British Journal of Nutrition (2014), **112**, 1478–1483
doi:10.1017/S0007114514002451
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Sleep patterns in male juvenile monkeys are influenced by gestational iron deprivation and monoamine oxidase A genotype

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(Submitted 6 January 2014 – Final revision received 7 July 2014 – Accepted 16 July 2014 – First published online 15 September 2014)

Abstract

Individual differences in sleep patterns of children may have developmental origins. In the present study, two factors known to influence behavioural development, monoamine oxidase A (*MAOA*) genotype and prenatal Fe-deficient (ID) diet, were examined for their influences on sleep patterns in juvenile rhesus monkeys. Sleep patterns were assessed based on a threshold for inactivity as recorded by activity monitors. Pregnant monkeys were fed diets containing either 100 parts per million (ppm) Fe (Fe sufficient, IS) or 10 ppm Fe (ID). At 3–4 months of age, male offspring were genotyped for polymorphisms of the *MAOA* gene that lead to high or low transcription. At 1 and 2 years of age, sleep patterns were assessed. Several parameters of sleep architecture changed with age. At 1 year of age, monkeys with the low-MAOA genotype demonstrated a trend towards more sleep episodes at night compared with those with the high-MAOA genotype. When monkeys reached 2 years of age, prenatal ID reversed this trend; ID in the low-MAOA group resulted in sleep fragmentation, more awakenings at night and more sleep episodes during the day when compared with prenatal IS in this genotype. The ability to consolidate sleep during the dark cycle was disrupted by prenatal ID, specifically in monkeys with the low-MAOA genotype.

Key words: Sleep: Non-human primates: Prenatal iron deprivation: Monoamine oxidase A genotype

Sleep in primates, including humans, is characterised by a consolidated sleep phase during the dark cycle[1,2]. Sleep locations protected from predators have been seen as the origin of this pattern, which contrasts with intermittent sleep periods, peaking in frequency according to the diurnal cycle, in rodents.

Disruption of the consolidated sleep pattern is the basis of common sleep disorders in humans, including delayed onset of sleep, waking during sleep, inability to resume sleep and daytime sleepiness[3]. Non-human primates, including rhesus monkeys, have been established as valuable models for human sleep based on observation, activity monitoring and electroencephalography[4–6].

Although environmental factors and disease state are becoming known through research as sources for sleep disruption, a developmental origin for sleep regulation variability is less studied. In the present study, we assessed the influence of prenatal Fe deficiency (ID) on sleep patterns in rhesus monkeys aged 1 and 2 years, approximately equivalent developmentally to children aged 4 and 8 years. ID is the most common single-nutrient deficiency worldwide, with pregnant women and infants being the most affected. The Center for Disease Control reports that 33·8 % of pregnant women in the USA develop anaemia (http://www.cdc.gov/pednss/pnsstables), with the highest frequency being observed in the third trimester. Because of the prolonged period of third-trimester fetal brain development in primates, monkeys, rather than rodents, are the more appropriate models for studying the consequences of third-trimester ID in children. To develop a model of third-trimester ID, their dams were fed a low-Fe diet *in utero*[7].

An additional variable in the present study was genotyping for monoamine oxidase A (*MAOA*) polymorphisms (high or low expression of the enzyme MAOA). A recent study has linked *MAOA* polymorphisms to daytime sleepiness, a possible reflection of sleep fragmentation, in humans[8]. Similar *MAOA* polymorphisms occur in monkeys and have previously been shown to interact with prenatal ID to influence behaviour in social and cognitive tests in this cohort of monkeys[9,10]. Both ID[11] and *MAOA* polymorphisms[12–14] influence monoamine neurotransmitter systems in the brain. We hypothesised that these two factors could interact during fetal brain development to produce long-term changes in sleep patterns.

Abbreviations: CNPRC, California National Primate Research Center; ID, Fe deficiency; IS, Fe sufficiency; MAOA, monoamine oxidase A; VGL, Veterinary Genetics Laboratory; VNTR, variable-number tandem repeats.

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British Journal of Nutrition

https://doi.org/10.1017/S0007114514002451 Published online by Cambridge University Press

British Journal of Nutrition

https://doi.org/10.1017/S0007114514002451 Published online by Cambridge University Press

Methods

Compliance with animal research guidelines

Animal husbandry was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the National Research Council. All protocols were approved before implementation by the UC Davis Institutional Animal Care and Use Committee. The California National Primate Research Center (CNPRC) is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Animal husbandry and veterinary medicine procedures were performed by specialised staff with advanced training in these areas.

Subjects, diets and genotyping

Pregnant rhesus (*Macaca mulatta*) dams were included in the study after screening for reproductive history. Experimental groups were balanced for dam age, weight and parity, but were otherwise randomly constructed. Pregnant dams were pair-housed in double cages (120 × 65 × 79 cm) in a cage room separate from the rest of the colony. They were fed the experimental diets twice a day in premeasured amounts, and water was available *ad libitum* from an automated system. Their care included daily cleaning of drop pans, twice daily feeding, biweekly cage changes to freshly sterilised cages, automatically controlled light cycles (lights on from 06.00 to 18.00 hours), and temperature control (20–25°C) and monitoring. Monkeys were observed each morning for health signs and referred to veterinarians for treatment if needed. An individual medical record was maintained for each animal. Monkey dams were time-mated and fed an ID (10 parts per million) or an Fe-sufficient (100 parts per million, IS) diet during gestation beginning at pregnancy confirmation by ultrasound. Pregnancies with male fetuses were identified during this time. Details regarding diet composition and feeding schedules have been reported previously[7]. This dietary regimen has been shown to cause anaemia in the third trimester (Fig. S1, available online). After birth, experimental diets were discontinued. Fe repletion was provided to any dams that were anaemic and even also to neonates in the ID group. Infants were reared by their mothers in double cages with another mother–infant pair until weaning at 5–6 months of age. They were then caged with a like-age peer during a 2-year assessment battery[9] including tests described below. During the test period, groups did not differ in body weight, an index of growth, or serum Hb, a marker of ID[9] (Fig. S2, available online).

Genotyping data were obtained from colony records. Genotyping for variable-number tandem repeat (VNTR) polymorphisms in the upstream regulatory region of the rhesus *MAOA* gene (*rhMAOA-LPR*) is conducted routinely in 3- to 4-month-old rhesus monkeys at the CNPRC by the Veterinary Genetics Laboratory (VGL) using PCR with MAOA-forward and MAOA-reverse primers[9,15,16]. All PCR included a negative (no DNA template) and two positive (*MAOA* genotypes 5/5 and 6/7) controls. Genotyping via fragment size analysis was performed using VGL's STRand software. The overall genotyping error rate at the VGL is less than 0·5 %.

MAOA is an X-linked gene with at least five different VNTR polymorphisms resulting in thirteen different female genotypes and five different male genotypes in rhesus monkeys. Males in the present study were identified as hemizygous for low-MAOA (seven VNTR) or high-MAOA (four, five or six VNTR) polymorphisms resulting in four groups: high-MAOA IS (n 5); high-MAOA ID (n 5); low-MAOA IS (n 5); low-MAOA ID (n 4). Due to the presence of an ambiguous *MAOA* VNTR, one infant in the ID group was excluded from the study.

Activity monitoring

Actimeters (ActiTrac, IM Systems) that record movement were placed on the back of a specially designed vest that the monkeys wore in their home cages for 48 h[17–20]. The home cage environment had a daily 12 h light cycle (lights on from 06.00 to 18.00 hours). The actimeter links to the computer to transfer data to ActiTrac software that provides measures of the onset, duration and level of each active and inactive period. An inactive period is defined as a 2 min period (epoch) for which that epoch and the epochs preceding and following it average to be below a software-defined activity threshold of 18 counts/2 min. This threshold has been validated as a measure of sleep in children, but not in monkeys. However, studies in monkeys show a good agreement between actimeter and electroencephalography indices of sleep[5].

Subgroups of six monkeys, balanced for group, were assessed over the weekend to minimise disturbance. Regular husbandry included daily cage cleaning and twice daily feeding. All entries to the room were recorded for potential exclusion of actimeter readings if necessary. This assessment was performed twice, at 1 and 2 years of age (14 months and 22·5 months). The second 24 h of the home cage monitoring period was used for analysis. The following sleep parameters were selected for analysis: time to sleep onset at night; number of awakenings at night; total awake time at night; number of sleep episodes during the day; total time asleep during the day.

Statistics and power estimates

Selected parameters were analysed with two-way ANOVA (genotype and ID diet) including the interaction (JMP, SAS Institute, Inc.). Planned comparisons were made to assess the effect of ID diet within genotype. Potential covariates (body weight, cage location, etc.) were screened for relevance to the sleep parameters, but none were significant. Datasets were screened for normal distribution before analysis.

Group sizes of 10 per diet group were selected for the present study based on behavioural effect sizes in a previous cohort. The effect sizes for the diet × genotype interaction could not be estimated. The effect size for our apical variable, the sleep fragmentation index, comparing the ID and IS groups with the low-MAOA genotype was $d = 1{\cdot}74$. Small-sample, non-human primate studies can detect smaller

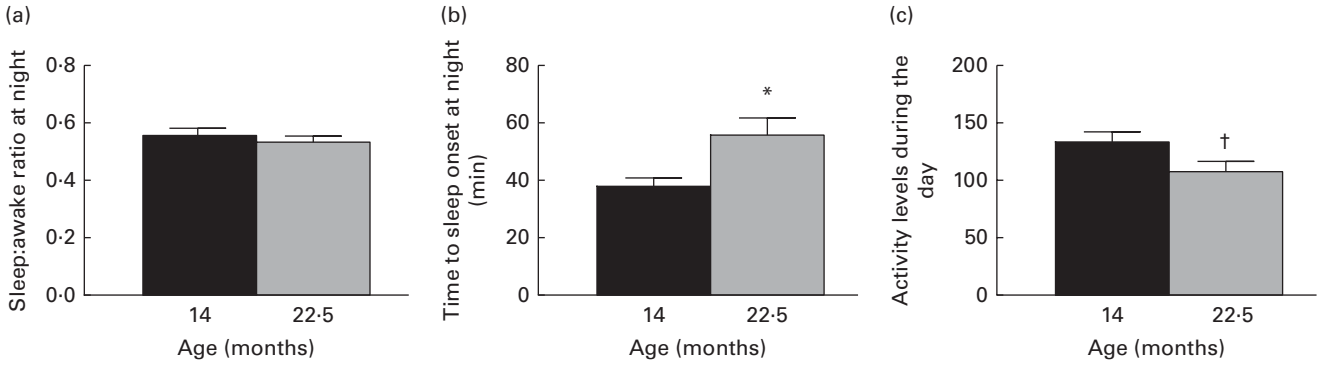

Fig. 1. Age differences in sleep patterns and activity levels at 1 and 2 years of age in male rhesus monkeys. (a) Ratio of sleep:awake time at night and (b) time to sleep onset at night. *Older monkeys took longer to fall asleep compared with younger monkeys ($P = 0{\cdot}006$). (c) Activity levels during the day. † Older monkeys were less active compared with younger monkeys during daytime hours ($P = 0{\cdot}009$).

effects compared with human studies due to strict environmental control and subject selection.

Results

Diet and genotype did not influence the growth or health of the test cohort as shown in Fig. S2 (available online). At night, about 55 % of the time was spent in the sleep (inactive) state at both ages. During the day, monkeys were inactive less than 5 % of the time, on average. The pattern of day–night activity as recorded by actimeters is shown in Fig. S3 (available online).

The time spent sleeping at night did not change with age in the test cohort as a whole (Fig. 1(a)), but older monkeys took longer to fall asleep after the onset of the dark period ($F(1,19) = 9{\cdot}72$, $P = 0{\cdot}006$; Fig. 1(b)) and had lower activity levels during the day ($F(1,19) = 8{\cdot}44$, $P = 0{\cdot}009$; Fig. 1(c)) compared with younger monkeys.

At 1 year of age, statistical trends suggested that low-MAOA monkeys slept more at night compared with high-MAOA monkeys in terms of more total inactive (sleep) time ($F(1,15) = 4{\cdot}11$, $P = 0{\cdot}061$) and greater percentage of inactive time ($F(1,15) = 4{\cdot}16$, $P = 0{\cdot}059$; Fig. 2(a) and (b)). In addition, low-MAOA monkeys were more active when they were awake at night ($F(1,15) = 8{\cdot}15$, $P = 0{\cdot}01$; Fig. 2(c)). Neither diet nor genotype influenced time to fall asleep, number of awakenings or time awake at night, or daytime sleep periods (data not shown).

At 2 years of age, the trend towards greater sleep at night in low-MAOA monkeys appeared to be reversed by ID. Awakenings at night were more frequent in low-MAOA ID monkeys than in low-MAOA IS monkeys (interaction $F(1,15) = 6{\cdot}35$, $P = 0{\cdot}023$, low-MAOA ID > IS, $P = 0{\cdot}048$; Fig. 3(a)). Conversely, low-MAOA ID monkeys had more frequent sleep episodes during the day (interaction $F(1,15) = 11{\cdot}69$, $P = 0{\cdot}004$; low-MAOA ID > IS, $P = 0{\cdot}008$; Fig. 3(b)). A significant interaction was also found for total sleep time during the day ($F(1,15) = 5{\cdot}69$, $P = 0{\cdot}031$), but no *post hoc* tests were significant. The pattern of means under the IS condition for both these endpoints suggested a difference between high-MAOA IS and low-MAOA IS monkeys. Specifically low-MAOA monkeys with adequate prenatal Fe supply had fewer nighttime awakenings (low-MAOA IS < high-MAOA IS, $P = 0{\cdot}133$) and fewer daytime sleep episodes (low-MAOA IS < high-MAOA IS, $P = 0{\cdot}008$) compared with their high-MAOA counterparts, but this pattern was reversed by prenatal ID, suggesting a fragmentation of the consolidated sleep pattern. A fragmentation index (number of awakenings at night + number of sleep episodes during the day) showed a *MAOA* × ID interaction ($F(1,15) = 9{\cdot}03$, $P = 0{\cdot}01$, low-MAOA

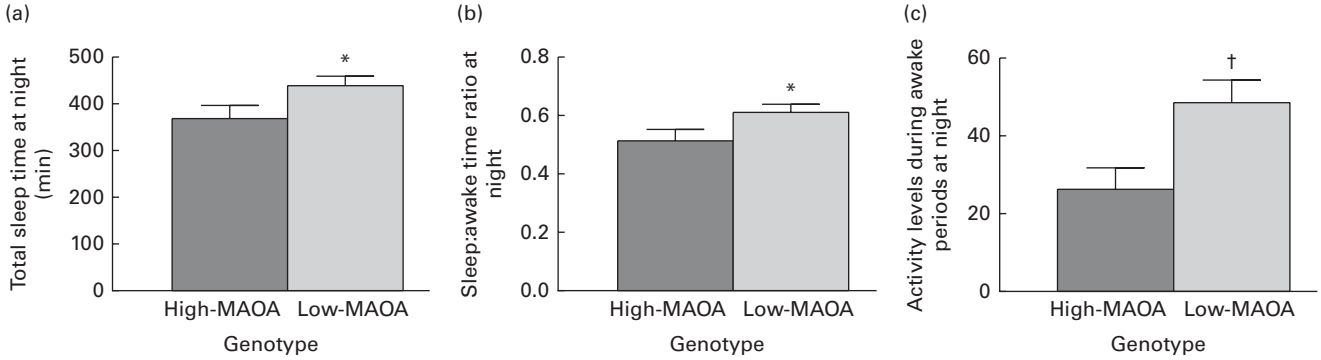

Fig. 2. Effects of monoamine oxidase A (*MAOA*) genotype on sleep patterns and activity levels in 1-year-old male rhesus monkeys. Analysis demonstrated a trend towards more sleep episodes at night and a significantly lower level of activity during the day in the low-MAOA genotype group. (a) Total sleep time at night. *A longer sleep time was observed in low-MAOA monkeys compared with high-MAOA monkeys ($P = 0{\cdot}06$). (b) Ratio of sleep:awake time at night. *A greater percentage of sleep at night was observed in low-MAOA monkeys than in high-MAOA monkeys ($P = 0{\cdot}06$). (c) Activity levels during awake periods at night. † More activity was observed in low-MAOA monkeys when they are awake at night compared with high-MAOA monkeys ($P = 0{\cdot}01$).

British Journal of Nutrition

https://doi.org/10.1017/S0007114514002451 Published online by Cambridge University Press

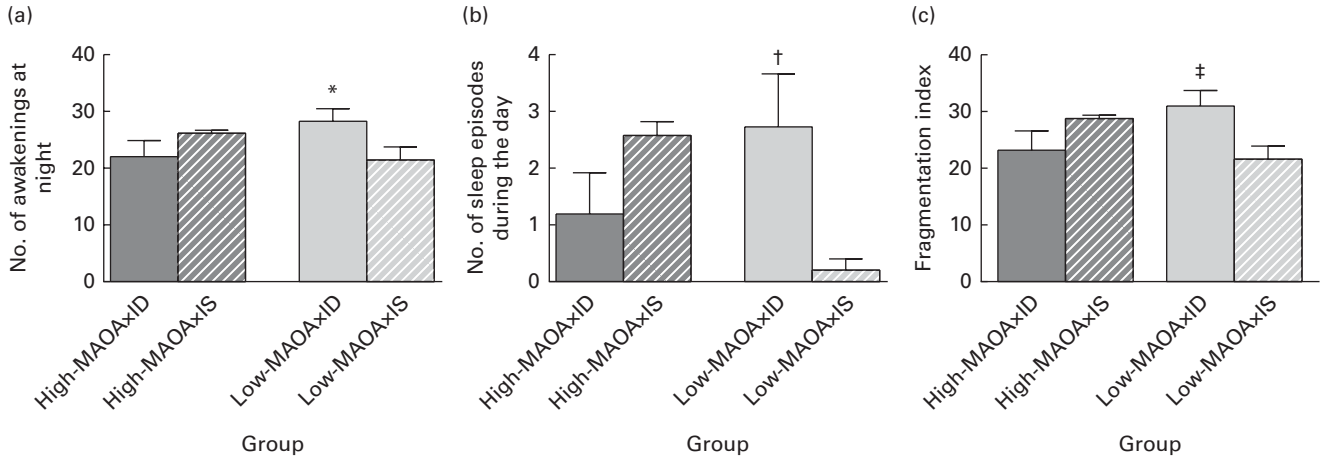

Fig. 3. Effects of the monoamine oxidase A (*MAOA*) × iron deficiency (ID) interaction on sleep patterns and activity levels in 2-year-old male rhesus monkeys. (a) Number of awakenings at night; a *MAOA* × ID interaction was observed for this parameter. *Low-MAOA ID monkeys exhibited significantly higher number of awakenings compared with low-MAOA iron-sufficient (IS) monkeys ($P = 0{\cdot}048$). (b) Number of sleep episodes during the day; a *MAOA* × ID interaction was observed for this parameter. † The low-MAOA ID group had more sleep episodes during the day compared with the low-MAOA IS group ($P = 0.08$). (c) Fragmentation index, the number of awakenings at night + the number of sleep episodes during the day. ‡ Low-MAOA ID monkeys exhibited more fragmented sleep compared with low-MAOA IS monkeys ($P = 0{\cdot}0244$).

ID > IS, $P=0{\cdot}02$; Fig. 3(c)). The pattern of means again suggested a difference between high-MAOA and low-MAOA IS monkeys (low-MAOA IS < high-MAOA IS, $P=0{\cdot}053$), which was reversed by ID.

Discussion

Research indicates that children differ widely in their sleep patterns. Although environmental conditions can be significant predictors[21], sleep patterns have trait-like stability across childhood[22]. Biological origins of trait-like sleep patterns may lie in genetics and/or early developmental influences. The present study suggests that *MAOA* polymorphisms and prenatal ID may play a role in the establishment of individual differences in sleep patterns.

The effect of prenatal ID on later sleep patterns has not been studied in children. The best-known association between ID and sleep has to do with treatment of restless leg syndrome with Fe, which is effective in children[23] as well as in adults. The levels of ferritin, an index of Fe status, were lower in the children who responded to Fe therapy for restless legs. Establishing direct links between the present study and the literature on restless leg syndrome and ID is difficult because (1) the test cohort of the present study was not ID at the time of sleep evaluation and (2) actimeters were placed on the trunk of the monkeys, rather than on a limb, so that limb movements were not recorded. However, links may be established if limb movements can be associated with night-time awakenings in juvenile monkeys with low-MAOA genotypes and prenatal ID. Also, it is possible to suggest that, during prenatal development, the fetus is receptive to the low-Fe environment and programmes its future regulation of activity to accommodate a projected lack of Fe in the postnatal environment. Rat studies have shown that Fe treatments during the developmental period of peak Fe uptake in the brain alter the expression of Fe regulatory genes in the brain throughout life[24].

Although prenatal ID has not been studied for an association with sleep regulation, follow-up studies with sleep assessment have been conducted in infants with ID, corrected by supplements. At 10 years of age, formerly ID children exhibited more awakenings at night and more leg movements as determined by electromyograph (EMG) during monitored overnight sleep[25]. Another recent study of activity in 5-year-olds after correction of infant ID has demonstrated differences in several actimeter measures including more sleep episodes during the day, more activity during sleep during the day, and greater variability in activity during sleep at night compared with controls[26]. An earlier actigraph study in this population as infants has recorded more awake time at night and more activity when awake at night in anaemic infants[27]. This finding was interpreted as reduced motor inhibition via nigrostriatal dopamine pathways, which are known to be influenced by ID[28]. The above research supports a potential role of developmental ID in the establishment of patterns of sleep regulation in children. A potential interaction between developmental ID and *MAOA* genotype could also be explored in children.

Rodent studies have shown that Fe levels in brain regions follow a diurnal pattern that is disrupted in ID[29]. ID also disrupts diurnal patterns of monoamine neurotransmitter systems[30]. MAOA levels were not measured in these studies, but indices of dopamine metabolism suggested an impact on the activity of this enzyme, which could provide a biological basis for the ID × *MAOA* interaction identified in the present study. As anticipated, *MAOA* polymorphisms have been shown to influence monoamine neurotransmitters as reflected in cerebrospinal fluid (CSF)[13]. Polymorphisms of the serotonin transporter gene, which also affect the levels of dopamine, as well as of serotonin, in the brain, have recently been shown to influence sleep patterns in adolescents[31].

British Journal of Nutrition

https://doi.org/10.1017/S0007114514002451 Published online by Cambridge University Press

Recently, a study in college students has linked low-MAOA polymorphisms to lower daytime sleepiness as assessed with a questionnaire[8]. This result, based on subjective reports of sleepiness, is generally consistent with the pattern of less daytime sleep in the control (IS) low-MAOA group compared with the control high-MAOA group in the present study (Fig. 3(b)). However, if the low-MAOA infants were Fe deprived as fetuses, the number of daytime sleep episodes increased dramatically.

The interaction pattern of means for sleep parameters suggests that ID differentially affected the two genotypes, but did not cause abnormal sleep. Although sleep fragmentation abnormalities can reach a level that signals sleep disorder, in the present study, the greater fragmentation in the low-MAOA ID *v.* IS monkeys did not lead to a level of fragmentation outside that observed in high-MAOA IS subjects. *MAOA* is X-linked so that high- and low-MAOA polymorphisms as determinants of *MAOA* transcription are clearly determined in males, but vary widely depending on allele combinations in females. Furthermore, the allele distribution of high- and low-transcribing polymorphisms in the human population is approximately 60/40, high-/low-MAOA, so that both polymorphisms are 'normal'. Thus, this interaction between *MAOA* gene transcription and fetal ID is more accurately identified as relevant to individual differences in sleep patterns than to sleep pathology. That said, this interaction in juveniles might be exaggerated in neonates or during ageing when sleep patterns are more volatile and sensitive to disruption.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0007114514002451

Acknowledgements

The authors thank the technical staff at the CNPRC for assisting with actimeter placements for monitoring. They also thank Alicia Bulleri for assisting with the technical aspects of the project and data management and paper preparation.

The present study was supported by the NIH grant P01 HD039386200 (to Betsy Lozoff (Principal Investigator); M. S. G. (Project 2 Principal Investigator)). The NIH had no role in the design and analysis of the study or in the writing of this article.

The authors' contributions are as follows: M. S. G. designed the study and wrote the manuscript; C. E. H. conducted the experimental procedures, developed the actimeter protocols and reviewed the manuscript.

The authors declare no conflicts of interest.

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