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A balanced clock: network plasticity in the central mammalian clock

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AGING AFFECTS THE CAPACITY OF PHOTOPERIODIC ADAPTATION DOWNSTREAM FROM THE MOLECULAR CLOCK

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ABSTRACT

3 Aging impairs circadian clock function, leading to disrupted sleep-wake patterns and a reduced capability to adapt to changes in environmental light conditions. This makes shift-work or the changing of time zones challenging for the elderly, and importantly, is associated with the development of age-related diseases. However, it is unclear what levels of the clock machinery are affected by aging, which is relevant for the development of targeted interventions. We found that naturally aged mice of >24 months had a reduced rhythm amplitude in behavior compared to young controls (3-6 months). Moreover, the old animals had strongly reduced ability to adapt to short photoperiods. Recording PER2::LUC protein expression in the suprachiasmatic nucleus revealed no impairment of the rhythms in PER2 protein under the three different photoperiods tested (LD: 8:16, 12:12 and 16:8). Thus, we observe a discrepancy between the behavioral phenotype and the molecular clock, and we conclude that the aging-related deficits emerge downstream of the core molecular clock. Since it is known that aging affects several intracellular and membrane components of the central clock cells, it is likely that an impairment of the interaction between the molecular clock and these components is contributing to the deficits in photoperiod adaptation.

1. INTRODUCTION

The continuing rise in life expectancy over the last decades has increased the attention for research on healthy aging. Even in healthy humans, aging is associated with fragmented sleep-wake patterns and declined circadian rhythms in eating patterns and hormone secretion, which in turn diminishes quality of life (Dijk and Duffy, 1999; Dijk et al., 1999; Carvalho-Bos et al., 2007; Froy, 2011). Aging reduces the capability of adapting to changes in light regimes, and exposure to abrupt changes in light-dark (LD) cycles even leads to a higher mortality rate in rodents (Davidson et al., 2006; Azzi et al., 2014). Aging humans show reduced season associated changes in behavior, and there is a seasonal effect on medical care needs and mortality in the elderly (Rolden et al., 2015; Cepeda et al., 2018). Moreover, several studies suggest a negative interaction between age-related neurodegenerative diseases and disturbances in circadian rhythmicity (Leng et al., 2019). It has been suggested that improving circadian rhythms in the elderly – for example with light therapy or melatonin treatment – can have beneficial effects on sleep-wake patterns. This will likely improve the quality of life, overall health and, moreover, could slow down the progression of neurodegenerative diseases (Most et al., 2010; Gaikwad, 2018).

The central circadian clock in mammals, the suprachiasmatic nucleus (SCN), is an interesting target for restoring circadian rhythms in the elderly. Age-related disruptions of rhythms in behavior and physiology can be restored by transplanting fetal SCN tissue near the hypothalamus of aged mice (Van Reeth et al., 1994; Cai et al., 1997). Moreover, it has been shown that disrupting circadian rhythms in the SCN (e.g. by knock-out of some core clock genes) induces various symptoms of premature aging in mice and rats (Kondratov et al., 2006; Dubrovsky et al., 2010). Knowledge on the functioning of the aging circadian clock will benefit the design of strategies for targeted interventions to enhance circadian rhythms in the elderly, improving their health and overall wellbeing.

A combination of molecular (e.g. clock gene expression), cellular (e.g. electrical activity), and network (e.g. neurotransmitters) elements underlie the proper functioning of the SCN. Electrical activity and neurotransmitters are important for synchronizing the SCN network, as well as for the output of the SCN to other brain areas. Aging is associated with reduced synchronization and amplitude of electrical activity rhythms in SCN neurons (Nakamura et al., 2011; Farajnia et al., 2012; Leise et al., 2013), partially due to the age-related decline in expression of important neurotransmitters, like vasoactive intestinal peptide (VIP) and γ -aminobutyric acid (GABA) (Kawakami et al., 1997; Nygard and Palomba, 2006; Palomba et al., 2008). Despite numerous studies, it is still unclear how aging affects the molecular clock in the SCN and to what extent core clock genes, like *Per2*, *Cry1*, and *Clock* are affected, while behavior is invariably been found to be affected (Asai et al., 2001; Weinert et al., 2001; Kolker et al., 2003; Wyse and Coogan, 2010; Nakamura et al., 2011; Chang and Guarente, 2013; Bonaconsa et al., 2014). Recent discoveries of small molecules with the potential to directly influence molecular clock components (Chen et al., 2018) increase the urgency to identify the best suitable components of the aging clock as targets for successful restoration of rhythmicity.

To study how aging affects circadian rhythms at the level of both the whole organism as well as the central circadian clock, we performed behavioral recordings of old (~24 months) and young (~5 months) PER2::LUC mice, and afterwards, recorded PER2::LUC gene expression characteristics in slices of the SCN. Under a 12:12 light-dark cycle (LD 12:12), we found that aging did not affect the molecular clock, as evidenced by unaltered PER2::LUC peak time and phase synchrony. Next, we investigated if challenging the circadian system by exposing mice to different photoperiods would induce differences at the behavioral level as well as the level of the molecular clock. We found that aging affected circadian behavior: old mice had reduced rhythm strength (LD 12:12) and were less able to adapt to short photoperiod (LD 8:16). However, old mice showed similar single-cell PER2::LUC rhythm characteristics after adaptation to long and short photoperiod as compared to young mice. These results suggest that the molecular clock of the aged SCN is still intact, while the behavioral phenotype is clearly affected.

2. MATERIALS AND METHODS

2.1. Animals and housing

The experiments performed in this study were conducted in accordance to the Dutch law on animal welfare. The permit (DEC 13198/PE. 16.039.001) was granted by the animal experiments committee Leiden. The homozygous PERIOD2::LUCIFERASE (PER2::LUC) mice were bred at the Leiden University Medical Center animal facility (see Buijink *et al.*, 2016). We have used young (4 – 8 months) and old (22 – 28 months) male PER2::LUC mice. The animals were kept in climate-controlled cabinets with full-spectrum diffused lighting with an intensity between 50-100 lux (Osram truelight TL), and *ad libitum* access to food and water throughout the experiment. Mice older than 20 months received, in addition to the regular food, hydration and nutritional gels as supportive care. Prior to behavioral assessment, mice were kept in groups of 2 – 5 mice in a 12h:12h light-dark (LD 12:12) cycle. During behavioral recordings, mice were kept in individual cages equipped with a passive infrared (PIR) sensor.

2.2. Behavioral analysis

Home cage activity was recorded with a PIR sensor throughout the experiment. First, behavior was recorded under LD 12:12 for at least 10 days. Then mice were exposed to either LD 16:8 or 8:16 for 28 days, which is referred to as photoperiod 1 (PP1). This was followed by a period of constant darkness (DD) for 11-14 days, before mice were exposed again to either LD 16:8 or 8:16 for at least 14 days referred to as photoperiod 2 (PP2), until the start of the bioluminescence recording of PER2::LUC. The photoperiod to which the mice were exposed was the same before and after DD. For the behavioral analysis we used: 1) the last 10 days of the LD 12:12 recordings, 2) the last 10 days of the first photoperiod exposure, 3) both 5 and 10 days from the second day of DD (marked in Figures) and 4) the last 10 days of the second photoperiod exposure, before the start of the Per2 recordings. For these time segments we determined the rhythm strength, the duration of activity (α) and resting (ρ), and relative activity level during α and ρ . Additionally, we determined the period

(τ) in DD over 10 days (free-running period). Time is expressed in projected external time (ExT), with ExT0 being the middle of the dark phase, and ExT12 the middle of the light phase.

We defined rhythm strength as the power of the F periodogram ($p = 0.05$ to peak). Alpha is defined by the interval between activity onset and offset. For this determination, activity recorded with the PIR sensors was averaged over 10 consecutive days (In DD, 5 days were used, and the period was corrected for τ , which was calculated over 10 days), and clustered in 10-minute bins. Activity onset and offset are less distinct in PIR recordings than in recordings of wheel running, on which standard methods for calculating alpha are developed. Therefore, we used the following characteristics to determine onset and offset for our recordings: the values of the highest average activity in a 10-hour bin of activity (M10), and lowest 5-hour bin (L5) was calculated (Witting *et al.*, 1990); activity was then smoothed for 2-hour bins; activity onset was defined as the first instance where the smoothed activity passed the $\frac{3}{4}$ value between L5 and M10 after the period with least activity; activity offset was defined as the last instance where the smoothed activity passed the $\frac{1}{2}$ value between L5 and M10 before the period with least activity (Figure S2).

To study the adaptation to photoperiod, we used the change in alpha from LD 12:12 to DD: Δ alpha. To obtain Δ alpha, the length of alpha for the period in LD 12:12 was subtracted from the length of alpha in DD (5 d).

2.3. Bioluminescence imaging and analysis

Slice cultures of the SCN were prepared as previously described (Buijink *et al.*, 2016). In brief, mice were killed by decapitation within one to three hours before lights off. The brain was dissected and placed in ice cold artificial cerebrospinal fluid (ACSF) with low Ca^{2+} and high Mg^{2+} . The ACSF contained (in mM): NaCl (116.4), KCl (5.4), NaH_2PO_4 (1.0), MgSO_4 (0.8), CaCl_2 (1.0), MgCl_2 (4.0), NaHCO_3 (23.8), D-glucose (15.1) and 5 mg/L gentamicin (Sigma-Aldrich, Munich, Germany), saturated with 95% O_2 -5% CO_2 and pH 7.4. From each brain, the hypothalamus, containing the SCN was isolated and sliced in 200 μm thick coronal slices with a VT 1000S vibrating microtome (Leica Microsystems, Wetzlar, Germany). The SCN was optically identified, cut out and both an anterior and posterior slice were placed on a Millicell membrane insert (PICMORG50, Merck – Millipore, Burlington, MA, USA) in a 35 mm petri dish. The dish contained 1.2 ml Dulbecco's Modified Eagle's Medium supplemented with 10 mM HEPES buffer (Sigma-Aldrich, Munich, Germany), 2% B-27 (Gibco, Landsmeer, The Netherlands), 5 U/ml penicillin, 5 $\mu\text{g}/\text{ml}$ streptomycin (0.1% penicillin-streptomycin; Sigma-Aldrich, Munich, Germany) and 0.2 mM D-luciferine sodium salt (Promega, Leiden, The Netherlands) and adjusted to pH 7.2 with NaOH.

The dish containing the slices was sealed with a glass cover slip and transferred to a temperature controlled (37 °C) and light-tight chamber (Life Imaging Services, Reinach, Switzerland), equipped with an upright microscope and a cooled CCD camera (ORCA –UU-BT-1024, Hamamatsu Photonics Europe, Herrsching am Ammersee, Germany). Bioluminescence images from the anterior and posterior slices were acquired consecutively with exposure time of 29 minutes resulting in image series with 1 h time resolution.

The bioluminescence image series were analyzed using a custom-made, MATLAB-based (Mathworks, Natick, MA, USA) program, as described in (Buijink *et al.*, 2016). In brief, we identified groups of pixels (ROIs) that showed characteristics of single-cells. Therefore, these ROI's are referred to as single-cells. The average bioluminescence was calculated for all pixels comprising the ROI's, for the image- series, resulting in bioluminescence traces representing PER2::LUC expression for each single-cell ROI. The raw traces were smoothed for further analysis of rhythm characteristics, like peak time and period. Phase distribution is defined as the standard deviation (SD) of peak time per slice, of the first cycle *in vitro*. The cycle-to-cycle interval is defined as the time difference between two consecutive half-maximum values of the rising edge of the PER2::LUC expression rhythm. The period variability is defined as the standard deviation (SD) of the cycle interval of individual cells, calculated for the first three cycles *in vitro*, and averaged per slice.

2.4. Community detection

We employed the community detection method we previously used for the identification of neuronal clusters in the SCN (Buijink *et al.*, 2016; Almog *et al.*, 2019). In short, a cross-correlation matrix was constructed from the multiple time series of PER2::LUC bioluminescence intensity traces. Followed by filtering out the local (neuron-specific) noise and global (SCN-wide) dependencies from the correlation matrix, using random matrix theory. The resulting communities have positive overall correlation within communities and negative overall correlation between communities, relative to the overall SCN activity. From a few slices the cluster locations could not be determined, this was the case for three slices in LP and one in SP (too few cells) in the anterior SCN and one slice in the posterior SCN in LP.

2.5. Statistical analysis

For the analysis of the data we used GraphPad Prism (San Diego, CA, USA). For comparing data from old and young mice we used a two-way ANOVA, followed by a Tukey's post hoc test. For the analysis of the data where we compared both old and young mice as well as LP and SP exposure, we used a one-way ANOVA, followed by a Sidak's multiple comparisons correction. Differences with $p < 0.05$ were considered significant.

3. RESULTS

3.1. PER2::LUC expression is not altered with aging under LD 12:12

Circadian rhythms in single-cell PER2::LUC expression were measured in slice cultures from old (21-28 months) and young (4-8 months) PER2::LUC mice maintained in LD 12:12 (for an example, see Figure 1A). We determined peak times and period of PER2::LUC rhythms from smoothed bioluminescence intensity traces of single SCN neurons. The average peak time was similar in slices from the SCN of young and old mice. Moreover, for both groups, peak time and period length did not differ between anterior and posterior slices (Figure 1B and C; Tukey's test, n.s.; table S1). Next, we tested whether aging affects the synchronization of PER2::LUC rhythms between SCN cells by using the standard deviation (SD) of peak times as a measure of phase distribution within slices.

In slices of the SCN from both young and old mice, PER2::LUC rhythms were synchronized to a similar degree (Figure 1D; Tukey's test, n.s.; table S1). The fluctuation of the cycle-to-cycle period was higher in the anterior SCN compared to the posterior SCN, both in slices from young and old mice (Figure 1E; Tukey's test, young: anterior vs posterior: $P < 0.05$, old: anterior vs posterior: $P < 0.05$; table S1). Combined, these results show that the PER2 expression in the SCN is unaltered by aging in the LD 12:12 light regime. Therefore, we sought to challenge the SCN in old mice using different photoperiods.

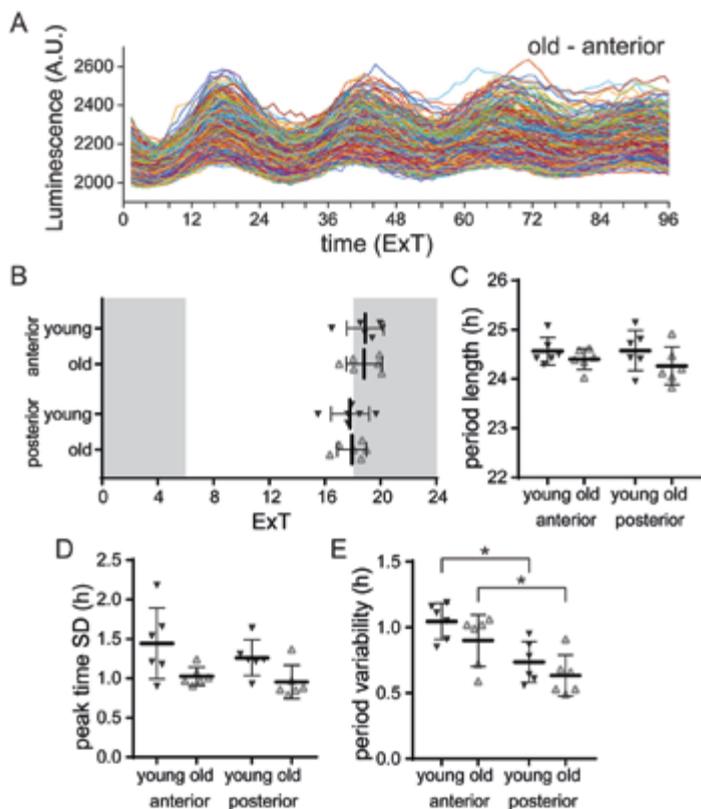


Figure 1. PER2::LUC expression is not altered with aging under LD 12:12. A. Examples of raw traces of bioluminescence intensity representing PER2::LUC expression from single cells in the anterior SCN of an old mouse (anterior, $n = 242$ cells; other examples in Suppl. Figure. S3). B. Average peak time of PER2::LUC rhythms per slice of the anterior and posterior SCN from young and old mice, plotted as external time (ExT). Shaded areas represent the projected dark phase. C. The average period length of the first 3 cycles in vitro is shown for the anterior and posterior SCN from young and old mice. D. Phase distribution is defined as the standard deviation (SD) of peak time of the first cycle in vitro and was calculated per slice. Phase distribution is shown for the anterior and posterior SCN from young and old mice. E. Period variability of single cells, averaged per slice from the anterior and posterior SCN from young and old mice. Filled triangles represent young mice ($n = 6$), and open triangles represent old mice ($n = 6$). Bars indicate mean \pm SD. * $P < 0.05$, 2-way analysis of variance, corrected for multiple comparison with Tukey's test.

3.2. Ability to behaviorally adapt to photoperiod is compromised in old mice

Both young and old mice were exposed to a seasonal adaptation protocol for either long or short photoperiod (LP and SP, respectively; Figure 2A and S2). Activity patterns in old mice were more fragmented, showing more activity during the day and a lower activity/rest-ratio, compared to young mice (Figure S1C). As a consequence, the behavioral rhythm strength in old mice was reduced in LD 12:12 when compared to young mice (Figure 2B; t-test: $P < 0.05$; table S2). Moreover, the free-running period in DD was longer after exposure to SP than after LP for young mice and this known after-effect of photoperiod (Pittendrigh and Daan, 1976) was absent in old mice (Figure 2C; Sidak's test, young: LP vs SP, $P < 0.01$, old: LP vs SP, n.s.; table S2). To examine if the mice were able to adapt to either long or short photoperiod, we determined the duration of locomotor activity (alpha) for the different light regime segments (Figure 2D) and subtracted the length of alpha in DD from its length in LD 12:12 as a measure for photoperiod-induced change in alpha (Δ alpha; Figure 2E). As expected, young mice showed an expansion of their activity profile under SP and a compression under LP, with an after-effect in the subsequent DD period (Figure 2D and S1; Refinetti, 2002). Old mice, however, were less capable of adapting to a different photoperiod. The alpha of old mice did not change after transition from LD 12:12 to SP nor from SP to DD, suggesting that old mice do not adapt to SP (Figure 2D and E, table S2). In contrast to the SP condition, old mice did show adaptation to LP, with their average alpha in DD following LP being lower than in LD 12:12 (Figure 2E and S1). The level of adaptation to LP – Δ alpha – did not significantly differ between old and young mice (Figure 2E and S1; Sidak's test, LP: young vs old, n.s.; table S2), however, the data from the old mice show a high variation, with some mice displaying no change of alpha in DD. This suggests that some old individuals were less able to adapt to LP. Taken together, the ability to adapt locomotor behavioral patterns to changing photoperiods is reduced in old mice.

3.3. Response of the molecular clock-network to photoperiod is unaltered in the aged SCN

We next investigated whether the reduced capability for seasonal adaptation in old mice is the result of a more rigid, less adaptive, molecular clock. Therefore, we measured PER2::LUC expression in SCN cultured slices of old and young mice after the mice were re-exposed for at least two weeks to either LP or SP following the DD period. We have previously shown that exposing young mice to a long photoperiod (LD 16:8) causes a wider phase distribution of peak times and a higher cycle-to-cycle period variability of single-cell PER2::LUC rhythms in the anterior part of the SCN, compared to short photoperiod (LD 8:16; Buijink et al., 2016). Since we see deficits in behavioral adaptation to photoperiod in old mice, and photoperiod can affect PER2 rhythm distribution, we may expect that the reduction in the capability to adapt to photoperiod is also seen at the molecular level.

Surprisingly, the PER2::LUC rhythms were remarkably similar in the SCN of young and old mice. The average peak time was about two hours later in the anterior slice, compared to the posterior slice, for all photoperiods in the SCN of both young and old mice (Figure 3B; Sidak's test, young vs old: n.s.; table S3). After adaptation to SP, we observed in the SCN of young mice a significantly shorter period length of PER2::LUC rhythms in the posterior part, compared to anterior part

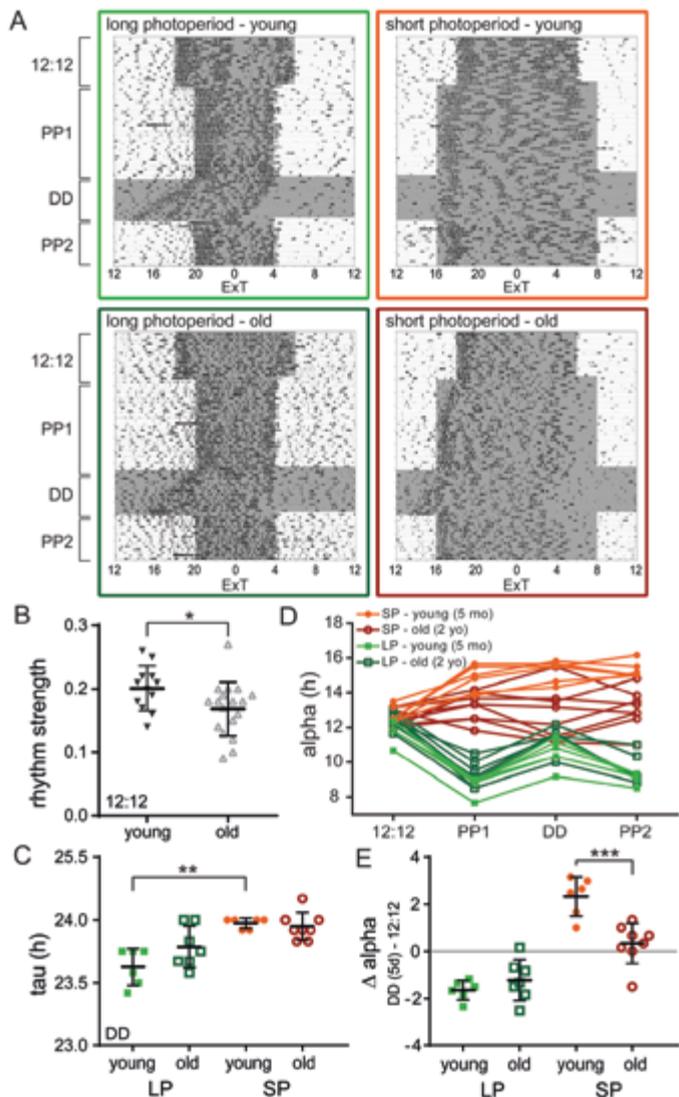


Figure 2. The ability to behaviorally adapt to photoperiod is reduced in old mice. A. Single-plotted actograms showing representative passive infrared recordings from activity of young (upper panels) and old (lower panels) mice, adapted to long photoperiod (LP; left) and short photoperiod (SP; right). Shaded areas represent the dark period. The time on the x-axis is given in external time (ExT). B. Rhythm strength of the LD 12:12 period for young (filled triangles; $n = 12$) and old (open triangles; $n = 18$) mice. C. Period (τ) of free-running behavioral rhythm during the period of constant darkness (DD; first 10 days) for young and old mice after adaptation to LP and SP. D. Activity period (α) for each segment of the entrainment protocol: LD 12:12, photoperiod 1 (PPI; LP or SP), constant darkness (DD; first 5 days), and PP2 (LP or SP), with each trace representing 1 mouse. E. Degree of adaptation to photoperiod represented by Δ alpha, which is determined by calculating the difference in alpha between LD 12:12 and the DD (5-day) period. Δ alpha is given for young and old mice adapted to LP and SP. Filled circles represent SP, young ($n = 6$); open circles represent SP, old ($n = 8$); filled squares represent LP, young ($n = 6$); and open squares represent LP, old ($n = 7$). Bars indicate mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, 1-way analysis of variance, corrected for multiple comparison with Sidak's test.

(Figure 3C; Sidak's test, young SP: anterior vs posterior, $P < 0.01$; table S3), and this was similar in the SCN of aged mice (Figure 3C; Sidak's test, posterior SCN SP: young vs old, n.s.; table S3). Consistent with previous studies, the anterior SCN of young mice adapted to LP showed a wider phase distribution of peak times, compared to SP condition (Figure 3D; Sidak's test, young anterior SCN: LP vs SP, $P < 0.001$; table S3). This difference between LP and SP was also present in SCN slices from old mice (Figure 3D; Sidak's test, old anterior SCN: LP vs SP, $P < 0.001$; table S3). Furthermore, in both the anterior and posterior part there was no significant difference in phase distribution between SCN slices from young and old mice (Figure 3D; Sidak's test, LP anterior: young vs old, n.s., LP posterior: young vs old, n.s., SP anterior: young vs old, n.s., SP posterior: young vs old, n.s.; table S3). Thus, even in the SCN of old mice, photoperiod still had a clear effect on the phase distribution of peak times.

We analyzed the fluctuations in period from cycle-to-cycle of individual cells in SCN slices from old and young mice as a measure for coupling strength (Herzog et al., 2015). The standard deviation (SD) of the cycle intervals of the first three cycles of PER2::LUC rhythms was determined for individual cells, and averaged per slice. Consistent with our previous study (Buijink et al., 2016), the average variability in single-cell period in the SCN of young mice adapted to LP was increased in the anterior part, compared to the posterior part (Figure 3E; Sidak's test, young LP: anterior vs posterior, $P < 0.01$; table S3) as well as to the (anterior) SCN of mice adapted to SP (Figure 3E; Sidak's test, young anterior SCN: LP vs SP, $P < 0.01$; table S3). This increase in single-cell cycle-to-cycle variability in the anterior SCN was similar in slices from old mice (Figure 3E; Sidak's test, old LP: anterior vs posterior, $P < 0.001$, old anterior SCN: LP vs SP, $P < 0.01$; table S3). There was no difference in the magnitude of increase of single-cell period variability between SCN slices from young and old mice (Figure 3E; Sidak's test, LP anterior SCN: young vs old, n.s.; table S3).

3.4. Degree of behavioral adaptation to photoperiod is related to PER2 period variability.

So far, we have shown that old mice have a reduced ability to adapt to changing photoperiods, while on the other hand they are still capable to adjust their molecular clock as do young mice. However, there is a higher level of variability in both behavioral and PER2::LUC data from the old, compared to young mice (Figure 2E and 3D). We wondered whether old mice that showed little adaptation in their alpha to LP also exhibited a smaller distribution in PER2::LUC peak times. Therefore, we correlated PER2::LUC period variability in the anterior SCN with the adaptation to SP or LP (Δ alpha). For the individual groups there is not a clear correlation between behavior and PER2::LUC expression rhythms (Figure S4). However, when all groups are plotted together, there appears to be an association between period variability and the adaptation of alpha to photoperiod (Figure S4; $R^2 = 0,74$).

3.5. Small changes on the level of functional clusters of neurons in the aged SCN

We have previously shown that there is a difference in functional cluster characteristics between the SCN of mice exposed to short and long photoperiod (Buijink et al., 2016). We wondered if

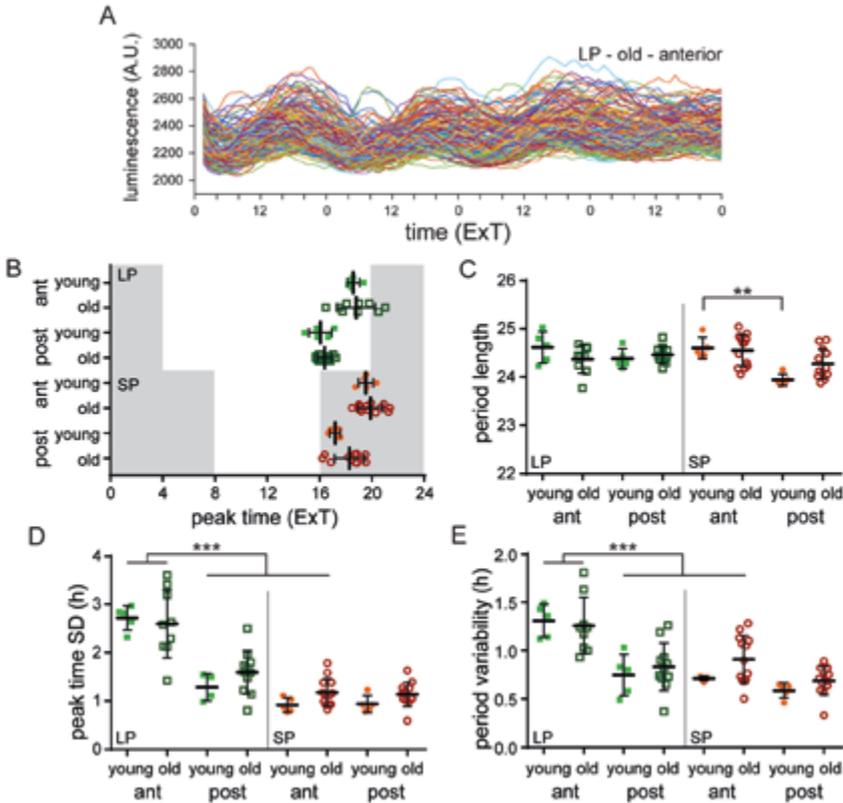


Figure 3. The molecular clock in the SCN of old mice can still adapt to different photoperiods. A. Examples of raw traces of bioluminescence intensity representing PER2::LUC expression from single cells from the anterior SCN of an old mouse ($n = 130$ cells). B. Average peak time of PER2::LUC rhythms per slice of the anterior and posterior SCN from young and old mice, adapted to long photoperiod (LP) and short photoperiod (SP) plotted as external time (ExT). Shaded areas represent the projected dark phase. C. Average period length of the first 3 cycles in vitro is shown for the anterior and posterior SCN from young and old mice entrained to LP and SP. D. Phase distribution of peak times per slice in the anterior and posterior SCN of young and old mice adapted to LP and SP. E. Single-cell period variability per slice in the anterior and posterior SCN of young and old mice adapted to LP and SP. LP: anterior: young: $n = 5$, old: $n = 9$, posterior: young: $n = 5$, old: $n = 11$; SP: anterior: young: $n = 5$, old: $n = 13$, posterior: young: $n = 5$, old: $n = 12$. Filled circles represent SP, young; open circles represent SP, old; filled squares represent LP, young; open squares represent LP, old. Bars indicate mean \pm SD. ** $P < 0.01$, *** $P < 0.001$, 1-way analysis of variance, corrected for multiple comparison with Sidak's test.

aging would affect any aspects of these clusters of SCN neurons, either in their spatial pattern, or their rhythm characteristics. Functional clusters were defined from time series data of PER2::LUC rhythm by an unbiased community detection algorithm (see Methods). We found that there is no difference in the location of the clustered cells in the SCN; there is a clear spatial distribution in the anterior and posterior SCN in both old and young mice, similar to that of our previous study. In LP, in both the young and old SCN, the VM/M cluster peaked earlier than the DL/L cluster, while in SP this was only the case in the old SCN (Figure 4B; Sidak's test, VM cluster vs DL cluster $P < 0.05$;

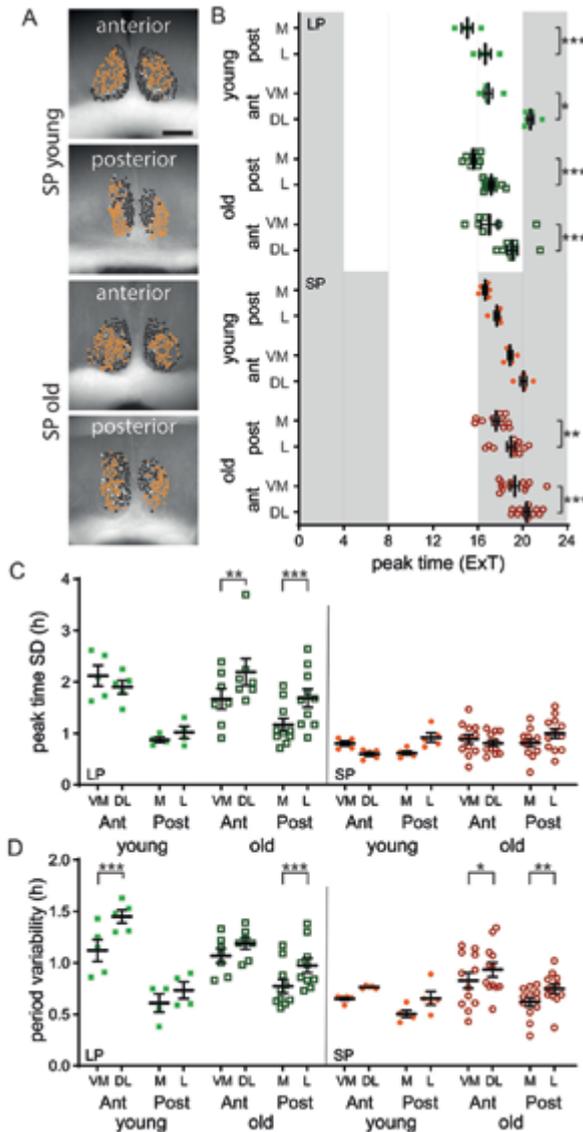


Figure 4. Functional cluster characteristics are similar in the old and young SCN. **A.** Cell cluster location projected on bright-field image of the SCN. The different shades represent the different clusters. Examples are given for the short photoperiod (SP) of young and old mice of both the anterior and posterior slice. Scale bar marks 200 μm . **B.** Average peak time of PER2::LUC rhythms for the ventromedial (VM) and medial (M), as well as the dorsolateral (DL) and lateral (L) cluster of the SCN of young and old mice entrained to either the long photoperiod (LP) or SP, plotted as external time (ExtT). **C.** Phase distribution of peak times for the VM/M and DL/L cluster in the anterior and posterior SCN of young and old mice adapted to the LP and SP. **D.** Single-cell period variability for the VM/M and DL/L cluster in the anterior and posterior SCN of young and old mice adapted to the LP and SP. LP: anterior: young: $n = 5$, old: $n = 7$, posterior: young: $n = 4$, old: $n = 10$; SP: anterior: young: $n = 5$, old: $n = 12$, posterior: young: $n = 5$, old: $n = 12$. Filled circles represent SP, young; open circles represent SP, old; filled squares represent LP, young; open squares represent LP, old. Bars indicate mean \pm SD. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, 1-way analysis of variance, corrected for multiple comparison with Sidak's test.

table S4). Interestingly, like in our previous study there is a significant difference between de VM and DL cluster in young mice exposed to LP. However, this difference is absent in old mice (Figure 4D; Sidak's test, anterior SCN LP: in young, DL vs VM cluster, $P < 0.01$; in old, DL vs VM cluster, *n.s.*; table S4). Taken together it seems that there are small changes in PER2 rhythm characteristics of clusters of neurons in the old compared to the young SCN.

4. DISCUSSION

It is well known that with aging sleep patterns and circadian rhythms get increasingly disturbed (Dijk and Duffy, 1999; Dijk et al., 1999). Moreover, elderly humans seem less capable to adjust to shift work and changing of the seasons (Harma et al., 1994; Cepeda et al., 2018). However, it remains unclear which processes regulating circadian rhythms underlie this decreased flexibility. Therefore, we investigated the plasticity of both behavior and PER2::LUC expression rhythms in the young and old SCN under different lighting conditions. We show that aging does not affect PER2::LUC expression rhythms in an equinoctial light regime, while – in line with previous findings – behavioral rhythm strength is affected, (Asai et al., 2001; Yamazaki et al., 2002; Kolker et al., 2003; Nakamura et al., 2011; Sellix et al., 2012; Leise et al., 2013). Considering that the old SCN neuronal network might be more rigid, we wanted to assess its adaptability by exposure to changes in day-length, since this requires plasticity of the SCN network (VanderLeest et al., 2007; Porcu et al., 2018). We show that aged mice are less capable of adapting their locomotor behavioral pattern to changes in photoperiod, compared to young mice. Surprisingly, the PER2::LUC expression rhythms in the SCN of old mice show similar levels of phase distribution and single-cell period variability as in young mice, with a wider phase distribution and higher period variability under long photoperiod, compared to short photoperiod. We do see more variability in these parameters in aging mice, and a general relationship between behavioral adaptation to photoperiod and PER2::LUC period variability. These results indicate that most of the plasticity of the molecular clock remains intact in the old SCN, and deficits in photoperiod adaptation arise downstream from the molecular clock.

4.1. Aging affects behavioral adaptation to photoperiod

To our knowledge, this is the first study that investigates the effect of exposure to long and short photoperiods on the behavior and PER2 expression in old mice. Our results on locomotor behavior in different photoperiods corroborate previous studies reporting impairment of circadian entrainment in aged rodents. Scarbrough and colleagues showed that in Syrian hamsters aging attenuates the effect of short photoperiod, as it did for the mice in our study (Scarbrough et al., 1997). Aging is shown to reduce the sensitivity to light, which might explain the impairment in circadian entrainment (Zhang et al., 1996; Benloucif et al., 1997; Biello et al., 2018), and in re-entrainment to changes in the light-dark cycle that we and others report (Valentinuzzi et al., 1997; Farajnia et al., 2012; Sellix et al., 2012).

4.2. Aging has little effect on PER2::LUC rhythms in the SCN in different photoperiods

In line with our results, previous studies have found no effect of aging on the peak time of PER2 rhythm in the SCN (Asai et al., 2001; Yamazaki et al., 2002; Kolker et al., 2003; Nakamura et al., 2011; Sellix et al., 2012; Leise et al., 2013), and no effect on peak time distribution (Sellix et al., 2012). Studies that examined PER2 expression in both the SCN and peripheral clocks have found that, while the SCN retains its phase, organs like the spleen and thymus peak significantly earlier in old, compared to young mice (Sellix et al., 2012; Leise et al., 2013). In addition, the SCN of old mice still responds relatively similar to a phase shift in the light-dark cycle as young mice, while their behavioral response, as well as PER2 expression in peripheral tissue is markedly delayed (Sellix et al., 2012; Leise et al., 2013). The molecular clock of the SCN only started to show signs of decay when exposed to constant darkness or constant light (Nakamura et al., 2015; Polidarova et al., 2017). Therefore, in this study we wanted to provoke the SCN of old mice by exposure to a naturally recurring challenge of the SCN network, namely the seasonal changes in photoperiod (Buijink et al., 2016). We found no effect of this challenge in overall rhythm characteristics of PER2::LUC. In young and old mice, and in both LP and SP, the PER2::LUC expression in the anterior SCN peaks later than in the posterior SCN, and peak time SD and period variability show a similar increase in the anterior SCN for both young and old mice (Figure 3).

Studies on aging clock have previously revealed a reorganization of the neuronal (Farajnia et al., 2012) and molecular networks (Chen et al., 2016). We therefore performed a detailed analysis of clusters of neurons in the SCN, using an unbiased method we used previously to show differences in neuronal networks in the SCN (Buijink et al., 2016; Almog et al., 2019). This analysis reconfirms the results from our previous study, and also reveals some small differences in peak time and period variability between the young and old SCN. These data suggest that there are some small changes on the network level of the SCN. Taken together, both our and previous studies on PER2 expression in the SCN have not found rigorous effects of aging, which remains surprising, given that there are strong effects of aging on other SCN neuronal components, as well as on PER2 expression in peripheral clocks.

4.3. Diverse effects of aging on the molecular clock of the SCN

Previous studies investigating other core clock genes in the SCN have failed to decisively show deficits in the molecular clock due to aging. Studies consistently show no age-related changes in the expression of the clock gene *Per1* and a decay in expression of the clock gene *Bmal1* (Asai et al., 2001; Weinert et al., 2001; Yamazaki et al., 2002; Kolker et al., 2003; Wyse and Coogan, 2010; Chang and Guarente, 2013; Bonaconsa et al., 2014). On the other hand, these studies have reported contradicting results on the effect of aging on the expression level of other clock genes, like *Per2*, *Cry1*, *Cry2*, and *Clock* (see Banks et al., 2016 for a detailed account). For PER2::LUC, there are two recent studies that did find an effect of aging on PER2::LUC expression. Nakamura and colleagues found that constant darkness results in a faster decay of the amplitude, and a spread of single-cell phases of PER2 expression rhythms in the SCN of old compared to young mice, although only

after more than 24 hours *in vitro* (Nakamura et al., 2015). Polidarova and colleagues (2017) found that after exposure to constant light, there was a higher incidence in arrhythmicity in PER2::LUC expression. However, only 7 out of 15 old animals showed arrhythmic patterns (up from 3/15 in young animals) and only in one of the two slices extracted per animal (Polidarova et al., 2017). Interestingly, these two studies found no aging-induced deficits in PER2 rhythms in the regular LD 12:12, only after challenging the system with constant light or constant darkness. These data suggest that the molecular clock in the old SCN is robust enough to adapt to the substantial changes in photoperiod we used, nonetheless, the unaffected molecular clock does not alleviate the deficits seen in behavior.

4.4. Aging affects cellular and network properties of the SCN

Despite the apparent lack of effects of aging on the molecular clock we have reported here, there is mounting evidence that the SCN plays an important role in aged-related disruptions of circadian behavior on multiple levels. (1) The observation that age-related alterations in behavioral rhythmicity can be reversed by transplanting fetal SCN tissue in aged animals, suggests that the SCN plays an important role in inducing aging associated behavioral changes (Van Reeth et al., 1994; Cai et al., 1997; Hurd and Ralph, 1998). (2) The amplitude of the SCN multiunit electrical activity signal, both *in vivo* and *ex vivo*, decreases in aged mice (Watanabe et al., 1995; Nakamura et al., 2011; Farajnia et al., 2012). This decrease in amplitude can be the result of a change in phase synchrony, since a subpopulation of SCN neurons peaks in anti-phase to the main activity peak in SCN slices of old mice (Farajnia et al., 2012). (3) Other electrical properties in SCN neurons also change with age, with alterations in the circadian regulation of ionic currents and cellular membrane deficits, which likely add to the decrease in the SCN's electrical rhythm amplitude (Farajnia et al., 2012; Farajnia et al., 2015). (4) The number of SCN neurons in aged rats remains the same (Rooyendaal et al., 1987; Miller et al., 1989; Madeira et al., 1995), which is in line with our results (Figure S3). However, several morphological changes have been reported, like reduced dendritic thickness and a loss of synapses (Machado-Salas et al., 1977; Palomba et al., 2008). (5) Despite the overall number of neurons being unaffected in the old SCN, the number of arginine vasopressin (AVP) and vasoactive intestinal polypeptide (VIP) expressing neurons is decreased (Rooyendaal et al., 1987; Chee et al., 1988) and the functionality of the main neurotransmitter in the SCN, GABA, is reduced with aging (Palomba et al., 2008; Farajnia et al., 2012). If we assume that the molecular clock is the least affected by aging as the data here and in previous studies suggest, the age-related deficits in clock function leading to the circadian phenotype seem to comprise an impairment of communication between the molecular clockwork and other intracellular clock-components of SCN neurons. This may subsequently lead to neuronal network alterations, resulting in the observed deficits in the circadian behavior.

4.5. Weakened link between the molecular clock and the SCN network in aging

The available data suggest that with aging the molecular clock continues to function normally in SCN neurons, while the SCN network is weakened at the level of electrical activity and neurotransmitters. The lower amplitude output-signal of the SCN reduces its ability to drive peripheral circadian

rhythms. However, this raises the question how the molecular clock can become dissociated from the other clock-components of the SCN and at what level communication between the SCN network and the molecular clock is altered in aging. Interestingly, a recent study showed a functional dissociation between the molecular clock in the SCN, and its downstream targets: in lactating mice, rhythms in electrical activity within the SCN as well as in the periphery were dampened, while molecular oscillations were unchanged, retaining the ability for circadian timekeeping (Abitbol et al., 2017). In addition, a modeling study of the SCN neuronal network predicts that differential GABAergic signaling can dissociate the electrical activity of SCN neurons and their molecular clock (DeWoskin et al., 2015). Other recent studies show that with the loss of the SCN network, circadian rhythms in electrical activity, calcium, and the molecular clock can become dissociated from each other (Enoki et al., 2017a; Enoki et al., 2017b; Noguchi et al., 2017). When the network strength is reduced between SCN neurons by either blocking action potentials or a lack of connections in low-density neuronal cultures, electrical activity has less influence on calcium and PER2 expression rhythms (Noguchi et al., 2017).

In SCN neurons, calcium is an important mediator of signals from the membrane to the molecular clock and vice versa (Lundkvist et al., 2005; Enoki et al., 2017b). We have previously shown that aging reverses the rhythm in intracellular calcium levels in SCN neurons with higher values in the night instead of the day (Farajnia et al., 2015). Therefore, we suggest that calcium homeostasis is disturbed in the aged SCN, leading to a weakening of the link between the molecular clock and the SCN network in both directions. However, the question remains how the SCN neurons are still able to adjust their phase distribution when their main modes of communication are disrupted. Further studies will be needed to elucidate the effect of aging on communication between network and the molecular clock, and its effect on calcium homeostasis to determine if this could be a target for strengthening circadian rhythmicity.

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SUPPLEMENTARY MATERIALS

3

Table S1. Summary results PER2 expression in SCN slices from young and old mice in LD 12:12

Fig.	Location	Young			Old		
		<i>n</i>	ANOVA ant-post	ANOVA ant-post	<i>n</i>	ANOVA ant-post	ANOVA ant-post
1B	Anterior	6	18.90 ± 1.33	n.s.	6	18.83 ± 1.28	n.s.
	Posterior	6	17.81 ± 1.37	n.s.	6	17.95 ± 1.04	n.s.
1C	Anterior	6	24.57 ± 0.28	n.s.	6	24.40 ± 0.21	n.s.
	Posterior	6	24.58 ± 0.41	n.s.	6	24.27 ± 0.38	n.s.
1D	Anterior	6	1.45 ± 0.45	n.s.	6	1.03 ± 0.12	n.s.
	Posterior	6	1.26 ± 0.23	n.s.	6	0.95 ± 0.21	n.s.
1E	Anterior	6	1.05 ± 0.14	<i>p</i> < 0.02	6	0.90 ± 0.20	<i>p</i> < 0.05
	Posterior	6	0.74 ± 0.16		6	0.63 ± 0.16	

Data represent mean ± SD. n.s. = not significant.

Table S2. Summary results behavioral adaptation to different photoperiod in young and old mice

Fig.	rhythm strengt	alpha (h)	Tau (h)	Δ alpha 12:12 - DD (h)	Young				Old						
					12:12	LP	n	ANOVA	SP	LP-SP	12:12	LP	n	ANOVA	SP
2B	0.2 ± 0.04	12	11.81 ± 0.61	6	p < 0.0001	12.72 ± 0.49	6	p < 0.0001	12.56 ± 0.50	8	n.s.	12.56 ± 0.50	8	n.s.	p < 0.05
2C	PP1	6	8.69 ± 0.51	6	p < 0.0001	14.80 ± 0.76	6	p < 0.0001	13.17 ± 0.82	8	n.s.	13.17 ± 0.82	8	n.s.	n.s.
	DD	6	10.17 ± 0.63	6	p < 0.01	15.06 ± 0.81	6	n.s.	12.90 ± 1.00	8	n.s.	12.90 ± 1.00	8	n.s.	n.s.
	PP2	6	9.08 ± 0.31	6	p < 0.0001	15.31 ± 0.46	6	n.s.	13.08 ± 1.11	8	n.s.	13.08 ± 1.11	8	n.s.	n.s.
2D	23.63 ± 0.15	6	23.63 ± 0.15	6	n.s.	23.97 ± 0.04	6	p < 0.001	23.95 ± 0.11	8	n.s.	23.95 ± 0.11	8	n.s.	n.s.
2E	-1.64 ± 0.42	6	-1.64 ± 0.42	6	n.s.	2.34 ± 0.84	6	p < 0.001	0.34 ± 0.86	8	n.s.	0.34 ± 0.86	8	n.s.	p < 0.001
2B	0.17 ± 0.04	18	12.48 ± 0.46	7	p < 0.0001	12.48 ± 0.46	7	p < 0.0001	12.48 ± 0.46	7	p < 0.0001	12.48 ± 0.46	7	p < 0.0001	n.s.
2C	PP1	7	9.31 ± 0.75	7	p < 0.0001	11.26 ± 0.57	7	p < 0.0001	11.26 ± 0.57	7	p < 0.0001	11.26 ± 0.57	7	p < 0.0001	n.s.
	DD	7	11.26 ± 0.57	7	p < 0.0001	9.48 ± 0.85	7	p < 0.0001	9.48 ± 0.85	7	p < 0.0001	9.48 ± 0.85	7	p < 0.0001	n.s.
	PP2	7	9.48 ± 0.85	7	p < 0.0001	23.79 ± 0.17	7	p < 0.0001	23.79 ± 0.17	7	p < 0.0001	23.79 ± 0.17	7	p < 0.0001	n.s.
2D	23.79 ± 0.17	7	23.79 ± 0.17	7	n.s.	-1.22 ± 0.87	7	n.s.	-1.22 ± 0.87	7	n.s.	-1.22 ± 0.87	7	n.s.	n.s.
2E	-1.22 ± 0.87	7	-1.22 ± 0.87	7	n.s.										n.s.

Data represent mean ± SD. n.s. = not significant.

Table S3. Summary results PER2 expression in SCN slices from young and old mice under different photoperiod

Fig.	Location	Young				Old			
		LP	ANOVA ant-post	SP	n	LP	ANOVA ant-post	SP	n
3B	anterior	18.58 ± 0.46	p < 0.001	19.54 ± 0.57	5	18.79 ± 1.48	p < 0.001	19.88 ± 0.95	9
	posterior	16.05 ± 0.88		17.18 ± 0.41	5	16.38 ± 0.56		18.29 ± 1.16	11
3C	anterior	24.61 ± 0.32	n.s.	24.60 ± 0.22	5	24.37 ± 0.29	n.s.	24.51 ± 0.33	9
	posterior	24.38 ± 0.21		23.94 ± 0.12	5	24.46 ± 0.17		24.27 ± 0.31	11
3D	anterior	2.72 ± 0.25	p < 0.001	0.92 ± 0.15	5	2.60 ± 0.70	p < 0.001	1.18 ± 0.29	9
	posterior	1.28 ± 0.28		0.94 ± 0.17	4	1.59 ± 0.45		1.14 ± 0.25	11
3E	anterior	1.31 ± 0.17	p < 0.001	0.71 ± 0.02	5	1.26 ± 0.29	p < 0.001	0.87 ± 0.23	9
	posterior	0.75 ± 0.21		0.59 ± 0.08	5	0.83 ± 0.25		0.66 ± 0.17	11
3B	anterior								
	posterior								
3C	anterior								
	posterior								
3D	anterior								
	posterior								
3E	anterior								
	posterior								

Data represent mean ± SD. n.s. = not significant.

Table S4. Summary results PER2 expression in SCN slices from young and old mice under different photoperiod

Fig.	Location	Young						Old					
		LP		ANOVA		SP		LP		ANOVA		SP	
		VM-DL / M-L	n	VM-DL / M-L	n	VM-DL / M-L	n	VM-DL / M-L	n	VM-DL / M-L	n	VM-DL / M-L	n
4B	anterior - VM	16.93 ± 0.36	5	p < 0.001	5	18.89 ± 0.21	n.s.	n.s.	5	n.s.	n.s.	n.s.	
	anterior - DL	20.69 ± 0.28	5		5	20.09 ± 0.29	n.s.	n.s.	5	n.s.	n.s.	n.s.	
	posterior - M	15.06 ± 0.45	4	p < 0.05	5	16.66 ± 0.20	n.s.	n.s.	5	n.s.	n.s.	n.s.	
	posterior - L	16.65 ± 0.50	4		5	17.70 ± 0.22	n.s.	n.s.	5	n.s.	n.s.	n.s.	
	anterior - VM	2.12 ± 0.20	5	n.s.	5	0.81 ± 0.04	n.s.	p < 0.001	5	n.s.	p < 0.001	n.s.	
	anterior - DL	1.90 ± 0.13	5		5	0.60 ± 0.04	n.s.	p < 0.001	5	n.s.	p < 0.001	n.s.	
4C	posterior - M	0.88 ± 0.05	4	n.s.	5	0.62 ± 0.04	n.s.	n.s.	5	n.s.	n.s.	n.s.	
	posterior - L	1.02 ± 0.12	4		5	0.92 ± 0.09	n.s.	n.s.	5	n.s.	n.s.	n.s.	
	anterior - VM	1.12 ± 0.11	5	p < 0.001	5	0.65 ± 0.02	n.s.	n.s.	5	n.s.	p < 0.01	n.s.	
	anterior - DL	1.45 ± 0.06	5		5	0.76 ± 0.01	n.s.	p < 0.001	5	n.s.	p < 0.001	n.s.	
	posterior - M	0.61 ± 0.09	4	n.s.	5	0.50 ± 0.03	n.s.	n.s.	5	n.s.	n.s.	n.s.	
	posterior - L	0.74 ± 0.08	4		5	0.66 ± 0.07	n.s.	n.s.	5	n.s.	n.s.	n.s.	
4D	anterior - VM	17.03 ± 0.78	7	p < 0.001	12	19.23 ± 0.39	p < 0.001	p < 0.001	12	p < 0.001	p < 0.001	n.s.	
	anterior - DL	19.08 ± 0.47	7		12	20.37 ± 0.26	p < 0.001	p < 0.001	12	p < 0.001	p < 0.001	n.s.	
	posterior - M	15.61 ± 0.17	10	p < 0.001	12	17.63 ± 0.31	p < 0.001	p < 0.001	12	p < 0.001	p < 0.001	n.s.	
	posterior - L	17.21 ± 0.22	10		12	18.96 ± 0.36	p < 0.01	p < 0.01	12	n.s.	p < 0.01	n.s.	
	anterior - VM	1.67 ± 0.20	7	p < 0.01	12	0.89 ± 0.09	p < 0.001	p < 0.001	12	n.s.	p < 0.001	n.s.	
	anterior - DL	2.19 ± 0.26	7		12	0.81 ± 0.06	p < 0.001	p < 0.001	12	n.s.	p < 0.001	n.s.	
	posterior - M	1.17 ± 0.13	10	p < 0.001	12	0.81 ± 0.08	p < 0.001	p < 0.001	12	n.s.	p < 0.05	n.s.	
	posterior - L	1.69 ± 0.18	10	n.s.	12	1.00 ± 0.09	n.s.	p < 0.001	12	p < 0.05	p < 0.001	n.s.	
	anterior - VM	1.07 ± 0.07	7		12	0.83 ± 0.07	n.s.	p < 0.01	12	p < 0.05	n.s.	n.s.	
	anterior - DL	1.19 ± 0.05	7		12	0.93 ± 0.07	n.s.	n.s.	12	n.s.	n.s.	n.s.	
	posterior - M	0.77 ± 0.07	10	p < 0.001	12	0.62 ± 0.05	p < 0.001	p < 0.01	12	p < 0.01	n.s.	n.s.	
	posterior - L	0.97 ± 0.07	10		12	0.75 ± 0.05	n.s.	n.s.	12	n.s.	n.s.	n.s.	

Data represent mean ± SD. n.s. = not significant.

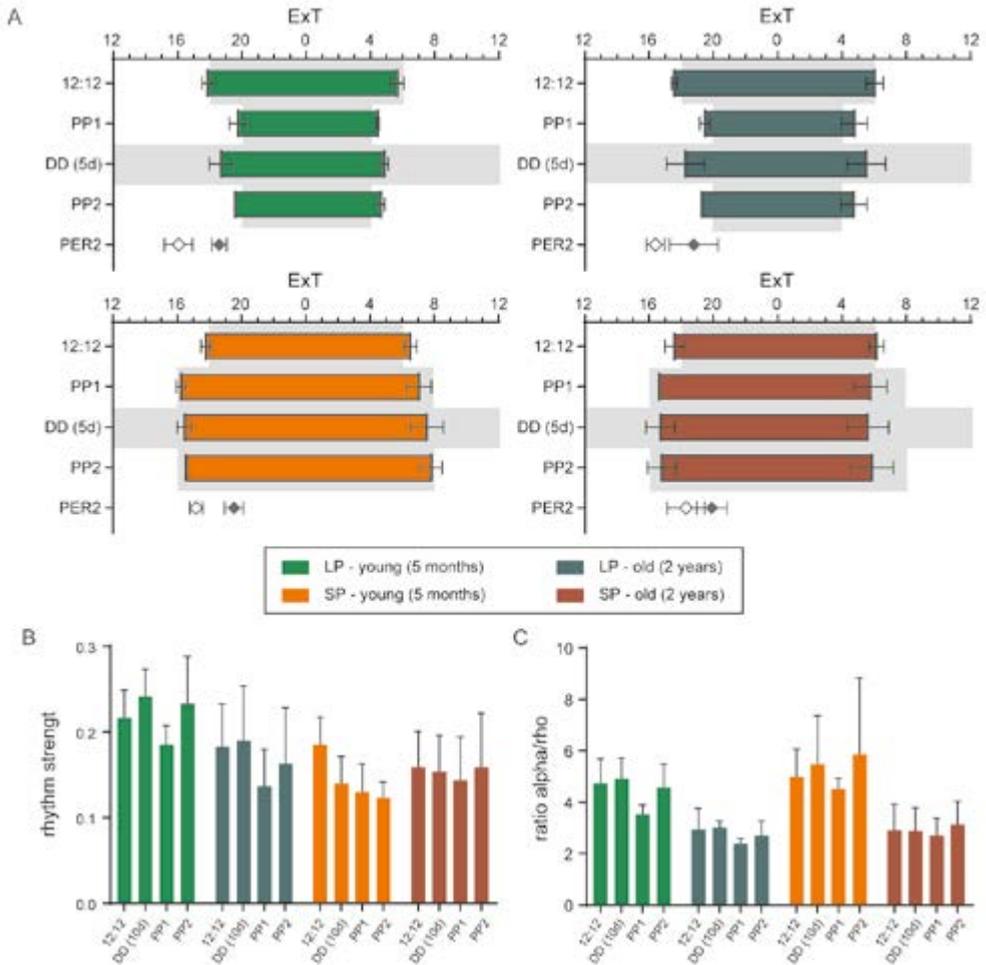


Figure S1. Behavioral changes in aging under different photoperiods. A. Average timing of activity onset and offset \pm SD given for LP (upper panels) and SP (lower panels) and for young (left panels) and old (right panels) during the different photoperiods, plotted with average PER2::LUC peak time for the anterior (filled diamond) and posterior (open diamond) SCN. B. Rhythm strength for the consecutive photoperiods, per experimental condition. C. Ratio of activity to rest (α/ρ) for all light regimes. Black bars indicate mean \pm SD.

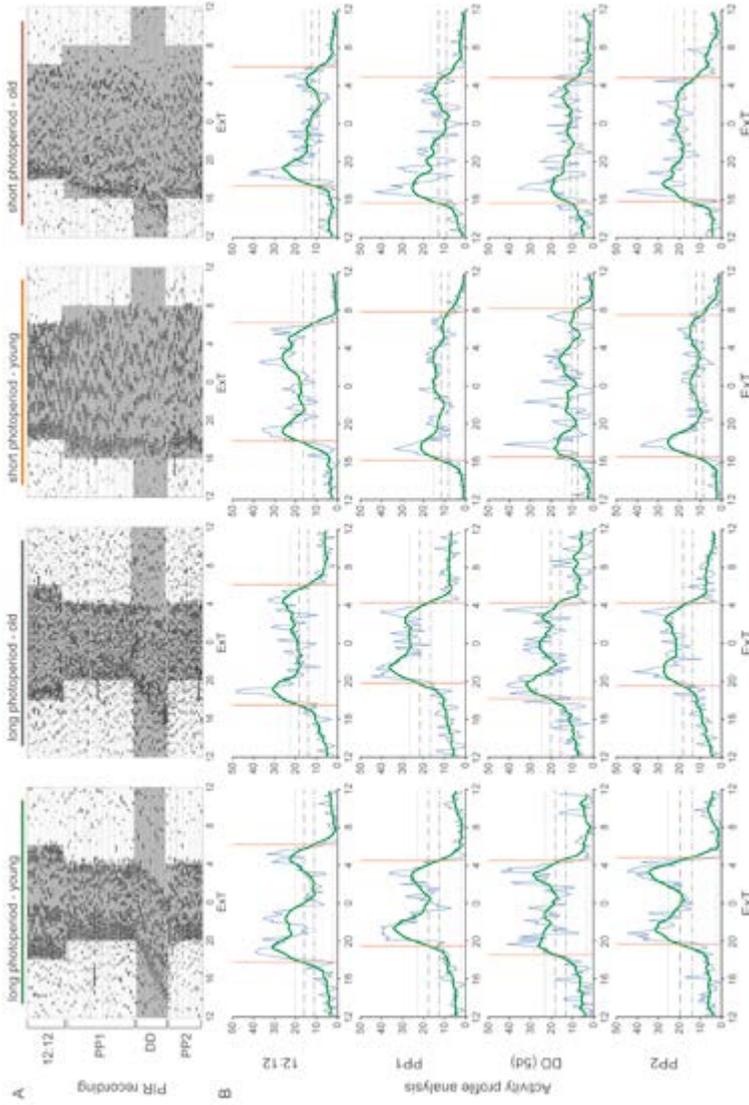


Figure S2. Analysis of activity profiles from PIR recordings for the consecutive photoperiods. A. Single-plotted actograms showing representative PIR recordings of activity from young and old mice adapted to either LP or SP. Shaded areas represent the dark period. Time on the x-axis is given in external time (EXT). B. Activity profiles of 10 consecutive days in LD 12:12 (top row), LP/SP (PP1; second row), 5 days of constant darkness (DD; corrected for Tau; third row), and 10 days on LP/SP (PP2; bottom row). Dark blue lines represent activity profiles (10 min bins), green lines represent smoothed activity profiles (2 hours), lower dotted lines represent L5; (lowest average activity in a 5 h interval), top dotted lines represent M10 (highest average activity in a 10 h interval), dash-dot lines denote $\frac{1}{2}$ value from L5 to M10 (used to determine offset of activity), dashed lines denote $\frac{1}{4}$ value from L5 to M10 (used to determine onset and offset (see Methods)). Red vertical lines show determined onset and offset (see Methods). Time on x-axis given in external time (EXT).

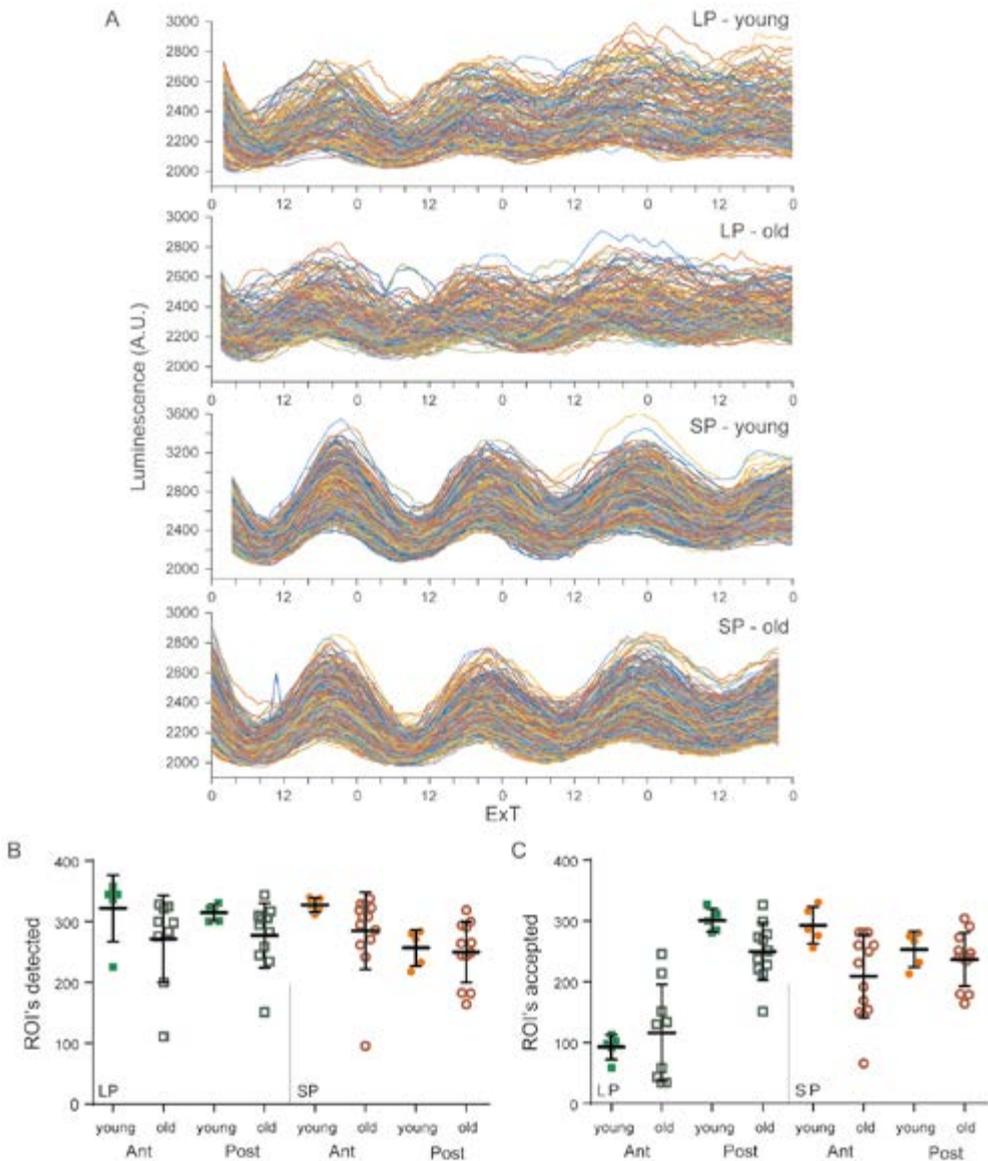


Figure S3. Number of PER2::LUC expressing cells is not altered with aging. A. Examples of raw traces of bioluminescence intensity representing PER2::LUC expression from single cells from the anterior SCN of young and old mice in LP (upper two panels; $n = 113$ cells and $n = 130$ cells) and SP (lower two panels; $n = 316$ cells and $n = 281$ cells). B. Number of ROI's detected in bioluminescence recording per slice of the anterior and posterior SCN from young and old mice, adapted to LP and SP. C. Number of ROI's that were accepted for further analysis per slice of the anterior and posterior SCN from young and old mice, adapted to LP and SP. Black bars indicate mean \pm SD.

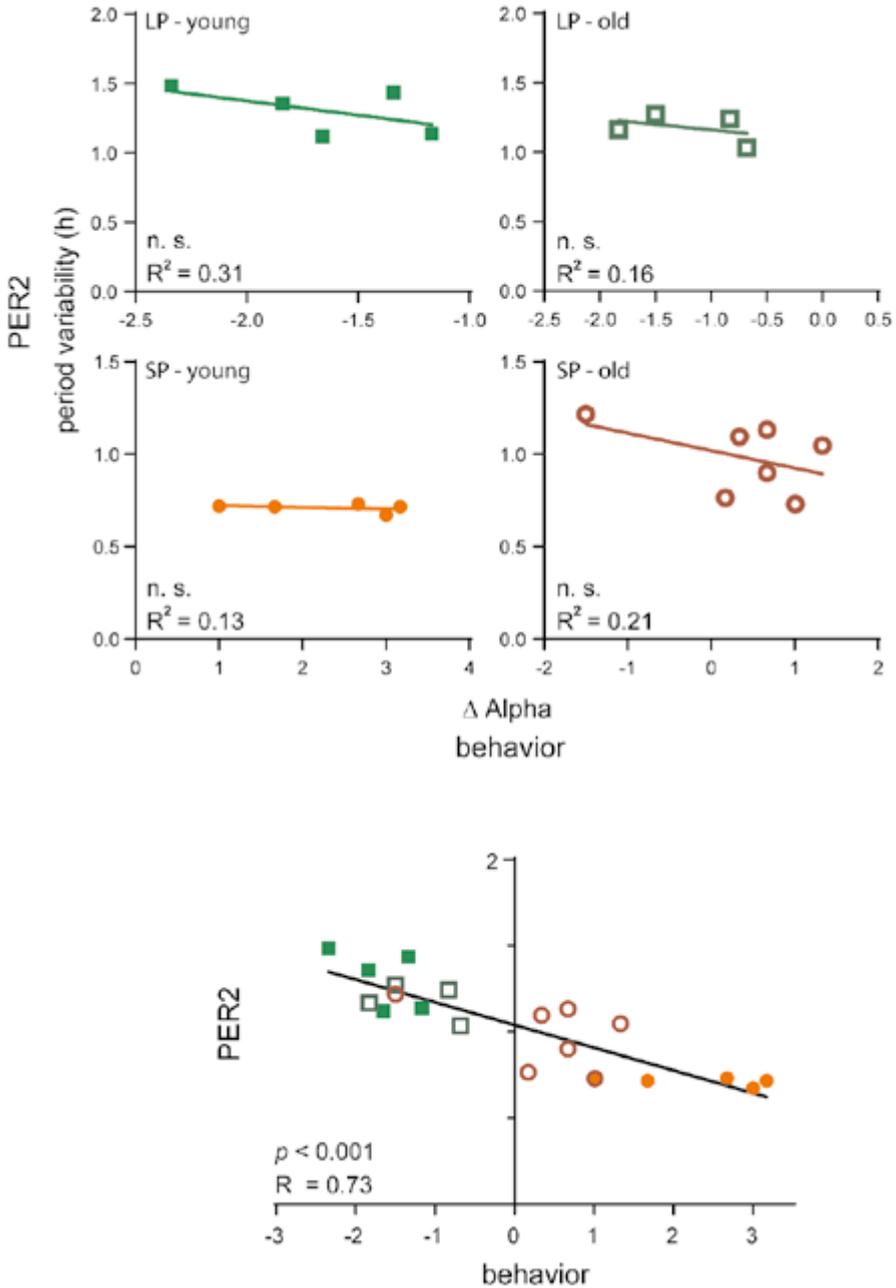


Figure S4. Association between behavior and molecular clock. A. When groups are considered separately, there is no significant correlation between behavior and PER2::LUC expression B. Taking the groups together, there is a clear correlation between the adaptation to photoperiod (alpha) and period variability in PER2::LUC expression in the SCN. Correlations are shown between PER2::LUC phase distribution (peak time SD; see Figure 3D) in the anterior SCN and behavioral adaptation to photoperiod (Δ alpha: LD 12:12 – DD; see Figure 2E) for young LP (upper right panel), old LP (upper left panel), young SP (lower right panel), and old SP (lower left panel). n. s. = slope of correlation not significantly different from zero.