Evidence for Internal Desynchrony Caused by Circadian Clock Resetting

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Circadian disruption has been linked to markers for poor health outcomes in humans and animal models. What is it about circadian disruption that is problematic? One hypothesis is that phase resetting of the circadian system, which occurs in response to changes in environmental timing cues, leads to internal desynchrony within the organism. Internal desynchrony is understood as acute changes in phase relationships between biological rhythms from different cell groups, tissues, or organs within the body. Do we have strong evidence for internal desynchrony associated with or caused by circadian clock resetting? Here we review the literature, highlighting several key studies from measures of gene expression in laboratory rodents. We conclude that current evidence offers strong support for the premise that some protocols for light-induced resetting are associated with internal desynchrony. It is important to continue research to test whether internal desynchrony is necessary and/or sufficient for negative health impact of circadian disruption.

INTRODUCTION

Our physiology and behavior are shaped by our circadian rhythms, which are driven by a master biological pacemaker in the suprachiasmatic nuclei (SCN) of the hypothalamus. Light exposure is the major source of circadian synchronization in the vast majority of studied organisms, and as such it can also drive circadian disruption when irregularly timed. The SCN is synchronized by the daily light-dark cycle, with lighting information transmitted to the SCN through pathways from the retina [1]. Both rod/cone and melanopsin photoreceptors play important roles in circadian responses [2]. The SCN then uses redundant mechanisms to relay this information to organs and tissues throughout the body, leading to temporal coordination of physiological functions and behaviors [3]. Molecular circadian rhythms are generated by a set of genes which comprise a regulatory feedback loop, and

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†Abbreviations: CCD, charge-coupled device; CT, Circadian Time; DD, constant darkness; EEG, electroencephalography; EMG, electromyography; LD, light:dark; PMT, photomultiplier tube; SCN, suprachiasmatic nuclei; VIP, Vasoactive Intestinal Polypeptide; ZT, Zeitgeber Time

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circadian expression is widespread throughout the body because of these intracellular molecular clocks. It is now evident that most peripheral organs and tissues can express circadian oscillations independently and respond differently to entraining signals, while still responding to inputs generated by the SCN \textit{in vivo}. Recent evidence has shown that during circadian clock resetting the body may be in a state of internal desynchrony among the central and peripheral clocks, a form of circadian disruption that may be linked to poor health outcomes.

\textbf{CIRCADIAN DISRUPTION HAS BEEN LINKED TO MARKERS OF POOR HEALTH}

The literature on the negative health costs of circadian disruption is substantial, describing a variety of effects occurring at cell, tissue, and whole organism levels. The SCN coordinates endocrine and autonomic systems throughout the circadian cycle, such as fluctuations in insulin sensitivity throughout the day [4]. Environmental phase shifts can affect physiology in many ways, such as a reduction in leptin, an increase in glucose and blood pressure, or by reducing sleep efficacy [5]. These physiological effects result in metabolic disruptions, cardiovascular and immune dysfunctions, mood disorders, increased risks of cancer [6], and even an increase in mortality rate in aged mice when subjected to chronic phase shifts [7,8].

Because of the negative effects of disrupted circadian rhythms, it is important to consider carefully what is known about the state of circadian disruption. The term “circadian disruption” has been used to describe various scenarios of misalignment [9,10], such as misalignment between the internal circadian system and external environmental cues (\textit{e.g.}, light-dark cycle) or desynchrony among the rhythms within the organism. For example, studies in humans show the effect of short-term circadian disruption on alignment between physiological circadian rhythms and behavioral cycles. It has been shown that phase markers of SCN rhythms, such as melatonin and clock protein rhythms, were not greatly shifted by night shift work, but many circulating plasma metabolites became dissociated from the SCN rhythm and became aligned with the shifted behavioral cycles [9]. Evidence of desynchrony such as this might explain how shift work and metabolic diseases may be associated.

\textbf{OVERVIEW OF THE ORGANIZATION OF THIS REVIEW}

In this paper we will review measures of internal
desynchronization that characterize circadian resetting in laboratory rodents. We will focus mostly on evidence arising from studies of gene expression rhythms during re-entrainment to phase shifts of the light-dark schedule. The review is organized by technique. We first highlight studies that measured clock gene expression at selected times of euthanasia (cross-sectional studies), and then describe studies using continuous measurements in transgenic reporter mouse models (longitudinal studies). We separately discuss in vitro methods using explanted tissues, and in vivo bioluminescence measures in free moving mice (Figure 1). We discuss pros and cons of the different techniques currently in use to determine genetic rhythms of circadian genes both ex vivo and in vivo, as well as critical assumptions of these techniques.

WHAT IS CIRCADIAN resetting AND HOW DO WE STUDY IT IN THE Lab?

Under normal conditions, the rhythms controlled by the circadian system maintain what we assume is an optimized phase relationship with environmental cues and with each other. The SCN master pacemaker sends diverse signals throughout the organism to maintain this orchestration. When a sudden change in phase of an external environmental time cue occurs (if we fly from New York to Paris, or vice versa; or when our work shift changes), the system resets to the new phase over several days.

The process of resetting the circadian system can easily be reproduced in experimental settings using rodents and other animal models. Laboratory animals are typically housed under a 24 h cycle of light and darkness (e.g., 12 h of light followed by 12 h of darkness; LD). By convention, we define Zeitgeber Time 12 (ZT12) as the moment when the dark interval begins; in a symmetrical LD cycle, ZT0 is coincident with the lights-on time. A shift in the phase of the LD cycle (i.e., phase shift) occurs by shifting the onset and offset of darkness by a determined number of hours. Under these controlled environmental conditions, we can study the mechanisms by which circadian rhythms are reset to the new phase.

Experimental phase shifts of the LD cycle can vary in magnitude and direction (advanced or delayed relative to the original phase) and can be achieved through different protocols. Advances can be performed by shortening either the light or the dark intervals of the cycle by the desired number of hours. Likewise, delays can be performed by lengthening either interval. Additional considerations for chronic phase shifting schedules include the latency between subsequent shifts, and the pattern of phase shifts (e.g., repetitive chronic phase advances or alternating advances and delays). Experiments can also involve variations in the characteristics of the light stimulus (e.g., intensity, wavelength, duration). These features allow researchers to work with a large number of experimental scenarios, but also give rise to considerable inter-experimental variation when trying to compare and integrate findings from different studies.

In some studies, a researcher may choose to release studied animals into constant darkness (DD) after a number of cycles of the shifted LD in order to assess their internal phase independently from the continual effects of light exposure. For example, 4 days following an 8 h phase advance of a 12:12 LD cycle, wildtype C57BL/6 male mice released into DD showed no apparent phase shift in locomotor activity measured with an infrared sensor, and only a small shift in locomotor activity if they were housed with a running wheel (see supplementary data Figure S4 in [11]). Delays of the LD cycle shifted the locomotor activity circadian rhythm more effectively in mice, producing a ~5 h delay in circadian rhythms after 1-2 days of an 8 h delay in the LD [11].

POST-DISSECTION MEASUREMENT OF GENE EXPRESSION (FIGURE 1A): EVIDENCE FOR INTERNAL PHASE MISALIGNMENT DURING PHASE RESETTING

The circadian system is now well understood at the molecular level. Transcriptional-translational loops between inter-related “canonical” clock genes generate daily cycles in both core clock genes (such as Per1, Per2, Cry1, Cry2, Bmal1, Clock) as well as tissue-specific clock-controlled output genes in the SCN and peripheral tissues. The sequence of this core loop involves the activation of Period (Per1, Per2, and Per3) and Cryptochrome (Cry1 and Cry2) genes transcriptions by a BMAL1/CLOCK heterodimer and, after a suitable delay built in by post-transcriptional mechanisms, the subsequent inhibition of this process by complexes containing PER and CRY proteins [12]. An additional loop involves the nuclear receptors Rev-erb and Ror, which can translocate into the nucleus to modulate Bmal1, Clock, and Npas2 transcription via opposing actions on a RORE sequence located in their promoter regions. These molecular feedback loops occur in cells throughout the body, and have a near-24 h cycle length.

What does jetlag look like from the vantage of the SCN as a whole? Circadian clock genes (Per1, Per2, Bmal1, and the “output” gene Dhp) measured in male mouse SCN tissue punches show days of misaligned clock gene expression within the SCN following a phase advance of the LD cycle. More specifically, after a 6 h advance of the LD cycle in mice, a dissociation occurs between Per and Cry, Dhp, Bmal1, and Rev-Erb genes, in which the Per genes show a fast adjustment to the new
rhythms among regions of the SCN. Cells in the ventral SCN are light-responsive and show strong induction of the light-responsive gene Per1 (e.g., see samples from 3:00 on days 1 through 6 following the shifted LD cycle in Figure 3B). Cells in the dorsal portion of the SCN do not shift for multiple days and shift at a rate correlated with behavioral shifts (compare samples from 15:00 on days 2 through 13 in Figure 3B). On the other hand, the ventral SCN rhythm in sensitivity to light-induced c-fos mRNA shifts phase slowly following a light cycle shift, readjusting at a rate similar to that of behavioral rhythms [16]. Internal desynchrony may be altered, depending upon cellular communication within the SCN. The speed of internal resetting across cell groups in the SCN, and thus the speed of resetting of downstream rhythms, can be increased by alterations in the expression of vasopressin receptors. Mice lacking the arginine vasopressin receptors showed faster phase resetting as measured in behavior, body temperature, and in SCN and peripheral tissue gene expression [11]. Both V1a and V1b receptors appear to play a role, and knocking out both receptors generates a phenotype of very rapid resetting in C57BL/6 mice. Experiments in which these receptors were pharmacologically blocked in wild type mice confirmed these results. This suggests that V1a- and V1b-mediated cell communication in the SCN plays a role in regulating the rate of re-entrainment. Other research shows that specifically timed treatment with vasoactive intestinal polypeptide (VIP) can speed resetting to an 8h advance of the LD cycle, perhaps attributable to the effect of this peptide treatment on cellular synchrony [17].

![Figure 2](image_url). Gene expression rhythms after an 8 h advance jetlag. Using qPCR, expression of clock genes was measured every 4 h after the phase shift in laser-microdissected tissue samples. Dbp gene expression rhythm in the SCN (black; left side Y-axis) and in the liver (red; right side Y-axis) with quantification of gene expression normalized to 36b4 expression, units of mmol/mol 36b4 mRNA. Adapted from Yamaguchi et al. (2013) [11]; Courtesy of Drs. Okamura and Yamaguchi.
light can be recorded continuously to infer temporal patterns of expression of the gene of interest.

TISSUE EXPLANT EX VIVO APPROACH (FIGURE 1B): EVIDENCE FOR INTERNAL PHASE MISALIGNMENT DURING PHASE RESETING

While in situ hybridization and immunohistochemistry methods can give information on the level of expression of a specific gene or protein, they can only provide a snapshot of a specific time of dissection. Transgenic techniques and the advent of genetically modified rodents, as well as virally expressed reporter genes that can be introduced into otherwise wild-type animals, have allowed the development of models carrying "reporter" genes that can be tracked continuously in real time. The gene for luciferase (Luc), the bioluminescent enzyme from the firefly, can be inserted into a mouse genome under the promoter of a circadian gene of interest, or even fused to the protein product generated from the rhythmically expressed gene. Tissues of interest can then be dissected and cultured in vitro in the presence of luciferase substrates, and emitted light can be recorded continuously to infer temporal patterns of expression of the gene of interest.

A seminal study presenting evidence of disruption in peripheral and central phase alignment during phase resetting was performed using a transgenic rat line in which luciferase was expressed under the control of the mouse Per1 (mPer1) promoter [18]. Tissue explants of SCN and various peripheral tissues (skeletal muscle, lung, and liver) were harvested, and the rhythmicity of the mPer1-controlled luciferase light emission was recorded. This study demonstrated that the SCN and peripheral tissue explants all showed rhythmic expression of the mPer1-driven reporter, with the peripheral tissue rhythms phase-lagging the SCN by approximately 7 to 11 hours. To investigate whether abrupt changes of the light cycle led to disorganization of circadian rhythms, rats had their 12:12 LD cycle either advanced or delayed by 6 h. The SCN explants shifted within two circadian cycles after shifts in either direction, while peripheral tissues shifted at different, slower rates, indicating a desynchronized
Most studies of mice use highly inbred, ~99 percent genetically identical strains, with a majority of studies utilizing male C57BL/6 mice. The C57BL/6 strain, as reviewed above [9], typically shows relatively slow (5-10 day) shifts of locomotor rhythms following large (6-8 h) advances of the LD cycle. On the other hand, a different inbred strain, BALB/c, shows almost no evidence for jetlag in locomotor rhythms or in temporal sensitivity to light-induced Fos expression in the SCN, achieving large (up to 10 h) phase advances within 1-2 circadian cycles [22]. The BALB/c and C57BL/6 strains show similar circadian-day patterns of phase shifts to brief (1-3 h) pulses of light (“phase response curves”), and the BALB/c mice cannot entrain normally to short (< 3 h) photoperiods (e.g., 2L:22D) or skeleton photoperiods (light cycles defined by several short light pulses, e.g., at dawn and dusk, 1L:10D:1L:12D). These two lines of evidence support the hypothesis that the significantly larger magnitude phase shifts achievable by BALB/c mice are highly dependent on the duration of light exposure [23], unlike C57BL/6J and most other mouse models.

Some F2 hybrids of BALB/cJ and C57BL/6J mice display intermediate rates of re-entrainment of locomotor rhythms, while others show rapid or slow phenotypes.
cycle (LD10:10), to which C57BL/6J mice are unable to entrain, in order to induce disruption to the circadian system through light at night and phase resetting. Control animals were housed under LD12:12. The time of dissection strongly influenced circadian phase of PER2::LUC rhythms in the SCN from mice housed under LD10:10, while the effect on control mice was minimal (Figure 5B). Dissection time reset the phase of adipose tissue regardless of the lighting cycle, but it did not reset the phase of thymus explants. These studies indicate circadian disruption can disrupt the coordination of phase within the SCN and between the SCN and peripheral tissues, and may thereby alter network sensitivity to stimuli related to explantation procedures to which they are usually unresponsive. In light of these studies, results from ex vivo gene expression experiments involving prior disrupting or resetting conditions should be interpreted with considerable caution.

IN VIVO APPROACH (FIGURE 1C): EVIDENCE FOR INTERNAL PHASE MISALIGNMENT DURING PHASE RESETTING

Techniques have now been developed to measure circadian gene expression from the SCN as well as other tissues in the body in vivo in freely moving animals. These techniques allow us to record circadian rhythms and the effect of light-induced phase shifts in real-time. Although recordings of bioluminescence and fluorescence enable

**Figure 5.** (A) Relationship between peak time and dissection time of the SCN explants prepared from mice entrained to LD or LDLD bifurcated light periods, dissected at various time points across the day. Each gray diamond indicates a dissection time (ZT) and peak time of a single explant culture. Black circles indicate average peak times for each dissection time group. Data are quadruple plotted to aid visualization of the resetting pattern (from Noguchi et al., 2018). (B) Similar results found in our recent work using PER2::LUC mice, showing the effects of time of dissection on resetting of phase shifts. Each dot represents the time of dissection of the explanted SCN tissue and the peak time relative to Circadian Time (a timeframe based on the locomotor activity under constant dark conditions; CT) prior to dissection for that tissue. Adapted from Leise et al., 2018 [25].
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**Per2** gene expression in the SCN of mice housed under a full LD cycle. (Unlike luciferase bioluminescence measurements, fluorescence measurements can be conducted without interference from environmental lighting). After an 8 h advance of the LD cycle, the **Per2**-Venus rhythms of the sub-population of VIP-expressing SCN neurons were suppressed and then phase shifted (Figure 6A), confirming the results of Yamaguchi et al. (Figure 2) with added cell-specificity. The study also confirmed that this cell population within the ventral SCN shifts more rapidly than behavioral rhythms (Figure 6C). Mei et al. determined that **Per2** and **Cry1** expression show similar rates of resetting (see Figure 6B), and a different group using a similar approach reported that **Per1** and **Bmal1** rhythms re-entrain at different speeds and are thus desynchronized during resetting [28].

The potential of these techniques can be appreciated in a recent paper by Mei and collaborators that studied the impact of an LD phase shift on a subpopulation of SCN neurons through *in vivo* fluorescence recording [27]. These researchers transduced SCN neurons with a fluorescent reporter for **Per2-Venus**, which is expressed selectively in SCN neurons expressing vasoactive intestinal polypeptide (VIP). A fiber optic guide implanted directly above the SCN delivered the excitation light as well as collected the emitted light, which was then measured by a photomultiplier tube (PMT). In this way, they could record **Per2** gene expression in the SCN of mice housed under a full LD cycle. (Unlike luciferase bioluminescence measurements, fluorescence measurements can be conducted without interference from environmental lighting). After an 8 h advance of the LD cycle, the **Per2-Venus** rhythms of the sub-population of VIP-expressing SCN neurons were suppressed and then phase shifted (Figure 6A), confirming the results of Yamaguchi et al. (Figure 2) with added cell-specificity. The study also confirmed that this cell population within the ventral SCN shifts more rapidly than behavioral rhythms (Figure 6C). Mei et al. determined that **Per2** and **Cry1** expression show similar rates of resetting (see Figure 6B), and a different group using a similar approach reported that **Per1** and **Bmal1** rhythms re-entrain at different speeds and are thus desynchronized during resetting [28].

These *in vivo* tools for recording gene expression provide new experimental possibilities. Saini et al. (2013) recorded the expression of clock genes in the liver of mice with SCN lesions and showed that hepatocytes synchronized more quickly to inverted feeding cycles than in control mice, suggesting that the SCN can “hold” the phase of the liver, resisting the signals of misaligned feeding rhythms [29]. Other groups have monitored rhythms of genes in regions of the brain other than the SCN. For example, in response to a single 8 h delaying
light pulse, Per1-luc bioluminescence rhythms in the olfactory bulb shifted more rapidly than did the cerebral cortex and the skin [26]. Internal desynchrony during re-entrainment to a phase shift can also be observed at the behavioral level in laboratory rodents, as shown in Figure 3A. The resetting of rhythms in sleep stages of rats after a 6 h phase delay of the LD cycle was studied using electroencephalography (EEG) and electromyography (EMG) recordings [30]. The study determined that slow-wave sleep rhythms were immediately re-entrained to the new LD phase, while rapid-eye movement sleep rhythms took 3 to 5 days to return to their original phase. The main consequence of this transitory desynchronization of sleep stages is an abnormally high propensity for rapid-eye movement sleep during the early subjective day, which is normalized after full resetting of sleep stages rhythms. The authors also show that this desynchronization of sleep stages has its origin in the differences in the speed of re-entrainment between the ventrolateral and the dorsomedial SCN [30].

CONCLUSION AND OUTLOOK: EVIDENCE FOR INTERNAL PHASE MISALIGNMENT DURING PHASE RESETTING

Our current understanding is that jetlag involves malaise that has two sources: during the period of re-entrainment, external lighting cues fall at biologically inappropriate parts of the circadian cycle (external desynchronization) and parts of the body shift their circadian rhythms at varied rates (internal desynchronization). Several studies stand out as providing strong evidence for the hypothesis that circadian phase resetting is accomplished after a transient internal desynchrony of many body clocks.

For example, we highlight the heroic study by Yamaguchi et al. (2013) [11], in which mice were euthanized every 4 h, before and up to 11 days after a shift of the LD cycle. After collecting 54 time points of gene expression measures, the authors showed that the SCN loses amplitude after a light cycle shift, then achieves the new phase several days prior to the liver or the kidney. The liver’s circadian Per2 expression across a population of mice takes over 10 days to shift in response to an 8 h LD phase advance, lagging the SCN which shifts faster [11]. Although the measures using this technique are necessarily static and population-based, the results provide strong support for this hypothesis.

We highlight an important complication in descriptions of SCN response. The ventral subpopulation of cells in the SCN shows rapid shifting, whereas the dorsal subpopulation lags [15]. Thus, the SCN is of “two minds” when it comes to adjusting to a new time zone. When a study analyzes tissue punches of SCN, we must recognize that results that do not support a strong rhythm may be signifying a diversity of responses within the SCN itself. New techniques are being developed that allow us to track the phase resetting dynamics of sub-populations of SCN cells [27].

Limitations

Techniques: Some of the strongest evidence we have highlighted here arises from studies using techniques that average responses across animals by collecting “snapshots” of circadian gene expression at set times (Figure 1A). Although this technique does not reveal the variable responses within a population of animals, when researchers collect frequent samples over long periods of time, results can provide detailed time series of resetting dynamics of multiple tissues within animals.

We have new concerns about veracity of past studies using ex vivo tissue explant techniques to measure circadian phase (Figure 1B). Recent research has shown that dissection itself may act as a stimulus that can cause phase resetting in tissues from mice previously exposed to circadian-disruptive conditions [24,25]. These results suggest that exposure to some forms of circadian disruption may desynchronize SCN neurons, thereby increasing network sensitivity. Tissues with weakened coupling, or with little to no coupling, as may be true of some peripheral tissues, will show larger resetting effects by the explantation procedure. Exposure to light at inconsistent circadian times on a recurring weekly basis disrupts locomotor activity rhythms and alters sensitivity of the SCN pacemaker to dissection time effects. Time of peak of Per1-luc rhythms recorded in vivo differed from those reported in ex vivo studies [26]. These results indicate we should be quite cautious in interpreting phase measurements from ex vivo circadian gene expression experiments. On the other hand, single-cell resolution is more feasible when working in vitro. An in vitro preparation of the entire Drosophila brain allowed visualization of detailed dynamics of desynchrony within neural components of circadian pacemakers in response to a single light pulse [31].

Newer in vivo techniques allow long-term recordings of gene expression of tissues, and should allow simultaneous recordings of behavior and physiology (Table 1). Currently it is most feasible to record from one tissue site, limiting the ability to track internal alignment of rhythms within one animal (but see [26]). One important consideration for future development of in vivo techniques is the ability to provide an environment optimal for animal welfare. In some studies reviewed here, mice are constrained to small recording chambers (e.g., 200 mm diameter in Hamada et al., 2016 [26]), are connected to pumps delivering substrates, or are tethered by probes entering the brain [18,27,32]. Ideal approaches to long-term monitoring of in vivo rhythms will minimize restraint, tethering,
Table 1. Some key papers using different in vivo approaches to measure internal desynchrony of circadian gene expression.

| Citation                  | Animal Model                                      | Detection Method                                           |
|---------------------------|---------------------------------------------------|------------------------------------------------------------|
| Saini et al., 2013 [29]   | Viral delivered Adv-Bmal1-luc or RevErba-luc; PER2::LUC mice | CCD camera, or PMT                                         |
| Ono et al, 2017 [28]      | Per1-luc, Bmal1-Eluc mice                         | Implanted optical fiber connected to a PMT                 |
| Hamada et al., 2016 [26]  | Per1-luc, Bmal1-Eluc mice                         | 2 CCD cameras with scintillators used for quantification of signal from multiple sites |
| Mei et al., 2018 [27]     | Injection of Per1 and Cry1 transcription reporter virus Venus fluorophore | Implanted optical fiber delivering excitation light and connected to a PMT |

PMT - Photomultiplier Tube
CCD - Charge-coupled device

and surgical interventions.

**Subject variables:** As noted above, most studies use only a few common laboratory strains, such as the C57BL/6 mouse, and often researchers use only male mice. We described the dramatically different responses that can be observed when using BALB/c strain mice. Other subject variables may also be important. Few studies have been powered to compare males and females. Older animals show slower adjustment of phase in response to shifts [33]. It is important to consider a wider range of subject variables in order to better estimate the importance of our findings for the larger population of rodents, and extrapolation to other species, including humans, should be evidence-based.

**Environmental factors:** Timed food availability does not shift the SCN circadian rhythm, but can phase shift the liver circadian clock [34]. This shift can be accomplished more rapidly when the SCN is ablated [29] indicating that there are dynamic interactions between these components of the circadian system. Timed feeding can shift phase of other organs as well, such as heart, kidney, pancreas, and skin [34,35]. Interestingly, a somewhat extreme feeding protocol (24 h fast followed by restricting food to 2 h/day) at the time of new onset of darkness can accelerate the rate of re-entrainment to 6 h advance of the LD cycle in rats [36].

Social housing is a much less well-explored factor and offers an innovative future direction for our research. One study showed that housing mice in groups of 5, but not pairs, could influence circadian synchronization [37]. Housing mice in groups of 3 or 4 blocked effects of chronic circadian disruption on weight gain [38]. Impact of social factors highlights the importance of studies in ecologically relevant conditions [39-42].

We have shown that the use of running wheels can improve circadian phase resetting in older mice [33]. A running wheel alleviates the disruptive metabolic effects of a chronically advancing light schedule (6 h advance every 2 d) [38].

**Future studies:** We see several high priority directions for future research on this topic.

1. The need for technique development. The evidence presented in this review suggests that the field is at a point where we can utilize in vivo techniques for phase measures, to move forward in testing the impact of various environmental factors thought to alter circadian phase during resetting. To measure the phase misalignment of multiple internal circadian rhythms, we need to track multiple internally generated rhythms from an individual animal over several weeks. It is also possible that individual cells may show different resetting dynamics, and the rhythm from an individual tissue may show broadening of rhythm waveform, loss of amplitude, and lower signal:noise ratio during circadian phase resetting. These changes in waveform would be impossible to interpret clearly without in vivo techniques offering cellular resolution.

2. Health significance. It will be essential in the future to demonstrate whether internal desynchrony underlies the negative health impact of circadian disruption. If this can be established, we can aim to determine how to reset circadian phase while minimizing internal phase misalignment. We note that a shift of the LD cycle might also negatively impact health via effects of light during the subjective night [43], through changes in lipid metabolism [7], loss of sleep quantity or quality [44] or non-specific stress response. Researchers studying the impact of internal circadian phase misalignment will aim to manipulate just this aspect of the response to an LD shift (as, for example, accomplished through a genetic approach by van der Vinne et al., 2018 [41]). Such research is key to determine the health significance of internal desynchrony in gene expression rhythms as distinct from external desynchrony.
3. Understanding of SCN network responses. Our understanding of the neuronal network of the SCN suggests the ventral cells respond quickly to light but the dorsal cells are less responsive, taking longer to shift phase. Dorsal SCN cells appear to shift at the same rate as locomotor activity rhythms [15], but this should be verified with other techniques that allow measurements of both rhythms in the same animal. New papers suggest astrocytes play important roles in the SCN network [45-48], but their roles are as yet unclear in terms of light-mediated shifts and internal desynchrony.

Overall, we highlight here the past research studies of gene expression studies in lab rodents. Such highly controlled studies demonstrate the state of internal desynchrony that accompanies circadian clock phase resetting. As circadian clock resetting is associated with adverse health consequences in animal models as well as in epidemiological studies in humans, understanding the contribution of internal desynchrony to these effects is of experimental interest and public health importance.

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