c-Fos rhythm in subdivisions of the rat suprachiasmatic nucleus under artificial and natural photoperiods

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Jáč, Martin, Alena Sumová, and Helena Illnerová. c-Fos rhythm in subdivisions of the rat suprachiasmatic nucleus under artificial and natural photoperiods. Am J Physiol Regulatory Integrative Comp Physiol 279: R2270–R2276, 2000.—Recent studies have shown that the waveform of the rhythm of c-Fos photoinduction in the ventrolateral (vl) part of the suprachiasmatic nucleus (SCN) and that of the rhythm in the spontaneous c-Fos production in the dorsomedial (dm) part of the SCN in rats released into constant darkness depend on the photoperiod under which the animals were previously maintained. The aim of the present study was to find out how the rhythms of c-Fos immunoreactivity in both SCN subdivisions are affected by actual light-dark (LD) cycles with various photoperiods, either artificial or natural ones, that animals may usually experience. Rats were maintained under artificial LD cycles, with either a long (16-h photoperiod) or a short (8-h photoperiod) or under natural daylight. In the latter case, c-Fos rhythms were followed in the summer when the photoperiod lasted about 16 h or in winter when it lasted only 8 h. The rhythms of c-Fos immunoreactivity under natural daylight did not differ significantly from those under corresponding artificial photoperiods. Under a long photoperiod, the morning c-Fos rise in the dm- as well as in the vl-SCN occurred about 4 h earlier than under a short one. In both SCN subdivisions, the interval when the nighttime c-Fos immunoreactivity was low, was shorter under a long than under a short photoperiod by roughly 6 h. The morning c-Fos rise in the dm-SCN always preceded that in the vl-SCN. Whereas in the former one the rise was due to the endogenous dm-SCN rhythmicity, in the latter one the rise was induced by the morning light onset. The results show that whereas c-Fos rhythmicity under actual LD cycles is affected by the photoperiod in both SCN subdivisions, mechanism of c-Fos induction in the dm-SCN differs from that in the vl-SCN.

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rings. Rats were maintained under LD regimens with a long or a short photoperiod, and the SCN c-Fos rhythms were followed under both regimens. In addition to these artificial square-wave LD cycles with abrupt transitions between lights on and lights off, rats also were maintained under natural daylight and the SCN rhythms of c-Fos immunoreactivity were followed in the summer when the photoperiod was long and in the winter when the photoperiod was short. Photoperiodic entrainment of the circadian pacemaker may be just a special case of photic entrainment, and the latter may be affected by changes in the amount of light and its spectral composition during the dawn and dusk twilight (1, 20, 29). In actual nature, however, rats may experience just a very small part of natural photoperiod as they have access to burrows.

METHODS

Animals and experimental paradigms. Sixty-day-old male Wistar rats (Velas, Prague, Czech Republic) were housed at a temperature of 23 ± 2°C with free access to food and water. For at least 4 wk prior to experiments, animals were maintained under a long photoperiod (LD 16:8) per day with lights on from 0400 to 2000, under a short photoperiod (LD 8:16) with lights on from 0800 to 1600, and under a natural daylight. In the latter case, experiments were performed either on June 21 when the photoperiod lasted around 16 h or on December 20 when the photoperiod lasted only around 8 h (Fig. 1). Under artificial LD regimens, illumination intensity provided by overhead 40-W fluorescent tubes was between 50 and 200 lx, depending on cage position. Under natural daylight, cages were close to windows and intensity of light changed in dependence on the time of day (Fig. 1). For c-Fos immunohistochemistry, rats at various day times, either in light during the light period or in darkness during the dark period, were perfused through the ascending aorta with heparinized saline.

To confirm the inducing effect of light at the time of the dark-light transition on c-Fos production in the vl- but not in the dm-SCN, rats maintained in LD 16:8 and in LD 8:16 did not experience the usual morning light onset at 0400 and at 0800, respectively. Instead, they were left in darkness and exposed to a 30-min light pulse at the time of the usual light onset and 1, 2, and 3 h thereafter, respectively. Then they were returned to darkness and perfused 30 min later. Control rats were just perfused in darkness with no previous light exposure.

Immunohistochemistry. Rats were deeply anesthetized with pentobarbital sodium (50 mg ip) and perfused through the ascending aorta with heparinized saline followed by PBS (0.01 M sodium phosphate-0.15 M NaCl, pH 7.2) and then freshly prepared 4% paraformaldehyde in PBS. Brains were removed, postfixed for 12 h at 4°C, and cryoprotected in 20% sucrose in PBS overnight at 4°C. Coronal 30-μm-thick sections were cut and processed for immunohistochemistry. For c-Fos determination, the avidin-biotin method with diaminobenzidine as the chromogene was used as described (4). The primary antiserum was generated against the amino acids 2—17 of the NH2-terminal peptide sequence of c-Fos and characterized elsewhere (30). Labeled cell nuclei in the whole SCN, in the dm-SCN, and in the vl-SCN were counted irrespective of the intensity of staining by an independent observer using an image analysis system (IMAGE PRO, Olympus) as described elsewhere (25, 26).

Data analysis. Data were analyzed by two-way ANOVA for group and time differences and by one-way ANOVA for time differences and subsequent pairwise comparisons by the Student-Newman-Keuls multiple-range test. When the two-way ANOVA was used, just time-matched values of two studied groups were analyzed. In addition, data on c-Fos induction by a light pulse were also analyzed by the Student’s t-test.

RESULTS

Rhythms of c-Fos immunoreactivity in the whole SCN, in the dm-SCN, and in the vl-SCN under an artificial long photoperiod (LD 16:8) and a short photoperiod (LD 8:16) are shown on Fig. 2. Because the rhythm in the complete SCN is composed of the dm- and of the vl-SCN rhythm, just the rhythmicity of both SCN subdivisions is further discussed.

For the dm-SCN rhythm, the two-way ANOVA revealed a highly significant difference between LD 16:8 and LD 8:16 (F = 38.2, P < 0.01), as well as a significant difference of time (F = 43.3, P < 0.01) and a significant interaction effect (F = 13.1, P < 0.01). A significant evening decrease of c-Fos immunoreactivity under LD 16:8 occurred at 2000 vs. 1400 (P < 0.01) and a significant morning increase at 0330 vs. 0300 (P < 0.05); the increase continued to 0400 vs. 0330 (P < 0.05, Fig. 2B). Hence, the rise occurred spontaneously well before the morning light onset, and the interval between the evening decrease and the morning increase, i.e., the period of low c-Fos immunoreactivity, lasted for about 7.5 h. Under LD 8:16, a significant evening decrease occurred at 1700 vs. 1400 (P < 0.01) and continued further to 2400 vs. 2000 (P < 0.05) and a significant morning rise occurred at 0700 vs. 0500 (P < 0.05); after 0800, c-Fos immunoreactivity did not increase any more. The interval between the significant evening decline and the morning rise, i.e., the period of low c-Fos immunoreactivity, lasted for about 14 h and was thus ~6.5 h longer than the period under LD 16:8. Whereas in the evening at any time point, c-Fos immunoreactivity under LD 8:16 did not differ from that under LD 16:8, in the morning, the c-Fos immunoreactivity under LD 8:16 was significantly

Fig. 1. Changes of light intensity at the cage level around the time of dusk and dawn on June 21 (●) and December 20 (●). Solid bars, dark periods; shaded bars, periods of twilight. Evening declines and morning rises of light intensity highly correlated with those of photon flux density (data not shown).
lower than that under LD 16:8 at 0400, 0500, and 0600 ($P < 0.01$). Apparently, the main difference between the dm-SCN rhythmicity under a long photoperiod and that under a short photoperiod occurred in the morning hours.

For the vl-SCN rhythm in c-Fos immunoreactivity, the two-way ANOVA revealed also a highly significant difference between LD 16:8 and LD 8:16 ($F = 19.0, P < 0.01$), as well as a significant difference of time ($F = 48.9, P < 0.01$) and a significant interaction effect ($F = 15.5, P < 0.01$). A significant evening c-Fos decrease under LD 16:8 occurred at 2000 vs. 1400 ($P < 0.05$) and a significant morning increase at 0400 vs. 0330 ($P < 0.05$); the increase continued to 0600 vs. 0400 ($P < 0.01$, Fig. 2C). The interval between the evening decrease and the morning increase thus lasted 8 h. Under LD 8:16, a significant evening decline occurred at 1700 vs. 1400 ($P < 0.01$) and continued further to 2000 vs. 1700 ($P < 0.01$); a significant morning rise occurred at 0730 vs. 0600 ($P < 0.05$) and further continued until 1000 vs. 0800 ($P < 0.01$). The interval between the significant evening decline and morning rise, i.e., the period of low c-Fos immunoreactivity, thus lasted for about 14.5 h and was 6.5 h longer than that under LD 16:8. In the morning, c-Fos immunoreactivity under LD 8:16 was significantly lower than that under LD 16:8 at 0400, 0500, and 0600 ($P < 0.01$); in the evening, there was no difference between both photoperiods. Hence, even in the vl-SCN, the main difference between the long and the short photoperiod rhythmicity occurred in the morning hours.

Under LD 16:8 as well as under LD 8:16, ANOVA with repeated measurements revealed a highly significant difference between the dm- and the vl-SCN ($F = 243.3, P < 0.01$ and $F = 96.5, P < 0.01$, respectively), as well as a significant difference of time ($F = 22.8, P < 0.01$ and $F = 91.1, P < 0.01$, respectively) and a significant interaction effect ($F = 3.6, P < 0.01$ and $F = 2.8, P < 0.01$, respectively). Average values of c-Fos immunoreactivity were higher in the dm- than in the vl-SCN. During the morning c-Fos rise, the immunoreactivity in the dm-SCN was significantly higher than that in the vl-SCN at 0300, 0330, and 0400 ($P < 0.01$) under LD 16:8 and at 0700 and 0730 ($P < 0.01$ and 0.05, respectively) under LD 8:16. Importantly, a significant morning rise in c-Fos immunoreactivity occurred by 0.5 h earlier in the dm- than in the vl-SCN (Fig. 2). The data suggest different mechanisms of the morning c-Fos rise in the two SCN subdivisions. In the dm-SCN, the rise might be driven by an endogenous pacemaker and occur spontaneously, whereas in the vl-SCN, the rise might be induced by the morning light onset.

To test the suggestion, a response of the vl- and of the dm-SCN to a morning light pulse was followed in rats maintained in LD 16:8 (Fig. 3, A and B), as well as in those maintained in LD 8:16 (Fig. 3, C and D). On the day of the experiment, the morning light was not turned on and animals were either exposed to a 30-min light pulse around the time of the usual light onset or they were left untreated in darkness. In the dm-SCN (Fig. 3, A and C), a light pulse administered at the time of the usual light onset or 1, 2, or 3 h later did not induce any increase of c-Fos immunoreactivity above the already high morning levels be it in rats maintained previously in LD 16:8 (Fig. 3A) or in LD 8:16 (Fig. 3C). In the vl-SCN (Fig. 3, B and D), however, a light pulse administered at the time of the usual light onset and 1, 2, and 3 h later induced a significant c-Fos increase above low morning control levels in darkness, both in rats maintained previously in LD 16:8 ($P < 0.001$, 0.001, 0.001, and 0.01, respectively; Fig. 3B) and in those maintained in LD 8:16 ($P < 0.001$, Fig. 3D). The increase was the highest after a pulse administered at the time of the usual light onset and declined progressively with the later time of the pulse administration. In LD 16:8 the induced c-Fos immunoreactivity was significantly higher after a light pulse at 0400.
than after a pulse at 0500, 0600, and 0700, respectively ($P < 0.01$) and after a pulse at 0500 than after a pulse at 0700 ($P < 0.05$). In LD 8:16, the induced c-Fos immunoreactivity was significantly higher after a light pulse administered at 0800 than after a pulse at 0900, 1000, and 1100, respectively ($P < 0.01$), and after a pulse at 1000 than after a pulse at 1100 ($P < 0.05$).

Rhythms of c-Fos immunoreactivity in the whole SCN, in the dm-SCN, and in the vl-SCN in summer and in winter are shown in Fig. 4. For the dm-SCN, the two-way ANOVA also revealed a significant difference between summer and winter ($F = 55.2$, $P < 0.01$), as well as a significant difference of time ($F = 12.3$, $P < 0.01$) and a significant interaction effect ($F = 11.3$, $P < 0.01$). A significant evening decline of c-Fos immunoreactivity in summer occurred at 2000 vs. 1200 ($P < 0.05$) and a significant morning rise at 0500 vs. 0300 ($P < 0.05$); the interval of low c-Fos immunoreactivity thus lasted for about 9 h. In winter, c-Fos immunoreactivity declined at 1800 vs. 1500 ($P < 0.05$) and increased again at 0700 vs. 0500 ($P < 0.05$), the interval of low c-Fos immunoreactivity thus lasted for about 15 h and was 6 h longer than that in summer. Whereas in the evening there was no

Fig. 3. The effect of a morning light exposure on c-Fos immunoreactivity in the dorsomedial (A and C) and in the ventrolateral (B and D) SCN. Rats were maintained either in LD 16:8 (A and B) or in LD 8:16 (C and D). On the day of the experiment, light was not turned on as usual, i.e., at 0400 in LD 16:8 (A and B) and at 0800 in LD 8:16 (C and D). Rats were exposed to a 30-min light pulse at the time of the usual morning light onset and 1, 2, and 3 h thereafter, respectively. They then were returned to darkness and perfused 30 min later (●). Control animals experienced just darkness and were perfused at the same times as the experimental ones (■). Each point represents mean ± SE from 4 animals.
significant difference between a summer and a winter c-Fos immunoreactivity in the dm- as well as in the vl-SCN at any time point studied, in the morning, c-Fos immunoreactivity was higher in summer than in winter at 0400, 0500, and 0600 (P < 0.05) in both SCN subdivisions and at 0700 (P < 0.05) only in the vl-SCN. Hence the dm- and vl-SCN c-Fos immunoreactivity profiles in summer differed from those in winter mostly in the morning hours. A significant morning c-Fos rise in summer as well as in winter occurred 1 h earlier in the dm- than in the vl-SCN, due probably to c-Fos endogenous rhythmicity in the dm-SCN and c-Fos photoinduction in the vl-SCN.

In summer, when the night lasted ~8 h (Fig. 1), the evening c-Fos decline and the morning rise in both SCN subdivisions occurred also at about the same time as under a corresponding artificial LD 8:16 photoperiod (compare Figs. 2 and 4). A main difference in c-Fos immunoreactivity between long and short days, whether under artificial or natural photoperiods, occurred in the morning hours.

**DISCUSSION**

Under an actual long artificial photoperiod as well as under a short one, rhythms in the dm-SCN c-Fos immunoreactivity resembled those of rats maintained under a long or a short photoperiod and then released into darkness (25). The morning c-Fos rise under both photoperiods occurred well in advance of the usual light onset, as was the case in rats released into darkness. A light pulse administered around the time of the dark-light transition did not increase further a high c-Fos immunoreactivity present already at the time of the usual light onset. Hence, under actual LD cycles, the morning rise was spontaneous and not induced by light, as was the case in darkness (25, 26). The interval of low c-Fos immunoreactivity in the dm-SCN was about 6 h longer in rats maintained in LD 8:16 than in those maintained in LD 16:8, again as was the case in rats maintained in short or long days and then released into constant darkness (25). In rats maintained under natural daylight, the interval of low c-Fos immunoreactivity in the dm-SCN was also about 6 h longer in winter than in summer. In all the above-mentioned cases, the difference in the interval duration between a long and a short photoperiod was mostly due to a delayed c-Fos rise under a short photoperiod compared with that under a long one; significant differences in c-Fos immunoreactivity between long and short days were always found only in the morning hours (Ref. 25 and the current study).

In the vl-SCN, rhythms of c-Fos immunoreactivity were robust in rats maintained under actual long or short photoperiods, either artificial or natural ones, in contrast to just faint rhythms of c-Fos immunoreactivity observed in rats maintained under various photoperiods and then released into darkness (25, 26). At first glance, the vl-SCN rhythms looked almost the same as the dm-SCN ones. Difference in duration of a low c-Fos interval between a long and a short photoperiod was about 6 h, and the morning c-Fos rise started around the time of the morning light onset. However, the morning c-Fos rise under a long or a short photoperiod, either artificial or natural, occurred always later in the vl- than in the dm-SCN. Whereas in the dm-SCN, the c-Fos rise occurred spontaneously before the light onset and a light exposure around the time of the dark-light transition did not induce any further increase in c-Fos immunoreactivity, in the vl-SCN, the morning c-Fos rise might be mostly induced by the light onset. A photic stimulus administered around the time of the dark-light transition induced a robust c-Fos increase from a low nighttime level in darkness to a high morning level in light. The data suggest a differ-
ent mechanism of the morning c-Fos rise in the dm- and in the vl-SCN; in the former, the rise is due to the endogenous dm-SCN rhythmicity, whereas in the latter, the rise is mostly due to c-Fos photoinduction. A small c-Fos rise in the vl-SCN observed at the time of light onset might be due partly to a faint spontaneous c-Fos increase in this part of the SCN but partly also to the high morning c-Fos rise in the dm-SCN inasmuch as accurate separation of both the SCN subdivisions is very difficult. Interestingly, c-Fos immunoreactivity in the vl-SCN of rats maintained either under artificial or natural long or short photoperiods remained elevated during the light period of the day and declined only in the late afternoon, as was the case in the SCN of rats maintained under an actual LD 12:12 cycle (22, 23), although theoretically a light stimulus administered during the subjective night but not during the day induces high c-Fos immunoreactivity in the SCN (13, 22, 27). However, our results show that a light pulse administered as late as 3 h after the expected morning light onset is still capable of inducing a small, but significant c-Fos increase above baseline levels in darkness and hence it is possible that continuous light exposure during the light period of the day may repetitively and additively induce an increased c-Fos production.

Under actual LD cycles, both the dm- and the vl-SCN c-Fos rhythms contributed to the overall SCN rhythm of c-Fos immunoreactivity; the dm-SCN c-Fos rise always preceded the vl-SCN one. Because the presence and amount of c-Fos may serve as an indicator of neuronal activity (21), it appears that under an entrained state, the dm-SCN neurons start to be active earlier in the morning than the vl-SCN ones. Although the vl-SCN projects densely to the dm-SCN, there is just little reciprocal innervation (15). Hence, information on the early morning dm-SCN neurons activation may not necessarily reach and affect the vl-SCN. Vice versa, information on a light exposure at night, conveyed, besides other ways, also by c-Fos photoinduction in the vl-SCN (13, 22, 28, 31) and hence information on activation of the vl-SCN neurons, might reach and affect the dm-SCN neurons more easily.

Rhythms of c-Fos immunoreactivity in the dm- as well as in the vl-SCN of rats maintained under a long or a short artificial photoperiod did not markedly differ from those of rats maintained under a natural long or short photoperiod and experiencing thus twilight at dawn and dusk; the time of the morning c-Fos rise, of the afternoon decline, as well as difference in the interval of low c-Fos immunoreactivity between a long and a short photoperiod were almost the same under artificial square-wave LD cycles with abrupt dark-light and light-dark transitions as under natural twilight cycles with gradual transitions. The experiments were, however, performed on animals entrained to artificial photoperiods or maintained under natural daylight for a long time. As dynamics of photoperiodic responses and of photic entrainment may depend on the way of changing LD cycles and on presence of twilight transitions (1, 6), the data on similarity of the SCN c-Fos rhythms under an artificial and a natural photoperiod cannot exclude a possibility that adjustment of the rhythms to a change of the photoperiod may proceed in a different way under an artificial and a natural photoperiod. In contrast to rats, the SCN rhythm of c-Fos photoinduction in gerbils maintained under a square-wave LD cycle differs from that in animals maintained under a natural daylight with twilight transitions (2). Whereas under the former regimen, the rhythm of c-Fos photoinduction exhibits a morning peak as in rats, under the latter regimen, the rhythm exhibits, in addition to the morning, also an evening peak; no such evening peak has been observed in rats. The discrepancies may indicate a difference in the SCN c-Fos photoinduction among species.

In conclusion, under an actual long or short photoperiod, either an artificial or a natural one, c-Fos immunoreactivity increases from low nighttime values before the time of the morning light onset in the dm- and at the time of the onset in the vl-SCN; the late day decline occurs in both the SCN parts at about the same time. Difference in the interval of low c-Fos immunoreactivity in both the SCN subdivisions between a long and a short photoperiod, either artificial or a natural one, is around 6 h. This difference may be a part of the SCN photoperiod modulation, which is subsequently transduced to other photoperiodic signals.

**Perspectives**

Actual photoperiods modulate rhythms of c-Fos immunoreactivity in both SCN dm and vl subdivisions. Protein c-Fos as a transcriptional factor may either be a part of the SCN entraining and circadian pacemaking system, or expression of its gene may be itself clock-controlled. Recently, more of mammalian clock genes have been cloned (for review, see Ref. 3) and some of their proteins, namely mPER1, mPER2 (8, 14), as well as mCRY1 and mCRY2 (14) exhibit marked circadian rhythms in the SCN. Our results suggest that rhythms of these clock proteins also may be modulated by an actual photoperiod and hence the whole SCN core clock mechanism may be photoperiod dependent. Recent results on the Per1 rhythm in the Syrian hamster SCN show that this indeed may be the case (16). Hence, it appears that the SCN pacemaking system may provide a daily as well as a seasonal program for the whole organism, as was suggested by the late Colin S. Pittendrigh (19).

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