Neurobiology of Aging 34 (2013) 1589–1598

Contents lists available at SciVerse ScienceDirect

Neurobiology of Aging

journal homepage: www.elsevier.com/locate/neuaging

Aging-like circadian disturbances in folate-deficient mice

Etienne Challet [a,*], Stéphanie Dumont [a], Madah K.M. Mehdi [a], Caroline Allemann [a], Tiffanie Bousser [b], Sylviane Gourmelen [a], Dominique Sage-Ciocca [c], David Hicks [a], Paul Pévet [a], Bruno Claustrat [b]

[a] Department of Neurobiology of Rhythms, Institute of Cellular and Integrative Neurosciences, CNRS UPR3212 associated with University of Strasbourg, Strasbourg, France
[b] Department of Chronobiology, Institute "Stem cell and brain", INSERM U846, Bron, and Hormone Laboratory, Hospices Civils de Lyon, France
[c] Chronobiotron, UMS3415, CNRS and University of Strasbourg, Strasbourg, France

ARTICLE INFO

Article history:
Received 26 June 2012
Received in revised form 30 August 2012
Accepted 26 November 2012
Available online 25 December 2012

Keywords:
Circadian rhythm
Folic acid
Homocysteine
Aging
Photic resetting
Jet-lag

ABSTRACT

The elderly population shows various circadian disturbances, including dampened amplitude of rhythmicity and decreased responsiveness to light. The common poor folate status in the elderly might account for these aging-related circadian disturbances. To test this hypothesis, we investigated whether folate deficiency in mice affects circadian oscillations of the master clock in the suprachiasmatic nuclei, and the shifting responses to light. Mice fed a diet without folate for 6 weeks displayed markedly reduced (4.5-fold) erythrocyte folate concentration and increased (2.3-fold) homocysteinemia compared with control mice. Folate deficiency decreased the circadian amplitude of vasopressin and the clock protein PERIOD 2 (PER2) in the master clock, slowed the rate of re-entrainment of behavioral rhythms after delayed light-dark cycle and reduced light-induced phase-delays, without detectable morphologic changes in the retina, such as the number of melanopsinergic ganglion cells, that might have impaired photodetection. In conclusion, folate deficiency and consecutive hyperhomocysteinemia led to dampened PER2 and vasopressin oscillations in the master clock and reduced responsiveness to photic resetting, which constitute hallmarks of aging effects on circadian rhythmicity.

1. Introduction

Folate, or vitamin B_9, is a water-soluble cofactor involved with vitamin B_{12} in metabolism of one-carbon units, allowing remethylation of homocysteine to methionine and synthesis of S-adenosyl-methionine, a methyl donor used in many biochemical transmethylation reactions (Selhub, 1999). As a consequence, folate deficiency leads notably to reduced global DNA methylation, thus affecting transcription and DNA repair (Kane, 2005), and hyperhomocysteinemia, a recognized risk factor for thrombosis (Graham et al., 1997). Considered as the most prevalent vitamin deficiency in the world, folate deficiency, either because of insufficient intake or malabsorption, is common in the elderly and certain pathophysiological conditions like alcoholism (Allen, 2008; Hamid et al., 2009; Rosenberg et al., 1982). Patients deficient in folate are prone to neuropsychiatric disturbances, such as mood disorders and Alzheimer's dementia (Kronenberg et al., 2009; Reynolds, 2006).

The circadian timing system allows behavior and physiology to cycle in phase with daily environmental changes. The sleep/wake cycle is controlled by a master clock in the suprachiasmatic nuclei of the hypothalamus, mainly reset by light cues perceived by the retina. Secondary clocks present in peripheral organs including the liver, are synchronized by the suprachiasmatic clock and can be influenced by various extracellular signals, in particular those associated with feeding (Golombek and Rosenstein, 2010). At the molecular level, central and peripheral clocks involve clock genes such as Clock, Bmal1, Period1-2, Cryptochrome1-2, Rev-erbα–β and Rorα–β, to generate self-sustained rhythmicity (Dardente and Cermakian, 2007; Ko and Takahashi, 2006). Folate biosynthesis is functionally interconnected with the molecular clockwork (Zhang et al., 2009). Mammalian cryptochromes are circadian transcriptional repressors that might use folate as cofactor (Ozgur and Sancar, 2003). Furthermore, folate-deficient rodents display markedly decreased rhythms of melatonin secretion, a major hormonal component of the circadian system (Fournier et al., 2002), and dampened day-night variations in a number of hepatic genes, especially those involved in fatty acid metabolism and DNA synthesis (Champier et al., 2012). Aging is associated with several circadian alterations, including decreased amplitude of rhythmicity, change in endogenous period, and decreased responsiveness to light (Benloucif et al., 1997; Skene and Swaab, 2003; Touitou, 1995; Weinert, 2000). Together, these findings suggest that poor folate status might negatively affect the circadian timing system, thus

* Corresponding author at: CNRS and University of Strasbourg, Institute of Cellular and Integrative Neurosciences, Department of Neurobiology of Rhythms, 67000 Strasbourg, France. Tel.: (+33) 3 88 45 66 93; fax: (+33) 3 88 45 66 54.
E-mail address: challet@inci-cnrs.unistra.fr (E. Challet).

0197-4580/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.neurobiolaging.2012.11.021

favoring disruption of circadian rhythms and possibly accounting for aging-related circadian disturbances.

The first hypothesis to be tested is whether deficit in dietary folate can impair circadian rhythmicity and photic synchronization. The second hypothesis is to test if folate deficiency mimics aging-associated circadian perturbations. For these purposes, we investigated circadian oscillations not only of the master clock but also of a secondary clock (i.e., liver), and the circadian responses to light in folate-deficient mice.

2. Methods

2.1. Animals, housing, and diet

Six-week-old male C57BL6JRj mice were purchased from Janvier Breeding Centre (Le Genest Saint Isle, France). This strain of mice hardly synthesizes melatonin at night (Vivien-Roels et al., 1998). Mice were housed in individual cages with running wheels, kept at 21 ± 1 °C under a 12-hour light-dark cycle (lights on at 7:00 AM) and had ad libitum access to food (standard low-fat diet, 105, SAFE, Augy, France) during 3 weeks of baseline. Thereafter, mice were randomly assigned to the control group ($n = 40$), which received a synthetic diet (SAFE) containing 8 mg of folic acid plus 10 g of succinylsulfathiazole per kilogram diet, or to the folate-depleted group ($n = 40$), which was fed an otherwise identical diet, but without folate. To inhibit de novo folate synthesis by the gut microflora, succinylsulfathiazole, a non-absorbable antibiotic drug, was added to the diet to enhance the level of folate depletion, as previously described (Champier et al., 2012; Fournier et al., 2002). The nutritional treatment lasted 4 weeks before the onset of chronobiologic studies and was maintained for 2 weeks until tissue sampling. Body mass and food intake were measured weekly. Mice were maintained under a light-dark cycle (12 hours each; lights on 7:00 AM, lights off 07:00 PM) unless stated otherwise.

2.2. Experimental design

In a first series, 40 mice were exposed either to phase advance of the light-dark cycle ("jet-lag" test with advance shift; $n = 10$ per nutritional treatment) or to phase delay of the light-dark cycle ("jet-lag" test with delay shift; $n = 10$ per nutritional treatment). On the day of the advance shift, time of lights on was phase-advanced by 6 hours, so that the dark period was shortened to 6 hours ("long day") to reach a new light-dark cycle (12 hours each; lights on 1:00 AM, lights off 1:00 PM). On the day of the delay shift, time of lights on was delayed by 6 hours, so that the dark period was lengthened to 18 hours ("short day") to reach the new light-dark cycle (12 hours each; lights on 1:00 PM, lights off 1:00 AM). Two weeks later, mice deeply anesthetized with overdose of intraperitoneal pentobarbital were killed every 6 hours starting at Zeitgeber time (ZT) 0, with ZT12 defining lights offset ($n = 5$ per time point and nutritional treatment).

In animals exposed to a light-dark cycle, synchronization of the suprachiasmatic clock to light mainly occurs at light-dark and dark-light transitions (i.e., dawn and dusk). In nocturnal animals housed in constant darkness, the active period defines the subjective night. During that daily period, discrete pulses of light produce phase-delays or advances when applied in early or late subjective night, respectively. In a second series, 40 mice were transferred to constant darkness (DD). A first group of 20 mice was used for actimetry ($n = 10$ per nutritional treatment). On the first subjective night, 10 animals ($n = 5$ folate-deficient mice and $n = 5$ control mice) were exposed to a 30-minute fluorescent white light pulse (200 lux recorded at the level of the animals) at projected ZT (pZT) 13 (pZT12 corresponding to the time of light offset the day before) and 10 other animals ($n = 5$ folate-deficient mice and $n = 5$ control mice) were exposed to this light pulse at pZT22. Their free-running activity was recorded 8 days in constant darkness. A second group of 20 mice was used for c-FOS (i.e., Fbj OsteoSarcoma viral oncogene homolog) immunohistochemistry ($n = 10$ per nutritional treatment). On the first subjective night, 10 animals ($n = 5$ folate-deficient mice and $n = 5$ control mice) were exposed to light pulses at pZT13 or pZT22 as above, and these mice were killed in darkness 1 hour after the beginning of the light pulse.

2.3. Sample collection

After overdose of intraperitoneal pentobarbital, intracardiac blood was collected into plastic tubes containing ethylene diaminetetraacetic acid (EDTA). An aliquot of whole blood was taken to measure erythrocyte folate, and the remainder was centrifuged, and the plasma stored at −20 °C until assayed. The animals were then euthanized by decapitation, and a liver aliquot was frozen in a tube immersed into liquid nitrogen and stored at −80 °C for further studies. Brains and eyes were also removed, fixed in 0.1 M phosphate buffer containing 4% paraformaldehyde for 24 hours. Thereafter, eyes were rinsed and stored in 0.1 M phosphate buffered saline (PBS). Brains were further cryoprotected in 30% sucrose for 72 hours, frozen in pre-cooled isopentane, and stored at −80 °C.

2.4. Biochemical analyses

Erythrocyte folate concentrations were measured by RIA Kit SimulTRAC-SNB (MP, Diagnostics Division; reference #06B264806). The limit of sensitivity of folate assay was 0.5 ng/mL (i.e., 1 nM). Intra-assay coefficients of variation were 7% at a 40 ng/mL concentration. Plasma total homocysteine concentrations were measured by high performance liquid chromatography, using the fluorometric method described in (Ubbink et al., 1991). Sensitivity of the homocysteine assay was 0.5 μM. Intra-assay coefficients of variation were 5% at a 10 μM concentration. For both assays, all samples were analyzed in the same run.

2.5. RNA extraction and quantitative real-time PCR

Samples of frozen livers were homogenized in lysis buffer supplemented with β-mercaptoethanol and total RNA was extracted according to the manufacturer's protocol (Absolutely RNA Miniprep Kit, Stratagene, Agilent Technologies). The RNA samples were further purified by precipitation with sodium acetate and isopropyl alcohol. RNA quality was evaluated with the Bioanalyzer 2100 (Agilent Technologies; RNA integrity number for all samples was >7). RNA quantity was measured using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies; A260/A280 and A260/A230 values were >1.8). cDNAs were synthesized from 1 μg of total RNA using the High Capacity RNA-to-cDNA Kit (Applied Biosystems). Quantitative real-time polymerase chain reaction (PCR) was performed using the 7300 Real-time PCR instrument and the TaqMan gene expression assay system (Applied Biosystems). PCR conditions were 10 minutes at 95 °C followed by 40 cycles of 15 seconds at 95 °C, and 1 minute at 60 °C.

RNA levels for *Per1* (ref. Mm00501813_m1), *Per2* (ref. Mm00478113_m1), *Cry1* (ref. Mm00514392_m1), and *Dbp* (ref. Mm01194021_m1) were normalized to *Tbp* (ref. *Mm00446971_m1*) and analyzed using the comparative delta Ct method as previously described (Asher et al., 2010). A dilution curve of the pool of all cDNA samples was used to calculate the amplification efficiency for each assay.