. One reason for this is that in most cases, melatonin profiles never reached the expected positions after advance phase shifts. We now know from our photoperiod data that phase angles may indeed change after a phase shift, and will consider this fact in future experiments and in reevaulation of advance shift data. Furthermore, even with delay shifts, a 3h, 4h, and 6h phase shift generally cannot be completed within one month. To combat this issue, we can focus only on 1h and 2h advance and delay shifts for outbred rats. To obtain information on how greater magnitude of shift affects rate of reentrainment, inbred rats with extremely early melatonin onsets can be used to promote faster reentrainment to 3h, 4h and 6h LD shift paradigms.

4.6.2 Administration of exogenous melatonin

This chapter shows that earlier endogenous melatonin synthesis results in faster reentrainment. Individuals with smaller melatonin onset phase angles represents the entrained circadian phase under the LD condition, but early melatonin itself may play a role in promoting reentrainment by acting on the SCN. We can administer a nightly dose of exogenous melatonin to artificially advance the melatonin onset timing, and monitor its effect on the speed of reentrainment.

4.6.3 Limit of melatonin entrainment to short night

Figure 4-2 showed that rat melatonin synthesis rhythm could not stably entrain to an 8h night. In future studies, we can decrease the photoperiod (increase dark time) in steps until we identify the photoperiod limit in which melatonin profile can stably entrain. The identification of this limit will provide valuable insights into the mechanism of entrainment at the central pacemaker, timing of entraining signals from the SCN to the

pineal gland, as well as more detailed time course for nightly melatonin synthesis onset and offset.

Chapter 5

Dynamics of photoperiod reentrainment of rat melatonin rhythm

Previous chapters have shown that melatonin synthesis rhythm entrains to different photoperiods with dramatically different profiles. When reentraining to a new photoperiod, the expansion or compression of darkness can occur either bidirectionally or unidirectionally. Data in Chapter 4 demonstrated that melatonin profiles under the same photoperiod may differ depending upon the paradigm used to reach the new photoperiod. This chapter, adapted from a recently submitted manuscript, describes the dynamics of melatonin reentrainment to different photoperiod change paradigms.

5.1 Introduction

Photoperiod dependent adjustment of melatonin secretion has been extensively studied in both photoperiodic animals and non-photoperiodic animals. In photoperiodic animals, melatonin plays an essential role in regulating seasonal phenotypes (Reiter, 1987; Goldman, 2001). In non-photoperiodic animals like rats, the dynamic responses of melatonin secretion provide many insights in deciphering entrainment mechanisms of the central circadian pacemaker in the absence of reproductive and coat changes (Herbert, 1989; Illnerova and Sumova, 1997).

5.1.1 Background

Analysis of photoperiodic regulation of melatonin secretion has been performed under artificial lighting of varying durations (Illnerova and Sumova, 1997). The expansion of the dark period increases the duration of locomotor activity (Puchalski and Lynch, 1986; Hastings et al., 1987; Gorman et al., 1997), melatonin secretion (Hoffmann et al., 1986; Puchalski and Lynch, 1986; Hastings et al., 1987), and activity of N-acetyltransferase (AANAT) (Illnerova et al., 1986) which is the key enzyme in melatonin production (cf. Chapter 1; Klein et al., 1992; Liu and Borjigin, 2005b).

5.1.1.1 Photoperiodic change in melatonin duration triggers seasonal phenotypes

In photoperiodic animals, change in melatonin duration under different photoperiods influences sleep distribution (Deboer and Tobler, 1996), induces molting (Lincoln et al., 2006) as well as seasonal reproduction in mammals (Anderson et al., 2003; Masumoto et al., 2010) and birds (Yoshimura et al., 2003). In hamsters, the expansion of melatonin duration triggers weight loss, gonadal regression, changes in pelage, immunity to diseases, among other changes (Hoffmann and Illnerova, 1986; Bartness and Goldman, 1988; Goldman, 1991).

5.1.1.2 History dependent response to photoperiod

Beyond melatonin duration, response to photoperiod also depends on lighting history. Identical photoperiod and melatonin profiles can elicit opposite effects on coat color, body weight, and reproduction in Djungarian hamsters depending on whether they are previously entrained to a long or short photoperiod (Hoffmann et al., 1986; Niklowitz et al., 1994). The reentrainment speed of AANAT activity (Illnerova et al., 1986; Hastings et al., 1987) is also influenced by lighting history. In response to dark expansion, the

speed of reentrainment is relatively slow in rats, with adjustments to dark expansion into the morning being faster than dark expansion into the evening (Illnerova et al., 1986). Reentrainment to dark compression from a 16h night to an 8h night was found to be a rapid process (Illnerova, 1991; Sumova et al., 1995).

5.1.1.3 Directional differences in photoperiod reentrainment

The majority of photoperiod studies of melatonin rhythm focus on animals' responses to short photoperiods; very little information is available on the directional dependent adjustment of rhythms to long photoperiods. Recently however, the induction of both thyroid stimulating hormone beta (TSHB), and Eya3 have been shown to depend on the direction of light extension (Masumoto et al., 2010). TSHB is a hormone induced in the pars tuberalis by long-day environment (Nakao et al., 2008; Ono et al., 2008) and Eya3, a transcription factor and a putative regulator of TSHB (Masumoto et al., 2010), both regulate vertebrate photoperiodism. While light extension into the evening hours had a delayed effect on the induction of TSHB and Eya3 expression in the pars tuberalis, light extension into the morning hours produced immediate effect in melatonin-proficient mice (Masumoto et al., 2010). The pars tuberalis is packed with melatonin receptors (Morgan et al., 1994), but it is not yet known whether the direction-dependent induction rate of TSHB is due to a differential response of melatonin in long days.

5.1.1.4 Limitations of previous studies

Thus far, photoperiod studies of melatonin synthesis rhythm consisted mostly of data compiled from multiple animals (Illnerova et al., 1984; Illnerova et al., 1986; Illnerova, 1991; Elliott and Tamarkin, 1994). Longitudinal studies of single individuals have mainly been performed using behavioral data (Zhang et al., 2000; Gorman and Elliott,

2004), or with melatonin sampled at every few hours (Alila-Johansson et al., 2001; Herwig et al., 2006). Furthermore, although animal facilities employ a 12h light and 12h dark schedule (LD12:12) from which animals are reentrained in photoperiod studies, only few publications explore melatonin's response to a new photoperiod from LD12:12 (Zhang et al., 2000).

5.1.2 What this chapter offers

Here, we offer a thorough depiction of melatonin's distinct reentrainment dynamics, exploring contraction and expansion of photoperiod as well as direction of photoperiod change. Rats under LD12:12 were introduced to 16h nights (LD8:16) or 8h nights (LD16:8) either symmetrically or unidirectionally (Figure 5-1). Using pineal microdialysis and high-pressure liquid chromatography (HPLC) system (Borjigin and Liu, 2008), we monitored individual melatonin concentration of freely moving rats every 20min for over a month. This powerful technique has been used to investigate circadian dynamics of melatonin in rats under LD (Chapter 3, Chapter 4, (Liu and Borjigin, 2006)), constant darkness (DD) (Chapter 3, (Liu and Borjigin, 2005a)) and shifting situations (Chapter 4, (Liu and Borjigin, 2005c)). These studies along with data from other laboratories demonstrate that duration as well as onset and offset phase angles of melatonin synthesis are subject to photic regulation of the central pacemaker. The daily melatonin profiles presented in this chapter detail the dynamics of melatonin reentrainment to long and short photoperiods, and display surprising and dramatic directional dependent differences in the final reentrained states.

5.2 Materials and Methods

5.2.1 Animals:

Two months old male Sprague Dawley (SD) rats born and raised in LD12:12h were purchased from Harlan (Indianapolis, IN). They were locally housed in light controlled chambers (LD12:12h) for 15-18 days before implanted with pineal microdialysis probes (for details on microdialysis and HPLC analysis see Chapter 2 and (Borjigin and Liu, 2008)). Post-surgical rats were housed individually with food and water available ad libitum. After 1 day of recovery from surgery, the entrained melatonin onset and offset of the freely moving rats were observed online for 3-5 days before reentrainment to a different photoperiod. Data output from HPLC systems were analyzed by Shimadzu Class-VP software, and then exported into excel forms for follow-up analysis.

5.2.2 Photoperiod reentrainment:

The compression and expansion of the photoperiod in this study was achieved in one of six ways, with two rats to each approach (Figure 5-1). All rats were entrained to LD12:12h since birth, with light-off at ZT 18:00 and light-on at ZT 6:00. From the LD12:12h schedule, the 12h dark period was expanded to 16h (LD8:16h) either symmetrically, or unidirectionally by acutely advancing light-off (evening dark expansion), or delaying light-on (morning dark expansion). Dark period compression from LD12:12h to LD16:8h was likewise accomplished either symmetrically, or unidirectionally by delaying the light-off (evening dark compression) or by advancing the light-on (morning dark compression). Illumination was supplied by white fluorescent lamps controlled by timers (400 lux at cage level).

5.2.3 Data analysis:

All melatonin data were analyzed in 20 min bins. Melatonin onset and offset timings were defined as when the concentration of melatonin rose above or fell below 20% of the daily maximum value. Melatonin duration was the time interval between onset and offset. Complete reentrainment to the new photoperiod was defined when both the onset and offset remained constant for three consecutive days following the change in photoperiod. Two rats underwent each of the six photoperiod conditions outlined in Figure 5-1, with the exception of dark expansion by delaying light-on, in which only one rat successfully lasted through the microdialysis experiment.

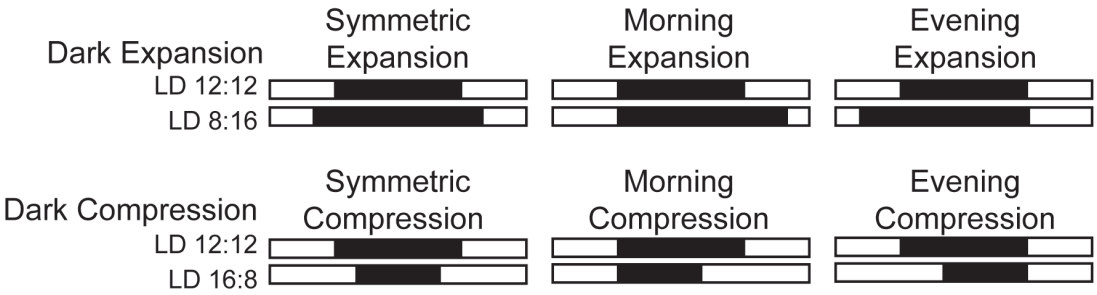

Figure 5-1 Photoperiod change paradigm

All rats were kept in LD12:12h where light turned off at 18:00 and turned on at 6:00. The entrained melatonin profiles were recorded for several days before the photoperiod was acutely changed to either a 16h night (LD8:16h) or an 8h night (LD16:8h) via one of the 6 paradigms. Dark compression or expansion occurred either symmetrically with a 2h shift of both the light-on and light-off, or unidirectionally with a 4h shift of only the light-on or light-off. (Zhang et al., manuscript in preparation).

5.3 Results

Adult rats used in this study all had similar melatonin profiles in the initial LD12:12 condition prior to photoperiod changes (Figure 5-4A and Figure 5-7A). Melatonin onset occurred at either 20:00 or 20:20 for all rats, with an onset phase angle of 2h or 2h20min. Offset occurred between 4:40 and 5:20, so that the offset phase angle fell between 40min and 1h20min before light-on. Following a change in photoperiod, melatonin onset and offset shifted independently, then gradually stabilized over a number of days (Figure 5-2 and Figure 5-6).

5.3.1 Dark expansion from LD12:12h to LD8:16h

5.3.1.1 Daily dynamics of melatonin reentrainment

The expansion of the dark period from 12h to 16h resulted in markedly different melatonin reentrainment dynamics and final profiles depending on the direction of dark expansion. For symmetrical dark expansion (Figure 5-2A), both rats' onset delayed by 40min and offset delayed by 1h40min. The offset shifted on the first night following introduction of the new photoperiod, whereas onset remained constant until day 3 for rat ID4593 and day 4 for ID4592. Although onset and offset seemed to have reached their reentrained position by day 9, it wasn't until day 24 (ID4593) and day 25 (ID4592) that they established relatively stable phase relationships (Figure 5-2A).

For unidirectional dark expansion into the morning (Figure 5-2B), only one rat's pineal microdialysis lasted well into the experiment. That rat's onset delayed by 1h40min and offset delayed by 2h. Both the onset and offset did not shift until day 2 following the abrupt change in photoperiod; it then only took 12 days to reentrain to the new schedule (Figure 5-2B).

When we expanded the dark period by only advancing the light-off time (Figure 5-2C), both onset and offset shifted on the first night. The melatonin profile then remained destabilized for 29 days before surprisingly returning to the original entrained positions under LD12:12. Although final melatonin timing shifted the least after evening dark expansion, it took 29 days for melatonin profile to stably reentrain to the new photoperiod. In comparison, melatonin onset and offset shifted the most following morning dark expansion (Figure 5-2B), yet reentrainment was completed in only 12 days.

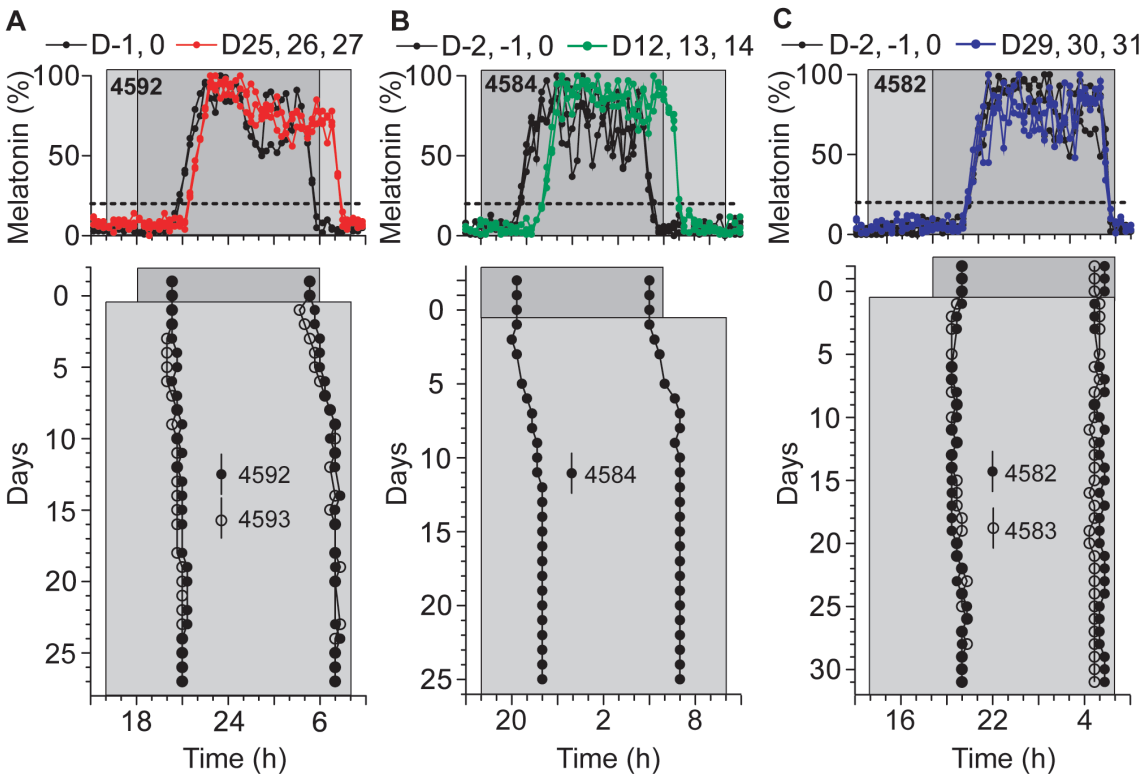

Figure 5-2 Temporal profiles of melatonin adaptation to dark expansion

The top graphs in panels A, B, and C depict the melatonin profiles of an individual rat both before and after a change in photoperiod. Black traces depicts the three consecutive days immediately prior to the change in photoperiod, and in red are the first three consecutive days when melatonin synthesis rhythm has reentrained to the new photoperiod. The shaded box represents the duration of darkness before and after the change. The bottom graphs in panels A, B, and C plot out the daily melatonin onset and offset dynamics of both rats in each condition throughout the course of the experiment. Each panel depicts a dark expansion paradigm with: **(A)**. symmetric dark extension, **(B)** morning dark extension, **(C)** evening dark extension. (Zhang et al., manuscript in preparation).

5.3.1.2 Melatonin phase angles

After reentrainment to LD8:16h, the phase angles were dramatically different depending on the direction of dark expansion (Figure 5-3A, B, and Figure 5-4B). Onset phase angle was the longest after evening dark expansion (6h), followed by symmetric dark expansion (5h), with morning dark expansion resulting in the shortest onset phase angle (4h) (Figure 5-3A and Figure 5-4B). In these cases, the amounts of dark extension into the morning are 0h, 2h, or 4h respectively. Thus, there appears to be a negative linear correlation between the magnitude of morning dark expansion and the reentrained melatonin onset phase angle (Figure 5-3).

Similarly, melatonin offset phase angle (Figure 5-3B and Figure 5-4B) showed changes that correlate with magnitude of morning dark extension. The offset phase angle remained the same when there was no dark extension in the morning, but did increase by a small amount when dark period was extended for 2h into the morning following symmetric dark expansion (Figure 5-3B). Extending the dark period 4h into the morning resulted in an offset phase angle that increased dramatically by 2h (from 1h to 3h; Figure 5-3B).

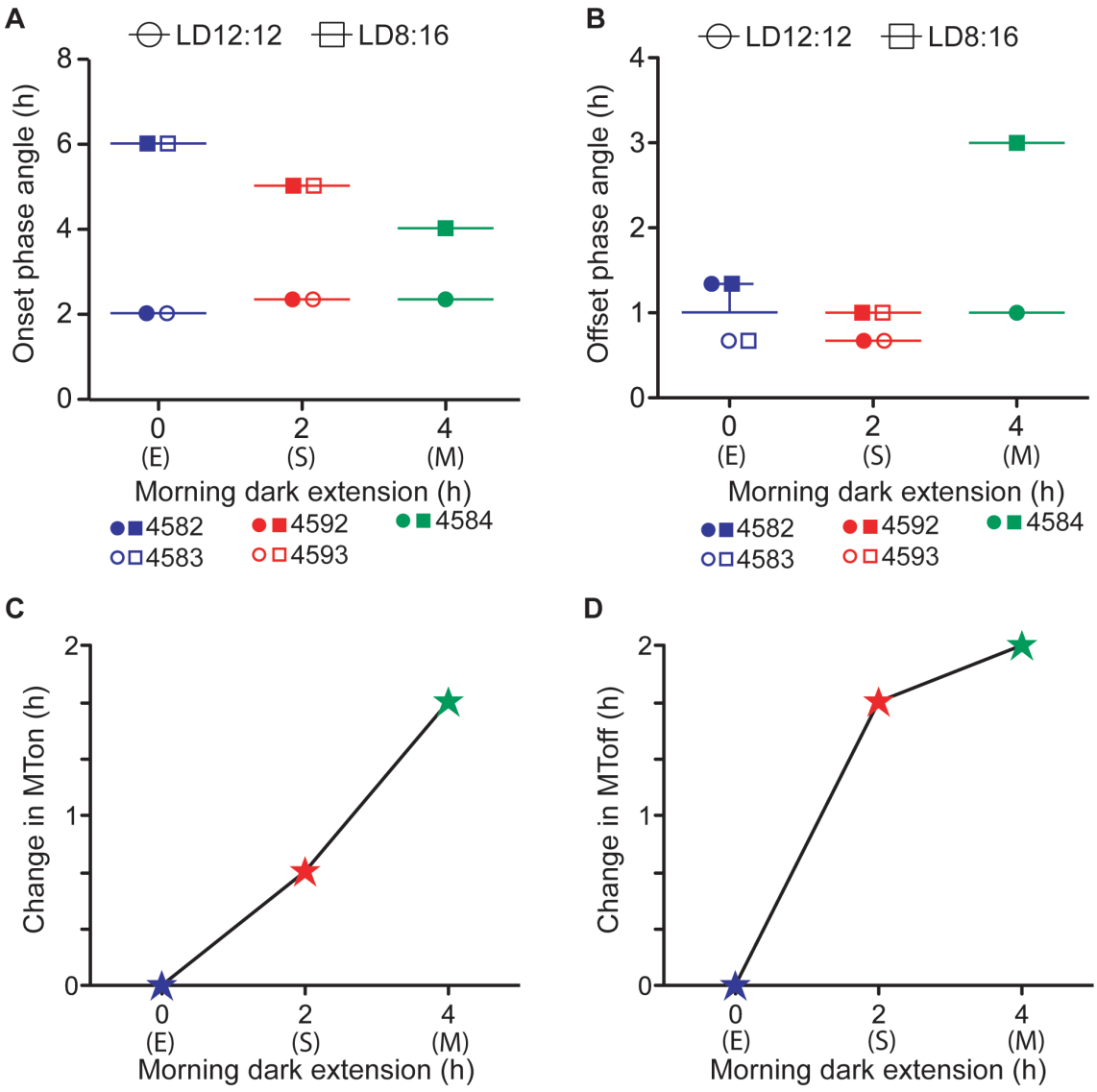

Figure 5-3 Melatonin phase angles in short photoperiod depend on the magnitude of morning dark extension

(A) Melatonin onset phase angles in LD12:12h and LD8:16h relative to the amount of morning dark extension. **(B)** Melatonin offset phase angles in LD12:12h and LD8:16h relative to the amount of morning dark period extension. **(C)** The amount of melatonin onset (MTon) change following dark period extension. **(D)** The amount of melatonin offset (MToff) change following dark period extension. (Zhang et al., manuscript in preparation).

Beyond phase angle relationship, the amount of change in melatonin onset and offset timing show correlation with the magnitude of morning dark extension (Figure 5-3C and D). Melatonin onset reentrained to the same position when the light-on extension was zero (following evening dark expansion). When the dark period was extended by 2h in the morning following symmetric dark expansion, onset delayed by 40min. After a 4h morning extension of darkness, melatonin onset delayed by 1h40min (Figure 5-3C). For melatonin offset, with no dark extension into the morning, there was no difference between original and final offset (Figure 5-3D). A 2h morning extension of darkness delayed offset by 1h40min, and a 4h extension delayed offset by 2h (Figure 5-3D).

5.3.1.3 Melatonin duration

Animals used in the dark expansion experiments had similar durations of melatonin secretion prior to photoperiod change, with a difference within the range of 40min (Figure 5-4A and C). The increase in melatonin duration following dark expansion was highly dependent on direction of photoperiod change (Figure 5-4B and D). After symmetric dark expansion, the melatonin duration of both rats increased by 1h from 9h to 10h; following morning dark expansion, duration increased by 20min from 8h40min to 9h (Figure 5-4C and D). For evening dark expansion, although the original melatonin duration of the two rats differed by 40 min in LD12:12h, both rats retained their original durations after reentrainment to LD8:16h (9h20min and 8h40min; Figure 5-4C and D).

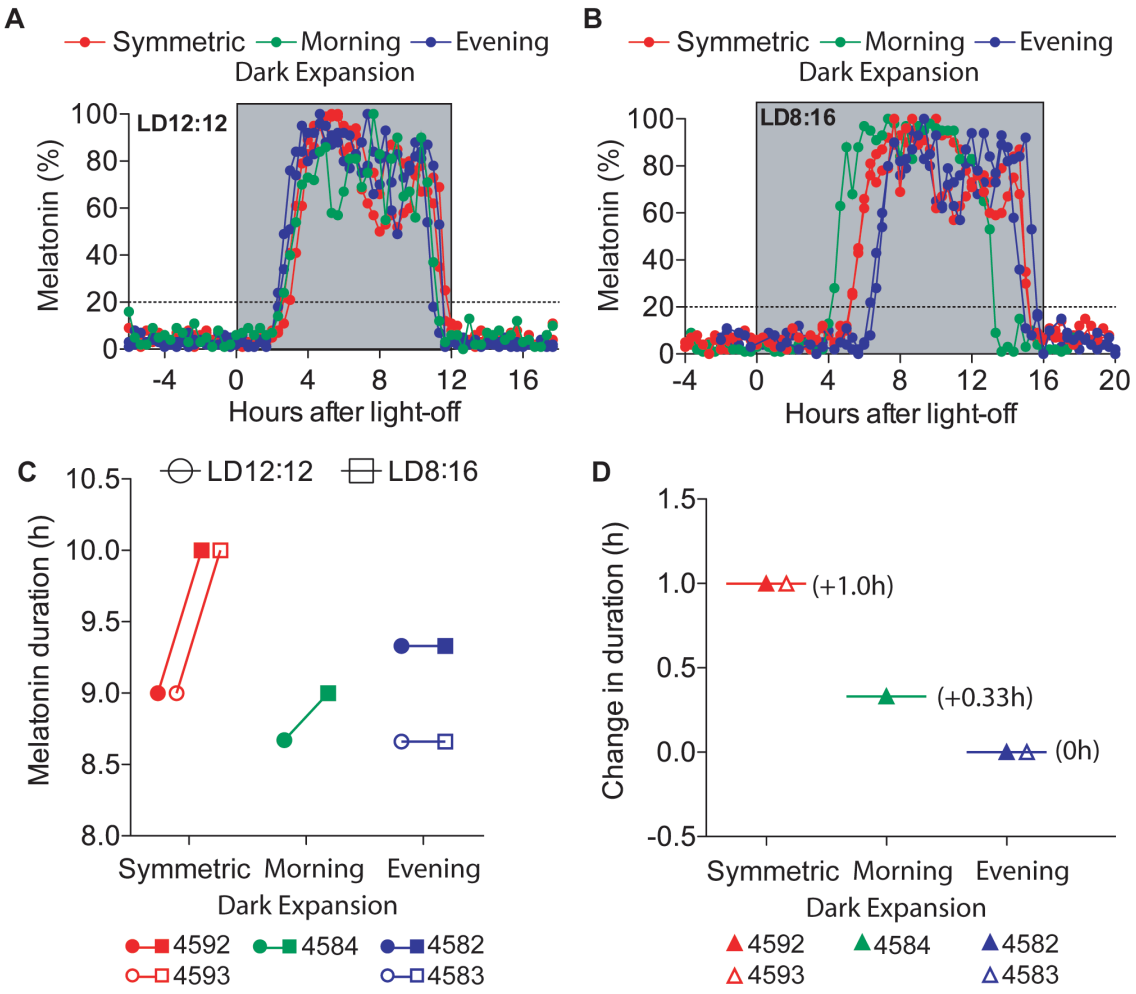

Figure 5-4 Entrained melatonin durations before and after dark expansion

(A) Original melatonin profiles under LD12:12h. Red traces depict individuals used for symmetric dark expansion, green traces show morning dark extension, and blue traces indicate evening dark extension studies. **(B)** Reentrained melatonin profiles under LD8:16h. In both panels A and B, the shaded boxes represent the duration of darkness. **(C)** Melatonin durations before and after dark extension. Circles represent melatonin durations under LD12:12h prior to photoperiod change, whereas squares depict melatonin durations in LD8:16h after reentrainment to short photoperiod. **(D)** Change in melatonin duration following extension of dark period from LD12:12h to LD8:16h. As with phase angles, melatonin duration is heavily influenced by the direction in which the photoperiod was changed. (Zhang et al., manuscript in preparation).

Through continual melatonin monitoring following dark expansion, we found that the duration of melatonin underwent large changes earlier on, followed by days of stabilization towards a final reentrained state (Figure 5-5). With symmetrical expansion of dark period, melatonin duration gradually expanded then stabilized to 111% of the original duration. With morning expansion of darkness, the rat initially exhibited large melatonin expansion up to 111% of the original value during the first 10 days, before stabilizing at a duration that's 104% of the original. Rats with evening expansion of darkness also showed a general expansion in duration before stabilizing back to the original duration (100%); the period of instability was the longest amongst dark expansion paradigms. In summary, melatonin duration change was greatest after symmetric dark expansion, followed by morning dark expansion, with no change after dark expansion into the evening.

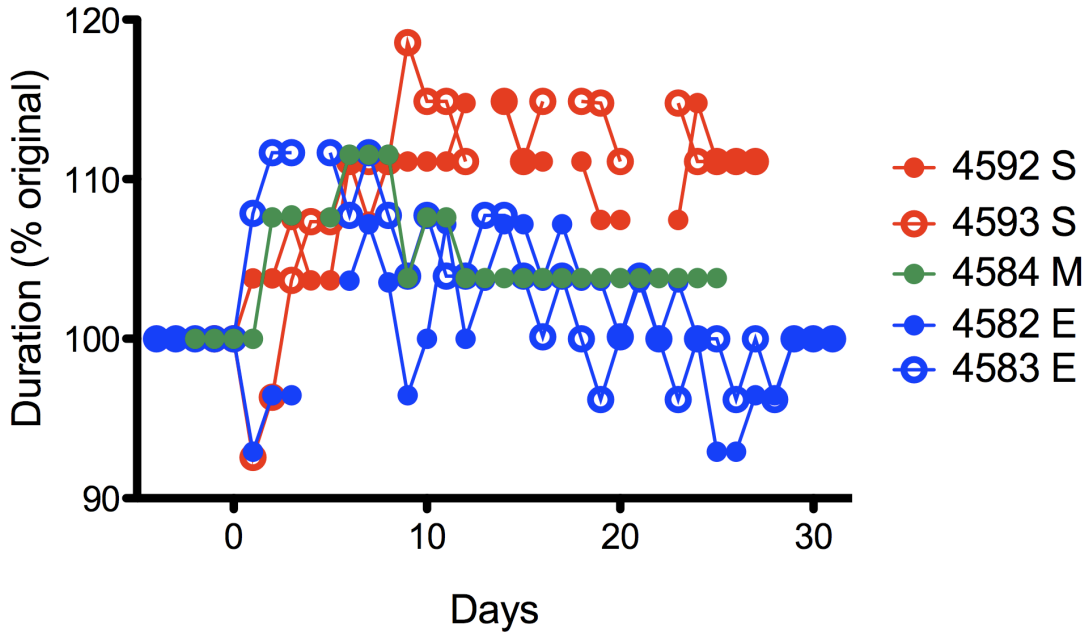

Figure 5-5 Adaptation of melatonin duration to LD8:16h

Melatonin duration following dark expansion in each rat was expressed as percentage change from their original values under LD12:12. Dark period was expanded symmetrically (S; red traces), into the morning by delaying light-on (M; green traces), or into the evening by advancing light-off (E; blue traces). The dynamics of duration change depends on the direction of photoperiod expansion. (Zhang et al., manuscript in preparation).

5.3.2 Dark compression from LD12:12h to LD16:8h

5.3.2.1 Melatonin reentrainment dynamics

For dark compression from LD12:12h to LD16:8h, the melatonin profiles of all rats shifted on the night of the photoperiod change. While melatonin profile generally reentrained faster compared to dark expansion, melatonin onset and offset were not as stable under LD16:8 even after 30 days. Following reentrainment to symmetric dark compression (Figure 5-6A), onset delayed by 1h40min (ID4586) or 1h20min (ID4587), offset advanced by 1h20min (ID4586) or 1h40min (ID4587). It took the rats only 3 or 4 days to reentrain to symmetric dark compression; but as with rats under LD16:8, both onset and offset continued to make minor adjustments for the remainder of the experiment (Figure 5-6A). For the morning dark compression (Figure 5-6B), onset on the first night was not affected. When the light turned on, melatonin production was abruptly cut off. Melatonin onset then proceeded to first shift in the delay direction then in the advance direction. Upon reentrainment on day 18 (ID4588) or day 14 (ID4589), onset has advanced from its original timing by 20min (ID4588) or 40min (ID4589). Melatonin offset for both rats remained at the dark/light junction until around day 8. It then made adjustments for two cycles before settling at the final position, having advanced 4h (ID4588) or 3h40min (ID4589) from its original position (Figure 5-6B). With evening dark compression (Figure 5-6C), both rats' onset delayed by 3h40min. Melatonin offset first shifted considerably in the delay direction, then gradually returned so that total shift following reenrainment was 40min. Melatonin onset and offset reached reentrainment requirements on day 19 (Figure 5-6C).

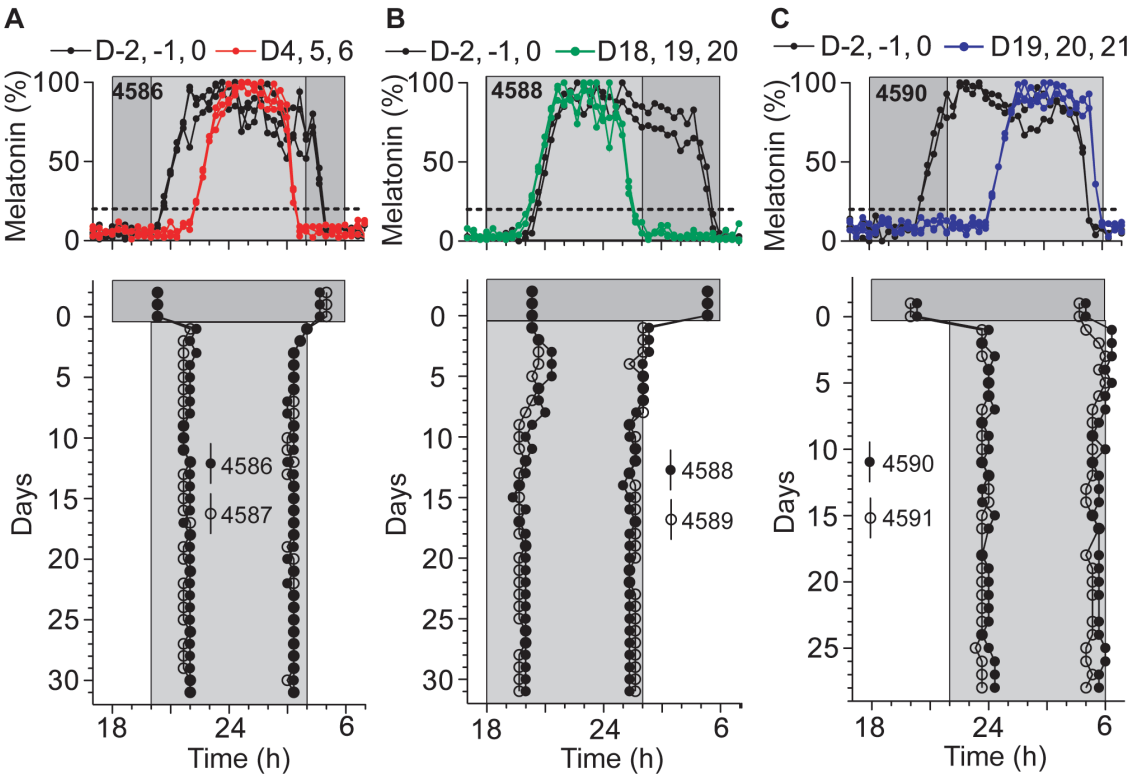

Figure 5-6 Temporal profiles of melatonin adaptation to dark compression

Panels A, B, and C are organized similar to Figure 2: **(A)** symmetric dark compression, **(B)** morning dark compression, **(C)** evening dark compression. (Zhang et al., manuscript in preparation).

5.3.2.2 Melatonin duration

Unlike with dark expansion, reentrainment to an 8h night through three separate paradigms did not result in significantly different melatonin profiles (Figure 5-7B). The reentrained onset phase angle for all rats in LD16:8h was either 2h or 1h40min, and the offset phase angle was either 20min or 40min (Figure 5-7B). Regardless of slight individual differences under LD12:12h, melatonin duration decreased by 3h for all animals, with the exception of rat ID4588 who's duration decreased by 3h40min after advancing light-on (Figure 5-7C and D).

Although the final melatonin durations of all rats under LD16:8h were not significantly different, the direction of dark compression does seem to affect the reentrainment dynamics of melatonin duration in the initial few days (Figure 5-8). Evening dark compression resulted in a dramatic decrease in melatonin duration of down to 52% of the original value by day 4 for both rats involved. For symmetric dark compression, the shortest melatonin duration was only 60% of the original on day 3 for ID4586, and 62% on day 10 from ID4589. In response to evening dark compression, the duration went down to 58% on day 15 for ID4590 and day 13 for ID4591. In summary, the direction of dark compression determines the magnitude of the initial response of melatonin duration, with dark compression in the morning resulting in the most dramatic decrease in melatonin duration. Following the initial differentiated response to dark compression (Figure 5-8), the melatonin duration of all animals eventually stabilized to LD16:8h with very similar reentrained profiles (Figure 5-7B).

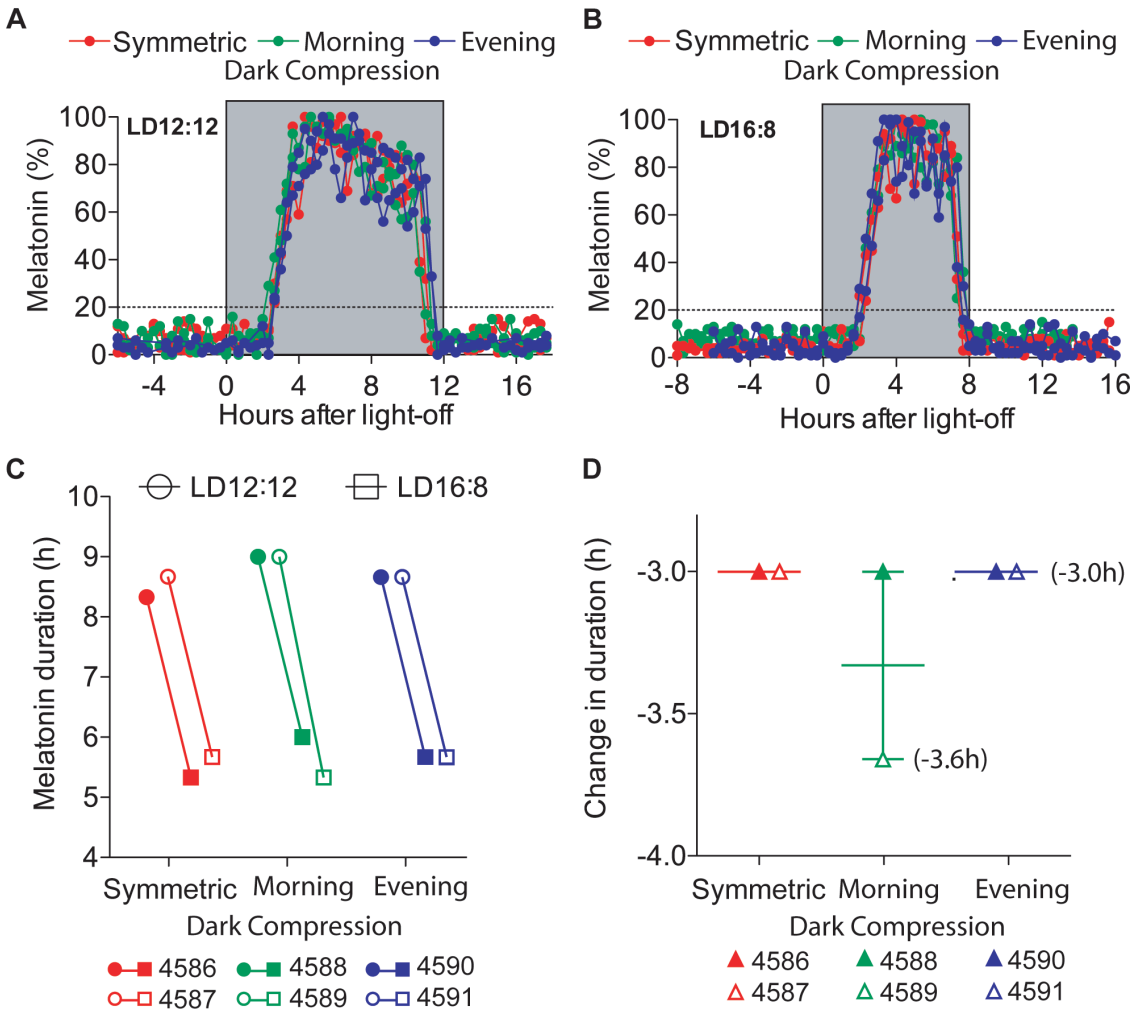

Figure 5-7 Entrained melatonin durations before and after dark compression

(A) Original melatonin profiles under LD12:12h. **(B)** Reentrained melatonin profiles under LD16:8h. The shaded box represents the duration of darkness in both panels A and B. **(C)** Melatonin duration under LD12:12h and after reentrainment to LD16:8h. **(D)** Change in melatonin duration after reentrainment. Negative change indicates shortening of melatonin duration. (Zhang et al., manuscript in preparation).

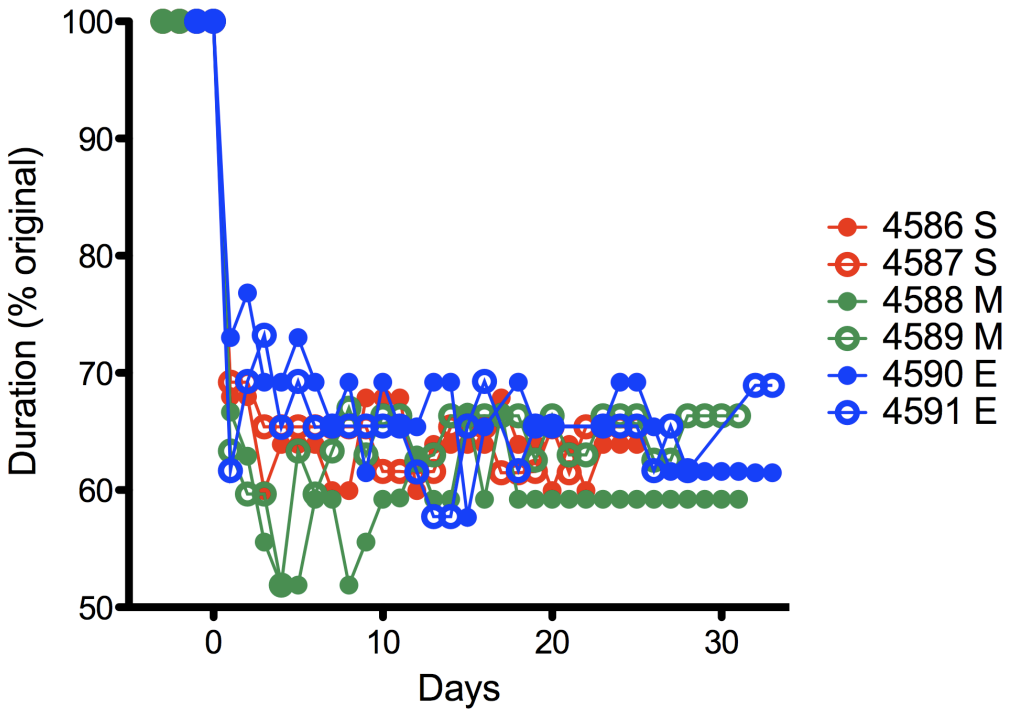

Figure 5-8 Adaptation of melatonin duration to LD16:8h

Melatonin duration following dark compression in each rat was expressed as percentage change from their original values under LD12:12. Dark period was compressed symmetrically (S; red traces), in the morning by advancing light-on (M; green traces), or in the evening by delaying light-off (E; blue traces). Morning dark compression resulted in the most dramatic decrease in the initial stages of reentrainment. (Zhang et al., manuscript in preparation).

5.4 Discussion

Exposing rats to short nights generated very similar final melatonin profiles, regardless of how the dark period was compressed. Interestingly, the melatonin profiles of animals could not stably entrain under LD16:8h, with stability defined as having the same melatonin onset and offset timing each day measured in 20min bins (cf. section 4.2.2). Reentraining animals to LD8:16h on the other hand, resulted in melatonin profiles that differed markedly depending on the direction of dark expansion. While symmetric and morning dark expansions altered onset, offset, and duration of melatonin, evening dark expansion had little effect on these parameters in the reentrained animals. Furthermore, there appeared to be a correlation between the amount of dark extension into the morning and the phase angles of melatonin in the new photoperiod. As the dark period was extended further into the morning, melatonin onset phase angle became smaller and offset phase angle became larger.

5.4.1 Direction dependent adaptation of melatonin profile to LD8:16h

5.4.1.1 Change in melatonin duration

Depending on the direction of dark expansion, significant differences in reentrained melatonin duration were observed. In previous publications where the dark period was expanded from LD16:8h to LD8:16h, the durations of melatonin synthesis in both hamsters (Hoffmann and Illnerova, 1986) and rats (Illnerova et al., 1986) were longer when the dark period was expanded into the morning than if it was expanded into the evening. Furthermore, the duration of locomotor activity was also greater by 1h following morning dark expansion when compared to evening dark expansion (Gorman et al., 1997).

Consistent with the published data (Hoffmann and Illnerova, 1986; Illnerova et al., 1986; Gorman et al., 1997), our longitudinal studies show that unidirectional dark expansion into the morning resulted in longer melatonin duration than expansion into the evening. However, unlike previous publications, we find that melatonin duration increased the most after symmetrical dark expansion. This result contradicts earlier data from Illnerova's group, which demonstrated that a morning dark expansion resulted in longer duration of AANAT activity than with symmetric or evening dark expansions (Illnerova et al., 1986). Since AANAT activity should closely represent melatonin synthesis rhythm, the difference in our findings may be due to the fact that this study looks at dark expansion from LD12:12h and not LD16:8h to LD8:16h. The more dramatic change in photoperiod may have led to different reentrainment responses.

5.4.1.2 Direction dependent adaptation of melatonin onset and offset to long night

In addition to melatonin duration, melatonin onset and offset phase angles were also markedly affected by the direction of dark expansion. Under the same photoperiod, greater magnitude of morning dark extension was associated with smaller melatonin onset phase angle and larger offset phase angle (Figure 5-3A and B). At the same time, melatonin onset and offset both delayed by larger amounts from their original timing in response to greater morning extension of dark period (Figure 5-3C, 3D). We were unable to find similar reports in published literatures for this type of melatonin behavior. A systematic analysis using inbred rats with little interindividual variation in melatonin profiles may be needed to probe this interesting phenomenon.

5.4.1.3 Nonresponsive feature of evening dark expansion

In this study, the melatonin profiles of rats reentrained to LD8:16h via evening dark expansion were no different from the melatonin profiles under LD12:12h (Figure 5-2, Figure 5-4 and Figure 5-5). Curiously, similar non-responsive traits have been seen in hamsters. Although hamsters are photoperiodic animals, a subpopulation of hamsters do not undergo an increase in the duration of activity (Gorman et al., 1997) or melatonin secretion (Puchalski and Lynch, 1986), and correspondingly do not exhibit other photoperiodic traits when transferred to a short day environment. Among Djungarian and Siberian hamsters that were non-responsive, activity duration increased transiently only if the dark period was extended into the morning. When dark period was extended into the evening, activity duration was unchanged despite shortened photoperiod (Puchalski and Lynch, 1986; Gorman et al., 1997), agreeing with the melatonin data from our SD rats. The lack of melatonin and activity expansion in short photoperiods have not been found in other species until this current study of melatonin rhythm in rats.

Indeed, the non-responsive feature towards evening dark expansion is not exhibited in Wistar (WI) rats (Illnerova et al., 1986) and Syrian hamsters (Hastings et al., 1987). In these animals, evening expansion of dark period from LD16:8h to LD8:16h resulted in a notable expansion in the duration of AANAT activity (Illnerova et al., 1986), melatonin, and locomotor activity rhythms (Hastings et al., 1987). Various possibilities could account for the discrepancy between our studies. First, the lack of melatonin expansion in response to evening dark extension may be species or strain specific. In our case, the SD rats were non-responsive; whether strains other than WI rats would show marked change in melatonin duration following evening dark expansion can be tested in future

experiments. Secondly, reentrainment history prior to the dark expansion may also account for some of the discrepancies. In the studies that demonstrate a non-responsive feature towards evening dark expansion, the animal subjects were born and raised under the original photoperiod prior to dark expansion (LD12:12h in this study, or LD16:8h in Puchalski and Lynch, 1986; Gorman et al., 1997). In contrast, animals that displayed expansion of AANAT, melatonin, or activity duration upon evening dark expansion were entrained first from LD12:12h to LD16:8h for only 17 days (Illnerova et al., 1986), or 1 month (Hastings et al., 1987) before reentrainment to LD8:16h. It is possible that response of melatonin to dark expansion may be sensitive to history of light entrainment as well as direction of dark expansion. Light-history dependency of melatonin secretion has been demonstrated in past publications (Hoffmann et al., 1985, 1986; Niklowitz et al., 1992; Niklowitz et al., 1994; Gorman and Zucker, 1997; Goldman and Goldman, 2003) and may be responsible for some of the discrepancies between this study and other published works.

5.4.2 Direction dependent adaptation of melatonin profile to LD16:8h

We show here that melatonin synthesis rhythms reentrained to LD16:8h are very similar regardless of the direction of dark compression (Figure 5-7B). However, the initial response of melatonin profile is very much dependent on the direction of dark compression – namely, morning dark compression results in the greatest percent decrease in melatonin duration in the first few days of photoperiod change (Figure 5-8).

The directional difference in initial melatonin duration response may serve as a functional explanation for the differences in TSHB and Eya3 induction rate mentioned in section 5.1.1.3. For TSHB and Eya3, dark compression into the morning resulted in

immediate increase in induction rate, where as evening dark compression led to eventual increase in induction after several days (Masumoto et al., 2010). Since the induction study was conducted in melatonin–proficient mice, there is a distinct possibility that differentiation in melatonin response shown in this study could be driving the difference in induction rate of TSHB and Eya3.

5.4.3 Rate of reentrainment

With only two or even one animal per sample group, the rate of reentrainment cannot be determined with statistical significance. However, by considering the reentrainment progression traces (Figure 5-2 and Figure 5-6) and considering when the melatonin duration became stable after the photoperiod change (Figure 5-5 and Figure 5-8), we can make some inferences towards differential reentrainment rates. In general, rats reentrained faster to dark compression than to dark expansion. Such difference in reentrainment rate have been shown in flight activity rhythms of mosquitos (Jones et al., 1967; Taylor and Jones, 1969), as well as activity offset of pinealectomized rats (Quay, 1970). Within dark expansion, reentrainment to morning expansion was the fastest, with the single rat's melatonin profile reentraining in only 11 days. The same result has been shown for rat AANAT rhythm in response to dark expansion from LD16:8h to LD8:16h (Illnerova et al., 1986). For dark compression, our data suggest that reentrainment to symmetric compression appeared to be the fastest.

5.5 Conclusion

This chapter examined the adjustment dynamics, and final profiles of melatonin secretion following photoperiod alteration from LD12:12h to LD8:16 or LD16:8. Depending on direction of dark expansion, melatonin profiles under the same

photoperiod differed systematically from each other. Symmetric dark expansion from 12h to 16h night led to the most substantial elongation of melatonin duration. Dark expansion into the morning hours was most effective in delaying both melatonin onset and offset. Dark expansion into the evening hours destabilized the melatonin profile transiently, but surprisingly caused no change in final melatonin profile despite four hours of increase in dark duration. Dark compression on the other hand produced similar melatonin profiles regardless of the direction of compression. Since melatonin's response to photoperiod induces various seasonal phenotypes, the detailed depiction of light-history dependent response of melatonin in single individuals presented here provides important implications for future photoperiod studies. Additionally, because melatonin closely reflects the rhythm of the central pacemaker, this study provides insights on how the pacemaker responds to photoperiodic changes.

5.6 Future directions

To achieve statistic significance in our findings, we need to repeat this study with more rats in each experiment group, and to use inbred rats to reduce interindividual differences. Beyond the experiments covered in this chapter, the resultant data gave rise to some other interesting areas to explore.

5.6.1 Maximum expansion of melatonin duration

This study showed that melatonin duration increased the most after symmetric dark expansion (Figure 5-2A). Furthermore, our data showed that melatonin duration tends to shift in the delay direction (Figure 5-2). Although duration increased up to 11% (Figure 5-5), the dark period can only be extended by 2h in both directions from LD12:12h to LD8:16h. Because melatonin cannot be secreted in the presence of light, it may be

possible that melatonin offset may extend further if entrained under an even smaller photoperiod such as LD6:18h or LD4:20h. Free-running melatonin data presented in Chapter 3 (Figure 3-1) demonstrated that melatonin duration in DD is actually not as long as when entrained under a short photoperiod of LD8:16h. It would be interesting to identify the physiological maximum of nightly melatonin synthesis duration under entrained conditions.

5.6.2 Phase angle versus rate of entrainment to photoperiods

The rats used in this study had similar phase angles under LD12:12h, which allowed us to discern the difference between photoperiod shift paradigms. It would also be interesting to look at how interindividual differences in phase angle may affect photoperiod reentrainment. Knowing that early birds prefer to sleep early and wake up early, one may assume that early bird chronotypes would reentrain faster to evening dark expansion and morning dark compression. The opposite may be assumed for night owls. If individual phase angle does play a role in direction of photoperiod reentrainment, it may further explain some of the differences we see between our study and past publications.

5.6.3 Gradual change in photoperiod

In this study, artificial changes in photoperiod occur instantaneously. In nature, photoperiod gradually expands and compresses bidirecitonally through the annual seasons. Melatonin may respond differently to gradual change in photoperiod so as to optimize internal representation of seasons under natural conditions. The dynamics of melatonin adjustment to gradual change in photoperiod remain to be seen.

Chapter 6

Conclusion

This dissertation focused on the phase angle of melatonin secretion and its relationship to various circadian components. Chapter 3 described the lack of a strong relationship between phase angle and free-running period. Chapter 4 further established that phase angle is independent of period in response to phase shifts, and that a smaller phase angle predicted faster reentrainment regardless of direction of shift. In both Chapters 3 and 4, photoperiod was used to manipulate the phase angle of melatonin, however the phase angle of melatonin under the same short photoperiod of LD8:16h also depends on how the LD schedule was shifted from LD12:12h. Chapter 5 details the adjustment of melatonin profiles to long and short photoperiods, and provides a backbone that supports the photoperiod manipulation of melatonin rhythm in previous chapters.

6.1 General future directions

6.1.1 Application to jet lag study in humans

Chapter 4 showed that individuals with smaller melatonin onset phase angles reentrain faster to both advance and delay shifts in schedule. Throughout this dissertation I also showed that melatonin phase angle decreases with longer photoperiods (shorter nights). Thus, by only manipulating the photoperiod, we can shrink the onset phase angle of melatonin and speed up the reentrainment process. This finding has possible therapeutic implications on the treatment of jetlag in humans. By identifying that smaller melatonin onset phase angles result in faster reentrainment, pharmacological and behavioral

methods may be tested to decrease the duration of jetlag. In particular, administration of exogenous melatonin can be specifically scheduled to advance the daily melatonin onset timing. Subjects can take melatonin late in the subjective afternoon or early in the subjective evening at their new schedule to see which is more affective in decreasing the duration of jetlag.

Furthermore, studies can be performed to see if restricting the dark period alone can help minimize the duration of jetlag without the use of pharmacological agents. In Chapter 5 we showed that the melatonin phase angle after dark compression was not significantly different between the three photoperiod change paradigms. Thus, dark restriction from the subjects' normal schedule can be performed to match the subjective night of the new time zone without concern of direction of shift. When returning to a normal LD schedule, morning expansion of dark period should result in the smallest melatonin onset phase angle (cf. Chapter 5), and thus further facilitate the reentrainment process. The compression and expansion of dark period during phase shift would be interesting to test in laboratory animals; furthermore, whether or not the same results hold true in humans remains to be seen.

6.1.2 Abrupt versus gradual LD transitions (use of twilight)

Animals in laboratory settings are commonly kept in LD cycles where the light/dark transition occurs abruptly, as was the case for studies presented in this dissertation. In nature however, the transition between morning and night occurs gradually. Gradual light transitions can be setup in the laboratory to simulate the natural dawn and dusk LD transitions.

Significant differences have been found in locomotor activity and body temperature rhythms of squirrel monkeys entrained under abrupt versus gradual LD transitions (Boulos et al., 1996a). Under abrupt LD transitions, the diurnal monkey exhibited consistent activity and temperature levels during the day, whereas in gradual LD transitions, activity and temperature levels peaked during dusk, with a smaller secondary peak at dawn. It would be interesting to see if melatonin profiles exhibit similar differences under abrupt versus gradual LD transitions.

Under gradual LD transitions, squirrel monkeys had longer activity duration and later activity offset. Most interestingly, activity onset was consistently earlier under gradual LD transition than under abrupt LD transition. However, although monkeys entrained faster to an 8h advance shift than an 8h delay shift of the LD cycle, there was no difference in rate of reentrainment between abrupt and gradual LD transitions. From Chapter 4, we know that an earlier melatonin onset indicates faster reentrainment rate in rats. It would be interesting to see if melatonin onset is earlier under gradual LD transitions when compared to abrupt transitions, and if so, whether rats would reentrain faster to an LD shift under gradual LD transitions.

In another study using hamsters, gradual LD transition was shown to increase the zeitgeber strength of the LD cycle by promoting entrainment to longer than 24h periods (Boulos et al., 1996b). As the LD period increased from a 24h day to a 26h day, all hamsters under gradual LD transition remained entrained, while animals under abrupt LD transition began to free-run. Similar entrainment stability may be applied to photoperiods. In this dissertation, I showed that melatonin rhythm can not stably entrain to long photoperiod of LD8:16h with abrupt LD transitions. It may be possible that by

entraining animals to gradual LD transitions, melatonin profiles would exhibit identical nightly onset and offsets even under LD8:16h conditions. It would be interesting to compare melatonin release while increasing the limits of entrainment to long and short photoperiods between abrupt and gradual LD transitions.

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