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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; Chattoraj et al., 2009). Melatonin is a reliable circadian marker for both animal (Illnerová and Vanecek, 1987; Drijfhout 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 (Zeitzer et al., 2000; Brainard et al., 2001) and animals (Chattoraj et al., 2009; Huang et al., 2010). A shift of the LD cycle can dramatically alter the patterns of melatonin secretion (Drijfhout et al., 1997; Liu and Borjigin, 2005; Kennaway, 1994) or activities of melatonin synthetic enzyme, N-acetyltransferease (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 (Drijfhout 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 (400 lx 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 (6 h) advance (A) shifts of the LD cycle (**4 × 4DxLD6hA**). 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 × 4DxLD6hD**). 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 μL/min flow rate with an artificial cerebrospinal fluid (CSF) solution consisting of NaCl (148 mM), KCl (3 mM), $CaCl_2{\cdot}2H_2O$ (1.4 mM), $MgCl_2{\cdot}6H_2O$ (0.8 mM), $Na_2HPO_4{\cdot}7H_2O$ (0.8 mM), and $NaH_2PO_4{\cdot}H2O$ (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 × 4.6 mmm with 5 μm 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.