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Effects of age on circadian rhythms are similar in wild-type and heterozygous *Clock* mutant mice

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Abstract

The amplitudes of many circadian rhythms, at the behavioral, physiological, cellular, and biochemical levels, decrease with advanced age. Previous studies suggest that the amplitude of the central circadian pacemaker is decreased in old animals. Recently, it has been reported that expression of several circadian clock genes, including *Clock*, is lower in the master circadian pacemaker of old rodents. To test the hypothesis that decreased activity of a circadian clock gene renders animals more susceptible to the effects of aging, we analyzed the circadian rhythm of locomotor activity in young and old wild-type and heterozygous *Clock* mutant mice. We found that the effects of age and the *Clock* mutation were additive. These results indicate that age-related changes in circadian rhythmicity occur equally in wild-type and heterozygous *Clock* mutants, suggesting that the *Clock* mutation does not render mice more susceptible to the effects of age on the circadian pacemaker.

Keywords: Aging; Circadian rhythm; Clock; *Clock* mutation

1. Introduction

Aging is associated with changes in the mammalian circadian timing system. These changes include decreases in the amplitude of many overt rhythms, including the rhythms of locomotor activity, drinking, body temperature, and the sleep–wake cycle [19,24,28,29,34], as well as corresponding decreases in the amplitudes of at least two rhythms of suprachiasmatic nucleus (SCN, site of the master mammalian circadian pacemaker) physiology: the rhythms of neural firing rate and of glucose uptake [25,31,35]. Additionally, species-specific changes in the free-running period in constant darkness (τ) are often observed [15,21,27,28] Aging does not alter the size of the SCN or the number of neurons it contains [14,36], suggesting that the effects of age are due to changes in the pacemaking capabilities of the neurons or the connections between them.

Current models of the mammalian circadian clock suggest that individual neurons are capable of generating circa 24-h rhythms [33]. These rhythms are produced by interlocking molecular feedback loops of transcription, translation, and repression of transcription [22]. Recently, we [11] and others [1] reported that aging is correlated with decreased expression of several of the genes that make up these feedback loops, including both *Clock* and its binding partner, *Bmal1*. These results led us to hypothesize that animals which carry a mutation in a clock gene clock might be more susceptible to the effects of age on the circadian rhythms.

The *Clock* mutant mouse presents a model to test this hypothesis, as both old rodents and *Clock* mutant mice exhibit dampened circadian rhythms at the behavioral [20,30] and electrophysiological levels [7,17,31], as well as altered expression of circadian clock genes. *Clock* mutant mice carry a point mutation in the *Clock* sequence, which leads to the skipping of exon 19 in the mature mRNA, thereby producing a shorter protein [10]. The mutant form of the protein is able to dimerize with BMAL1, its binding partner, but cannot activate transcription of E-box-containing sequences. This leads to decreased accumulation of downstream protein products (including, but not limited to, the PER homologs) within the cell [8]. At the behavioral level, heterozygous

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Clock mutant mice have a circadian period approximately 25 h; homozygotes show 28-h rhythmicity for several cycles, followed by arrhythmicity in the circadian range [30].

Given the important role of *Clock* in generating circadian rhythms in young mice [30], and the fact that old rodents show decreased expression of several circadian genes, we hypothesized that animals with a defective circadian clock would be more susceptible to the effects of age on the circadian timing system. In the present set of experiments, we examined the circadian rhythm of locomotor activity in wild-type and heterozygous *Clock* mutant mice at 3 and 18 months of age. We found that the effects of age on the circadian timing system are essentially independent of *Clock* genotype. These results suggest that the age-related changes in circadian rhythms cannot be explained by changes in *Clock* activity alone.

2. Materials and methods

2.1. Animals

All animals were bred and born at the Center for Experimental Animal Resources at Northwestern University. Wild-type (C57BL/6J) and heterozygous *Clock* mutant mice (on a coisogenic C57BL/6J background [30]) were used. Animals were designated as either young (approximately 3 months old at the start of the experiment) or old (at least 18 months old at the start of the experiment). Some of the old animals had previously been exposed to varying light–dark (LD) cycles and running wheels when they were young.

2.2. Procedure

Animals were individually housed in cages equipped with a running wheel; a microcomputer running Chronobiology Kit software (Stanford Software Systems, Stanford, CA) recorded each revolution of the running wheel. The cages were kept in light-tight boxes equipped with a 40 W fluorescent lamp. Food and water were available ad libitum throughout the experiment. Animals were maintained in a 12 h/12 h LD cycle for at least 3 weeks. They were transferred to constant darkness (DD) by extension of the dark phase. Three weeks later, they were given a 6-h light pulse (40 W fluorescent lamp, 300–400 lx) beginning at circadian time CT 17; young *Clock* heterozygotes show significantly larger phase shifts to such light pulses [13]. They were returned to DD and after 10 days were sacrificed at CT 6. Brains were rapidly extracted, frozen on dry ice, and stored at −80 °C until in situ hybridization.

2.3. Probe template preparation

Total RNA was extracted from mouse brains with Trizol (Life Technologies, Bethesda, MD) following the manufacturer's protocol. Approximately 1 μg of RNA was reverse-transcribed (RT) with MMLV-RT (Promega, Madison, WI, 200 U final concentration) in the presence of dNTP (Promega, 500 μM), random hexamer primers (Promega, 2.5 ng/μl), RNAse inhibitor (Promega, 20 U), and RT reaction buffer (Promega, 1×). The RNA and primers were heated to 70 °C for 10 min and quenched on ice for 2 min. The remaining reaction components were added, incubated 10 min at room temperature, then for 60 min at 37 °C, and heat-inactivated for 5 min at 70 °C. An aliquot (1 μl) of this reaction product served as the template for PCR reactions. The following primer pairs, purchased from Integrated DNA Technologies (Coralville, IA), were used to PCR-amplify cDNAs with a T7 RNA polymerase recognition site immediately 5′ of the antisense sequence: *mPer1*: 5′-CCG GAA TTC AGC TCT GCT GGA GAC CAC TGA-3′ and 5′-TAA TAC GAC TCA CTA TAG GGA GAG TGT ATT CGG ATG TGA TAT GCT CC-3′; *mPer2*: 5′-ACG AGA ACT GCT CCA CGG-3′ and 5′-TAA TAC GAC TCA CTA TAG GGA GAA CAG CCA CAG CAA ACA TAT CC-3′. The amplification conditions were: 94 °C for 3 min, followed by 25 cycles of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 1 min, followed by a final incubation at 72 °C for 5 min. The PCR was carried out with AmpliTaq (Perkin-Elmer) in the presence of primers (250 nM each), dNTP (200 μM), and PCR buffer (1×). PCR products were electrophoresed on 1% agarose gels and the bands of the expected size were excised and gel-purified with QiaEX II (Qiagen, Valencia, CA).

2.4. In situ hybridization

The brains were sectioned on a cryostat at 20 μm through the SCN. Alternate sections were collected on separate slides for hybridization with either *mPer1* or *mPer2* antisense riboprobes. Sections were thaw-mounted onto gelatin coated slides and frozen at −80 °C until ready for hybridization. All reagents were from Sigma Chemical Co. (St. Louis, MO) except as indicated. Slides were thawed under cool air, fixed for 5 min in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). They were treated with 2× SSC for 5 min, dipped in DEPC-treated water, dipped in 0.1 M triethanolamine (TEA, pH 8.0), and acetylated for 10 min in 0.25% acetic anhydride in TEA. They were dipped briefly in 2× SSC, dehydrated through an ethanol series, and stored under vacuum until completely dry. ^{33}P-labeled riboprobes were transcribed from PCR templates (see above) with Ambion's MaxiScript kit (Ambion Inc., Austin, TX). Probe was diluted in hybridization solution (50% formamide (v/v), 300 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate (w/v), 750 μg/ml yeast RNA (Sigma R7125)) to a final concentration of approximately 5×10^7 cpm/ml. Sections were hybridized overnight at 48 °C in a humid chamber. The next day, coverslips were removed, and the sections were soaked in 4× SSC for 10 min twice. They were treated with RNAse A (20 μg/ml in 2× SSC) for 30 min at 37 °C, washed in 2×