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Light regulates expression of a Fos-related protein in rat suprachiasmatic nuclei

(circadian rhythm/transcription factor/hypothalamus)

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ABSTRACT **Mammalian circadian rhythmicity is endogenously generated by a pacemaker in the suprachiasmatic nuclei and precisely entrained to the 24-hr day/night cycle by periodic environmental light cues. We show that light alters the immunoreactive levels of a transcriptional regulatory protein, Fos, in the suprachiasmatic nuclei of albino rats. Photic regulation of Fos immunoreactivity does not occur in other retino-recipient brain areas except for the intergeniculate leaflet, which appears to be involved in mediating some of the complex effects of light on expressed circadian rhythms. Our results point to a promising new functional marker for the cellular effects of light and suggest that the expression of Fos or a related nuclear protein may be part of the mechanism for photic entrainment of the circadian clock to environmental light/dark cycles.**

Light-responsive neurons in the mammalian brain are organized into discrete circuits that are functionally, physiologically, and anatomically distinct. Thus, direct retinal projections to the superior colliculi are responsible for visually guided eye movements; visual inputs to the pretectum drive reflex pupillary function; and the optic pathway to the lateral geniculate nuclei relays information required for image formation. In addition to these classic connections, some retinal ganglion cells monosynaptically innervate the suprachiasmatic nuclei (SCN) in the anterior hypothalamus, site of an endogenous circadian clock (1). Activation of this retinohypothalamic tract appears to be both necessary and sufficient for precise entrainment of the period and phase of overt circadian rhythms to the natural day/night cycle (2). Such entrainment is achieved by light-induced phase shifts of the endogenous oscillation of the circadian pacemaker in the SCN; permanent advances or delays occur because the oscillator is differentially sensitive to light exposure at different phases of its free-running circadian cycle (3). The cellular mechanism of photic entrainment is unknown, but the search is underway (predominantly in organisms simpler than mammals) for molecular candidates at the membrane, cytoplasmic, and nuclear levels that might be a part of this signal-transduction pathway.

The nuclear phosphoprotein Fos, product of the c-*fos* protooncogene, is believed to be part of a sequence-specific DNA-binding protein complex that alters gene expression by regulating transcription (4). In cultured cells, c-*fos* is rapidly and transiently induced by a variety of extracellular signals and intracellular second messengers, and the concept has emerged that increased Fos expression serves to couple short-term membrane events to long-term changes in cellular structure and function (5, 6). In the brains of intact animals, Fos immunoreactivity is increased in the nuclei of specific neurons after seizures (7–9), cortical (10, 11) and peripheral (12, 13) stimulation, and water deprivation (10). Recently, exposure of rats to flashing lights was reported to increase immunoreactive Fos in some cells of the inner nuclear and ganglion cell layers of the retina (14). To determine whether regional brain Fos expression might also be modulated by light, we used an affinity-purified antibody against a synthetic peptide of Fos (amino acids 132–154, a sequence that includes the probable DNA-binding domain) to perform immunohistochemistry on sections from the brains of Sprague–Dawley rats. We now report that light increases the levels of immunoreactive Fos in the SCN and hypothesize that events at the transcriptional level are part of the mechanism for photic entrainment of circadian rhythms to environmental light/dark cycles. Some of these data have been reported previously in abstract form (15).

MATERIALS AND METHODS

Animals. Adult male Sprague–Dawley rats (Charles River Breeding Laboratories) were individually housed in wire-mesh cages, each within its own light-controlled isolation chamber. Light was provided by 15-W cool white fluorescent tubes delivering an intensity of ≈600 lux at the middle of each cage. Purina Rat Chow and water were freely available and were replenished once every 6–7 days. Animals were entrained to a 12 hr/12 hr light/dark cycle or to a reversed cycle, thus allowing all experiments to be conducted between 1000 and 1300, Eastern Standard Time.

Immunohistochemistry. At various times and lighting conditions, rats were deeply anesthetized with pentobarbital (30 mg, i.p.) and perfused through the ascending aorta with 35 ml of heparinized saline followed by 500 ml of freshly prepared, cold 2% paraformaldehyde/0.1% lysine/0.2% periodate fixative. Brains were removed and immersed in fixative for 2–4 hr at 4°C, and 60-μm coronal sections were cut on a vibratome. Tissue was treated with 2% nonimmune goat serum (NGS) and 0.1% Triton X-100 in 0.2 M Tris/saline (0.2 M Tris/0.15 M NaCl, pH 7.6) for 1 hr and then incubated in primary Fos antiserum diluted 1:80 to 1:90 in NGS with 0.1% Triton X-100 for 48–60 hr at 4°C. The antiserum was generated in rabbits to a synthetic Fos peptide (amino acids 132–154) conjugated to bovine serum albumin with carbodiimide and suspended in complete Freund's adjuvant. Antiserum was affinity-purified over CH-Sepharose 4B columns to which Fos-(132–154) was attached and the antibodies were eluted in 4.5 M $MgCl_2$. Sections were treated using the avidin–biotin method with diaminobenzidine as the chromagen, mounted on gelatin/chrome alum-coated ("subbed") slides, dehydrated, coverslipped, and examined with a Zeiss

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Abbreviations: SCN, suprachiasmatic nuclei; NGS, nonimmune goat serum; VIP, vasoactive intestinal peptide.

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Axioplan microscope. Limited studies were also performed using a previously characterized rabbit polyclonal anti-Fos-(127–152) antiserum diluted 1:100 (generously supplied by Tom Curran; ref. 16); the animals for these experiments were perfused with 4% paraformaldehyde. Antibody to vasoactive intestinal polypeptide (VIP) (Immuno Nuclear, Stillwater, MN) had been characterized for immunohistochemistry (17) and was used at a dilution of 1:800.

Antiserum Characterization. The anti-Fos-(132–154) antiserum was characterized by Western blot analysis of HeLa cell nuclear extracts. Cells grown to 5×10^5 cells per ml in a 500-ml suspension were treated with cytochalasin D (10 mg/ml) for 45 min at 37°C; this treatment is known to increase c-*fos* mRNA levels (18). Nuclear extracts (≈0.5 mg of protein per ml) were obtained using a modification (19) of the method of Dignam *et al.* (20), submitted to polyacrylamide gel electrophoresis (8% gel), and transferred to Zeta-Probe (Bio-Rad). The blot was treated with 3% NGS in 0.2 M Tris/saline (pH 7.6) for 1 hr at room temperature and then incubated in anti-Fos antiserum diluted 1:100 in NGS overnight at 4°C. After three 10-min washes in Tris/saline containing 0.5% Tween 20, affinity-purified anti-rabbit antibodies conjugated to alkaline phosphatase (1:5000 dilution; Boehringer Mannheim) was applied for 1 hr at room temperature. Alkaline phosphatase was reacted with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Kirkegaard and Perry Laboratories, Gaithersburg, MD). The anti-Fos-(132–154) antiserum recognized proteins with apparent molecular masses of 62–68, 45–48, and 35–37 kDa (N.A., unpublished observations). This pattern closely corresponds to published Western blot analyses of the anti-Fos-(127–152) antiserum with serum-stimulated fibroblasts, PC12 rat pheochromocytoma cells exposed to nerve growth factor, and mouse brains after electrically or pharmacologically induced seizures (21–23); immunohistochemically, this antibody stains neuronal nuclei, with maximal labeling 3–4 hr after stimulation (7, 10). Thus, although we refer to "immunoreactive Fos" throughout this report, the anti-Fos antibodies directed against the DNA-binding domain actually recognize both authentic Fos (62 kDa) and Fos-related antigens (46 and 35 kDa).

RESULTS

When rats were killed during the light phase of the light/dark cycle (4 hr after lights were turned on), Fos immunoreactivity was clearly detected in the nuclei of SCN cell bodies ($n = 5$) (Figs. 1*A* and 2). Deletion of primary antibody or preadsorption with synthetic peptide Fos-(132–154) at 1 μg/ml eliminated specific labeling. Staining was restricted to the ventrolateral subdivision of the SCN, the site of termination of retinal input (24). Labeling was also observed during the light phase as early as 1–2 hr ($n = 3$) and as late as 10–11 hr ($n = 3$) after lights-on. On the other hand, if lights were not turned on in the morning, and the rats remained in darkness until they were killed 4 hr later, both the number of cell nuclei stained and the intensity of their labeling were dramatically diminished ($n = 4$) (Fig. 1*B*). These findings were reproduced using the antiserum directed against Fos-(127–152) (examined only at the time point 4 hr after normal lights-on). Thus, immunoreactive Fos levels in the SCN were altered at this time of day by the presence or absence of environmental light. This property is not a general feature of all substances found in the SCN; for example, immunohistochemical staining for VIP, a neurotransmitter of the ventrolateral SCN (25), was present at this time of day irrespective of ambient lighting (N.A. and W.J.S., unpublished observations; see also ref. 26).

Fos immunoreactivity was similarly modulated by the natural alternation of light and darkness over the 24-hr day. Low levels were found in the SCN of rats killed during the dark phase of the light/dark cycle (4 hr after lights-off) ($n = 4$) (Fig. 1*C*). If instead lights remained on for these 4 hr, immunoreactive Fos expression was high and similar to that seen during the normal light phase ($n = 4$) (Fig. 1*D*). Thus, environmental light increased immunoreactive Fos levels regardless of time of day.

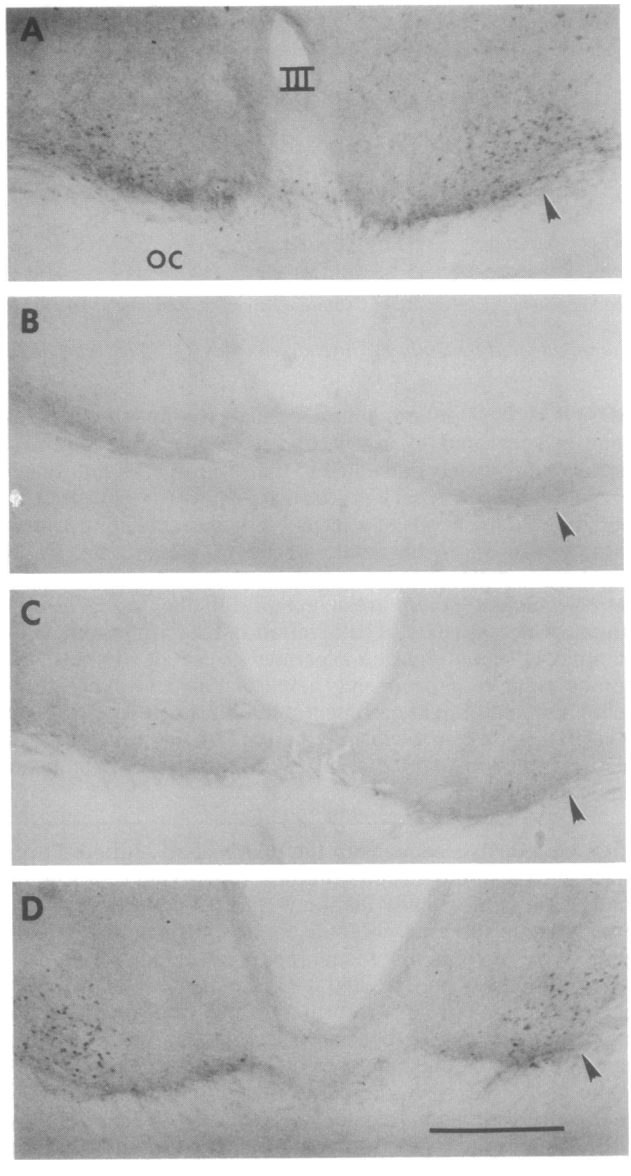

FIG. 1. Representative coronal brain sections from male Sprague–Dawley rats entrained to a 12 hr/12 hr light/dark cycle for 10–14 days and killed in the light phase, 4 hr after lights were turned on (*A*); at the same time as in *A*, but lights were not turned on in the morning and remained off for the next 4 hr (*B*); in the dark phase, 4 hr after lights were turned off (*C*); and at the same time as in *C*, but lights remained on for these 4 hr (*D*). Arrowheads, ventrolateral SCN; III, third ventricle; OC, optic chiasm. (Bar = 200 μm.)

These observations were substantiated by counts of immunoreactive cell nuclei (Table 1). The number of labeled cells was significantly higher in the presence of light (during the normal light phase or when the dark phase was illuminated) than in its absence (during the normal dark phase or when the light phase was unilluminated).

Labeling was not observed in the superior colliculi or dorsal/ventral lateral geniculate nuclei ($n = 5$), in agreement with findings from other laboratories using BALB/c mice (7) and Sprague–Dawley rats (27). In contrast, numerous cells of the intergeniculate leaflet expressed immunoreactive Fos;

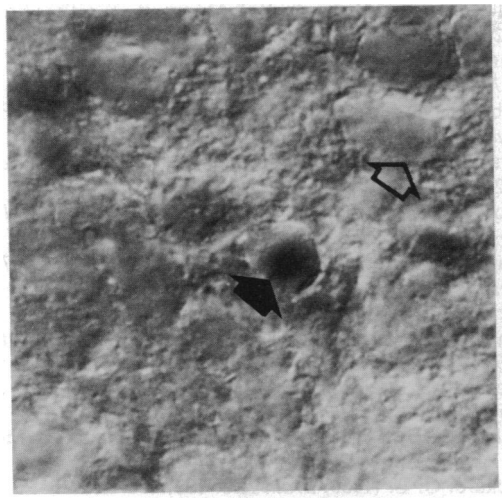

FIG. 2. Staining of the nucleus of a SCN cell body (solid arrow) viewed under Nomarski optics; an unstained nucleus in another cell body is shown by the open arrow. (×800.)

high levels during the normal light phase ($n = 5$) (Fig. 3*A*) were markedly reduced if lights were not turned on ($n = 4$) (Fig. 3*B*).

DISCUSSION

Our results show that Fos immunoreactivity is physiologically regulated in the SCN and intergeniculate leaflet by environmental lighting. Daily rhythmicity of this transcriptional regulatory protein reflects the external light/dark cycle and not an endogenously generated rhythm. Interestingly, the intergeniculate leaflet appears anatomically and functionally related more closely to the SCN than to the neighboring lateral geniculate nuclei. The leaflet projects to the ventrolateral SCN (28, 29), and both the leaflet and the SCN are innervated by bifurcating axons from individual retinal ganglion cells (30). Additionally, the leaflet has been implicated in mediating some of the complex effects of light on expressed circadian rhythmicity (31, 32). Thus, in this strain of albino rat, the regulation of immunoreactive Fos by light appears selective for the visual system for photic entrainment of circadian rhythms; no change in the low levels of Fos expression was detected in other retino-recipient areas responsible for reflex oculomotor function and image formation. Of note, the mammalian entrainment system is characterized by several additional special features that distinguish it from other visual systems, including its photoreceptive properties (33), lack of retinotopic organization (34), and electrophysiological characteristics of SCN neurons (35, 36).

Table 1. Counts of Fos-labeled cell nuclei in the SCN

Group (Fig. 1)	Phase of cycle	Ambient lighting	No. of cells (mean ± SEM)
A	Light	On	34 ± 5*
B	Light	Off	8 ± 2
C	Dark	Off	15 ± 3
D	Dark	On	32 ± 5*

Labeled cell nuclei in the ventrolateral SCN, irrespective of the intensity of staining, were counted without knowledge of lighting conditions. Values are for a single nucleus of the bilaterally paired SCN and are averages derived from four to eight sections from each rat, with four rats to each group except for group A with five rats. The intensity of the staining in groups B and C was just above the resolution of bright-field light microscopy (see Fig. 1).

*Cell counts from both groups with lights on are significantly higher than from each of the groups with lights off ($P < 0.01$ by a two-way analysis of variance followed by Boneferroni t statistic).

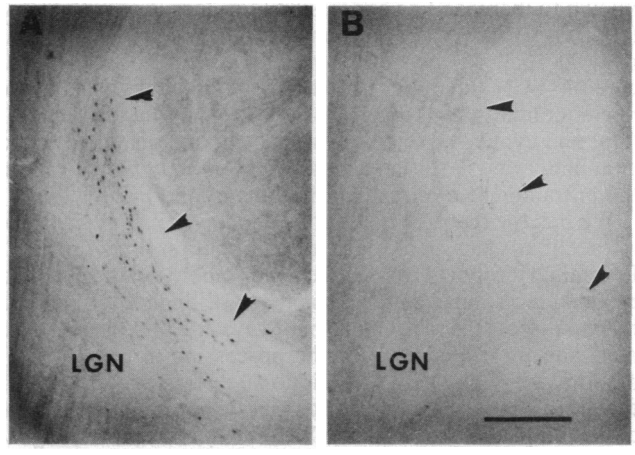

FIG. 3. Representative coronal brain sections from rats entrained to a 12 hr/12 hr light/dark cycle and killed in the light phase, 4 hr after lights were turned on (*A*) and at the same time as in *A*, but lights were not turned on in the morning and remained off for the next 4 hr (*B*). Arrowheads, intergeniculate leaflet; LGN, lateral geniculate nucleus. There were 62 ± 8 labeled cells in each of the leaflets from the five rats in *A*. (Bar = 200 μm.)

These properties probably reflect the different functions of the entrainment system, which appears specialized to respond to tonic background illumination (37) and not to the temporally and spatially restricted photic stimuli required for eye movements and pattern vision.

The mechanisms by which increased Fos levels affect gene transcription in the SCN are likely to be complex. In previous studies using simpler biological systems, the primary c-*fos* translation product was found to undergo considerable posttranslational processing, including phosphorylation (38). Furthermore, multiple proteins in addition to Fos interact with its DNA binding site, the consensus recognition sequence for the transcription factor AP-1 (39). The Fos antisera have revealed a series of Fos-related antigens that are individually expressed with different kinetics in response to an extracellular stimulus (21–23), and at least one of these proteins is encoded by a gene other than c-*fos* (40). It may be that some of the immunoreactivity that we have attributed to Fos over the course of the light phase or during the dark phase was due to the persistent expression of Fos-related antigens after authentic Fos had disappeared. Moreover, Fos appears unable to bind to its AP-1 site unless it is complexed with the protein product of another protooncogene, c-*jun*. Fos and Jun (also known as Fos-associated protein p39) form heterodimers by means of domains consisting of leucines spaced periodically at every seventh residue along parallel α-helices (the "leucine zipper"; refs. 41–44). Thus, further study of the possible posttranslational modifications and protein–DNA and protein–protein interactions of a host of transcriptional regulators will be required in order to fully understand the actions of Fos in the SCN. In this regard, it is interesting that mRNA coding for another transcriptional activator, Oct-2, has been localized to the SCN of adult rats (45), although its possible rhythmicity and regulation by light have not been studied.

It will be a challenging task to define the target genes in the SCN that are regulated by Fos. One obvious candidate (46, 47) is the gene coding for mRNA of the precursor of VIP, also present in the ventrolateral SCN (48, 49), although we first need to determine whether light-induced Fos expression is occurring in cells that synthesize VIP. It will also be important to elucidate the cascade of transmembrane and intracellular signals in the SCN that mediate the induction of the c-*fos* gene. Whereas these activating factors are probably tissue-specific, it is nonetheless noteworthy that some of the stimuli

known to increase Fos expression [e.g., nerve growth factor (50, 51), cyclic AMP (52), and cholinergic (53) and glutamatergic (23, 54) neurotransmitters] have also been implicated in SCN function. The ventrolateral subdivision of the nuclei contains high levels of nerve growth factor receptor (55); and cyclic AMP (56) as well as cholinergic (57, 58) and glutamatergic (59, 60) neurotransmission may play a role in the entrainment of circadian rhythms by the SCN.

We believe that Fos holds considerable promise as a novel intracellular marker of the effects of light on SCN function. Preliminary reports from three other laboratories appear to be reaching similar conclusions (61–63). Such a tool should help to dissect the anatomical pathways and pharmacological mechanisms responsible for the processing of photic information by the nuclei. Transcriptional events may be part of the intracellular machinery for photic entrainment of the circadian pacemaker in the SCN to the environmental light/dark cycle, and Fos or related nuclear proteins may be links in this signal-transduction mechanism.

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