

# Light-induced c-Fos Expression in the Mouse Suprachiasmatic Nucleus: Immunoelectron Microscopy Reveals Co-localization in Multiple Cell Types

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## Abstract

Although light is known to regulate the level of *c-fos* gene expression in the suprachiasmatic nucleus (SCN), the site of an endogenous circadian clock, little is known about the identities of the photically activated cells. We used light-microscopic immunocytochemistry and immunoelectron microscopy to detect c-Fos protein in the SCN of Sabra mice exposed to brief nocturnal light pulses at zeitgeber time 15–16. Stimulation with light pulses that saturated the phase-shifting response of the circadian locomotor rhythm revealed an upper limit to the number of photo-inducible c-Fos cells at about one-fifth of the estimated total SCN cell population. This functionally defined set was morphologically and phenotypically heterogeneous. About 24% could be labelled for vasoactive intestinal polypeptide, 13% for vasopressin–neurophysin, and 7% for glial fibrillary acidic protein. The remaining 56% of c-Fos-positive cells were largely of unknown phenotype, although many were presumptive interneurons, some of which were immunoreactive for nitric oxide synthase.

## Introduction

Daily behavioural, physiological and hormonal rhythms in mammals are governed by an endogenous circadian clock located in the suprachiasmatic nucleus (SCN) of the hypothalamus (for review see Klein *et al.*, 1991). Visual input to the SCN serves to synchronize the phases and periods of these rhythms to the environmental day–night cycle. Although the molecular mechanisms for such photic entrainment are unknown, activation of immediate-early genes in the SCN probably plays a role (for reviews see Kornhauser *et al.*, 1993; Abe and Rusak, 1994; Hastings *et al.*, 1995; Schwartz *et al.*, 1995).

The most intensively studied of these genes, *c-fos*, encodes a sequence-specific DNA-binding protein that alters the expression of target genes by regulating their transcription (for review see Morgan and Curran, 1991). Levels of *c-fos* mRNA and immunoreactive c-Fos protein in the retinorecipient zone of the rodent SCN are dramatically elevated (from essentially undetectable levels) by light. The threshold, magnitude and phase-dependence of this photic activation correlate with photically induced phase shifts of overt circadian rhythmicity. Light pulses, administered during the dark phase of the light–dark cycle or during the subjective night in constant darkness, evoke c-Fos (as well as delaying or advancing phase shifts of overt rhythms), whereas light pulses delivered during most of the subjective day elicit neither c-Fos nor phase shifts. Furthermore, in the hamster, pharmacological agents that block behavioural phase shifts also block the photic stimulation of c-Fos in specific regions of the SCN. Light-

induced phase delays of the rat locomotor rhythm are prevented by intracerebroventricular injections of antisense phosphorothioate oligodeoxynucleotides to both *c-fos* and *junB* (Wollnik *et al.*, 1995).

These findings have led to the idea that transcriptional mechanisms in general, and c-Fos in particular, are involved in the photic entrainment of circadian rhythmicity. Among the critical issues that need to be addressed are the elucidation of the signal transduction pathways for the photic activation of c-Fos and the target gene candidates for transcriptional regulation by c-Fos. Establishing the identity of the SCN cells that express c-Fos should contribute to addressing these questions, and attempts have been made to co-localize the immunoreactive c-Fos protein to known peptidergic neurons in the rat SCN (Daikoku *et al.*, 1992; Earnest *et al.*, 1993; Mikkelsen *et al.*, 1994; Romijn *et al.*, 1996; Speh and Moore, 1996). Photically induced c-Fos has also been co-localized to a subpopulation of calmodulin-containing cells in the hamster SCN (Silver *et al.*, 1996). However, it is noteworthy that only a minority of the entire c-Fos nuclear population in the SCN has thus far been attributed to any one identified neuronal phenotype, which has prompted the suggestion that some c-Fos-positive cells may be glial (Bennett and Schwartz, 1994).

Much of this previous work was inconclusive partly because the small size and high packing density of double-labelled cells in the SCN make their identification uncertain at the light microscope level.

Moreover, a submaximal photic stimulus might activate only a fraction of the entire c-Fos population, raising the possibility that different lighting regimes might excite different cellular subgroups rather than the whole set. Lastly, most of the co-localization data have come from the rat SCN, where c-Fos cells are limited to a ventrolateral distribution, raising the concern that conclusions might not be generalizable.

This paper seeks to resolve these issues, including presentation of the ultrastructural localization of the c-Fos protein in the SCN by immunoelectron microscopy. Photic activation of *c-fos* gene expression in the mouse SCN has been described previously by Colwell and Foster (1992) and Schwartz *et al.* (1996), and the Sabra mouse SCN, with whose ultrastructure we are familiar (Castel *et al.*, 1990, 1993), was chosen for the present study.

For immuno-ultrastructural demonstration of the c-Fos protein, the crystalline chromogen tetramethyl benzidine (TMB) served as a distinctive electron-dense marker for nuclear c-Fos, and was well suited also for dual immunocytochemical co-localization in combination with diffusely labelled cytoplasmic antigens. The latter included vasoactive intestinal polypeptide (VIP), vasopressin-associated neurophysin (VP-NP) and nitric oxide synthase (NOS), all of which are associated with messenger molecules in the SCN, and also glial fibrillary acidic protein (GFAP). Our present results, derived by the use of light microscope immunocytochemistry, indicate that there is probably an upper limit to the number of SCN cells that express c-Fos after photic stimulation, and immunoelectron microscopy shows that this functionally defined population is morphologically and phenotypically heterogeneous. Brief preliminary reports of these findings have been made (Castel and Belenky, 1994; Castel *et al.*, 1995).

## Materials and methods

### Animals

Adult male albino mice (25–30 g) of the Sabra strain were obtained from the Hebrew University Medical School and maintained individually in clear polycarbonate cages within a well-ventilated room (26°C) under a 12 h light:12 h dark cycle for at least 3 weeks before experiments. Light intensity was ~400–500 lux during the light phase; no light was present during darkness. Purina chow and water were provided freely, and cage handling was once weekly at random times.

For routine experiments photic stimulation was administered during the dark phase, 3–4 h after lights off [zeitgeber time (ZT) 15–16]. Individual mice were exposed to a single pulse of bright white light of various intensities. Different groups of mice were exposed to no light or to a single 15 min light pulse of 65 450 or 2300 lux, then returned to darkness for another 30 min. Animals were routinely killed 45 min after the onset of the pulse.

In order to assess the possible effect of different time schedules on the numbers of c-Fos-positive nuclei, pilot experiments at 65 450 or 2300 lux included: (i) exposure of mice to photic stimuli 9–10 h (instead of 3–4 h) after lights off, i.e. at ZT 21–22 (instead of ZT 15–16); (ii) killing the mice at ZT 15–16, 90 min after the light pulse (instead of 45 min).

A total of 74 mice were used for the present study, as follows: quantitative light microscope c-Fos immunocytochemistry at ZT 15–16, 45 min after light stimulation ( $n=22$ ); *in situ* hybridization ( $n=20$ ); light microscope immunocytochemistry for VIP, VP-NP, GFAP ( $n=3$ ); additional dual ultrastructural immunocytochemistry ( $n=6$ ); NOS immunocytochemistry ( $n=3$ ); pilot experiments at ZT 21–22 ( $n=12$ ), and 90 min after photic stimulation ( $n=8$ ).

### Immunocytochemistry

After photic stimulation, mice were deeply anaesthetized by i.p. injection of Nembutal under a dark-red safelight, and their eyes were covered with black insulation tape. Prior to perfusion, ~50 µl heparin (5000 IU/ml; Choay, Paris, France) was injected into the left cardiac ventricle. The circulation was perfused with PBS, then with freshly prepared fixative containing 4% *p*-formaldehyde, 0.1% glutaraldehyde (Electron Microscopy Sciences, Fort Washington, PA, USA) and 0.2% picric acid in 0.1 M phosphate buffer at pH 7.0. Brains were then removed and immersed in fixative for 2–3 h at room temperature, after which they were cut as 50 µm serial coronal sections on a vibratome into Tris-buffered saline (TBS), pH 7.0. Each mouse brain provided about eight to ten serial sections containing the SCN, of which alternate sections were taken for light and electron microscope immunocytochemistry or for cresyl violet staining.

Two different affinity-purified, polyclonal anti-Fos antibodies generated in rabbits were used, one directed against a synthetic Fos peptide (amino acids 128–153 of rat c-Fos) conjugated to keyhole limpet haemocyanin (1:400; kindly supplied by Dr Michael Iadarola, National Institutes of Health, Bethesda, MD) and a second directed against a different peptide (amino acids 4–17 of human c-Fos) (1:150; Ab-2; Oncogene Science, Cambridge, MA, USA). Also used were polyclonal anti-VIP (Incstar, Stillwater, MN, USA), monoclonal anti-VP-NP (Ben-Barak *et al.*, 1985) and rabbit polyclonal antiserum to GFAP raised against an antigen purified from human brain (Biomakor, Rehovot, Israel). Three rabbit antisera for NOS were used: against neuronal NOS (NOS-I), macrophage NOS (iNOS) and endothelial NOS (eNOS), all obtained from Affiniti Research Products, Minehead, EX, UK.

### Light microscopy

Interspersed by copious TBS washes, and using TBS as diluent, sections were sequentially treated with 25% methanol containing 0.3% H<sub>2</sub>O<sub>2</sub> for 30 min; 0.5% sodium borohydride for 30 min; 1% Triton X-100 for 1 h; blocker containing 0.5% Triton X-100, 2% egg albumin, 0.5% glycine and 0.5% lysine for 1 h; and primary antiserum with 0.5% Triton X-100 and 1% egg albumin for 12 h at room temperature. Labelling was visualized by the use of a biotin-avidin-peroxidase kit (ABC Elite, Vectastain; Vector Laboratories, Burlingame, CA) with diaminobenzidine-H<sub>2</sub>O<sub>2</sub> as chromogen. Sections were lifted onto subbed slides, dipped briefly in 0.1% osmium tetroxide, dehydrated in ethanol, cleared in xylene, and coverslipped with Entellan (Merck).

### Quantitation of c-Fos-immunoreactive nuclei

Counts of cell nuclei within the bilateral SCN were derived from five alternate serial coronal sections per mouse brain. The area within which the nuclei were counted was determined from corresponding cresyl violet stained sections (Fig. 1A), which served as templates from which overlays were drawn. Within these circumscribed areas, all cell nuclei labelled for c-Fos (both strongly and faintly stained) were counted with the use of a meshed graticule in a Zeiss microscope at a magnification of ×100, repeated twice independently (by M. B. and S. C.). Since the template overlays had been drawn on a meshed background that corresponded with the mesh of the graticule, this facilitated recording of nuclear counts. For each bilateral SCN, the counts from five alternate serial vibratome sections were pooled. Areas were digitized from the overlays with the use of an IBM PC-compatible computer, Summasketch Plus digitizer (Summagraphics, Fairfield, CT, USA) and SigmaScan software (Jandel Scientific, Corte Madera, CA, USA). The sum of the areas, derived from five

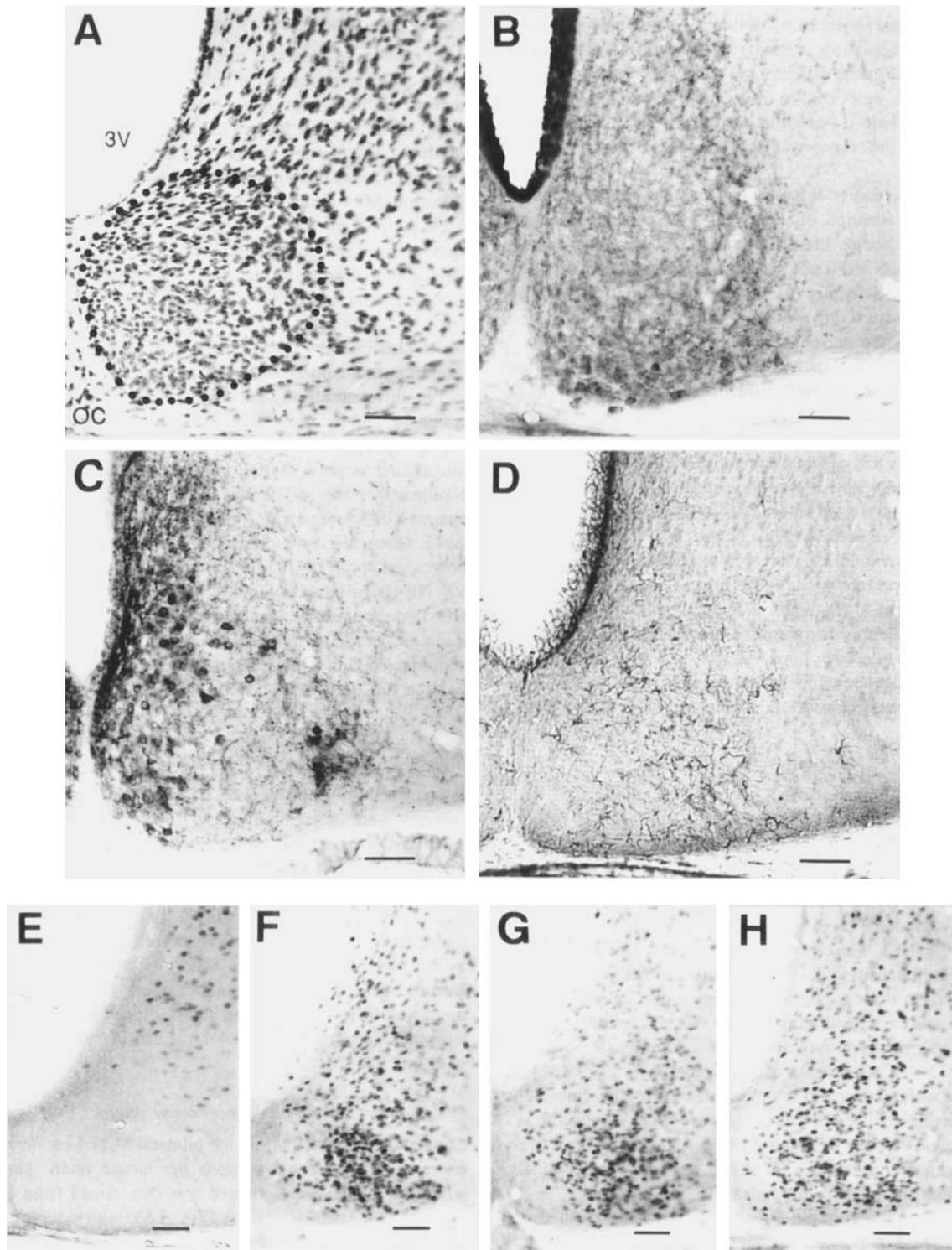


FIG. 1. Micrographs of coronal vibratome sections from the suprachiasmatic nucleus (SCN) of Sabra mice at a similar rostrocaudal level. (A) Cresyl violet staining emphasizes the rounded shape of the SCN and the close packing of the small cell nuclei. The dotted border circumscribes the area within which c-Fos-positive nuclei were counted in corresponding immunocytochemically labelled sections. (B) Vasoactive intestinal polypeptide immunoreactivity in ventral somata and in processes throughout the nucleus. (C) Vasopressin-neurophysin immunoreactivity mainly in dorsomedial somata, in a few ventrolateral somata, and in processes throughout the nucleus. (D) Glial fibrillary acidic protein immunoreactivity within the SCN, in small astrocytic somata and extensive processes. (E-H) c-Fos immunoreactivity in SCN sections from Sabra mice that had been exposed to a 15 min light pulse of different intensities at ZT 16. (E) Control, no light exposure. (F) 65 lux pulse. (G) 450 lux pulse. (H) 2300 lux pulse. Note the absence of c-Fos labelling within the SCN of the control, although there are some labelled cell nuclei dorsal to the SCN. All the light-exposed specimens reveal large numbers of c-Fos-immunoreactive cell nuclei throughout the SCN. 3V, third ventricle; OC, optic chiasma. Scale bars, 50  $\mu$ m.

rostrocaudal levels of the bilateral SCN, represented the total area from which nuclei were counted for a particular specimen. Since this area was generally close to 0.5 mm<sup>2</sup>, nuclear counts were calculated and presented as number of cell nuclei per 0.5 mm<sup>2</sup>.

#### Electron microscopy

Sections were processed as for light microscopy, except that methanol and Triton X-100 were omitted. The chromogen for visualizing c-Fos was TMB, followed by stabilization with diaminobenzidine, according to a modification of the Weinberg and Van Eyck (1991) procedure. Briefly, sections were washed in 0.1 M phosphate buffer (pH 6.0), preincubated in buffer containing 0.005% TMB and 0.5% ammonium paratungstate for 20 min, then incubated in this medium with 0.01% H<sub>2</sub>O<sub>2</sub> added. Dark-blue reaction product labelled c-Fos nuclei within the SCN within 5–7 min. For stabilization, sections were incubated in 1% paratungstate in 0.1 M phosphate buffer (pH 6.9) for 20 min, washed in buffer, and treated with a diaminobenzidine/cobalt/H<sub>2</sub>O<sub>2</sub> mixture in 0.1 M cacodylate buffer (pH 6.9), after which the reaction product turned greyish-blue. The entire TMB procedure was carried out at 4°C on a horizontal shaker. As required, alternate sections were dual-labelled for VIP, VP-NP, NOS or GFAP, with diaminobenzidine as the chromogen.

Immunoreacted sections were postfixed in 1% osmium tetroxide and 1.5% potassium ferricyanide in cacodylate buffer for 1 h at room temperature, dehydrated in ascending concentrations of ethanol, infiltrated with epoxy resin (EM-BED-812, Electron Microscopy Sciences, Fort Washington, PA, USA), flat-embedded in preformed resin blocks, and polymerized for 2 days at 60°C. Ultrathin sections were cut with a diamond knife on an LKB III Ultratome and picked up on 300-mesh thin-bar grids. Sections were contrasted with lead citrate for 2–3 min and ethanolic uranyl acetate for 5 min, and viewed in a JEOL 100CX or a Philips 300 electron microscope. Counting was done directly from the fluorescent screen and manually recorded on a mesh template corresponding to the thin-bar grid.

#### In situ hybridization

Mice were exposed to no light ( $n=6$ ) or to a single 15 min light pulse of either 65 ( $n=7$ ) or 2300 ( $n=7$ ) lux, returned to darkness for 30 min, then swiftly decapitated under a dark-red safe light. Brains were rapidly removed and frozen in 2-methylbutane cooled to –30°C with dry ice. Coronal cryostat sections (15 µm) through the SCN were mounted onto Vectabond-subbed slides, and processed for hybridization with a <sup>35</sup>S-labelled *c-fos* cDNA, as previously described (Schwartz *et al.*, 1996). Slices were exposed to Hyperfilm mp (Amersham, Arlington Heights, IL), and the optical density (OD) of the autoradiographic hybridization signal was measured using a Zeiss (Kontron) Image Processing System. The size of the cursor was 100 µm<sup>2</sup>, and when centred over a unilateral SCN it was slightly smaller than the autoradiographic image. For each SCN, bilateral readings were taken from at least three sections, so that OD data for each mouse represents the average of at least six readings. This was expressed as a relative OD (ratio of the OD of the SCN to that of the surrounding hypothalamus).

The *c-fos* cDNA probe was derived from a 2.2 kb rat cDNA insert subcloned into the *Eco*R1 site of pSP65 vector (Curran *et al.*, 1987; recombinant plasmid generously supplied by Dr Tom Curran, Roche Institute of Molecular Biology, Nutley, NJ). It was prepared by cutting the cDNA with DdeI, retrieving the fragments by phenol–chloroform extraction and ethanol precipitation, and labelling with [<sup>35</sup>S]dCTP by the random primed method (Boehringer Mannheim, Indianapolis, IN). Labelled probe was isolated by passage through a Sephadex G-25 spin column (Boehringer).

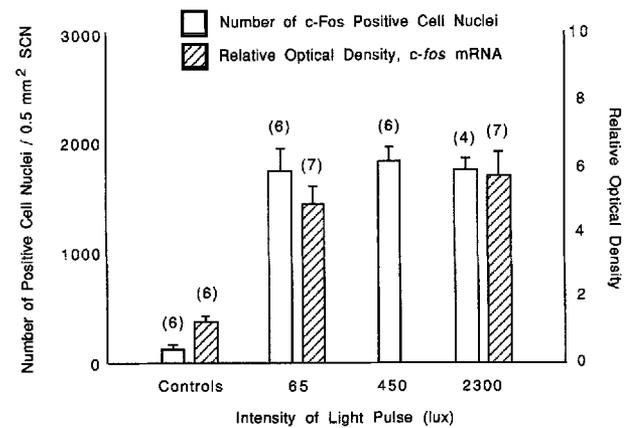


Fig. 2. Expression of immunoreactive c-Fos protein (open columns) and *in situ* hybridized *c-fos* mRNA (striped columns) in the Sabra mouse SCN after 15 min exposure at ZT 15–16 to a single light pulses of different intensity. The open columns indicate the numbers of c-Fos-immunoreactive cell nuclei in controls ( $n=6$ ) and after exposure to 65 ( $n=6$ ), 450 ( $n=6$ ) and 2300 ( $n=4$ ) lux. The mean cell counts for the four groups were significantly different ( $P = 0.006$ , Kruskal–Wallis one way analysis of variance on ranks), and pairwise comparisons using Dunn's method showed that each of the light-stimulated groups was significantly different from the control ( $P < 0.05$ ) but they were not significantly different from each other. The striped columns indicate optical density measurements from autoradiographs expressing *c-fos* mRNA levels in controls ( $n=6$ ) and after exposure to 65 ( $n=7$ ) and 2300 ( $n=7$ ) lux. The mean optical densities for the three groups were significantly different ( $P = 0.002$ , Kruskal–Wallis one-way analysis of variance on ranks), and pairwise comparisons using Dunn's method showed that both of the light-stimulated groups were significantly different from the control ( $P < 0.05$ ) but not from each other. Each column represents mean  $\pm$  SEM (see Materials and methods for details of counting and optical density procedures).

## Results

### Light microscopy

The microanatomical borders of the SCN in the Sabra mouse were demarcated from cresyl violet-stained vibratome sections (Fig. 1A), from which templates were drawn, as described under Materials and methods. Immunocytochemical labelling of corresponding sections showed that somata containing VIP were mostly located ventrally, including some embedded in the optic chiasma (Fig. 1B), while those containing VP-NP were clustered mainly dorsomedially and in a discrete aggregation near the ventrolateral border of the SCN (Fig. 1C). Processes, immunoreactive for these neuropeptides, ramified throughout the SCN. Astroglial labelling, defined by GFAP immunoreactivity, was much more prominent in the SCN than in the surrounding hypothalamic tissue (Fig. 1D), as has been described also in the SCN of other rodents (Morin *et al.*, 1989; Lavalie and Servière, 1993).

Immunoreactive c-Fos protein levels in the Sabra mouse SCN were robustly elevated after animals were exposed to a light pulse administered during the dark phase of the light–dark cycle (3–4 h after lights-off), whereas the levels were barely detectable at this time in control mice maintained in darkness (Fig. 1E–H). Labelled cell nuclei, distributed extensively throughout the full extent of the mouse SCN, overlapped subdivisional boundaries demarcated by VIP- and VP-NP-immunoreactive somata.

Counts derived from bilateral SCNs of mice that had been photically exposed at ZT 15–16 indicated on average ~1800 c-Fos-positive cell nuclei per 0.5 mm<sup>2</sup>, a total that did not increase further with increasing light intensity (Fig. 2). Pilot experiments in which mice were killed 90 min (instead of 45 min) after pulse onset yielded similar results

(not shown), further attesting to 'saturation' in numbers of c-Fos positive cells. *In situ* hybridization for c-fos mRNA was consistent with the immunocytochemical data. Exposure of animals to no light or to single 15-min light pulses or 65 or 2300 lux revealed that c-fos mRNA levels were not significantly different at the two light intensities (Fig. 2), which is compatible with similar data recently reported for C3H/HeN mice (Benloucif *et al.*, 1997).

Pilot experiments were also conducted during the latter part of the night, and showed that mice photically exposed to light at ZT 21–22 (not shown) yielded essentially similar nuclear counts to those exposed at ZT 15–16. This matches the observations of Colwell and Foster (1992) for *Mus domesticus* and of Masana *et al.* (1996) for C3H/HeN mice, but differs from recent results in the rat, where light exposure during early night (ZT 14) yielded lower counts than exposure later in the night (ZT 19) (Romijn *et al.*, 1996; Speh and Moore, 1996) (see Discussion).

#### Immunoelectron microscopy

At the ultrastructural level c-Fos-containing nuclei were identified by the presence of distinctive TMB/tungstate spicule-like crystals, mainly within the paler euchromatic region of the karyoplasm (Figs 3 and 4). Nucleoli were invariably unlabelled. The number of TMB/tungstate spicules per c-Fos-positive nucleus varied, while dual immunocytochemical procedures sometimes tended to reduce their electron density. Cytoplasmic labelling with TMB/tungstate was rare, but occurred occasionally near the Golgi region (Fig. 3D), which may indicate the ephemeral presence of c-Fos within the cytoplasm, prior to its accumulation within the nucleus. Non-specific background labelling was negligible.

In order to estimate at the ultrastructural level the proportion of SCN cell nuclei that were c-Fos-immunoreactive after photic stimulation, a total of 4838 nuclei were counted on 20 grids from various levels of the SCN derived from six mice, of which  $21 \pm 3\%$  (mean  $\pm$  SEM) were c-Fos-positive, ranging between 3 and 36% per individual grid. These counts were made from SCNs of mice exposed to a light pulse of 2300 lux, after pilot experiments indicated similar data for 65 450 and 2300 lux. In the absence of photic stimulation, only sporadic c-Fos labelling was found in the SCN of control mice.

A noteworthy feature of the cell population containing c-Fos-positive nuclei was its morphological and phenotypic heterogeneity. Most c-Fos-labelled cells appeared to be neurons (Figs 3A–D and 4A, B, D), while some were astroglial (Fig. 4C). A considerable proportion of c-Fos-positive neurons were peptidergic, judging by extensive rough endoplasmic reticulum and Golgi configurations and dense-core vesicles. Dual immunocytochemical labelling confirmed that ~24% of all c-Fos-positive nuclei were located within VIP-containing neurons and ~13% within VP–NP neurons. However, most VIP- and VP–NP-immunoreactive neurons were c-Fos negative. Occasional ciliated cells within the SCN also contained c-Fos-labelled nuclei (Fig. 3D).

However, the majority of all c-Fos-positive nuclei were located in presumptive interneurons, so designated because of their ubiquity, small size and organelle-poor cytoplasm lacking secretory vesicles. Interneurons are a prominent component of the dense local circuitry that exemplifies the SCN (Van den Pol, 1980, 1991). We found that these small cells with pale cytoplasm were not a uniform population. Some were closely associated with capillary endothelium (Fig. 3B) while most were not; the nuclei of some were smooth while others were highly pleomorphic (Fig. 3A–C). Furthermore, a considerable number of putative interneurons were c-Fos-negative. Preliminary evidence from dual immunocytochemical labelling indicated that nuclear c-Fos and cytoplasmic NOS were co-localized in some

interneurons (Fig. 5). However, the relative percentage of interneurons in which c-Fos and NOS were co-localized has not yet been determined. It is noteworthy that of the three NOS antisera that we used (NOS-I, iNOS, eNOS), only neuronal NOS-I gave a positive reaction.

Astroglial cells were identified by GFAP immunoreactivity (Figs 1D and 4C), at the ultrastructural level, seen as a thin rim of immunoperoxidase-labelled perinuclear cytoplasm, and also within extensive branching profiles insinuated between other elements of the neuropil. About 7% of all c-Fos-positive nuclei in the SCN were located within GFAP-immunoreactive glial cells. However, in the majority of identified astroglial cells c-Fos was not detected.

Dual immunocytochemical labelling also served to identify some of the synaptic inputs onto c-Fos-positive neurons. A common occurrence was VIP-immunoreactive axonal boutons impinging synaptically on c-Fos-labelled somata (Fig. 4D). It is not known whether the neurons from which these boutons originated expressed c-Fos themselves. Adjacent c-Fos-positive neuronal somata were often postsynaptic to common presynaptic boutons (Fig. 4D), suggesting functional synchrony between photically activated cells.

#### Discussion

Despite a literature of over 50 publications on the physiology, pharmacology and ontogeny of c-Fos induction in the rodent SCN, relatively little has been written about the identity of the cells expressing c-Fos. We have found that the SCN cells, immunoreactive for c-Fos after photic stimulation, are morphologically and phenotypically a mixed population. Furthermore, c-Fos expression was induced only in a relatively small proportion of total SCN cells, an upper limit (~1800 cells per 0.5 mm<sup>2</sup>, ~20%) that was not exceeded even after photic stimuli of supramaximal intensity.

#### Characterization of c-Fos-expressing cells

Our results confirm that, in the mouse, photically induced c-Fos labelling occurs within cell nuclei dispersed throughout the entire dorsoventral extent of the SCN (Colwell and Foster, 1992). This differs from the situation in the rat, in which the labelling is restricted to the ventrolateral SCN (Rea, 1989; Aronin *et al.*, 1990; Earnest *et al.*, 1990). These differences in c-Fos distribution match differences in the retinal input to the SCN, which in the mouse is more homogeneously distributed throughout the nucleus (Castel *et al.*, 1993), whereas in the rat the retinorecipient region is concentrated in the ventral subdivision of the SCN (Johnson *et al.*, 1988).

Previous attempts to establish the identity of c-Fos-expressing cells in the SCN at the light microscope level focused mainly on known phenotypes within the retinorecipient region of the rat SCN. In the rat, neuronal cell bodies immunoreactive for VIP or for gastrin-releasing peptide (GRP) are concentrated within the ventral SCN (Mikkelsen *et al.*, 1991), and in some instances the two neuropeptides coexist in the same neurons (Okamura *et al.*, 1986). Earnest *et al.* (1993) reported that 8% of c-Fos positive cell nuclei could be co-localized to GRP-containing neurons, but, contrary to our results, these authors found no co-localization of c-Fos nuclei in VIP perikarya. However, there have been other reports of 'many' (Daikoku *et al.*, 1992) or 'some' (Mikkelsen *et al.*, 1994) VIP neurons that were immunoreactive for c-Fos after photic stimulation. Phase-dependent distribution and co-localization of light-induced c-Fos expression within VIP and GRP neurons in the rat SCN has been reported recently (Romijn *et al.*, 1996; Speh and Moore, 1996). Complicating the issue is the fact that, while the light pulses used to

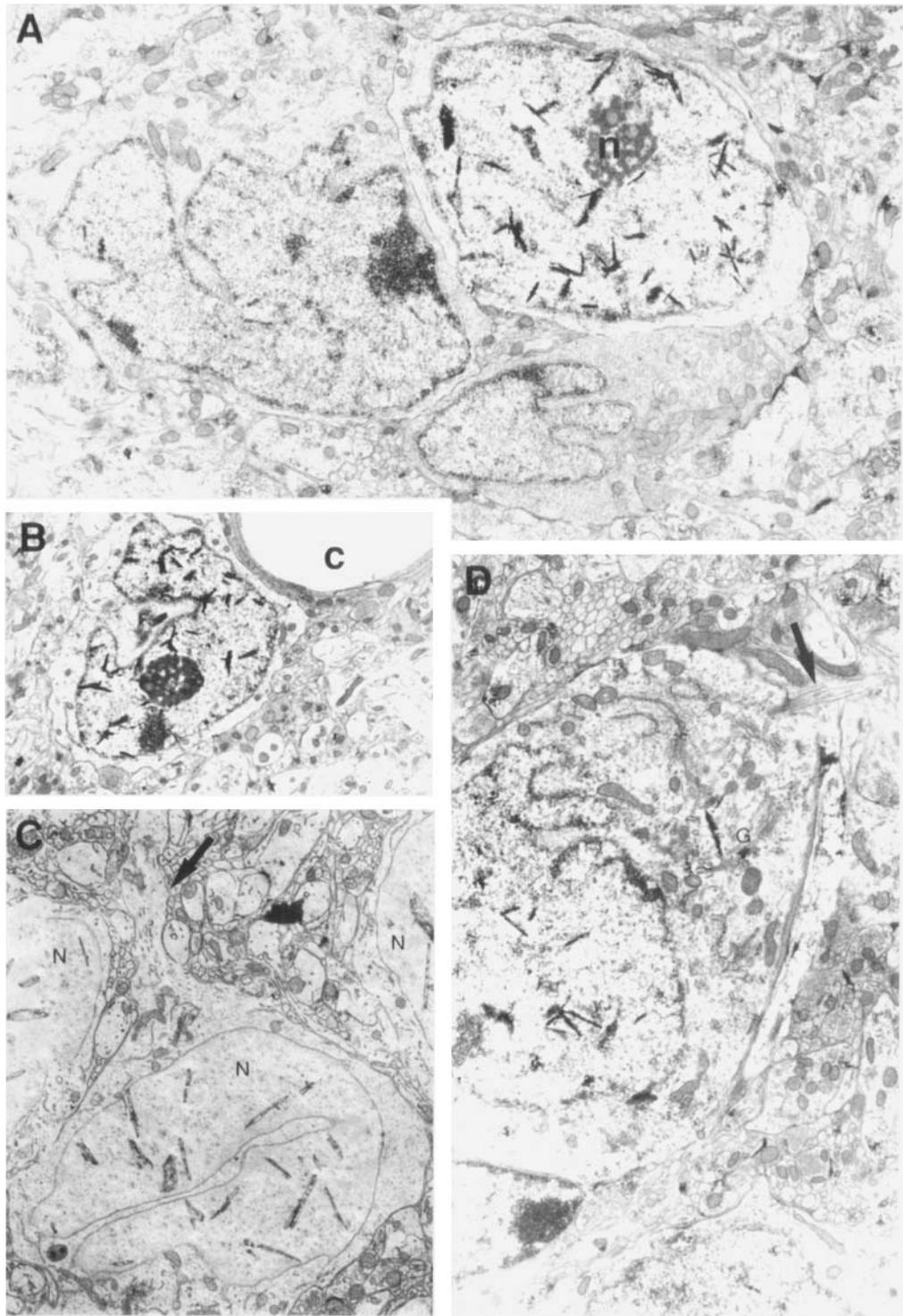


FIG. 3. Electron micrographs showing c-Fos immunoreactivity in the nuclei of various cell types in the Sabra mouse SCN. Mice were exposed to a 15 min light pulse at ZT 16 of the light-dark cycle. Immunoreactivity is indicated by electron-dense spicule-like crystals, the reaction product of the chromogen TMB/tungstate. (A) Profiles of three SCN somata are depicted, one of which displays reaction product denoting c-Fos protein within the karyoplasm of its nucleus. Spicules are associated mainly with pale euchromatin; the nucleolus (n) is unlabelled. The pale perinuclear cytoplasm, containing few organelles, suggests an interneuron. (B) Profile of a soma (interneuron?) adjacent to capillary (c) endothelium (upper right). The pleomorphic nucleus is c-Fos-immunoreactive. (C) A dendrite-like process (arrow) extends from the soma of a small putative interneuron, whose pleomorphic nucleus (N) is c-Fos-immunoreactive. Nuclei (N) of two neighbouring somata are also immunoreactive. (D) Profile of neuronal soma bearing a cilium (arrow). Spicule-like reaction product is seen in the karyoplasm; a spicule is also located in the cytoplasm close to Golgi configurations (G). Magnification,  $\times 15\,000$  in A,  $\times 7000$  in B,  $\times 12\,000$  in C,  $\times 18\,000$  in D.

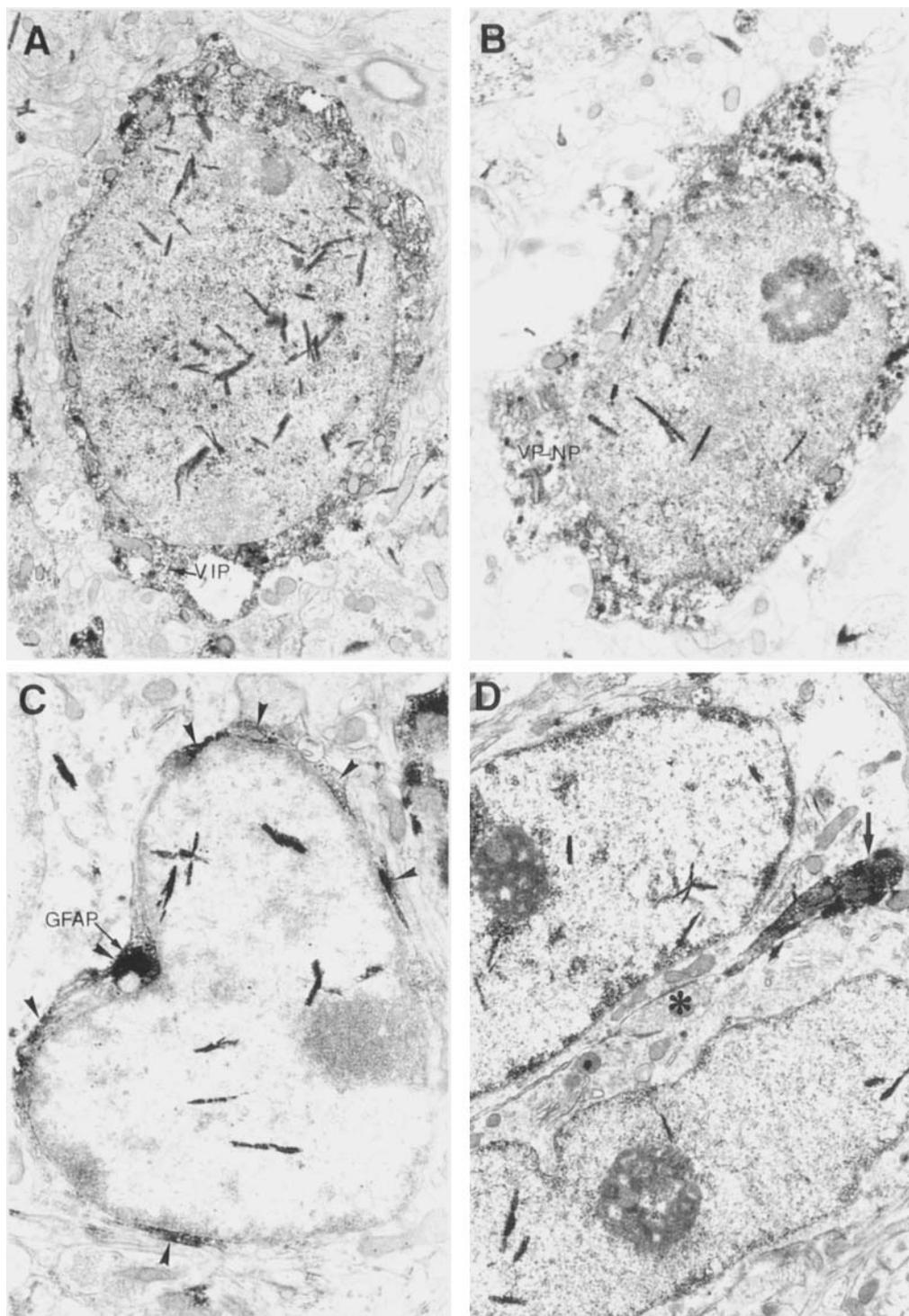


FIG. 4. Electron micrographs showing dual immunocytochemical labelling in SCNs derived from light-pulsed specimens. Immunoreactivity for c-Fos is denoted by TMB/tungstate crystals, and cytoplasmic ligands are indicated by diffuse diaminobenzidine reaction product. (A) Neuronal soma showing co-localization of nuclear c-Fos and cytoplasmic VIP. Note electron-dense secretory granules in the diffusely labelled cytoplasm; the mitochondrial matrix is unlabelled. (B) Neuronal soma showing co-localization of nuclear c-Fos and cytoplasmic VP-NP. (C) Profile of an astrocyte soma. The very thin rim of perinuclear cytoplasm contains patches of diffuse reaction product (arrowheads) denoting immunoreactivity for GFAP. The nucleus is c-Fos-immunoreactive. (D) Labelling with anti-c-Fos and anti-VIP; profiles of two c-Fos-immunoreactive neurons (of unknown phenotype), the upper one postsynaptic to a large VIP-containing bouton (arrow). Both c-Fos-positive neurons are also postsynaptic to a common unlabelled synaptic bouton (asterisk). Magnification,  $\times 15\,000$  in A and B,  $\times 19\,000$  in C,  $\times 18\,000$  in D.

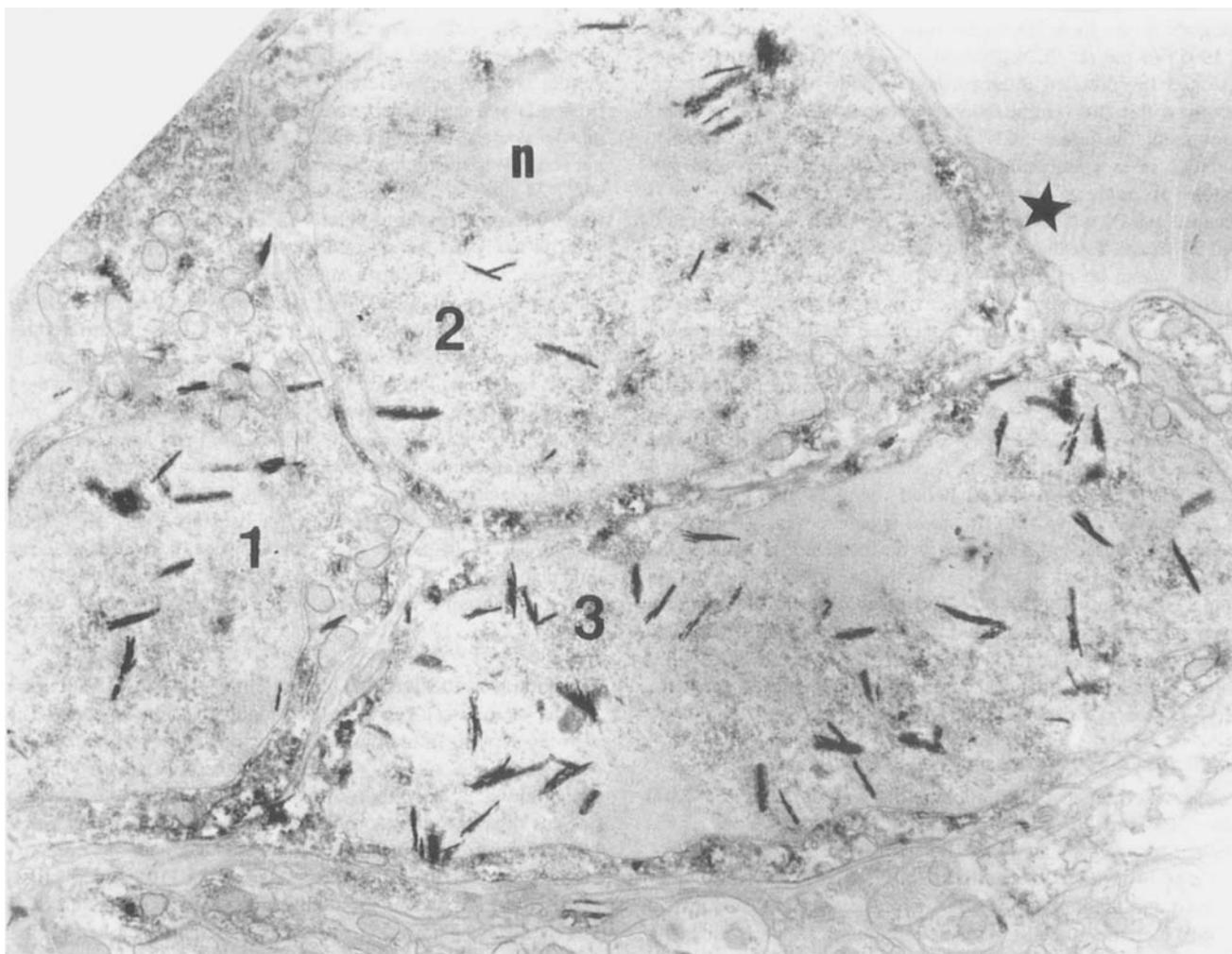


FIG. 5. Electron micrograph showing juxtaped profiles of several putative interneurons in the SCN of a light-pulsed Sabra mouse. Three of the presumptive interneurons (1, 2, 3) are dual-labelled for nuclear c-Fos and cytoplasmic NOS; TMB/tungstate crystals within the cell nuclei denote c-Fos immunoreactivity, and diffuse diaminobenzidine reaction product within the cytoplasm indicates immunoreactive NOS. Note unlabelled nucleolus (n) in cell 2. At the upper right is a portion of an unlabelled cell (star). Magnification,  $\times 20\,000$ .

activate c-Fos would be expected to increase GRP immunoreactivity, they would probably diminish immunoreactivity for VIP (Inouye and Shibata, 1994). In an effort to establish whether glia were among the c-Fos-expressing cells in the rat SCN, Bennett and Schwartz (1994) used an antibody to the calcium-binding protein S-100 and estimated that 5–10% of the photoinduced c-Fos-labelled nuclei were in identified astroglial cells. In the hamster, photic stimulation induces c-Fos-immunoreactivity within a specific subpopulation of calbindin-D28-containing cells in the core of the SCN (Silver *et al.*, 1996).

In the present study on the Sabra mouse, dual labelling revealed the phenotypes of almost half the c-Fos-expressing nuclei: 24% were in VIP-immunoreactive neurons, 13% in VP-NP neurons, and 7% were attributed to GFAP-immunoreactive astroglia. Co-localization of NOS within some c-Fos-positive interneurons was also revealed. In the dorsomedial SCN, co-localization of c-Fos within VP-NP somata may be peculiar to the mouse, where optic terminals gain access to cells throughout the SCN and c-Fos immunoreactivity is indeed widely distributed. It is noteworthy that our results, compared with those of the afore-mentioned authors, were achieved with a relatively brief stimulation paradigm (45 min after onset of a 15 min light pulse).

Hence, the common feature of c-Fos-expressing cells is not their peptide content or even their neuronal or glial character; perhaps a receptor subtype or second messenger mechanism is a unifying characteristic. Other data have also suggested that the c-Fos cells in the SCN constitute a heterogeneous population. For example, the inhibition of c-Fos photoinduction by pharmacological blockade is regionally specific within the hamster SCN (Abe *et al.*, 1992; Vindlacheruvu *et al.*, 1992; Colwell *et al.*, 1993; Rea *et al.*, 1993; Zhang *et al.*, 1993).

The precise function of c-Fos may differ in different cell types, and it will be important to learn whether the *c-fos* gene is differentially regulated in the variety of SCN cells that have been identified. Moreover, we do not know whether c-Fos in peptidergic neurons and in glia is expressed simultaneously or sequentially after onset of the light stimulus. Adding to this uncertainty is the current lack of knowledge concerning the precise identity of the neurons directly impinged upon by the retinal input.

In the present study, approximately half the total population of photoinduced c-Fos-expressing cells in the SCN were of unknown phenotype. Among these the most numerous were small putative interneurons with sparse, cytoplasmic membrane systems, which may

be analogous to the local circuit neurons described by Van den Pol (1980, 1991) in the rat SCN. Recently NOS immunoreactivity was demonstrated in so-called interneurons by both light and electron microscopy within the ventral retinorecipient region of the rat SCN, and throughout the entire SCN of the mouse (Wang and Morris, 1996). NOS is a calmodulin-dependent enzyme responsible for conversion of arginine into citrulline and nitric oxide (NO) (Garthwaite, 1991), which is believed to be a messenger molecule involved in signal transduction within the SCN (Amir, 1992; Ding *et al.*, 1994; Watanabe *et al.*, 1994, 1995). It is postulated that, within the circadian timing system, NOS activity is initiated by the influx of  $\text{Ca}^{2+}$  through NMDA receptors activated by the glutamatergic retinal input (Pape, 1995). In the hamster, retinohypothalamic projections have been shown to impinge on NOS-immunoreactive neurons in the ventral SCN (Decker and Reuss, 1994), although the precise relationship between NOS activity and c-Fos expression is not yet clear (Weber *et al.*, 1995). In the rat, Reuss *et al.* (1995) have reported that some SCN cells are double-labelled for NOS and VIP. In the mouse we present the first ultrastructural evidence clearly indicating that photically induced c-Fos protein is co-localized in some NOS-containing interneurons. It is therefore possible that these NOS-containing interneurons bear NMDA receptors, are activated by glutamate released from retinal boutons, and represent 'first-order' c-Fos-expressing cells. The rapid transmembrane effect of NO, within a 'spherical domain of about 100  $\mu\text{m}$ ' (Pape, 1995) may account for a variety of 'second order' c-Fos-expressing cells.

#### Quantitation of c-Fos-expressing cells

Within the wide range of light stimuli that we employed (65–2300 lux), the number of c-Fos-immunoreactive cells in the Sabra mouse SCN remained constant at  $\sim 1800$  per  $0.5 \text{ mm}^2$  per bilateral SCN. Previous reports have shown that the level of c-fos mRNA (Kornhauser *et al.*, 1990) and the number of c-Fos-expressing cells (Trávníčková *et al.*, 1996) increase in the SCN when light pulses of altered intensity or timing increase the amplitude of behavioural or hormonal phase shifts; in these studies the amount of c-Fos correlates with the magnitude of the phase shift. Here we exposed mice to light pulses of increasing intensity, but the magnitude of our dimmest pulse was similar to pulses that saturate the phase-shifting response of the murine circadian locomotor rhythm (Foster *et al.*, 1991; of the order of 1–10  $\mu\text{W}/\text{cm}^2$ ). In Sabra mice also, we found that the amplitude of their locomotor phase shifts was not augmented with light pulses greater than 65 lux (M. C. and W. J. S., preliminary observations); similar findings have now been reported in C3H/HeN mice (Benloucif *et al.*, 1997). The salient point is that our experimental setup has enabled us to define an upper limit to the number of c-Fos-expressing cells. There is an apparent ceiling to the size of the photo-inducible c-Fos population in the SCN, indicating that it is not indefinitely recruitable or expandable.

The maximum count of  $\sim 1800$  per  $0.5 \text{ mm}^2$  c-Fos-positive cell nuclei was based on light microscope counts from *alternate* serial vibratome sections, comprising approximately half of the rostrocaudal SCN. Therefore, it is not unreasonable to extrapolate that the actual number within the entire rostrocaudal SCN would be about double that count, close to  $2 \times 1800 = 3600$  c-Fos cells per SCN. The total number of cells in the mouse SCN is not known, but extrapolating from the rat SCN, where the total number of neurons and glia has been estimated at  $\sim 20\,000$  in the paired nuclei (Van den Pol, 1980, 1991), then 3600 c-Fos-immunoreactive cell nuclei in the mouse SCN would represent roughly 18% of the total. This tallies reasonably well with our on-grid counts at the ultrastructural level, where an average of 21% of cell nuclei were c-Fos-immunoreactive.

As for a possible influence of early versus late night on the number of photically induced c-Fos-positive nuclei in the Sabra mouse SCN, we did not find any difference between routine counts at ZT 15–16 ( $n = 22$ ) and preliminary counts at ZT 21–22 ( $n = 12$ ). Likewise, in *Mus domesticus*, Collwell and Foster (1992) reported 'that it was not possible to distinguish between the effect of a light pulse delivered at CT 24 and at CT 16 on Fos-like-immunoreactivity' (CT is circadian time). Moreover, in light-pulsed C3H/HeN mice c-Fos mRNA levels were virtually the same at CT 14 and CT 22 (Masana *et al.*, 1996). However, in the rat (Speh and Moore, 1996; Romijn *et al.*, 1996), light stimuli at ZT 19 induced significantly higher counts of c-Fos-immunoreactive cells than light stimuli at ZT 14. A differential was also reported in c-Fos/neuropeptide co-localization, so that c-Fos at ZT 19 (accompanied by phase advance of behavioural rhythmicity) appeared to be associated with both GRP and VIP neurons, while c-Fos at ZT 14 (accompanied by phase delay) was associated only with VIP neurons. Apart from the methodological differences between the studies discussed above, the results probably do indicate a real difference between mice and rats in light-induced c-Fos expression in the SCN. Of note, since mice typically exhibit free-running circadian periods of  $< 24 \text{ h}$  (Schwartz and Zimmerman, 1990), unlike rats, they entrain to light-dark cycles primarily by phase delays.

#### Technical considerations

Our immuno-ultrastructural observations add to a small group of studies examining c-Fos at the electron microscope level in the central nervous system: in the mouse hippocampus after Metrazole-induced seizures (Mugnaini *et al.*, 1989), in the rat neostriatum after intrastriatal quinolinic acid (Aronin *et al.*, 1991) and in the rat supraoptic nucleus after chronic intermittent salt loading (Lafarga *et al.*, 1993). Immunoelectron microscopy also showed c-Fos immunoreactivity in cortical cells of the zona fasciculata of ACTH-injected rats (Yang *et al.*, 1990). These studies, all of which visualized immunoreactive c-Fos by diffuse diaminobenzidine labelling, demonstrated localization within those subcellular nuclear domains that correspond to actively transcribing dispersed euchromatin. Within the SCN, using diaminobenzidine as the chromogen, c-Fos immunolabelling was not satisfactory (M. C. and S. C., unpublished observations), possibly because the amount of c-Fos generated by a physiological stimulus such as ambient light may be less than that induced by the pharmacological stimuli used in the aforementioned studies. However, use of the more sensitive TMB procedure, combined with tungstate augmentation, produced a readily visible and unambiguous crystalline electron-dense reaction product that could be discerned even at low magnifications. Furthermore, for dual immuno-ultrastructural labelling, TMB/tungstate marking of c-Fos and diaminobenzidine labelling of cytoplasmic antigens has the distinct advantage of two chromogens with distinguishing morphological features.

One of the drawbacks of TMB labelling is that the antigenic site that each crystalline spicule denotes is spatially more limited than the spicule itself, although this did not adversely affect the interpretation of results in the present study. Another drawback, relating to dual labelling, is that the TMB/tungstate procedure itself is somewhat damaging for tissue constituents, and since it was used to label the first antigen (c-Fos) it had a deleterious effect on the antigenicity of the cytoplasmic ligands that were subsequently labelled with diaminobenzidine. This would imply underestimation of co-localized ligands in our dual-labelling results, which was particularly apparent when NOS was the second antigen. Future studies will seek to alleviate this problem by the use of antibodies with higher affinity for cytoplasmic antigens, and modification of the TMB/tungstate procedure, which is protracted and carried out at acidic pH.

## Conclusion

c-Fos was photo-inducible in only about one-fifth of the total number of cells that constitute the SCN, and within this limited population various cell types were differentially represented, about half of which were characterized phenotypically. The temporal order of c-Fos induction in the SCN remains an open question: are all cell categories simultaneously stimulated by the photic input, or is a signal transduction cascade initiated via one type of cell and conveyed to others?

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## Abbreviations

GFAP	glial fibrillary acidic protein
GRP	gastrin-releasing peptide
NOS	nitric oxide synthase
SCN	suprachiasmatic nucleus
TMB	tetramethyl benzidine
VIP	vasoactive intestinal peptide
VP-NP	vasopressin-neurophysin
ZT	zeitgeber time

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