RESETTING OF CENTRAL AND PERIPHERAL CIRCADIAN OSCILLATORS IN AGED RATS

Alec J. Davidson[2], **Shin Yamazaki**[3], **Deanna M. Arble**[4], **Michael Menaker**, and **Gene D. Block**[1]
Department of Biology, University of Virginia, Charlottesville, VA 22904-4328

Abstract

The mammalian circadian timing system is affected by aging. Analysis of the suprachiasmatic nucleus (SCN) and of other circadian oscillators reveals age-related changes which are most profound in extra-SCN tissues. Some extra-SCN oscillators appear to stop oscillating *in vivo* or display altered phase relationships. To determine whether the dynamic behavior of circadian oscillators is also affected by aging we studied the resetting behavior of the *Period1* transcriptional rhythm of peripheral and central oscillators in response to a 6 hr advance or delay in the light schedule. We employed a transgenic rat with a luciferase reporter to allow for real-time measurements of transcriptional rhythmicity. While phase-resetting in the SCN following an advance or a delay of the light cycle appears nearly normal in 2-year old rats, resynchronization of the liver was seriously disrupted. In addition, the arcuate nucleus and pineal gland exhibited faster resetting in aged rats relative to 4-8 month-old controls. The consequences of these deficits are unknown, but may contribute to organ and brain diseases in the aged as well as the health problems that are common in older shift-workers.

Keywords

Aging; Rat; Period1; Per1-luc; synchronization; jet-lag; phase shift; liver; pineal gland; arcuate nucleus; Suprachiasmatic nucleus; SCN; core body temperature

1. INTRODUCTION

The rat circadian timing system is a hierarchical assembly of oscillators found throughout the body but is organized, temporally, by the central circadian clock, the suprachiasmatic nucleus (SCN) of the hypothalamus [10]. Each day, photoreceptors in the retina [15] convey information regarding environmental lighting conditions to the SCN via a specialized pathway from the optic tract. The SCN adjusts its phase in response to this light input thereby maintaining its synchronization with the external world. The SCN then plays a central role in organizing rhythmicity throughout the organism by communicating phase information to oscillators in tissues elsewhere in the brain, and in nearly all other tissues and organs.

Circadian timing systems are affected by the aging process. The mollusk, *Aplysia*, exhibits a reduced amplitude rhythm in optic nerve impulse frequency as a consequence of age [27] In

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[1]Corresponding author PO Box 400226 University of Virginia Charlottesville, VA 22904 Email: gdb@virginia.edu Phone: 434-924-3606 Fax: 434-924-1497.
[2]Current Address: Circadian Rhythms and Sleep Disorders Program Neuroscience Institute Morehouse School of Medicine 720 Westview Dr. SW Atlanta, GA 30310 adavidson@msm.edu
[3]Current Address: Department of Biological Sciences Vanderbilt University VU Station B, Box 35-1634 Nashville, TN 37235-1634
[4]Current Address: Center for Sleep and Circadian Biology Northwestern University 2205 Tech Drive Evanston, IL 60208-3520

mammals, old mice show delayed activity onsets, take longer to reentrain to phase shifts, and have increased fragmentation in their wheel running records [29,35]. Mice and hamsters display disruptions in their phase-shifting ability to photic [5,39] and non-photic [30] stimuli in advanced age, and their locomotor activity patterns show an increase in fragmentation [26]. Period shortening has also been reported [24,32] but there is some inconsistency in the literature (see [11,14]).

Fewer studies have been performed in rats but the results are similar. Circadian drinking behavior [7], locomotor activity rhythms [12], and body temperature rhythms [20] all have reduced amplitude in older rats, leading in some cases to the loss of some rhythms while others are spared [25]. Alterations in phase are also common in old rats [7] [20].

The effects of aging on circadian rhythms have implications for cognitive performance and physiological function. The fragmentation of circadian locomotor behavior in hamsters has been linked with impaired performance on a memory task [2]. Furthermore, longevity appears to be linked with the quality of the subject's rhythms; implantation of neonatal SCN implants into aged hamsters is reported to increase longevity, while non-invasive disruption of rhythmicity decreases longevity [16]. Human studies have also revealed negative effects of aging on circadian rhythms. There is evidence that disruption of sleep consolidation [13] in aged humans significantly impacts quality of life.

Because aging affects functions regulated by the SCN, it has been suggested that the SCN itself is the primary locus for age-related changes [37]. Indeed, the aged SCN shows a decrease in VIP expression [18], light-induced immediate early gene expression [5,28], and melatonin binding [6]. Electrical rhythmicity, an important output of the clock in the SCN, exhibits a reduced amplitude in slices containing SCN from old hamsters [34] and rats [25]. Also, individual SCN neurons cultured from older mice exhibit decreased amplitude of firing rate and increased variability in period when compared with those cultured from young mice[4]. However, it is presently uncertain whether these changes in the electrical properties *in vitro* are due to non-specific effects of aging on the stability and health of brain slices or of cultured neurons. Transplantation of young SCN into aged animals has yielded improvements in numerous rhythmic functions for the host animal [8,17,21,31,32]. Taken together these studies suggest that the SCN is a primary locus for age-related changes in the rodent circadian system.

Three recent studies have begun to address whether there are changes in molecular rhythm generation in aged animals. In all three studies Period 1 (*Per1*) and/or Period 2 (*Per2*) gene expression rhythms were unaffected in the aged SCN [3,19,37]. However, Kolker and colleagues [19] reported that both *Bmal1* and *Clock* expression in the SCN was reduced in aged hamsters. Furthermore induction of *Per1* in the SCN by a light pulse was also diminished in aged hamsters [19] and rats [3]. Yamazaki et al. [37] investigated rhythmicity in areas outside the SCN in rats using Per1-luciferase reporter gene technology. That study reported the intriguing finding that, *in vitro*, some tissues from old animals were rhythmic while others were not. The arrhythmic tissues became rhythmic when stimulated with forskolin, demonstrating that they were capable of rhythmicity and suggesting that they were not being effectively driven by the SCN *in vivo*. Furthermore, some tissues, such as the pineal gland, paraventricular hypothalamus, and kidney exhibited altered phase with respect to the light cycle (and the SCN) in old rats.

Because some tissues, although capable of rhythmicity *in vitro*, do not appear to be rhythmic *in vivo* [37] and there is evidence for a decrease in the amplitude of both the electrical output [4,25,34] and neurotransmitter expression [18], it is possible that the suprachiasmatic nuclei in aged animals is acting as a weak or unreliable *Zeitgeber* to peripheral oscillators.

The weakened signals might allow some of the peripheral oscillators to damp out and for others to exhibit altered phase relationships when entrained. We reasoned that if the SCN were providing weak entraining signals to peripheral oscillators, the dynamic behavior of these oscillators during re-entrainment to an altered light schedule might be slowed or become more irregular. To test this hypothesis, we subjected aged *Period1-luciferase* transgenic rats to 6 hour advances or delays in the light schedule. Subsets of animals were killed either before the shift or on the first or sixth day in the new light cycle and cultures were prepared from SCN, arcuate nucleus, pineal gland and liver, 4 tissues that were robustly rhythmic according to [37]. *Period1-luc* rhythms were measured *in vitro* in order to track the resynchronization of these circadian clocks that receive downstream signals from the SCN. The results of these experiments failed to reveal a uniform slowing of reentrainment by peripheral oscillators; rather we observed tissue-specific changes in the dynamic behavior suggesting that aging affects peripheral oscillators as well as the SCN.

2. METHODS

2.1 Animals and housing

Period1-luciferase (*Per*1-*luc*) Wistar rats [36] bred in our vivarium served as the experimental model in all experiments. A total of 64 rats were used to collect the data presented in this study: 28 4- to 8-month-old controls, and 26 rats aged to at least 24 months. Only rats that appeared healthy were chosen. All procedures and standards of care were approved by the University of Virginia Animal Care and Use Committee.

Rats individually housed in cages inside light-tight boxes equipped with lighting and airflow and had ad-libitum access to food and water. Phase-advances were accomplished by advancing the time of lights-on, while phase delays were accomplished by delaying the time of lights-off (Fig. 1A). Groups of rats underwent no shift (baseline), a six hour advance, or a six hour delay. Shifted rats were killed just before lights-off either 1 day or 6 days after the light cycle was changed (asterisks, Fig 1A). Day 6 rats were undisturbed for those days. Cultures were prepared from SCN, arcuate nucleus, pineal gland and liver.

2.2 Culture preparation & bioluminescence recording

Culture procedures are identical to those described in [36] and [1]. Briefly rats were killed with CO_2 overanesthesia followed by decapitation. The brain was dissected out and placed in chilled Hank's solution. The pineal gland and a small piece of liver were also excised and placed in Hank's. The brain was sliced in the coronal plane on a vibratome at a thickness of 300 μm. Sections containing the SCN and the arcuate nucleus (1.2mm further caudal) were isolated. These structures were cut from the slices using scalpels and placed at the liquid interface on membranes (Millicell-CM, PICM030-50, Millipore) in 35mm dishes containing 1.2ml recording media [serum-free, low sodium bicarbonate, no phenol red, Dulbecco's Modified Eagle's Medium (13000-021, Gibco)] supplemented with 10 mM HEPES (pH 7.2), B27 (2%; 17504-010, Gibco), and 0.1 mM luciferin (beetle luciferin, potassium salt, Promega) and antibiotics (25U/ml Penicillin, 25mg/ml Streptomycin). Pieces of liver (1mm^2 × 300μm) and pineal (whole, flattened) were placed in similar conditions. The dishes were sealed with a cover glass using vacuum grease and placed under photomultiplier tube assemblies inside light-tight 36.5°C environmental chambers. Bioluminescence was counted every minute from every dish for at least 3 days.

Bioluminescence records were detrended by subtraction of the 24h running average from the raw data, then smoothed with a 2h running average [9]. The first peak in the smoothed data after 24h in vitro was used as a phase marker (e.g. arrows, Fig 1B). The time of the peak in baseline animals was then set as 0, and data from advanced and delayed rats were plotted relative to the baseline phase for each tissue.

3. RESULTS

As previously reported [36], SCN of young animals reset rapidly following a phase-shift in the light schedule (Fig.1C). We observed that the SCN from aged rats was slower to advance than in young rats. While the aged SCN wasn't fully resynchronized on day 1 following the advance of the light cycle, the young rat SCN advanced rapidly, and as in earlier studies [22,23], "overshot" the steady-state phase by an average of nearly 2 hours. Delays were near maximum on Day 1 in SCN from young and old rats, indicating a very rapid resynchronization for both groups.

Liver resynchronization was highly disrupted in aged rats (Fig. 1 B & C). While young rat liver was fully resynchronized by the 6th day following an advance or delay of the light cycle, liver from aged rats showed an advance on Day 1 regardless of the direction of the light adjustment. While the delayed aged rat livers did manage to approach the target phase by Day 6, the liver from advanced aged rats was still completely unshifted by the 6th day in the new light cycle.

In contrast to the liver results, both arcuate nucleus and pineal gland explant cultures exhibited generally faster resynchronization in aged than in young animals. After a phase advance, pineal glands from aged rats were nearly reset by the first day (Fig 2) (Students t-test; $p<0.002$ vs. young controls), while pineals in young rats took longer to adjust. Arcuate nuclei also advanced ($p<0.002$ for Day 1) and delayed ($p<0.05$ for Day 6) faster in the aged rats.

Table 1 summarizes the results of the culture experiments including the group sizes. It is of note that the baseline phase for liver was later in explants from aged rats (peak time at ZT 22.4h) than from young (ZT 17.7; t-test $p<0.02$)). We have reported baseline phase differences among young and old rats previously [37]. In the earlier study young liver peaked earlier in young rats, but this difference did not reach significance. This may be due to the use in the present study of somewhat older (4-8-month old instead of 2-month old) control rats, or the exclusive use of the L2 line of homozygous Per1-luciferase rats rather than heterozygous and homozygous L1 rats in the earlier study.

Interestingly, following a phase-advance, 3 of 8 aged rats did not survive until the sixth day. No young rats nor any aged rats subjected to phase delays died following the shift.

4. DISCUSSION

In this study we report that the circadian system of the aged rat exhibits organ-specific changes in resetting kinetics. Specifically we found that while some tissues, such as liver, phase shift more slowly following a change in the light cycle in aged rats other tissues, such as arcuate nucleus, shifted more rapidly in aged animals. These findings build upon previous studies that have revealed other deficits in peripheral rhythms in aged rats including lower circadian amplitude or arrhythmicity, altered phase relationships and shortened period [37]. Although one could speculate that an increased rate of shifting for arcuate nucleus and pineal gland correlates with decreased circadian amplitude *in vivo*[33], indirect measurements of rhythm robustness for pineal gland did not reveal any age-related differences in amplitude [37]. It must be noted that culture preparation can affect the circadian phase of some cultured tissues [38]. However the low phase variance among the cultures within each group, and the fact that the mean peak phases were consistent with expected values for transients during resynchronization together suggest that our measurements reflect *in vivo* phases of these tissues.

4.1 Locus of age-related changes in the circadian system

Numerous studies have suggested that the SCN is affected by aging [4,5,18,28,34]. However changes also are observed downstream from the SCN.

Retrochiasmatic area and lung exhibit decreased amplitude, pineal gland and kidney exhibit altered phases with respect to the light cycle [37] , and we report in this study that, in aged animals, resynchronization is faster in some tissues, and slower in others. The increased speed of entrainment in some tissues is not consistent with observation that there is a weakening of some SCN output signals (e.g., impulse frequency and VIP expression) and a slowing of the phase advance of *Per1* rhythmicity measured photometrically over the entire SCN. It should be recognized, however, that the SCN is a temporally complex tissue, with different regions phase shifting at different rates [22,23] and thus one cannot rule out the possibility that the accelerated reentrainment of some extra-SCN oscillators could be due to age-related changes within the SCN and outputs that were not captured in the photometric recording technique employed in our experiments. Nonetheless, we believe that a more likely explanation for our data is that not only is the SCN itself affected by aging, but so also are other components of the mammalian circadian system: peripheral oscillators themselves, and potentially the routes of communication by which circadian organization is maintained.

4.2 Are phase advances different than phase delays?

Our data indicate that the *Per1* rhythm of the SCN, measured photometrically, is slower to advance in aged rats, whereas delays appear to be unaffected by aging. However, this age-related slowing in phase advances was not observed in other tissues. We found that the aged arcuate nucleus was shifted by more than 6h on the first day after a light schedule advance, while the young animal's arcuate rhythm had not yet shifted. Similarly in the pineal gland, *Per1-luc* rhythms showed a large phase advance on the first day, whereas young pineals had not yet begun to advance. Age-related changes in phase-delays were much less common in this study, limited to the pineal gland and the arcuate nucleus.

It is well established that phase shifts in light schedules lead to transiently altered phase relationships among oscillating tissues in young animals [36] [1] . However this alteration in circadian organization appears more profound and persistent in aged rats, and in the direction of a phase-advance. Given the marked internal desynchronization we observed in aged animals following phase advances it is perhaps not surprising that we observed a 37% mortality rate in aged rats that were exposed to a phase-advance and left undisturbed for 6 days. In contrast, no rats died following phase delays. The effects of light schedule changes on mortality appear remarkable and merit further study.

4.3 Final thoughts

These and earlier data taken together suggest that the effects of aging on circadian organization is complex. Oscillating tissues differ significantly with respect to age-related changes in amplitude, phase, period and resynchronization behavior. How many of the changes in peripheral oscillator behavior are due to intrinsic alterations within the tissues themselves and how many are due to changes in the entraining signals from the SCN and other timing sources (e.g., feeding) remains unclear. It will be important to understand the physiological role(s) played by oscillations in non-SCN tissues to better appreciate the importance of the breakdown in normal phase relationships. This may allow us to develop strategies to minimize the effects of aging on the circadian behaviors such as the sleep wake cycle and may also inform the use of chronotherapy in older adults.

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All procedures were approved by the University of Virginia IACUC.

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These data have not been submitted or published elsewhere.

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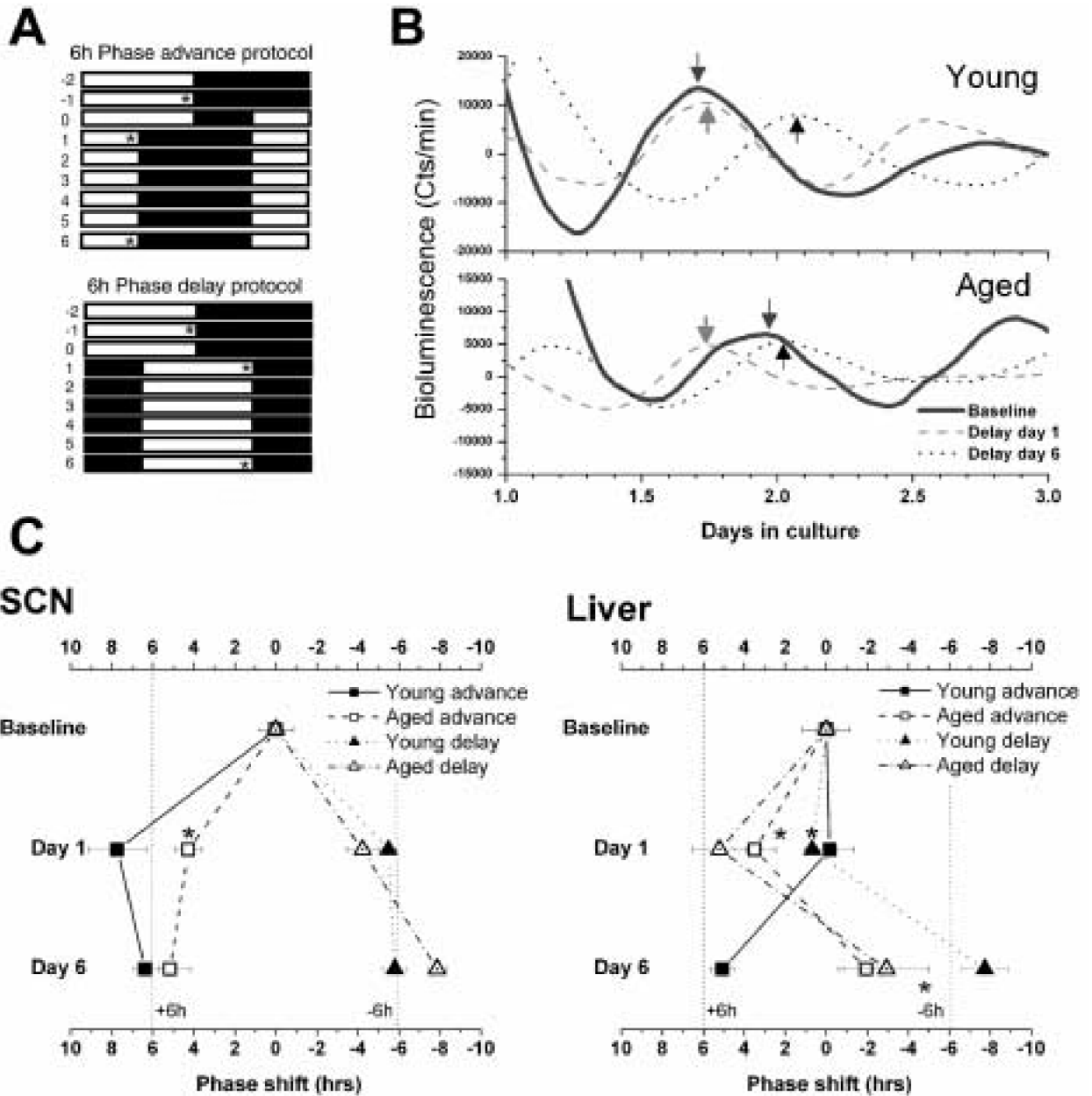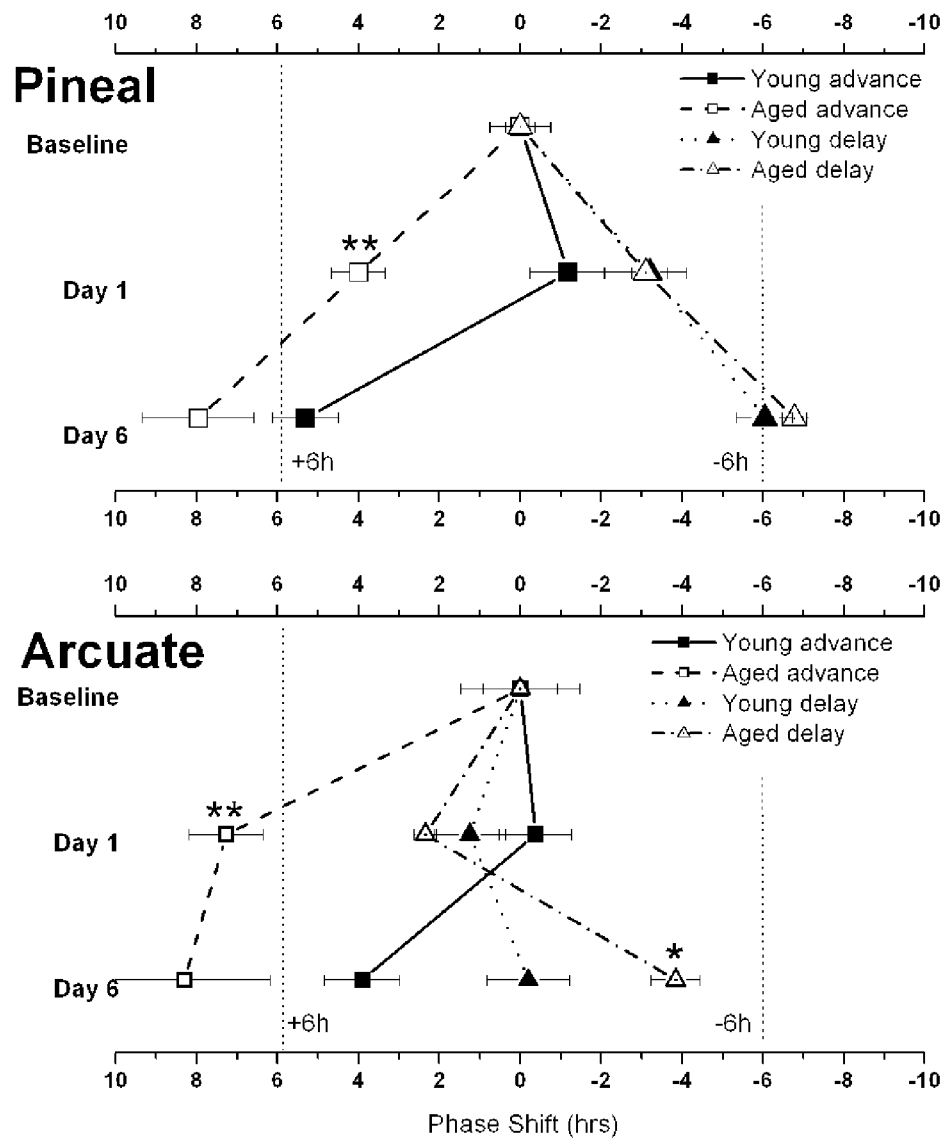
Figure 1.
Resynchronization of SCN and liver *Per1-luc* rhythms after phase-shifts A. Phase shift schedules. Asterisks indicate the times at which cultures were prepared for *Per1-luc* measurements. B. Typical examples of bioluminescence recordings from liver cultures prepared either before the shift, or on the first or 6th day following a phase-delay. The arrows indicate the peaks used as phase markers for subsequent analysis of rhythm phase. C. Peak-phases of SCN and liver cultures during resynchronization in young and aged rats. The phases from unshifted rats were normalized to 0, and subsequent phase measurements plotted relative to those baselines. The vertical dotted lines indicate the target phases that the tissues are moving towards. Students t-test; *p<0.05 relative to young controls.

Figure 2.
Resynchronization of Pineal gland and arcuate nucleus *Per1-luc* rhythms after phase-shifts in young and aged rats. Conventions are the same as in Fig. 2. Student's t-test; *p<0.05 and ** p<0.002 relative to young controls.

Table 1

Mean phases of tissue explant cultures after delays or advances of the light cycle. Reported here are the mean group peak phase times (hrs *in vitro*), standard errors of the means, and the group sizes (# of cultures contributing to the mean / # of cultures attempted). Some tissues were arrhythmic and therefore provided no phase data. Three of 8 aged animals intended for advance Day 6 cultures died prior to the sixth day, preventing culture preparation.

		YOUNG			AGED		
		Phase	SEM	n	Phase	SEM	n
SCN	Baseline	30.804	0.852	4/4	30.3904	0.202	9/9
	Advance Day 1	47.081	1.396	6/6	26.123	0.656	6/6
	Advance Day 6	24.433	0.624	4/4	25.224	0.999	5/5 +3 died
	Delay Day 1	36.293	0.23	5/5	34.62033	0.75988	8/8
	Delay Day 6	36.6043	0.608	4/4	38.254	0.442	4/4
Liver	Baseline	41.733	0.5043	4/4	46.409	1.13933	8/9
	Advance Day 1	41.9056	1.186	6/6	42.92	1.02466	6/6
	Advance Day 6	36.72224	0.5989	6/6	44.46	1.34119	4/5 + 3 died
	Delay Day 1	41.047	0.164	5/5	41.2	1.34226	9/9
	Delay Day 6	25.4667	1.1549	4/4	25.3504	2.026	2/2
Pineal gland	Baseline	43.6709	0.36	4/4	41.793	0.767	9/9
	Advance Day 1	44.852	0.929	3/6	37.8	0.66669	6/6
	Advance Day 6	38.361	0.811	6/6	33.84	1.3891	5/5 +3 died
	Delay Day 1	46.77	0.45	5/5	44.893	1.0116	9/9
	Delay Day 6	25.62084	0.695	4/4	24.571	0.3064	2/2
Arcuate nuc.	Baseline	42.52	0.916	4/4	43.656	1.477	5/5
	Advance Day 1	42.8999	0.90277	6/6	36.384	0.91263	5/6
	Advance Day 6	38.62	0.931	4/6	35.36	2.13016	3/5 + 3 died
	Delay Day 1	41.277	0.87	5/5	41.31252	0.27084	2/2
	Delay Day 6	42.7209	1.017	4/4	47.496	0.6114	3/3