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13. ABSTRACT (Maximum 200 words) On the basis of their putative role in the process of stimulus-transcription coupling, immediate-early genes, such as *c-fos* and *c-jun*, have been implicated as important components of signal transduction programs by which environmental stimuli regulate long-term cellular events. To explore the possibility that immediate-early genes may play a similar role in the transduction of light signals mediating the entrainment of the circadian pacemaker in the suprachiasmatic nucleus (SCN), immediate-early gene expression in the SCN was examined *in vivo* and *in vitro* for evidence of circadian regulation by photic stimuli. Immunocytochemical and molecular analyses demonstrate that light has an inductive effect on the expression of *c-fos* mRNA and Fos protein in the SCN, but only at critical times during the circadian cycle when light is capable of modulating the period of the circadian pacemaker and mediating its entrainment. In addition, this circadian-dependent induction of *c-fos* expression in the SCN by light was mainly localized in the retinorecipient or ventrolateral subfield of the nucleus within a substantial portion of the GRP-containing neurons located in this region. These data provide evidence for the correlative relations between the induction of *c-fos* gene expression in the SCN and the modulation of the SCN circadian pacemaker by light and suggest that immediate-early genes may be components of the signal transduction cascade by which light entrains circadian rhythms.

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In mammals, the generation of circadian rhythms in a variety of biochemical, physiological and behavioral activities is governed by an internal biological clock located in the suprachiasmatic nucleus (SCN) of the anterior hypothalamus (Moore, 1983). The endogenous timekeeping function of the SCN is complemented by its role in mediating the entrainment of circadian rhythms to the daily cycle of light and darkness. Although the functional anatomy and neurochemical organization of the SCN and the neural projections conveying light:dark signals to this circadian neural pacemaker have been described in some detail, limited information is available on the cellular pathway by which light entrains the SCN pacemaker and its circadian output. Since the integrated responses of SCN cells to light:dark signals mediating the photoentrainment of circadian rhythms presumably entail a cascade of transmembrane and cellular events, the primary objective of the present studies was to characterize discrete components of this signal transduction cascade.

Immediate-early genes, such as *c-fos* and *c-jun*, provided a focus in these studies because the protein products of these genes are components of a DNA-binding complex that: 1) regulates transcription; and 2) may represent a key component of other signal transduction programs coupling environmental stimuli to long-term cellular responses (Morgan and Curran, 1987; Morgan and Curran, 1989; Sheng and Greenberg, 1990). In particular, studies examined the possible role of the *c-fos* gene in the circadian timekeeping mechanism in the SCN and its regulation by photic signals. The specific aims of these experiments are: 1) to first determine whether *c-fos* gene expression within the SCN is regulated in a circadian fashion by light and 2) to determine whether the circadian regulation of the *c-fos* gene by light occurs in cells within the ventrolateral SCN that express specific peptidergic signals.

Photic and Rhythmic Regulation of *c-fos* Expression in the Rat Suprachiasmatic Nucleus *in situ*.

In view of observations indicating that a variety of extracellular stimuli induce *c-fos* expression in mature neurons in the brain, studies were conducted to determine whether light-dark signals influence the expression of Fos protein(s) and *c-fos* mRNA in the rat SCN since this structure governs the generation and photic regulation of circadian rhythms. The expression and distribution of Fos protein(s) within the SCN were examined in male Long-Evans rats that had been maintained in

constant darkness (DD) for 24 hours and then exposed to a 1-hour light pulse. In addition, analysis of the effect of a similar light treatment on levels of *c-fos* mRNA in the SCN was conducted on microdissected SCN tissue obtained from a separate group of animals.

Near the mid-subjective day (i.e., 1200h or circadian [CT] 6) and near the mid-subjective night (i.e., 2400h or CT 18), animals were exposed to 1-hour pulses of light and then perfused for immunocytochemistry. Control animals were maintained in DD conditions and perfused under darkness at the same circadian times as light-treated animals. Dual immunostaining for Fos protein(s) and neuropeptide Y (NPY) was conducted on brain sections using a modification of the avidin-biotin immunoperoxidase reaction. For *c-fos* mRNA analysis, similarly treated animals were sacrificed, SCN tissue was removed and later homogenized for extraction of total RNA. SCN content of *c-fos* mRNA was determined using a solution hybridization/ RNA protection assay.

With regard to immunocytochemical analysis, low levels of Fos immunostaining were found only within cell nuclei in a variety of brain loci, including the hypothalamic supraoptic and paraventricular nuclei, hippocampus, dentate gyrus, and cerebral cortex. Within these regions, no differences between control and light-pulsed animals were evident in the density and distribution of Fos-immunopositive cells. A small population of immunoreactive cells (5-8 cells/section) was also observed in light-pulsed, but not control, animals within the intergeniculate leaflet of the thalamus. In contrast to other brain regions, the distribution and level of expression of Fos protein(s) in the SCN was dependent not only on presence of light, but also on the circadian time during which the light pulse was administered. Irrespective of the time of sacrifice (i.e., CT 6 or CT 18), control animals experiencing no light stimulus consistently exhibited a small number of Fos-immunoreactive within the SCN. Immunopositive cells in the SCN of control animals were equally distributed between the two immunocytochemically distinct subfields of this structure. Animals exposed to a 1-hour light pulse at CT 6 showed no appreciable change in the distribution or density of Fos-immunoreactive cells within the SCN when compared to time-matched control animals; Fos immunostaining in the SCN was confined to a few cells scattered in both the dorsomedial and ventrolateral subdivisions. However, exposure to a 1-hour light pulse at CT 18 altered the expression and distribution of Fos

protein(s) in the SCN, such that immunoreactive cells in the SCN were greatly increased in number and density relative to time-matched dark-treated controls and were mainly confined to an area coextensive with the NPY-immunoreactive fibers distinguishing the retinorecipient or ventrolateral subfield. At CT 18, the density of Fos-immunoreactive profiles within the ventrolateral SCN was approximately fourfold greater in light-pulsed animals than in dark-treated controls.

Solution hybridization/RNA protection analysis of *c-fos* mRNA levels in the SCN of dark-treated control and light-pulsed animals yielded similar results. Low levels of *c-fos* mRNA were consistently observed in the SCN of dark-treated control animals, irrespective of the time of sacrifice. One-hour light pulses at CT 6 caused no detectable change in *c-fos* mRNA expression in the SCN relative to the basal levels observed in dark-treated controls. In contrast, *c-fos* mRNA content in the SCN was elevated in response to 1-hour light pulses at CT 18. Relative quantification of mRNA levels in bands containing the *c-fos* cRNA:mRNA protected fragments indicates that SCN content of *c-fos* mRNA in animals exposed to light at CT 18 was increased by $\approx 100\%$ relative to the content observed in control animals.

Collectively, the aforementioned experiments examining the time-dependent effects of light pulses on the expression of *c-fos* mRNA and Fos protein(s) in the SCN may have some implications for the functional role of the *c-fos* gene in the circadian timekeeping system. In this regard, it is noteworthy that the *induction of c-fos expression in the SCN occurring selectively in response to photostimulation during the subjective night coincides with the critical times when light also mediates the entrainment of circadian rhythms*. This coincidence between the time-dependent effects of light in regulating *c-fos* expression in the SCN and in mediating the entrainment of circadian rhythms suggests that this immediate-early gene may represent an important component of the signal transduction program by which light regulates SCN pacemaker function. In addition, the coextensive distribution of Fos immunoreactivity and the NPY-immunoreactive fibers of the geniculohypothalamic tract within the ventrolateral SCN of animals exposed to light at CT 18 serves to demonstrate the anatomical association between light-induced Fos expression and retinal innervation of the SCN,

suggesting further that changes in *c-fos* expression may be involved in the molecular pathway mediating the photoentrainment of the SCN pacemaker.

Consistent with the endogenous nature of SCN circadian timekeeping function, many indices of intrinsic neural activity expressed by SCN neurons oscillate with a circadian periodicity in the absence of light-dark cues. Consequently, experiments were conducted to determine whether the expression of Fos proteins within the SCN, like the endogenous neural activity of the SCN pacemaker, oscillates over the course of the circadian cycle under conditions of constant retinal illumination.

Male Long-Evans rats were exposed to constant illumination (LL) for 30 hours and beginning at CT 12 and at 4-hour intervals thereafter, small groups of animals were then perfused for immunocytochemistry. Brain sections were processed for dual immunostaining of Fos protein(s) and NPY.

During exposure to LL, Fos-immunoreactive nuclei were consistently identified within a number of cells in the SCN. As observed previously, Fos immunostaining was mainly confined to cells segregated in an area that was coextensive with the NPY-immunoreactive fibers characterizing the ventrolateral subfield. Although immunopositive nuclei were observed in other brain regions, the SCN was unique in that Fos immunostaining within this locus showed overt signs of variation over time (Earnest et al., 1992). This temporal variation in Fos immunostaining within the SCN was confirmed by analysis of the densities of immunoreactive cells within the ventrolateral subfield in animals sacrificed at four-hour intervals throughout the circadian cycle. Analysis of variance indicated that time-based determinations of immunopositive cell density within the ventrolateral subfield at CT 2, 6, 10, 14, 18, and 22 were significantly different ($F= 11.01$; $P<0.01$). Moreover, mean values for the densities of Fos-immunoreactive profiles within the ventrolateral SCN at time points during the subjective night (CT 14, 18 and 22) were significantly greater ($P<0.01$) than all of those values observed at times during the subjective day (CT 2, 6 and 10). In fact, the densities of immunopositive cells within the ventrolateral SCN throughout the subjective night were increased 2-fold relative to those observed during the subjective day. Although Fos immunostaining was evident within the dorsomedial SCN at all circadian times, the densities of immunopositive cells within this

subfield were highly variable among individual animals at any given time and showed no consistent pattern of temporal variation.

These data demonstrate that in the absence of daily light-dark cues the expression of Fos protein(s) oscillates in the SCN. Importantly, the SCN appeared to be unique in expressing this oscillation in immunostaining under conditions of constant retinal illumination. Even within other areas that receive retinofugal innervation, such as the intergeniculate leaflet of the thalamus and the superior colliculus, there were no overt signs of temporal variation in Fos immunostaining. Within the SCN, the oscillation in Fos immunostaining during exposure to LL was observed within the ventrolateral subfield and was characterized by low densities of immunopositive cells during the subjective day and peak densities throughout the subjective night. Importantly, the rhythmic variation in Fos immunostaining observed within the ventrolateral SCN does not appear to be correlated with the length of exposure to LL because the lowest densities of immunopositive cells in the SCN were observed both in animals exposed to LL for the shortest periods of time (i.e., 30 and 34 hr for CT 6 and 10, respectively) and in animals maintained in LL for the longest time interval (i.e., 50 hr for CT 2). Instead, the oscillatory pattern in Fos immunostaining within the ventrolateral SCN presumably reflects either an endogenous property of the circadian pacemaker mechanism or an output of the pacemaker that is regulated in a time-dependent fashion by light.

Application of an *in vitro* Model to Study the Photic Regulation of *c-fos* Expression

Although whole animal preparations utilized in these studies have proved useful in identifying the *c-fos* gene as a possible component of the pathway for the photoentrainment of the SCN pacemaker, application of *in vitro* approaches may provide a basis for not only complementing, but also extending the focus of these observations. Consequently, the objective of this study was to determine whether the cellular responses mediating the photic regulation of *c-fos* expression remain intact within the SCN *in vitro*. An existing *in vitro* model using perfused explants of the SCN was applied in these studies to examine the effect of optic nerve stimulation on *c-fos* mRNA content of the SCN.

SCN explants were dissected as described previously (Earnest and Sladek, 1986, 1987). The explants were maintained under constant environmental conditions in a perfusion culture system

within individual chambers equipped with a pair of small cuff electrodes to allow introduction of the trunks of each optic nerve and delivery of electrical impulses in isolation from external perturbations. To accommodate the inductive effect of dissection-related tissue trauma on *c-fos* expression (lasts for \approx 4hr; Herrera and Robertson, 1989) in the context of this study, SCN explants were allowed to equilibrate for 6-8 hrs prior to experimental manipulation. Optic nerve stimulation was conducted at CT 6 and CT 18 in separate experiments and stimulation parameters were based on those established previously in studies demonstrating that optic nerve stimulation evokes changes in the electrical activity of SCN neurons (Nishino *et al.*, 1976; Sawaki, 1979). In control explants, current was conducted into the medium surrounding the explants. Approximately 1 hour after optic nerve stimulation, explants were frozen and later homogenized for extraction of total RNA. *C-fos* mRNA content of the explants was determined using a solution hybridization/ RNA protection assay.

Consistent with observations on the photic regulation of *c-fos* expression in the SCN *in situ*, the effect of optic nerve stimulation on SCN content of *c-fos* mRNA was time-dependent. Control explants expressed low, but detectable, levels of *c-fos* mRNA at CT 6 and CT 18 (Fig. 1). Similar to control explants, SCN explants contained basal levels of *c-fos* mRNA following optic nerve stimulation at CT 6. However, optic nerve stimulation at CT 18 had an inductive effect on *c-fos* mRNA expression in SCN explants. Relative quantification of mRNA levels in bands containing the *c-fos* cRNA:mRNA protected fragments indicates that SCN content of *c-fos* mRNA in explants subjected to optic nerve stimulation at CT 18 was increased by \approx 300% relative to time-matched controls (Fig. 2). Irrespective of the time of optic nerve stimulation, no detectable changes were observed in the levels of β -actin and α -tubulin mRNAs in SCN explants.

These data demonstrate that optic nerve stimulation induces time-dependent increases in *c-fos* mRNA levels expressed by SCN explants in a fashion similar to those observed within the SCN *in situ* in response to light pulses, suggesting that the responses of the SCN to photic signals remain intact following isolation of the nucleus *in vitro*. Since the fibers of the retinohypothalamic tract represent the only viable pathway capable of transmitting photic signals to the explanted SCN, these

data also suggest that this visual pathway may mediate the photic regulation of *c-fos* expression in the SCN.

Neurochemical Identity of Individual Cells Exhibiting Light-Induced Fos Expression within the SCN.

The photic induction of Fos expression in the SCN during the subjective night is mainly localized within the ventrolateral subfield of the nucleus. Since this subfield is distinguished by a number of neuronal perikarya containing GRP and/or VIP (Mikkelsen *et al.* 1991; Okamura *et al.*, 1986) and since their close associations with retinofugal fibers innervating the ventrolateral SCN suggest that these peptidergic cells may be involved in the transduction of entraining light signals, double-labeling immunocytochemical analysis was conducted to determine whether the photoinduction of *c-fos* expression in the ventrolateral SCN occurs within the populations of GRP- and/or VIP-containing neurons located within this subfield.

Male Long-Evans rats that had been maintained under DD conditions (for \approx 24h) were exposed to light for 1 hour near the mid-subjective night and then perfused for immunocytochemistry. Brain sections (30 μ m) were processed for simultaneous immunocytochemical localization of Fos protein and GRP or VIP. In order to visualize the nuclear immunostaining for Fos and cytoplasmic immunostaining for VIP or GRP within *individual* neurons with the best possible morphological preservation, brain sections were embedded in resin after completion of double-labeling immunocytochemistry and cut at a thickness of 1-4 μ m on an ultramicrotome.

In all light-treated animals, SCN cells with Fos-positive nuclei were mainly segregated in an area coextensive with fiber plexuses and a small number of perikarya in the ventrolateral subfield that exhibited VIP- or GRP-immunoreactivity. Further examination of Fos-immunoreactive cells in thin sections revealed that in light-treated animals Fos-related proteins were expressed in 40% of the neurons with cytoplasmic immunostaining for GRP found in the ventrolateral subfield. Consistent with the general morphology of SCN peptidergic neurons, double-labeled cells in the SCN were characterized by large nuclei immunoreactive for Fos surrounded by a scant, concentric border of cytoplasm displaying GRP-immunoreactivity. GRP neurons expressing light-induced Fos immunoreactivity within the ventrolateral SCN were primarily distributed throughout the rostral two-

thirds of the nucleus. At these levels of the SCN, most perikarya with dual immunostaining for Fos-related proteins and GRP were aligned in the ventral margin of the nucleus or embedded in the optic chiasm. In the converse comparison, only a small portion of the cells in the ventrolateral SCN with nuclei expressing light-induced Fos-immunoreactivity ($\approx 8\%$) were found to also exhibit GRP-immunopositive perikarya. No sign of light-induced Fos expression was observed in VIP-positive neurons in the SCN. Simultaneous immunocytochemical localization of Fos protein(s) and GRP or VIP was never observed within individual neurons in brain regions outside of the SCN.

These results demonstrate that light induces *c-fos* expression in at least a subpopulation of the GRP-containing neurons in the SCN, suggesting that these cells may be involved in processing entraining light signals. In addition, homology between a nucleotide sequence motif in the promoter region of the GRP gene and the AP-1 consensus sequence (Franza *et al.*, 1988; Lebacqz-Verheyden *et al.*, 1990) raises the possibility that the gene encoding this peptide may represent a target gene for transcriptional regulation by light-induced Fos proteins. It is somewhat surprising that VIP-immunopositive cells in the ventrolateral SCN failed to show light-induced Fos immunoreactivity because: 1) GRP and VIP may be colocalized within SCN neurons in the ventrolateral subfield that receive retinal inputs (Okamura *et al.*, 1986); and 2) a sequence element in the promoter region of the VIP gene bears similarity to the AP-1 binding motif (Giladi *et al.*, 1990). One possible explanation for this is that VIP levels in the SCN may oscillate and analysis at a single time point during the mid-subjective night may have coincided with a period during which VIP levels were at a minimum. This explanation receives some support from data indicating that VIP-immunoreactivity in the SCN is low at CT 18 (Takahashi *et al.*, 1989), although the experiments were conducted under diurnal lighting conditions. Further analysis of the effects of light pulses at different times throughout the subjective night will be necessary to determine whether light-induced Fos immunoreactivity also occurs within VIP-containing neurons in the ventrolateral SCN.

FIGURE LEGENDS

FIGURE 1. Comparative analysis of *c-fos* mRNA content in the SCN *in vitro* at CT 6 and at CT 18 in control explants (C) experiencing electrical stimulation of the adjacent medium or explants experiencing stimulation of the optic nerves (ONS). Autoradiograms depict the fractionation of *c-fos* cRNA:mRNA protected fragments after solution hybridization of single-stranded *c-fos*-specific ^{32}P -labeled RNA probe to total RNA extracted from SCN tissue. Each lane represents the total nucleic acids pooled from 2 SCN explants. The specific band sizes of about 950 bases correspond to the expected size of the fragment protected by endogenous *c-fos* mRNA.

FIGURE 2. Relative quantification of *c-fos* mRNA content in the SCN *in vitro* at CT 6 (left panel) and at CT 18 (right panel) in control explants (CONT) experiencing electrical stimulation of the adjacent medium or explants experiencing stimulation of the optic nerves (ONS). Mean values (\pm SEM) of 8 explants per group are depicted and expressed as a percentage of the values expressed by time-matched control explants.

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c-fos →

SCNe
SCNoms
SCNoms

CT
6

SCNe
SCNoms
SCNoms

CT
18

c-fos cRNA



← 1018

← 517

