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Spontaneous *fos* expression in the suprachiasmatic nucleus of young and old mice

Jonathan P. Miller, J. Devin McAuley, Kevin C.H. Pang*

J.P. Scott Center for Neuroscience, Mind, and Behavior, Department of Psychology, Bowling Green State University, Bowling Green, OH 43403, USA

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Abstract

The senescence-accelerated mouse (SAMP8) is an animal model of aging that displays an array of circadian rhythm disruptions as early as 7 months of age. The present study explored the physiological basis for age-related changes in circadian rhythms by measuring c-Fos immunostaining. Cellular activity in the SCN "core" and "shell" was examined for 2-, 7-, and 12-month-old SAMP8 at circadian times (CTs) 2 and 14. Consistent with previous studies in rats, we observed higher levels of cellular activity at CT2 than at CT14, and higher levels of activity in the "shell" than in the "core" of the SCN. However, there was no effect of age on the pattern of cellular activity in either the "core" or the "shell" of the SCN. These results are discussed in the context of current research on spontaneous and light-induced c-Fos expression in the SCN of rodents.

Keywords: Circadian rhythm; Aging; Suprachiasmatic nucleus; SCN; c-Fos; Senescence-accelerated mouse; SAMP8

1. Introduction

Research on the biological basis of circadian rhythms has a well-established history. In mammals, the suprachiasmatic nucleus (SCN) of the anterior hypothalamus is believed to be a major component of the circadian clock [17,21]. One source of evidence for a role of the SCN in maintaining circadian rhythms is the daily cycles of metabolic and electrical activity that can be observed in in vivo and in vitro SCN preparations [13,41]. Supporting evidence that the SCN is important for the generation and maintenance of circadian rhythms comes from lesion studies [29,38,44] and fetal transplant studies [8,9,23–25,34]. Both complete and partial SCN lesions produce an array of rhythm disruptions including fragmentation of activity patterns, changes in the rate of re-entrainment following a phase shift, reductions in rhythm amplitude, and changes in the free-running period (τ) [8,9,25,29,38,44]. Conversely, transplants of fetal SCN tissue restore many aspects of these arrhythmic behaviors [20,23,24,34,35].

Age-related changes in the circadian rhythms of humans are similar in many ways to those observed following lesions in rodents. For example, common complaints of elderly individuals include fragmented sleep–wake patterns, high levels of night-time activity, reduced day-time cognitive performance, and more difficulty adjusting to a time shift (i.e., increased "jet lag") [7,37,45,53,54]. Age-related changes in other measures, such as τ, are less clear-cut and appear to vary with species. For example, some species display an age-related lengthening of τ, while other species show an age-related shortening of τ [4,12,33,63]. The similarity between the effects of SCN lesions and aging suggest that some age-related disruptions in circadian rhythms may be due to age-related changes to the SCN, or its outputs.

One model of aging used to examine the biological basis of age-related changes in circadian rhythms is the senescence-accelerated mouse (SAM) [51]. The P8 sub-line of the SAM model (SAMP8) has received attention for accelerated aging deficits in learning and memory and circadian rhythms

* Corresponding author. Tel.: +1 419 372 6974; fax: +1 419 372 6013.
E-mail address: kpang@bgnet.bgsu.edu (K.C.H. Pang).

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[50]. Age-related changes in the circadian rhythms of the SAMP8 appear as early as 7 months of age and are similar in many ways to those that have been reported in elderly humans [26,40,54]. Consistent age-related changes in the circadian rhythms of the SAMP8 are an increase in the proportion of wheel-running activity during the normally inactive light phase, a reduction in rhythm amplitude during both LD and constant darkness conditions, and overall increased fragmentation of wheel-running activity [26,30]. A somewhat unusual age-related change observed in the SAMP8 is the appearance of "split-like" activity rhythms, where two distinct bouts of activity occur approximately 12 h apart [26,30]. With respect to free-running period, the data are mixed; one study has suggested a lengthening of τ [4], while others have found no age-related changes in τ [26,30].

One possible physiological basis for the age-related changes in circadian rhythms of the SAMP8 is a disruption of the normal activity rhythms observed in the SCN. The SCN in rodents is divided into two major areas, which have been given different labels depending on the rodent species [1,27,28]. In the mouse, the two divisions are referred to as "core" and "shell" [1,27,28]. Although the immediate early gene *c-fos* and its protein c-Fos are likely to play a role in circadian rhythms [43], investigators have also used c-Fos as a marker of cellular activity in the SCN. Two different patterns of c-Fos expression have been observed in the "core" [8,15,42,43,49] and "shell" [15,43,46,47], suggesting that these two areas have different functions. In the "core," where photic inputs from the retina terminate, c-Fos expression is primarily light-induced [8,15,42,43,49]. This type of c-Fos expression follows the behavioral pattern of the animal's phase responses to light pulses suggesting that these SCN cells and the expression of c-Fos are involved in entrainment to the environmental light–dark cycle [3,10,43]. In contrast to the "core," the "shell" of the SCN does not receive photic input and displays a spontaneous rhythm of c-Fos expression in constant conditions. For this reason, some previous studies have proposed that the "shell" is important in the generation of free-running (endogenous) rhythms [14,43], whereas the "core" is important for the maintenance of these rhythms.

The expression of c-Fos in the "core" and "shell" differ in two other respects. First, the overall amount of spontaneous expression in the "shell" is much lower than the amount of light-induced expression in the "core" [15,47]. Second, the time course of expression is different. In the core, the time of maximum light-induced expression occurs during the dark phase, whereas in the shell, the time of maximum spontaneous rhythmic c-Fos expression occurs about 2 h into the animals' projected day [15,47], or circadian time 2 (CT2); CT0 corresponds to the time that lights would have come on in constant darkness conditions and CT12 corresponds to the time that lights would have gone off.

Previous studies of the effects of age on c-Fos expression in the SCN core have shown an age-related decrease in the amount of light-induced expression in the core and a corresponding reduction in an animal's ability to properly phase shift in response to a light pulse [6,48,64]. Conversely, aged animals that receive grafts of fetal SCN tissue show an increase in the amount of light-induced expression in the core and a corresponding improvement in an animal's ability to properly phase shift in response to a light pulse [8,55]. Combined, these data suggest that age-related changes in the activity of the SCN "core" may directly affect an animal's ability to properly phase shift, with increase (or decreases) in cellular activity corresponding to decreasing (or increasing) amounts of time for an animal to realign its circadian rhythm to a change in the light–dark cycle.

The effects of age on spontaneous activity in the SCN "shell" are less well understood. In young rats, "shell" activity in constant conditions, as measured by c-Fos expression, changes throughout the day [47], with activity peaks in the early morning and activity troughs during the night. If "shell" activity is important for the generation of circadian rhythms [14,43], then one possibility is that age-related reductions in rhythm amplitude are associated with an age-related decrease in the amount of spontaneous c-Fos expression in the SCN "shell." However, as far as the authors are aware, no previous research has examined this possibility.

The present study had two goals. The first goal was to examine the effects of age on spontaneous rhythmic activity of the SCN, with the expectation that age should reduce the amount of spontaneous activity in the SCN shell. Previous research on spontaneous rhythmic activity of the SCN was performed on rats, and so far, has not been examined in mice. Therefore, the second goal of the present study was to determine whether spontaneous c-Fos expression in the SCN of mice was similar to that previously found in rats, using the SAMP8 model and c-Fos expression as an activity marker.

2. Methods

2.1. Animals

Sixty naïve, male SAMP8 mice, ages 2 months ($n = 20$), 7 months ($n = 20$), and 12 months ($n = 20$) served as subjects for this study. Two-, seven-, and twelve-month-old SAMP8 were placed into one of two sacrifice times: CT2 (14 h after light offset) or CT14 (26 h after light offset). These times have been shown to be the highest (peak time) and lowest (trough time) points of spontaneous c-Fos expression in the SCN, respectively [47]. Dividing the animals by age and time of sacrifice resulted in six groups, with ten animals in each group. Mice were bred and raised in the animal research facility at Bowling Green State University. Two to four same-sex littermates were housed together, with food and water available ad libitum. Lights in the animal room were maintained on a 12 h light/12 h dark cycle (lights on at 7:30 a.m. local time). All animals were maintained in accordance with NIH animal care guidelines and all procedures were approved by the Bowling Green State University Institutional Animal Care and Use Committee.

2.2. Design

A 3 (Age) × 2 (Sacrifice time) × 2 (SCN location) mixed factorial design was implemented. SCN cells immunoreactive for the c-Fos protein (c-Fos-ir) were counted and compared across three ages of animals, sacrifice time, and SCN location.

2.3. Procedure

Procedures similar to Sumova et al. [47] were employed. The mice were removed from the animal colony room during the light phase (around 2:00 p.m. local time) and housed individually in a separate room. Lights in this room were turned off at the same time the lights went off in the colony room (7:30 p.m. local time), and the room was then maintained in a dark state. Animals remained in extended night until they were sacrificed. Care was taken so that mice were not exposed to white light. Red light (two Phillips 100 W incandescent red light bulbs provided 3 lux illumination at cage level) was used to illuminate the room while animals were being selected and transferred to a cage in which they were carried to the room where perfusions were performed. The red light was turned off upon exiting the room. Animals were sacrificed in groups of three. All animals were sacrificed within 20 min of the initial entry into the holding room, and all were sacrificed within 5 min of CT2 or CT14.

Immediately prior to perfusion, mice were anesthetized with a urethane solution (0.3 mg/10 mg body weight, i.p.) under dim lighting. Animals were perfused within 5 min of light exposure, but there is an estimated 60–90 min delay from the induction of the *c-fos* gene before c-Fos protein levels peak [18]. Perfusion was performed transcardially with 0.9% saline, at room temperature, followed by freshly prepared and chilled 4% paraformaldehyde in isotonic sodium phosphate buffer. Brains were removed by dissection, postfixed for 24 h at approximately 4 °C in the 4% paraformaldehyde solution, and then cryoprotected in 30% sucrose in 0.1 M phosphate buffer (PB), pH 7.4, for 3 days or until the brains sank into the solution.

Sections (70 μm) were cut on a freezing microtome throughout the whole SCN. The avidin-biotin (AB) method [19] with diaminobenzidine (DAB) as the chromogen was used. Sections were incubated with the rabbit-anti-c-Fos (dilution 1:1000; #sc-52: Santa Cruz Biotechnology, Santa Cruz, CA) in a 0.3% Triton X-100/1% normal donkey serum/0.1% sodium azide/PB solution for approximately 17 h at room temperature (22 °C) followed by three 5-min (3 × 5 m) washes in PB to rinse the tissue. The tissue was then incubated in biotinylated donkey-anti-rabbit serum (dilution 1:200; Jackson ImmunoResearch Laboratories Inc., West Grove, PA) in a 0.3% Triton X-100/1% normal donkey serum/0.1% sodium azide/PB solution for 2 h at room temperature (22 °C) followed by 3 × 5 m washes in PB. Following this, the sections were placed into AB reagent solution (1:200; standard ABC kit, Vector Laboratories, Inc., Burlingame, CA) for 2 h at room temperature (22 °C), again followed by 3 × 5 m washes in PB. Staining was visualized using a nickel-enhanced DAB reaction. Tissue was reacted for 20 min in a DAB (0.05% in PB) nickel solution (0.12% $NiCl_2$); then hydrogen peroxide was added to the solution (0.06% final concentration) and the reaction continued for approximately 6 min. Six 5-min washes in PB followed this final reaction. Sections were mounted on gelatin-coated glass sides, dehydrated, and cover-slipped using Permount. Tissue from the six animals sacrificed at either CT2 or CT14 during the same day were processed through the immunocytochemistry procedure together using the same solutions.

2.4. Data analysis

Cell counting was performed using standard stereology techniques [61]. Every section throughout the entire SCN was analyzed resulting in 5–6 total sections per animal. Stereological analysis using the optical fractionator methods was performed with the Stereo Investigator program (MicroBrightField, Colchester, VT) and a microscope with a motorized *x*-, *y*-, and *z*-axis stage (Bio Point 30, Ludl Electronic Products, Hawthorne, NY). Leading edges of c-Fos-ir cells were counted using a 63× objective lens (Carl Zeiss, 1.4 N.A.) The counting frame had an area of 8500 μm^2 and a height of 15 μm. A guard zone of 5 μm was used so that no cells were counted within 5 μm of the slice surface.

In the interest of counting as many SCN neurons as possible (since SCN was only in 5–6 sections) tissue sections were not withheld for stains other than c-Fos. The borders of the SCN were delineated on each section using anatomical markers (i.e. optical chiasm and third ventricle) and contrast differences in background staining. Visual estimates for the boundaries between the "core" and "shell" regions were created for counting (Fig. 1) by using the Stereo Investigator program to outline each of these areas; these boundaries were approximated based on comparisons of the current slice being considered and drawings from Abrahamson and Moore's [1] Fig. 11. Since the SCN of each animal varied slightly in size and shape, a rigid template of Abrahamson and Moore's figure was not feasible and definitions of the boundaries had to be approximated for each section. However, every attempt was made to match our slides with the drawings in terms of SCN borders and "core" versus "shell" boundaries. This method may not be sensitive to individual differences in "core" and "shell" boundaries between animals. Consequently, our method may have resulted in a few cells, primarily those cells located along the boundaries, being incorrectly added to either the "core" or the "shell" within an animal thereby adding variability to the cell counts.

The statistical analysis of the cell counts was conducted using SPSS for Windows (Version 11.0, SPSS, Inc., Chicago, IL). A 3 × 2 × 2 mixed-model ANOVA assessed differences in cell counts for the three ages, two sacrifice times, and two

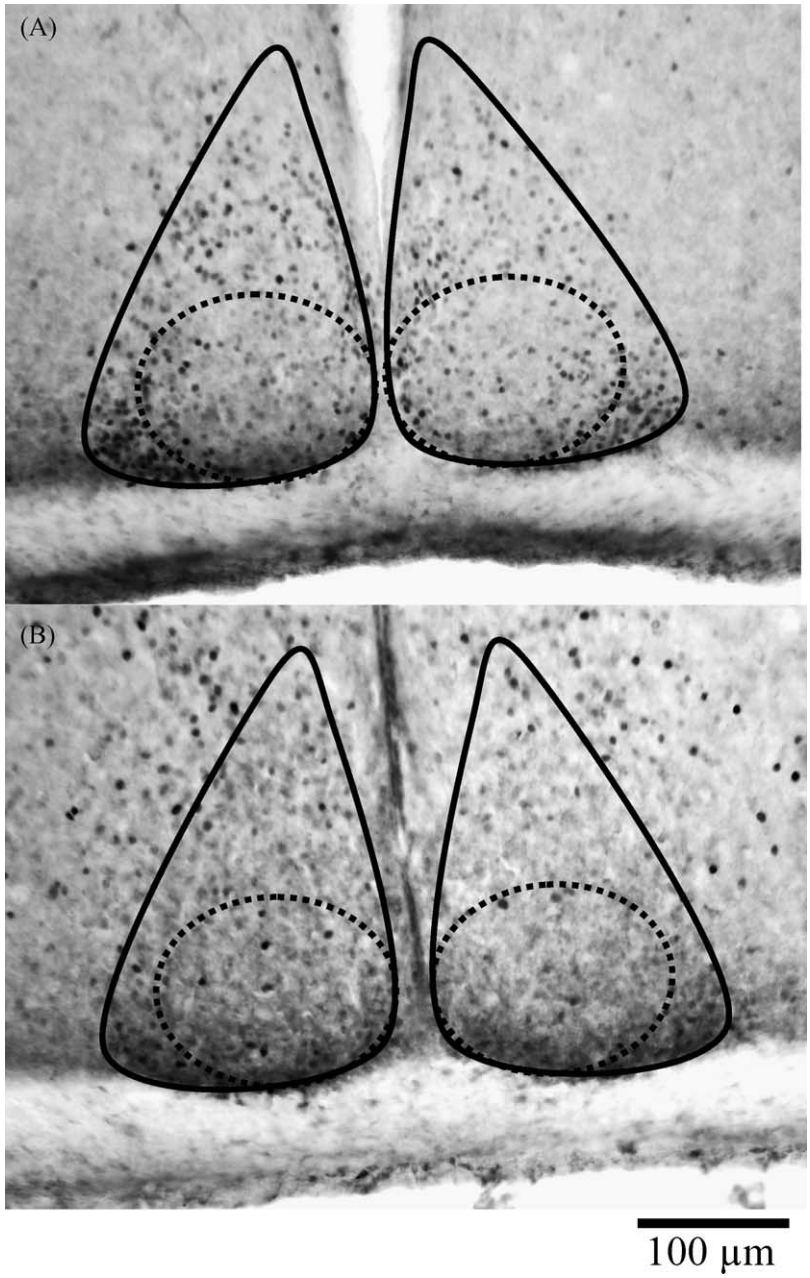

Fig. 1. Example of c-Fos immunoreactivity at CT2 (A) and CT14 (B) from 2-month-old SAMP8 mice. Coronal sections of the SCN are shown with boundaries for the "core" and "shell" regions (magnification = 10×).

SCN locations. Age and sacrifice time were between subjects factors, and SCN location was a within subjects factor.

To examine the possibility of hemispheric asymmetries in the amount of c-Fos expression, left- and right-hemisphere cell counts for the "shell" were plotted on *x*- and *y*-axes, respectively, for all three age group (2, 7, and 12 months of age) and both sacrifice times (CT2 and CT14). The "core" was left out of this analysis because, on the whole, its cell counts were lower and less variable than counts from the "shell" across the CT2 and CT14. A linear regression line was then fit through the resulting scatter plots for each age group. If the amount of c-Fos expression in the right and left hemisphere is on-average the same, then the regression line should have a positive slope (indicating the amount of expression is positively correlated between hemispheres). However, if there are systematic asymmetries in the amount of expression in the left and right hemispheres, then the regression line should have a negative slope (indicating the amount of expression is negatively correlated between hemispheres). Here, a negative slope would suggest that the spontaneous rhythms expressed in each hemisphere are out of phase. Finally, if some of the animals exhibit symmetric expression and others show asymmetric expression, then the slope of the line should be close to 0.0 (indicating no correlation between hemispheres).

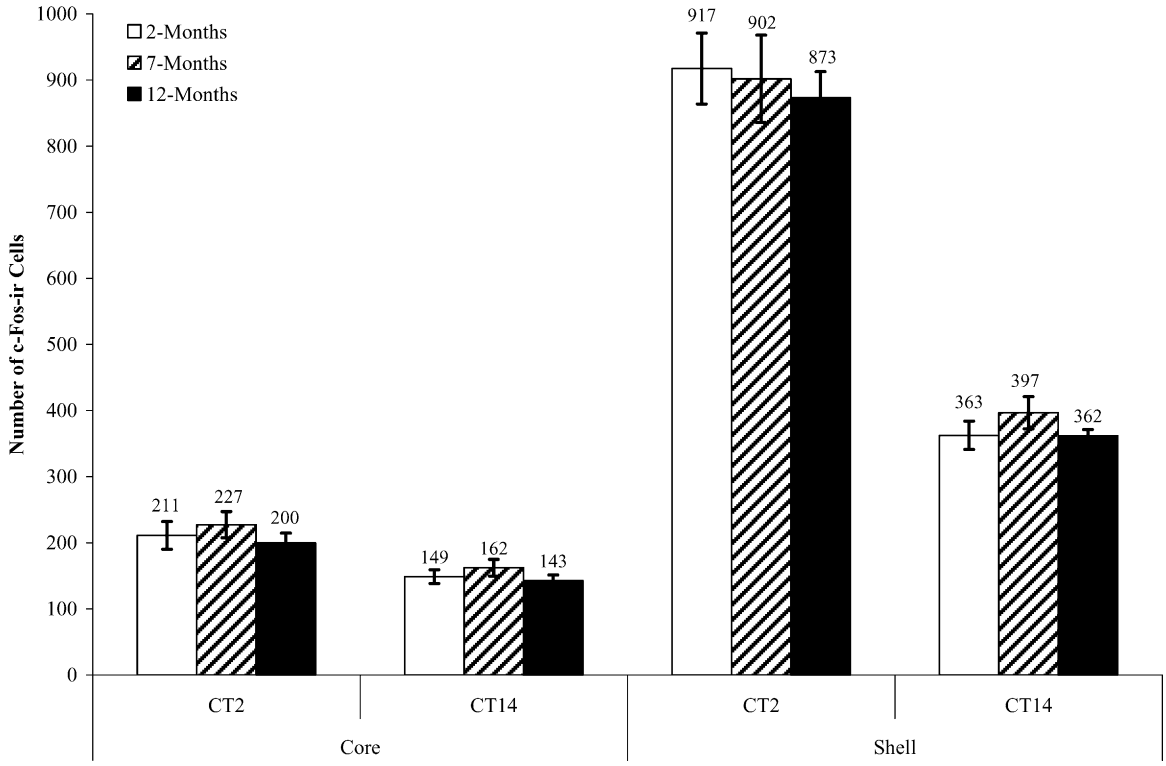

Fig. 2. Number of c-Fos-ir cells in the SCN for three ages of mice. No age-related differences in the number of c-Fos-ir cells were found. The three age groups (2, 7, and 12 months) had similar c-Fos-ir cell counts in each of the different conditions (time of sacrifice and SCN location). The number of cells in the "core" was less than in the "shell." In addition, there was a decrease in the number of cells from CT2 to CT14 sacrifice times. Error bars are expressed as standard error of the mean.

3. Results

The means of the cell counts for each group are shown in Fig. 2. Contrary to expectations, age did not affect the number of c-Fos-ir cells. All three age groups displayed similar counts of c-Fos-ir cells across conditions ($F_{(2,54)} = 0.60$, MSE = 12834.60, $p > 0.05$; Fig. 2). Additionally, the age × SCN location ($F_{(2,54)} = 0.08$, MSE = 6057.59, $p > 0.05$), age × time ($F_{(2,54)} = 0.15$, MSE = 12834.60, $p > 0.05$), and age × time × SCN location ($F_{(2,54)} = 0.31$, MSE = 6057.59, $p > 0.05$) interactions were not significant.

Overall, the number of c-Fos-ir cells was higher in the SCN "shell" than in the "core" as revealed by a significant main effect of SCN location ($F_{(1,54)} = 1019.16$, MSE = 6057.59, $p < 0.01$; Figs. 1 and 2). Additionally, a greater number of c-Fos-ir cells was observed at CT2 than at CT14 ($F_{(1,54)} = 200.12$, MSE = 12834.60, $p < 0.01$; Figs. 1 and 2). There was also a significant interaction between location and time ($F_{(1,54)} = 264.57$, MSE = 6057.59, $p < 0.01$). While the number of c-Fos-ir cells in the "core" and "shell" both appeared to decrease from CT2 to CT14, there was a greater decrease in the SCN "shell" than in the "core" (Fig. 2).

Fig. 3 shows left- versus right-hemisphere cell counts for the "shell" for all three age group (2, 7, and 12 months of age) and both sacrifice times (CT2 and CT14). As illustrated in this graph, the scatter plots produced regression lines with slopes close to 1 for each of the three age groups suggesting (a) that there are no hemispheric asymmetries in the amount of spontaneous expression and (b) that this finding is similar in 2-, 7-, and 12-month-old mice. The proportion of variance accounted for by each of the regression lines was 0.87, 0.95, and 0.97, respectively.

4. Discussion

In previous research, consistent circadian rhythm disruptions have been observed in SAMP8 as early as seven months of age. These disruptions include an increased proportion of activity during the normally inactive light-phase, reduced rhythm amplitude, and overall increased fragmentation of wheel-running activity during a regular light–dark cycle and during constant darkness [4,26,30]. One possible reason for the observed reduction in rhythm amplitude is a reduction in the number of spontaneously active cells in the SCN "shell" at times corresponding to peaks in rhythm amplitude.

To address this issue, the present study examined spontaneous cellular activity in the SCN of the SAMP8 using the IEG *c-fos* and its associated protein c-Fos as a marker of cellular activity. c-Fos expression in the SCN "core" and "shell" was assessed at two different times of day (CT2 and CT14) in 2-, 7-, and 12-month-old SAMP8. We found higher c-Fos-ir

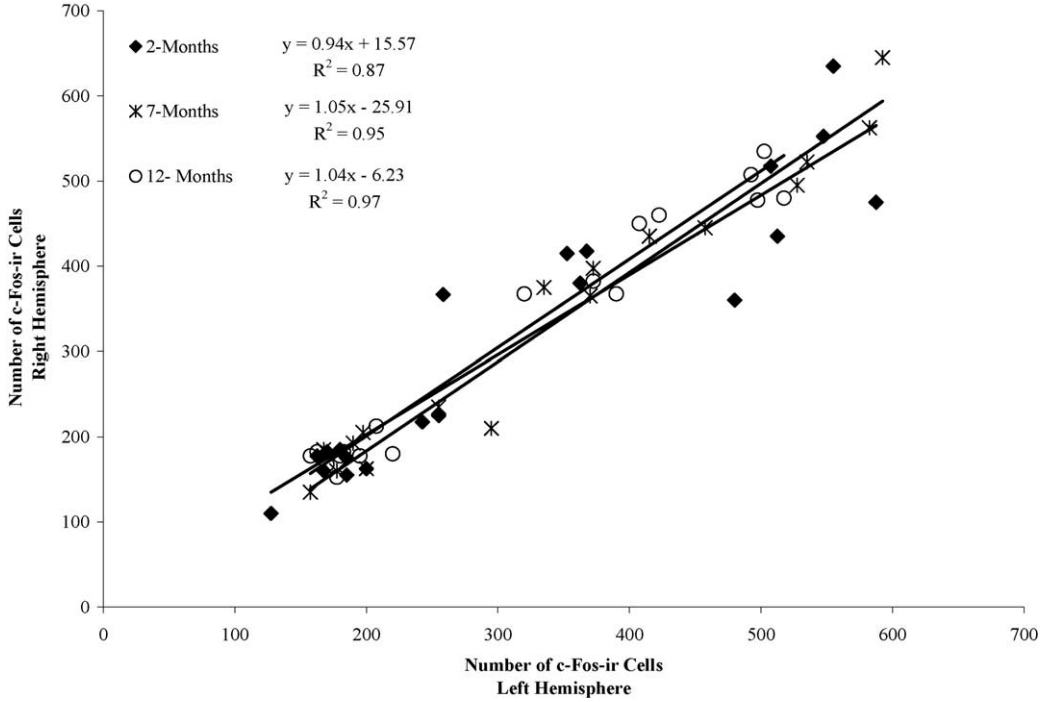

Fig. 3. No left vs. right hemisphere differences in the SCN "shell." Overall, the left and right hemispheres of the SCN "shell" had a similar number of c-Fos-ir cells. Regression slopes for each age group (2, 7, and 12 months) were all close to 1.0 suggesting that the hemispheres are similar in the phase of oscillation.

cell counts in the shell than in the core and higher c-Fos-ir cell counts at CT2 than at CT14. Moreover, the difference between the number of c-Fos-ir cells at CT2 and CT14 was greater for the "shell" than the "core." This pattern of results is consistent with previous reports on spontaneous c-Fos expression in rats [15,46,47]. Contrary to our expectation, we found no evidence for an effect of age on the number of c-Fos-ir cells in either the shell or core for both CT2 and CT14. Thus, the amplitude reduction of behavioral rhythms (i.e., wheel running) observed in aged SAMP8 is not due to a reduction in the number of spontaneously active cells in the SCN. One possible explanation is that the individual cells within the SCN are functioning properly, but aging compromises the ability of the SCN to consolidate these cellular signals together. This could result in a reduction in the strength of any output signals leaving the SCN. Another possibility is the change in the amplitude of the behavioral activity rhythm is due to age-related changes "downstream" from the SCN. Peripheral systems may be impaired in their ability to read or synchronize with an output signal from the SCN. One place to examine may be the subparaventricular zone since this area is one of the major projection sites of the SCN [57–59].

It is possible that an age-related change in the pattern of c-Fos expression was not observed because we assayed c-Fos expression at only two time points. We expected the change in the SCN that was associated with the age-related reduction of behavioral rhythms would be present at the peak or trough of activity (CT2 and CT14). We cannot discount the possibility that increasing the number of times for sacrifice might reveal a difference in the overall pattern of activity that was not captured by the two time points used in the current study.

There might also be an age-related difference in the amount (intensity) of c-Fos being expressed. Because we counted c-Fos-ir cells instead of assessing the intensity of c-Fos staining, the data from the present data do not address this possibility. It would be difficult to measure the amount of c-Fos expressed in SCN neurons using our data because all of the tissue was not reacted at the same time or in the same solutions. Future studies directed at examining this issue could measure levels of *c-fos* mRNA and c-Fos protein using more quantitative methods.

Despite the fact that we did not observe age-related changes in c-Fos expression in the SCN, age-related changes of the SCN (for review, see [52,60]) have been observed using measures other than c-Fos expression, including glucose metabolism [62], number of arginine-vasopressin (AVP) neurons [36], and in vitro neuronal activity [39,56]. Additionally, changes in the expression of the *Clock* gene seems to be related to changes in rhythm amplitude [2,22]. It is conceivable that alterations in some or all of the above may occur in the SCN with aging and may be responsible for the amplitude reduction in behavioral rhythms that occurs with age. So far, these neurobiological markers have not been studied in the SCN of SAMP8, and additional research would be needed to relate the behavioral disruptions of circadian rhythm in aged SAMP8 with these potential disturbances of the SCN.

An interesting age-related disturbance in the circadian rhythms of the SAMP8 is a "split-like" activity pattern con-

sisting of two distinct bouts of activity approximately 12 h apart. Evidence from analysis of *Per*, *Bmal*, AVP, and c-Fos in hamsters with "split" activity rhythm has shown that the left and right hemispheres of the SCN are operating 180° out of phase. This decoupling of the left and right hemispheres of the SCN was not observed in hamsters with "unsplit" locomotor rhythms [11]. If the age-induced "split" activity rhythms in the SAMP8 is similar those observed in the hamsters, then one might expect to see systematic differences in the amount of c-Fos expression in the left and right hemispheres of the SCN from aged SAMP8. However, the present study found no evidence for any hemispheric asymmetry in the number of c-Fos-ir SCN cells for all three ages of the SAMP8; scatter plots of left- versus right-cell counts produced regression lines with slopes close to one for all three age groups.

Based on our analysis of potential hemispheric asymmetries in expression, a tentative conclusion is that the split activity rhythms observed in SAMP8 [26,30] may not be the same phenomenon that has been reported in hamsters [5,11,16,31,32]. However, this conclusion may be premature for two reasons. First, the wheel-running behavior was not recorded in the present study to determine whether any of the animals were displaying "split" activity rhythms. However, based on our previous work, we would have expected approximately half of the aged animals to exhibit split rhythms [26,30]. Second, it is possible that hemispheric asymmetries may occur for SCN markers other than c-Fos. Previous reports on the biology of "split" activity rhythms in hamsters found asymmetries in levels of *Per*, *Bmal*, and AVP, in addition to c-Fos [11]. It would be interesting for future studies to examine levels of these other important markers of SCN activity in SAMP8 that exhibit age-related "split" activity rhythms.

Finally, the present study is the first to examine spontaneous c-Fos expression in the SCN "core" and "shell" of the mouse. In rats, the SCN "shell" shows a peak of expression around CT2 and lowest levels of expression around CT14. In addition, the SCN "core" in rats displays a pattern similar to the "shell." However there is significantly less spontaneous c-Fos expression in the "core" compared to the "shell" at both CT2 and CT14. The results of the present study with mice are consistent with these previous reports in rats [15,46,47] (see [47], Fig. 2B and C). One implication of this observation in mice is that investigators can use all of the genetic tools developed in mice to examine the endogenous circadian rhythms of the SCN, and the differential functions of the "core" and "shell."

In summary, age-related changes in spontaneous c-Fos expression were not observed in the SCN of SAMP8 mice. This result contrasts with the pronounced age-related disruptions of behavioral circadian activity rhythms and light-induced c-Fos expression in the SCN "core" observed in previous studies. The results suggest that the age-related reduction in rhythm amplitude either involves other processes apart from the production of c-Fos, or that the cause of this age-related change in rhythm amplitude is located outside of the SCN. Although spontaneous c-Fos expression was unaffected by age, the number of c-Fos-ir neurons in the SCN was influenced by time of day and location within subregions of the SCN.

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