

Research paper

Chronic circadian advance shifts abolish melatonin secretion for days in rats

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ABSTRACT

Melatonin deficiency has been proposed to underlie higher risks for cardiovascular and several other diseases in humans experiencing prolonged shiftwork. However, melatonin secretion has not been monitored longitudinally during consecutive shifts of the light:dark (LD) cycles in the same individuals (animals or humans) and the extent of melatonin deficiency is unknown in individuals experiencing consecutive LD shifts. We investigated the effect of consecutive LD shifts on melatonin secretion in adult F344 rats using continuous online pineal-microdialysis. The rats were entrained to the 12 h:12 h LD cycle before the shifts. The LD cycle was then advanced (n = 5) or delayed (n = 4) for six hours every four days for four consecutive times. The rats exhibited marked asymmetry in response to delay or advance LD shifts. While rats exposed to the repeated LD delay shifts always exhibited melatonin secretion throughout the entire periods, repeated LD advance shifts suppressed nocturnal melatonin secretion for several consecutive days in the middle of the 3-week period. Moreover, melatonin offset after LD delay and melatonin onset after LD advance determined the rate of circadian pacemaker reentrainment. Additionally, melatonin offset was phase locked at the new dark/light junctions for days following LD advance. These data demonstrate that chronic LD shifts are deleterious to melatonin rhythms, and that this effect is much more pronounced during advance shifts. These data may enhance our understanding of impact of LD shifts on our circadian timing system and benefit better design of shiftwork schedules to avoid melatonin disruption.

1. Introduction

Modern society demands circadian dysregulation. Almost 15% of Americans (> 20 million individuals) work full time on evening shift, night shift, rotating shifts, or other employer arranged irregular schedules. Shiftworkers have lower levels of melatonin (Hunter and Hershner-Figueiro, 2017), the neurohormone easily disrupted by the changes of the light:dark (LD) cycle (Papantoniou et al., 2014). Despite the association of its deficiency with multitudes of health issues in shiftworkers, melatonin levels have not been determined longitudinally at high temporal resolution in shiftworkers.

Melatonin production in the pineal gland is driven by the suprachiasmatic nucleus (SCN), the central pacemaker of the circadian system in the brain (Borjigin et al., 1999; Chatteraj et al., 2009). Melatonin is a reliable circadian marker for both animal (Illnerová and Vanecek, 1987; Drijshtout et al., 1997; Liu and Borjigin, 2005) and human (Lewy et al., 1999; Klerman et al., 2002; Benloucif et al., 2008) circadian studies. Melatonin production is initiated when clock-controlled norepinephrine is released from superior cervical ganglia. Light

exposure at night rapidly reduces melatonin production in both humans (Zeitler et al., 2000; Brainard et al., 2001) and animals (Chatteraj et al., 2009; Huang et al., 2010). A shift of the LD cycle can dramatically alter the patterns of melatonin secretion (Drijshtout et al., 1997; Liu and Borjigin, 2005; Kennaway, 1994) or activities of melatonin synthetic enzyme, N-acetyltransferase (Illnerová et al., 1987). Melatonin secretion has not been monitored before, during, and after multiple shifts of the LD cycle in these models.

Unlike other circadian markers (locomotor activity, temperature, cortisol, etc), melatonin rhythms exhibit well defined onset and offset phases in both animals and humans (Benloucif et al., 2008; Liu and Borjigin, 2006). Following a delay (Liu and Borjigin, 2005) or advance (Drijshtout et al., 1997; Illnerová et al., 1989; Van Cauter et al., 1998) shift of the LD cycle, melatonin onset and offset shift in different rates depending on the direction of the LD shifts. These data suggest the importance of monitoring both onset and offset phases of melatonin rhythm for accurate assessment of circadian phase. In majority of human circadian research including studies of shiftworks, however, melatonin onset, or dim light melatonin onset (DLMO), is the only

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marker used (reviewed in (Benloucif et al., 2008; Pandi-Perumal et al., 2007)). The role of melatonin offset in assessment of circadian phase has not been examined carefully. Our lab established a protocol wherein online pineal-microdialysis is coupled to high performance liquid chromatography (HPLC), which allows for weeks of melatonin sampling directly from the pineal gland of freely moving rodents in as little as 10–20 minute intervals. We employed this protocol to show that reentrainment of the circadian pacemaker following an LD cycle delay in rats is a much slower process than previous studies suggested, and that melatonin onset and offset must both be taken into account in circadian studies (Liu and Borjigin, 2005). In this study, the pineal microdialysis/HPLC technique was extensively utilized to probe the impact of repeated shifts of LD cycles on the secretion of melatonin in adult rats in an effort to further the understanding of the effects of shiftwork on melatonin rhythms in humans.

2. Materials and methods

2.1. Ethic statement

All experiments were conducted in accordance with the University of Michigan Animal Care and Use Committee policies.

2.2. Subjects

Adult (209–224 days of age at start of experiment) male inbred Fischer 344 rats ($n = 9$) were purchased from Harlan (Harlan, Indianapolis, IN) and acclimatized in our housing facility for at least one week prior to experimentation. All animals were housed in a LD cycle of 12:12 h (lights on at 6 am) and provided with *ad libitum* food and water. Shifts of the LD cycle were achieved by adjusting periods of illumination as supplied by white fluorescent lamps (400lx at cage level). For delay LD shifts, the duration of the light period was *lengthened* for 6 additional hours in the first cycle during each of the 4 shifts, while the duration of dark period was maintained at 12 h. For advance LD shifts, the duration of the light period was *shortened* by 6 h in the first cycle during each shift, while the duration of dark period was maintained at 12 h. The rats were divided into two groups for the experiment. Five rats were subjected to chronic (every 4 days, or 4D, for 4 times) six hour (6h) advance (A) shifts of the LD cycle (4 × 4D × LD6hA). The additional 4 rats were subjected chronic (every 4 days, or 4D, for 4 times) six hour (6h) delay (D) shifts of the LD cycle (4 × 4D × LD6hD). The University of Michigan Committee on Use and Care of Animals approved all experimental procedures.

2.3. Construction of the pineal microdialysis probe

Probes were constructed from blunt tip needles of two different configurations to provide an outer support shaft for prevention of bending during dialysis. A 25-gauge needle 1.0 in. in length – to come into direct contact with dialysate – was inserted into a 21-gauge needle 0.5 in. in length and glued to the base of a plastic luer with epoxy. Two such needle shafts were then bent forming a left and right-side probe. A 1.5 in. semipermeable microdialysis hollow fiber was inserted into the right probe followed by a 2.0 in. tungsten rod to function as a guide. This configuration was fixed with epoxy glue.

2.4. Surgical implantation of the pineal microdialysis probe

The surgical implantation of probes for pineal microdialysis was conducted on each rat using a method modified from a published protocol (Borjigin and Liu, 2008) and under strictly aseptic conditions. The rats were anesthetized lightly first using a combination of ketamine (10 mg/kg, i.m.) and xylazine (2 mg/kg, i.m.). The animal's head was shaved and positioned in a stereotaxic instrument with the head flat. For the rest of the surgery, anesthesia was provided by 1.8% (1.5–2%)

isoflurane. The skull was exposed by a 2 cm coronal incision between the two ears along the interaural line. Three stabilizing stainless steel screws 1 mm in diameter were placed to allow the positioning of the probes on the skull. Two small burr holes were gently created on both sides of the skull. The smaller hole on the right side was ~0.5 mm in diameter, which prevented the tip of the 25-gauge dialysis needle from penetrating the skull, whereas the larger hole on the left side was ~1 mm in diameter and allowed the probe to easily exit the skull during implantation. Next, the right probe was carefully pushed into the brain tissue through the pineal from the right side of the skull leaving the epoxy ball outside of the skull. Following the completion of probe insertion, the epoxy on the left side was removed using a cautery and the tungsten rod was then carefully pulled out of the probe. The excess dialysis fiber was cut and the hollow fiber tip was then secured to the tip of the second part of the probe using epoxy. The probe setup was fixed to the anchor screws on top of the skull with dental cement. Finally, the muscles and skin were sutured. The entire procedure took less than two hours per animal. The animals were returned to their cages, housed individually, and allowed to recover from the surgery for more than 4 days before microdialysis recording proceeded.

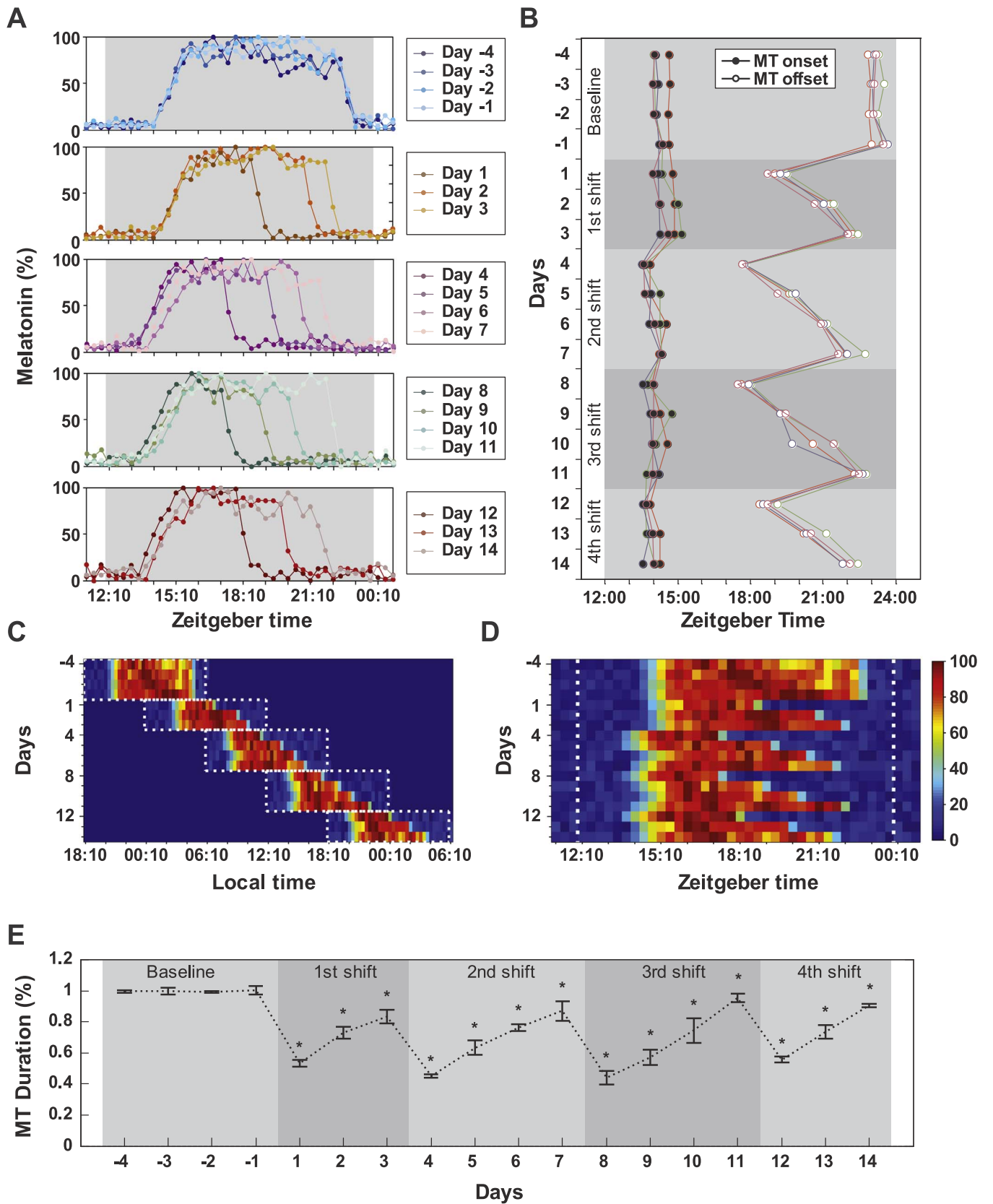
2.5. In vivo measurement of melatonin secretion

An automated system combining microdialysis with real-time high-performance liquid chromatography (HPLC) was utilized for measurement of melatonin secretion in subjects. The HPLC system consisted of one Shimadzu SCL-10A VP controller, two Shimadzu LC-20AD isocratic pumps, a CTO-20AC column oven containing 2 Supelco C18 reversed phase columns, two RF-10AXL detectors, two VICI Cheminert® sample injectors (2-position/10-port actuator), and a VICI digital sequence programmer.

Rats were first implanted with pineal microdialysis probes as described above. Each system was designed to analyze data from four rats, with two rats to each detector. Following recovery, animals were placed within a light-controlled microdialysis chamber that held 2 cages. Rats were allowed to move freely throughout due to a swivel mounted on a counterbalance arm. The 21-gauge needle base was connected with PEEK tubing and a syringe to link two rats each to an Instech peristaltic pump. Microdialysis was performed at a continuous 2 μ L/min flow rate with an artificial cerebrospinal fluid (CSF) solution consisting of NaCl (148 mM), KCl (3 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1.4 mM), $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (0.8 mM), $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ (0.8 mM), and $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$ (0.2 mM). Pineal dialysates were collected for two rats 10 minutes at a time and delivered to a sample loop (Instech, Plymouth Meeting, PA, USA) during which time previously collected samples for two rats were injected by a VICI Cheminert® sample injector (2-position/10-port valve) into a reversed phase C18 column, 250 \times 4.6 mm with 5 μ m packing (Sigma, St Louis, MO, USA) maintained at 45 °C. A Shimadzu LC-20AD isocratic pump (Shimadzu, Tokyo, Japan) delivered the mobile phase, which consisted of 34% methanol with ~10 mM sodium acetate, at 1.5 mL per minute. Staggering sample collection and analysis in this way allowed for each rat to be analyzed every 20 minutes online by a Shimadzu fluorescence detector (excitation: 280 nm; emission: 345 nm). The automated control was carried out with an external computer using Shimadzu chromatography software. Melatonin is a naturally fluorescent indole, allowing for direct detection by the fluorescent detectors. Data collection and sequence processing was performed on CLASS-VP firmware from Shimadzu. Melatonin data was collected for 20 days for all subjects.

3. Results

Rats' responses to repeated LD shifts were displayed in 5 different panels in both Figs. 1 and 2.



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Fig. 1. Four consecutive 6-hour delays of the LD cycle ($4 \times 4DxLD6hD$ shifts). **A.** Melatonin secretion pattern of one representative rat during 4 days of baseline (Day -4 to Day -1; blue tracings in the top panel), 3 days of 1st LD6hD shift (Day 1 to Day 3; orange colored tracings in the 2nd panel), 4 days of 2nd LD6hD shift (Day 4 to Day 7; pink colored tracings in the 3rd panel), 4 days of 3rd LD6hD shift (Day 8 to Day 11; green colored tracings in the 4th panel), and 3 days of 4th and the last LD6hD shift (Day 12 to Day 14; brown colored tracings in the 5th panel). Melatonin levels were normalized in each 24 h cycle to its nocturnal peak levels. **B.** Melatonin secretion patterns of 4 individual rats before (Baseline) and after each consecutive LD6hD shift. Melatonin levels are determined at 20% of the maximum nocturnal values for each night and for each rat. Circles with black filling denote melatonin onset time and those with white filling denote melatonin offset time. **C.** Melatonin secretion patterns are shown as a heat map with color indicating levels of secretion: warmer colors indicate higher melatonin secretion. The progression of melatonin's daily response was marked in local time. **D.** Same as in C., except that the progression of melatonin's daily response was marked in zeitgeber time. **E.** Melatonin duration change (marked at 20% max) for all 4 rats, before (days -4 to -1) and following each of the 4 LD6hD shifts. Melatonin duration showed significant reduction from the baseline values for each of the 14 days ($p < 0.05$).

3.1. 6-hour delays of the LD cycle: $4 \times 4DxLD6hD$ shifts

Under baseline conditions (days -4 to -1), rats were stably entrained to the LD cycle, as evident from the nearly identical melatonin secretion profile and reproducible melatonin onset and offset timing (Fig. 1A-D). Immediately following the 1st 6 h delay shift of the LD cycle (LD6hD) on day 1, melatonin (MT) onset delayed rapidly to a position very close to its expected phase angles in the new LD condition (Fig. 1B and D). Melatonin offset shift was slower (Fig. 1A, B, and D), such that melatonin duration was shortened markedly for one cycle (Fig. 1E). For the next two days (days 2 and 3), while melatonin onset remained the same, melatonin offset made large shifts (Fig. 1A, B, and D), such that melatonin duration was nearly restored to the baseline levels (Fig. 1E).

Following the 2nd, 3rd, and 4th LD6hD shifts, melatonin secretion followed a similar pattern of change: it was shorter immediately following the LD delay, which gradually restored to baseline values over the course of 4 days. All 4 rats showed consistent patterns of shifts (Fig. 1B and E): shorter melatonin duration on day 1 in the new LD schedule, which gradually lengthened due to the delay shifts of melatonin offset (Fig. 1A and B). When a heat-map was used to track the shifts of melatonin secretion (Fig. 1D), melatonin onset phase angle was reproducibly phase advanced, relative to the time of dark onset, at the beginning of the 2nd, 3rd, and 4th delay shifts, which showed further delay during the subsequent days (Fig. 1B and D).

3.2. 6-hour advances of the LD cycle: $4 \times 4DxLD6hA$ shifts

Similar to the cohort of rats exposed to LD delay shifts in Fig. 1, rats in the advance shift cohort also showed stable entrainment to the LD cycle before the LD shifts in days -4 to -1 (Fig. 2): both onset and offset of melatonin secretion appeared with reproducible phase angles relative to the onset of darkness (Fig. 2A-D). Following the 1st LD advance shift of 6 h (LD6hA), melatonin offset was truncated by the earlier light onset (day 1 in panels A-D), where it remained for 3 more days (days 2–4 in panels A-D). On the other hand, melatonin onset made little move from its baseline position for three days and begun to shift on day 4 (Fig. 2C). Following the 2nd LD6hA shift on day 5, melatonin secretion was nearly abolished for one rat (Fig. 2A and B), absent in four of the five rats tested (Fig. 2B), and was entirely absent in all five rats for 3 additional nights (days 6–8) (Fig. 2B). After the 3rd LD6hA shift on day 9, melatonin continued to be absent (Fig. 2A) and this finding was reproduced in all five rats (Fig. 2B). Two of the rats, shown in light blue and orange traces in Fig. 2B, continued to have no melatonin secretion on day 10, the first two days during the 3rd shift. On day 10, melatonin reappeared with phase positions not predicted by baseline phases (days -4 to -1) for both onset and offset (Fig. 2A and B). Days 11 and 12 were marked by shifts of both onset (advance) and offset (delay), resulting in a partial recovery of melatonin secretion duration (days 10–12) (Fig. 2A-D). On day 12, the last day during the 3rd advance shift, melatonin onset was very close to the expected phase angle of melatonin onset at baseline (Fig. 2A and B), while offset was still phase advanced relative to the light onset in comparison with baseline profile (Fig. 2A). Following the last LD6hA shift, melatonin secretion appeared to reproduce patterns seen for the 1st LD6hA shift, with shorter melatonin duration on first day of the shift followed by gradual advance shifts of onset (days 13–16; Fig. 2A-E). Melatonin offset was

never reentrained by the end of 4 days in each of the advanced LD schedules (Fig. 2A, B, and D).

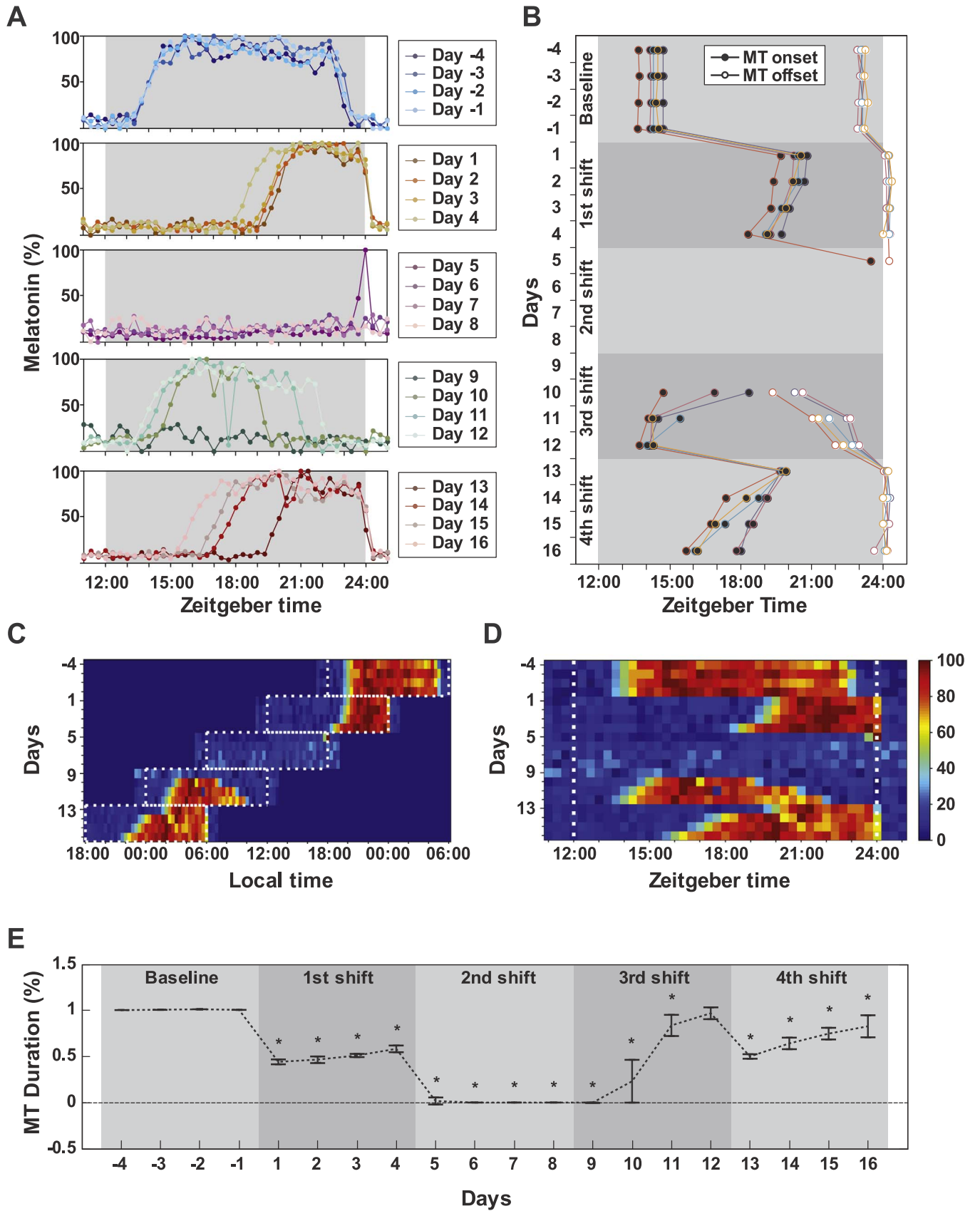
4. Discussion

The effects of simulated shiftwork on melatonin secretion were investigated in an inbred strain of rats in this study. To our knowledge, this is the first study that followed melatonin secretion longitudinally at high resolution (every 20 minutes) before, during, and following repeated shifts of LD schedule. This study revealed several new features of circadian response to repeated shifts of the LD cycle that were previously unknown: (1) delay and advance shifts of the LD cycle produce a markedly asymmetric outcome on melatonin secretion; (2) repeated advance shifts of the LD schedule can cause a complete suppression of melatonin secretion for days; (3) when the LD cycle was delayed, melatonin *offset* marks the rate of reentrainment, while during LD advance, melatonin *onset* marks the rate of entrainment of melatonin rhythm.

Reentrainment to a single delay shift of the LD cycle of 3, 6, and 12 hours was previously reported by our laboratory in outbred Sprague-Dawley (SD) rats (Liu and Borjigin, 2005). The immediate shift of onset and rapid shift of offset of melatonin secretion was seen, regardless of the magnitudes of the LD delay shift. The inbred F344 rats in the present study responded to the LD6hD shift in a very similar manner, suggesting this phenomenon is common in all rats. Thus, for delay LD shift, single or successive shifts, melatonin *offset* sets the rate of reentrainment. Currently, both melatonin onset (DLMO) and offset (DLMOFF) are used to define circadian phases only in a few studies (Barger et al., 2004; Smith and Eastman, 2008). In a majority of human studies, DLMO is the only circadian phase marker used (Benloucif et al., 2008; Pandi-Perumal et al., 2007). Routine use of both DLMO and DLMOFF may promote better understanding of melatonin as the hands of the circadian clock.

Reentrainment of melatonin rhythms to a single advance shift of the LD cycle of 8 h was previously reported (Kalsbeek et al., 2000). Five days following LD advance of 8 h, Wistar rats appeared to have been reentrained to the new LD cycle at melatonin onset, while offset is far from the expected phase position (Fig. 2A in (Kalsbeek et al., 2000)). Following 5 additional days in the new LD cycle, while melatonin offset got closer to the expected phase, melatonin onset appeared to have been over-shooted with a shorter phase angle of onset (Fig. 2B in (Kalsbeek et al., 2000)). This data suggests that reentrainment to 8-h advance shift of the LD cycle is a slow process in Wistar rats. Melatonin onset of the Fisher rats tested in our study showed very little advance shifts 4 days after the first LD6hA shifts. If we were to estimate the time these rats may take to complete melatonin onset shift, based on the slope of onset shift from day 1 to day 4 (Fig. 2B), 8–12 additional days appear to be needed. Even then, it is unknown how quickly melatonin offset could reestablish the expected phase angle in the new LD cycle. These data suggest that reentrainment of melatonin rhythms to LD advance shifts of 6–8 h is a slow process (> 12 days) in rats regardless of the strain.

The most striking finding of our study, shown in Fig. 2, is the complete disappearance of melatonin secretion for multiple nights during days 5–9 after the first advance shift. It is worth noting that when the dark period was shifted to 6 am–6 pm on day 5, the light-on



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Fig. 2. Four consecutive 6-hour advances of the LD cycle ($4 \times 4DxLD6hA$ shifts) in five rats. **A.** Melatonin secretion pattern of one representative rat during 4 days of baseline (Day -4 to Day -1; blue tracings in the top panel), 4 days of 1st LD6hA shift (Day 1 to Day 4; orange colored tracings in the 2nd panel), 4 days of 2nd LD6hA shift (Day 5 to Day 8; pink colored tracings in the 3rd panel), 4 days of 3rd LD6hA shift (Day 9 to Day 12; green colored tracings in the 4th panel), and 4 days of 4th and the last LD6hA shift (Day 13 to Day 16; brown colored tracings in the 5th panel). Melatonin levels in each 24 h cycle were normalized to its nocturnal peak levels. **B.** Melatonin secretion patterns of 5 individual rats before (Baseline) and after each consecutive LD6hA shift. Melatonin levels are determined at 20% of the maximum nocturnal values for each night and for each rat. Circles with black filling denote melatonin onset time and those with white filling denote melatonin offset time. **C.** Melatonin secretion patterns for one rat are shown as a heat map with color indicating levels of secretion: warmer colors indicate higher melatonin secretion. The progression of melatonin's daily response was marked in local time. **D.** Same as in C., except that the progression of melatonin's daily response was marked in zeitgeber time. **E.** Melatonin duration change (marked at 20% max) for all 5 rats, before (days -4 to -1) and following each of the 4 LD6hA shifts. Melatonin duration showed significant reduction from the baseline values for each of the 16 days ($p < 0.05$).

time (6 pm) in the new LD cycle coincided with the timing of melatonin onset on day 4, which occurred at 6 pm (Fig. 2A and C). Melatonin secretion during day 5 could be interpreted as light-mediate suppression. However, despite the continued availability of darkness in the next three cycles (days 5–9), no melatonin release was observed. The behaviors of this later cycles are difficult to interpret using the same argument. In a study from my lab (unpublished), we exposed entrained rats to 24 h constant light for a single cycle and returned them to 12:12 h LD cycle afterwards. As expected, melatonin rhythms disappeared during the night of light exposure, and recovered partially as soon as the LD cycle was restored. In a marked contrast, the rats in the present experiment lost melatonin secretion for more than 4 days. We believe that the absence of melatonin secretion in this study is a reflection of the impact of repeated shifts of LD cycle on the circadian timing system, rather than a simple masking effect.

Previous studies reported distinct reentrainment profiles of pineal rhythms in response to an 8-h advance of the LD cycle in outbred rats (Illnerová and Vanecek, 1987; Drijfhout et al., 1997). Pineal rhythms were abolished 2 days following the LD8hA shift in all tested rats (Illnerová and Vanecek, 1987; Drijfhout et al., 1997). Five days later, some rats still had no melatonin rhythms at night, while others regained melatonin secretion with a narrower melatonin duration (Drijfhout et al., 1997). Melatonin duration expanded further during the next five days following the LD shift, largely due to the shortening of phase angle of melatonin offset (Figs. 1–3 in (Drijfhout et al., 1997)). This data demonstrates the large inter-individual variation of the strategies outbred Wistar rats adopt for reentrainment to LD advance shifts. Our data suggest that the inter-individual variation in reentrainment rate, though exists (see days 5, 10, and 16 in Fig. 2B), is much smaller in inbred Fisher rats than those reported in outbred rats.

Repeated shifts of LD cycle in aged mice are associated with increased mortality (Davidson et al., 2006). Intriguingly, mortality is significantly higher in mice exposed to advanced, rather than delayed, LD shifts (Davidson et al., 2006). Our data, which demonstrate the association of higher degree of melatonin disruption with advance LD shifts compared to LD delays, validate the asymmetric impact of LD shifts on rodent physiology observed in Davidson's study.

While directional impact of single LD shift on pineal melatonin synthetic enzyme (N-acetyltransferase) rhythms has been reported (Illnerová et al., 1989), our study is the first demonstrating the directional impact of repeated LD shifts on disruption of melatonin secretion: chronic advance shifts resulted in dramatically more deleterious effect than chronic delay shifts. The mechanism underlying the asymmetric impact of repeated LD shifts (delay vs. advance) should be explored in future studies. Furthermore, LD shifts that resemble more realistic shiftwork scheduling should be conducted in future studies.

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Conflict of interest

None.

I declare that there are no conflicts of interest associated with this project and its authors.

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