

Effect of Aging on Daily Rhythms of Lactate Metabolism in the Medial Prefrontal Cortex of Male Mice

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Abstract—Aging is associated with reduced amplitude and earlier timing of circadian (daily) rhythms in sleep, brain function, and behavior. We examined whether age-related circadian dysfunction extends to the metabolic function of the brain, particularly in the prefrontal cortex (PFC). Using enzymatic amperometric biosensors, we recorded lactate concentration changes in the PFC in Young (7 mos) and Aged (19 mos) freely-behaving C57BL/6N male mice. Both Young and Aged mice displayed diurnal and circadian rhythms of lactate, with the Aged rhythm slightly phase advanced. Under constant conditions, the Aged rhythm showed a reduced amplitude not seen in the Young mice. We simultaneously observed a relationship between arousal state and PFC lactate rhythm via electroencephalography, which was modified by aging. Finally, using RT-qPCR, we found that aging affects the daily expression pattern of Glucose Transporter 1 (GLUT-1). © 2020 IBRO. Published by Elsevier Ltd. All rights reserved.

Key words: neurometabolism, circadian, sleep, biosensor, solute transporters.

INTRODUCTION

Reduced dendritic branching in the prefrontal cortex (PFC) (de Brabander et al., 1998), and deficits in working memory (Mattay et al., 2006) and executive function (Singh-Manoux et al., 2012) are features of aging in humans and other vertebrates. Normal aging is also accompanied by changes in circadian rhythms and sleep, including blunted temperature (Weitzman et al., 1982) and melatonin rhythms (Reiter et al., 1980), and increased sleep fragmentation (Spira et al., 2017). More severe circadian dysfunction is associated with diseases of old age, including Alzheimer's Disease (Musiek et al., 2015) and Parkinson's Disease (Videnovic and Golombek, 2013).

Another hallmark of aging is alterations in neurometabolism, such as a reduction in cerebral blood flow (Fabiani et al., 2014) and changes in the concentration of various metabolites (Harris et al., 2014). Much like changes in circadian rhythms, greater severity of neurometabolic disruption is associated with diseases of old age. Reduced glucose utilization in the brain has been observed in both Alzheimer's Disease (Hoyer, 1991)

and Parkinson's Disease (Berti et al., 2013). This neural hypometabolism can be detected before the appearance of brain atrophy and clinical symptoms (Mosconi et al., 2006), making it a potentially important target for early diagnosis and treatment of age-related diseases.

Fine control of neurometabolism is necessary for brain function, because neuronal firing produces dynamic changes in local energy demand. The Astrocyte Neuron Lactate Shuttle Hypothesis (ANLS) (Pellerin and Magistretti, 1994) provides one way of understanding how these changing needs are met. In this model, neuronal activity increases extracellular glutamate, which stimulates increased glucose uptake and glycolysis in astrocytes. Within astrocytes, lactate is produced from pyruvate in a reversible manner by the lactate dehydrogenase enzyme (Ross et al., 2010). The astrocytes then release this lactate, increasing its extracellular concentration. Important to the ANLS hypothesis, and the current study, lactate can be used by nearby neurons as an energy source (Bélanger et al., 2011). Consistent with the ANLS hypothesis, lactate concentration in the frontal cortex increases during wake and REM sleep, when cortical firing rates are higher (Vyazovskiy et al., 2014), and decreases during NREM sleep (Naylor et al., 2011; Wisor et al., 2013; Lundgaard et al., 2017). Additionally, a 24 h rhythm of lactate has been observed in the somatosensory cortex, with higher lactate levels during the animal's active phase (Shram et al., 2002).

Given that changes in the PFC, circadian rhythms, and neurometabolism are all associated with the aging process, we investigated whether the PFC displays a

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Abbreviations: ANLS, Astrocyte Neuron Lactate Shuttle; CT, circadian time; GLUT-1, Glucose Transporter 1; LDH, lactate dehydrogenases; PFC, prefrontal cortex; ZT, zeitgeber time.

rhythm of extracellular lactate, and whether this rhythm is affected by aging. Additionally, since neurometabolic processes are directly related to neural activity, we examined the association between sleep state and lactate, and whether this relationship was different in Aged mice. Finally, we probed the PFC for changes in the expression of genes that are related to metabolic processes.

EXPERIMENTAL PROCEDURES

Animals

All mice were male C57BL/6N mice obtained from the National Institute of Aging aged colony at Charles River Laboratories. Aged mice arrived at our facility at 17–18 months of age. At recording, Control were 7 months old, and Aged mice were 19 months old. Both Control and Aged mice were maintained on a 12:12 light/dark cycle. All mice were single-housed and had food (Purina LabDiet 5001) and water available *ad libitum*, with 5 g of nesting material, in sound attenuated and ventilated isolation cabinets (Phenome Technologies, Chicago, IL). Since mice that underwent surgery needed to be single-housed, all mice were single-housed for at least 8 weeks before experiments to mitigate this potential confound. Light at cage level was maintained at 30–35 lux using white LEDs. Room temperature was maintained between 21 and 23 °C. All mice were acclimated to our facilities for at least 1 week prior to any procedures. All experimental procedures were approved by the Washington State University Animal Care and Use Committee.

RT-qPCR

Transcripts were analyzed using TaqMan® Low Density Array (TLDA) RT-qPCR. Mice were euthanized at one of four zeitgeber times (ZT1, 7, 13, or 19) by rapid, unanesthetized decapitation. Four to five mice were used in each distinct group/time-point. For standard RT-qPCR, whole brains were removed and frozen in powdered dry ice and stored at –80 °C until tissue punching. Tissue punches were taken using a biopsy coring tool (0.5 mm ID), as previously described (Kinlein et al., 2015). Total RNA was extracted from the tissue using Direct-Zol™ RNA MicroPrep Kit (Zymo Research, cat #R2060). cDNA was synthesized using the RNA samples as templates and the High-Capacity cDNA Reverse Transcription Kit with RNase Inhibitor (Life Technologies, cat. # 4374966). RT-qPCR was run with obtained cDNA using TaqMan Fast Advance Master Mix (Life Technologies, cat # 4444963), and TLDA Array Card 24 (Life Technology, cat # 4342249, Design ID RTGZFCW) with 24 TaqMan Gene Expression Assays situated on the card. 18s rRNA, Rn18s, and B2m were used as housekeeping genes (since B2m was the most stable of the housekeeping genes, it was used in all analyses). Cards were run on an Applied Biosystems ViiA7 RT-PCR machine. C_T values were produced, and raw data were analyzed using ViiA 7 RUO Software v1.2 (Applied Biosystems). Relative gene expression (Fold Change) was calculated using the comparative $\Delta\Delta C_T$ method (Schmittgen and Livak, 2008).

Surgical procedures

Mice were prepared for electrophysiology and lactate measurements as previously described (Clegern et al., 2012). Under isoflurane anesthesia, the skull surface was exposed, a stereotaxic apparatus was used to determine the placement of the cannula (1.95 mm anterior to Bregma, 0.25 mm lateral to the midline, –0.7 mm from skull surface), for which a hole was drilled using a 0.5 mm drill bit. Four stainless steel screw electrodes were implanted, two over the frontal lobe, and two over the parietal lobe. Two additional screws were implanted above the parietal lobe to anchor the head stage. The frontal electrodes were used to measure electroencephalogram (EEG), while the parietal electrodes were used as reference and ground. These electrodes were then soldered to a printed circuit board (PCB) with a plastic 6-pin connector (Pinnacle Technology, Inc., Lawrence, KS). Two stainless steel wires attached to the PCB were inserted into the neck muscle to record electromyogram (EMG). The electrodes and PCB were then enclosed in a light-activated flowable composite resin (Prime-Dent). Mice were then allowed at least 1 week recovery before beginning recordings.

Extracellular lactate and sleep recordings

All lactate and sleep recordings were longitudinal (within animal), such that each mouse was normalized to its own baseline. During recording, mice were single-housed in a circular 10-inch diameter sleep chamber. Food and water were available *ad libitum*. Mice were tethered through a commutator to the sleep recording system (Pinnacle Technology, Inc.) for a 24 h baseline sleep recording. For recordings in constant darkness (DD), a separate group of mice were kept in their light cycle until ZT12 (time of lights off) on day 2, after which the lights remained off. Mice that underwent recording entirely under a light dark cycle are referred to as “LD”, while those that spent the second half of the recording in darkness are referred to as “DD”. Lactate recordings began immediately after the baseline sleep recording. Lactate biosensors (Pinnacle, Inc.) were pre-calibrated with known concentrations of lactate and ascorbic acid, as per manufacturer guidelines. Approximately 1 in 5 biosensors was discarded due to ascorbic acid response. After calibration, the probes were immediately placed into the cannula of each mouse, and the mouse was re-tethered. Sleep and lactate data were acquired and archived using Sirenia Acquisition (Pinnacle Technology, Inc.). Recordings continued for ~96 h, after which the biosensors were removed from their cannulas and post-calibrated. Final group sizes for these recordings were: LD Control ($n = 8$), LD Aged ($n = 9$), DD Control ($n = 7$), and DD Aged ($n = 10$).

Sleep scoring

Archived data were loaded into Sirenia Sleep Pro (Pinnacle Technology, Inc.) for analysis. Data were parsed into 10 s epochs, and the power spectrum was calculated for EEG and EMG signals. Sleep scoring

was done using a cluster cutting technique based on the density clusters of EEG and EMG power (Rector et al., 2009). Briefly, scatter plots were generated with EEG power on the y-axis and EMG power on the x-axis. Clusters associated with low EEG amplitude and high EMG amplitude were labeled wake, high EEG amplitude with low EMG amplitude were considered NREM, and low EEG amplitude with low EMG amplitude were considered REM. This scoring was visually confirmed based on the same criteria used to cluster score. Sleep scoring was conducted by multiple individuals who were blinded to different aspects of the conditions. Spot-checks were also performed by comparing the scores to scores from someone completely blinded to all the conditions.

Data analysis

Scored sleep files were analyzed using the Sirenia SleepPro bout analysis as well as a custom MATLAB script to determine the number and length of bouts during the second day of recording (ZT18 to ZT18). Sirenia SleepPro was used to determine the number of minutes spent in each state overall, as well as during the Light and Dark periods specifically. For all sleep data, differences between groups and states or light conditions were compared using two-way ANOVAs, and further post hoc comparisons were made using Tukey's or Sidak's multiple comparisons tests as appropriate.

Lactate data were exported from Sirenia Acquisition into Excel, trimmed, and imported to MATLAB, where outlying points were eliminated and the downward trend was removed. For rhythm analysis, data were binned into 1 h intervals and a total of 48 h of data were used for further analysis. Some data points were excluded when computer issues caused a loss of more than 30 epochs per hour. Binned data were transferred into GraphPad Prism for cosinor analysis, with a user-defined equation of $Y = \text{Mesor} + \text{Amplitude} * \cos(2 * \pi * [x - \text{Acrophase}] / 24)$. This returns three parameters: the MESOR (Midline Estimating Statistic of Rhythm) which is the mean value across cycles, the amplitude of the rhythm, and the acrophase, which represents the time at which the rhythm peaks. To evaluate differences between groups, the cosinor analysis was run two groups at a time using the extra sum-of-squares *F* test, which uses sums-of-squares and degrees of freedom to compute whether data are better fit by a simpler model or a more complex model. In our analyses, the simpler model had one shared/global parameter between the groups, while the more complex model had entirely separate parameters for each group. For each pair of groups, two comparison cosinor analyses were run, one in which amplitude was the shared parameter, and another for which acrophase was the shared parameter. In all cases, differences were considered significant at $p < 0.05$. Acrophases computed by the cosinor analysis were transformed such that they are expressed in ZT.

To assess the sleep-state-dependency of lactate changes, both lactate and sleep scores were maintained in their original 10 s bins. Bouts of NREM or Wake lasting longer than 5 min on the second day of recording (ZT18 to ZT18) were used. Each animal had multiple bouts that were included in the analysis. These bouts were loaded into Prism, where the average lactate signal of the minute preceding the state transition was set to 1 and the following points were expressed as a percentage of this starting value, combining the bouts from one animal into a single average bout. After this baseline correction, average traces from animals were placed into their respective groups and a linear regression was run to determine and compare their slopes (beta coefficients). These data were then moved into RStudio, where they were plotted using the local regression smoothing method, with shaded areas representing SEM. For gene expression data, Two-way ANOVAs followed by Tukey HSD post-hoc tests were used to compare differences between groups and time points. In the case of unequal variance, a Kruskal–Wallis test was used. In all cases, means were presented $\pm$ standard error, and results were considered statistically significant at the $p < 0.05$ level.

RESULTS

Sleep fragmentation is increased by aging

Sleep state was determined by visual analysis of EEG/EMG patterns, and group comparisons were made using the number of bouts (Fig. 1A), length of NREM bouts (Fig. 1B), and the percentage of bouts that were shorter than 1 min (Fig. 1C) or longer than 5 min (Fig. 1D). The higher percentage of bouts shorter than 1 min in the Aged mice indicates that they have more fragmented sleep than their Control counterparts.

Time in sleep states is altered by aging

Aging affected the overall time spent in each sleep state (two-way ANOVA; sleep state, $p < 0.0001$; age, $p = 0.6910$; interaction, $p < 0.0001$). Aged mice spent significantly less time awake (Control, mean = 775.7 ± 26.2 ; Aged, mean = 661.7 ± 24.13 , Sidak's multiple comparisons test; Wake, $p = 0.0008$), and more time in NREM sleep (Control, mean = 609.4 ± 28.09 ; Aged, mean = 707.3 ± 20.23 , Sidak's multiple comparisons test, NREM, $p = 0.0045$) than Control mice (Fig. 2A). This effect was driven by differences in the dark period (two-way ANOVA; sleep state, $p < 0.0001$; age, $p = 0.5725$; interaction; $p < 0.0001$; Sidak's multiple comparisons test; Wake, $p = 0.0002$; NREM, $p = 0.0040$; Fig. 2B) while no significant differences were observed during the light period (two-way ANOVA; sleep state, $p < 0.0001$; age, $p = 0.9988$; interaction, $p = 0.0123$; Sidak's multiple comparisons test; Wake, $p = 0.1279$; NREM, $p = 0.0726$; Fig. 2C). There were no significant differences in REM sleep (Sidak's multiple comparisons test; Overall REM, $p = 0.9988$; Dark REM, $p > 0.9999$; Light REM, $p = 0.9922$).

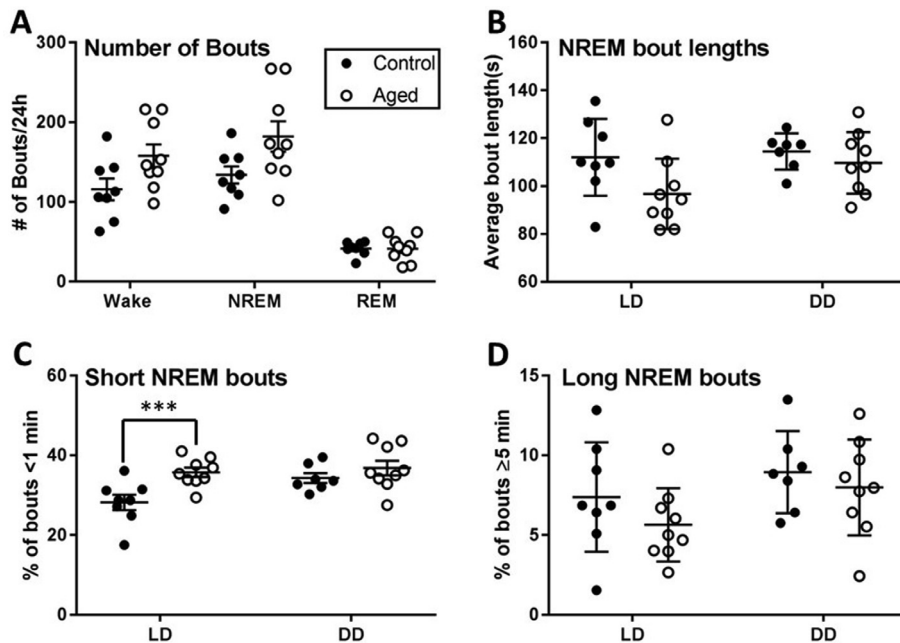


Fig. 1. Some measures of sleep fragmentation are affected by aging. **(A)** The number of bouts of each state over 24 h is not significantly different between Control and Aged mice (two-way ANOVA, sleep state, $p < 0.0001$; group, $p = 0.0052$; interaction, $p = 0.1233$; Tukey's multiple comparisons test; Wake, $p = 0.1864$; NREM, $p = 0.0922$; REM, $p > 0.9999$). **(B)** The average length of NREM bouts over 24 h is affected by age (Two-way ANOVA; light condition, $p = 0.1113$; age, $p = 0.0409$; interaction, $p = 0.2686$), but comparisons between Control and Aged mice within light conditions were not significant (Tukey's multiple comparisons test; LD, $p = 0.1092$; DD, $p = 0.8952$). **(C)** During LD (but not DD), Aged mice have a higher percentage of bouts that are less than one minute in length (Two-way ANOVA; light, $p = 0.0347$; age, $p = 0.0042$; interaction, $p = 0.1299$; Tukey's multiple comparisons test; LD, $p = 0.0043$; DD, $p = 0.4958$). **(D)** There are no statistically significant differences in the percentage of bouts longer than or equal to five minutes (Two-way ANOVA, light $p = 0.0608$ age, $p = 0.1887$; interaction, $p = 0.6985$). Error bars = SEM.

Extracellular lactate is rhythmic in the PFC and is affected by aging and light

In Control mice ($n = 8$), there was a diurnal rhythm of lactate in the PFC (cosinor analysis; Control LD amplitude = 0.007758, acrophase = 19.922; Fig. 3A).

In Aged mice ($n = 9$), the lactate rhythm was significantly phase advanced as compared to Control mice (cosinor analysis with F test, Aged LD amplitude = 0.007888, acrophase = 17.55; acrophase, $p = 0.0012$; Fig. 3B), but the amplitude was not significantly different (cosinor analysis with F test; amplitude, $p = 0.9303$). For DD experiments, after recording over one light cycle, the lights went off at ZT12 and remained off for the rest of the experiment. Under DD conditions, just like in LD conditions, the amplitude did not differ between Control and Aged groups (cosinor with F test; amplitude, $p = 0.0613$) but the acrophase of the Aged mice was still significantly phase advanced as compared to controls (cosinor analysis with F test; acrophase, $p = 0.0405$). In Control mice ($n = 7$), DD conditions did not significantly change the waveform of extracellular lactate (cosinor analysis with F test; Control DD amplitude = 0.006564, acrophase = 18.403; amplitude, $p = 0.3513$; acrophase, $p = 0.0716$; Fig. 4C). However, in Aged DD mice ($n = 10$), the amplitude of this rhythm was significantly blunted compared to the Aged rhythm in LD (cosinor analysis with F test; Aged DD amplitude = 0.004072, acrophase = 16.39; amplitude $p = 0.0032$) but the acrophase was not significantly changed (acrophase $p = 0.2054$).

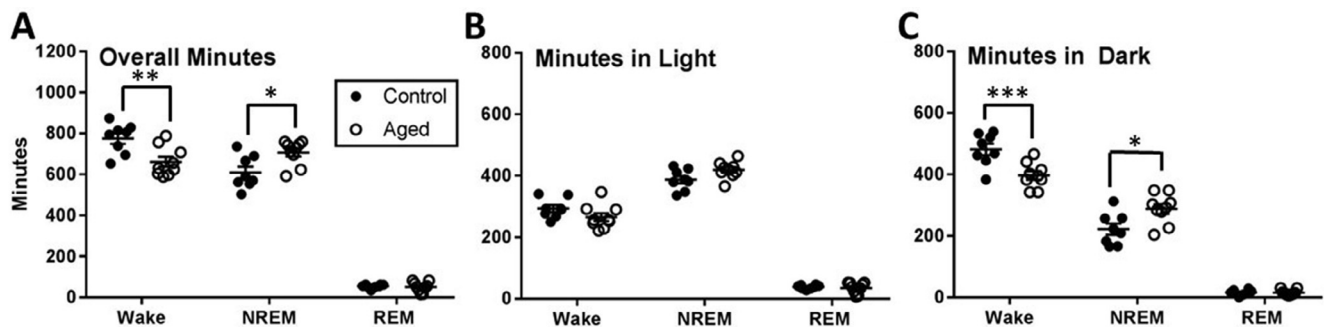


Fig. 2. Aging affects the time spent in sleep states. **(A)** Over both light and dark periods, Aged mice spend significantly less time awake and more time in NREM than Control mice (Two-way ANOVA, sleep state, $p < 0.0001$; age, $p = 0.6910$; interaction, $p < 0.0001$; Sidak's multiple comparisons test; Wake, $p = 0.0008$; NREM, $p = 0.0045$; REM, $p = 0.9988$). **(B)** There was no significant effect of Age in the light phase (Two-way ANOVA; sleep state, $p < 0.0001$; age, $p = 0.9988$; interaction, $p = 0.0123$; Sidak's multiple comparisons test; Wake, $p = 0.1279$; NREM, $p = 0.0726$; REM, $p = 0.9922$). **(C)** The overall differences were driven by differences during the Dark period (Two-way ANOVA; sleep state, $p < 0.0001$; age, $p = 0.5725$; interaction, $p < 0.0001$; Sidak's multiple comparisons test; Wake, $p = 0.0002$; NREM, $p = 0.0040$; REM, $p > 0.9999$). Error bars = SEM.

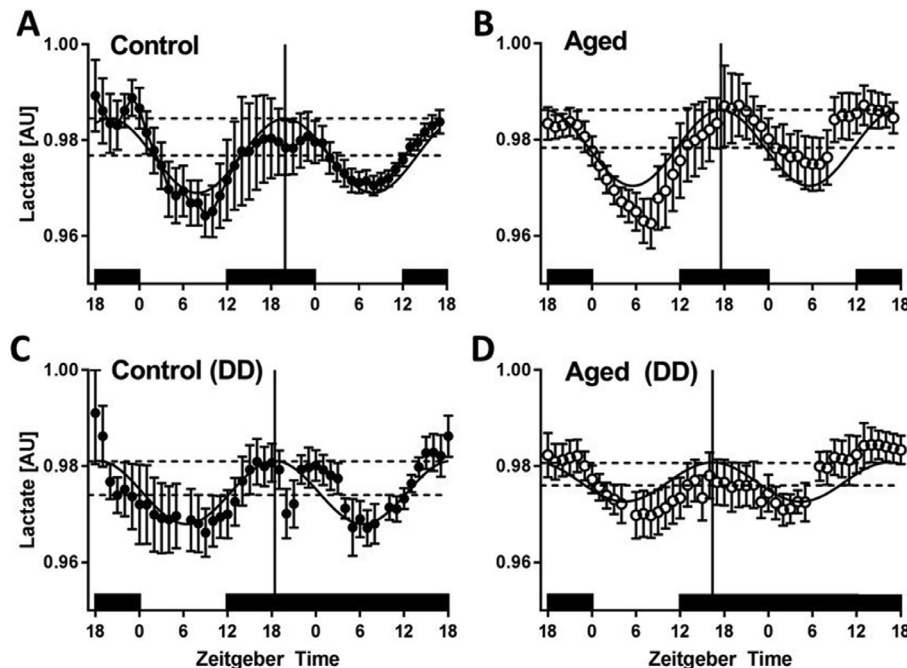


Fig. 3. A circadian rhythm of extracellular lactate is present in the PFC, and this rhythm is affected by Aging and exposure to constant darkness (DD). **(A)** Across 48 h in a 12:12 LD cycle, Control mice ($n = 8$) displayed a rhythm of extracellular lactate (cosinor analysis; amplitude = 0.007758, acrophase = 19.922). **(B)** Aged mice ($n = 9$) in a 12:12 LD cycle also displayed a rhythm (amplitude = 0.007888, acrophase = 17.55). The amplitude of Control and Aged rhythms was not significantly different (cosinor analysis with F test; $p = 0.9303$), however the acrophase was significantly phase advanced by Aging (cosinor analysis with F test; $p = 0.0012$). **(C)** A separate cohort of mice were housed as previously, but after the first light cycle, the lights were turned off at ZT12 and did not come back on for the remainder of the experiment. Across 48 h, Control mice ($n = 7$) continued to display a significant rhythm of extracellular lactate (amplitude = 0.006564, acrophase = 18.403). For Control mice, exposure to DD did not result in significant changes to the amplitude or acrophase (cosinor analysis with F test; amplitude, $p = 0.3513$; acrophase, $p = 0.0716$). **(D)** Aged mice ($n = 10$) continued to display a lactate rhythm in DD (amplitude = 0.004072; acrophase = 16.39). Under DD conditions, just like in LD conditions, the amplitude did not differ between Control and Aged groups (cosinor with F test; amplitude, $p = 0.0613$) but the acrophase of the Aged mice was significantly phase advanced as compared to controls (cosinor analysis with F test; acrophase, $p = 0.0405$). Under DD conditions, the amplitude of the Aged rhythm was significantly blunted as compared to Aged LD (cosinor with F test; amplitude, $p = 0.0032$). There was no significant difference in the acrophase of Aged LD and Aged DD (cosinor with F test; acrophase, $p = 0.2054$). Acrophases calculated by cosinor analysis are presented in ZT. Due to artifacts caused by a computer failure, some data points were removed from the graphs (see Methods for specifics). Error bars = SEM.

The relationship of sleep state and lactate is influenced by aging and light

Lactate concentration is known to change based on sleep state, with concentration increasing during Wake and REM sleep, and decreasing during NREM sleep (Wisor et al., 2013). Scored sleep from the EEG/EMG recordings was analyzed along with lactate for bouts of Wake and NREM lasting more than 30 epochs (5 min). In both Aged (LD $n = 9$, DD $n = 10$) and Control mice (LD $n = 8$, DD $n = 7$), there was a decrease in lactate during NREM sleep under both LD (linear regression, Control LD slope = -0.2536 ± 0.02154 ; Aged LD slope = -0.292 ± 0.01598 ; Fig. 4A) and DD conditions (linear regression, Control DD slope = -0.2907 ± 0.01086 ; Aged DD slope = -0.3888 ± 0.01777 ; Fig. 4B). There was no statistically significant difference in the slope between Aged and Control NREM under LD conditions ($p = 0.1576$),

but there was a difference under DD conditions ($p < 0.0001$). For Wake, there was an increase in lactate under both LD (linear regression, Control LD slope = 0.1375 ± 0.02013 ; Aged LD slope = 0.2448 ± 0.02219 ; Fig. 4C) and DD conditions (linear regression, Control DD slope = 0.191 ± 0.01384 ; Aged DD slope = 0.1901 ± 0.02889 ; Fig. 4D). There was a statistically significant difference in the slope between Aged and Control Wake under LD conditions ($p = 0.0006$) but not under DD conditions ($p = 0.9779$).

Aging affects the daily expression pattern of lactate dehydrogenase enzyme mRNAs

There are two isoforms of lactate dehydrogenase: LDHA, which preferentially converts pyruvate to lactate, and LDHB, which converts lactate to pyruvate. LDHA functions optimally in anaerobic environments and can be found in muscle and astrocytes, while LDHB functions best in aerobic environments and can be found in the heart and neurons (Ross et al., 2010). To more specifically assess the effects of aging in the PFC, we took fine PFC punches at the same coordinates as the location of the biosensors (in a separate, non-surgically manipulated set of mice). Though expression of LDHA was somewhat dynamic in Control mice ($n = 4$ /time), the difference between times was not statistically significant (Control Kruskal–Wallis

test, $p = 0.1731$; Aged one-way ANOVA, $F_{3,15} = 0.2093$, $p = 0.8884$; Fig. 5A). The expression of LDHB does not change significantly across the day in either group (one-way ANOVA, Control $F_{3,12} = 0.5996$, $p = 0.6275$; Aged, $n = 5$ /time, $F_{3,15} = 0.5371$, $p = 0.6640$; Fig. 5B).

Aging affects the daily expression pattern of mRNAs related to solute transport

Solute transporters are essential to neurometabolism, moving glucose across the blood–brain barrier (e.g. Glucose Transporter 1 (GLUT-1), (Pardridge et al., 1990)), transferring lactate out of astrocytes and into the extracellular space (e.g. Monocarboxylate Transporters 1 and 4), and transporting extracellular lactate into neurons for use (e.g. Monocarboxylate Transporter 2

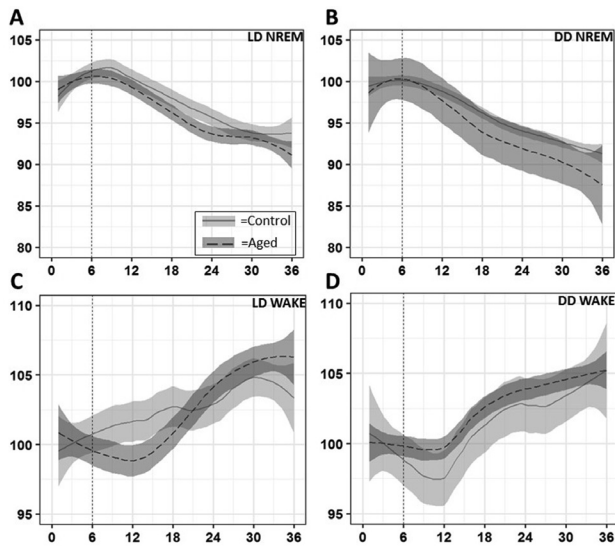


Fig. 4. The relationship between sleep state and lactate is modified by Aging and exposure to constant darkness (DD). **(A)** During NREM sleep, lactate levels fall in both groups (Control $n = 8$, Aged $n = 9$). There is no significant difference in the slope (beta coefficient) between Aged and Control mice in LD conditions ($p = 0.1576$, 95% CI of Control slope = -0.2974 to -0.2099 ; 95% CI of Aged slope = -0.3244 to -0.2595). **(B)** In DD during NREM sleep, lactate levels still fall in both groups. There is a significant difference in the slope between Control ($n = 7$) and Aged ($n = 9$) mice ($p < 0.0001$; 95% CI of Control slope = -0.3128 to -0.2686 ; 95% CI of Aged slope = -0.4249 to -0.3527). **(C)** During Wake, lactate levels rise in both groups. There is a significant difference in the Wake slope between Control and Aged mice under LD conditions ($p = 0.0006$; 95% CI of Control slope = 0.09664 – 0.1784 ; 95% CI of Aged slope = 0.1997 – 0.2899). **(D)** In DD, both groups continue to show an increase in lactate during Wake. Under DD conditions, the lactate slope of Control and Aged mice is not significantly different ($p = 0.9779$, 95% CI of Control = 0.1629 – 0.2191 ; 95% CI of Aged = 0.1314 – 0.2488). Shading = SEM.

(Bergersen, 2007)). The daily expression pattern of *Slc2a1* (GLUT-1) is dynamic in Control mice (One-way ANOVA, $F_{3,12} = 3.969$, $p = 0.0353$; Fig. 6A) and is modified by aging (Two-way ANOVA, Interaction $F_{3,28} = 3.075$, $p = 0.0438$, % of total variation = 20.74, Fig. 6A). The expression pattern of several other solute transport genes are visually different in Aged mice ($n = 5$ /time), although the differences did not reach statistical significance (Fig. 6B, Control $n = 4$ /time).

DISCUSSION

Our findings document a robust rhythm in extracellular lactate in the PFC of male mice, which persists even in constant conditions. Moreover, we demonstrate that in Aged mice, this rhythm is altered, which we posit may be related to the documented changes in PFC structure and function observed in aging. Further, we replicated previous findings that demonstrated a relationship between sleep state and change in lactate concentration (Shram et al., 2002; Naylor et al., 2011; Clegern et al., 2012; Wisor et al., 2013), and extended this to demonstrate that aging affects this dynamic relationship as well. Finally, we identified an altered expression pattern of a solute transport gene in the PFC of aged mice.

The active process by which neurons fire action potentials, and restore and maintain membrane potentials comes at a high energetic cost (Attwell and Laughlin, 2001). It is also noteworthy that neurons themselves do not possess large reserves of energetic substrates, nor the biochemical machinery that would enable rapid generation of useable energy (Cataldo and Broadwell, 1986). Thus, the energetic support for neural function likely requires complex metabolite exchange between cell types (e.g. glia). While glucose has long been considered to be the primary energy source of the brain, under some circumstances, neurons show a preference for lactate over glucose (Itoh et al., 2003). This preference is somewhat puzzling, as neurons show very slow rates of glycolysis, and cannot increase this rate without compromising intracellular anti-oxidant potential (Bolaños et al., 2010). Astrocytes, on the other hand, can rapidly produce lactate, and release it into the extracellular space (Walz and Mukerji, 1988), supporting the ANLS hypothesis (Pellerin and Magistretti, 1994). In this model, neuronal activity leads to an increase in extracellular glutamate, which stimulates glucose uptake and glycolysis in astrocytes, which release lactate into the extracellular space where it can be used by nearby neurons (Bélanger et al., 2011). This is accomplished via a suite of proteins including lactate dehydrogenases (LDH) which convert between lactate and pyruvate (Cahn et al., 1962), the solute transporters (MCTs) that shuttle metabolites in and out of cells (Bergersen, 2007), and glutamate sensors and regulators (Bélanger et al., 2011).

The PFC is a key neural regulator of executive function (Hupalo and Berridge, 2016; Jett et al., 2017) and PFC dysfunction is implicated in many mental illnesses (O'Mahony et al., 2010; Rive et al., 2013; Duman and Duman, 2015; Eden et al., 2015). In the current study, we showed that extracellular lactate in the PFC fluctuates over the 24 h day. Lactate peaks during the dark phase, when nocturnal mice are most active. This finding aligns with the ANLS model's prediction that higher levels of lactate should correspond with higher neural activity (Pellerin and Magistretti, 1994). Though a putative diurnal rhythm of lactate was previously identified in the cortex of the rat (Shram et al., 2002), that study was somewhat limited by the voltammetric recordings, which were limited in length (only 8–12 h long). A strength of our approach is that we have demonstrated this rhythm within single individuals, continuously, over several days. We also characterized this rhythm under constant conditions to determine if the rhythm was endogenously generated (i.e. circadian). When Control mice were placed in DD, the lactate rhythm displayed no significant changes in amplitude, nor acrophase. This finding further supports our hypothesis that PFC lactate has an endogenously generated circadian rhythm. Future studies should examine the mechanisms by which this rhythm is generated, and determine the relative contributions of local PFC clocks versus the SCN master clock.

Aged mice showed a phase advance in the PFC lactate rhythm, a result that echoes other work showing that sleep onset and plasma cortisol levels are both

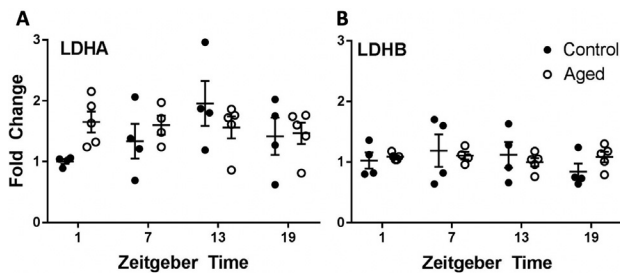


Fig. 5. Lactate dehydrogenase enzyme transcripts are not strongly rhythmic. **(A)** In Control mice ($n = 4/\text{time}$), the expression of LDHA mRNA appears dynamic, while in the Aged mice it remains similar throughout the day ($n = 5/\text{time}$). This result was not statistically significant (Interaction $p = 0.1369$). **(B)** There does not appear to be a diurnal pattern of LDHB mRNA expression in either Control or Aged mice. Error bars = SEM.

phase advanced in aged humans (Yoon et al., 2003). This effect was observed under both LD and DD conditions. Though aging is associated with reduced amplitude of several rhythms, including body temperature (Weitzman et al., 1982), melatonin secretion (Reiter et al., 1980), and sleep (Spira et al., 2017), we did not find a reduced amplitude of PFC lactate in aged mice housed in an LD cycle. However, when placed into DD, the rhythm of Aged mice was significantly blunted, as compared to Aged mice in LD conditions. Thus, our results suggest that the robust lactate rhythm observed in Aged mice in LD may in part be related to a direct effect of the light cycle known as “masking”. Masking refers to the acute responses to light or darkness that can supersede the effect of circadian rhythms. For example, nocturnal mice become more active in response to darkness, and less active in response to light (Shuboni et al., 2012). Thus, behavioral responses to the light/dark cycle can support and maintain other physiological rhythms, but when this cycle is removed, the underlying circadian rhythm is revealed. In this case, it appears that the circadian mechanisms driving lactate production are impaired in Aged mice, but this can only be seen under constant conditions. This impairment could reflect underlying problems with the molecular clock. Considering that 43% of all genes display a circadian rhythm in their transcription (Zhang et al., 2014), small changes in the function of the molecular clock could result in widespread dysfunction. Previous studies have shown that aged mice display significant differences in SCN clock gene expression, but these differences were only apparent after housing in DD for 10 days (Nakamura et al., 2015). Since the rhythm in PFC lactate is significantly blunted during just 24 h of DD, this suggests that endogenous timing mechanisms within the PFC, or synchronizing signals from the SCN to the PFC, may be impaired in the Aged mice.

Previous studies have identified a relationship between extracellular lactate, neuronal activity (as measured by EEG/EMG) and arousal state. Lactate levels rise during Wake and REM sleep, and drop during NREM sleep (Shram et al., 2002; Naylor et al., 2011; Clegern et al., 2012; Wisor et al., 2013). This finding is well aligned with the ANLS hypothesis, which predicts that extracellular lactate levels would rise during

wakefulness to support higher neural activity (Pellerin and Magistretti, 1994; Bélanger et al., 2011). In addition, during NREM sleep (and anesthesia), lactate is actively cleared from the brain by the glymphatic system (Lundgaard et al., 2017). Thus, the NREM-associated reduction of brain lactate is not simply a result of halting astrocytic lactate production, but in addition requires active transport. This may explain why lactate levels continue to climb during sleep deprivation (Naylor et al., 2011), rather than reaching a plateau. In Control mice, we replicated and extended the findings of other groups, finding a lactate increase during Wake and a decrease during NREM sleep. We found that during LD conditions, there was no significant difference in lactate during NREM sleep between Control and Aged mice (Fig. 4A). However, during DD conditions, there was a significant difference between groups in NREM lactate (Fig. 4B). This makes some sense in the context of our 48 h lactate findings. The amplitude of the lactate rhythm was blunted in Aged DD mice as compared to Aged LD mice. This may be influenced by the lactate response to NREM sleep, which has higher variability in Aged DD mice. For Wake lactate, the opposite relationship was true, with Control and Aged mice displaying significant differences during LD (Fig. 4C) but not DD (Fig. 4D). Thus, the production and clearance of lactate appear to be differentially affected by the light cycle to which an Aged mouse is exposed.

Previous studies in both humans (Kaneshwaran et al., 2019) and mice (Hasan et al., 2012; Soitani et al., 2019) have provided evidence of increased sleep fragmentation in aging. In the current study, we found evidence of increased sleep fragmentation in the Aged mice during LD but not DD (Fig. 1A). Previous research has shown that sleep fragmentation (without sleep deprivation) leads to increases in sleep pressure, indicating that the quality of sleep is reduced by fragmentation (Baud et al., 2015). This increase in sleep pressure may, in turn cause the longer NREM sleep time seen in our Aged mice (Fig. 2). The increased sleep fragmentation observed in older animals may be a result of the changes in their neurometabolism. This relationship has been observed in aged *Drosophila*, in which the manipulation of genes involved in fatty acid metabolism modulate the observed aged fragmentation phenotype, and can restore sleep and circadian parameters to those seen in healthy young flies (Laranjeira et al., 2016).

We also investigated how aging affected the diurnal expression of two genes closely related to lactate production: *Ldha* and *Ldhb*, as well as several genes involved in metabolite transport. LDHA, is an enzyme that converts pyruvate to lactate (Cahn et al., 1962). We observed a somewhat dynamic change in *Ldha* mRNA species in Control mice over the day, although it was not statistically significant (Fig. 4A). However, in Aged mice, *Ldha* expression did not appear to change throughout the day, and appeared somewhat higher. This higher *Ldha* expression in Aged mice replicates the findings of an earlier study, which found elevated levels in the brains of both aged and prematurely aging transgenic mice (Ross et al., 2010). The tissue in that study was collected

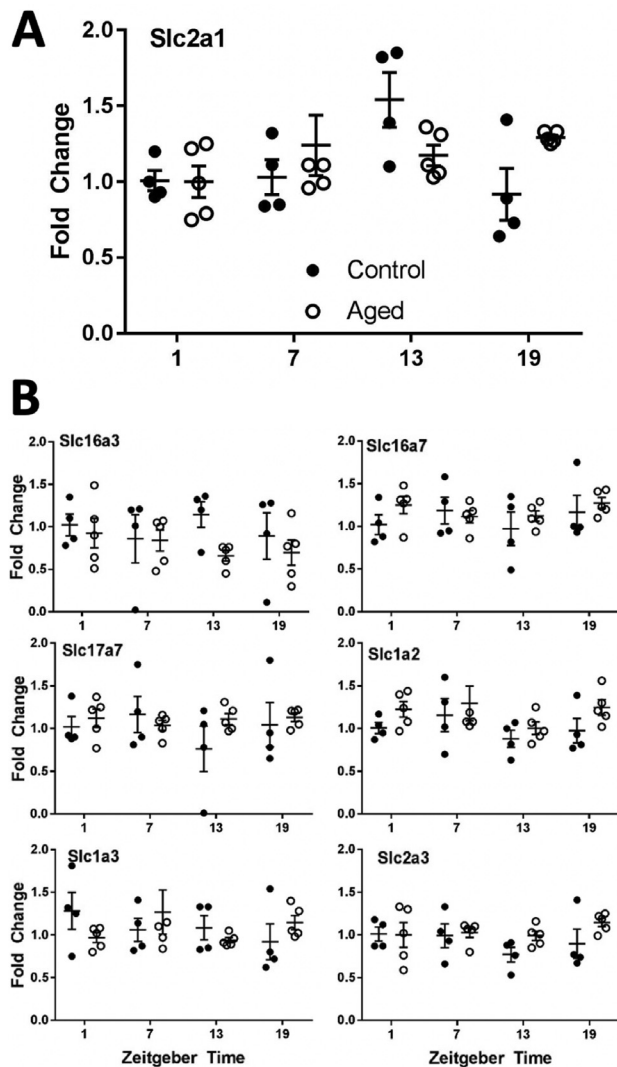


Fig. 6. The daily expression pattern of *Slc2a1* mRNA is affected by aging. **(A)** The expression of *Slc2a1* mRNA (Glucose Transporter 1) in Aged mice ($n = 5/\text{time}$) is significantly different from Controls (Control $n = 4/\text{time}$, Interaction $p = 0.0438$). **(B)** Other solute transporters have statistically non-significant, but potentially biologically relevant, differences in their daily gene expression pattern as a result of increased age. Error bars = SEM.

during the light phase (personal communication, Ross, J. M.) which corresponds with the pattern of expression that we observed, corroborating our own findings. While the previous study labeled high brain lactate as a “hallmark of aging” and implicated high *Ldha* expression as the cause, our findings add some nuance to this view. While we can neither support nor refute changes in the absolute concentration of brain lactate in old age, our findings show that the expression of *Ldha* may be time-dependent, so the higher expression in older mice does not necessarily hold at all times of day. *Ldha*, on the other hand, does not appear to have a daily rhythm in Control or Aged mice.

In Control mice, *Slc2a1* (GLUT-1) was expressed in the PFC with a robust rhythm, with higher expression during the dark period. We found that this rhythm was significantly blunted in Aged mice. GLUT-1 is

responsible for transporting glucose across the blood brain barrier (Pardridge et al., 1990). Like many other aspects of neurometabolism, GLUT-1 expression has been found to be associated with Alzheimer’s disease. People in the early stage of Alzheimer’s disease have decreased expression of GLUT-1 in their brain-derived endothelial cells (Vogelsang et al., 2018). Additionally, defects in GLUT-1 exacerbate symptoms of Alzheimer’s disease. Mice that are haploinsufficient for GLUT-1 (*Slc2a1*^{+/-}) have lower glucose in the cerebrospinal fluid and reduced cerebral blood flow. When these haploinsufficient mice also overexpress human amyloid β (*Slc2a1*^{+/-} *-APP*^{Sw/0}), they accumulate more amyloid in the brain, have increased cognitive impairment, and show greater neurodegeneration than mice with normal GLUT-1 (*APP*^{Sw/0}) (Winkler et al., 2015).

Strengths and limitations

A strength of our approach is the use of enzyme biosensors which provide improved temporal resolution over most microdialysis protocols (Wilson and Johnson, 2008). The biosensor approach also enabled longer recordings, in contrast to a previous study that identified a rhythm of lactate in the rat brain. While that study was limited to 8–12 h recording periods (Shram et al., 2002), we were able to record two full circadian cycles within the same individual. A limitation of our approach when compared to microdialysis is that we were not able to determine absolute values of lactate, but instead changes in relative concentrations. Thus our study cannot provide information about absolute differences in baseline concentration across groups. The current study was also limited by using only male mice. It is known that circadian and sleep parameters can be different in females, and males and females respond differently to perturbations of either. Thus, future studies should extend these experiments to female mice and allow for direct comparisons between the sexes.

An additional caveat is that our study design does not completely rule out the contribution of peripheral lactate, though strong evidence suggests this is likely not a critical consideration in the present preparation. Two recent studies (Takado et al., 2018; El Hayek et al., 2019) provide evidence that lactate from the periphery can pass through the blood–brain barrier. However, both of these studies examined this phenomenon in the presence of very high levels of lactate in the blood, from 13 to 20 mM (Takado et al., 2018; El Hayek et al., 2019), compared to the normal lactate concentration in mouse blood of 4.6 mM (Iversen et al., 2012). These high concentrations were achieved by bolus injection or voluntary exercise that lasted for 30 d (Takado et al., 2018; El Hayek et al., 2019). Because our mice do not have access to a running wheel, it is highly unlikely that peripheral lactate would reach sufficient concentrations to influence brain lactate. Moreover, the temporal relationship between lactate and arousal state occurs on a time scale that rule out a peripheral source.

Our findings provide evidence of a complex relationship between circadian rhythms, neurometabolic processes in the PFC, and normal aging. We found that

healthy young mice express a robust circadian rhythm of lactate neurometabolism in the PFC but found evidence that this rhythm is weakened in aging, as its amplitude is reduced during exposure to constant darkness. Future studies can expand on this work by examining absolute lactate concentration, and also by observing aged mice that have been kept in constant conditions for longer periods of time. In addition, follow-up studies should aim to elucidate the mechanisms by which this rhythm in lactate is generated within the PFC, and the contribution of local circadian clocks in its expression.

The developed world has a rapidly aging population, and a concomitant increase in age-related neurological disorders. Given that circadian and sleep rhythms degrade with age, and occur in parallel with increased neurocognitive deficits and abnormalities in affect, identifying links between these processes is critical. Neurometabolic function has also become a focus for probing underlying cellular mechanisms of disease, and evidence suggests that changes in brain metabolism may be able to predict Alzheimer's disease before cognitive symptoms become apparent (Mosconi et al., 2006). Our work provides a window into the relationships between circadian rhythms, sleep, and neurometabolic processes. This could contribute to the further development of non-invasive neurological risk biomarkers such as brain metabolism over time, as well as potential supportive or mitigating therapies. While conjecture at this point, it is important that we gain a more mechanistic understanding of the links between circadian rhythms, sleep, neurometabolism, and aging.

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AUTHOR CONTRIBUTIONS

I.N.K. and N.K.W. conceived the study and prepared the manuscript. N.K.W. performed surgeries, collected lactate data, collected tissue, and completed data analysis. F.P. scored EEG/EMG data and collected tissue for qPCR. M.S. ran RT-qPCR and assisted in its analysis.

COMPETING INTERESTS STATEMENT

The authors report no competing interests.

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