Photic Regulation of Fos-Like Immunoreactivity in the Suprachiasmatic Nucleus of the Mouse

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ABSTRACT

In mammals, the suprachiasmatic nucleus (SCN) of the hypothalamus functions as the primary pacemaker of the circadian system. Light has been shown to induce Fos-like immunoreactivity (Fos-LI) in the SCN of rats and hamsters. The purpose of the present study was to evaluate extensively the effect of light on Fos-LI in the mouse SCN. Brief pulses of light administered to animals otherwise in constant darkness were found to induce Fos-LI. This photic induction was unaffected by the *rd* mutation, which causes the profound loss of photoreceptors but fails to affect circadian responses to light. Light regulation of Fos-LI was dependent upon the phase of the circadian cycle in which the light pulse was administered. Phases at which light causes phase shifts of the circadian system were permissive for Fos-LI induction (CT 16 and 24), while phases in which light does not cause phase shifts were not permissive (CT 6 and 9). The time course of the induction at CT 16 was also described. In a light/dark cycle, Fos-LI was found to be rhythmically expressed with Fos-LI elevated soon after the lights came on but remaining low throughout the rest of the cycle. However, this rhythm is a direct consequence of the light because in constant darkness Fos-LI was always low. These results have implications regarding the possible functional roles of Fos in the circadian system and add to our understanding of light regulation of circadian physiology in the mouse.
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Key words: retinal degenerate (*rd/rd*) mouse, circadian rhythm, SCN, photic, Fos

Circadian rhythms are endogenously generated oscillations with periods of approximately 24 hours. In order to function adaptively, the endogenous oscillators responsible for these rhythms must be synchronized (or entrained) to the 24 hour period of the environment. The daily light-dark cycle, acting through light-induced advances and delays of phase of the endogenous oscillation, is the primary cue responsible for the entrainment of these oscillators to the environment. Thus, characterization of the mechanisms by which light influences the phase of circadian oscillators is one of the major problems which must be addressed in order to understand circadian organization.

In mammals, the suprachiasmatic nucleus (SCN) functions as the dominant pacemaker of the circadian system. Bilateral destruction of the SCN abolishes a large number of physiological and behavioral circadian rhythms (Moore and Eichler, '72; Stephan and Zucker, '72; Meijer and Rietveld, '89). Further, the region of the hypothalamus that contains the SCN is capable of sustaining circadian oscillations *in vitro* (Green and Gillette, '82; Earnest and Sladek, '86) and there is recent evidence that disassociated SCN cells in culture retain this property (Murakami et al., '91). In addition, it was shown that transplanting SCN from one animal to another whose SCN had been lesioned restored rhythmicity and also imposed the period of the donor animal's circadian rhythm on that of the host (Ralph et al., '90; Ralph and Lehman, '91).

The effects of light on the SCN are mediated by unknown photoreceptors, located in the retina, which project to the hypothalamus, at least in part, via a monosynaptic fiber tract known as the retinohypothalamic tract (RHT) (Moore, '83; Foster et al., '91). Although the transmitter(s) at the RHT/SCN synaptic connection remains unidentified, a variety of evidence now suggests that excitatory amino acids are involved (Rusak and Bina, '90; Colwell et al., '91). The protein Fos (the product of the c-fos gene) is induced in neurons in response to a number of stimuli including excitatory amino acids (Morgan and Curran, '91). Recent work has shown that light causes an increase in Fos-like immunoreactivity in the SCN (Rea, '89; Aronin et al., '90; Earnest et al., '90; Rusak and Bina, '90). This increase in immunoreactivity appears to be a consequence of light-

induction of c-fos mRNA (Kornhauser et al., '90; Rusak et al., '90). Further, the induction by light of c-fos mRNA in the SCN shows a similar phase dependence and threshold as light induced phase shifts of the circadian oscillator. This work suggests that Fos induction can be used as a cellular marker of photic input to the SCN and raises the possibility that Fos induction may play a causal role in the phase shifting of the circadian system by light.

We feel that the mouse, which offers both a well-characterized circadian system and the possibility of genetic manipulation, will prove to be an advantageous preparation to address questions about the role of Fos in the circadian system. In the present study, we characterized light-induced changes in Fos-LI in the mouse SCN. We then compared light-induction of Fos-LI in a retinal degenerate (rd/rd) and wild-type mouse. Finally, we described the temporal profile of Fos-LI in the SCN of mice maintained on either a light/dark (LD) cycle or in constant darkness (DD).

METHODS
Experimental animals

Adult male mice (Mus domesticus, C57BL/6J, rd/rd or +/+), obtained from our breeding colony at 60 days of age, were housed individually and their wheel-running activity recorded. The animals were exposed to a 12:12 LD cycle for 2 weeks. They were then either left in the LD cycle or placed in DD for at least 10 days to assess their freerunning locomotor activity period. The mice had free access to food and water.

The light stimulus used in these experiments was a 15 minute pulse of monochromatic light (515 nm; half-peak bandwidth 10 nm) at an irradiance of $1 \times 10^{-1} \mu W/cm^2$. The irradiance of the light pulse was measured before each trial with a UDT radiometer (United Detector Technology, CA). The light stimulus parameters (duration, irradiance, and wavelength) were chosen to produce a 60-70 % of maximal phase delay at Circadian Time 16 (see Foster et al. '91). Following the light pulse, the mice were returned to constant darkness until the time of perfusion. All handling and treatment of animals before perfusion was carried out in complete darkness with the aid of an infrared viewer (FJW Industries, Elgin, IL).

Tissue preparation and immunocytochemistry

For perfusion, animals were removed from their light-tight boxes and anaesthetized with a lethal dose of halothane by inhalation. Then, under dim red light, the mice were perfused intracardially first with 4-5 ml physiological saline (0.9% w/v) containing 150 IU heparin/10 ml, followed by 200 ml 2% w/v paraformaldehyde in phosphate-buffered saline (PBS; pH 7.2-7.4). After perfusion, the brain was removed and postfixed overnight at 4°C. Serial frontal sections (50 μm thick) were cut into cooled phosphate buffered saline (PBS) using an Oxford Vibratome. Free-floating sections were washed in fresh PBS for 2-3 hours at 4°C and transferred to PBS containing 0.2% Triton X-100 (PBS-T) for 30-40 minutes, before immunocytochemical processing.

Sections were transferred to a solution of normal goat serum (1/30 in PBS-T) for 30 minutes and then directly into primary antiserum. Two Fos antisera were used in this study. We initially used a rabbit polyclonal antiserum (anti-Fos 132-154), which was generously donated by Dr.

Steven Sagar (University of California, VA Medical Center, San Francisco). The working dilution for this antibody was 1:100 for 72 hours at 4°C. All of the results obtained with this antibody were duplicated with a rabbit polyclonal antiserum (anti-Fos 4-17) purchased from Oncogene Science (Manhasset, NY). This antibody was used at a dilution of 1:250 for 72 hours at 4°C. We found no differences between the results obtained with either of the two antibodies. The sites of the antibody:antigen binding were visualized with an avidin-biotin-peroxidase procedure (Elite ABC kit, Vector Labs, Burlingame, CA). Sections were washed in Tris buffer (pH 7.2, 15 minutes) before incubation for 10 minutes in 0.025% diaminobenzidine (DAB) containing 0.003% (w/v) peroxide. Sections mounted on gelatin-coated slides were dehydrated through graded alcohols into xylene and left overnight then rehydrated to distilled water and immersed in 0.2% (w/v) osmium tetroxide solution (2 minutes) to intensify the DAB reaction. Histological analysis and photomicrographs utilized a Zeiss Axiophot photomicrographic system.

To minimize variability, we used a standardized immunocytochemical protocol, which kept incubation times constant and used a single batch of antisera and other reagents. In addition, we processed mouse tissue for immunocytochemistry in large groups of 10 or 12 brains which constituted a self-contained experiment. Two types of immunocytochemical controls were performed. Tissue sections were either processed for immunocytochemistry, replacing the primary antiserum with normal rabbit serum, or incubated with "pre-absorbed" primary antibody. To pre-absorb the antibody, 1 μg of peptide (Oncogene Sciences, Manhasset, NY) was added to 1 ml of diluted antibody and incubated at 4°C overnight before being added to the brain sections. No staining was observed in either case.

Analysis of Fos immunostaining within the mouse SCN

A series of immunostained sections were counterstained with toluidine blue in order to define Fos immunostaining within the histological borders of the SCN. Non-counter stained sections were observed by phase-contrast microscopy to confirm the distribution of Fos staining within the SCN. Camera lucida drawings of Fos-stained perikarya within the SCN were made. It was possible to accurately identify and count immunostained perikarya. Cell number was recorded in every section by an observer who did not know the treatment history of the animal. To determine whether there were differences in cell number between the animals with different treatments, the counts were analyzed by a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison procedures where appropriate. Values were considered significantly different if $P < 0.05$.

RESULTS

Light caused an increase in Fos-LI in the SCN of mice (Fig. 1). All of the mice exposed to a light pulse at CT 16 and killed 90 minutes after the onset of the 15 minute treatment (n = 16) showed robust staining in the SCN. A schematic diagram of a representative pattern of Fos-LI in the SCN after a light pulse is shown in Figure 2. Stained cells were found in the dorsal and lateral borders of the rostral SCN; however, most staining was seen in the central
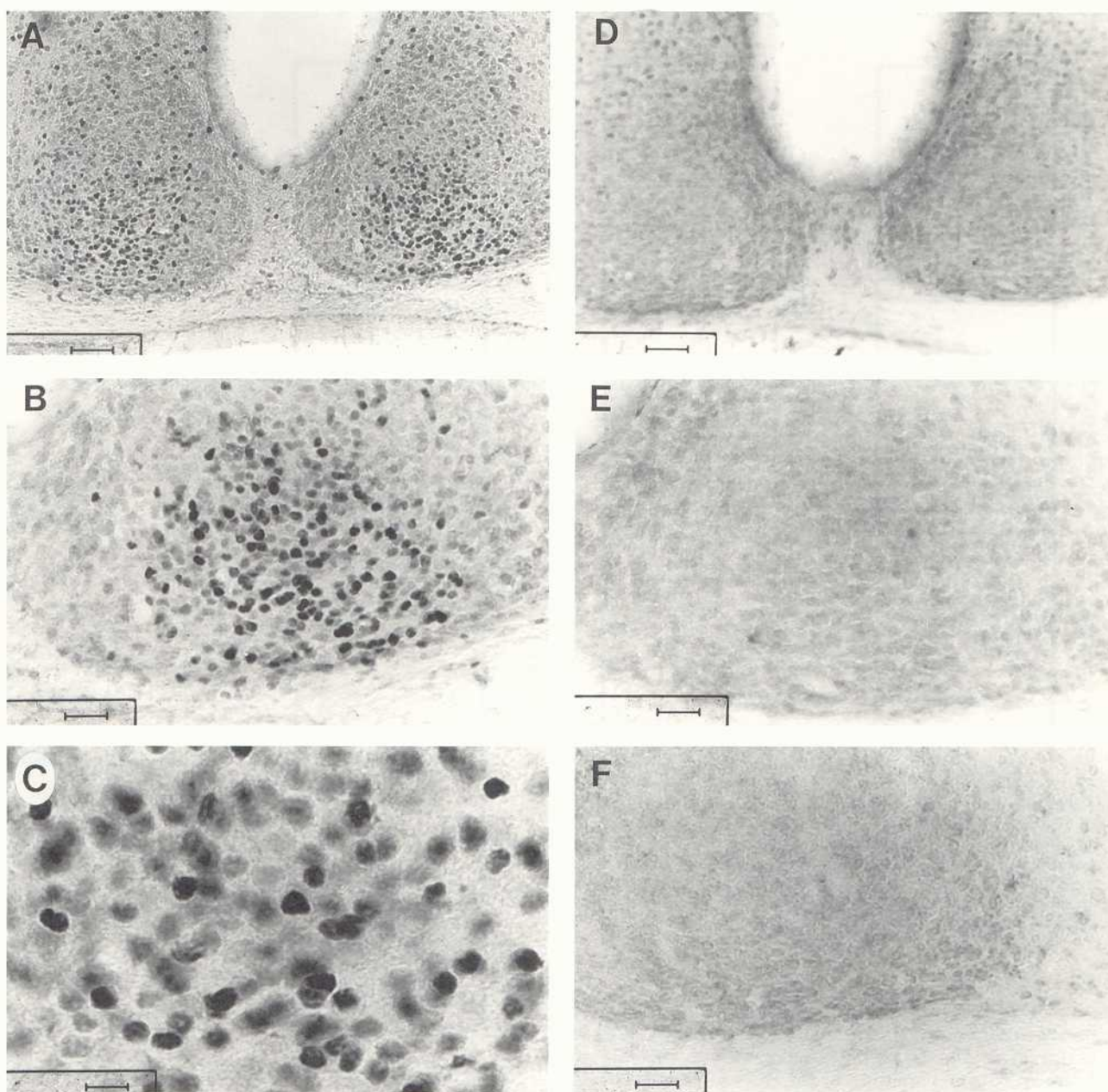
Fig. 1. Photomicrographs of coronal tissue sections through the suprachiasmatic (SCN) region of a mouse (C57BL/6J, *rd/rd*) which have been stained for Fos-LI. Mice in constant darkness were exposed to either 15 minutes of light 4 hours after activity onset at circadian time (CT) 16 (**A, B,** and **C**), or handled but not exposed to light (**D,E**). Animals were perfused 90 minutes after the beginning of the light treatment. There was no evidence for staining in preabsorption controls (shown in F) which received a light pulse but were also incubated with the Fos peptide. Scale bar = 50 μm for A and D, 25 μm for B,E,F, and 10 μm for C.

region of the SCN. Control animals, which were handled but not exposed to the light, showed little or no staining in the SCN (n = 8).

We examined light-induction of Fos-LI in the SCN of both retinal degenerate and wild-type C57 mouse. We found no differences in either the distribution or number of Fos-LI cells between the two strains. Figure 3 shows a schematic diagram which illustrates representative patterns of Fos and other immunoreactivities in the SCN as

well as counts of the number of immunoreactive cells in the SCN of both retinal degenerate and wild-type mice. There were no significant differences between the results obtained from the two genotypes.

The time course of this photic induction of Fos-LI was investigated by collecting brains from mice at various intervals after exposure to the light pulse (Fig. 4A,B); 30 minutes after the beginning of exposure to a 15 minute light pulse, Fos-LI could not be detected in the SCN (n = 4).
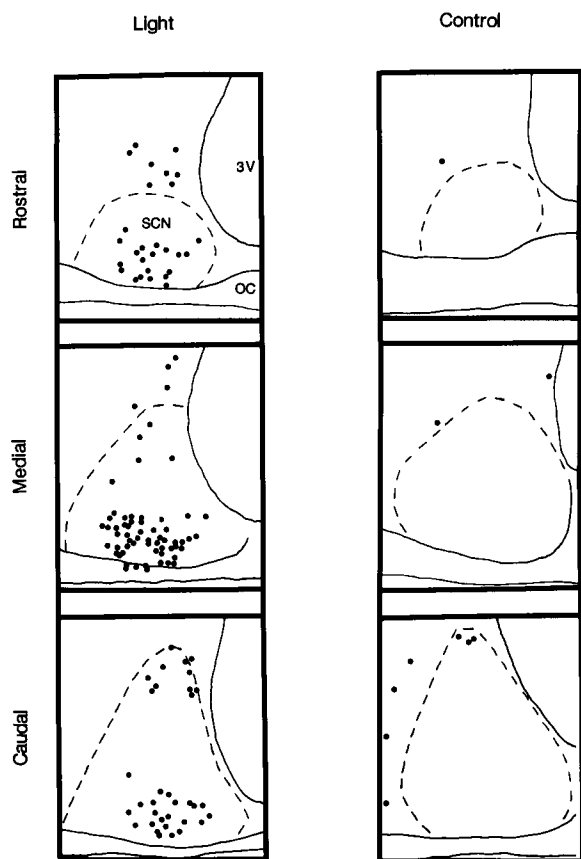
Fig. 2. Schematic diagrams illustrating the distribution of Fos-LI through the rostral, intermediate, and caudal aspects of the mouse SCN. The animal on the left (labeled "Light") was exposed to 15 minutes of light at CT 16 while the animal on the right (labeled "Control") was handled but not exposed to light. The animals were perfused 90 minutes after treatment. Immunoreactive perikarya are represented by black dots and the Nissl-defined SCN is shown with the dashed line. OC, optic chiasm; 3V, third ventricle.

Staining was clearly detected as early as 45 minutes after exposure to a light pulse and persisted clearly for as long as 4 hours after the light pulse. It was not possible to distinguish between staining in the SCN of mice killed 45 (n = 4), 60 (n = 5), 90 (n = 10), 120 (n = 3), and 240 (n = 3) minutes after the beginning of the light pulse. However, by 6 hours after the beginning of the light pulse, the Fos-LI could not be distinguished from untreated controls in three out of four animals. In one animal, Fos-LI could still be detected in the SCN 6 hours after the light pulse.

The effect of a light pulse on Fos-LI in the SCN was found to depend on the phase at which the light pulse was delivered (Fig. 5). At CT 16, the light pulse was found to cause both a phase delay in the circadian rhythm of locomotor activity as well as an induction of Fos-LI in the SCN. This was also true at CT 24 (n = 3) when light causes a phase advance in the behavioral rhythm. In fact, it was not possible to distinguish between the effect of a light pulse delivered at CT 24 or at CT 16 on Fos-LI. However, the same light treatment delivered at CT 6 (n = 3) or CT 9 (n = 3) did not cause a significant phase shift, nor did it result in
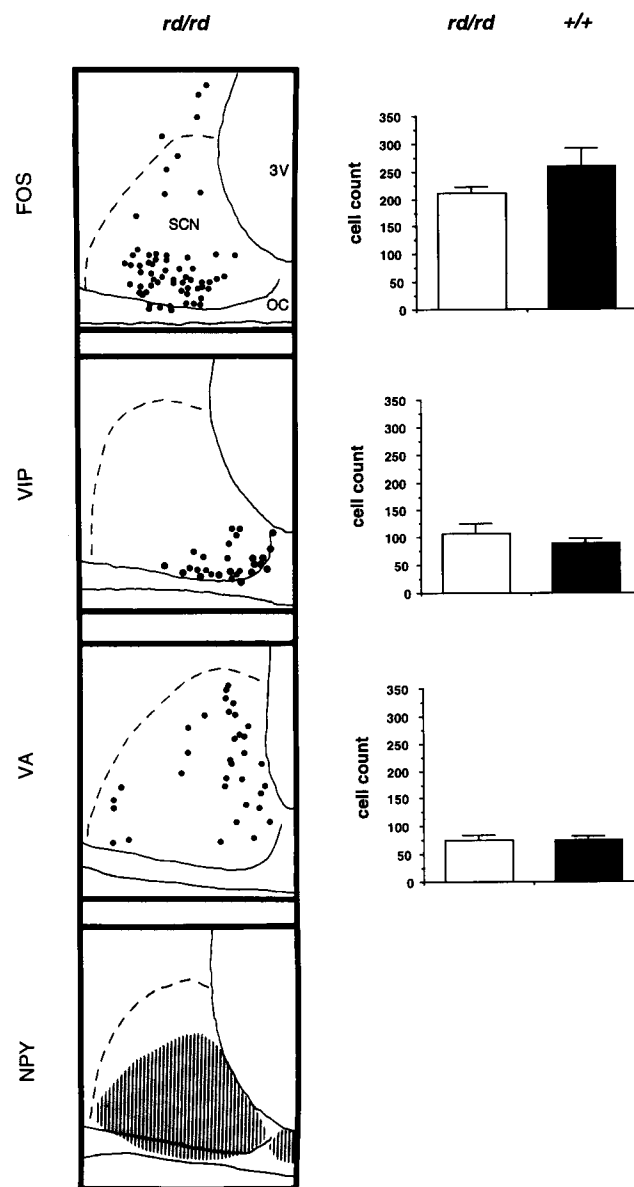
Fig. 3. Left column: schematic representation of immunocytochemical distributions in the medial SCN of the mouse. Immunoreactive perikarya are represented by black dots, immunoreactive processes are represented by black stripes, and the Nissl-defined SCN is shown with the dashed line. FOS, Fos-like immunoreactivity; VIP, vasoactive intestinal peptide-like immunoreactivity (Peninsula Labs, 1:1,000); VA, vasopressin-like immunoreactivity (Incstar, 1:500); NPY, neuropeptide Y-like immunoreactivity (Peninsula Labs, 1:1000); OC, optic chiasm; 3V, third ventricle. Right column: mean number of immunoreactive cells in the SCN of retinal degenerate and wild-type mice. There were no significant differences between the two strains. n = 4–6 for all points; vertical bar represents S.E.M.

a detectable increase in Fos-LI compared to controls which were handled but not exposed to light.

The SCN of mice maintained on a LD cycle showed a daily rhythm in Fos-LI (Fig. 6). When mice were killed during the light phase of the LD cycle, Fos-LI was detected in the SCN 1 hour after the beginning of the light phase (lights-ON).
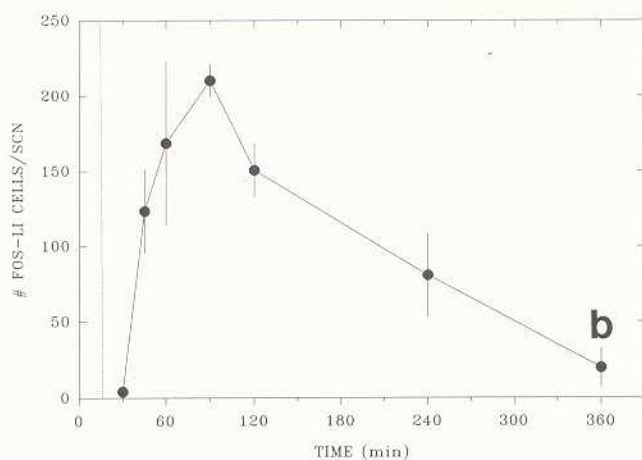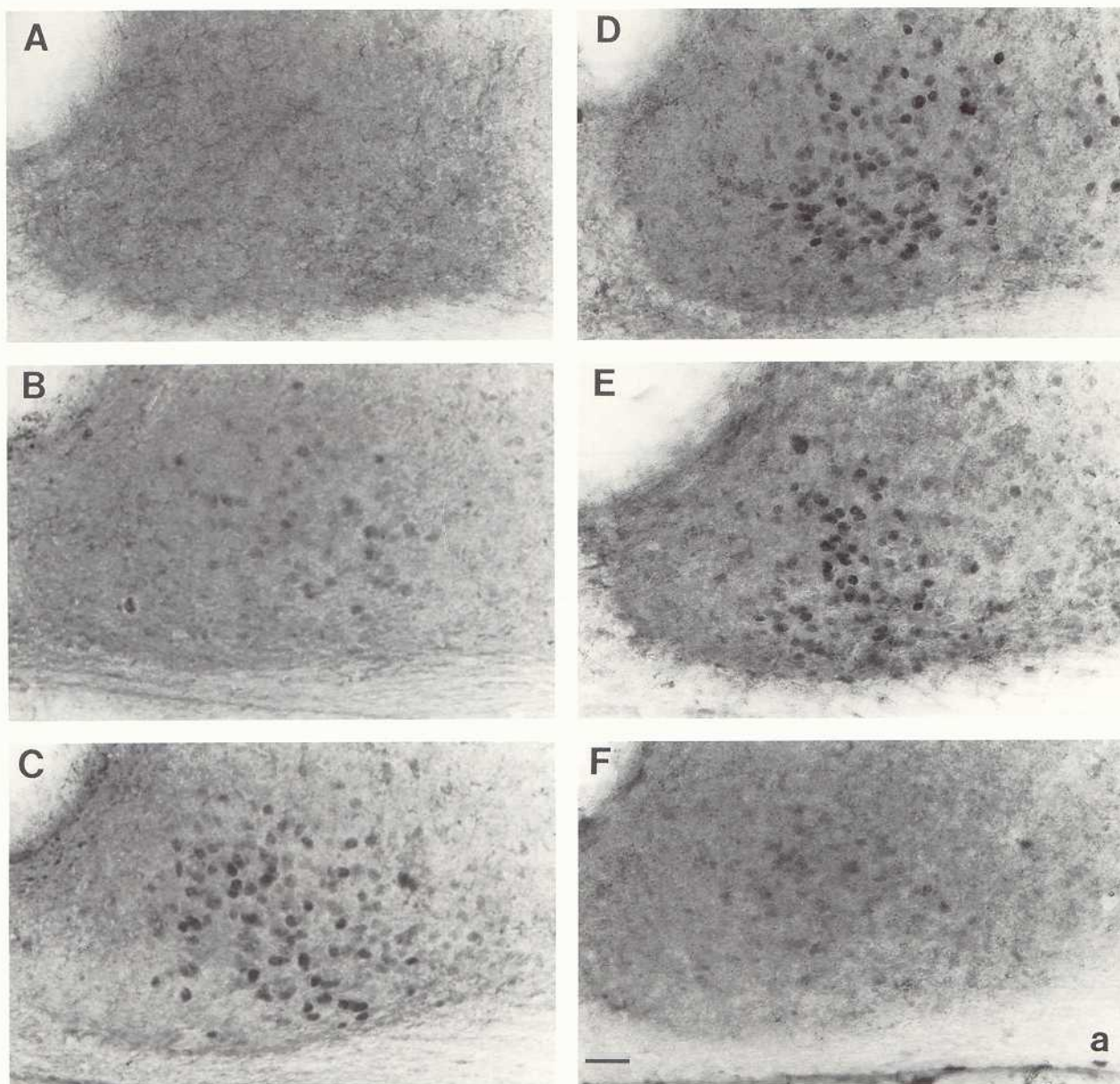
Fig. 4. **a:** Photomicrographs of coronal sections through the SCN region of mice that have been stained for Fos-LI. Mice in constant darkness were exposed to 15 minutes of light at CT 16 and were killed at various intervals after the light pulse: A = 30 minutes after the beginning of the light pulse, B = 45 minutes, C = 60 minutes, D = 120 minutes, E = 240 minutes, and F = 360 minutes. Scale bar = 25 µm. **b.** Mean number of Fos-positive cells in the SCN at various intervals after the light treatment. The light pulses were delivered at CT 16 (time = 0 in the figure; the end of the 15 minutes light pulse is represented by the dotted line). n = 3-6 for all points; vertical bar represents S.E.M.

However, by 4 hours after lights-ON, both the number of SCN cells stained and the intensity of staining was dramatically reduced. There was no detectable Fos-LI in the SCN of mice killed at 6 hours and 11 hours after lights-ON or at 1, 4, 6, and 11 hours after the beginning of the dark interval of the LD cycle (lights-OFF). Animals maintained on a LD cycle were capable of responding to a light pulse with an
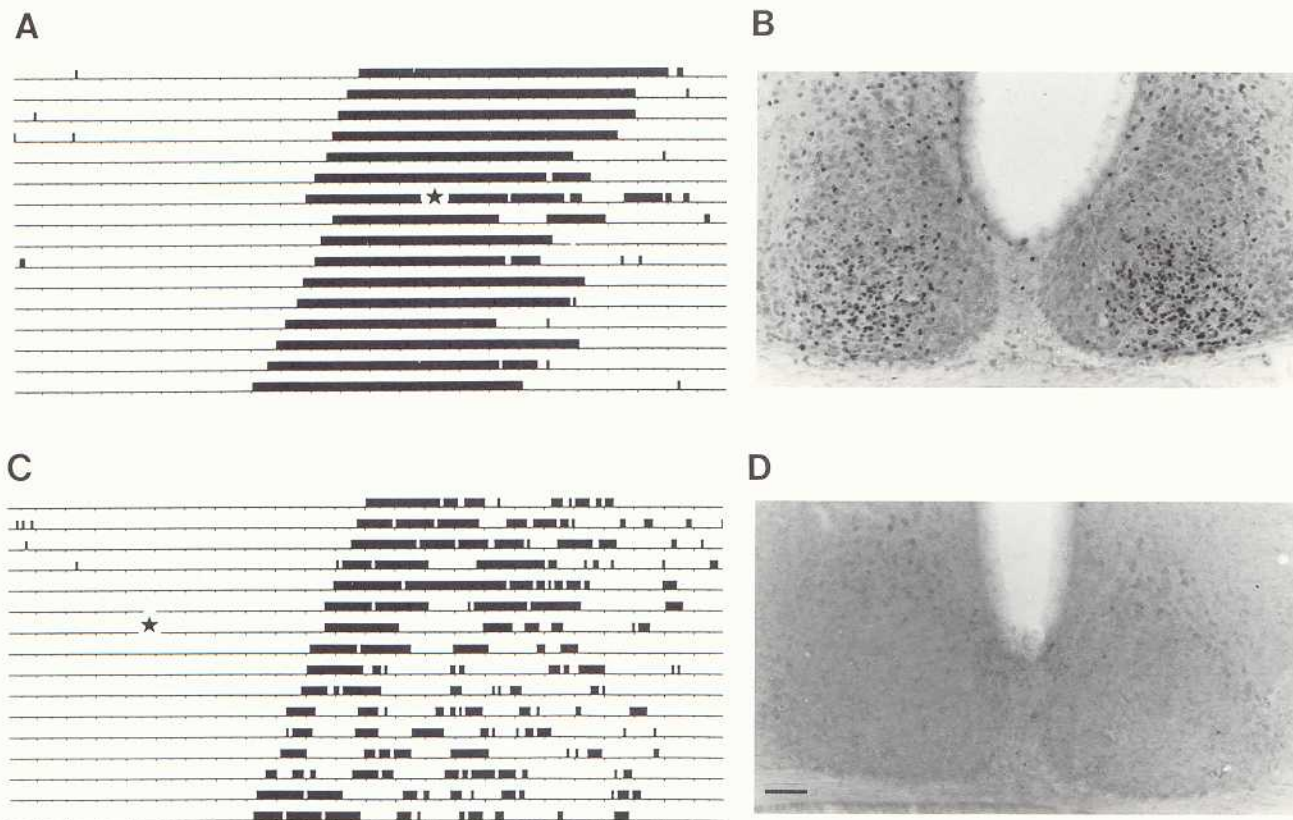
A

B

C

D

Fig. 5. Photomicrographs of coronal sections through the SCN region of mice that have been stained for Fos-LI. Mice in constant darkness were exposed to 15 minutes of light at either CT 6 (shown in D) or CT 16 (shown in B) and were killed 90 minutes later. The left panels illustrate the effect of the same light treatments on the circadian rhythm of wheel-running activity at CT 6 (shown in C) and CT 16 (shown in A). Scale bar = 50 μm.
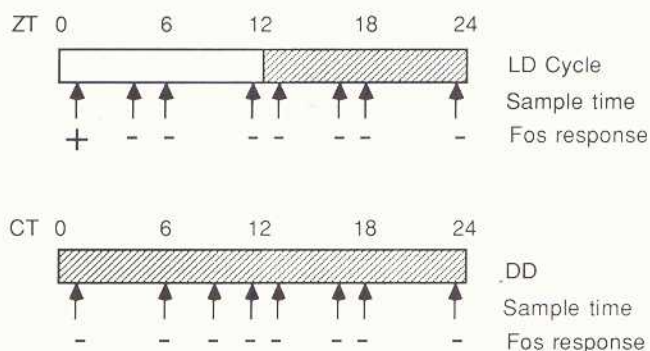

Fig. 6. Schematic diagram illustrating the expression of Fos-LI in the SCN of animals maintained in a light-dark cycle (LD) or in constant darkness (DD). The phases in which Fos levels were sampled are indicated by the arrows. The presence of Fos-LI in the SCN is indicated by a plus (+) while the lack of Fos-LI is indicated by a minus (−). n = 2–6 at each point.

increase in Fos-LI. Four hours after lights-OFF, mice responded to a light pulse (15 minutes in duration) with a clear increase in both the intensity and number of cells stained compared to untreated controls when measured 1 hour after the light pulse.

When animals were placed into DD, there was no evidence that the SCN showed a daily rhythm in Fos-LI (Fig. 6). The daily rhythm found when animals were maintained on a LD cycle was not endogenous in nature. Mice were killed at various phases of their locomotor activity cycle: CT 1 (n = 3), CT 6 (n = 3), 9 (n = 3), 11 (n = 2), 16 (n = 3), 18 (n = 4) and 24 (n = 3). No clear Fos-LI was found in the SCN at any of these phases. However, in some animals the dorsal border of the SCN contained some Fos-LI cells. This staining was not consistent nor did it correlate with any particular phase.

DISCUSSION

During a LD cycle, the SCN of mice showed a daily rhythm in Fos-LI. Fos-LI was found in the SCN in the beginning of the day. However, by 6 hours after lights-ON, Fos-LI had declined and remained low throughout the rest of the day and night. We have shown that, in DD, Fos-LI is always low. We were unable to detect any evidence for a circadian rhythm in Fos-LI. Therefore, the rhythm seen in LD is a direct consequence of the environmental lighting conditions and is not a reflection of endogenous rhythmicity. This is an important result which addresses the possible roles of Fos in the circadian system. The fundamental components of an oscillator must vary with the period of the oscillation. Since we have no evidence that Fos shows a

circadian rhythm, this suggests that transcriptional regulation involving the Fos protein is not part of the central mechanism by which the circadian oscillation is generated. However, Fos could play an important role in coupling the oscillating components of the pacemaker or in coupling the environment to the oscillator.

Under constant conditions, mice show a circadian rhythm of wheel-running activity with a period less than 24 hours. In order for these animals to be synchronized or entrained to a 24 hour LD cycle, the light signal must cause a daily phase delay in the endogenous oscillation. In mice, as in all other organisms, light causes phase delays in the early subjective night and phase advances in the late subjective night (Daan and Pittendrigh, '76; Colwell et al., '91). Thus, if Fos couples the SCN pacemaker to the photic environment, we would expect Fos levels to be high late in the day or in the early night. However, when we examine the expression of Fos-LI in an LD cycle, we do not find any evidence for Fos levels being elevated in the night and only find Fos-LI in the beginning of the day. This result is not consistent with Fos acting in a simple way as part of the mechanism by which light causes phase shifts of the circadian system.

A brief pulse of light was found to result in an increase in Fos-LI both in DD and during the dark interval of a LD cycle. Light pulses have been found to cause an increase in Fos-LI in the SCN of hamster and rat (Rea, '89; Aronin et al., '90; Earnest et al., '90; Rusak et al., '90) and in the mouse (present study). These increases in Fos protein are paralleled by increases in c-fos mRNA (Kornhauser et al., '90; Rusak et al., '90). In general, the pattern of light-induced staining includes areas of the SCN that receive retinal input via the RHT. In the hamster and rat, retinal input is generally to the ventral/lateral subdivision of the SCN (Johnson et al., '88) and there is a corresponding pattern of light-induced Fos-LI (Rusak et al., '90). On the other hand, in mouse, both the RHT innervation (Cassone et al., '88) and Fos staining in the SCN are more diffuse. However, there does not appear to be a complete overlap between regions of the SCN which receive retinal input and those which react to a light pulse with an increase in Fos-LI. For example, in the mouse and hamster, there appears to be light-induced staining dorsal to the Nissl-defined SCN. Further, in the mouse, the pattern of RHT input appears to include a much broader region of the SCN than that which responds to a light pulse with an increase in Fos-LI.

The ability of light to increase Fos-LI is phase dependent. In the mouse, the same light pulse which led to Fos-LI induction at CT 16 and 24 had no effect at CT 6 and 9. The same general result has been obtained in both hamster and rat. Such phase dependence is significant because it mirrors the phase dependence of the circadian system itself. In general, circadian oscillators respond to light with phase shifts during the subjective night and do not respond to the same light treatment given during the subjective day. In the mouse, light pulses given at CT 16 and 24 result, respectively, in phase delays and advances while they produce no phase shift at CT 6 and 9 (Colwell et al., '91). This phase dependence demonstrates that light's action in inducing Fos-LI is gated or regulated by a circadian oscillator.

Light treatment was found to lead to relatively long-lasting increases in Fos-LI. At CT 16, a 15 minute pulse of light resulted in stimulation of Fos-LI in the SCN for 4 hours and in one case 6 hours after the pulse had started. In

other systems, workers have found stimuli which led to increases lasting 2–4 hours (e.g., Sonnenberg et al., '89). It may be that some of the Fos-LI is a reflection of Fos-related antigens which are known to be expressed with different kinetics than Fos itself. The antibody used for this part of the study was directed against amino acids 132–154 of a synthetic Fos peptide. An antibody directed against a similar region of the Fos peptide (amino acids 127–152) has been shown to recognize the Fos protein as well as two Fos-related proteins. This antibody shows maximal staining 3–4 hours after stimulation in other brain areas (Morgan et al., '87; Sagar et al., '88). However, in a preliminary study, we obtained a similar time course of light induced Fos-LI with an antibody directed against amino acids 4–17 of the Fos peptide.

In the present study, we used a retinal degenerate mouse (rd/rd). This mutation affects rod photoreceptor cGMP-phosphodiesterase activity and results in the profound loss of visual photoreceptors (Bowes et al., '90). Previous studies have shown that light induced phase shifts of the circadian system are unaffected by this mutation (Colwell et al., '91, Foster et al., '91). In the present study, we found no difference between the photic induction of Fos-LI in the SCN of wild-type and retinal degenerate mice. Collectively, these results support the remarkable conclusion that light input to the SCN is unaffected by the loss of normal photoreceptors caused by the rd/rd mutation. We view the retinal degenerate mouse as a "reduced" preparation with which to examine questions about photic regulation of the circadian system.

Fos is a transcriptional regulatory factor which is normally involved in the transduction of extracellular signals into changes in gene expression (Morgan and Curran, '91). Thus it is likely that a light pulse which induces Fos protein is consequently altering transcription in the SCN. The functional significance of this regulation is currently unclear. It is exciting to speculate that some transcriptional event is part of the cascade by which light impacts on the circadian pacemaker in the SCN. In some circadian systems, protein synthesis inhibitors may inhibit light-induced phase shifts (e.g., Raju et al., '90; Johnson et al., '90). However, interpretation of these experiments is complicated by the fact that the inhibitors themselves cause phase shifts of the circadian system (e.g., Takahashi and Turek, '87; Raju et al., '91). New tools will need to be brought to bear on this problem. The mouse, with a well characterized circadian system and possibility of genetic manipulation, may prove to be an advantageous preparation to address questions about the roles of Fos and other transcriptional regulatory factors in the circadian system. The power of this animal model is illustrated by a recent study in which a transgenic mouse line containing a fos-lacZ fusion gene was described in which β-galactosidase activity can be used as a marker to identify cell populations expressing c-fos (Smeyen et al., '92). These workers found that a light pulse leads to increased β-galactosidase activity in the SCN during the night but not during the day. The use of transgenic mice should help to elucidate the signal transduction cascade that mediates the photic regulation of Fos in the SCN.

ACKNOWLEDGMENTS

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