UNIVERSITY OF CALIFORNIA, SAN DIEGO

The Influence of Circadian Waveform Manipulation on
Re-entrainment and Memory

A dissertation submitted in partial satisfaction of the requirements for the
degree of Doctor of Philosophy

in

Psychology

by

Elizabeth Mary Harrison

Committee in charge:

Professor Michael Gorman, Chair
Professor Stephan Anagnostaras
Professor Sonia Ancoli-Israel
Professor Sean Drummond
Professor Don MacLeod
Professor David Welsh

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The Dissertation of Elizabeth Mary Harrison is approved, and it is acceptable in quality and form for publication on microfilm and electronically:

                                              
                                              
                                              
                                              
                                              
                                              
                                              
                                              Chair

University of California, San Diego

2014
DEDICATION

To Leon.
EPIGRAPH

There is a crack, a crack in everything
That’s how the light gets in.
- Leonard Cohen

These interlocking rhythmic things are really fun.
- Danny Elfman
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VITA

Education

University of California, San Diego- La Jolla, California
  Doctor of Philosophy in Experimental Psychology, 2014
  Master of Arts in Experimental Psychology, 2010

University of California, Berkeley
  Bachelor of Arts in Sociology, December 2002

Publications


ABSTRACT OF THE DISSERTATION

The Influence of Circadian Waveform Manipulation on Re-entrainment and Memory

by

Elizabeth Mary Harrison
Doctor of Philosophy in Psychology
University of California, San Diego, 2014
Professor Michael Gorman, Chair

Circadian disruption, specifically in shiftwork, entails significant health risks including metabolic and sleep-related disorders. Most current interventions involve the manipulation of the phase, or timing, of circadian rhythms, while those involving the waveform, or shape, of rhythms have received relatively little attention. The studies in this dissertation characterize
behavioral, physiological, and cognitive effects of a novel circadian waveform manipulation, both under steady state conditions and under simulated jet lag paradigms. Here we clearly demonstrate that both a natural, photoperiodic change in waveform, and an artificial, bifurcated circadian waveform confer a more than two-fold advantage in adjustment to 6 equidistant time zones. Further, while mammals in long days typically re-entrain to new light schedules at the rate of ~1 h/day, we show here that bifurcated animals can entrain to light cycles 12 h apart (i.e., on opposite sides of the world) in three days. Thus, manipulating waveform before phase shifts utilizing only simple changes in the daily pattern of light exposure increases plasticity in this system to an unprecedented extent. Here we also show that the bifurcated entrainment state involves a unique configuration of rhythmic clock genes that is apparently functional and readily-reversible. Recently, bifurcation has been suggested as a potential entrainment state for human shiftworkers. Here we find that bifurcation can be maintained under a simulated rotating shift schedule, and that it does not impair memory, while simulated jet-lag does. Both of these findings of intact learning and memory and resilience to perturbations of the light/dark schedule are key pieces in the applicability of bifurcation as an entrainment state for humans exposed to shiftwork or other challenging schedules.
CHAPTER 1. General Introduction

Circadian rhythms are daily fluctuations in mammalian physiology and behavior that are orchestrated by a master pacemaker located in the hypothalamus known as the suprachiasmatic nucleus (SCN). Environmental light is the primary Zeitgeber, or timekeeping cue, for the system (Duffy, Kronauer, & Czeisler, 1996; Murphy & Campbell, 1996; Reppert & Weaver, 2002). Light information travels through the eye via the Retino-Hypothalamic-Tract (RHT), which projects directly to the SCN. A molecular transcription/translation feedback loop (TTFL) of rhythmic gene expression contributes to the generation of daily oscillations within the organism. Core clock genes include the positive elements Clock and Bmal, which induce transcription and translation of the negative elements, Per and Cry. Per and Cry then negatively feed back to repress transcription of Clock and Bmal. This transcription/translation feedback loop (TTFL) is the primary model for the molecular mechanism by which circadian rhythmicity is generated at an organismal level, though the actual mechanism is more complex and involves rhythmic post-translational modification as well (McClung, 2011).

Clock genes are rhythmically expressed, not only in the SCN, but also in peripheral tissues, via similar TTFLs (Ko & Takahashi, 2006; Panda et al., 2002; Storch et al., 2002). Peripheral oscillators in organs such as the liver (Yamazaki et al., 2000) and kidney (Yoo et al., 2004) are synchronized by the
SCN via neuronal and hormonal signaling (Perreau-Lenz, Pevet, Buijs, & Kalsbeek, 2004; Schibler, Ripperger, & Brown, 2003). In the SCN of a nocturnal rodent, *Per* and *Cry* expression levels are high during the day, when the animal is behaviorally inactive, whereas *Bmal* and *Clock* are high at night, during the active phase. The timing of the expression of individual clock genes relative to one another is conserved in peripheral tissues, though peak expression times lag behind the SCN by ~5-8 h (Yan, Wang, Liu, & Shao, 2008). Thus the phase of expression in the peripheral tissues relative to the light/dark cycle differs from that in the SCN.

Within the organism, then, exists an endogenously generated *subjective day* and *subjective night*. These physiological states persist even in the absence of all light cues (i.e., in constant dark (DD)). Under typical conditions (i.e., a light/dark cycle), this internal time is synchronized to the external light schedule via small, daily light-induced shifts in the *phase*, or timing, of the rhythms. In humans and other animals, the phase-shifting actions of light are greatest during subjective night: even brief pulses of light at the beginning and end of subjective night are sufficient to delay and advance the pacemaker, respectively (Aschoff, 1999). Light input rapidly triggers transcriptional activation in the SCN – light acutely induces SCN expression of *Per1* and *Per2* (Albrecht, Zheng, Larkin, Sun, & Lee, 2001; Shigeyoshi et al., 1997), core clock genes that are part of the TTFL, in addition to other genes
that are part of the signaling pathway, such as \( pERK \) and \( cFos \) (Albrecht, Sun, Eichele, & Lee, 1997).

As alluded to above, the effects of light on the circadian system change as a function of subjective time of day. The magnitude and direction of a phase shift elicited by a given stimulus (e.g., light, or some other \( Zeitgeber \)), can be plotted as a phase-response curve (PRC). PRCs are typically generated while an organism is under constant conditions, such as DD, to minimize the effect of other \( Zeitgebers \) on the system. PRCs have been constructed for a variety of photic stimuli, across different wavelengths and durations of light exposure. Evidence from PRCs indicates that in both nocturnal and diurnal animals, light stimuli are most potent during subjective night (Golombek & Rosenstein, 2010; Reppert & Weaver, 2002). Light at the beginning of the subjective night will shift the system later (\( delay \)), whereas light at the end of subjective night will shift it earlier (\( advance \)). Further, the mammalian circadian system is capable of relatively large shifts (~2 h) in response to even very brief (i.e. 15 m) pulses of light (Daan & Pittendrigh, 1976). Conclusions drawn from these studies in both humans and other mammals form the basis of current recommendations for adjustment to challenging schedules (Golombek & Rosenstein, 2010).

Entrainment theory based on behavioral observations posits that the alignment of the approximately-24 h internal rhythms of an organism with the
24 h external light/dark schedule is achieved either via parametric or non-parametric effects of light on the circadian system. Under non-parametric entrainment theory, the effects of light at dawn and dusk are critical for entrainment, whereas under the parametric theory, entrainment depends on long-duration light input across the whole 24 h cycle. Evidence for parametric effects of light includes increasing stability of behavioral entrainment under longer, full photophases, and the tendency under constant conditions for an organism’s period, or subjective circadian cycle, to increase as light intensity increases (Aschoff, 1979). Conversely, evidence for non-parametric actions of light include the phase-shifting effects of brief light pulses under constant conditions, as well as the maintenance of behavioral entrainment under “skeleton” photoperiods, wherein full photophases are replaced with short light pulses that bracket subjective night (Pittendrigh & Daan, 1976). There is, however, emerging evidence that parametric and non-parametric effects use different pathways or mechanisms of action, and thus one may be more relevant than the other under certain conditions. A recent paper showed remarkably-enhanced phase-shifting responses in mice lacking vasopressin receptors, but only under full photophases (parametric), and not in response to brief light pulses (non-parametric) (Yamaguchi et al., 2013). Additionally, the enhanced phase-resetting effects of dim nocturnal illumination apply only
under conditions wherein full photophases are shifted (Evans, Elliott, & Gorman, 2007).

**Circadian Manipulations**

The most commonly-attempted circadian manipulation in both basic and applied circadian biology is a shift in the phase or timing of subjective day and night. In circadian research in rodents, protocols utilizing brief pulses of light to measure non-parametric phase-shifting (or \textit{phase-resetting}) effects of light are commonly used to assess circadian function. In human research, however, the focus tends to be on more ecologically valid protocols using shifts of the full light/dark cycle to simulate changing work schedules or flight across multiple time zones.

Phase-shifting is commonly recommended for adjustment to challenging schedules in humans in spite of serious limitations in application (Crowley, Lee, Tseng, Fogg, & Eastman, 2003; Lee, Smith, & Eastman, 2006; Smith, Fogg, & Eastman, 2009). Under laboratory conditions, humans and non-human animals can phase-shift successfully in either direction (advance or delay). Outside of the laboratory, humans rapidly crossing several time zones eventually adopt a phase aligned with the new location, though it may take several days, as resetting typically occurs at the rate of about 1 h/day (Sack et al., 2007). In a context such as rotating shiftwork, however, those
days (in some cases up to 12) required for adjustment can create problems for the health and performance of the worker. Further, effective phase-shifting in such a setting requires complex schedules of light (Dumont, Blais, Roy, & Paquet, 2009). As the phase-resetting actions of light are greatest during subjective night (Aschoff, 1999), inappropriately timed light can not only undo an achieved phase-shift, but actually move an individual’s clock further from the desired schedule. In particular, bright morning light at the end of a night shift has strong phase-advancing effects that counteract the desired shift (Crowley et al., 2003). To combat these complicated effects in the real world, one of the most highly successful interventions outside of the lab thus far utilized scheduled exposure to bright light and darkness in conjunction with sunglasses, early sleep, and timed ingestion of melatonin, with mixed results (Crowley et al., 2003).

Circadian Disruption

As the precise synchrony and timing of circadian rhythms are determined by the SCN in response to input from light/dark schedules, synchrony between external and internal time is disrupted after abrupt schedule changes such as travel across time zones, or a rotation from day shift to night shift. During this transition, individual rhythms may dampen or continue to oscillate robustly but fall out of alignment with the light schedule
and/or one another. This physiological state is termed *dysrhythmia*. An example of an organism remaining internally synchronized, but out of alignment with the environmental light/dark cycle, is a night-shift worker that maintains a stable and regular work schedule, but never fully adjusts physiologically to it. Unfortunately, this is more common than might be expected – less than 3% of permanent night-shift workers fully adjust as measured by melatonin rhythms (Folkard, 2008). A rotating shiftworker, on the other hand, provides an example of an individual subjected to repeated schedule changes that occur too rapidly for the system to adjust. Under these changing conditions, individual rhythms may dissociate, as well as be acutely influenced by the timing of behaviors such as sleep/wake and feeding. Both forms of circadian disruption have negative consequences for health and performance in mammals (Evans & Davidson, 2013).

There have been a number of rodent studies examining the effects of jet-lag on re-entrainment in behavior and various tissues and genes. The SCN in rats bearing a luciferase-reporter *Per1* gene shifted faster than liver, lung and muscle following 6 h phase shifts (Yamazaki et al., 2000), indicating differential rates of resetting for SCN and peripheral tissues. By contrast, utilizing a *Per2* reporter in mice, Davidson and colleagues illustrated that after a 6 h phase advance SCN slices take 3 d to adjust by some measures, but up to 8 by others (Davidson, Castanon-cervantes, Leise, Molyneux, & Harrington,
In the same experiment, they showed that many SCN cells show dampened rhythmicity on the first day after the shift. Likewise, the phase of Per2-expressing neurons in the SCN widens post-shift, reflecting differential rates of resetting in cells within dorsal and ventral subregions of the SCN, with the ventral portion shifting more rapidly than the dorsal. Further, variability between animals in SCN peak expression likewise increases post-shift, indicating that animals respond differentially to phase-shifts (Davidson et al., 2009).

After a shift in the light/dark cycle, not only might rhythms in individual tissues dissociate, but within each tissue, individual clock genes can reset at different rates. Using in situ hybridization, Reddy and colleagues found that Per1 SCN expression shifts more quickly than both Cry and behavior during an advance, but not a delay, leading to the postulation that Cry may be a rate-limiting factor for behavioral adjustment (Reddy, Field, Maywood, & Hastings, 2002). In one of the most elaborate examinations of disruption of the coordination of clock gene expression after a phase shift, Kiessling and colleagues found that the rate of reentrainment of clock gene mRNA expression differed across SCN and peripheral tissues, as well as in different genes within the same tissue (Kiessling, Eichele, & Oster, 2010). As in Reddy et al, Period genes entrained more quickly than other genes in SCN and most peripheral tissues examined. Further, reentrainment of activity followed the
slower kinetics of the non-Per genes. This lack of coordination in an organism’s physiology may underlie the symptomatology associated with jet lag. Persistent circadian perturbations cause deficits in reproductive, cardiovascular, and immune function, as well as increase mortality in rodents (Davidson et al., 2006; Evans & Davidson, 2013).

Circadian rhythms in learning and memory

In addition to the health costs of dysrhythmia, circadian disruption has implications for cognition. In rodents and humans, many cognitive tasks show a circadian peak in performance, including acquisition and recall of memories (Gerstner et al., 2009; Wright, Lowry, & LeBourgeois, 2012). Although in many experimental paradigms it is difficult to dissociate the individual circadian effects of these two stages of memory, a number of recent studies have elegantly succeeded in doing so. In a fear conditioning paradigm, mice trained either in the external or subjective day freeze at higher rates during acquisition than mice trained in the night, whereas recall for both conditioned and cued fear peaks in the day independent of training time (Chaudhury & Colwell, 2002). In another study, mice trained in the evening exhibited lower rates of contextual freezing at 12 h, but not 24 h, post-training, while mice trained in the morning show no such phase-dependence (Cai, Shuman, Gorman, Sage, & Anagnostaras, 2009). In a third paradigm, mice phase-shifted immediately
before training performed best 24, rather than 18 or 32, h post-training (Loh et al., 2010). Taken together, these results likely reflect a “time-stamp” for circadian phase (and not environmental time) of recall, found previously in hamsters in a conditioned place preference protocol (Ralph et al., 2002). Circadian rhythms have also been observed in long-term potentiation (LTP) in areas such as the hippocampus that are critical for many forms of learning (Chaudhury, Wang, & Colwell, 2008; Nakatsuka & Natsume, 2014).

Performance on memory tasks is subject to impairment following perturbations of the circadian clock or the light/dark schedule in many (Craig & Mcdonald, 2008; Devan et al., 2001; Legates et al., 2012; Loh et al., 2010; Neto, Carneiro, Valentuzzi, & Araujo, 2008) but not all paradigms (Craig & Mcdonald, 2008; Neto et al., 2008). Moreover, arrhythmic Siberian hamsters show deficits in long-term object recognition and spatial learning (Ruby et al., 2013). In addition to behavioral decrements, free-running mice exposed to a 7-h day show decrements in hippocampal LTP (LeGates et al., 2012), and two recent experiments indicate that chronic phase advances impair hippocampal neurogenesis (Gibson, Wang, Tjho, Khattar, & Kriegsfeld, 2010; Kott, Leach, & Yan, 2012). Finally, mutations in core mammalian clock components including Cry and Per result in learning deficits (for review, see Evans & Davidson, 2013). It is unknown to what extent several aspects of circadian disruption contribute to specific learning deficits – e.g., exposure to light during subjective
night; reduction in rhythm amplitude or synchrony; repeated phase-shifting, etc. Besides direct clock effects, various downstream physiological processes that are typically under circadian control such as sleep and activity may also contribute learning and memory deficits (Cai et al., 2009; Hagewoud et al., 2010; Holmes, Galea, Mistlberger, & Kempermann, 2004; Kohman et al., 2012).

**Human Shiftwork**

Dysrhythmia in humans is a common problem in our 24 h society. An estimated 17-60% of Americans work outside the regular daytime shift (McMenamin, 2007; Rosekind, 2005), resulting in annual costs exceeding $200 billion (Kerin & Aguirre, 2005). This estimate includes two cost categories: first, due to the mismatch between work schedule and alertness, night work carries an increased incidence of injuries, accidents, and errors compared to day work (Folkard, 2003; Mitler et al., 1998); second, due to the physiological consequences of dysrhythmia, including mistimed sleep and/or light exposure, shift-work results in serious deficits in health (reviewed in Evans & Davidson, 2013) including increased cancer risk (Costa, Haus, & Stevens, 2010). Mitigation of the harms of this mismatch between environment and physiology therefore has the potential to increase the safety, productivity, and health of the shift-worker.
The paramount problem faced by human shift-workers is that work, typically requiring sustained attention and precluding sleep, is demanded of them during their subjective night. To a significant extent, shift-workers can consciously override the influences of the circadian pacemaker to remain awake at night and sleep during the day, albeit at reduced levels of alertness and quality, respectively (Akerstedt, 1998). Critically, this volitional adjustment of behavior does not produce a corresponding shift in the rhythm of the circadian pacemaker: only 1 in 4 shift-workers with a permanent schedule outside of the regular 9-5 workday are able to even partially shift circadian phase as measured by melatonin, while only 3% fully shift (Folkard, 2008). The sluggish rate of phase resetting underscores a significant barrier to effective phase-resetting in the work context: many shift-workers are on rotating shifts, requiring that the pacemaker be shifted both rapidly and repeatedly. Likewise, when a shift-worker is given time off, as on a weekend, the new schedule competes with the old (Smith et al., 2009). In fact, it has been advised for night shift workers with weekends off to adapt a “compromise phase position,” entraining to a phase between the two conflicting schedules, without being well-matched to either (Lee et al., 2006; Smith et al., 2009).

Night shiftwork poses additional problems. Light at night has been shown to increase the incidence of cancer and growth of tumors, and has been identified as a probable carcinogen by the World Health Organization.
(WHO; Straif et al., 2007). Empirically, even in individuals with permanent schedules, for whom a stably altered phase may be reasonably expected, the clock generally remains entrained to a phase typical of day workers (Folkard, 2008). Thus, despite a solid understanding of how the human circadian rhythm responds to light, the lack of practically implemented strategies for shift-workers underscores the need for an alternative target of circadian manipulation that may be more responsive and more tractable than phase.

**Waveform & Bifurcation**

The *waveform* of a circadian rhythm is simply its shape across the ~ 24 h cycle. The waveform of the pacemaker, for example, has been commonly conceptualized as a simple (i.e., a square-wave) alternation between biological night and day (Harrison & Gorman, 2012). That is, the physiology of the organism during subjective day is quite distinct from that in subjective night, and the alternation between these states is typically cued by transitions from light to dark or darkness to light. Compared to phase (or period), waveform has received scant attention in the circadian literature (e.g., in PubMed, “circadian” and “waveform” yield under 2% of the hits generated by “circadian” and “phase”).

One manipulation of circadian waveform that has been examined is the naturally-occurring phenomenon of *photoperiodism*. In non-equatorial regions,
the ratio of daylight to dark varies across the annual cycle, and photoperiodism is the physiological adjustment in organisms to these seasonally varying daylengths. In rodents, the waveform of a given photoperiod is encoded in the SCN itself; mice in long winter-like nights show alterations in clock gene expression, increased photoinduction of c-fos, and altered phase relationships between neurons in SCN sub-regions (Meijer, Michel, Vanderleest, & Rohling, 2010; Schwartz, de La Iglesia, Zlomanczuk, & Illnerova, 2001; Sumová, Bendová, Sládek, Iková, & Illnerová, 2004). These changes in the SCN reflect a change in pacemaker waveform; namely, the expansion of subjective night in response to long environmental nights. As a direct result of this expansion, the waveform of downstream outputs is altered. In particular, the duration of two markers of subjective night, melatonin and behavior, both expand (Elliott & Tamarkin, 1994; Goldman & Elliott, 1988; Sumová, Kováciková, & Illnerová, 2007). In rodents, other aspects of physiology also change in response to the altered light signal, including reproduction, body weight, and pelage. There is accumulating evidence that humans share this capacity for an adjustment of pacemaker waveform to seasonal changes in relative length of night (Beck-Friis, Rosen, Kjellman, Ljunggren, & Wetterberg, 1984; Kauppila, Kivela, Pakarinen, & Vakkuri, 1987; Stokkan & Reiter, 1994; Vondrasova & Hajek, 1997; Wehr, 1991; Wehr, Schwartz, et al., 1995).

Photoperiodic waveform manipulation appears to modulate light
response. Specifically, the magnitude of phase-shifts elicited by a single acute, light pulse is increased in rodents previously held under winter-mimicking lighting conditions with longer nights and fewer daylight hours (Evans, Elliott, & Gorman, 2004; Glickman, Harrison, Elliott, & Gorman, 2014; Goldman & Elliott, 1988). This same photoperiod manipulation increases by approximately 1.5 log units the SCN clock’s threshold sensitivity to acute light pulses (Glickman et al., 2012). Further, these non-parametric effects of light on the photoperiodic system are dissociable from other effects of light, such as melatonin suppression (Glickman et al., 2014). Whether prior manipulation of pacemaker waveform has translational utility remains to be determined. For the human shift-worker or time-zone traveler outside of the laboratory, circadian re-entrainment is generally necessitated by abrupt shifts in full and repeated light:dark cycles (parametric) rather than by exposure to a single short light pulse (non-parametric). It is has been argued based on behavioral (Goldman & Elliott, 1988) and electrophysiological (vanderLeest, Rohling, Michel, & Meijer, 2009) evidence from rodents that these phase-shifting effects are a result of altered coupling in the circadian system.

In a recently discovered entrainment paradigm termed “bifurcation,” exposure to a light/dark/light/dark (LDLD) schedule in rodents facilitates a reorganization of the circadian system into two periods of alternating activity and rest per 24 hours. The typically-unimodal circadian waveform of the
organism is thus “bifurcated” and the organism experiences two subjective days and nights per 24 h period. Extensive behavioral studies indicate that this reflects a *bona fide* entrainment state: bifurcation can be maintained under skeleton photoperiods (Gorman & Elliott, 2003, but see Evans et al., 2011) and despite perturbations in the light schedule (Gorman & Steele, 2006; Harrison & Gorman, 2012; Fig 1.1) In addition to behavior, other rhythms that are markers of subjective day and night -- melatonin, light responsiveness and SCN function -- are bimodally expressed in bifurcated animals (Gorman & Elliott, 2003; Raiewski, Elliott, Evans, Glickman, & Gorman, 2012; Watanabe et al., 2007; Yan, Silver, & Gorman, 2010). Recent evidence of antiphase PER1 protein expression in bifurcated Syrian hamster SCN core and shell subregions suggests that this may reflect a dissociation between oscillators in the SCN itself (Yan et al., 2010).

While the twice-daily activity bouts in bifurcated animals represent two subjective days and nights per 24 h in the organism, there is strong evidence from behavioral studies that the two bouts are not functionally equivalent. First, the phase of activity onsets relative to lights off under entrained conditions commonly differs in a consistent manner between the two (Fig 1.2). Second, when released into DD at the beginning of either scotophase, the phase of activity onsets relative to prior lights off differs as well, indicating the dissociated oscillators exert differential effects on one another (i.e., have
different coupling mechanisms; Evans et al., 2011). The two bouts rejoin after a few days in constant conditions, and altering the phase angle between the bouts changes the robustness of the entrainment state (Gorman & Steele, 2006). The light during the photophases counteracts these coupling mechanisms and holds the two oscillators in place apart from one another via parametric actions of light (Evans et al., 2011; Gorman & Elliott, 2003).

While much is known regarding effects of shifting phase of circadian rhythms on cognition, little is known about the consequences of changing the waveform of these rhythms. There is evidence of altered cognitive and affective outputs in mammals after a natural seasonal variation in circadian waveform, i.e. exposure to long winter nights. Rats and hamsters exposed to short photoperiods mimicking long winter nights display more depressive and anxiety-like behavior than animals exposed to simulated long summer days (Prendergast & Kay, 2008; Prendergast & Nelson, 2005), though a recent study in nocturnal rats found the opposite pattern (Dulcis, Jamshidi, Leutgeb, & Spitzer, 2013). Additionally, exposure to long winter nights results in reduced hippocampal volume, decreased hippocampal LTP, and impaired spatial learning and memory in white-footed mice (Walton, Haim, Spieldenner, & Nelson, 2012). Further, plastic song production occurs later in sparrows under longer nights (Whaling, Soha, Nelson, Lasley, & Marler, 1998). It is unclear, however, whether these observed changes are a direct result of reorganization
of the circadian system, or are instead an indirect effect of seasonal physiological and reproductive changes in the organism induced by the light schedule. Further, photoperiodism is but one form of waveform manipulation. Thus, the effects of circadian waveform manipulation *per se* on cognition are as yet poorly understood.

**Bifurcation as a potential entrainment state for shift-workers**

Bifurcation has been suggested as a potential entrainment state for shift-workers, as it offers several significant advantages over an exclusive phase-shifting strategy (Harrison & Gorman, 2012). First, because it would align the pacemaker with the behaviorally modified output of the sleep/wake cycle, there is the strong expectation that alertness would be optimized both during the night-time work shift and the active-time away from work, despite these times falling 12 h apart at opposite phases of the natural day (Fig 1.3). Correspondingly, the programming of sleep during intervals of subjective night would plausibly enhance sleep efficiency. The non-pharmacological enhancement of worker alertness and sleep efficiency has obvious benefits related to optimization of productivity and minimization of accidents, and perhaps the physiological and behavioral problems commonly seen in the shift-working population.
For a bifurcated entrainment protocol to be useful to human shift-workers it must be robust to schedule variation. While days off tend to undo shifts of non-bifurcated rhythms (Smith & Eastman, 2008), bifurcation is immediately re-established after perturbations in the lighting cycle, including replacement of light with darkness (Gorman & Steele, 2006) or darkness with light (Harrison & Gorman, 2012; Fig. 1.1). Moreover, bifurcation takes advantage of morning light, which typically impedes entrainment of the shift-worker, but actually reinforces the bifurcated entrainment pattern, holding the system in place (Evans et al., 2011; Gorman & Steele, 2006). If desired, the bifurcated state can, however, be instantly reverted upon exposure to a unimodal LD cycle (Fig. 1.1). Additionally, the two intervals of darkness in each 24 h can range anywhere from 1-6 h; the two dark periods need not be of equal duration (Harrison & Gorman, 2012; Fig 1.1), nor occur 12 h apart (Gorman & Steele, 2006). Further, as discussed, phase shifts are generally achieved very slowly in humans, requiring successive timed bright light exposures to generate a controlled shift in entrainment. Bifurcation, in contrast, can be reliably elicited in rodents within a single cycle in nearly every individual without transient entrainment states (Yan et al., 2010). Finally, while bifurcation has not been formally tested in humans, there is strong evidence for a conserved, flexible, multi-oscillatory circadian system in humans (reviewed in Harrison & Gorman, 2012). Given these commonalities the
presumption should be that the human pacemaker, like those of other mammals, will be dissociable under permissive conditions.

**Summary:**

The circadian system is a complex, hierarchical system that can be easily perturbed on multiple levels by abrupt and/or chronic shifts in the light/dark schedule. Such disruption has negative consequences for the health and physiology of mammals. Targets for minimizing disruption typically focus on the phase of the rhythm, while waveform is generally ignored. Waveform manipulation has inherent value in understanding the mechanisms behind the multi-oscillatory mechanisms in the circadian system and how they are coupled. Further, waveform manipulation may provide a more promising target than phase for application to human schedule changes.

Specifically, these studies aim to answer the following:

1. Does a naturally-occurring waveform manipulation enhance behavioral re-entrainment in response to shifts in the light/dark cycle?
   If so,
2. Does an experimentally-induced waveform manipulation, bifurcation, likewise enhance behavioral re-entrainment?
3. Does bifurcation enhance re-entrainment of central and peripheral clocks in the circadian system?

4. Can entrainment be maintained under a more applied, simulated rotating shift system?

5. Are there consequences of bifurcation for learning and memory?

Figure 1.1. Bifurcation can be maintained under a variety of LDLD cycles and in spite of perturbations of the light/dark schedule. Each line represents a 24 h day (x axis) with 11 days of activity plotted below one another in each panel. Shaded areas represent times of relative darkness. Schematics below panels transpose the hamster activity pattern into a hypothetical human entrainment paradigm. (A) Nocturnal wheel running is stably divided between two 5 h nights with intervening 5 and 9 h intervals of light. When one light phase is replaced with darkness (arrow), the subsequent activity bout is advanced, but the bifurcated pattern is quickly restored. The analogous human exposure would be a shift-worker sleeping through the environmental night on a day off or having a long sleep during the day. Note that scotophases do not have to be evenly spaced within 24 h. Removal of a photophase (A) – analogous to a shift-worker sleeping in on a day off) or a scotophase (B) – analogous to a shift-worker staying up all day on a day off) does not compromise the stability of the bifurcated state of entrainment. (C) Scotophases do not have to be of equal length, and bifurcation remains stable even when the bouts are asymmetrical and greatly reduced in length. (D) Bifurcated animals can revert to a normal, diurnal rhythm within one cycle.
Figure 1.2. Asymmetric but stable entrainment of a mouse to an LDLD 6:6:6:6 schedule. Activity patterns in each of the two scotophases may differ under LDLD cycles. Each appears to be driven by a distinct oscillator with a 24 h rhythm, cycling in antiphase. As above, white bars represent periods of light (photophase), and gray bars represent periods of darkness (scotophase), while black patterns illustrate activity.
Figure 1.3. Entrainment and dysrhythmia (opposite). (A) Normal entrainment with day work. Sleep coincides with subjective night when melatonin is elevated (solid line) and alertness is low. Work occurs in subjective day when alertness (dashed line) is elevated and melatonin is basal. Each panel begins at midnight of the environmental day. The pacemaker is represented as a clock, while activity, an output of the pacemaker, is represented as the hands of a clock. (B) Dysrhythmia in shift-work. Work is scheduled to coincide with an unaltered subjective night when melatonin is elevated. Sleep is commonly divided into episodes before and after the work shift, but efficiency is low during the subjective day. Behavior is thus out of synchrony with underlying pacemaker. Note that the schedule illustrated here involves a shift of both subjective nights into the former subjective day. Other schedules (e.g., split shifts etc) would not necessarily require this inversion. (C) Hypothetical bifurcated entrainment to shift-work. With subjective day and night bifurcated into two components each with presumed changes in alertness, sleep efficiency and melatonin secretion, work could be profitably scheduled during environmental nighttime hours. The worker’s night shift now occurs during the first and longer subjective day, with a second subjective day in which to conduct business and interact with family. The light between bouts, which includes morning light, stabilizes the bifurcation (see Figure 1.1 A for more detail). Sleep occurs in two episodes bracketing a short subjective day in which to interact with family or conduct business. Behavior is always consistent with underlying pacemaker phase.
CHAPTER 2. Circadian waveform manipulation enhances behavioral re-entrainment in response to shifts in the light/dark cycle

Summary Paragraph:

Daily rhythms in mammalian physiology and behavior are generated by a central pacemaker located in the hypothalamic suprachiasmatic nuclei (SCN), the timing of which is set by light from the environment (Klein, Moore, & Reppert, 1991). When the cycle of light and dark is shifted, as occurs with rapid travel across time zones, the SCN and myriad output rhythms must reset or re-entrain their phases to match the new schedule. In humans, like other mammals, this phase resetting occurs slowly at the rate of about an hour a day (Lockley, 2009). Beyond the capacity for phase-resetting, the circadian pacemaker may also be reset with respect to its waveform (i.e., the shape of the 24 h oscillation) allowing for tracking of seasonally varying daylengths (Schwartz et al., 2001). Introducing a global assay of circadian adjustment, we here demonstrate an unprecedented acceleration in re-entrainment to simulated time zone travel as a result of prior manipulation of circadian waveform. Following exposure to long summer days typical of laboratory housing and human voluntary light exposure (16 h light, 8 h dark), we demonstrate the characteristically slow re-entrainment of Syrian hamsters to time zone shifts and proportionately longer re-entrainment periods for larger shifts. By comparison, two distinct waveform manipulations prior to simulated
travel greatly reduced the mismatch between endogenous and local time. After adaptation to winter waveforms via exposure to an extended night (8 h light, 16 h dark), rhythms of animals shifted twice as far in the first three days after travel. Moreover, a bifurcated waveform induced by exposure to a novel 24 h light:dark:light:dark cycle (LDLD) permitted animals to adjust within 1-3 days to destination time at any of 6 equidistant global meridians representing an up to 71% reduction in jetlag. Thus, a marked enhancement of phase-shifting can be induced via non-pharmacological, non-invasive manipulation of the circadian pacemaker waveform in a model species for mammalian circadian rhythmicity. Given the evidence of conserved flexibility in the human pacemaker waveform (Wehr et al., 1993), these findings raise the promise of flexible resetting applicable to circadian disruption in shift-workers, frequent time-zone travelers, and any individual forced to adjust to challenging schedules.

Entrainment of circadian rhythms to a given light cycle is achieved via small, daily phase shifts in response to light, in particular around subjective dawn and dusk. Light presented early in the night will induce the steady state oscillation to resume with a delayed phase whereas late-night light will advance the phase of the clock (Lockley, 2009). Through extensively characterized mechanisms, pulses of bright light at night perturb expression
levels of elements of an elaborated transcription/translation feedback loop in the SCN (Kuhlman, Silver, Le Sauter, Bult-Ito, & McMahon, 2003). Acute phase shifts additionally induce a temporary misalignment of otherwise typically synchronized internal rhythms during the period of readjustment (Davidson et al., 2009) that generally increases with the magnitude of the shift. Circadian dysrhythmia is prevalent in shift-workers and frequent time-zone travelers and has deleterious effects on performance and health including increased mortality in animal models (Davidson et al., 2006).

Previously, it has been shown that the magnitude of phase-shifts elicited by a single, acute light pulse is increased in rodents previously held under winter-mimicking lighting conditions with longer nights and fewer daylight hours (Evans et al., 2004; Glickman et al., 2014). The resulting alteration of the circadian pacemaker’s waveform, termed photoperiodism, is reflected in SCN electrical and molecular rhythms and the pacemaker’s control of important outputs including locomotor activity and melatonin secretion (Goldman & Elliott, 1988; Schwartz et al., 2001). This same photoperiod manipulation increases by approximately 1.5 log units the SCN clock’s threshold sensitivity to acute light pulses (Glickman et al., 2012). Whether prior manipulation of pacemaker waveform has translational utility remains to be determined. For the human shift-worker or time-zone traveler outside of the laboratory, circadian re-entrainment is generally necessitated by abrupt
shifts in full and repeated light:dark cycles rather than by exposure to a single short light pulse. Additionally, any practical enhancement of circadian resetting would ideally be general with respect to distance and direction of the required shift. Therefore, here we employ a novel assay using full photoperiods to assess whether two manipulations of circadian waveform -- one natural and one highly artificial -- enhance circadian adjustment following simulated travel to six time-zones around the world 4 hours apart from one another.

In Experiment 1, Syrian hamsters were housed in either long days (LD; 16 h light, 8 h dark, n=45) or short days (SD; 8 h light, 16 h dark, n=52) for a period of nine weeks to allow for full and stable circadian adjustment. These photoperiods correspond to summer and winter daylengths at approximately 48° latitude, and for expository purposes, are designated in terms of nearby geographical referents (LD = “London, UK” and SD = “Falkland Islands, Argentina”. Subsets of hamsters from each condition were exposed to shifts in the lighting cycle to simulate LD conditions at six equidistant meridians (London, UK; Aral Sea, Kazakhstan; Beijing, China; Adak, Alaska, USA; Seattle, USA; Halifax, Canada). Consequently, three readily identified markers of circadian pacemaker phase – subjective night onset, subjective midnight and subjective day onset – are required to re-entrain to 4 h increments across the globe. As can be seen in representative activity
records (Fig. 2.1) and group mean data (Figs. 2.2 & 2.3), to all Northern hemisphere destinations with the exception of London, SD (Falklands) animals adjusted more rapidly than their LD (London) counterparts when measured by activity onset, a robust marker of the start of subjective night. The London cohort showed both the expected sluggish ~1.3 h/day response to time zone travel with greater mismatch between circadian time and local time as simulated travel distance increased. Falklands animals, by contrast, showed a much-attenuated jetlag under the same destination schedules (Figs. 2.1-2.2). Averaging across all destinations, including those requiring an 8 or 12 h shift, the onset of subjective night of the Falklands cohort was only 2.7 h from the respective destinations in the first three days after travel and fully entrained within 5 days. The London cohort, in contrast, averaged a discrepancy of 4.8 h and required 7.5 days to entrain (Welch-corrected $t_{(70)}=4.63$, $p<0.0001$; Fig. 2.3). The same enhancement was observed in the other two measures of phase: mid-subjective night and onset of subjective day (Fig. 2.4). Figure 2 illustrates the longer time-course of adjustment -- in subsequent days, animals in all groups converged on the new destination light schedule. On average, onsets for animals in both groups were within 1 h of the new light schedule by days 13-15, with the exception of onsets for the LD animals shifted 12 h (Fig. 2.2).
While entrainment to long, 16 h nights enhanced phase-resetting in hamsters, direct translation for human application is likely impractical. It requires not only a lengthy adaptation period but also a strict avoidance of artificial light that, outside of the laboratory, maintains people in a chronic summer entrainment state regardless of season (Wehr, Giesen, Moul, Turner, & Schwartz, 1995). We therefore tested whether entrainment could be accelerated following an alternative waveform manipulation, termed “bifurcation,” that requires neither a lengthy adaptation time nor a reduction in daily light exposure. Briefly, exposure to permissive 24 h light:dark:light:dark conditions (LDLD), can rapidly and reliably re-entrain circadian timing systems of rodents to generate two subjective days and two intervening subjective nights in each 24 h period (Gorman & Elliott, 2003). This bifurcated waveform, with bimodal expression of activity, melatonin, gene expression, and other measures of subjective day and night (Gorman & Elliott, 2003; Raiewski et al., 2012; Yan et al., 2010), effectively represents a temporal chimera of London and Adak phases.

To determine whether this form of waveform manipulation likewise modulates the phase-resetting response to the same long summer day destinations as above, in a second experiment hamsters were entrained to 16:8 (n=33) or were bifurcated for a period of two weeks to an 8:4:8:4 light cycle (n=36). Hamsters were then challenged with the phase-resetting assay
described above. Long day hamsters again demonstrated a dose-response function to phase-shifts of increasing magnitudes, whereas London/Adak chimeras adjusted rapidly, requiring on average less than half of the time required by long day animals to entrain to their destination light schedule (Fig. 2.5). Across all destinations, including those requiring an 8 or 12 h shift, the onset of subjective night of the London/Adak cohort was only 1.6 h from the respective destinations in the first three days after travel and fully entrained within 3 days, a drastic reduction of circadian mismatch compared to the long day London animals (Welch-corrected $t_{51}=3.61$, $p<0.001$; Fig. 3). This same pattern was seen for mid-subjective night and for the onset of subjective day (Fig. 2.5). Importantly, 4 animals under LDLD conditions failed to bifurcate, and resetting rates for those animals resembled those of long day animals (Fig. 2.7).

In a third experiment, 35 hamsters were bifurcated under optimized conditions and subjected to the same protocol, resulting in only a 1.4 h mismatch from respective destinations in the first three days (Figs. 2.3 & 2.6). Notably, while exposure to either short days or LDLD light cycles induces profound changes in circadian waveform, animals in all groups rapidly adopted a long day waveform after transfer to their destination light schedule (Fig. 2.8).

We next verified that the rapid adjustment of locomotor activity to transmeridian destination schedules seen after bifurcation reflects entrainment
of the endogenous circadian pacemaker, and not an acute masking response to the new lighting conditions. As above, six hamsters were bifurcated for two weeks and challenged with shifts simulating trips to London or to Adak. We confirmed that by the second normal LD cycle, wheel-running closely matched both of the anti-meridian conditions. After three complete LD cycles, animals were kept in constant darkness for 7 days where they exhibited waveforms that were indistinguishable from those of long day animals, indicating that the bifurcated state is readily reversible. Establishing that the pacemaker had been entrained by the three days in LD, activity onsets of the free-running, endogenously generated activity rhythm projected to a phase that in no case deviated more than 90 min from the prior time of lights out. Thus, no more than three days, and perhaps fewer, are required to re-entrain a previously bifurcated pacemaker to phases matching opposite meridians. Using an analogous protocol, we further determined that no more than 72 hours in a bifurcated state is required to achieve this effect (Fig. 2.9).

Here we show an unprecedented enhancement in the rate of phase-resetting as a result of two types of circadian waveform manipulation. The enhanced resetting dynamics that evolved in connection with photoperiodism, although impractical for direct human translation, may be exploited using an artificial bifurcation of waveform. The novel global assay and results described here illustrate the remarkable potential of this waveform manipulation. Unlike
long-night adaptation, bifurcation is a manipulation of waveform that can be experimentally-induced in hamsters within a single day and allows for conservation of eight hours of darkness/24 h day. Moreover, in spite of the plasticity induced by bifurcation, it is relatively stable as an entrainment state and is resilient to light schedule perturbation (Gorman & Steele, 2006; Harrison & Gorman, 2012). Further, while bifurcation has not yet been formally tested in humans, evidence of photoperiodic responsiveness and other evidence of dissociable circadian components suggests that human bifurcation is a plausible entrainment strategy, and it has therefore been proposed as a model for adjustment to shift-work and other challenging schedules (Harrison & Gorman, 2012).

In summary, we show that the mammalian pacemaker is capable of adjusting essentially to opposite sides of the world within 3 cycles using a non-pharmacological, non-invasive manipulation of circadian waveform that is readily inducible in nearly every animal under permissive environmental conditions. Thus the efficacy of commonly adopted practices in the treatment of circadian disorders may have the potential to be vastly improved by simple manipulations of light, and apparent constraints of phase-shifting can be lifted with waveform manipulations.
Methods Summary:

Animals. Experiments were conducted with prior approval of the UCSD Institutional Animal Care and Use Committee. Data are from 206 male Syrian hamsters aged 5-6 wks at initial entrainment (Experiment 1: 45 LD, 52 SD, Harlan; Experiment 2: 33 LD, 36 LDLD; Experiment 3: 34 LDLD, bred in-house) and include all animals that successfully entrained to baseline conditions. Six animals (3 LD, 3 SD) were excluded due to a technical failure. DD data are from a subset of additional purchased hamsters.

Protocol. In Experiment 1 animals were group-housed during the five-week baseline period, then given individual cages with running wheels for four weeks before “traveling” to one of 6 equidistant LD scotophases. In Experiments 2 and 3 animals were group-housed under a 16:8 or 20:4 LD cycle for 9 (Expt 2) or 21 days (Expt 3), then transferred to individual cages with wheels under either a 16:8 photoperiod or an LDLD 8:4:8:4 cycle, maintained for 2 weeks before “travel.”

Behavioral analysis: Activity was recorded via wheels with magnetic contacts and compiled into 6 min bins by VitalView (MiniMitter, Sun River, OR). Actograms were analyzed using Clocklab (Actimetrics, Evanston, IL). Daily
onsets, midpoints and offsets were established via eye-fit for each animal and averaged over intervals of three days to obtain 5 values for each animal for each measure over days 1-15 post-shift. Distance from re-entrainment for onset, midpoints, and offsets was defined as distance from the new lights off, mid-scotophase, and lights on, respectively. Animals were excluded from analysis if they failed to entrain to the destination light schedule within 14 days (Expt 1 n=1/49 LD; 1/77 SD), if they were non-adaptors to short days, failing to show onsets within 2 h of lights off in baseline (Expt 1 n=21/77 SD), or if they failed to bifurcate under LDLD (Expt 2 n=3/37, Expt 3 n=1/35). Statistical analyses were performed using SPSS software.

**Illumination:** Photophase illumination was achieved via white fluorescent bulbs (~100 lux) and for Experiments 1 and 3, dim scotophase illumination (<0.1 lux) via medium-wavelength LEDs.

Acknowledgements: Chapter 2 is currently being prepared for submission for publication. Rapid adjustment of circadian clocks to time zones across the globe. Harrison, Elizabeth; Gorman, Michael. The dissertation author was the primary investigator and author of this paper.
Figure 2.1. Sluggish re-entrainment observed under summer photoperiods is accelerated under winter and LDLD photoperiods. Representative double-plotted actograms (i.e., each horizontal line plots 48 h of data, and horizontal lines begin at 24 h intervals, so that all data are plotted doubly) of wheel-running activity in Syrian hamsters exposed to our novel assay. Gray shaded areas represent times of darkness. Clock times are plotted relative to British Standard Time (BST).
Figure 2.2. Radar plots of effective mismatch at progressive 3 day intervals (opposite). Each axis of the radar plot represents a shift of varying magnitude to a new LD 16:8 schedule, represented by white and black bars for periods of light and dark, respectively. The white hexagon in the center of each plot represents the “target” time of lights off in the new scotophase. Each colored polygon represents the absolute value of the average three-day deviation of the mean onset from the new lights off in hours. Animals in LDLD have a clear, immediate advantage.
Figure 2.3. **Effective mismatch in onsets days 1-3 after phase shifts.** Phase resetting was measured here as the absolute value of the deviation of the mean onset from the new lights off in hours, averaged over the first three days post-shift ± SEM. Shift magnitude was calculated relative to baseline lights off. For LDLD animals, this was lights off in the original 20:4 condition. Data are averaged over all shifts for each waveform. All conclusions in this paper are robust with respect to comparisons of multiple phase markers.
Figure 2.4. Effective mismatch in all phase markers days 1-3 after phase shifts in long days vs. short days. Phase resetting was measured here as the absolute value of the deviation of the mean onset from the new lights off in hours, averaged over the first three days post-shift ± SEM. Shift magnitude was calculated relative to baseline lights off. All conclusions in this paper are robust with respect to comparisons of multiple phase markers.
Figure 2.5. Effective mismatch in all phase markers days 1-3 after phase shifts in long days vs. bifurcation. Phase resetting was measured here as the absolute value of the deviation of the mean onset from the new lights off in hours, averaged over the first three days post-shift ± SEM. Shift magnitude was calculated relative to baseline lights off. For LDLD animals, this was lights off in the original 20:4 condition. All conclusions in this paper are robust with respect to comparisons of multiple phase markers.
Figure 2.6. Effective mismatch in all phase markers days 1-3 after phase shifts in bifurcation under an optimized protocol. Phase resetting was measured here as the absolute value of the deviation of the mean onset from the new lights off in hours, averaged over the first three days post-shift ± SEM. Shift magnitude was calculated relative to baseline lights off. For LDLD animals, this was lights off in the original 20:4 condition. All conclusions in this paper are robust with respect to comparisons of multiple phase markers.
Figure 2.7. Phase-shifting kinetics of animals that do not bifurcate under LDLD are comparable to those of long day controls. Actograms from Syrian hamsters housed under LDLD cycles with un-bifurcated activity rhythms illustrate that it is bifurcation, and not the LDLD cycle, that is responsible for enhanced phase-resetting responses. Un-bifurcated animals shift slowly, similarly to animals housed under LD.
Figure 2.8. The length of the activity rhythm, alpha, returns to an LD-like pattern rapidly after a change in waveform (opposite). Actograms from Syrian hamsters housed under LDLD cycles with un-bifurcated activity rhythms illustrate that it is bifurcation, and not the LDLD cycle, that is responsible for enhanced phase-resetting responses. Un-bifurcated animals shift slowly, similarly to animals housed under LD.
Figure 2.9. Only 3 LDLD cycles are required for rapid resetting to anti-phase schedules. Representative actograms illustrating wheel-running activity in constant darkness (DD) after simulated travel to one of two destinations 12 h in anti-phase. Actograms are plotted here across 48 h. When behavioral rhythms are unmasked in constant darkness (DD), it is revealed that animals are in fact able to entrain to light/dark schedules that are 12 h in anti-phase within 3 light/dark cycles. Furthermore, this rapid resetting can be accomplished with as few as 3 days of activity bifurcation prior to the shift in schedule. Phase was determined via a line fit to 7 days of free-running activity, skipping 3 days to allow for transients, then projected back to Day 1 of DD.
CHAPTER 3. Rapid re-entrainment of central and peripheral circadian clocks and reorganization of the periphery in bifurcation

INTRODUCTION

In Chapter 2, incredibly rapid resetting was observed in animals that had been bifurcated for 14 days, and then shifted to a new light/dark cycle for a period of two weeks. It is yet unknown, however, how many days of that non-bifurcated, LD cycle after a shift from a bifurcated entrainment state are required for pacemaker adjustment. In preliminary data from Syrian hamsters released into constant darkness (DD) four days after being challenged to shift from a bifurcated state to one of two un-bifurcated (LD) schedules 12 h in anti-phase, activity onset remained within ~1.5 h of lights off, indicating that the pacemaker was able to adjust to light schedules 12 h apart from each other within 4 days (Chapter 2). We sought to extend these data to mice, and to determine whether the previously-bifurcated clock could adjust in just one day.

In the first experiment, the number of days required for the pacemaker of bifurcated mice to entrain to a new LD cycle was assessed via a behavioral assay which is commonly employed to assess circadian phase: namely, activity onsets in constant darkness, or DD (Aschoff, 1979). Bifurcated animals were either released immediately into DD from one of the twice-daily scotophases, or they were shifted to one of two times zones, 12 h apart.
Shifted animals were released into DD after 1 or 3 full photocycles in the new time zone.

Given that bifurcation is a relatively new entrainment paradigm that has been studied largely at the behavioral level, little is known about the organization of the circadian system in this entrainment state. Two independent studies have found that Per1 expression oscillates in antiphase in the core and shell of the SCN under bifurcation (Watanabe et al., 2007; Yan et al., 2010). Watanabe and colleagues also examined the liver, and found that expression of Per1 and Per2 are bimodal, while Bmal1 remains unimodally rhythmic albeit at a reduced amplitude, and other clock genes including Cry1 were considered arrhythmic (2007). Based on these findings, the authors conclude that feeding behavior is likely responsible for the bimodality in hepatic Per gene expression. Here, in Experiment 2, we sought to extend this characterization of the organization of the peripheral circadian system under bifurcation to include other tissues (kidney and lung), other clock genes of interest (Cry2 and Clock), and further analyses of clock functionality and organization, including phase relationships and correlations between genes in each tissue. We further sought to examine re-entrainment in the peripheral circadian system, as after phase shifts the circadian system can become transiently disorganized, and the phases of individual tissues in the periphery can dissociate from the central clock, activity, and from each other. Thus, for
this experiment, mRNA expression levels across 24 h were determined in 6 core clock genes in three peripheral tissues in bifurcated and Long Day animals both in steady state conditions and on the third day following 12 h (LD) or antiphase (LDLD) shifts of the light/dark cycle.

Possible Outcomes:

Experiment 1

If only 3 days of a new photocycle is required for adjustment to one of two new time zones 12 h apart, then we would expect that activity onsets in DD would similarly be 12 h apart from one another (i.e., the phase of onset for animals that traveled to “Seattle” would be 12 h apart from the phase of those that traveled to “Moscow”). The same logic applies to those animals shifted after only one full photocycle. Further, we would expect that the phase would differ significantly from those of the animals that were moved immediately to DD from a bifurcated entrainment state. Follow-up studies can utilize the optimal number of days as a starting point to examine this paradigm under more challenging schedules (i.e., 4, 6 or 8 h phase shifts).

Experiment 2

It is of interest to characterize clock gene expression in the periphery in the bifurcated steady-state, as bifurcation induces a reorganization of the
circadian system, and it has been recommended as a potential entrainment state for shift-workers. It may be the case that peripheral clock gene expression is bimodal only in some genes, such as those that are acutely induced by feeding (Per genes), but not others. Similarly, we may find that the bimodal activity and/or feeding rhythms in bifurcation cause bimodality in gene expression in some tissues, but not in others. It may also be the case that the clock is disrupted under bifurcation, and genes that typically oscillate together in a predictable manner in the same organism (e.g., Clock and Bmal1) may become disassociated, or stop oscillating altogether. Thus, in addition to measures of rhythmicity and phase, the relationships between genes within subjects will be examined via correlation. Finally, it is possible that bifurcation may induce a reorganization that nonetheless preserves functionality. Most of these alternatives are not mutually exclusive, and a final possibility may be some combination of any of these outcomes.

A secondary goal of Experiment 2 was to characterize re-entrainment in the periphery in animals that have been previously bifurcated compared to long day controls. If mRNA levels in the peripheral tissues indicate that bifurcated animals can entrain to a new light-dark schedule within 72 h, as is suggested by activity data from prior studies, then the unprecedented resetting seen behaviorally in bifurcated animals does in fact reflect a cohesive shift in the entire circadian system. Further, by comparing the peripheral circadian
system of un-shifted bifurcated animals to shifted ones, we can examine the transition of the circadian system as it is moved from one waveform and entrainment state (LDLD) to the other (LD).

METHODS

General Methods

For both experiments, prior to baseline, C57BL/6J mice were housed in groups of 4-5 in polypropylene cages under a 14:10 LD cycle. At the start of each experiment, animals were moved to individual cages modified for height to accommodate running wheels, and this was done within 30 min of the time of lights off under the new light dark schedule. Throughout both protocols, animals received photophase illumination of ~100-200 lux, and dim scotopic illumination via ~500 nm LEDs at <0.01 lux. Locomotor activity rhythms were monitored with a Vitalview data collection system (Version 4.2, Minimitter, Bend OR) that compiled the number of half wheel revolutions into 6-minute bins. Throughout both experiments, food and water were available ad libitum. All experiments were done with the approval of the UCSD Institutional Animal Care and Use Committee.

Experiment 1

Experimental Design
30 male C57BL/6J mice aged 4-5 weeks were purchased from Jackson Laboratory (Sacramento, CA) (6 conditions, n=5/group). After 3 d of adaptation to the vivarium schedule (LD 14:10; lights off 8pm PST), animals were bifurcated under an LDLD 8:4:8:4 schedule, with introduction of the running wheel coinciding with the start of the new scotophase (lights off 8am PST). After two weeks in LDLD, mice were shifted to an LD 16:8 light cycle, either to Seattle (lights off 8pm PST) or Moscow (lights of 8am PST). The Seattle designation was given to the time zone consistent with lights off in LD prior to bifurcation (8pm), while the Moscow designation was given to the other (8am). The shift was achieved by skipping the previous scotophase on the day of the shift and extending the subsequent one (see Fig. 3.1). Half of the animals were kept in the new LD cycles for 3 days (3 full photocycles), and the other half for only 1 day, before being released into DD. Two further groups of animals were moved directly from LDLD to DD from the time of lights off in either Seattle or Moscow, without being shifted to a 16:8 schedule (Fig. 3.1).

Behavioral analysis

To analyze behavior in DD, free-running period was determined from least-squares regression lines to eyefit activity onsets in ClockLab (Actimetrics, Wilmette IL). Skipping three circadian cycles after release into DD to allow for transients, regression lines were fit to the estimated onsets of free-
running activity during the subsequent seven days (days 4-10 after release). From the fitted line, the projected phase of activity onset on the day of transfer to DD could be estimated by projecting backwards to the first cycle in DD to determine activity onset at the beginning of free-running conditions. Thus, the timing of the start of subjective night could be estimated and plotted for each mouse, and its phase compared with that of the environmental night. The Rayleigh test for non-uniformity of circular data was used to test for randomness of the distribution of these projected activity onsets. As we expected activity onset to occur somewhat close to the time of lights off, a complementary approximation of phase across groups was used by measuring the deviation of the projected line on the day of release from that value.

**Experiment 2**

Experimental Design

In Experiment 2, both males and female mice aged between 4-9 weeks were bred in-house from Jackson stock and used in approximately equal numbers ($n=138$; 24-30/group). One half of the animals in each group were placed on a reverse LD light/dark cycle for two weeks before baseline entrainment to facilitate later collection of tissues across multiple time points. Animals were then moved to individual cages affixed with running wheels at the beginning of
lights off (8am or 8pm PST), where they remained in either LD 16:8 or LDLD 8:4:8:4 for a minimum of two weeks. A subset of mice remained in LD (n=30) and LDLD (30), while the remaining mice underwent a simulated jet lag paradigm. As above, the Seattle designation was given to the time zone consistent with lights off in LD prior to bifurcation (8pm), while the Moscow designation was given to the other (8am). For the LD Moscow group (n=30), the 12 h shift in the LD cycle was accomplished by extending the photophase (see Fig. 3.2). The two LDLD groups, LDLD Seattle and LDLD Moscow (n=24 each), were shifted as described in Experiment 1 (see Fig. 3.2).

Behavioral analysis

Post-shift adjustment rates to Seattle or Moscow were assessed by calculating the absolute deviation in hours between the new lights off (8am or 8pm) and activity onsets on days 1-3 as determined via eyefit in ClockLab (Actimetrics, Wilmette IL). The absolute deviations were then averaged to yield one number per animal for days 1-3. Student’s t-tests with Welch’s df corrections for non-equal variances were conducted to determine group differences.

Tissue Sampling & q-RT-PCR
Kidney, liver, and lung samples were collected from 4-5 animals per group every 4 hours across 24 h (24-30 animals per group) on the third day of the new LD schedule. Tissue was collected immediately following cervical dislocation, placed on dry ice, and stored at -80 C until processed. Tissue was homogenized and RNA purified using an RNEasy Mini Kit (Qiagen). cDNA was synthesized using the Super Script III First-Strand Synthesis System for RT-PCR (Invitrogen). qPCR was performed on a CFX384 Real-Time Detection System with SYBR Green (Bio-Rad) according to the manufacturer’s instructions.

Raw mRNA expression levels were generated as an expression of the number of cycles required to reach the user-defined fluorescence level threshold of 500. Two determinations were made for each gene for each tissue sample. Where the difference between those determinations exceeded a value of 1 cycle, the determination that most closely represented the mean for that time point was included in the analysis (58/5796 determinations). Values were then normalized to expression levels for the same sample for the control gene Actb, using the formula \((2^{(#ActCycles)/2(#ProbeCycles)})\) (Pfaffl, 2001). Data were normalized via the X fold method, whereby the lowest mean value for each series was given the value 1. Two samples with expression levels > 3 s.d. from the mean in three genes in the lung of control animals were excluded from analysis for all genes in the lung.
Analysis of mRNA Expression levels/Statistics

Least-squares sine curves were fit to the normalized expression profiles of six clock genes (Cry1, Cry2, Per1, Per2, Clock and Bmal1) in each of the three peripheral tissues for each experimental group using GraphPad Prism (Version 5.0b, La Jolla, CA). Sine fits generated R² values, as well as estimated values and 95% confidence intervals for three parameters of interest: phase, amplitude, and baseline (mesor). Circadian rhythmicity of expression profiles was evaluated using the R² value and the degrees of freedom for each series. Chi-square analyses were performed on the number of rhythmic genes in each group across all three tissues. As LDLD data were frequently not well-described by parametric sine functions, non-parametric analysis of rhythmicity at 12 h was calculated for all series using JTK_CYCLE (Hughes, Hogenesch, & Kornacker, 2010). Five genes in LDLD and one in LDLD Seattle were found to be rhythmic at 12 h using this method, and a phase estimation in whole hours was derived from the fit. JTK-derived phases are utilized in this paper only in the general discussion of clock function and determination of ZT in LDLD animals, and are not included in any statistical analysis. For all other genes across groups, phase was derived from statistically significant sine fits, as a sine function is an appropriate fit for LD control data and we were interested in comparing deviations from this state caused by the re-entrainment paradigm across groups. Further, sine fits allow
for greater temporal resolution (on the order of 0.01 h) than that in JTK (1 h).

Extra-sums-of-squares $F$ tests on fit curves allowed for intra-group comparisons. Amplitude for each fit was calculated by summing the absolute value of fit-generated baseline and amplitude values. Extra-sums-of-squares $F$ tests were conducted on the phases and amplitudes of sine fits for each gene in a given tissue that was rhythmic in both LD and the comparison group. Chi-square analyses were conducted on the number of genes in each tissue per group that, based on sine-fits: 1) were rhythmic, and 2) differed from LD controls in phase, and 3) amplitude.

For comparison of group differences, sine-fit-derived amplitude was calculated as a percent of the LD control values in each gene for each tissue. In general, non-parametric tests were used to accommodate unequal variances across groups due to scaling or, in the case of phase comparisons, differences in sample size. Differences in amplitude and phase across groups were investigated using Kruskal-Wallis ANOVA with Wilcoxon signed-rank tests on paired comparisons. For a measure of phase in traveling groups relative to LD controls, we compared the absolute magnitude of the deviation from LD control phase for each gene in each tissue. In all LD 16:8 conditions, including the Seattle and Moscow scotophases in the re-entrainment groups, lights off was designated as ZT12 (Johnson, Elliott, & Foster, 2003). For LDLD mice, the lights off that coincided with lights off in their prior LD schedule (8pm,
“Seattle”) was similarly designated as ZT12. Pearson product-moment correlations were conducted on normalized gene expression values within each tissue for each group in order to compare clock function across groups, relative to LD controls. All statistical tests described above and all plotted data pertain to normalized (X-fold) data, with one exception. As we hypothesized that bifurcation may dampen rhythmic expression of some genes, raw (normalized only to Actb, not X-fold) expression levels were compared across groups, utilizing t-tests with Welch’s correction for unequal variance.

RESULTS

Experiment 1

Analysis of DD data and Rayleigh tests of vector plots indicated that after 3 full photocycles, activity onsets in DD were 12 h in antiphase: Moscow and Seattle vectors were at clock times 5:30 and 17:45, respectively. Mean vector lengths were 0.98 and 0.97, indicating a high level of clustering (both ps<0.01; Fig. 3.3A). The same analysis on activity from animals that only received one full photophase in the new location yielded vectors that were also significantly clustered, but were only ~7.5 hours apart (clock times 3:12 and 19:48, respectively; vector lengths 0.99 and 0.82, both ps<0.05). In animals put directly into DD from a bifurcated state, we observed a pattern similar to that seen with one day, though vectors were even more advanced relative to
time of release into DD (clock times 1:48 and 18:06 and vector lengths 0.90 and 0.77, both ps<0.05). With the exception of the single-photophase Seattle group, the phases of animals released into DD in all conditions on average phase-led, or were advanced relative to, dark onset. The advance of the mean vector time relative to lights off increased in a dose-dependent manner for Moscow (8am) groups, as did the absolute value of the deviation from lights off (Fig. 3.3B). In Moscow, all values, independent of group, were advanced by 1.5 h or more relative to 8am, whereas across Seattle groups, though the majority were advanced, 2 were delayed by 2 h and a further four were advanced by less than 1.5 h. There was also more variability in the timing of Seattle (average vector = 0.85 vs Moscow 0.96). Thus the manner in which the two oscillators respond in DD differs significantly, with the oscillator controlling the Moscow component of the bifurcated rhythm arguably a weaker oscillator, as indicated by the uniformity of phase within group in response to DD, and the differential effect of the number of days in the new photocycle (though the one-photocycle group did not differ statistically from the group that went straight into DD (p=0.17; Fig. 3.3B)). This advance relative to the old light-dark transition was also observed in hamsters (Chapter 2), a species with a longer endogenous period (~24.1), but was specific to the introduced, Moscow scotophase (Evans et al., 2011).
Experiment 2
LD v LDLD
Quantification of Rhythmicity

Figure 3.4 plots the daily rhythms in LD and LDLD mice of six canonical clock genes in kidney, liver and lung. Data are plotted as individual data points with lines representing the mean rhythms, with the sine fits for those same data plotted underneath. In LD controls, 17/18 genes (6 genes x 3 tissues) assayed were described well by a sine fit, and met criteria for rhythmicity at 24 h. *Cry1, Cry2, Per1, Per2,* and *Bmal1* were significantly rhythmic in all three tissues. In spite of being highly expressed in kidney, *Clock* was rhythmic only in liver and lung. In bifurcated (LDLD) mice, fewer genes (8/18; \( \chi^2(1) = 10.60, p<0.01; \) Table 3.1 & Fig. 3.5) met criteria for rhythmicity at 24 h based on goodness of sine fit: *Clock* and *Bmal1* in all three tissues, *Cry2* in kidney, and *Cry1* in liver. Neither *Per1* nor *Per2* were rhythmic in LDLD mice in any tissue at 24 h. Five genes were significantly rhythmic at 12 h as determined by JTK analysis: *Per1* in both kidney and liver, *Per2* in liver, *Cry1* in lung, and *Clock* in lung. Thus, combining both analyses, 12/18 genes in bifurcated mice were rhythmic, either at 12 h, 24 h, or both (*Clock* in lung). This is still statistically fewer than the 17/18 found in LD \( \chi^2(1) = 4.43, p<0.05 \). An ANOVA of R-squared values for all sine fits for all 18 genes revealed significant group differences \( F_{(4,85)}=10.87, p<0.0001 \). LD mice had higher R-squared values on
average than both LDLD (p<0.001) and LD Moscow (p<0.05) mice. LDLD mice likewise had lower values than all three re-entrainment groups (LD Moscow, p<0.05; LDLD Seattle, p<0.001; and LDLD Moscow, p<0.01). No differences were found between any of the re-entrainment groups (Fig. 3.6).

Phase determinations

Figure 3.7 is a graphic depiction of peak times of all genes found to be rhythmic both in LD and LDLD. Genes are color-coded by peak time in LD. Phase is plotted in angular coordinates. The slight variation in radial coordinates does not reflect any actual differences between data points and is plotted as unequal only for ease of viewing. In this figure only, data points include phase determinations of rhythmicity at 12 h via JTK_CYCLE. In LD mice, phase relationships between genes stayed relatively consistent across tissue type, with \textit{Per1} peaking just before or shortly after lights off, \textit{Per2} and the \textit{Cry} genes during the scotophase (though \textit{Cry2} peaked earlier in lung), and \textit{Clock} and \textit{Bmal1} shortly after lights on (Fig. 3.7). This phase relationship between genes is consistent with other reports of peripheral gene expression in mice under 12:12 (Liu, Cai, Sothern, Guan, & Chan, 2007; Yamamoto et al., 2004), and appears in spite of the shorter scotophase in our 16:8 manipulation (i.e., in 12:12, \textit{Bmal1} and \textit{Clock} peak before lights off, between CT 20-0, in liver, kidney, and lung). This indicates that animals under a 16:8 light/dark
schedule align their 24 h clock relative to lights off, rather than lights on (see discussion for more details). In all tissues, the peak phase of Cry2 did not differ statistically from that of Per1 and Per2.

In LDLD mice, as in LD mice, Clock and Bmal1 peaked together, around mid-scotophase (or ~2 h before lights on) in the “Seattle” 8pm scotophase. Further, just as in LD controls, peak times for Clock and Bmal1 within LDLD mice did not statistically differ (i.e., they cycled together in all tissues, with the same exception; all ps > 0.1). The only exception is the 12 h oscillation of Clock in the lung, which peaked in the middle of both scotophases (2 h before lights on in each case). Of course, due to the nature of the light treatment, Bmal1 and Clock peaked earlier in LDLD mice, though this difference was only statistically significant for Bmal1 in the lung (p<0.05).

While in LDLD Clock and Bmal1 peaked within a few hours of lights on, as in LD, due to the short nature of the scotophases (i.e., mid-scotophase is only two hours away from both lights on and off), it is difficult to say definitively that the phase in LDLD is equivalent to LD. The absolute value of the deviation, in ZT hours (ZT 12 = 8pm lights off in both groups), of each series from the phase of LD controls for the same series, summed for each group, yielded statistically significant differences between LD and LDLD groups for all genes that were rhythmic at 24 h in LDLD (Fig. 3.8A). When we examined JTK_CYCLE-derived phase for the bimodal genes, Per1 peaked near lights off
(0, 12) in both LDLD kidney and liver, similar to LD controls. Per2 in liver peaked at 4 and 16, which as in LD controls, is ~4 h after lights off and the peak of liver Per1. As in LD lung, Cry1 and Clock cycled together in LDLD mice in both liver, where it was unimodally expressed, and lung, where it was bimodally expressed. In liver, Cry1 peaked significantly earlier in LDLD compared to LD (~1.5 h before lights on for LD but just before lights off in LDLD). Also, in this and other studies, phase is similar across tissues for the same gene in LD mice. This appears to hold true, at least for Bmal1, Clock, and Per1, in LDLD mice (Fig. 3.7).

The phase relationship between the Per genes and the positive elements Clock and Bmal1 appears to be modified in LDLD. Both gene pairs peak relative to Scotophase 2, or Seattle (wherein lights off matches lights off from prior LD). The Per genes, however, are generally phase-locked to lights off (at least in kidney and liver), whereas Bmal1 and Clock are phase-locked to lights on for the same scotophase. Thus the clock appears to be tracking a 20:4 light cycle in some genes, and an 8:4:8:4 in others. An alternative is that the organism is interpreting the schedule as one long scotophase (from ZT0-16). The placement of Cry and Bmal1 could indicate that a long scotophase starts with Scotophase 1 (Moscow) and continues through to the end of Scotophase 2. However, the phase of Bmal1 is still slightly altered for this scenario to be accurate, and the peak phase of Cry1 is at a time that would
not be predicted by either scenario. Additionally, unpublished behavioral evidence from our lab indicates that interpretation of this schedule as a short day is unlikely, as rodents that do not bifurcate under LDLD tend to cluster activity around one 4 h scotophase, suggesting a signal of one extremely long day.

Amplitude & Raw Expression Levels

Figure 3.9 is a graphic depiction of the peak times as a function of amplitude for all genes found to be rhythmic in LD (thus Clock in kidney is excluded), with phase plotted in angular coordinates and amplitude in radial coordinates. For the LD group, amplitude is set to a value of 1 for all series, whereas in LDLD amplitude is plotted as a percent of LD, with values that meet or exceed those in LD given an amplitude value of 1. JTK_CYCLE 12 h phase determinations are not included in Figure 3.8. Comparison of amplitude (peak-to-trough height) utilizing the Extra-sums-of-squares F test in series that were significantly rhythmic in both groups revealed significantly lower amplitude in LDLD than LD in 7/7 (Table 3.1). LDLD mice had significantly higher Clock expression levels in lung compared to LD mice, and Clock was rhythmic in LDLD kidney whereas it was not in LD. There were no differences between the two groups in Cry2 and Per1 expression in lung. Figure 3.8B depicts the mean amplitudes for series that were significantly rhythmic in both
LD and LDLD. Comparison of amplitudes across groups yielded significant differences, with LDLD having significantly lower amplitude than LD mice across all tissues (Fig. 3.8B).

In addition to the amplitude, differences were found in non-normalized expression levels collapsed across time points and compared between groups: t-tests revealed LDLD mice had overall lower levels of kidney $\text{Cry1}$ and $\text{Cry2}$, as well as lung $\text{Per2}$ and $\text{Clock}$, compared to LD mice. By contrast, kidney $\text{Clock}$ expression was significantly higher in LDLD than LD (Table 3.1). There were no changes in liver, and overall, no consistent pattern of either reduced or increased expression levels in LDLD.

Correlations

Pearson product-moment correlations were conducted on normalized gene expression values within each tissue for each group in order to compare clock function in LD vs LDLD (Fig. 3.10). In typical, LD animals, certain clock genes within the same organism are related (i.e, $\text{Per1}$ negatively correlated with $\text{Bmal1}$, $\text{Clock}$ positively correlated with $\text{Bmal1}$). Relationships between cycling clock genes can be used as a marker of clock functionality (Bracci et al., 2014; Kennaway, Varcoe, & Mau, 2003; Oishi, Sakamoto, Okada, Nagase, & Ishida, 1998). If clock gene cycles are disordered or at a tonic level in LDLD, we would not expect to observe the same relationships that we do in LD. We
found that many relationships observed in LD controls were maintained in
LDLD (4/7 in kidney, 6/9 in liver, 6/9 in lung; blue-shaded boxes in Fig. 3.10).
Some group differences were observed (pink-shaded boxes). In some cases,
positive correlations were observed in LDLD that were not observed in LD, and
in other cases, gene pairs that were related in LD were not in LDLD. All
differences between these two groups were attributable to a lack of statistical
significance in one or the other, and in no case was there a statistically
significant relationship in the opposite direction. Thus, expected relationships
between genes are largely preserved in LDLD.

Re-entrainment groups

Activity

Representative actograms in Figure 3.2 depict extremely rapid
behavioral resetting (by the first or second day) of activity onsets in previously
bifurcated groups (LDLD Seattle and LDLD Moscow) compared to shifted LD
mice (LD Moscow group). Comparison of the absolute deviation of onsets from
lights off yielded significant differences between groups, with activity patterns
of previously-bifurcated mice occurring closer to the new lights off than in LD
mice shifted 12 h (Fig. 3.11). There was a trend for LDLD Seattle to be farther
away from the goal of lights off than LDLD Moscow, but it did not reach
significance (Welch’s t_{39} = 1.92; 1.75 +/- 0.41 for LDLD Seattle, 0.81 +/- 0.27
for LDLD Moscow; p=0.062). Activity data from both previously-bifurcated groups were thus combined. Previously-bifurcated mice shifted their behavior to within 1.5 h of the new lights off in the first 3 d after a shift, whereas onsets of control mice shifted 12 h were 7 h away from lights off (Fig. 3.11; Welch’s t(48) = 11.32; LD mean +/- SEM = 7.00 ± 0.44, N=30); LDLD mean (1.28 ± 0.25, N=48).

Quantification of Rhythmicity

Figures 3.12-14 plot the daily rhythms in both LD and each respective re-entrainment group of six canonical clock genes in kidney, liver and lung. Data are plotted as individual data points with lines representing the mean rhythms, with the sine fits for those same data plotted underneath. In the LD mice shifted 12 h (LD Moscow group), 15/18 genes assayed on the third day after the re-entrainment paradigm were described well by a sine fit, and met criteria for rhythmicity at 24 h (Table 3.1). This is not statistically different from the 17/18 found in LD ($\chi^2(1) = 1.13, p=0.29$; Fig. 3.5). Per2 in liver, and Cry2 and Per2 in lung were not significantly rhythmic. As in LDLD, but not LD, Clock was rhythmically expressed in the kidney.

In the two groups subjected to a jet-lag paradigm after two weeks of bifurcation (LDLD Seattle and LDLD Moscow), 15/18 genes were found to be rhythmic at 24 h in LDLD Seattle mice, and 16/18 in LDLD Moscow (Fig. 3.5
and Table 3.1). In LDLD Seattle mice, *Clock* in kidney, *Cry2* in liver, and *Per1* in lung were not considered rhythmic. As mentioned above, one gene (*Clock* in kidney) was found to be rhythmic at 12 h using JTK. In LDLD Moscow, *Cry2* was not found to be rhythmic in liver or lung (Table 3.1).

Restoration of waveform & phase

The percent of rhythmic series that had phases that were statistically different from those in LD did not differ between re-entrainment groups (Fig. 3.5; all *p* > 0.05). When the absolute deviation of peak phase from that of the respective control series was calculated, however, peak times of gene expression in peripheral tissues in previously-bifurcated mice was more aligned with LD than the LD group subjected to a 12 h shift (Fig. 3.8A; \( K = 16.06, p < 0.01 \)); paired Wilcoxon signed-rank tests revealed that both previously-bifurcated groups were closer to the target phase on average than both LDLD and LD Moscow animals (all *p* < 0.05).

In LD Moscow, several genes were found to be at a different phase from LD controls, though no differences for kidney were found, indicating kidney adjusted fastest in LD Moscow. In liver, *Per1* and *Cry* genes peaked earlier than LD controls (*Per2* was not significantly rhythmic). *Cry2* peaked almost 7 h earlier than LD controls. *Per1* was \(~3.5\) h and *Cry1* 2.5 h earlier than LD controls. In some cases, large confidence intervals (intra-group
variability and/or poor fit to a sine function) may interfere with determining how out of phase rhythms were (Clock in liver is 10.5 h out of phase with LD controls, but not statistically significant). Finally, in lung, Bmal1 was 3.17 h and Cry1 2.33 earlier than LD controls, respectively.

The profile of LDLD Seattle did not differ greatly from that of LD Moscow. A similar number of genes differed in phase, and peaked earlier, than LD controls. Unlike LD Moscow, phase differences in kidney were found, with Per2 and both Cry1 and Cry2 peaking significantly earlier than controls. Per2 and Cry1 also peaked earlier in the liver. Finally, Bmal1 peaked earlier in lung in LDLD Seattle compared to controls. Kidney Clock expression in LDLD Seattle (but not LD, LDLD or LDLD Moscow) was bimodal.

The profile of LDLD Seattle and LDLD Moscow groups differed markedly from one another. Under LDLD Moscow, only two genes were found to be out of phase with LD controls, and in both cases, they were found to be <2 h delayed relative to controls. Due to the altered phase of Bmal1 and Clock to lights off in LDLD and to the nature of the antiphase re-entrainment, to re-align phase of Bmal1 and Clock with LD, mice in the LDLD Moscow group had to advance ~3-4 h, whereas in LDLD Seattle they had to delay ~ 6-7 h. The Pers, while peaking at the same time(s) as LD, had to become unimodal, so phase differences between these two previously-bifurcated groups may reflect these changes.
Amplitude & Raw Expression Levels

The percent of rhythmic series that had amplitudes that were statistically different from those in LD did not differ between traveling groups (Fig. 3.5 & Table 3.1; all $p$ values $>0.05$). Comparison of amplitudes across groups yielded significant differences, with LD Moscow having significantly lower amplitude than previously-bifurcated mice across all tissues (Fig. 3.8B).

T-tests on non-normalized expression levels collapsed across time points and compared between groups revealed that expression levels of *Per1* and *Clock* in the kidney were higher in all re-entrainment groups relative to LD controls. There were few changes in liver in any of the groups, with lower expression of *Cry2* in LD Moscow mice, and higher levels of *Cry2* and the *Per* genes in LDLD Moscow, relative to controls (Table 3.1). In the lung, expression levels in the previously-bifurcated groups was lower than LD controls for all genes except *Per1*, whereas levels of *Per1* and *Cry2* in LD Moscow increased relative to controls.

Correlations

Figure 3.11 depicts Pearson product-moment correlations conducted on normalized gene expression values within each tissue for each group in order to compare clock function. As in LDLD, all differences between previously-bifurcated groups and LD controls were attributable to a lack of statistical
significance in one or the other, and in no case was there a statistically significant relationship in the opposite direction. By contrast, one opposite relationship was found in LD Moscow. Additionally, there were more light pink squares denoting a mismatch with LD controls in LD Moscow (25/45) compared to the previously-bifurcated groups (15/45 for LDLD Seattle, $\chi^2_{(1)} = 4.50$, $p<0.05$; 14/45 for LDLD Moscow, $\chi^2_{(1)} = 5.48$, $p<0.05$). Similarly, there was a trend for fewer blue squares, denoting relationships shared with LD controls, for LD Moscow, though this did not reach significance (11/25 for LD Moscow compared to 17/25 for both LDLD Seattle and LDLD Moscow; $\chi^2_{(1)} = 2.92$, $p>0.05$ for each comparison).

**DISCUSSION**

Behavioral evidence from past experiments (Chapter 2) indicated that, in previously-bifurcated animals, three full photocycles in a new time zone after a shift was sufficient for rapid behavioral adjustment. Here we examined: 1) whether on a behavioral level, only one photocycle is sufficient for rapid adjustment, 2) how the circadian reorganization that takes place under bifurcation affects clock gene expression in the periphery, and 3) whether clock gene expression in peripheral tissues reflects the rapid behavioral resetting observed in previous experiments.
In Experiment 1, we found evidence that while in previously-bifurcated animals, rapid behavioral re-entrainment occurs under a light/dark cycle as measured by activity onsets, one day in a new time zone is not sufficient for pacemaker entrainment as reflected in activity onsets in constant darkness (DD). We further discovered that while the rapid behavioral resetting observed after 3 d in the new time zone does in fact reflect that dissociated oscillators within the pacemaker are oscillating in anti-phase, the system phase-leads dark onset by approximately 2.5 h when it is released into DD. It is unknown whether this reflects underlying coupling mechanisms of oscillators that were kept apart under an LDLD light regimen but exert their effects in DD, drawing the two bouts together at an intermediate phase. We think this may be unlikely, given that behavioral onsets of bifurcated hamsters released directly into DD at opposite scotophases are phase-leading prior lights off at one time point (equivalent to “Moscow” in our experiment), but not at the opposite one (equivalent to “Seattle” here (Evans et al., 2011). Here we replicated this in mice, with activity phase-leading prior dark onset in the 0 day Moscow group more than the Seattle one. Exposure to three full photocycles in the new Moscow LD schedule reduced the difference between the two anti-phase groups, though both were still advanced relative to lights off. Whether this species difference reflects the shorter endogenous period of the mouse or some species-specific physiological change under bifurcation is not known.
Future studies should examine clock gene expression in the SCN itself, both under LDLD and in the days following release into DD as the system reorganizes into a unimodal behavioral pattern. Similarly, future studies should examine whether two days is sufficient for rapid re-entrainment.

Our primary goal for Experiment 2 was to characterize peripheral clock gene expression under steady-state bifurcated entrainment in bifurcated mice. Clock genes cycled as expected in LD controls, with expression levels of all genes peaking throughout subjective and environmental night (lights off 8pm PST). In bifurcated mice, we found evidence that clock gene expression in peripheral tissues is altered, with some genes oscillating every 24 h, and some every 12. We found no such bimodality in LD controls. These bimodal genes were not limited to early-inducible *Per* genes, but rather included *Cry1* and *Clock* in the lung. Further, we observed a reorganization of the relationships between peak times of clock genes under LDLD conditions. While it is not immediately clear which of the two scotophases should be interpreted as lights off in LDLD, the fact that several genes were unimodally expressed was able to provide some clues as to how the clock was tracking the light schedule. Expression times for most genes appeared clustered around one scotophase ("Seattle"; lights off 8pm PST), while genes that were expressed bimodally peaked throughout both scotophases.
Though bimodal *Per1* has been found in the SCN of bifurcated rodents (Watanabe et al., 2007; Yan et al., 2010), we do not know if the temporal patterns found here in peripheral tissues will extend to the SCN of bifurcated mice. Much of the work examining how the phase relationships of clock genes change as a function of changing light schedules has been done in the context of photoperiodism, and most of it in the SCN. In sheep SCN, *Per1* extended in long days, while *Bmal1* shortened (Lincoln, Messager, Andersson, & Hazlerigg, 2002) and Per 1, -2 and Cry1 tracked dawn (Hazlerigg, Ebling, & Johnston, 2005; Johnston, Ebling, & Hazlerigg, 2005). Similar results occurred in *Per1* and -2 and *Cry2* in hamster SCN (Carr et al., 2003), as well as in rat SCN, with *Per1* and *Cry1* onset and *Bmal1* decline phase-locked to time of lights on during a transition to short days (Sumová, Já, Sládek, Šauman, & Illnerová, 2003). The effect of the light schedule on the SCN involves interactions and reorganization of a complex network, which may not apply to the peripheral tissues. To our knowledge only a handful of experiments examine photoperiodic changes in clock gene expression in peripheral tissues, and even fewer examine genes other than *Per1* (Andersson, Johnston, Messager, Hazlerigg, & Lincoln, 2005; de la Iglesia, Meyer, & Schwartz, 2004; Messager, Ross, Barrett, & Morgan, 1999; Morgan et al., 1998; Nuesslein-Hildesheim, O’Brien, Ebling, Maywood, & Hastings, 2000).
Consistent with the SCN findings in mice and sheep reported above, in the livers of Syrian hamsters Per1 peaked near beginning of the scotophase in both photoperiods, while Bmal1 peaked near the end, though this did not meet criteria for rhythmicity in short days by their methodology (ANOVA; Maronde, Pfeer, Glass, & Stehle, 2007). Similarly, in the liver of sheep, the phase relationship between Per2 and Bmal1 was altered in long compared to short days (Andersson et al., 2005). Other evidence similarly suggests that in hamsters in both short and long days, Bmal in liver tracks lights off, while Per1 in the pars tuberalis tracks lights on (Maronde et al., 2007; Tournier et al., 2007). Thus Per and Bmal1 may track and “frame” subjective night under different light schedules, both in the SCN and the periphery. Our findings in this study indicate this is likely true in bifurcated mice, though Per genes in kidney and liver preceded both scotophases, while Bmal1 and Clock cycled at the end of the Seattle scotophase only. Thus, it appears that under bifurcation, some genes, such as Bmal1 and Clock (with the exception of bimodal Clock in lung) track the light signal as a long, 20:4 photocycle around the Seattle scotophase, whereas others interpret it as a 12 h day. Behavioral evidence from Experiment 1 and past experiments in our lab indicate that the two oscillators dissociated under bifurcation respond differentially to light input, and that activity in “Seattle,” the scotophase wherein lights off is phase-locked with the lights off from the prior scotophase, tends to reflect the stronger and
more stable oscillation (Evans et al., 2011; Chapter 3). This stronger oscillator may have thus remained phase-locked to lights off.

Thus it is largely unknown how the phase relationships between genes change with waveform, particularly in the periphery, and the changes thus far observed in the periphery under different photoperiods may be a function of acute light-induction of Per and/or photoperiodic physiological changes in the organism. One study speaks to this point directly: while gene expression in the SCN, heart, and lung of Syrian hamsters maintained under short days differs from that of animals housed under long days, in refractory hamsters wherein reproductive and endocrine systems revert to a long-day state, gene expression in the SCN follows the light/dark cycle (i.e. looks similar to hamsters housed in short days), whereas gene expression in the periphery follows the physiology (i.e., looks similar to hamsters housed in long days). Thus patterns of gene expression in the periphery of photoperiodic animals may not be a consequence of external coincidence, but rather more likely reflect internal coincidence. Support for this is also in Lincoln and colleagues’ findings wherein gene expression in the pars tuberalis of sheep appears to track melatonin and SCN the light dark schedule (Hazlerigg et al., 2005; Lincoln, Andersson, & Loudon, 2003), and in a study in mice wherein feedback from extended activity under short days is posited to be responsible for observed differences between SCN and liver in the rate and mechanism of
adjustment of several clock genes in the transition from long to short days (Sosniyenko, Parkanová, Illnerová, Sládek, & Sumová, 2010). In all five experiments, the photoperiodic response of \textit{Per} genes in the periphery seems not to differ from SCN, whereas other genes respond differently.

While peripheral oscillators typically receive neural or humoral signals from the SCN, they can also be readily entrained independently of SCN phase by changes in feeding schedules, hormonal signals (glucocorticoid receptor agonists), as well as daily fluctuations that simulate changes in body temperature (Balsalobre et al., 2000; Brown et al., 2002; Schibler et al., 2003; Vollmers et al., 2009). Under bifurcation, both melatonin and body temperature are bimodal (Raiewski et al., 2012; Rosenthal, Vakili, Evans, Elliott, & Gorman, 2005). The mice we used in our study, however, do not produce melatonin, so we cannot attribute the changes in the steady-state relationships between the \textit{Per} and \textit{Cry} genes observed here to the melatonin signal, as in Lincoln (2002). It is quite possible, however, that other humoral or behavioral signals are responsible. Perhaps of particular relevance to our study, Damiola and colleagues have reported food-induced entrainment to an antiphase schedule in SCN and periphery in multiple genes and tissues (Damiola, Minh, Preitner, Fleury-olela, & Schibler, 2000). In their study, entrainment of the periphery to a phase 12 h from that of the light schedule and SCN proceeded faster in liver
than other tissues, but phases in all tissues were similar after 1 wk of restricted feeding.

Preliminary data from our lab indicate that LDLD mice feed twice daily, during their active period (unpublished observations). It is thus possible that the clock gene rhythms observed in our LDLD mice are not driven by the SCN, but rather driven by the 12 h pattern of feeding, or activity (for example, indirectly via body temperature feedback). In the above-mentioned study, neither the lung nor the genes Clock and Bmal were examined. Thus we cannot directly compare our bimodal results in the lung of bifurcated mice. However, unlike the mice in Damiola’s study, our mice were not undergoing a restricted-feeding paradigm; that is, while it is plausible to argue that eating during the introduced scotophase (Moscow) is a novel time that may provide physiological input to the circadian system, the animals are in fact eating ad libitum, and this occurs bimodally, coinciding with both active periods (unpublished data). It is unknown based on their paradigm whether feeding at both a novel time (Moscow) and during their prior subjective night (Seattle) would induce a strong enough resetting signal (whether ad libitum feeding that occurs at both a novel and regular time can reset the clock). Two experiments wherein two daily bouts of restricted feeding occurred in antiphase had no effect on the timing of kidney or liver (Hirao et al., 2010; Kuroda et al., 2012).
While we have only preliminary evidence from SCN under bifurcation, we have additional behavioral evidence that the two activity bouts in bifurcated animals represent two subjective nights. Damiola and colleagues posit that the feeding-induced changes in temperature may shift the less resilient peripheral tissues, but preserve SCN phase. In their paradigm, however, restricted feeding during subjective day depresses body temperature. Under bifurcation, not only do bimodal activity patterns elevate body temperature, but the body temperature remains bimodal independent of activity (Rosenthal et al., 2005). It is nonetheless possible that the bimodal feeding pattern may still contribute to the bimodality seen in the *Per* genes in the liver, as feeding also acutely suppresses *Per1* and increases *Per2* in the liver such that *Per1* peaks right before feeding onset, and *Per2* after offset, even in mice without a functional clock (Vollmers et al., 2009).

Generally, amplitude of clock gene rhythms was markedly dampened in bifurcated animals, with the exception of *Clock* and *Per1*. This could either reflect that the clock is actually running with decreased amplitude, or due to the between-subjects design, that animals are desynchronized from one another in some, but not all, genes. We do not believe that these 12 h oscillations reflect two subpopulations with 24 h rhythms within our sample, oscillating in antiphase. The number of rhythmic genes, relationships to light transitions, and the synchronous peak times of *Bmal* and *Clock* across
tissues, all speak to the fact that there is likely clock organization, albeit somewhat altered, under bifurcation. Further, this likely occurs in most, if not all, animals. Thus the reduced amplitude is likely a reflection of the steady-state oscillation.

Does this imply that the clock is “broken” under bifurcation? In addition to the above findings, we also found consistent correlations between genes in the same tissue. Correlations have been used in other studies to determine clock function (Bracci et al., 2014; Kennaway et al., 2003; Oishi et al., 1998). While none of these things alone (rhythmicity, phase within a tissue, phase across tissues, correlations of gene expression levels within a tissue) indicate a functional clock, taken together, they seem to. Thus, we argue that the circadian system is intact, but re-organized. Therefore, in a steady state, different arrangements of cycling patterns may be possible. As the LDLD schedule inherently has twice as many light transitions and photophases and scotophases of shorter duration than a typical light/dark schedule, one might expect gene expression peaks to be quite brief in some instances. More frequent sampling (i.e. more time points) in future experiments may serve to better characterize the true shape of these rhythms.

Circadian dysrhythmia has been implicated in a number of metabolic disorders (for review, see Evans & Davidson, 2013). Though not yet formally examined, preliminary bodyweight data from animals entrained to LDLD cycles
does not indicate a negative effect of bifurcation on metabolism (unpublished data). Future experiments should screen for phenotypes based on disrupted metabolism, perhaps examining processes such as glucose metabolism. Similarly, we have preliminary data that suggest that feeding behaviors are not altered (i.e., occur during subjective night, albeit twice daily), reproduction is not impaired (unpublished), and memory is not impaired (Chapter 5).

In Experiment 2, we also sought to characterize resetting of peripheral oscillators in previously-bifurcated animals relative to LD controls. We observed a more rapid rate of behavioral resetting in animals shifted from a bifurcated state compared to LD controls after only two full photophases in the new time zone (Fig. 3.11), replicating and extending in mice our results from hamsters in Chapter 2. Further, we found indications in all measures that the organizational changes that take place in the peripheral circadian system in bifurcated animals are readily reversible and largely indistinguishable from LD controls after only two full days in the new light/dark cycle. While we did not measure expression rhythms over time, our observed rapid change in waveform just 3 days after the shift is in marked contrast to photoperiodic changes in the waveform of clock gene expression, which occur quite gradually, even in peripheral tissues (Sosniyenko et al., 2010). Finally, we found evidence that while there are some phase and amplitude differences in gene expression in peripheral tissues after a jet-lag manipulation, the
peripheral systems of previously-bifurcated mice were better adjusted to a new
time zone three days after the shift than LD counterparts (Fig. 3.8A).

It is not clear what the mechanism is for the enhanced re-entrainment
rates observed in bifurcated animals, nor is it clear whether it is driven by the
SCN and behavioral rhythms, or intrinsic to the clock gene cycle in the
peripheral tissues. The coordination of circadian rhythms in the periphery
occurs via multiple synchronization pathways (Kornmann, Schaad, Bujard,
Takahashi, & Schibler, 2007). Circadian oscillators in peripheral tissues have
an altered PRC, are responsive to phase resetting throughout the day
(Balsalobre et al., 2000), and re-entrain to shifts in the light/dark schedule at
different rates than the SCN (Davidson et al., 2009; Kiessling et al., 2010).

While we found a reduction in amplitude of most genes under LDLD, it
is unclear what the relationship is between clock gene expression amplitude
and rate of re-entrainment. Amplitude of clock gene expression is lower
following a phase shift (Davidson et al., 2009; Yamaguchi et al., 2013), though
this likely reflects desynchronization among individuals (Davidson et al., 2009),
rather than amplitude per se. There is evidence that a disrupted clock can
increase phase-shifting capability. Mice lacking vasopressin receptors adjust
more quickly (Yamaguchi et al., 2013), and mice homozygous for Cry1 and
Cry2 null alleles immediately adapt their behavior to new LD cycles (van der
Horst et al., 1999). Additionally, Clock mutants that have reduced amplitude of
Per1 and Per2 circadian expression (but not inducibility) have a higher-amplitude phase-resetting response (Vitaterna et al., 2006). When the reduction in amplitude is not caused by a dampening of rhythmicity, but rather by dissociation between SCN networks, reduced amplitude may increase phase-resetting responses: a report by Herzog & colleagues suggests that VIP causes the rhythms of individual cells to desynchronize, or “phase tumble,” resulting in enhanced phase-resetting responses (An et al., 2013). While this is predicted by limit-cycle theory, it is in contrast to findings regarding SCN network in short- and long-photoperiods, wherein phase coherence increases response to light (Meijer et al., 2010).

As alluded to above, evidence of the effects of different photoperiods on amplitude is also mixed, and most research focuses on the amplitude of network oscillations in SCN, and not on the amplitude of clock gene oscillations. Some groups have reported higher Per expression in SCN under short days (Lincoln, Messager, Andersson, & Hazlerigg, 2002; Steinlechner et al., 2002; Sumová et al., 2003), whereas others have reported lower (Messager et al., 1999). While we did not measure clock gene expression in the periphery under SD conditions, we did observe reduced amplitude of expression in peripheral tissues under bifurcation relative to LD controls. Other experiments have showed reduced amplitude in peripheral gene expression rhythms under short-day manipulations (Andersson et al., 2005; Carr et al.,
Importantly, while *Per1* expression is higher in the pars tuberalis under long days, in SCN, it is higher under short days (Lincoln et al., 2002). Thus while the implications of reduced amplitude remain unknown, they may differ as a function of tissue (and perhaps entrainment signal). Consistent with those bi-directional effects, lower amplitude in hamster *Per, Cry, Clock* and *Bmal1* gene expression was found in liver under short days (Maronde et al., 2007). Finally, in an experiment that looked at both SCN and peripheral tissues in photo-refractory hamsters, Carr and colleagues found that the reduced amplitude in peripheral gene expression observed under short days disappears when the hamsters revert to a long-day physiology under short day lighting conditions (Carr et al., 2003). Thus, the effects of waveform change observed in SD animals may reflect physiology, rather than properties of the pacemaker, not only for phase (discussed above), but also for amplitude (2003). How these effects generalize to animals with a bifurcated waveform remains to be seen. As suggested above, it may be of interest to obtain clock gene expression profiles in a variant of the experiments conducted in Chapter 2, both in SCN and in the periphery.

We observed characteristically slow behavioral resetting in the LD Moscow group, but surprisingly few statistically significant differences in phase of peripheral clock gene expression relative to controls. Other studies have
shown slower resetting in the periphery than we observed (Davidson et al., 2009; Kiessling et al., 2010), though the time-course design of those studies offers a higher resolution than we have here (i.e., we are unable to observe re-entrainment kinetics). In the LD Moscow group, Cry2 peaked almost 7 h earlier in liver than LD controls. Reddy et al found that SCN Cry2 tended to move more slowly than Per, and in conjunction with activity rhythms (2002). We found on average, behavioral rhythms in LD Moscow group over the first three days after the shift were 7 hr from time of lights off. We cannot rule out the possibility that either the dim light or the 16:8 manipulation employed here affected rates of resetting in all groups. Dim light no brighter than starlight or moonlight has been shown to alter the coupling mechanisms under manipulations of waveform and phase (Evans et al., 2007; Evans, Elliott, & Gorman, 2009). Further, gradual twilight light/dark transitions have been shown to modify phase relationships of Per gene expression in both different cell populations in the SCN and relative to the dark onset in different photoperiods (Sosniyenko, Hut, Daan, & Sumová, 2009).

The difference in phase relationships between the two travelling LDLD groups and LD controls indicates that they “travel” in opposite directions, i.e., the two bouts are not equivalent in terms of jet lag adjustment, at least in two peripheral tissues. The fact that LDLD Moscow appeared to entrain more quickly than LDLD Seattle is interesting, given that the light/dark transition to
the “Seattle” scotophase occurs at the time of prior lights off. One would expect, then, that unimodal components of the clock, such as \textit{Bmal}, would operate similarly to LD controls (i.e., reset slowly), but that does not seem to be the case. These data are consistent, however, with our behavioral data wherein the Moscow oscillator appears to be weaker and more easily perturbed (Chapter 2; Evans et al., 2011). LDLD Moscow also appeared to behave more like previous reports of gene expression in LD phase-shifted mice, in that \textit{Bmal1} was the last gene to adjust. The profile of relative phase in LDLD Seattle differed largely from that observed in LDLD Moscow. While \textit{Per2} was found to be fastest in other papers, particularly in the liver (Kiessling et al., 2010), it was farthest out of phase for LDLD Seattle in both liver and kidney, in spite of being bimodal under LDLD. \textit{Cry2} in LDLD Seattle kidney was 2.5 h earlier than LD controls. These altered patterns of re-entrainment in previously-bifurcated mice, both relative to LD controls and to one another, are most certainly a product of the altered peripheral organization observed under bifurcated steady-state conditions.

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Figure 3.1. Experiment 1 representative actograms and experimental design. Actograms are double-plotted, with 48 h on the X axis and days on the Y axis. Black marks indicate wheel-running activity, and scotophases are indicated by gray shading. Bifurcation was maintained for 14 days before mice “traveled” to one of two LD cycles (16:8) representing time-zones 12 h apart (Seattle or Moscow; n=5/group). The Seattle designation was given to the time zone consistent with lights off in 20:4 (8pm), while Moscow designation was given to the other (8am). After 3 or 1 full photocycles in the new time zone, animals were placed in constant darkness (DD) to determine their phase of entrainment to the new light/dark schedule. Two further groups of animals were moved directly from LDLD to DD from the time of lights off in either Seattle or Moscow (n=5/group).
Figure 3.2. Experiment 2 representative actograms and experimental design. Actograms are double-plotted, with 48 h on the X axis and days on the Y axis. Black marks indicate wheel-running activity, and scotophases are indicated by gray shading. Non-shifted animals (LD and LDLD) were entrained for a minimum of two weeks before tissue samples were collected (4-5/time point) at time points 4 h apart beginning 2 h after lights off, indicated by green stars. For shifted groups (LD Moscow, LDLD Seattle, and LDLD Moscow) samples were collected on the third day following the shift to the new light/dark cycle.
Figure 3.3. Behavioral results from Experiment 1 indicate that previously-bifurcated mice entrain to antiphase time zones in 3, but not 1, day (opposite).

Individual data points and mean vectors for projected phase on Day 1 of DD. Numbers around the outside of the plot denote clock time. Time of release into DD was at prior lights off for each group (8pm for Seattle, 8am for Moscow). The pacemaker of bifurcated mice can be shifted 12 h within 3 light/dark cycles: Projected phase of activity in DD is 11.6 h apart for mice that “traveled” to Seattle and Moscow for 3 days, but only ~7.5 h apart for those that traveled for 1 or for those that did not travel. Symbols for the Seattle groups are black and whereas Moscow groups are gray. Triangles, diamonds and circles denote 0, 1 and 3 days in the new time zone, respectively. Vector lines are dotted for the 0 groups, dashed for the 1 day groups, and solid for the 3 day groups.
Figure 3.4. Clock gene mRNA expression in peripheral tissues of bifurcated mice. Raw expression levels and sine-fits from normalized (X-fold) data are plotted for each clock gene assayed in each of three peripheral tissues: kidney, liver and lung ($n=4$-5 animals/time point). For mean graphs, each circle represents a single sample, and lines connect means for each time point. Solid lines are used for LD controls, and dashed for LDLD. For sine plots, the solid gray line represents the best fit for LD controls. Solid colors are used for LDLD fits, with confidence intervals indicated by black dashed lines. For all graphs, gray shading indicates the scotophase for LD controls (16:8). Black bars indicate the two scotophases for the animals in LDLD (8:4:8:4). For LD, the light–dark transition was designated as CT12. For LDLD, the light-dark transition that coincided with the light-dark transition from prior LD cycle was designated as ZT12 (see text for further details). In general, rhythms appear to be dampened and/or bimodal under bifurcation.
Figure 3.5. Chi-square analysis of peripheral rhythmicity. Fewer clock genes in the peripheral tissues of bifurcated mice (LDLD group) are rhythmic at a 24 h period relative to other groups ($\chi^2(4) = 18.47$, $p<0.001$). Two days after a shift to one of two LD times zones 12 h apart, the number of rhythmic genes in the periphery of previously-bifurcated mice did not differ from those of LD controls (*$p$ value <0.05; and ** <0.01). Shown are the percent of all assayed series considered rhythmic by group (18 were assayed: 6 genes each in kidney, liver and lung).
Figure 3.6. R-squared values for sine fits to peripheral clock gene expression profiles across groups. R-squared values for all sine fits for all 18 genes (6 genes x 3 tissues). An ANOVA revealed significant group differences ($F_{(4,85)} = 10.87$, $p < 0.0001$). Significance values are for Bonferroni-adjusted comparisons; * denotes a $p$ value $<$0.05; ** $<$0.01; and *** $<$0.001). LD mice had higher R-squared values on average than both LDLD and LD Moscow mice. LDLD mice likewise had lower values than all three re-entrainment groups (LD Moscow, LDLD Seattle, and LDLD Moscow). No differences were found between any of the re-entrainment groups.
Figure 3.7. Visual representation of phase of peripheral mRNA expression in long day and bifurcated mice. Polar plots of the phase of mRNA expression of rhythmic clock genes assayed in each of the three peripheral tissues, with phase represented in angular coordinates. Phase is plotted in *Zeitgeber* time (ZT) for all animals (see text for estimation of ZT in LDLD mice), and includes genes that were rhythmic both by sine fit and JTK_CYCLE analysis. The slight variation in radial coordinates does not reflect any actual differences between data points and is plotted as unequal only for ease of viewing. Genes are color-coded by phase of peak time in LD. The light/dark schedule is represented by shading in the circle on the outside of the plot. Dark is represented as gray, as all animals received dim (<0.01 lux) illumination throughout the scotophase.
Figure 3.8. Phase and amplitude of peripheral gene expression across groups.

Clock gene expression in the peripheral tissues of bifurcated mice shifted to time zones twelve hours apart (LDLD S & LDLD M groups) adjusted to the shift more rapidly than long day animals shifted 12 h (LD M group). Further, both phase and amplitude were significantly different from that of bifurcated mice that were not shifted (LDLD group). For both A and B, values were derived from sine-fits, and only values from series with significant rhythmicity at 24 h in both LD controls and each respective series are considered (LDLD, \( n = 7 \); LD M, \( n = 14 \); LDLD S, \( n = 15 \); LDLD M, \( n = 15 \)). Due to uneven sample sizes, comparison of phase and amplitude relative to LD controls was carried out using Kruskal-Wallis ANOVA with post-hoc non-parametric Wilcoxon signed-rank tests on paired comparisons; * denotes a \( p \) value <0.05; ** <0.01; and *** <0.001). A) The absolute value of the deviation, in hours, of each series from the phase of LD controls for the same series, summed for each group (\( K = 16.06, p<0.01 \)). LDLD S and LDLD M groups have phases significantly closer to that of the LD group than either the LD shifted group (LD M) or bifurcated non-shifted mice (LDLD). B) Amplitude was calculated as a percent of LD control amplitude for each series, and summed for each group (\( K = 16.06, p<0.01 \)). Rhythms from LDLD S and LDLD M groups have significantly higher amplitudes than both the LD shifted group (LD M) and bifurcated non-shifted mice (LDLD).
Figure 3.9. Visual representation of peripheral mRNA expression relative to controls (opposite). Polar plots of the phase of mRNA expression of each clock gene assayed in each of the three peripheral tissues, with amplitude represented in radial coordinates and phase in angular coordinates. Genes are color-coded by phase of peak time in LD. The light/dark schedule is represented by shading in the circle on the outside of the plot. Dark is represented as gray, as all animals received dim (<0.01 lux) illumination throughout the scotophase. Phase is plotted in zeitgeber time (ZT) for all animals (see text for estimation of ZT in LDLD mice). For amplitude, LD values were fixed at 1, and values for all other groups are represented as a percentage of the LD value for that series. Where values for experimental groups met or exceeded LD values they are represented as 1. *Clock* is not shown for LD Kidney, as it was not found to be rhythmic. For purposes of visual comparison only, all values from other groups are plotted, including values from series that were not found to be rhythmic. While phase cannot be determined accurately in the absence of rhythmicity, non-rhythmic values plotted as a function of LD amplitude should be close to 0 and therefore provide an illustration of changes in clock function. For all statistical analyses across groups in this paper, non-rhythmic values were not considered. Rhythmicity in LDLD is markedly dampened, and phase relationships between genes within each tissue altered. For the three groups subjected to a phase shift, clock function is likewise altered, though it appears to be less so in mice that had been bifurcated before the shift (LDLD S and LDLD M groups) compared to mice that had not (LD M group). Lung appears to be the tissue that is farthest from adjusted in all shifted groups.
Figure 3.10. Correlations between clock genes in the periphery of long day, bifurcated, and re-entraining mice (opposite). Pearson product-moment correlations were derived for normalized (X-fold) expression levels between genes in the same tissue for each group. A “+” and “-” indicates a significant positive and negative correlation, respectively. The intersection of genes that were not statistically significant were left blank. For the other groups, shading indicates the consistency of the intra-group relationships with that of LD controls. Boxes are shaded in blue if the relationship found is the same, light pink if a relationship was found either only in LD or only in the comparison group, and dark pink if a relationship was found in the comparison group that was in the opposite direction of that found in LD. Many between-gene relationships found in LD are also present in LDLD. Across all tissues in the three traveling groups, LDLD S and LDLD M groups have more blue and fewer pink squares than LD M, particularly in the liver. LD M is the only one with a relationship opposite to that found in LD.
Figure 3.11. Activity onsets in long day and bifurcated mice after a phase shift.
The absolute value of the deviation, in hours, of activity onsets for each animals over
the first 3 days after the phase shift. Deviations from LD animals shifted 12 h (LD
Moscow) are represented by the black bar. Data were collapsed across both groups
that shifted from LDLD to an LD time zone 12 h apart (LDLD Seattle or LDLD
Moscow) and are represented by the gray bar. Activity onsets for LD animals shifted
12 h (Moscow) are >5x farther from the time of lights off than those for LDLD animals
shifted to one of two times zones 12 h apart (Seattle or Moscow).
Figure 3.12. Clock gene mRNA expression in peripheral tissues 3 d post-shift: LD Moscow group (12 h shift to lights off 8am). Raw expression levels and sine-fits from normalized (X-fold) data are plotted for each clock gene assayed in each of three peripheral tissues: kidney, liver and lung (n=4-5 animals/time point) three days post-shift. For mean graphs, each circle represents a single sample, and lines connect means for each time point. Solid lines are used for LD controls, and dashed for LD Moscow. For sine plots, the solid gray line represents the best fit for LD controls. Solid colors are used for LD Moscow fits, with confidence intervals indicated by black dashed lines. For all graphs, gray shading indicates the scotophase, both for LD non-shifted controls (16:8) and for the new time zone ("Moscow").
Figure 3.13. Clock gene mRNA expression in peripheral tissues 3 d post-shift: LDLD Seattle group (shift from LDLD to LD lights off 8pm). Raw expression levels and sine-fits from normalized (X-fold) data are plotted for each clock gene assayed in each of three peripheral tissues: kidney, liver and lung (n=4 animals/time point) three days post-shift. For mean graphs, each circle represents a single sample, and lines connect means for each time point. Solid lines are used for LD controls, and dashed for LDLD Seattle. For sine plots, the solid gray line represents the best fit for LD controls. Solid colors are used for LDLD Seattle fits, with confidence intervals indicated by black dashed lines. For all graphs, gray shading indicates the scotophase, both for LD non-shifted controls (16:8) and for the new time zone (“Seattle”).
Figure 3.14. Clock gene mRNA expression in peripheral tissues 3 d post-shift: LDLD Moscow group (shift from LDLD to LD lights off 8am). Raw expression levels and sine-fits from normalized (X-fold) data are plotted for each clock gene assayed in each of three peripheral tissues: kidney, liver and lung (n=4 animals/time point) three days post-shift. For mean graphs, each circle represents a single sample, and lines connect means for each time point. Solid lines are used for LD controls, and dashed for LDLD Moscow. For sine plots, the solid gray line represents the best fit for LD controls. Solid colors are used for LDLD Moscow fits, with confidence intervals indicated by black dashed lines. For all graphs, gray shading indicates the scotophase, both for LD non-shifted controls (16:8) and for the new time zone “Moscow”).
Table 3.1. Rhythmicity, phase, amplitude and expression of clock genes in the periphery of experimental groups relative to controls. Comparison of clock gene expression in peripheral tissues for each group, relative to LD controls. For rhythmicity, a ✔ indicates that a given gene was found to be statistically rhythmic at 24 h via sine-fit, whereas an “n.s.” indicates that no circadian rhythm was detected. For phase, arrows indicate a significant difference was found using the Extra-sums-of-squares F-test on sine fits, with arrows pointing to the left and right indicating an earlier and later peak phase, respectively, relative to LD controls, whereas “n.s” indicates no statistical differences were found between the phases. For amplitude, similar conventions were followed, with an up and down arrow indicating higher and lower amplitudes, respectively. Finally, for expression levels, non-normalized values were considered, relative to controls, and compared using paired t-tests. Of note is that the patterns observed for LDLD S and LDLD M appear to differ from those of LDLD, and that the phase relationship between LD controls and LDLD S (earlier) and is opposite that with LDLD Moscow (later).

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CHAPTER 4. Maintenance of bifurcation throughout a simulated rotating shift schedule in mice.

INTRODUCTION

If shift-workers were better able to tolerate the regular inversion of subjective night and day by actually entraining to the imposed work schedule, many of the harms associated with shift-work would presumably be mitigated. Recently, bifurcation of the circadian waveform has been suggested as a potentially useful entrainment state for night- and rotating shiftworkers due to the enhanced resetting rate seen under bifurcation, along with the robustness of the rhythm against changes in schedule and the coincidence of periods of subjective day with work hours (Harrison & Gorman, 2012). Therefore, rather than using a jet-lag paradigm to facilitate phase-shifting from a bifurcated to long day state (Chapters 2 & 3), here we employed a more practical series of phase-shifts simulating a rotating shiftwork schedule to determine if bifurcation can be maintained under a more applied protocol. Using a simulated “Dupont” schedule, common in U.S. manufacturing, we subjected both long day (LD) and bifurcated (LDLD) mice to a series of simulated rotating 12 h day and night shifts. To determine the ideal protocol for shifting bifurcated rhythms, LDLD mice were divided further into three groups that differed only in the timing of the scotophases on “days off” between shifts. One bifurcated group
remained in a variant of a 12:4:4:4 schedule for the whole protocol (Phase group), a second group was subjected to more frequent shifts of a smaller magnitude (Step group), and a third was shifted into an antiphase, 8:4:8:4 schedule where possible (i.e., on “days off”).

Possible Outcomes

Given the enhanced behavioral phase-resetting responses observed in bifurcated animals in Chapters 2 & 3, we anticipated that animals under shifting LDLD conditions may be able to adjust to 12 h shifts rapidly, faster than LD counterparts. Conversely, a simulated rotating 12 h shift necessitates a 12 h photophase. Therefore, under this paradigm, on “work days,” the bifurcated system is by necessity on a 12:4:4:4 light/dark cycle. As described above, the activity patterns of bifurcated animals are robust against brief perturbations of the light/dark schedule, but coupling forces exert themselves in the absence of all light cues (Chapter 3; Evans et al., 2011). We therefore hypothesized that the regular and rapid phase-shifts in this protocol, particularly in addition to the short length (4 h) of one photophase for the majority of the manipulation (i.e., on the 12 h “work days”), may result in behavioral rejoining of the two bouts of activity. This rejoining would then be followed by either an entrained or a non-entrained, free-running unimodal behavioral pattern.
Between bifurcated groups, if entrainment is maintained, we anticipated that there may be differences in resetting rates, as well as in the symmetry of the behavior in each of the scotophases. Generally, we might expect exposure to fewer schedule changes, longer photophases, and/or antiphase scotophases where possible within the protocol’s simulated “days off” to predict which groups are more successful at maintaining symmetrical bifurcation throughout the protocol (Anti and Phase groups). Conversely, more gradual shifts, rather than abrupt ones, have been recommended for human rotating shift-workers (Eastman, 1990). Thus, we might expect mice that are shifted more gradually on “days off”, in a series of 2 h “steps” rather than abrupt 4 h shifts of each scotophase, to maintain greater stability under the shifting LDLD schedules (Step group).

METHODS

Protocol

48 male C57/B6 mice 4-5 weeks of age were individually housed in propylene cages affixed with running wheels. Lighting in the photophase was provided by white tube fluorescent lights providing illumination intensity ranging from 30-100 lux inside individual cages. To facilitate bifurcation, scotophases for all groups were dimly illuminated by green LEDs at an intensity of <0.1 lux (cite). Animals were exposed to a either a 16:8 LD (n=6) or
a 8:4:8:4 LDLD (n=18) cycle for 4 weeks, after which they were moved to a simulated “DuPont” rotating 12 h work shift, with nights and days on alternating weeks for three weeks with one final week off (Fig. 4.1). The Dupont schedule as employed in U.S. manufacturing typically consists of three or four alternations between day (e.g., 8am-8pm) and night (8pm-8am) shifts across a month, with one to three days off between transitions (Circadian 24/7 Workforce Solutions, 2014). During the four-week “work schedule” we employed in this protocol, scotophases under LD conditions were selected based upon self-reported behaviors of typical shift-workers (i.e., maintaining nocturnal sleep after an afternoon shift, and sleeping upon returning home after a night shift) (Akerstedt, 1998). The three different LDLD groups (n=6 per group) were exposed to slightly different light schedules on days off in an effort to determine the optimal protocol for maintenance of bifurcation under shifting schedules. The three LDLD groups were as follows: 1) in the “Anti” group, scotophases occurred 12 h in antiphase wherever possible; 2) in the “Phase” group, the twice-daily scotophases remained consistently in some form of 12:4:4:4; and 3) in the “Step” group, the transitions between schedules took place in the form of more gradual 2-hr, rather than abrupt 4-hr, shift. While mice are a nocturnal rodent, and are thus active during the scotophases, or dark periods, for all conditions in this experiment, the 12 h “work” schedule occurred during the photophase, as it would occur in diurnal humans. All
experiments were conducted in compliance with the rules and regulations of the Institutional Animal Care and Use Committee, University of California, San Diego.

**Activity Analysis**

Activity data were collected using Vital View software and analyzed using Clocklab software (Actimetrics). Because of the variability in the given schedules, activity data were analyzed for quality of entrainment on two measures: 1) the phase relationship between lights off and activity onset, and 2) the percent of total daily activity that occurred in the photophases. For activity onset analysis, we employed a variant of the method described in Chapters 2 & 3; as the number of shifts varied between groups, an absolute deviation from the new lights off was calculated for each animal for Days 1-3 after each phase shift, and then averaged for each animal over the length of the protocol. Percent of all activity in the photophases was similarly calculated for Days 1-3 after each phase shift. These two measures were considered a means of quantifying *acute* disruption. The same two values were also calculated for all 28 days of the protocol, as a measure of *chronic* disruption. For all other measures, including those described below, data from all 28 days of the shifting protocol were used.
For the purposes of comparing entrainment across schedules that were constantly changing and differed across groups, activity was converted into 32 h actogram records via insertion of 8 h of null data points into each 24 h day. This served to align scotophases for direct comparison of the symmetry of the two bouts of activity between groups (Fig. 4.3). For the Anti group, 32 hrs of wheel running activity (24 h of actual data) were removed from the record before the analyses were run, as the animals had atypical activity during this period due to a light failure during the experiment. In addition to the measures described above, for comparison among the bifurcated groups three measures of the symmetry of the bifurcated entrainment state were calculated. First, a Bifurcation Index (BI) was generated from the ratio of Lomb-Scargle periodogram values for the aligned actograms at 16 over those for 16 and 32 combined (Bifurcation Index = 16/16+32). Higher values indicate a more symmetric pattern of entrainment (i.e., the waveform of the behavior in the first scotophase compared to the behavior in the second). Second, asymmetry of activity in both photophases was defined as the ratio of activity in the scotophase with higher counts over the lower minus 1; asymmetry of scotophase activity was calculated similarly (Asymmetry Index = (higher/lower)-1). Higher values thus indicate a more asymmetric pattern of bifurcated entrainment. Group comparisons were done in GraphPad Prism.
using ANOVAs with Bonferroni-adjusted t-tests for post-hoc comparisons. All tests were evaluated at the $p < 0.05$ significance level.

**RESULTS**

**Long Day and Bifurcated entrainment during shifting schedules**

Visual inspection of the actograms indicated that animals in all groups entrained remarkably well to the varied schedules, with the majority of activity constrained to scotophases. Analysis of the deviation of onsets in hours on Days 1-3 post-shift, averaged over all shifts, indicated that Long Day animals were farther from entraining to local time than all Bifurcated groups both in the acute measure ($F_{(3,20)} = 20.90$, $p < 0.0001$, all Bonferroni-adjusted $ps < 0.0001$; Fig. 4.4A), and in the chronic measure, though the magnitude of the effect was much smaller ($F_{(3,20)} = 3.18$, $p < 0.05$, all Bonferroni-adjusted $ps < ns$; un-corrected $t$-tests yielded significant differences between all bifurcated groups and the LD controls groups at $p<0.05$; Fig. 4.4A). The percent of total activity in the photophase, however, did not differ between any of the groups, either acutely ($F_{(3,20)} = 3.37$, $p = 0.06$; Fig. 4.4C) or chronically ($F_{(3,20)} = 0.91$, $p = 0.45$; Fig. 4.4D).

**Three LDLD protocols for maintenance of bifurcation during shifting schedules**
The photophase asymmetry index differed across LDLD groups ($F_{(2,15)} = 4.18$, $p < 0.05$; Fig. 4.5A), with higher values for the Phase group than the Step group (Bonferroni-adjusted $p < 0.05$). A similar pattern was observed for the scotophase asymmetry index ($F_{(2,15)} = 4.79$, $p < 0.05$, Bonferroni-adjusted $p < 0.05$; Fig. 4.5B). The same pattern of more stable bifurcated entrainment in the Step group was also observed in the Bifurcation Index (BI): ($F_{(2,15)} = 3.82$, $p < 0.05$, Bonferroni-adjusted $p < 0.05$; Fig. 4.5C). Collapsed across all groups, BI during the 28 days of the shifting protocol was negatively correlated with both the acute ($Pearson’s r = -0.58$, $p < 0.05$; Fig. 4.5D) and the chronic onset deviation measure ($Pearson’s r = -0.47$, $p < 0.05$; not shown).

**DISCUSSION**

Bifurcation has the ability to enhance behavioral phase-resetting responses, not only when animals are shifted from LDLD to LD, as in Chapters 2 and 3, but when bifurcation is maintained throughout multiple phase shifts (up to 11, in the case of the Step group). While we observed a difference between re-entrainment in LD animals and LDLD animals based on the phase of activity onsets relative to the shifting light/dark cycle (Fig. 4.4A & B), no such difference was observed by the measure of percent of activity in the photophases (Fig. 4.4C & D).
We observed here that bifurcation appears to also confer a phase-resetting advantage over long days controls, even when mice are shifted from one LDLD cycle to another (as compared to shifted from LDLD to LD, as in Chapters 2 and 3). It is unknown precisely why bifurcation enhances phase-resetting. One hypothetical mechanism for the enhanced rate of resetting observed in bifurcated animals shifted from an LDLD cycle to a LD cycle (Chapters 2 & 3) is the strong coupling effects exerted by the dissociated oscillators in the bifurcated pacemaker. Our evidence here, however, suggests that this is not the case.

Before this study, it was unknown to what extent the bifurcated state could be maintained while being repeatedly shifted over a long period of time. Prior studies in bifurcated animals suggest that aspects of the entrainment state are quite resistant to perturbations of light (Gorman & Steele, 2006; Harrison & Gorman, 2012). There are, however, strong coupling mechanisms observed in hamsters that tend to drive the system to rejoin when it is perturbed too much (Gorman & Steele, 2006), or when it is moved into constant conditions (Evans et al., 2011). Thus, we may have expected the system to rejoin and become unimodal after repeated shifts. By contrast, we show here, based on visual inspection of the actograms, asymmetry measures and the bifurcation index, that the twice-daily activity rhythms in bifurcated mice can remain quite stable under a simulated rotating shift paradigm.
Further, the manner in which the LDLD cycle was shifted (i.e., the timing of the two scotophases on “days off” between “shifts”) appeared to be important. If total number of shifts predicted resetting rates independent of entrainment state (i.e., repeated phase-shifts decreased entrainment stability), then we would have expected the LD group to entrain the fastest (4 shifts), followed by the Phase group (5 shifts), then the Anti (8 shifts), and finally the Step (11 shifts). In fact, what we observed when considering all measures was effectively the opposite pattern. Thus, while continued disruption over time serves to impair resetting under a typical, long day waveform, a bifurcated waveform appears to protect against this effect. Further, shifting gradually, rather than abruptly, may serve to keep the dissociated oscillators from rejoining. While it is known that repeated circadian perturbations have serious consequences for health, it is unknown what aspect of disruption is specifically responsible for those effects (i.e., being subjected to multiple phase shifts, being unable to entrain to those shifts, exposure to light during subjective night, etc). Thus it remains unclear whether or not bifurcation is protective against such effects.

Another prediction based on prior findings would be that anti-phase LDLD light schedules would confer the most stability. Prior behavioral studies of the relationship between the two scotophases suggest that as they approach one another (i.e., as one of the photophases shortens), the
likelihood of the two bouts rejoining increases (Gorman & Steele, 2006). Our findings, however, were that the Anti group assumed an intermediate position between Step and Phase, and was statistically different from neither on any measure.

While the physiological consequences of long-term bifurcation remain unknown, that bifurcation can be maintained throughout such a protocol is encouraging for application to human shiftwork and other challenging schedules. If bifurcation is going to be considered as a potential entrainment state for shift-workers, however, future studies should examine the effect of bifurcation on cognitive measures, as well as physiological ones such as sleep and metabolism.

Acknowledgements: Chapter 4 is currently being prepared for submission for publication. Maintenance of bifurcation throughout simulated rotating shift schedules in mice, Harrison, Elizabeth; Gorman, Michael. The dissertation author was the primary investigator and author of this paper.
Figure 4.1. Schematic of the simulated rotating shift for LD controls and three different bifurcated groups. The simulated rotating shift schedule for each experimental group is plotted here across 24 h (horizontal axis) and days of the experiment (vertical axis) for easier visualization of the work schedule. Gray bars represent the “work periods” around which light/dark schedules were structured, which are identical for all groups. White and black bars represent periods of light and dark, respectively. Animals were first entrained to either a LD or LDLD schedule, then challenged to complete a commonly-used rotating shift schedule – the Dupont schedule (a series of 12 h work shifts alternating every 3-7 days between day and night shift). A) LD control and LDLD B) Anti, C) Phase and D) Step groups.
Figure 4.2. Double-plotted representative actograms from all groups. Actual wheel-running activity from representative mice from each of the four groups, plotted here over 48 h to more easily view response to repeated perturbations. Black markings represent daily wheel-running activity across 48 h (horizontal axis) and over subsequent days (vertical axis). Gray shading represents periods of darkness. A) LD control and LDLD B) Anti, C) Phase and D) Step groups. The light failure that affected the Anti group can be observed.
Figure 4.3. Representative actogram from the Step group illustrating how scotophases were aligned for analysis. Actual wheel-running activity from the same animal depicted in Figure 4.2D (Step group), plotted here with 8 h of daily null data inserted in order to align scotophases for analysis. Black markings represent aligned daily wheel-running activity across one 24 h period, represented as 32 h (horizontal axis), and over subsequent days (vertical axis).
Figure 4.4. Bifurcated mice show enhanced resetting in a simulated rotating shift paradigm. Bifurcated mice in all three LDLD groups show faster resetting of activity onsets in the three days after each shift than do the LD mice (Panel A; $F_{(3,20)} = 20.90, p < 0.0001$, all Bonferroni-adjusted $p$ $s$ $< 0.0001$), and show less disruption throughout the protocol (Panel B, $F_{(3,20)} = 3.18, p < 0.05$, all Bonferroni-adjusted $p$ $s$ $=$ $ns$; un-corrected t-tests yielded significant differences between all bifurcated groups and the LD controls groups at $p<0.05$). The percent of total activity in the photophase, however, did not differ between any of the groups, either acutely (Panel C; $F_{(3,20)} = 3.37, p = 0.06$) or chronically (Panel D, $F_{(3,20)} = 0.91, p = 0.45$).
Figure 4.5. Shifting incrementally under bifurcation is more stable. Mice in the LDLD Step group had less asymmetry in both the photophases (Panel A; $F_{(2,15)} = 4.18, p < 0.05$) and scotophases (Panel B; $F_{(2,15)} = 4.79, p < 0.05$), as well as a higher Bifurcation Index (Panel C; $F_{(2,15)} = 3.82, p < 0.05$, all Bonferroni-adjusted $ps < 0.05$). The bifurcation indices of all LDLD mice correlated with how quickly onsets were reset (Panel D; Pearson’s $r = -0.58, p < 0.05$).
CHAPTER 5. Circadian phase-shifting, but not waveform bifurcation, impairs memory for cued fear in mice.

ABSTRACT

In mammals, memory acquisition and recall can be affected by time of day, as well as by manipulations of the light/dark cycle. Under bifurcation, a manipulation of circadian waveform, two subjective days and nights are experimentally-induced in rodents. Using a Pavlovian fear conditioning protocol in C57BL/6J mice, we tested whether the bifurcated entrainment state impairs learning and memory. Here we demonstrate that bifurcation of the circadian waveform produces a small decrement in acquisition, but without the negative effect on recall seen in a jet-lag paradigm. The results have implications for those attempting to adjust to shift-work or other challenging schedules.

INTRODUCTION

In rodents and humans, many cognitive tasks show a circadian peak in performance, including acquisition and recall of memories (Gerstner et al., 2009; Wright et al., 2012). Performance on memory tasks is subject to impairment following perturbations of the circadian clock or the light/dark schedule (Craig & Mcdonald, 2008; Devan et al., 2001; LeGates et al., 2012; Loh et al., 2010; Neto et al., 2008; Ruby et al., 2013). In contrast to effects of
shifting the timing, or *phase*, of circadian rhythms, little is known about the consequences of changing the shape, or *waveform*, of these rhythms for learning and memory (see Chapter 1).

In a recently discovered entrainment paradigm termed “bifurcation,” exposure to a light/dark/light/dark (LDLD) schedule in rodents facilitates a reorganization of the circadian system into two periods of alternating activity and rest per 24 hours. In addition to behavior, other rhythms that are markers of circadian day and night -- melatonin, light responsiveness and SCN function -- are bimodally expressed in bifurcated animals (Gorman & Elliott, 2003; Raiewski et al., 2012; Watanabe et al., 2007; Yan et al., 2010). Bifurcation enables a steady state variation in waveform without the confounding effects inherent in exposure to winter nights, including reproductive changes and light duration. Further, bifurcation of the waveform results in a relatively stable entrainment state that can be rapidly induced (Yan et al., 2010), and is robust against perturbations of the light/dark schedule (Gorman & Steele, 2006; Harrison & Gorman, 2012). As such, it has been suggested as a potential entrainment state for shift-workers or others with challenging schedules (Harrison & Gorman, 2012).

Thus, we aimed to assess whether the reorganization of the circadian system observed in bifurcated animals has negative cognitive effects similar to those observed during or after circadian disruption utilizing a simulated jet-lag
paradigm. Pavlovian fear conditioning is well-suited to this task for a number of reasons, including a well-defined neurobiology (Anagnostaras, Gale, & Fanselow, 2001; Anagnostaras, Maren, & Fanselow, 1999; Gale et al., 2004). In Pavlovian fear conditioning, animals are placed in a novel environmental context wherein a tone is paired with a shock. After training, rodents exhibit fear by freezing when returned to the training context or when presented with the tone in a novel context. Contextual and cued fear conditioning are dissociable: contextual fear is a prominent animal model of declarative memory (Anagnostaras et al., 2001) and evidence suggests it is dependent on both hippocampus and amygdala, whereas cued fear depends solely on the amygdala (Gale et al., 2004). Given that conditioned fear is a paradigm with a relatively short, discrete time course for both training and testing and can therefore be measured during circadian manipulations without disrupting the independent variable of the light/dark schedule, it is an ideal paradigm for testing our hypotheses.

Behavioral evidence suggests that the twice-daily rhythms observed in bifurcated animals do not represent one 12 h rhythm, but rather two 24 h rhythms, both originating from the SCN but oscillating in anti-phase. Within the same animal, activity in the two bouts may differ in waveform, amplitude and/or response to light (Evans et al., 2011; Gorman & Elliott, 2003), and is therefore hypothesized to reflect oscillations in distinct subregions of the SCN.
Thus, although much of behavior and physiology of bifurcated mice recurs on a 12 h basis, its underlying clock substrate is organized in terms of 24 h. Thus a secondary aim was to assess whether the bifurcated mouse demonstrated a 12 or 24 h rhythm in learning and/or memory.

**Hypotheses & Objectives:**

In two experiments, we compared Pavlovian fear conditioning in bifurcated and non-bifurcated mice. In Experiment 1, the performance of bifurcated animals was contrasted with that of animals after repeated phase advances in a simulated jet-lag paradigm (Fig. 5.1A). Experiment 2 investigated the contributions of entrainment state, circadian phase of training, and train-test interval (Fig. 5.1B).

**Hypothesis 1:**

As bifurcation constitutes a restructuring of circadian organization it will result in impaired recall as seen in jet lag or other difficult schedules. Alternatively, because bifurcation is a stable entrainment state, learning impairments seen in other circadian manipulations will be avoided. In Experiment 1, bifurcated animals were directly compared to animals that had undergone a simulated jet-lag paradigm (*Advancing* group). In both
experiments, mice from Bifurcated and Control groups were compared 24 hours after training.

Hypothesis 2:

The two subjective days and nights in bifurcated animals may differentially contribute to learning and memory. Within bifurcated animals, there are a number of reasons to suspect that the two activity/rest bouts observed within one 24 h period might have differential effects on cognition. As discussed above, each of the two subjective days and nights in bifurcated animals are distinct in various ways from the other, including behavior (Fig. 5.2B). It may be the case, therefore, that they affect downstream outputs, such as cognition or sleep, differentially. Alternatively, we might expect performance in bifurcated animals to have two peaks, one for each of the two subjective days per 24 h. Consequently, in Experiment 2, bifurcated animals trained immediately before the first subjective day were directly compared to animals trained immediately before the second subjective day both 12 h and 24 h after training to determine the contribution of each of the distinct 24 h oscillations.

Materials and Methods

General Methods
138 C57BL/6J mice aged 5-8 wks were used with approximately equal numbers of males and females balanced across groups. Mice were at least 9 weeks of age at training and were purchased directly from Jackson (West Sacramento, CA; Experiment 1, n=42) or bred in-house from inbred stock from Jackson (Experiment 2, n=96). Mice were group housed 2-5 per cage at 22±2°C in polypropylene tubs (17.8cm x 25.4cm x 15.2cm) under baseline lighting conditions for two weeks. Food (Purina Rodent Chow No. 5001, St. Louis, MO) and water were provided ad libitum for the entirety of the study. Lighting in the photophase was provided by white tube fluorescent lights providing illumination intensity ranging from 30-100 lux inside individual cages. To facilitate bifurcation, scotophases for all groups were dimly illuminated by green LEDs at an intensity of <0.1 lux (Evans et al., 2007). All experiments were conducted in compliance with the rules and regulations of the Institutional Animal Care and Use Committee, University of California, San Diego.

After two weeks of baseline lighting conditions (12:12 for Control and Advancing animals, 18:6 for animals to be bifurcated), mice were transferred to individual cages with wire running-wheels (4.5” diameter) in polypropylene tubs modified for additional height to accommodate wheel revolutions. Control and Advancing mice remained in 12:12 and were transferred to wheels immediately before lights out (Fig. 5.2). Upon the transition to individual cages, mice in the Advancing group were exposed to a repeated phase-shifting
simulated jet-lag paradigm wherein the light schedule was shifted 8 h earlier (advanced) every 3 days. The first advance coincided with exposure to novel wheels and the last shift was on day 15 of the protocol (Fig. 5.2C). To induce bifurcation, mice housed under 18:6 baseline conditions were transferred to a 6:6:6:6 light/dark (LD) cycle, with introduction of the wheel coinciding with the new dark period (scotophase; Fig. 5.2B).

Locomotor activity rhythms were monitored with a Vitalview data collection system (Minimitter, Bend OR) that counted the number of electrical closures triggered by a half wheel revolution. Activity counts were compiled into 6-minute bins and entrainment was verified using ClockLab Software (Actimetrics, Wilmette IL).

Experimental conditions

All animals in Experiment 1 were trained immediately before a dark period, and tested 24 h later. The advancing group ended the protocol on the same schedule as the control group and stayed there for one full photocycle before training and subsequent testing 24 h later (Fig. 5.1A and 5.2C).

In Experiment 2, to test the effect of diurnal phase on acquisition, control animals were trained at the beginning of the day (AM) or 12 hours later at the end of the day (PM; Fig. 5.1B). While bifurcated animals were likewise trained at the same two time points separated by 12 h (AM or PM), due to the
nature of the entrainment state, in both cases training for bifurcated animals took place immediately before a light period/subjective day. To test the effect of the consolidation interval, control and bifurcated animals were tested either 12 or 24 h later.

Fear Conditioning

All training and testing took place in the light and within one hour of a scheduled light transition. During lighting transition periods in a 12:12 cycle, animals generally begin to become active and training and testing took place during this time to minimize disruption. Cage changes for all groups occurred one week before training and mice were handled for 5 days prior to training. Four mice were tested concurrently in individual conditioning chambers. Fear conditioning was conducted as described previously (Carmack, Howell, Rasaei, Reas, & Anagnostaras, 2014) with the following exceptions: Training began with a 2-min baseline, followed by three tone–shock pairings at minutes 3, 4 and 5, consisting of a 30-sec tone (2.8 kHz, 85 dBA) that co-terminated with a 2-sec scrambled, AC foot shock (0.75 mA, RMS). Tone testing consisted of a 2-min baseline, followed by a 3 30-sec tone presentations at minutes 3, 4 and 5 (2.8 kHz, 85 dBA).

Statistical Analysis

Statistical analyses were conducted using SPSS (IBM, New York) and
Graphpad Prism (La Jolla CA). In Experiment 1, female mice froze more during acquisition and during the tone test. Sex was therefore covaried in all analyses for Experiment 1. Sex was considered as a factor and had no effect on outcomes for Experiment 2. All tests were evaluated at the alpha = 0.05 significance level. Greenhouse-Geisser corrections were applied in instances where homogeneity of variance was violated.

RESULTS

Entrainment

As expected, control animals exhibited activity patterns typical of entrainment to a standard 12:12 light/dark cycle, with wheel-running activity concentrated in the single 12 h scotophase (Fig. 5.2A). In contrast, within a few days of exposure to the LDLD schedule, most animals in the Bifurcation group divided their wheel-running activity between the two 6 h scotophases, albeit sometimes with more activity in one of the two (Fig. 5.2B). Four animals in the LDLD entrainment condition (2 in Expt 1 and 2 in Expt 2) maintained a unimodal pattern of wheel-running activity (i.e., they did not bifurcate), and were thus excluded from analyses. Mice in the Advancing group in Experiment 1 exhibited advancing patterns of wheel-running activity, with high amounts of transients and activity in the photophases, typical of exposure to a changing light/dark cycle (Fig. 5.2C).
Fear conditioning

Experiment 1

Freezing during the five minutes of training reflects learning, or acquisition, of fear memory. In our protocol, tone-shock pairings occurred at minutes 3, 4 and 5. In Experiment 1, a Two-way Repeated Measures ANOVA with Minutes and Entrainment State as factors and sex as a covariate revealed that Control, Bifurcated and Advancing animals demonstrated acquisition during training (RM ANOVA, p<0.001 for Minute) with no group differences across the five minutes of training (p=0.77 for Group and p=0.23 for Minute x Group Interaction; Fig. 5.3A). Sex did not have any effect when other factors were considered, though there was a trend (p=0.09 for Sex and p=0.09 for Minute x Sex Interaction). There were no differences across the three groups in baseline locomotor activity or shock reactivity (Two-way ANOVA for Group x Sex, p<0.05 for Sex in both measures; Group and Interactions for both measures, all ps > 0.05).

Immediate memory in our protocol is the level of freezing during the five minute period without stimuli that immediately follows training. It is less well understood than other learning measures but is thought to reflect short-term memory for the association between the context and shock (Fanselow, 1986). In Experiment 1, there was no effect of group on immediate memory (One-Way ANOVA for Group with Sex as a covariate, p=0.49; Fig. 5.3B).
In Experiment 1, there were no significant differences in levels of freezing to context by group (One-Way ANOVA with Sex as a covariate, p=0.72; Fig. 5.4A). However, there was an effect of group on tone (One-Way ANOVA with Sex as a covariate, p<0.05; post-hoc t-tests indicate animals that were chronically shifted (Advancing) showed impaired memory for tone compared to both Control (p<0.05) and Bifurcated animals (p<0.05; Fig. 5.4B).

Experiment 2

In Experiment 2, a two-way repeated measures ANOVA with Minute, Training Phase and Entrainment State as factors demonstrated that Bifurcated animals exhibited a small but significant decrement in acquisition compared to the Control groups. While all groups showed increased freezing across subsequent tone-shock pairings (p<0.0001 for Minute), Bifurcated animals showed significantly lower rates of freezing during acquisition than both control groups in Minutes 4 and 5 of training (p<0.001 for Entrainment State, and p<0.001 for Minute x Entrainment State interaction; Bonferroni-adjusted post-hoc tests for Minutes 4 and 5 significant at p<0.05; Fig. 5.3C). Control animals trained in the PM showed significantly higher rates of freezing during acquisition compared to Control animals trained in the AM and to Bifurcated animals trained at either phase (p<0.05 for Phase, but p=0.16 for Minute x Phase; Bonferroni-adjusted post-hoc tests for Minute 3 for PM Controls vs AM
Controls and Bifurcated groups all significant at $p<0.05$ or less). There was no significant interaction of Minute x Training Time x Entrainment State ($p=0.25$), or for Entrainment State x Training Time ($p=0.09$). There were no differences across the four groups in baseline locomotor activity or shock reactivity (both $ps > 0.05$).

For immediate memory in Experiment 2, a two-way ANOVA with Entrainment State and Training Phase as factors yielded a main effect of Phase ($p<0.05$), a trend for Entrainment State ($p=0.07$) and no significant interaction ($p=0.42$; Fig. 5.3D). Post-hoc t-tests reveal an effect of Phase for Control animals (AM-trained control animals showed lower rates of freezing during the immediate memory test compared to animals trained at the PM phase ($p<0.05$), while Bifurcated animals did not ($p=0.37$). While this apparently conflicts with the lack of interaction, post-hoc tests suggest there may be a time of day dependence in Control but not Bifurcated mice, perhaps due to insufficient power to detect an interaction. Taken together, these results appear to indicate that the two subjective days per 24 h seen in bifurcated animals may not differentially affect acquisition of conditioned fear.

In Experiment 2, bifurcated mice again show recall of conditioned contextual and cued fear comparable to long day controls. To control for the significant difference in context memory as a result of phase of training (AM v PM) for control groups tested 12, but not 24, h post-training, we examined the
effect of entrainment state in groups tested 24 h later only (though outcomes remained the same if all groups were included). For groups tested 24 h post-training, there was no effect of entrainment state on tone memory (p=0.19, Fig. 5.4D), nor was there an effect on context memory, although there was a non-significant trend for greater recall in Controls (p=0.09; Fig. 5.4C).

In addition to the phase effects found in control animals for acquisition and immediate memory, phase of training had an effect on recall as well. AM-trained Control animals showed impaired recall for context compared to animals trained at the PM phase when the train-test interval was 12 h (p<0.05), but not when it was 24 h (p=0.86; Fig. 5.5A). There were no phase differences in recall for tone (all p values >0.05; Fig. 5.5C). Unlike Controls, Bifurcated groups trained in the AM did not differ from those trained in the PM in expression of conditioned contextual or cued fear when tested 12 (AMvPM t-test for context, p=0.71, Fig. 5.5B; for tone, p =0.32, Fig. 5.5D) or 24 h (AMvPM t t-test for context, p=0.32; for tone, p=0.49) post-training.

Contrary to findings in other labs (Cai et al., 2009; Chaudhury & Colwell, 2002; Loh et al., 2010), recall did not change over time in Control animals trained in the PM (PM 12v24 t-test for context, p=0.92, Fig. 5.5A; tone, p=0.44; Fig. 5.5C), though it did for context in Control AM mice (AM 12v24, context, p<0.05, tone, p=0.06; Fig. 5.5A). Recall did not change over time for
Bifurcated animals (AM 12v24 t-test for context, p=0.79, Fig. 5.5B; tone, p=0.28 Fig. 5.5D; PM 12v24 t-test for context, p=0.28; tone, p=0.54).

**DISCUSSION**

**Bifurcation does not disrupt long-term memory to the extent of a jet-lag paradigm.**

In Experiment 2, bifurcated animals showed a small but significant decrement in acquisition and immediate memory compared to control animals. Despite this, learning is intact in bifurcated animals, as shown by the increased freezing over Minutes 3 through 5. Additionally, they show no impairment in recall for either contextual or cued fear compared to control animals in either Experiment 1 or Experiment 2. This implies that the reorganization of the SCN that takes place under a bifurcated entrainment state need not disrupt learning and memory. Despite the possibility of poorer acquisition (and presumably encoding), the deficits observed in acquisition and immediate memory did not persist into long-term recall. Bifurcation may thus protect against negative effects of phase in context fear (Fig. 5.5).

By contrast, animals that were chronically shifted (Experiment 1), while trained and tested at comparable phases of the LD cycle, showed impaired memory for tone compared to both control and bifurcated animals (Fig. 5.4B). Previous studies have found that while circadian phase affects training and
recall of contextual fear in C57BL/6 mice, tone memory appears to be independent of circadian phase in some (Valentinuzzi et al., 2001), but not all (Chaudhury & Colwell, 2002) paradigms. Therefore, it is probable that the deficit for recall of cued memory seen in the Advancing group is a direct consequence of circadian disruption, rather than an effect of testing at a non-comparable phase. Bifurcated animals freeze to the tone at levels comparable to control animals and greater than the chronically-advanced mice (Fig. 5.4B). This suggests that the steady-state reorganization of the circadian system in bifurcated mice does not disrupt recall to the extent of a jet-lag paradigm.

**Phase effects are found for control, but not bifurcated, mice.**

In our study, control animals (12:12) trained during the transition from night to day showed impaired acquisition, immediate memory, and recall for context 12 h post-training compared to control animals trained during the transition into night. Like other studies we find phase effects in our control mice for phase of training and testing when holding the train-test interval constant. As in Chaudhury and Colwell (2002), phase affected rates of freezing during acquisition (Fig. 5.3C) and contextual recall (Fig. 5.5C), though we observed no such difference in cued recall (Fig. 5.5D). Similarly, as in other studies (Cai et al., 2009; Chaudhury & Colwell, 2002; Ralph et al., 2002), our results support evidence for a “time stamp” for learning, wherein recall is
higher when training and testing occur at the same time of day (Fig. 5.5C). However, our findings differ from these studies in a number of ways. In Cai et al. (2009), higher rates of freezing 12 h post-training were seen in 129B6 mice trained before subjective day, and not night. Similarly, Chaudhury and Colwell (2002) reported higher rates of freezing in C-3H and C57BL/J6 mice when training and testing occur during subjective day. These differences may be attributable to differences in strain, fear-conditioning protocol (i.e., time between context and tone tests; the number, timing, or intensity of shocks), or to the fact that our mice had access to running wheels. Importantly, in a report by Valentinuzzi and colleagues (2001) wherein phase of fear conditioning was examined in C57BL/6J mice with access to running wheels, animals trained and tested early in subjective night, rather than day, showed higher rates of freezing to context 24 h post-training. In rats, performance on a novel location recognition task, which like the context test in our paradigm is hippocampal-dependent, likewise peaked at night rather than day (Takahashi, Sawa, & Okada, 2013).

We found no evidence that the two subjective days per 24 h seen in bifurcated animals differentially affect acquisition or consolidation of conditioned fear; bifurcated animals trained 12 h apart showed no differences in acquisition, immediate memory, or recall for context or tone fear (Experiment 2). These findings do not exclude a phase dependency that could
be detected with more frequent sampling (i.e., every 6 h instead of 12). The effect of training and testing bifurcated animals before subjective day vs night was never explicitly examined in these experiments: In Experiment 2, all bifurcated animals, whether trained in the environmental AM or PM, were trained during the transition to a photophase, whereas in Experiment 2, training and testing always occurred during the transition to a scotophase.

**Interval effects were found for control mice, but not bifurcated mice.**

In previous work, animals with only a 12 h subjective night between training and test showed a recall decrement compared to other groups (Cai et al., 2009). While this same pattern was not observed in the present results, we demonstrated a different interaction between phase and interval (Fig. 5.5A). By convention, we have induced bifurcation by introducing the animals to a novel wheel at the start of one of the scotophases (Gorman & Elliott, 2003), and therefore wheels were used in our protocol. Running wheels have been shown to change the organization of sleep (Welsh, Richardson, & Dement, 1988), increase learning (Greenwood, Strong, Foley, & Fleshner, 2009; Kohman et al., 2012) increase synaptic plasticity (Patten, Sickmann, & Hryciw, 2013), and rescue induced learning deficits (Christie et al., 2005; Fardell, Vardy, Sha, & Johnston, 2011). While it is unknown whether the wheels may have compensated for small decrements dependent on sleep or other
variables, the fact that our protocol was sensitive to differences in circadian manipulation (Experiment 1) and phase (Experiment 2) in control animals indicates that the wheels did not create a general ceiling effect.

Conclusions

Despite a major reorganization of the circadian timing system, learning and memory is intact in bifurcated animals. While control animals have a slight significant advantage in acquisition and immediate memory over bifurcated animals trained immediately before the end of night (Experiment 2), these do not persist through recall of the memory, and are not present when bifurcated animals are trained and tested prior to a scotophase (Experiment 1). The lack of a phase effect in bifurcated animals in Experiment 2 suggests that AM and PM training, while 12 h apart, most likely occurred at a functionally equivalent circadian phase in bifurcated animals. Therefore, in terms of acquisition and recall of conditioned fear, each subjective day and each subjective night may be equivalent in these mice. In sum, it appears that the memory for conditioned fear in bifurcated mice is intact and superior to that observed in mice undergoing a simulated jet-lag paradigm. Therefore, unlike effects of chronic jet-lag, dissociation of oscillatory circadian components may not impair recall per se. These results have implications for understanding the
organization and flexibility of the circadian system, and do not preclude bifurcation as a potential model for application in human shift-work.

Acknowledgements: Chapter 5 is currently being prepared for submission for publication. Circadian phase-shifting, but not waveform bifurcation, impairs memory for cued fear in mice, Harrison, Elizabeth; Carmack, Stephanie; Block, Carina; Sun, Jonathan; Anagnostaras, Stephan; Gorman, Michael. The dissertation author was the primary investigator and author of this paper.
Figure 5.1. Experimental schema for Experiments 1 (A) and 2 (B). Black and white bars denote the light/dark schedule. In Experiment 1, mice were entrained under 12:12, 6:6:6:6 lighting conditions, or a chronically phase-advancing light schedule. In Experiment 2, mice were entrained under 12:12 or 6:6:6:6 lighting conditions only. All mice were trained and tested within one hour of a light transition. Boxes labeled “AM” or “PM” represent training times, whereas circles labeled “12” or “24” represent train-test intervals of 12 or 24 hours, respectively.
Figure 5.2. Representative single-plotted actograms from groups in Experiment 1. Wheel-running activity patterns from an animal in 12:12 (A), 6:6:6:6 (B), and the chronically phase-advanced group (C). Actograms are plotted across 24 h on the X axis and days on the Y axis. Gray shading indicates hours of darkness. Note the stability of the bifurcated activity pattern in B, although the two daily dark phases differ in the amount of activity expressed.
Figure 5.3. Acquisition and Immediate Memory for Experiments 1 (A&B) and 2 (C&D) (opposite).

**A & C. Acquisition.** For acquisition graphs, percent (%) freezing over minutes 1 through 5 of training for are shown for Experiment 1 (A) and 2 (C). For both experiments, clear symbols represent control groups, and filled symbols indicate bifurcated groups. Lightning bolts indicate the administration of tone-shock pairings at minutes 3, 4 and 5. For experiment 1, the advancing group is represented by gray squares. There are no differences between groups in acquisition in Experiment 1. For Experiment 2, groups trained in the morning (AM) are represented by triangles, while groups trained at night (PM) are represented by circles (clear symbols for control groups, and filled symbols for bifurcated). Bifurcated animals showed significantly lower rates of freezing during acquisition than both control groups in Minutes 4 and 5 of training, indicated by the symbol (*) (post-hoc Bonferroni-adjusted p<0.05; Fig. 3A). Control animals trained in the PM showed significantly higher rates of freezing during Minute 3 of acquisition than both control animals trained in the AM and than bifurcated animals trained at either phase, indicated by the symbol (#) (post-hoc Bonferroni-adjusted p<0.05).

**B & D. Immediate Memory.** Percent (%) Freezing in the five-minute period that immediately follows the five-minute training session. Clear bars are control groups, filled bars are bifurcated groups, and the gray bar is the advancing group. There were no differences between groups in immediate memory for Experiment 1. For Experiment 2, a Two-way ANOVA indicated an effect of Phase (p<0.05), but not Entrainment State (p=0.07) and no significant interaction (p=0.42; Post-hoc t-tests indicate significantly higher freezing in the PM group relative to the other 3 groups (ps<0.5), indicated by a (*).
Figure 5.4. Freezing during Context and Tone recall tests by entrainment state for Experiments 1 (A & B) and 2 (C & D). Percent (%) freezing during recall tests for control (white bars), bifurcated (black bars), and advancing animals (gray bars).
A. In Experiment 1, the advancing group froze less in response to the tone presentation than both the controls and the bifurcated animals (D). There were no group differences for contextual fear (C). In Experiment 2, for each entrainment state, data from both the AM- and PM-trained 24 h interval groups were combined to directly compare controls to bifurcated animals over recall measures. No group differences were found.
Figure 5.5. Phase effects in LD12:12 control, but not bifurcated, animals for context (A&B) and not tone (C&D). Percent (%) freezing during recall tests for control (white bars) and bifurcated (black bars) animals. Control animals trained in the AM show less freezing after a 12 h train-test interval than a 24 h one indicated by the symbol (*).
CHAPTER 6: General Discussion

In the circadian literature, rhythmic waveform has been examined relatively little compared to phase, in spite of limited but convincing evidence that a natural manipulation of waveform, photoperiodism, enhances phase resetting responses (Evans, Elliott, & Gorman, 2010; Glickman et al., 2014; Goldman & Elliott, 1988). Here we examined the impact of this change in waveform and a novel, experimentally-induced waveform manipulation, termed bifurcation, on re-entrainment under simulated jet-lag paradigms. We additionally examined the effects of bifurcation on learning and memory. The studies in this dissertation were specifically aimed at answering the following:

1. Does a naturally-occurring waveform manipulation enhance behavioral re-entrainment in response to shifts in the light/dark cycle?

If so,

2. Does an experimentally induced waveform manipulation, bifurcation, likewise enhance behavioral re-entrainment?

3. Does bifurcation enhance re-entrainment of central and peripheral clocks in the circadian system?

4. Can entrainment be maintained under a more applied, simulated rotating shift system?
A naturally occurring waveform manipulation enhances behavioral phase re-entrainment in response to shifts in the light/dark cycle

Photoperiodism is a naturally-occurring response to seasonal changes in daylength. Experiments examining the effects of photoperiod on phase-shifting indicate that animals under short, winter-like days have a higher amplitude PRC and thus respond to brief light pulses with phase-shifts of greater magnitude than long day counterparts (Evans et al., 2010; Glickman et al., 2014; Goldman & Elliott, 1988). This enhanced phase-resetting is thought to be a result of changes in pacemaker network organization wherein there is a higher level of synchrony in the phase of SCN neurons (reviewed in Meijer et al., 2010). It has been hypothesized that this greater synchrony results in a more uniform response from the network of cells as the light stimulus is transmitted to the neurons at similar phases of their PRC (Mickman, Stubblefield, Stubblefield, Harrington, & Nelson, 2008). Further, recent work in our lab has demonstrated that there is an enhanced acute light response in the SCN following these brief pulses of light during subjective night (Glickman et al., 2012). Yet, all of this work examines the nonparametric (i.e., acute) effects of light on the circadian system, which are dissociable and likely differ in mechanism from the parametric (i.e. tonic) effects of light (Evans et al.,
Further, due to the constraints of the circadian system under this often-utilized paradigm, known effects are limited to phase shifts of a smaller magnitude (i.e., ~2-4 h). Finally, in order to compare phase shifts of full light/dark cycles, a global assay ranging across all magnitudes is required, as there is an inherent ambiguity in the size of any single phase shift when waveform changes (i.e., when both LD and SD are shifted to a new 16:8 LD cycle that is a 4 h advance of lights off, it is also a 4 h advance of lights on for the LD group, while it is an 8 h advance of lights on for the SD group).

Thus, in Chapter 2 we extended this body of work to include parametric effects of light, utilizing shifts of full photocycles ranging in magnitude from 4-12 h. We found that Syrian hamsters under short photoperiods were able to adjust to phase shifts of all magnitudes twice as quickly on average as long day counterparts.

**An experimentally-induced waveform manipulation, bifurcation, likewise enhances behavioral phase re-entrainment in response to shifts in the light/dark cycle**

In Chapter 2 we extended extant data on phase-resetting under different photoperiods to include re-entrainment to phase shifts of full photocycles of varying magnitudes. In Chapter 3, we utilized the same protocol to extend those findings to include a novel, experimentally-induced
form of waveform manipulation, bifurcation. Numerous behavioral and a smaller number of molecular studies indicate that bifurcation is a *bona fide* entrainment state wherein oscillating components of the core circadian clock in the SCN are dissociated and oscillating in anti-phase (Gorman & Steele, 2006; Gorman & Elliott, 2003; Watanabe et al., 2007; Yan et al., 2010). Here we show that this reorganization of the circadian system facilitates phase-shifts of varying magnitude and direction in a simulated jet lag paradigm. The wheel-running activity of Syrian hamsters shifted from a bifurcated state to one of 6 LD time zones adjusted more than twice as quickly as long-day counterparts. Further, daily activity onsets of previously-bifurcated hamsters shifted to antiphase times zones are 12 h apart when released into DD after three full photocycles in the new time zone. This indicates that the master pacemaker itself of the animals is shifted into antiphase time zones in only a matter of days, confirming that the rapid behavioral resetting seen after bifurcation is not a behavioral artifact of masking.

**Bifurcation enhances phase-resetting of central and peripheral circadian clocks**

In Chapter 2, enhanced re-entrainment rates were observed in previously-bifurcated Syrian hamsters, and it was determined that three full photophases in a new light/dark schedule are sufficient for antiphase
entrainment to occur. In Chapter 3, we sought to determine if bifurcation could confer this plasticity on the system in as little as one day. A subsidiary aim was also to extend those findings to a mouse model. In Experiment 1, we found that one day in the new scotophase was not sufficient for previously-bifurcated animals to demonstrate anti-phase entrainment. We did, however, succeed in extending our findings in hamsters to a new species: mice that received three full photophases had behavioral rhythms that were 12 h in antiphase when released into DD.

Finally, we sought to characterize the peripheral circadian system under bifurcation and determine if the enhanced rate of re-entrainment is observed at the level of simple tissues, such as liver, kidney and lung. Peripheral tissues can reset independently of the SCN, and likewise receive synchronizing cues independently of the SCN, such as through behavior (Damiola et al., 2000; Vollmers et al., 2009). Thus, in Experiment 2, we examined 6 core clock genes in the peripheral tissues of bifurcated animals relative to controls, both in steady-state entrainment, and after a phase-shift to an LD cycle 12 h in antiphase. We found a unique pattern of clock gene expression in the peripheral circadian system of bifurcated animals. Similar to our findings, Watanabe and colleagues found expression of *Per1* and *2* was bimodal in the liver of mice under a 7:5:7:5 LDLD cycle, whereas *Bmal1* was unimodal and other clock genes were classified as arrhythmic (Watanabe et al., 2007). Our
results varied slightly from theirs, most likely due to procedural differences in lighting conditions and in classification of rhythmicity. In addition to replicating their findings, we were able to extend them to include a wider profile of core clock genes, in three peripheral tissues. Further, we found that while amplitude of clock gene expression was generally much lower under bifurcation, many of the genes were rhythmically expressed, some unimodally, and some bimodally. Further, we found that to a great degree, gene pairs cycled together (positive with positive, and negative with negative), and the expression of many genes had the same relationships in LDLD as they did in LD. Thus we conclude that the clock is likely reorganized, but functional, under bifurcation. Finally, we found a slight advantage for re-entrainment of the peripheral circadian system after antiphase shifts of the light/dark cycle for previously-bifurcated mice compared to LD controls. While the strong effect of bifurcation observed in behavior was not mirrored to the same extent on the molecular level in the periphery, we anticipate that it most likely will be in expression patterns in SCN.

**Bifurcation can be maintained under challenging, applied rotating schedules**

Bifurcation has been suggested as a potential entrainment state for shiftworkers (Harrison & Gorman, 2012). In Chapter 4, we examined whether
bifurcation could be maintained under a simulated 12 h rotating shift schedule that is commonly used in U.S. manufacturing. We further examined whether or not the manner in which the two bouts of activity were shifted played a part in the stability of the bifurcation, utilizing three groups that differed in the manner in which they shifted to antiphase cycles (i.e., from “day” to “night” shift). We found that bifurcation is resilient to repeated perturbations, and remained robust in all three groups, with a slight advantage for the Step group over the Phase group, and intermediary values for the Anti group. Thus, phase-shifting in smaller increments of 2 h, rather than 4, does appear to be advantageous under LDLD. Further, we found that the LD control group was on average twice as far as all LDLD groups from the target phase in the first three days following shifts of the schedule. Thus, bifurcation may confer a re-entrainment advantage, even when shifted under LDLD cycles.

**Bifurcation does not impair learning and memory to the extent of a jet lag protocol**

As bifurcation has been suggested as a potential entrainment state for shift-workers, it was of interest to determine whether there are negative consequences of bifurcation on cognition. Our understanding of the effect of manipulations of waveform on cognition has been limited to experiments examining differences in photoperiodic species under long vs short days. It is
unclear whether those effects are a result of the waveform manipulation itself or of the physiological and reproductive state that the animal is in. Here we examined the effects on learning and memory of a novel and artificially-induced manipulation of waveform and found that while a simulated jet lag paradigm has negative effects on fear memory, bifurcation does not. We also found no differences in bifurcated animals tested 12 and 24 h post-training at either time of day (AM or PM), suggesting there may be no distinction in cognitive function between the two dissociated oscillators under bifurcation.

**Conclusions**

Together, these studies characterize behavioral, physiological, and cognitive effects of a novel waveform manipulation, both under steady state conditions and under simulated jet lag paradigms. Here we clearly demonstrate that a both natural, photoperiodic change in waveform, and an artificial, bifurcated circadian waveform confer an advantage on the system with regard to re-entrainment to full light/dark schedules. The rapid rate of re-entrainment observed here is remarkable, specifically considering that it is induced via a simple, non-invasive manipulation of the light dark cycle. Most work demonstrating such enhanced resetting is a result of pharmacological and/or genetic manipulations (Hatori et al., 2014; Vitaterna et al., 2006; Yamaguchi et al., 2013). There are, however, two examples of enhanced
resetting via light manipulations alone. Previous work from our lab has demonstrated enhanced re-entrainment after 4 h advances and delays utilizing only dim nocturnal illumination (Evans, Elliott, & Gorman, 2009). Importantly, in Experiment 1 of Chapter 1, both LD and SD hamsters received dim nocturnal illumination, while no nocturnal illumination was employed in Experiment 2. In those experiments, we also examined phase-shifts of not only 4, but also 8 and 12, h in magnitude. Thus, the waveform-induced results observed here likely exceed those found utilizing only dim light. Other work has shown an enhanced phase resetting response upon presentation of constant darkness after 27 h or more of continuous light in mice (Chen, Seo, Bell, von Gall, & Lee, 2008). Under such conditions, the manipulation is likely driving the circadian system, and the resetting response is therefore likely not equivalent to the enhanced re-entrainment to a new, full light/dark cycle we observed here.

As a supplement to our behavioral data, molecular data showing the kinetics of shifting clockwork in SCN and periphery for all three waveforms examined here may help determine the mechanism for the enhanced re-entrainment we observed. While candidate mechanisms (i.e, network phase synchrony and increased acute light response) have been proposed for effects observed in short days, little is yet known about the mechanism for resetting in bifurcation. One candidate mechanism may be the acute induction, by light,
feeding, or activity, of some clock genes (i.e. Per1) in antiphase to those programmed by the SCN. This regularly-induced expression of core parts of the clock may likely stimulate expression of other clock genes in anti-phase. Thus, some genes may be expressed every 12 h, while others every 24. This antiphasic expression of Per could potentially facilitate shifting clock in any direction (Chapter 3), as acute induction of Per is mechanistically involved in adjustment to phase-shifts (Yan & Silver, 2002). Another candidate may be the reduced amplitude observed in most clock genes in the periphery in LDLD (Chapter 3). While it is unknown what aspect of disruption (i.e., reduced amplitude, disrupted phase, modified coupling) is responsible for the negative effects of phase-shifts on the circadian system, there is evidence that in some cases, reduced amplitude in SCN can enhance phase-resetting responses (An et al., 2013). More extensive work utilizing our assay here with phase shifts of various magnitudes following manipulation of waveform would be valuable not only for determining a mechanism for the effects of bifurcation, but also for extending current work on gene expression in jet lag; (Davidson et al., 2006; Kiessling et al., 2010; Reddy et al., 2002; Yamaguchi et al., 2013 ; Chapter 3) to include phase shifts of different magnitudes.

The unique pattern of gene expression observed in under a bifurcated entrainment state may be the result of a number of things. It is possible, for example, that acute induction of Per1 and 2 via bimodal feeding (or some
other stimulus) then induces expression of the other handful of clock genes expressed bimodally in the peripheral tissues examined here. It is also possible that certain genes are in fact reflecting, in essence, a 12 h SCN clock. Another possibility is that we are observing harmonics of 24 h rhythms, as multiplicative regulation by core clock genes can generate harmonics (Korenčič et al., 2014). Liu and colleagues found significant 12 h components in clock gene expression in under LD conditions in peripheral tissues, including mPer1 in kidney and liver, and found a combined 12 and 24 h cosine model best fit their data (2007). We, however, did not observe any significant rhythms at 12 h for any gene in any tissue examined here for either the LD, LD Moscow, or LDLD Moscow groups. Indeed, in spite of their previous bifurcation, we found only 1 bimodal clock gene, Clock, in the LDLD Seattle group. Thus we believe that the rhythms detected here in the LDLD group reflect true oscillations induced by the LDLD condition. Recently, our lab has developed a de-masking paradigm that also controls for duration of light exposure with which future experiments may probe whether the light schedule is acutely inducing the pattern of expression observed here (Sun & Gorman, in prep). Restricted feeding paradigms should be able to dissociate whether most genes are cycling unimodally, while others are induced in antiphase via bimodal feeding behavior. Certainly, follow-up studies should include a profile of the same genes in SCN.
In any case, it is clear that the entire clock is not cycling twice daily. This is worth addressing, as bifurcation has been suggested as a potential entrainment state for human shiftworkers. It is yet possible, however, that entrainment of the entire circadian system to one long day with a short scotophase and entrainment of only parts of the system to the additional scotophase may still be less harmful than repeatedly perturbing an intact, unimodally-organized oscillating system. Of note is the consideration that if most of the system is in fact in a long-day state, we would expect that phase-shifting may be more sluggish than in 16:8. Yet that was not what we observed.

We found antiphasic re-entrainment occurred in just three full photocycles in previously-bifurcated animals. These findings should be extended to behavioral and molecular assays after only two days in new photocycle to definitively determine the number of days required for adjustment. Importantly, the activity was advanced relative to the light/dark cycle, indicating that while anti-phase entrainment did occur, it may not have been precisely aligned with the prior light/dark schedule. Future experiments should examine this in greater detail. Additionally, we elected to use hamsters in our Chapter 2 experiments, and mice in subsequent experiments. It is possible that our results would be different had we not switched species. Behavioral evidence indicates some small but perhaps meaningful differences
in the manner in which the bifurcation is induced and maintained in the two species. Under protocols optimized for hamsters, Syrian hamsters tend to bifurcate more rapidly and precisely than mice (Gorman & Elliott, 2003). Further, mice tend to have more symmetric patterns of activity in the two bouts (unpublished observations). It is therefore possible that there are differences between the two species under the entrainment state. However, molecular evidence from the SCN in the two species, though somewhat limited, yields similar results (Watanabe et al., 2007; Yan et al., 2010).

It is quite remarkable that bifurcation can be maintained under shifting schedules. This resilience to perturbations of the light/dark schedule is a key piece in the applicability of bifurcation as an entrainment state for humans exposed to shiftwork or other challenging schedules. Circadian disruption, particularly shiftwork, increases risk of numerous health disorders, particularly metabolic and sleep-related disorders. These studies should be extended to include a longer time course, and metabolic markers such as body weight and glucose metabolism should be assayed to determine if bifurcation is advantageous in this regard relative to a long-day state. Further, sleep under both bifurcated steady-state and shifting conditions should be assessed.

Finally, we show here that in a simple paradigm of learning and memory, bifurcation does not impair memory, while simulated jet-lag does (Chapter 5). That we were able to demonstrate no deficits under bifurcation
utilizing a paradigm that was sensitive to effects of circadian disruption of cognition implies that a bifurcated entrainment state may be advantageous relative to a unimodal waveform (i.e., LD) that is constantly shifting, as in a rotating shift schedule. Future studies should examine additional forms of learning, as well as other behavioral measures of cognitive state, including depression and anxiety, as in a forced-swim or elevated plus maze.

In conclusion, waveform manipulation has inherent value in understanding the mechanisms behind the multi-oscillatory circadian system and how individual yet interdependent oscillators are coupled. Manipulating waveform before phase utilizing only simple changes in the daily pattern of light exposure increases plasticity in this system to an unprecedented extent. Further, we show here that waveform manipulation may provide a more promising target than phase for application to human schedule changes.
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