

DIFFERENTIAL REGULATION OF *fos* FAMILY GENES IN THE VENTROLATERAL AND DORSOMEDIAL SUBDIVISIONS OF THE RAT SUPRACHIASMATIC NUCLEUS

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Abstract—Extensive studies have established that light regulates *c-fos* gene expression in the suprachiasmatic nucleus, the site of an endogenous circadian clock, but relatively little is known about the expression of genes structurally related to *c-fos*, including *fra-1*, *fra-2* and *fosB*. We analysed the photic and temporal regulation of these genes at the messenger RNA and immunoreactive protein levels in rat suprachiasmatic nucleus, and we found different expression patterns after photic stimulation and depending on location in the ventrolateral or dorsomedial subdivisions. In the ventrolateral suprachiasmatic nucleus, *c-fos*, *fra-2* and *fosB* expression was stimulated after a subjective-night (but not subjective-day) light pulse. Expression of the *fra-2* gene was prolonged following photic stimulation, with elevated messenger RNA and protein levels that appeared unchanged for at least a few hours beyond the *c-fos* peak. Unlike *c-fos* and *fra-2*, the *fosB* gene appeared to be expressed constitutively in the ventrolateral suprachiasmatic nucleus throughout the circadian cycle; immunohistochemical analysis suggested that Δ FosB was the protein product accounting for this constitutive expression, while FosB was induced by the subjective-night light pulse. In the dorsomedial suprachiasmatic nucleus, *c-fos* and *fra-2* expression exhibited an endogenous circadian rhythm, with higher levels during the early subjective day, although the relative abundance was much lower than that measured after light pulses in the ventrolateral suprachiasmatic nucleus. Double-label immunohistochemistry suggested that some of the dorsomedial cells responsible for the circadian expression of c-Fos also synthesized arginine vasopressin. No evidence of suprachiasmatic nucleus *fra-1* expression was found.

In summary, *fos* family genes exhibit differences in their specific expression patterns in the suprachiasmatic nucleus, including their photic and circadian regulation in separate cell populations in the ventrolateral and dorsomedial subdivisions. The data, in combination with our previous results [Takeuchi J. *et al.* (1993) *Neuron* **11**, 825–836], suggest that activator protein-1 binding sites on ventrolateral suprachiasmatic nucleus target genes are constitutively occupied by Δ FosB/JunD complexes, and that c-Fos, Fra-2, FosB and JunB compete for binding after photic stimulation. The differential regulation of *fos* family genes in the ventrolateral and dorsomedial suprachiasmatic nucleus suggests that their circadian function(s) and downstream target(s) are likely to be cell specific. © 2000 IBRO. Published by Elsevier Science Ltd. All rights reserved.

Key words: AP-1, circadian rhythms, *fos*, immediate-early genes, suprachiasmatic nucleus, vasopressin.

The mammalian suprachiasmatic nucleus (SCN) in the anterior hypothalamus is the site of an endogenous circadian clock.³⁴ The clock governs an array of behavioral, physiological and hormonal rhythms, and synchronizes their phases and periods to the environmental light–dark cycle. The body of evidence now implicating the SCN as a clock is so compelling that the strength of this functional localization is unsurpassed by that of any other structure in the CNS.

Current research is beginning to identify the molecular processes that constitute the clock mechanism, and it has become clear that transcriptional control plays an important role.¹³ In mammals, light pulses can stimulate the expression

of transcriptional regulatory proteins in the rodent SCN. The most intensively studied of these proteins has been c-Fos (for reviews, see Refs 26, 36 and 58). Light-induced *c-fos* gene expression in the SCN has the same threshold, magnitude⁶⁴ and phase dependence as do light-induced phase shifts of behavioral or hormonal rhythms; pharmacological agents that block behavioral phase shifts also block the photic activation of c-Fos in specific regions of the SCN.

Once expressed, c-Fos affects the transcription of target genes by preferentially binding to a specific *cis*-acting regulatory DNA element [the activator protein-1 (AP-1) binding site], but only when it is complexed as a heterodimer, especially to a member of the *jun* family of DNA-binding proteins (for recent reviews, see Refs 19, 33 and 50). Expression of the Jun proteins (c-Jun, JunB and JunD) is not co-ordinately regulated in the SCN, resulting in the generation of AP-1 binding complexes with constant, as well as variable, protein components.^{17,63} JunD appears to be a fixed constituent of complexes both in darkness and in light, whereas binding by JunB and c-Fos occurs only after photic stimulation. Since the constitutive JunD and inducible JunB proteins can be co-localized within individual c-Fos-positive SCN cell nuclei, we proposed that light acts to change the protein composition

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Abbreviations: AP-1, activator protein-1; AVP, arginine vasopressin; CRE, cyclic-AMP response element; CT, circadian time; EDTA, ethylenediaminetetra-acetate; GRP, gastrin-releasing peptide; OD, optical density; PBS, phosphate-buffered saline; SCN, suprachiasmatic nucleus; SSC, sodium citrate/sodium chloride; VIP, vasoactive intestinal polypeptide.

of AP-1 binding complexes in the SCN.⁶³ Compositional changes can alter the stability and binding affinity of the complexes, and along with changes in total binding activity,^{17,37} may ultimately influence the transcriptional regulation of SCN genes with AP-1 sites on their promoters.

Like the *jun* genes, there is a family of *c-fos*-like genes that are structurally related to *c-fos*, including *fra-1*,¹¹ *fra-2*⁴⁶ and *fosB*.⁶⁹ The proteins encoded by these genes (Fra-1, Fra-2, FosB and Δ FosB), like c-Fos and the Jun proteins, all share the same structural motif ("bZIP"), consisting of a leucine repeat domain (for dimerization with other bZIP nuclear proteins) and a domain of highly basic amino acids lying N-terminal to the leucine repeat (for binding to DNA). Despite their conserved structure, *fos* family gene products show differences in their expression and transcriptional activities in various experimental systems. Compared to *c-fos*, relatively little is known about their expression in the SCN, although their possible differential regulation has been suggested by two reports that describe an immunohistochemical pattern of constitutive FosB expression that differs from the inducible c-Fos pattern.^{16,52}

In an effort to understand the relationship of SCN *fra-1*, *fra-2* and *fosB* to *c-fos* and circadian clock mechanisms, we analysed the photic and temporal regulation of these genes at the mRNA and protein levels, using *in situ* hybridization and immunohistochemistry on tissue sections of rat SCN. Our results show that this regulation is complex, with differential expression of the genes after their photic stimulation and in their location in the ventrolateral or dorsomedial subdivisions of the SCN.

EXPERIMENTAL PROCEDURES

Animals

Adult male Sprague–Dawley rats (Harlan Sprague–Dawley, Indianapolis, IN) were housed in clear polycarbonate cages contained within light-proof environmental compartments isolated in an animal facility. The conditions within the compartments included 12 air changes per hour, a thermostatically controlled internal temperature of $72 \pm 1^\circ\text{F}$ and a relative humidity matching that of the animal room outside (maintained at 60%). Light within each compartment was provided by 15-W cool-white fluorescent tubes automatically controlled by a 24-h timer; intensity varied within the cages but was of the order of 300–400 lux at the mid-cage level. No light was present during darkness. When necessary, a single 15-W safe light with a dark red (Kodak series 2) filter was used to allow for routine care; rats were exposed to approximately 30 lux maximally and <1 lux usually. Food and water were freely available and replenished once every week at irregular hours.

Lighting cycles within the different compartments could be set so that every animal (irrespective of circadian phase or lighting condition) could be killed within the same 2–3-h interval of real time, allowing us to treat all the brains simultaneously with identical reagents. In this way, tissue sections representing different points of the environmental lighting cycle or exposure to light pulses at different circadian phases were processed concurrently.

All experiments were approved by the University of Massachusetts Animal Care and Use Committee and the American Veterinary Medical Association, and all efforts were made to minimize the number of animals used and their suffering.

In situ hybridization

Rats were decapitated (guillotine), brains were rapidly removed and frozen in 2-methylbutane cooled to -30°C with dry ice, and 15- μm -thick coronal sections through the SCN were cut on a cryostat and mounted on slides coated with Vectabond (Vector Laboratories, Burlingame, CA). Sections were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 10 min, treated in 0.1 M

triethanolamine hydrochloride (pH 8.0) with 0.25% acetic anhydride for 10 min, rinsed in 2 \times sodium citrate/sodium chloride (SSC), dehydrated with alcohol and delipidated with chloroform. Sections were incubated in pre-hybridization buffer (10% dextran sulfate, 50% formamide, 0.3 M NaCl, 10 mM Tris, 1 mM EDTA, 1 \times Denhardt's solution) for 2 h at 55°C, followed by buffer and 10⁶ c.p.m./ml of the appropriate ³⁵S-labeled cRNA probe, 200 mM dithiothreitol and 0.5 mg/ml yeast tRNA overnight at 55°C. The sections were then washed in 2 \times SSC with 10 mM dithiothreitol for 15 min at 22°C and 2 \times SSC for 30 min at 22°C; RNase A (20 $\mu\text{g}/\mu\text{l}$) in buffer (500 mM NaCl, 10 mM Tris, 1 mM EDTA, pH 8.0) for 30 min at 37°C and buffer alone for 30 min at 37°C, 2 \times SSC for 30 min and 0.5 \times SSC for 30 min at 22°C, and 0.1 \times SSC for 30 min at 22°C and 30 min \times 2 at 65°C. Slides were dehydrated in alcohol, dried and exposed to Hyperfilm- β max (Amersham, Arlington Heights, IL). All solutions were prepared RNase-free with hydrated diethyl pyrocarbonate.

Linearized recombinant plasmids were used as templates for the generation of antisense cRNA probes [*c-fos* from a 2.3-kb cDNA insert in pSP65 (Promega, Madison, WI), linearized with HindIII; *fra-2* from a 0.98-kb insert in pCR3 (Invitrogen, San Diego, CA), linearized with HindIII; *fra-1* from a 1.5-kb insert in pBluescript KS(+) (Stratagene, La Jolla, CA), linearized with SpeI; and *fosB* from a 1.8-kb insert in pGEM1 (Promega), linearized with SmaI; all enzymes from Promega]. Probes were transcribed in the presence of [³⁵S]CTP with the appropriate RNA polymerases (T7 for *c-fos*, T3 for *fra-1*, and SP6 for *fra-2* and *fosB*) using the MaxiScript *in vitro* transcription kit (Ambion, Austin, TX). The corresponding sense cRNA probes were also synthesized and gave no hybridization signal on tissue sections. The integrity and size of labeled riboprobes was confirmed autoradiographically using 3.5% polyacrylamide gel electrophoresis with Ribomark™ markers (Promega).

The optical density (OD) of the autoradiographic hybridization signal was measured using a Zeiss (Kontron) Image Processing System. The average OD for each rat SCN was derived from at least two sections through the nucleus, each expressed as a relative OD (ratio OD SCN/OD surrounding hypothalamus).

Immunohistochemistry

Rats were deeply anesthetized with pentobarbital (50 mg, i.p.) and perfused through the ascending aorta with 500 ml of freshly prepared cold 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS). Brains were removed and immersed in fixative for 2–4 h at 4°C, and 60- μm -thick coronal sections were cut on a Vibratome. Tissue was blocked sequentially in 0.3% hydrogen peroxide in 0.15 M PBS for 15 min and 10% non-immune goat serum in 0.15 M PBS for 30 min before incubation in rabbit polyclonal antisera in 2% non-immune goat serum and 0.4% Triton X-100 in 0.15 M PBS at 4°C. The anti-c-Fos₃₋₁₇ antiserum (SC52, Santa Cruz Biotech.) was diluted 1:2000 and incubated overnight; the anti-FosB₇₉₋₁₃₁[N] and anti-FosB₂₄₅₋₃₁₅[C] antisera, raised against TrpE/FosB fusion proteins and well characterized,^{44,45} were each diluted 1:10,000 and incubated for 48 h, and the anti-Fra-2₆₈₋₉₆ antiserum (no. 2605), with specificity confirmed by supershift and peptide-blocking assays (Baler R., unpublished observations), was diluted 1:12,000 and incubated for 48 h. Sections were treated with biotinylated goat anti-rabbit secondary antibody and the avidin–biotin method (Vector Laboratories, Burlingame, CA), with diaminobenzidine (Kirkegaard & Perry, Gaithersburg, MD) as the chromogen, mounted on gelatin/chrome alum-coated slides, dehydrated, coverslipped and examined with a Zeiss Axioplan microscope. On three to four sections per animal through the middle of the SCN, labeled cell nuclei (irrespective of the intensity of staining) were counted by one of us (N.A.) without knowledge of lighting conditions or time of day.

For double-label immunohistochemistry, sections were blocked in 5% normal horse serum and 0.1% Triton X-100 in 0.2 M PBS for 30 min before incubation in mouse monoclonal antibody to arginine vasopressin-associated neurophysin (PS 45; gift from Dr Harold Gainer, NINDS, Bethesda, MD) diluted 1:500 in 2% normal horse serum and 0.1% Triton X-100 in 0.2 M PBS overnight at 22°C. Sections were treated with a biotinylated horse anti-mouse secondary antibody and the avidin–biotin method, with Vector® SG substrate (SK-4700) as the chromogen. The sections were processed as described for anti-c-Fos₃₋₁₇, without the hydrogen peroxide step.

Statistical analysis

Multiple comparisons were done by one- and two-way ANOVA, and subsequent pairwise comparisons by Bonferroni *t*-statistics with statistical significance set at $P < 0.05$. Single comparisons were done by Student's *t*-test with statistical significance set at $P < 0.05$.

RESULTS

Ventrolateral subdivision of the suprachiasmatic nucleus

Figure 1 illustrates the characteristic features of *c-fos* gene expression in the rat SCN, as described previously by us and others (for reviews, see Refs 26, 36 and 58). Levels of *c-fos* mRNA were dramatically elevated (from essentially undetectable levels) after a light pulse was administered during the "subjective night" (in constant darkness, subjective night represents the time interval when the lights would have been off during the preceding light-dark cycle). The hybridization signal was restricted to the ventrolateral subdivision of the SCN, in a distribution similar to the termination of visual inputs.³² The photic stimulation of *c-fos* mRNA levels depended on circadian phase, with a phase dependence similar to that already well described for light-induced phase shifts of overt locomotor rhythmicity. A light pulse delivered during the subjective night robustly elevated *c-fos* mRNA levels (measured after 30 min of light), whereas relatively little expression was evoked by a pulse during the subjective day. A two-way ANOVA showed that the main effects of light ($P < 0.001$, $F = 108.66$) and circadian time ($P < 0.001$, $F = 65.22$) were significant, as was the interaction between light and time ($P < 0.001$, $F = 57.31$). Pairwise comparisons using Bonferroni *t*-statistics showed that the value after the subjective-night light pulse was significantly greater than the value in darkness ($P < 0.05$) or after the subjective-day light pulse ($P < 0.05$).

Of the other *fos* family genes, only *fra-2* exhibited all these *c-fos*-like properties in the SCN (Fig. 1). Like *c-fos*, *fra-2* mRNA was mostly undetectable in darkness, clearly elevated by the subjective-night light pulse, and confined to the SCN's ventrolateral subdivision. A two-way ANOVA showed that the main effects of light ($P = 0.002$, $F = 12.48$) and circadian time ($P < 0.001$, $F = 25.75$) were significant, as was the interaction between light and time ($P < 0.001$, $F = 16.46$). Pairwise comparisons using Bonferroni *t*-statistics showed that the value after the subjective-night light pulse was significantly greater than the value in darkness ($P < 0.05$) or after the subjective-day light pulse ($P < 0.05$).

However, there were also some notable differences between the patterns of *fra-2* and *c-fos* gene expression. The stimulatory effect of light appeared less for *fra-2* (less than a 2.5-fold signal increase) than for *c-fos* (nearly a 7.5-fold signal increase) when measured after 30 min of light. The time-courses for the photically induced elevations of *fra-2* and *c-fos* mRNAs also differed significantly (Fig. 2). When a 4-h light pulse was given during the late subjective night [onset at circadian time (CT) 19, Fig. 2, middle panel], the response of *c-fos* was rapid, peaking 30 min after light onset, and transient, with reduced signal by 1 h and return to baseline by 2 h. The activation of *fra-2* was equally fast, but signal remained high for at least 2 h before it declined. A two-way ANOVA showed that the main effects of time ($P < 0.001$, $F = 66.65$) and mRNA type ($P < 0.001$, $F = 20.35$) were significant, as was their interaction ($P < 0.001$, $F = 7.78$). Pairwise comparisons using Bonferroni *t*-statistics showed

that *fra-2* and *c-fos* signal intensities differed significantly only at the 1- and 2-h time-points ($P < 0.05$). This pattern was also found if the 4-h light pulse was given instead during the early subjective night (onset at CT 14, Fig. 2, top panel). The persistent expression of *fra-2* mRNA after the light pulse did not depend on the continued presence of light, since a similar result was obtained after a 30-min light pulse (onset at CT 19, Fig. 2, bottom panel).

The differing time-courses of *fra-2* and *c-fos* gene expression were reflected in the expression of their immunoreactive proteins. During subjective night, Fra-2 and c-Fos immunoreactivities were not detectable in darkness, but they were clearly elevated in the ventrolateral SCN after a light pulse (Fig. 3). Although the level of immunoreactive Fra-2 expression appeared less than that of c-Fos, it was persistent and appeared unchanged for at least 4 h. Between 2 and 4 h after light onset, the number of c-Fos-immunoreactive cells in the ventrolateral SCN decreased by 31%, from 106 ± 10 to 73 ± 6 labeled cells per unilateral SCN per tissue section (mean \pm S.E.M., $n = 8$ animals at each time-point; $P = 0.016$ by Student's *t*-test), whereas the number of Fra-2-immunoreactive cells remained stable over this interval, from 44 ± 7 to 43 ± 6 labeled cells per unilateral SCN per tissue section ($n = 6$ animals at each time-point).

The expression patterns of *fra-1* and *fosB* in the SCN were different from those of *c-fos* and *fra-2* (Fig. 4). No *fra-1* signal was visible in the SCN, either in darkness during subjective day or night or after light pulses. In contrast, *fosB* hybridization signal was constitutively present, even in darkness, during both subjective day and night, with only a modest elevation (less than 50%) in the ventrolateral SCN after the subjective-night light pulse. As with *c-fos* and *fra-2*, a two-way ANOVA showed that the main effects of light ($P < 0.001$, $F = 40.04$) and circadian time ($P < 0.001$, $F = 125.98$) were significant for *fosB*, as was the interaction between light and time ($P < 0.001$, $F = 49.92$). Pairwise comparisons using Bonferroni *t*-statistics showed that the value after the subjective-night light pulse was significantly greater than the value in darkness ($P < 0.05$) or after the subjective-day light pulse ($P < 0.05$).

Importantly, *fosB* mRNA is known to undergo a unique post-transcriptional modification, in which 140 nucleotides of the primary transcript may be removed by alternative splicing to create a truncated protein (Δ FosB) containing the first 237 amino acids of FosB. To distinguish between these two *fosB* gene products, we employed two specific rabbit polyclonal antibodies; one (FosB[N]) was raised against amino acids 79–131 of the N-terminus of FosB and recognizes both FosB and Δ FosB, while the other (FosB[C]) was raised against amino acids 245–315 of the C-terminus of FosB (missing from Δ FosB) and so recognizes FosB alone. When rats were killed in darkness during either subjective day or night, immunoreactivity was detected in the ventrolateral SCN throughout the circadian cycle, but only with the FosB[N] antibody (Fig. 5, Table 1). A two-way ANOVA showed that the main effect of antibody was significant ($P < 0.001$, $F = 59.48$), but not the main effect of circadian time ($P = 0.132$, $F = 1.99$) or the interaction between antibody and circadian time ($P = 0.708$, $F = 0.47$). Since both antibodies did recognize a FosB-like immunoreactivity after a 2-h light pulse (Table 1), the results suggest that Δ FosB is the species constitutively expressed in darkness, with FosB ($\pm \Delta$ FosB also) induced by the subjective-night light pulse.

Dorsomedial subdivision of the suprachiasmatic nucleus

During the course of our experiments, we discovered that SCN *c-fos* and *fra-2* mRNA levels also exhibited endogenous circadian rhythms in constant darkness. In contrast to photo-inducible gene expression in the ventrolateral SCN, the circadian *c-fos* and *fra-2* signals were localized to the dorsomedial subdivision (Fig. 6), with high levels during the early subjective day and low levels during the subjective night (Fig. 7). For *c-fos*, the mean values for the 15 time-points in Fig. 7 were significantly different ($P < 0.001$, $F = 6.29$, one-way ANOVA), and pairwise comparisons using Bonferroni *t*-statistics showed that the subjective-day values for CT 0, 0.5, 6 and 8 were significantly greater than the subjective night values for CT 13, 18, 22 and 23 ($P < 0.05$). For *fra-2*, the mean values for the 15 time-points were also significantly different ($P = 0.013$, one-way ANOVA on ranks), but pairwise comparisons failed to isolate significant differences between individual time-points.

Although our *in situ* procedure did not provide a quantitative assay, the level of dorsomedial circadian gene expression was clearly much lower than the level of ventrolateral photic gene expression. As shown in Fig. 8, the film exposure time required for demonstrating dorsomedial *c-fos* mRNA expression at CT 0.5 in darkness was at least six times longer than the exposure time required for demonstrating ventrolateral *c-fos* mRNA expression in light during a light–dark cycle (30 min after lights on at dawn). These observations probably account for our previous report that SCN *c-fos* mRNA levels are rhythmic in rats entrained to a light–dark cycle (peaking 30 min after dawn) but not in free-running rats in constant darkness.⁵⁹ Given the relatively long film exposure time, and since *fosB* hybridization signal was constitutively present in the ventrolateral SCN during both subjective day and subjective night in constant darkness (Fig. 4), it was not possible to assay for any circadian expression of *fosB* using these methods. No *fra-1* signal was detected at these long exposure times.

The circadian rhythm of gene expression in the dorsomedial SCN was reflected at the protein level, at least for c-Fos (Fig. 9, Table 2). The mean values for the four time points in Table 2 were significantly different ($P = 0.008$, $F = 8.14$, one-way ANOVA), and pairwise comparisons using Bonferroni *t*-statistics showed that the subjective day values for CT 1 and 9 were significantly greater than the subjective night value for CT 21 ($P < 0.05$). For *Fra-2*, differences in the mean values for the four time-points failed to reach statistical significance ($P = 0.365$, one-way ANOVA).

Since some cells in the dorsomedial SCN synthesize arginine vasopressin (AVP; for review, see Ref. 30), we employed double-label immunohistochemistry to document possible c-Fos/AVP co-localization, using antibodies to c-Fos and AVP-associated neurophysin on single tissue sections from rats killed at CT 0.5 in darkness. Neurons with dual labeling for nuclear c-Fos and cytoplasmic AVP–neurophysin were clearly identified (Fig. 10). The percentage of AVP–neurophysin cell bodies that were also c-Fos positive was $15 \pm 3\%$

(mean \pm S.E.M.; $n = 4$ animals, 831 AVP–neurophysin cells counted in total). However, such cells appeared to represent only a tiny proportion of all c-Fos-containing perikarya. The percentage of c-Fos-positive nuclei that were in the AVP–neurophysin cell bodies was only $3 \pm 1\%$ ($n = 4$ animals, 5335 c-Fos nuclei counted in total).

DISCUSSION

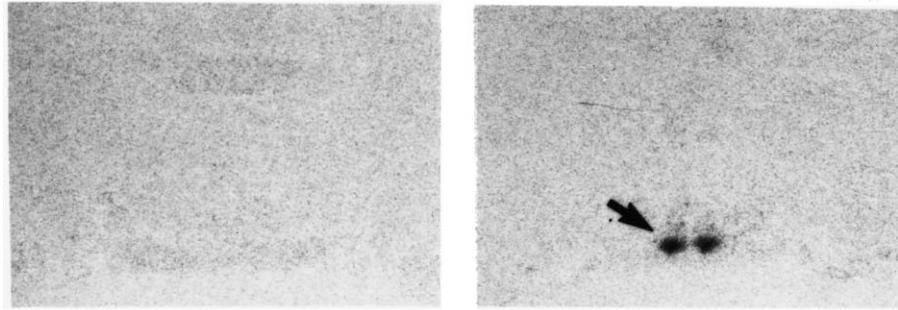
The data presented here provide a comprehensive analysis of *fos* family gene expression in the rat SCN. We found that *c-fos*, *fra-2* and *fosB* exhibit differences in their specific expression patterns, including their photic and circadian regulation in separate cell populations in the ventrolateral and dorsomedial subdivisions of the SCN. No SCN *fra-1* expression was found using our cRNA probe and *in situ* hybridization technique.

Photic regulation of fos family genes in the ventrolateral rat suprachiasmatic nucleus

Phase-dependent photic stimulation of *c-fos* gene expression in the ventrolateral SCN is now well known (for reviews, see Refs 26, 36 and 58). We found that both *fra-2* and *fosB* share this property, with significant elevations of mRNA and immunoreactive protein levels after a subjective-night (but not subjective-day) light pulse. The fact that photoinduction is phase dependent means that these genes are targets of the circadian pacemaker (i.e. they are clock controlled); the mechanism that gates their activation to the time of day must itself be one of the pacemaker's output pathways. This mechanism is uncertain but appears to be specific to the SCN. In the intergeniculate leaflet, another retino-recipient component of the circadian visual system, photic stimulation of *c-fos* mRNA and immunoreactive protein levels does not depend on the circadian phase of stimulation.^{5,49,53} Moreover, circadian control of c-Fos protein expression persists in the SCN stimulated as a tissue slice *in vitro*.^{3,15} Thus, neither the retina nor the rest of the brain is a required constituent of the gating mechanism (although they might contribute to it).

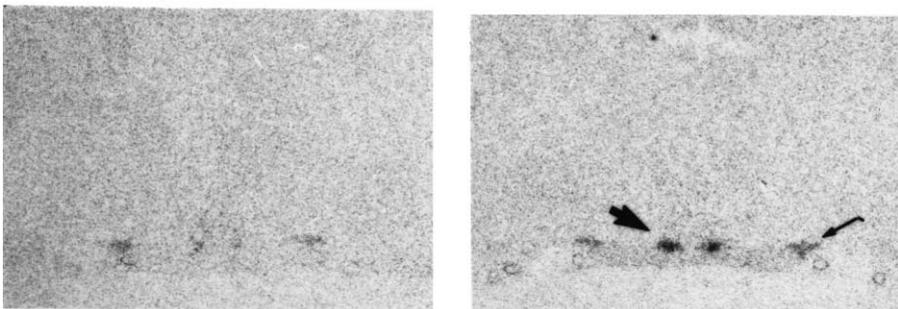
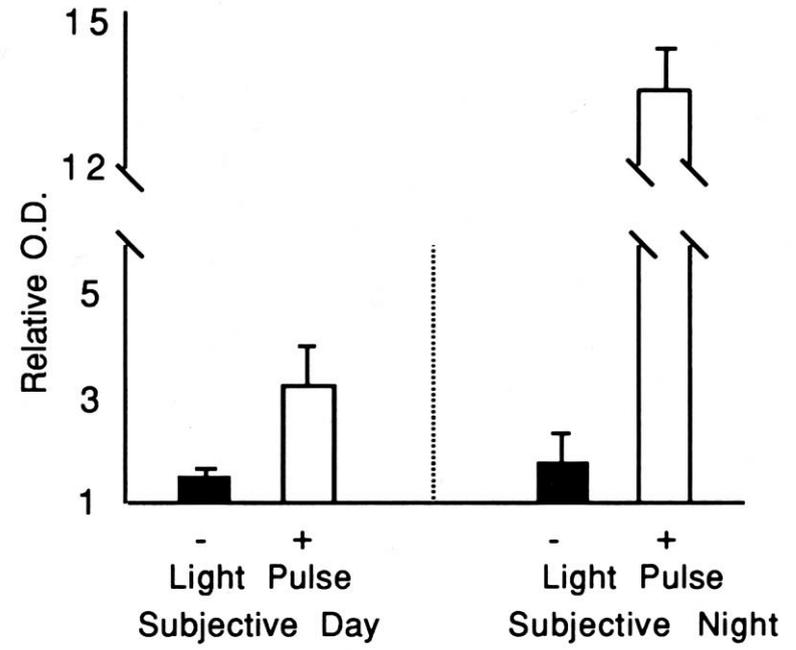
Notably, details of the *fra-2* and *fosB* expression patterns differ from the pattern established for *c-fos*. Expression of the *fra-2* gene is prolonged following photic stimulation, with elevated mRNA and immunoreactive protein levels that appear unchanged in the ventrolateral SCN for at least a few hours beyond the *c-fos* peak. The significance of this variable ratio of c-Fos/*Fra-2* levels over time is unknown. *Fra-2* may act as either an activator or a repressor of gene transcription, depending on the identity of its Jun binding partner⁶² and perhaps also on its phosphorylation state.²¹ One possibility is that genes *trans*-activated by c-Fos early after light onset might then be *trans*-repressed by *Fra-2* late after light onset. Another possibility is that c-Fos/Jun and *Fra-2*/Jun complexes might exist simultaneously and bind preferentially to different promoters, perhaps depending on the identity of the response elements or other sequences flanking the canonical AP-1 binding site.

Fig. 1. Photic stimulation of *c-fos* and *fra-2* expression in the ventrolateral SCN. Left: autoradiographs (*in situ* hybridization) of representative coronal brain sections from rats killed during subjective night (CT 19), either in darkness or after a single 30-min light pulse. Large arrows: ventrolateral SCN; small arrow: constitutive *fra-2* signal in the supraoptic nucleus. Scale bar = 1 mm. Right: mRNA levels (*in situ* hybridization) graphed as relative OD of the ventrolateral SCN on autoradiographs of tissue sections from rats killed during subjective day (CT 7) or night (CT 19), either in darkness ($n = 5$ rats at CT 7, $n = 6$ rats at CT 19) or after a single 30-min light pulse ($n = 5$ rats at CT 7, $n = 7$ rats at CT 19). Each column represents mean \pm S.E.M.



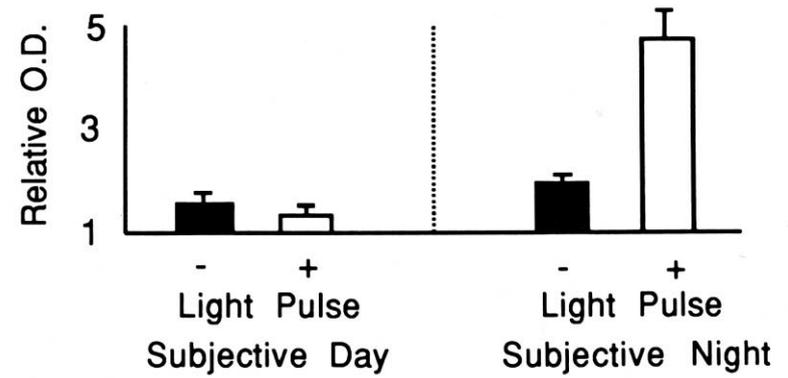
- +
Light Pulse
Subjective Night

c-fos



- +
Light Pulse
Subjective Night

fra-2



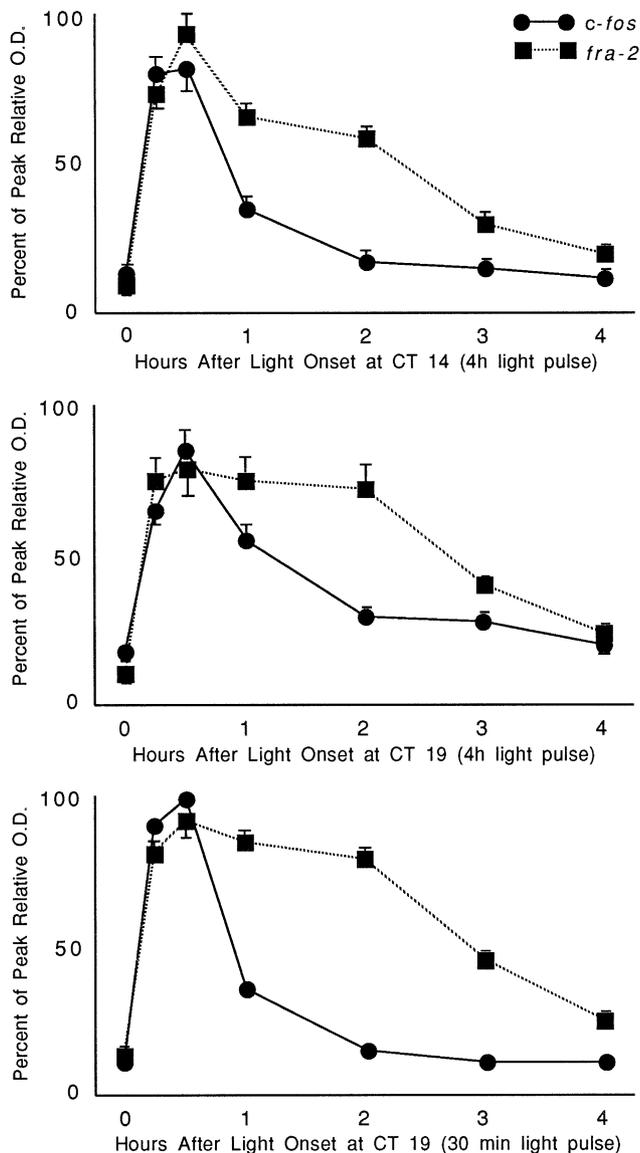


Fig. 2. Time-course of photo-inducible *c-fos* and *fra-2* expression in the ventrolateral SCN. Messenger RNA levels (*in situ* hybridization) graphed as relative OD of the ventrolateral SCN on autoradiographs of tissue sections from rats killed during subjective night, exposed to either a 4-h light pulse from CT 14 to 18 (top), a 4-h light pulse from CT 19 to 23 (middle), or a 30-min light pulse from CT 19 to 19.5 (bottom). The two probes were used on alternating SCN sections ($n = 4$ rats, except for *c-fos* in the bottom plot, with $n = 2$ rats). For each time-point, values are expressed as a percentage of the peak relative OD for that mRNA. Each symbol represents mean \pm S.E.M.

Unlike *c-fos* and *fra-2*, the *fosB* gene appears to be expressed constitutively in the ventrolateral SCN throughout the circadian cycle in constant darkness. Our immunohistochemical analysis suggests that Δ FosB is the protein product accounting for this constitutive expression, while FosB (and perhaps also Δ FosB) is induced by the subjective-night light pulse. This interpretation accounts for the results of three previous reports. Peters *et al.*⁵² and Ebling *et al.*¹⁶ used two different antibodies that should both recognize Δ FosB, one raised against a bacterially expressed fusion protein containing amino acids 4–338 of FosB (in Peters *et al.*) and the other raised against a 15-residue synthetic peptide corresponding to the N-terminal sequence of FosB (in Ebling *et*

al.). Both antibodies demonstrated constitutive immunoreactivity in the ventrolateral SCN of rats and hamsters, respectively, housed in constant darkness; Peters *et al.* also detected a significant elevation after a subjective-night (but not subjective-day) light pulse. Guido *et al.*,²² using a 45-mer oligonucleotide complementary to the C-terminal region of the primary *fosB* transcript (spliced out in Δ FosB), found a robust, phase-dependent photo-induction of *fosB* mRNA in the ventrolateral hamster SCN after subjective-night light pulses, but they detected no constitutive expression in darkness. The significance of Δ FosB expression in the SCN is unknown; however, this splice variant lacks FosB's C-terminal region involved in transcriptional activation, and in some experimental situations it appears to act as a dominant-negative inhibitor of AP-1-mediated transcription.^{43,44,68}

Based on our present results, together with our previous demonstration of differential Jun protein expression and the co-localization of c-Fos with Jun proteins and FosB in SCN cell nuclei,^{52,63} we suggest that AP-1 binding sites on ventrolateral SCN target genes are constitutively occupied by Δ FosB/JunD complexes, and that c-Fos, FosB and JunB (as well as Fra-2, if co-localized) compete for binding after photic stimulation. This view is in partial agreement with the recent work of François-Bellan *et al.*¹⁷ Using gel mobility shift assays of SCN protein extracts, they also implicated JunD as a constitutive component of AP-1 binding complexes in darkness, observed c-Fos and JunB only after photic stimulation, with modest increases of Fra-2, c-Jun and Jun D, and failed to detect Fra-1. However, they also failed to detect FosB and concluded that AP-1 binding complexes in darkness represent heterodimers of Fra-2 and JunD. The reasons for this discrepancy are not resolved, but they may relate to different lighting conditions and/or reagents. It is not clear from François-Bellan *et al.*'s report whether their animals were housed in constant darkness or in dim red light. Their immunohistochemistry using a commercial Fra-2 antibody (epitope not specified) showed widespread Fra-2 immunoreactivity throughout the SCN in darkness, with only a slight (20%) light-induced elevation of undetermined statistical significance. It is noteworthy that Δ FosB/JunD complexes have been implicated previously as components of a "long-term" AP-1 binding activity in other brain regions following seizures, drugs or lesions.^{10,28,40,51}

Circadian regulation of fos family genes in the dorsomedial rat suprachiasmatic nucleus

In an earlier publication,⁵⁹ we had commented on the presence of c-Fos-immunoreactive cells in the dorsomedial SCN during the early light phase of the light–dark cycle (2 h after lights on at dawn). This labeling was variable and much less prominent than the labeling of cells in the ventrolateral subdivision at this phase (the ventrolateral labeling was eliminated if lights were not turned on at expected dawn and the rats remained in darkness). In our current experiments, using cRNA probes and long autoradiographic film exposure times, an endogenous circadian rhythm of *c-fos* and *fra-2* mRNA levels is clearly evident in the dorsomedial SCN in constant darkness. The mechanism by which the circadian pacemaker controls these two rhythms is not known, but it is likely to involve changes in transcription. Importantly, transcriptional co-regulation of these two genes is not a universal phenomenon, e.g., in the pineal gland.²

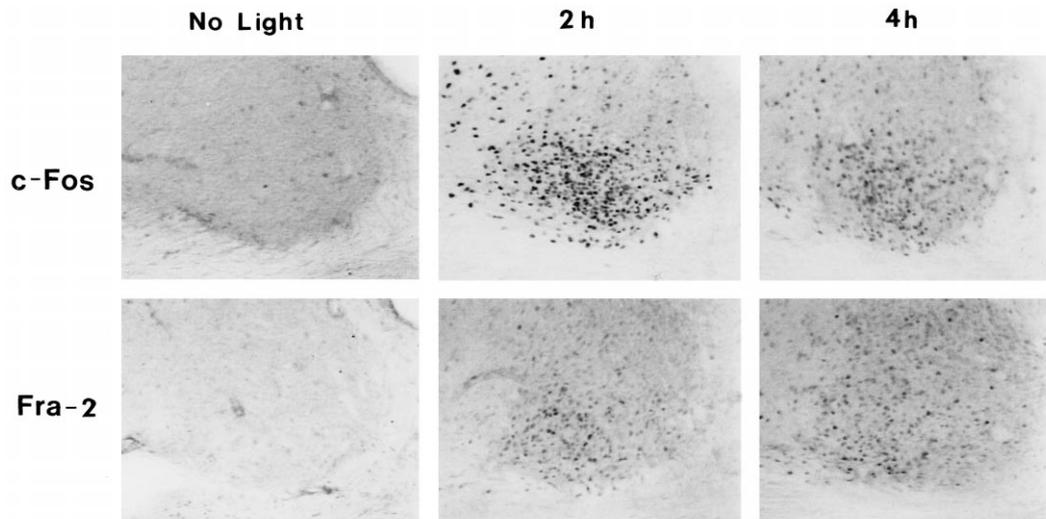


Fig. 3. Photic stimulation of *c-Fos* and *Fra-2* immunoreactivities in the ventrolateral SCN. Representative coronal brain sections of the unilateral SCN from rats killed during subjective night, either in darkness or after a single 2-h (CT 19–21) or 4-h (CT 19–23) light pulse, and processed for immunohistochemistry. Scale bar = 0.1 mm.

There are now several precedents for the differential regulation of *c-fos* gene expression between SCN subdivisions. Koibuchi *et al.*³⁵ first reported a rhythm of *c-Fos* immunoreactivity in the SCN of rats enucleated two months before perfusion, with higher levels during the subjective day; in their published photomicrograph, the dorsomedial SCN appears to be the subdivision involved. Rhythms of *c-fos* mRNA⁶⁵ and immunoreactive *c-Fos* protein⁴ levels have been described in the SCN of neonatal and middle-aged rats, respectively. The immunoreactive protein rhythm was clearly documented as circadian and in the dorsomedial subdivision by Sumová *et al.*⁶¹ As in our study, Sumová *et al.* also noted that the level of dorsomedial circadian expression was significantly less than the level of ventrolateral photic expression. In hamsters, circadian rhythms of SCN *c-fos* gene expression have been reported in the rostral and dorsal SCN,^{9,23} in a distribution distinct from the region in the caudal and ventral SCN that responds to photic stimulation. A spontaneous rhythm of SCN *c-fos* mRNA levels might even persist *in vitro*.⁵⁴ Most recently, a novel demonstration of rhythmic *c-fos* transcription *in vitro* has been accomplished by monitoring bioluminescence from SCN slice cultures derived from a transgenic *fos/luc* mouse line expressing the human *c-fos* promoter linked to a firefly luciferase reporter.¹⁸

It is known that several neuropeptides in the rat SCN exhibit rhythmic properties that are consonant with the functional compartmentalization of the SCN into dorsomedial and ventrolateral subdivisions (for review, see Ref. 30). AVP or somatostatin are expressed by some of the dorsomedial cells, and the levels of these mRNAs and peptides exhibit circadian rhythmicity, with higher levels during the subjective day. In contrast, the mRNA and peptide levels of gastrin-releasing peptide (GRP) and vasoactive intestinal polypeptide (VIP)—which are co-localized in some ventrolateral neurons—show oppositely phased rhythms during the light–dark cycle, with high levels of GRP during the light and VIP during the dark. In intact animals, these rhythms are not present in constant darkness.

c-Fos, arginine vasopressin and circadian clock genes in the suprachiasmatic nucleus

Our double-label immunohistochemical observations suggest that at least some of the dorsomedial cells responsible for the circadian expression of *c-Fos* also synthesize AVP. This is in contrast to the identity of the ventrolateral cells responsible for the photic expression of *c-Fos* in rat and hamster SCN, some of which contain GRP and/or VIP,^{1,12,14,41,55} but not AVP. Co-localization of *c-Fos* and AVP immunoreactivities has also been reported during the early light phase of the light–dark cycle (4 h after lights on at dawn) in the SCN of *Arvicanthis niloticus*, a diurnal rodent.⁵⁶ However, it is not yet clear whether the *c-Fos* expression in this species represents a circadian rhythm or a direct response to light, since retinal inputs are not restricted to the ventrolateral SCN in *A. niloticus*.⁶⁰

It is conceivable that the circadian expression of *c-Fos* in dorsomedial cells may contribute to the circadian rhythm of SCN AVP synthesis. Nuclear run-on analysis has demonstrated that rhythmic levels of AVP mRNA in the SCN are regulated by transcription.⁶ Besides a putative AP-1 binding site, the AVP gene promoter contains a putative cyclic-AMP response element (CRE).⁴² This element can bind *c-Fos* in conjunction with other CRE-binding proteins,^{24,57} and CRE-mediated reporter gene expression exhibits a circadian rhythm in the SCN.⁴⁷

The AVP promoter also contains an E box enhancer, an element that positively drives transcription of the circadian clock genes *period* (*per*) and *timeless* (*tim*) in *Drosophila*.²⁵ In mammals (for review, see Ref. 13), *per* is represented as a family of three homologs (*per1*, *per2* and *per3*), each encoding mRNAs that oscillate with a circadian rhythm in the SCN. When two basic helix–loop–helix (bHLH) transcription factor proteins (CLOCK and BMAL1) dimerize and bind to the E box, *per* transcription is activated. PER proteins accumulate in the cytoplasm, associate with proteins encoded by *tim* and the cryptochrome (*cry1* and *cry2*) genes, and translocate to the nucleus to negatively regulate their own transcription by interfering with CLOCK–BMAL1 dimers.^{20,39}

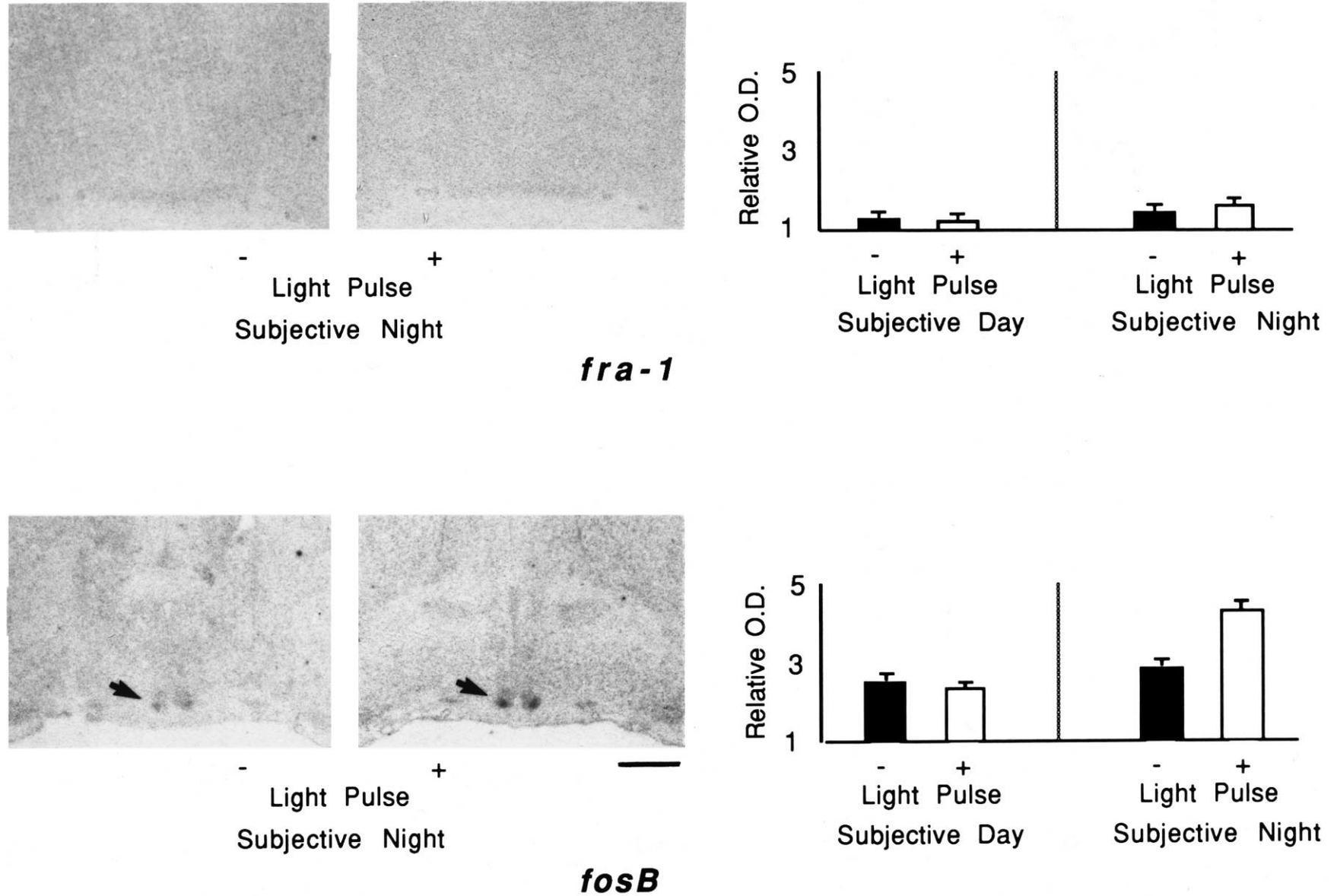


Fig. 4. Photic stimulation of *fra-1* and *fosB* expression in the ventrolateral SCN. Left: autoradiographs (*in situ* hybridization) of representative coronal brain sections from rats killed during subjective night (CT 19), either in darkness or after a single 30-min light pulse. Large arrows: ventrolateral SCN. Scale bar = 1 mm. Right: mRNA levels (*in situ* hybridization) graphed as relative OD of the ventrolateral SCN on autoradiographs of tissue sections from rats killed during subjective day (CT 7) or night (CT 19), either in darkness ($n = 3$ rats at CT 7, $n = 4$ rats at CT 19) or after a single 30-min light pulse ($n = 4$ rats at CT 7, $n = 4$ rats at CT 19). Each column represents mean \pm S.E.M.

α -FosB(N) **α -FosB(C)**

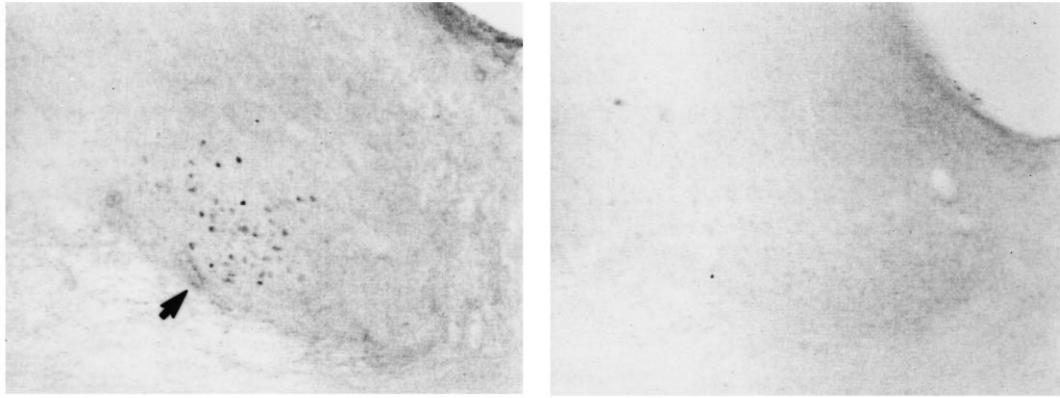


Fig. 5. FosB immunoreactivity in darkness in the ventrolateral SCN. Representative coronal brain sections of the unilateral SCN from rats killed during subjective night in darkness and processed for immunohistochemistry. Immunoreactivity is detected using the FosB[N] antibody only (arrow). Scale bar = 0.1 mm.

Table 1. FosB immunoreactivity in the ventrolateral suprachiasmatic nucleus in constant darkness

Circadian phase	Antibody	
	α -FosB[N]	α -FosB[C]
CT 2 (<i>n</i> = 4)	23 \pm 6	3 \pm 1
CT 9 (<i>n</i> = 4)	18 \pm 4	5 \pm 2
CT 15 (<i>n</i> = 4)	20 \pm 4	2 \pm 1
CT 21 (<i>n</i> = 10)	27 \pm 3	7 \pm 1
CT 21 (<i>n</i> = 6) (after light pulse from CT 19 to 21)	95 \pm 9	95 \pm 7

Values represent means \pm S.E.M. of the number of labeled cell nuclei per section in the unilateral ventrolateral SCN. Antibodies [N] and [C] were used on alternating SCN sections from each of "n" rats.

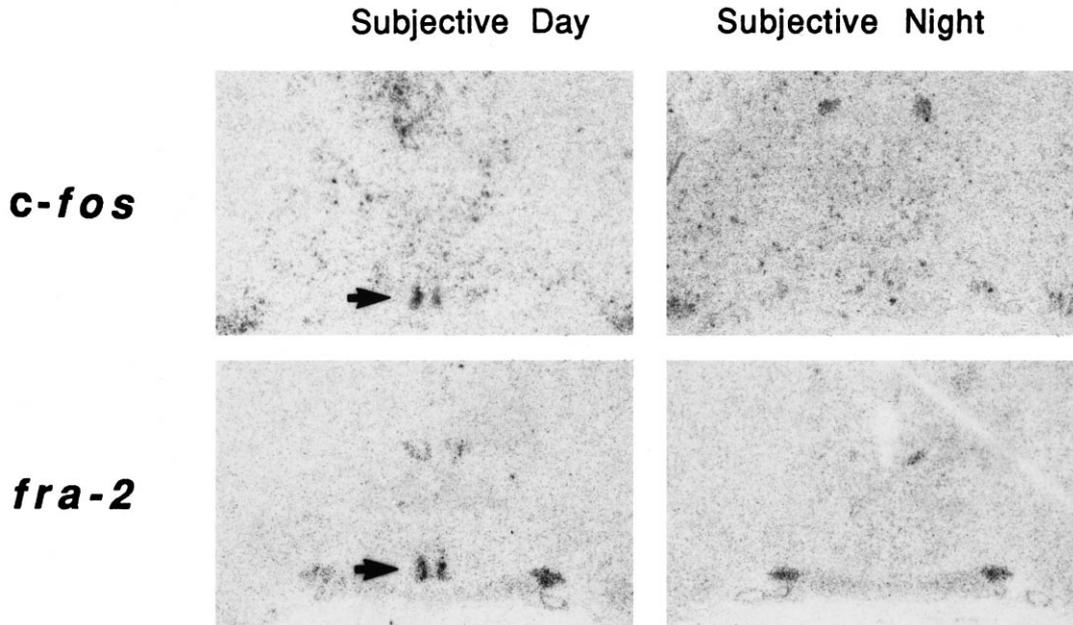


Fig. 6. Endogenous circadian rhythm of *c-fos* and *fra-2* expression in the dorsomedial SCN. Autoradiographs (*in situ* hybridization) of representative coronal brain sections from rats killed during subjective day (*c-fos*: CT 1; *fra-2*: CT 0.5) or subjective night (*c-fos*: CT 20; *fra-2*: CT 15) in darkness. Large arrows: dorsomedial SCN. Scale bar = 1 mm.

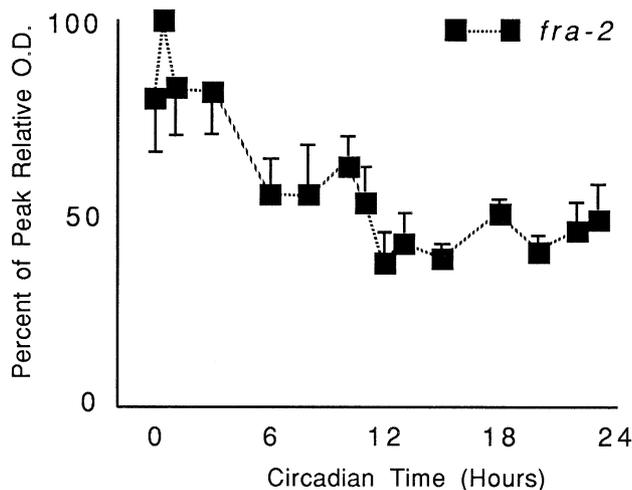
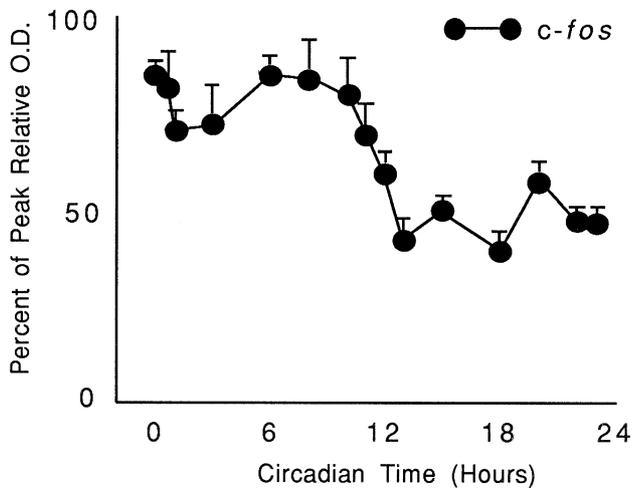


Fig. 7. Time-course of circadian *c-fos* and *fra-2* expression in the dorsomedial SCN. mRNA levels (*in situ* hybridization) graphed as relative OD of the dorsomedial SCN on autoradiographs of tissue sections from rats killed throughout the circadian cycle in darkness. For each time-point, values are expressed as a percentage of the peak relative OD for that mRNA. Each symbol represents mean \pm S.E.M. ($n=4$ rats for *c-fos*, $n=3$ rats for *fra-2*).

Recent studies in mice have shown that AVP cells in the SCN express the PER1 protein²⁷ and that CLOCK–BMAL1 heterodimers act through the E box to activate AVP gene transcription, while PER and TIM proteins are inhibitory.³¹ Thus, the dorsomedial AVP cell may be a useful system to elucidate the relationship between SCN c-Fos and the transcriptional feedback loops that are believed to constitute the core circadian clock mechanism. It is important to note, however, that c-Fos expression in mouse SCN AVP cells can be photically induced (unlike in rat and hamster SCN).⁸ Retinal input to the mouse SCN is not limited to the ventrolateral subdivision, but is more homogeneously distributed throughout the entire dorsoventral extent of the nucleus.⁷

Some implications of the complex regulation of fos family genes in the suprachiasmatic nucleus

The photoinducibility of SCN *c-fos*, *fra-2* and *fosB* may

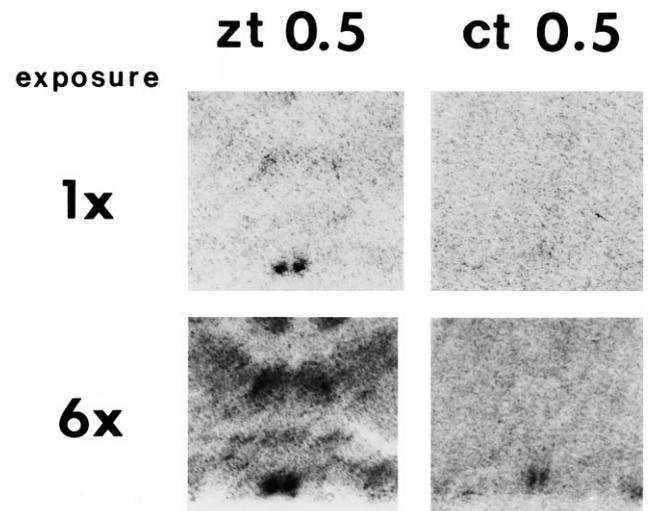


Fig. 8. Relative levels of photic and circadian *c-fos* expression in the ventrolateral and dorsomedial SCN. Autoradiographs (*in situ* hybridization) of a coronal brain section from a rat killed in the light during a light–dark cycle, 30 min after lights on at dawn [left upper and lower images, zeitgeber time (ZT) 0.5] and from a rat killed in darkness during subjective day, 30 min after subjective dawn (right upper and lower images, CT 0.5). The film exposure time required for demonstrating circadian *c-fos* expression in the dorsomedial SCN at CT 0.5 (right lower image) was six times longer than the exposure time required for demonstrating photic *c-fos* expression in the ventrolateral SCN at zeitgeber time 0.5 (left upper image).

Table 2. c-Fos and Fra-2 immunoreactivities in the dorsomedial suprachiasmatic nucleus in constant darkness

Circadian phase	Antibody	
	α -c-Fos	α -Fra-2
CT 1 ($n=3$)	56 \pm 10	48 \pm 10
CT 9 ($n=3$)	52 \pm 8	36 \pm 6
CT 15 ($n=3$)	39 \pm 5	39 \pm 12
CT 21 ($n=3$)	13 \pm 3	25 \pm 3

Values represent means \pm S.E.M. of the number of labeled cell nuclei per section in the unilateral dorsomedial SCN. Antibodies to c-Fos and Fra-2 were used on alternating SCN sections from each of “ n ” rats.

help to explain the data of Honrado *et al.*,²⁹ who reported that mice homozygous for a *c-fos* null mutation nevertheless entrained to a light–dark cycle and generated phase-dependent phase shifts of their wheel-running rhythms to light pulses. In their paper, Honrado *et al.* speculated that other proteins might compensate for the loss of c-Fos in these animals; our data implicate Fra-2 and FosB as plausible candidates for such a role. Indeed, in studies of dividing Swiss 3T3 fibroblasts, intracellular microinjection of various antibodies against Fos family proteins suggests that the activity of multiple proteins must be inhibited in order to effectively block DNA synthesis.³⁸ In contrast, similar experiments using antibodies against Jun family proteins indicate that the activity of each single Jun protein is essential for cell cycle progression. This requirement for Jun proteins may account for the results of Wollnik *et al.*,⁶⁶ who reported that the intracerebroventricular injection of antisense oligodeoxynucleotides to both *c-fos* and *junB* prevented light-induced phase delays of the rat

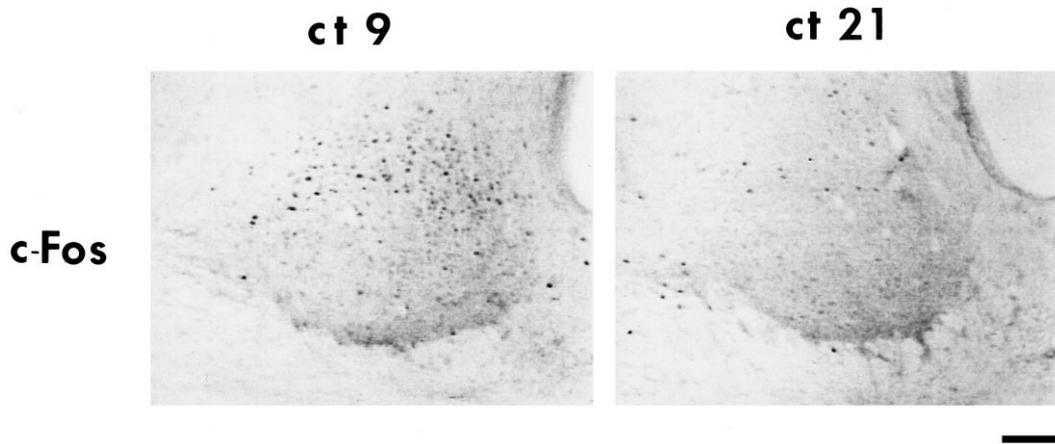


Fig. 9. Endogenous circadian rhythm of c-Fos immunoreactivity in the dorsomedial SCN. Representative coronal brain sections of the unilateral SCN from rats killed during subjective day (CT 9) or subjective night (CT 21) in darkness and processed for immunohistochemistry. Scale bar = 0.1 mm.

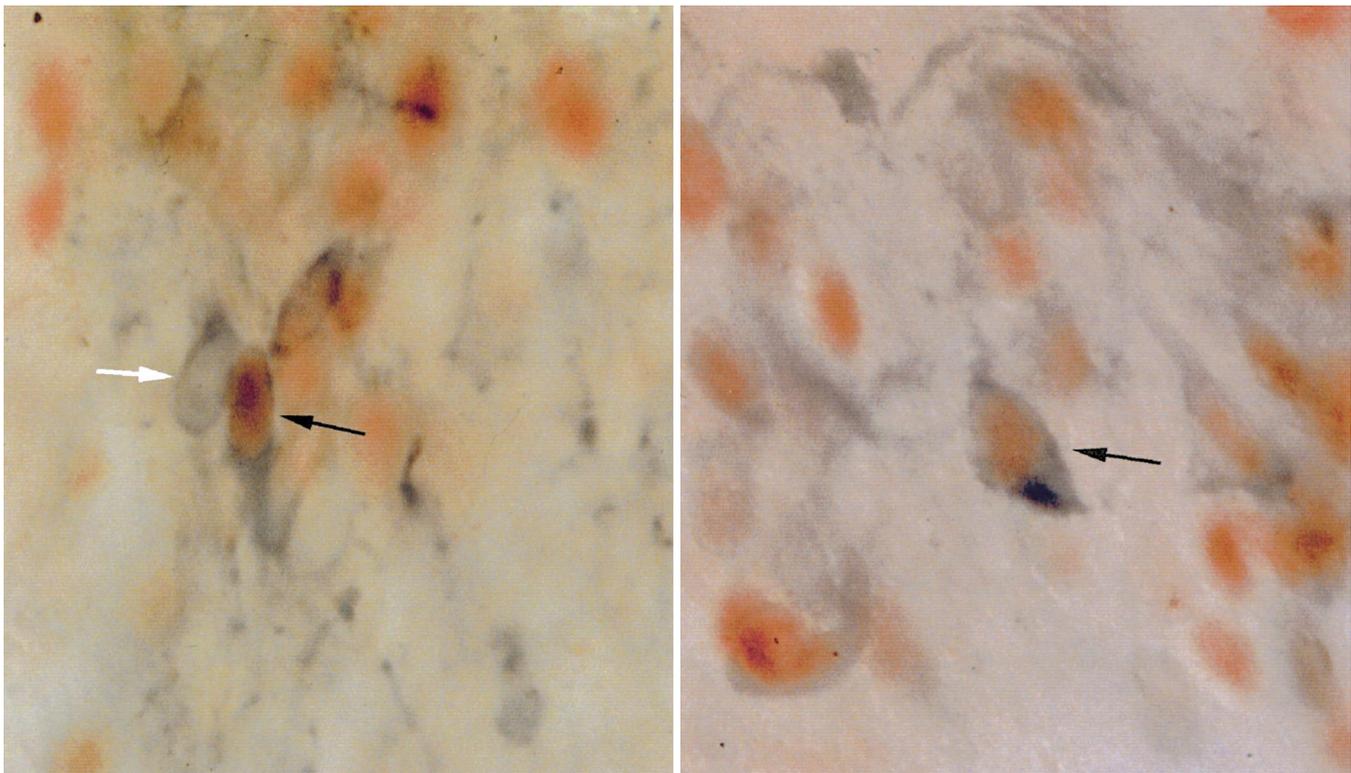


Fig. 10. c-Fos-positive cell nuclei in the dorsomedial SCN co-localize with AVP-associated neurophysin. Dual-label immunohistochemical demonstration of c-Fos-positive cell nuclei (brown) also stained for cytoplasmic AVP-associated neurophysin (blue-gray; black arrows). Also shown for comparison is an AVP-associated neurophysin cell without a c-Fos-positive nucleus (white arrow).

locomotor rhythm. Thus, it may be that the inhibition of *junB* is the indispensable part of Wollnik *et al.*'s experimental design.

The topographic differences in the regulation of *fos* family genes within the SCN imply that their function in circadian timekeeping is likely to be cell specific. The levels of *c-fos* and *fra-2* are photically induced and endogenously rhythmic, but these two patterns are occurring in separate SCN cell populations. The same may be true of other regulatory molecules. For example, *per* gene expression in rat SCN is photically induced in ventrolateral neurons, whereas its circadian expression occurs preferentially in dorsomedial neurons.⁶⁷

Similarly, the photic and circadian regulation of p44/42 mitogen-activated protein kinase activity seems to occur in different regions of the mouse SCN.⁴⁸ This complex regulation means that the analysis of the functional interactions of putative SCN "clock" genes will need to be conducted at the cellular level.

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