Differences in Colocalization Between Fos and PHI, GRP, VIP and VP in Neurons of the Rat Suprachiasmatic Nucleus After a Light Stimulus During the Phase Delay Versus the Phase Advance Period of the Night

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ABSTRACT

Two groups of four rats each received a 15-minute light stimulus during the first part of the night (ZT14) and the second part (ZT19), respectively. After 45–60 minutes, the animals were killed by perfusion fixation. Adjacent Vibratome sections through the suprachiasmatic nucleus (SCN) were double-immunostained for the presence of peptide histidine isoleucine (PHI), gastrin releasing peptide (GRP) or vasoactive intestinal peptide (VIP) with Fos by using fluorophore-conjugated secondary antibodies. A few sections were triple-immunostained for PHI, GRP or VIP with vasopressin (VP) and Fos. Sections were analyzed with a confocal laser scanning microscope. It turned out that the ZT19 light stimulus induced 4.2 times more nuclear profiles in the SCN immunoreactive for Fos than the light stimulus given at ZT14. The SCN of control animals did not show any Fos immunoreactivity. After the ZT14 light stimulus, approximately 33% of the Fos profiles showed colocalization with a perikaryal profile immunoreactive for PHI, GRP or VIP, whereas at ZT19, this percentage had doubled to approximately 65%. After the light stimulus at ZT14, the relatively low Fos induction was numerically and proportionally most prominent in the PHI-immunoreactive perikarya. As compared with ZT14, the increase of Fos after the ZT19 light stimulus was most pronounced in the GRP-immunoreactive perikarya (21 $\times$) followed by VIP (15 $\times$) and PHI (5 $\times$). This outcome suggests that at least three different cell groups characterized by, respectively, PHI alone, GRP, and VIP fully or partly colocalized with PHI, play a prominent role during light-induced phase shifts: the PHI neurons during light-induced phase delays, the GRP and VIP/(PHI) neurons during light-induced phase advances. © 1996 Wiley-Liss, Inc.

Indexing terms: phase shift, fos, neuropeptides

On both sides of the third ventricle, just above the optic chiasm, the mammalian brain contains a relatively small nucleus termed the suprachiasmatic nucleus (SCN). Both nuclei are mutually connected by contralaterally projecting nerve fibers (Buijs et al., 1994) and together they form the "biological clock." The latter means that when animals, e.g., the rat, are exposed to constant darkness and are additionally isolated from any other time-giving stimulus, several physiological and behavioral activities, e.g., locomotor activity, keep showing a day-night cycling, although the period length is then often somewhat shorter or longer than 24 hours. These behaviors lose their circadian rhythmicity if the SCN or its output to the hypothalamic nuclei

controlling these behaviors is lesioned. Normally, the endogenous circadian rhythmicity of the SCN (a high neuronal firing frequency during the day and a low one during the night) is daily synchronized to the environmental photoperiod by retinal information entering the SCN at its ventral side via the retinohypothalamic tract (RHT).

A special phenomenon is that upon a brief exposure to light during the animal's first part of the night (or the subjective night if the animal is housed in constant dark-

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ness), the SCN-driven behaviors show a phase delay. Light exposure during the second part of the (subjective) night, by contrast, results in phase advance, whereas light exposure during the middle of the night as well as during the (subjective) day has no effect (Summer et al., 1984). This shows that, depending on the internal state, the biological clock reacts differentially to light. A corresponding feature of these light-induced phase shifts is an enhanced activity of the immediate early gene *c-fos* and its transcription protein Fos in neurons located in the ventral part of the SCN (Rea, 1989; Aronin et al., 1990; Earnest et al., 1990, 1992; Kornhauser et al., 1990; Rusak et al., 1990 in rat and hamster; Colwell and Foster, 1992 in mouse; Rea, 1992; Chambille et al., 1993 in hamster; Sutin and Kilduff, 1992 in rat).

The SCN contains different neurotransmitters. The ventral part, where most of the RHT fibers have their endings, is characterized by, among others, neurons containing vasoactive intestinal peptide (VIP), gastrin-releasing peptide (GRP) or peptide histidine isoleucine (PHI). Some of these neurons show colocalization for two or three of these peptides (Okamura et al., 1986; Albers et al., 1991, 1992; Reghunandanan et al., 1993). The mediodorsal part of the SCN is mainly characterized by neurons containing vasopressin (VP) and somatostatin (SOM). Gamma aminobutyric acid (GABA) has been reported to be a cotransmitter of most of these neurons (Moore and Speh, 1993). Normally, these five peptides and GABA show a diurnal rhythm, but, during constant environmental conditions, only VP and SOM keep showing circadian rhythmicity (Takahashi et al., 1989; Inouye and Shibata, 1994; Shinohara et al., 1993; Okamura and Ibata, 1994). Experimental evidence suggests that such an endogenous circadian rhythmicity also holds for the inducibility in the expression of Fos by light (Earnest et al., 1992; Sutin and Kilduff, 1992).

Since little is known about the cellular organization underlying phase shifts in the biological clock, we aimed to investigate whether the induction of a phase delay or a phase advance can be immunocytochemically distinguished from one another on the basis of the amount of Fos production and the pattern of colocalization with one of the above-mentioned peptides in the SCN.

MATERIALS AND METHODS

Light exposure, fixation and sectioning

From birth onwards, twelve male rats (random-bred Wistar Hsd/Cpd: Wu strain), weighing approximately 300 g each, were kept on a 12:12-hour light-dark cycle (light on at 7 a.m.). Without being taken out of the cage, one group of four animals (numbered 15–18) received a 15-minute light stimulus during the first part of the night (at 9 p.m. ~ ZT14); another group of four animals (numbered 19–22) received such a light stimulus during the second part of the night (at 2 a.m. ~ ZT19). The light stimulus consisted of the normal fluorescent “day light” the animals were used to, with an intensity of approximately 150–200 lux at the center of the animal cages. Previous studies (unpublished) had shown that such light stimuli induce behavioral phase shifts. Forty-five minutes after the end of the light stimulus, the animals were killed by perfusion fixation under deep i.p. Nembutal anesthesia. The fixation consisted of 4% freshly depolymerized paraformaldehyde in 0.1M phosphate buffer (pH 7.4). The time span between perfusion of the first and the fourth animal was 15 minutes. Two control

Fig. 1. Scheme of coronal Vibratome sectioning of the rat suprachiasmatic nucleus in a random systematic way. Routinely, the first and second sections of each series were double-immunostained for Fos and peptide histidine isoleucine (PHI), all third and fifth sections for Fos and gastrin-releasing peptide (GRP), and all fourth and sixth sections for Fos and vasoactive intestinal peptide (VIP). A few sections, however, were triple-immunostained, i.e., for Fos, vasopressin (VP) and PHI, GRP or VIP.

groups of two animals each, which had not received a light stimulus, were fixed at the same time as the ZT14 and the ZT19 experimental animals. After perfusion, the brains were dissected and postfixed in the same fixation for 30 hours.

Antibodies

Polyclonal antisera against the neuropeptides PHI, GRP, and VIP were raised in rabbits and subsequently tested for their specificity in our institute. Dot spot experiments, for example, did not show any cross-reactivity between anti-PHI and the peptides GRP and VIP, anti-GRP and the peptides PHI and VIP, and anti-VIP and the peptides PHI and GRP (see for details and the procedure followed: Pool and Buijs, 1988; Van der Sluis et al., 1988; Buijs et al., 1989). The monoclonal mouse VP antibody PS41 was a generous gift from Drs. H. Gainer and S.H. Key (Bethesda, MD). The sheep polyclonal antiserum against the *c-fos* protein was purchased from Cambridge Research Biochemicals (batch no. 09046; for characterization and specificity, see the product specification of CRB). Multiple labeled fluorophore-conjugated secondary antibodies, i.e., donkey anti-sheep-conjugated-Cy5, donkey anti-rabbit-conjugated-Cy3, donkey anti-mouse-conjugated-Cy3 and donkey anti-rabbit-conjugated fluorescein isothiocyanate (FITC), were purchased from Jackson ImmunoResearch Laboratories.

Sectioning and labeling for immunofluorescence

Per animal, 4–6 series of six consecutive 35-µm-thick coronal Vibratome sections were cut through the region of the SCN (Fig. 1). All first sections of each series were put together in one vial which was allocated number 1; all second sections in another vial allocated number 2 etc., so that eventually six vials were obtained, each containing 4–6 sections at a mutual distance of 210 µm. Since it was impossible to see during sectioning which section contained the most frontal aspect of the SCN, the sections in each vial

represented a random systematic sample of the SCN. The sections were stored in Tris-buffered saline (TBS), pH 7.4–7.6. Of each animal, the sections numbered 1 and 2 (two vials) were processed for double-label immunofluorescence of Fos and PHI, those numbered 3 and 5 for Fos and GRP, and those numbered 4 and 6 for Fos and VIP. However, of the animals numbered 15 and 19, the first, third and sixth section of the second series was processed for triple-label immunofluorescence of, respectively, (i) Fos, PHI and VP, (ii) Fos, GRP and VP, and (iii) Fos, VIP and VP.

For double-label immunofluorescence, free-floating Vibratome sections were routinely incubated in a mixture of sheep anti-Fos (1:20,000) with one of the rabbit antibodies against PHI, GRP or VIP (1:2,000) overnight at 4°C. For triple-labeling, mouse anti-VP (1:200) was added to the incubation mixture. Subsequently the sections were incubated for 1 hour at room temperature in a mixture of donkey anti-sheep-Cy5 (1:400) and donkey anti-rabbit-Cy3 (1:400) in case of a double-labeling, while in the case of a triple-labeling the mixture consisted of donkey anti-sheep-Cy5 (1:400), donkey anti-rabbit-FITC (1:200) and donkey anti-mouse-Cy3 (1:400). The vehicle for all incubations was a mixture of 0.25% gelatin and 0.5% Triton X-100 in TBS (pH 7.4–7.6); intermediate rinsing steps were done in TBS alone. Sections were mounted on gelatin-coated slides and coverslipped with the antifading agent Vectashield® (Vector Laboratories).

Confocal laser scanning microscopy

We used a Zeiss 410 confocal laser scanning microscope (CLSM) equipped with three different lasers emitting at 488, 543 and 633 nm to excite, respectively, FITC, Cy3, and Cy5. With two photomultipliers and appropriate filter settings, the double- and triple-labeled Vibratome sections could be analyzed for each fluorophore separately without any cross-talk. With the aid of a 40× objective (Zeiss, plan-neofluar NA 1.3, oil) and the green light of an HBO-50 lamp for conventional fluorescence of Cy3 (neuropeptides), the right SCN was identified in a Vibratome section. Subsequently, the SCN was x/y-scanned along the Z-axis with the 543 nm laser beam in order to get some information about histology and staining quality and to adjust offset and gain for minimal under- and overflow. Since the scanned area was $320 \times 320 \mu\text{m}^2$, it was possible to capture in one optical section almost the whole ventral part of the SCN, even at its largest diameter, in the x/y direction. This region was characterized by the presence of PHI-, GRP-, VIP-, and a few VP-immunoreactive (IR) neuronal perikarya; only the most mediodorsal part of the SCN, characterized by the presence of some VP-IR neurons, fell outside. With optimal scanning settings, a random optical section was then made. If this neuropeptide immunofluorescence image of the SCN contained five or more immunolabeled neuronal perikarya with at least part of a nuclear profile, it was temporarily stored in the video-RAM memory. The same optical section was then scanned with the 633 nm laser beam and the pertaining Fos image was also stored. Thereafter, both images were superimposed and stored as a composite image on the hard disk. In the same way, the left SCN was scanned and this procedure was repeated for all sections. In case of a triple-labeled section, three images were combined and stored as a composite image on the hard disk.

Quantitative analysis

For quantitation analysis, a composite image of an optical section through the SCN was retrieved from the hard disk and stored in the video-RAM memory, after which the neuropeptide/Cy3 image (in case of double-labeling) or the neuropeptide/FITC image (in case of triple-labeling) was displayed on the monitor. All profiles of labeled neuronal perikarya were then marked and automatically counted by using the mouse to place a white square around them in the overlay (Fig. 2a). Then the pertaining Fos/Cy5 image was displayed together with the former overlay image of white squares, and all Fos-labeled nuclear profiles located with a square (indicating colocalization) were marked and counted by placing a white circle around them (Fig. 2b). Finally, the Fos and the peptide image, together with both overlays, were combined again and displayed in pseudocolors (peptides, green; Fos, red) for a second check (Fig. 2c). In a separate session, the total number of Fos-IR nuclear profiles was counted. In this way, the stored images were analyzed in a random order to minimize bias.

Data processing

Of each double- or triple-immunolabeled section, the number of perikaryal profiles immunoreactive for one of the neuropeptides and counted in the right and left SCN were averaged in order to normalize for a few sections in which only one SCN profile could be observed. Thereafter, the averaged values of sections immunolabeled for the same neuropeptide were added up for each animal. Thus, sections numbered 1 + 2, 3 + 5 and 4 + 6 resulted in an arbitrary, relative measure for the total number of PHI-, GRP- and VIP-IR perikarya per animal, respectively. The same was done for the nuclear profiles immunoreactive for Fos as well as for the number of colocalizations. This resulted, per animal, in three estimates, i.e., (1) the total number of perikaryal profiles, (2) the total number of nuclear profiles, and (3) the total number of colocalizations. Estimates (1) and (3) were used to calculate, per animal, the percentage of PHI-, GRP-, and VIP-IR perikarya showing colocalization with FOS-IR nuclear profiles. Finally, the estimates (1), (2), (3) and the calculated percentages of colocalization were each averaged over the four ZT14 and the four ZT19 animals and presented by three tables and one graph, respectively.

Statistical analysis

Group differences expressed as percentages (Fig. 5; factors: ZT and peptides) were assessed by two-way ANOVA followed by comparison of pairs using the Student's *t* test corrected according to Bonferroni. The data of Tables 1, 2 and 3 were analyzed by the nonparametric Wilcoxon-Mann-Whitney test, one-tailed. Some groups were not mutually compared because of morphometric constraints or other reasons, casting serious doubt on the meaning of such a comparison.

RESULTS

Qualitative analysis

Since the scanned area was $320 \times 320 \mu\text{m}^2$, it was possible to capture in one optical section nearly the whole ventral part of the SCN, even at its largest diameter, in the x/y direction. This region was characterized by the presence

Fig. 2. Confocal laser scanning microscopic images of a rat suprachiasmatic nucleus (SCN) one hour after exposure to a 15-minute light stimulus during the second half of the night (ZT19). A Vibratome section was double-immunostained with a mixture of primary antibodies against PHI and Fos followed by a mixture of Cy3- and Cy5-conjugated secondary antibodies. Laser scanning took place with a 543 and a 633 nm laser beam resulting in two corresponding pictures, one for PHI (Cy3) and one for Fos (Cy5), of the same optical section (± 0.7 nm thick). (a) shows PHI-IR perikaryal profiles (marked by white squares), nerve fibers and endings. (b) shows Fos-IR nuclear profiles part of which are located within a white square indicating colocalization with a PHI-IR perikaryal profile (marked by white circles). c: Composite illustration of the PHI and Fos image displayed in pseudocolors (PHI, green; Fos, red). Observe that a few neurons are fully embedded in the optic chiasm (bottom side). Medial side is right. Scale bars = 25 μ m.

Fig. 3. Confocal laser scanning microscopic image of a rat SCN 1 hour after exposure to a 15-minute light stimulus during the second part of the night (ZT19). A Vibratome section was double-immunostained with a mixture of primary antibodies against GRP and Fos

followed by a mixture of Cy3- and Cy5-conjugated secondary antibodies. Laser scanning took place with a 543 and a 633 nm laser beam, whereafter the corresponding pictures (of the same optical section) were combined and displayed in pseudocolors: GRP-immunoreactive perikaryal profiles, nerve fibers and endings, green; Fos-immunoreactive nuclear profiles, red. Observe that some perikaryal profiles show Fos immunoreactivity. Medial side is left. Scale bar = 50 μ m.

Fig. 4. Confocal laser scanning microscopic image of a rat SCN 1 hour after exposure to a 15-minute light stimulus during the second part of the night (ZT19). A Vibratome section was double-immunostained with a mixture of primary antibodies against VIP and Fos followed by a mixture of Cy3- and Cy5-conjugated secondary antibodies. Laser scanning took place with a 543 and a 633 nm laser beam whereafter the corresponding pictures (of the same optical section) were combined and displayed in pseudocolors: VIP-immunoreactive perikaryal profiles, nerve fibers and endings, green; Fos-immunoreactive nuclear profiles, red. Observe that some perikaryal profiles show Fos immunoreactivity. Medial side is left. Scale bar = 50 μ m.

of PHI-, GRP-, VIP- and a few VP-IR neuronal perikarya; only the most dorsal part of the SCN (characterized by the presence of some VP-IR neurons) fell outside. What was striking was that the light stimulus given at ZT19 induced substantially more FOS-IR nuclear profiles than the light stimulus given at ZT14 (Figs. 2–4). The SCN of the control animals for ZT14 and ZT19 did not show any Fos-IR nuclear profiles. After both light stimuli, Fos-IR nuclear profiles were seen to colocalize with perikaryal profiles immunoreactive for PHI, GRP or VIP (Figs. 2–4). A small part of the FOS-IR profiles was often found scattered outside the boundaries of the SCN, particularly in the dorsolateral direction where perikarya immunoreactive for PHI, GRP or VIP were not observed. These Fos nuclei were thus not included in our quantitative analysis. In those cases where sections had been triple-stained, the VP-IR neurons did not show any immunoreactivity for Fos (in only one case a single neuron showed colocalization with Fos at ZT19).

The triple-stained sections showed that the four classes of neurons immunoreactive for PHI, GRP, VIP and VP,

respectively, formed a dense network with many synaptic-like connections (not shown). In some sections, a few PHI-, GRP- or VIP-IR perikarya were found to be fully embedded in the optic chiasm (Figs. 2–4).

Quantitative analysis

Table 1 shows that a light stimulus given at ZT19 induced about 4.2 times more nuclear profiles in the SCN immunoreactive for the early immediate gene product Fos than a light stimulus given at ZT14. Despite this dramatic difference in Fos induction, the mean number of perikaryal profiles in the SCN immunoreactive for PHI, GRP or VIP were of the same order of magnitude for both ZT times, although the number of GRP profiles showed a small decrease ($P < 0.02$) at ZT19 (Table 2). After the light stimulus at ZT14, a small number of all three classes of neuronal perikarya immunoreactive for PHI, GRP and

Figures 2-4 (Continued.)

TABLE 1. Mean Number of Fos-IR Nuclear Profiles ($\bar{X} \pm$ S.E.M.) Counted in Vibratome Sections of the SCN Double-Immunostained for One of the Neuropeptides PHI, GRP or VIP¹

	In sections stained for:			$\bar{X}$
	FOS + PHI	FOS + GRP	FOS + VIP	
ZT14 contr (2)	0	0	0	0
ZT19 contr (2)	0	0	0	0
ZT14 light (4)	61 $\pm$ 12]*	48 $\pm$ 6]*	46 $\pm$ 5]*	51.7 $\pm$ 4.8
ZT19 light (4)	237 $\pm$ 42]3.9 $\times$	212 $\pm$ 19]4.4 $\times$	204 $\pm$ 31]4.4 $\times$	218.1 $\pm$ 17.2

¹The table illustrates that (i) roughly the same number of Fos profiles were observed regardless for which peptide was stained, and (ii) the numerical increase of Fos profiles after the ZT19 light stimulus relative to the ZT14 light stimulus was 3.9 $\times$, 4.4 $\times$ and 4.4 $\times$, with a mean of 4.2 $\times$. Data were obtained from: two control animals for both ZT14 and ZT19, four animals after a light stimulus at ZT14, and four animals after a light stimulus at ZT19. The column furthest to the right gives the averaged values $\pm$ S.E.M. Note 1: all numbers represent arbitrary, relative indices and should only be used for mutual comparison. Note 2: the actual numbers counted per animal were twice as high as presented in the table since the values for the left and right SCN were averaged per section.

* $P < 0.02$, Wilcoxon-Mann-Whitney test, one-tailed.

TABLE 2. Mean Number of PHI-, GRP- and VIP-IR Perikaryal Profiles ($\bar{X} \pm$ S.E.M.) Counted in Vibratome Sections of the SCN Double-Immunostained With Fos¹

	In sections stained for:		
	FOS + PHI	FOS + GRP	FOS + VIP
ZT14 contr (2)	2	2	2
ZT19 contr (2)	2	2	2
ZT14 light (4)	195 $\pm$ 17	97 $\pm$ 4]*	145 $\pm$ 9
ZT19 light (4)	178 $\pm$ 24	81 $\pm$ 5]*	145 $\pm$ 12

¹Data were obtained from four animals after a light stimulus at ZT14 and four animals after a light stimulus at ZT19.

²Sections of control animals were not included in this analysis. Note 1: all numbers represent arbitrary relative indices and should only be used for mutual comparison. Note 2: the actual numbers counted per animal were twice as high as presented in the table, since the values for the left and right SCN were averaged per section.

* $P < 0.02$, Wilcoxon-Mann-Whitney test, one-tailed (statistical analysis between peptides per ZT was not considered meaningful).

TABLE 3. Mean Number of Colocalizations between Fos-IR Nuclear Profiles and PHI-, GRP- or VIP-IR Profiles ($\bar{X} \pm$ S.E.M.) Counted in Double-Immunostained Vibratome Sections in the SCN of Four Animals After a Light Stimulus at ZT14 and Four Animals After a Light Stimulus at ZT19¹

	In sections stained for:			Total
	FOS + PHI	FOS + GRP	FOS + VIP	
ZT14 light (4)	13.3 $\pm$ 1.7]*	2.0 $\pm$ 0.9]*	2.5 $\pm$ 0.5]*	17.8
ZT19 light (4)	64.8 $\pm$ 15.9]*	37.3 $\pm$ 6.3]*	37.5 $\pm$ 6.5]*	139.6

¹The column furthest to the right gives the total numbers. Note 1: all number represent arbitrary relative indices and should only be used for mutual comparison. Note 2: the actual numbers counted per animal were twice as high as presented in the table, since the values for the left and right SCN were averaged per section.

* $P < 0.02$, Wilcoxon-Mann-Whitney test, one-tailed.

VIP, respectively, showed some Fos immunoreactivity of their nuclear profiles (Table 3); the highest number was found for the PHI-IR neurons. After the light stimulus at ZT19 the situation was quite different. The general increase of FOS-IR nuclear profiles (approximately 4.2 $\times$, Table 1) was dramatically reflected in a higher number of colocalizations for all three neuropeptides (Table 3). These data are again presented in Figure 5, but then expressed as the percentage of PHI-, GRP- and VIP-IR perikarya showing Fos immunoreactivity. It shows that the Fos induction was numerically most enhanced in the GRP-IR perikarya (approximately 21 $\times$) followed by the VIP-IR perikarya (about 15 $\times$) and the PHI-IR perikarya (approximately 5 $\times$). By combining for ZT14 and ZT19 separately the averaged

Fig. 5. Mean percentage of PHI-, GRP- and VIP-IR perikaryal profiles ($\bar{X} \pm$ S.E.M., $n = 4$) showing colocalization with nuclear profiles immunoreactive for Fos in the SCN after a light stimulus at ZT14 (light bars) and ZT19 (dark bars). The SCN of the control animals for ZT14 ($n = 2$) and ZT19 ($n = 2$) did not show any Fos immunoreactivity. Two-way ANOVA indicated a significant ZT effect: $F(1,6) = 37.5$, $P < 0.001$, and a significant effect of peptides: $F(2,12) = 15.9$, $P < 0.001$. Post hoc comparison of pairs was done by using Student's t test (corrected according to Bonferroni). If the three different peptides were mutually compared per ZT, this resulted in a significant difference for only PHI vs VIP at ZT14 ($\dagger P < 0.01$). Mutual comparison per peptide of the ZT14 and ZT19 values showed for each peptide a significant difference (* $P < 0.01$).

number of FOS-IR nuclear profiles (Table 1: 51.7 and 218.1) with the total number of colocalizations (Table 3: 17.8 and 139.6), for each ZT time the percentage of FOS-IR nuclear profiles showing colocalization with any of the three neuropeptides PHI, GRP or VIP could be calculated. This resulted in 33% for ZT14 and 65% for ZT19.

DISCUSSION

Methodology

We realize that calculating percentages and ratios from perikaryal and nuclear profiles is a rapid method which does not fully meet the demands of an unbiased morphometric analysis (Gundersen et al., 1988). However, the short-term and subtle character of both experimental conditions on the one hand, and the large numbers of profiles counted in sections of the same anatomical orientation together with the big experimental effects and small variations (partly expressed as percentages) on the other, make it very plausible that the latter are physiologically significant and not due to any swelling or shrinkage of the organelles in question.

Experimental results

In keeping with other reports (Rea, 1992; Sutin and Kilduff, 1992), the present results show that after 45–60 minutes, a light stimulus given during the second part of the night results in substantially more Fos induction than a light stimulus given during the first part of the night. Actually, the difference in number of Fos-IR nuclei amounted to 4.2 $\times$. The SCN of control animals did not show any Fos-IR nuclear profiles (Table 1). Striking was

that the calculated percentage of Fos-IR nuclear profiles showing colocalization with any of the three neuropeptides PHI, GRP or VIP doubled from approximately 33% at ZT14 to 65% at ZT19. This suggests that a good deal of the Fos-IR neurons do not synthesize one of these three neuropeptides or, if they do, the peptidergic content of their perikarya must have stayed below the immunocytochemical detection level of our method.

Considered from the side of the peptidergic neurons in the SCN, the PHI-IR neuronal perikarya showed the highest percentage of colocalization with Fos-IR perikarya ($6.9 \pm 0.9\%$) after the ZT14 light stimulus, followed by GRP and VIP (Fig. 5). After the ZT19 light stimulus, all three types of peptidergic neurons showed a substantial increase of their percentage of colocalization with Fos which was numerically and proportionally most prominent for the GRP-IR neurons ($21\times$) followed by VIP ($15\times$) and PHI ($5\times$; Fig. 5). However, two comments should be made here. First, although the mean number of PHI-, GRP- and VIP-IR neuronal profiles counted in the Vibratome sections was of the same order of magnitude for both ZT times (Table 2), the lower number of GRP-IR profiles counted after the ZT19 light stimulus relative to the ZT14 light stimulus appeared to be significant ($P < 0.02$). This difference may reflect the normal nocturnal decline in GRP immunoreactivity in the SCN (Shinohara et al., 1993; Okamura and Ibata, 1994; see also Zoeller et al., 1992). We do not know, however, to what extent the present nocturnal GRP decrease was affected by the ZT19 light stimulus, nor whether the Fos induction only occurs in the GRP-IR (thus visible) neuronal cell bodies or also in those GRP neurons that drop below the immunocytochemical detection level. In the hypothetical case that the ZT19 Fos induction only takes place in the immunocytochemically visible GRP neurons, the percentage of GRP-IR profiles showing colocalization with Fos (45.6%; see Fig. 5) requires a downward correction. Thus, to calculate the percentage of colocalization for ZT19, the ZT14 GRP value (i.e., 97) and not the ZT19 value (i.e., 81) of Table 2 should be used. In this most "unfavorable" case, the percentage then drops approximately 16% to 38.3%, which is, however, still the highest value in comparison to those of PHI and VIP, and still leads to the most prominent increase in colocalization with Fos ($17.4\times$).

The second comment concerns the reported cellular cosynthesis of PHI and VIP from the same precursor protein (Tatemoto and Mutt, 1981; Nishizawa et al., 1985), which explains the reported colocalization of these peptides in SCN neuronal cell bodies (Daikoku et al., 1985; Moore et al., 1985; Okamura et al., 1986; see also Card et al., 1988). Some of these PHI/VIP-containing neurons were observed to additionally contain GRP, which is derived from a separate gene (Okamura et al., 1986; Albers et al., 1991). If we refer now to our own results presented in Table 2, it is not clear whether the higher numbers of PHI-IR profiles as compared to the numbers of VIP-IR profiles reflect the existence of a group of neurons containing only PHI in addition to neurons containing both PHI and VIP. Indeed, the numerical difference between PHI-IR and VIP-IR profiles per ZT time could also have been caused by differences in binding affinities between the two antibodies with their respective peptide. However, the data presented in Table 3 exclude such an explanation since the ratio between the number of colocalizations after the ZT14 light stimulus for Fos and PHI (13.3 ± 1.7) on the one hand, and Fos and VIP

(2.5 ± 0.5) on the other, is substantially higher than that between the corresponding number of PHI- and VIP-IR profiles (Table 2). Even more important is the differential increase between the number of colocalizations after the ZT19 light stimulus relative to the ZT14 light stimulus for PHI (approximately $5\times$) and VIP (approximately $15\times$). Together, these data indicate that PHI is not only present in cells also containing VIP in more or less equal amounts (as suggested by the afore-mentioned data on colocalization), but also in a separate cell group lacking VIP. The possibility that there is a third cell group containing VIP without PHI remains open. In other words, our experimental results combined with some data indicate that upon a light stimulus given during the first or the second part of the night at least three different cell populations are activated that are characterized by, respectively, PHI alone, GRP, and VIP fully or partly colocalized with PHI.

That at least three neuropeptides, i.e., PHI, GRP and VIP, are involved in the induction of phase shifts in the SCN is also the outcome of physiological experiments done in other laboratories. In these experiments, Albers et al. (1991, 1995) and Piggins et al. (1995) were able to induce behavioral phase delays and/or advances by injecting PHI, GRP and VIP, either separately or combined, into the SCN of the hamster. However, their detailed results are not fully in concert with each other and we will therefore not discuss them here in relation to our experimental outcome.

Our results also corroborate those of Earnest et al. (1993), who likewise observed a high percentage of GRP-IR neurons in the rat SCN (approximately 40%), showing Fos expression upon a light stimulus at ZT18. However, according to this author, VIP-IR neurons did not show any Fos coexpression, which is inconsistent with our results and those of others (e.g., Daikoku et al., 1992). Recently, Wollnik et al. (1995) reported that an intracerebroventricular injection of antisense oligonucleotides directed against c-fos and JunB, prior to a ZT15 light stimulus, prevents the occurrence of a phase delay. This suggests the existence of a causal relationship between the activities of both immediate early genes on the one hand, and resetting of the endogenous clock time and concomitant circadian behavior on the other. All this raises questions as to what is the cellular mechanism behind the differential induction of Fos upon a light stimulus given during the phase delay versus the phase advance period of the (subjective) night and, secondly, whether the Fos-IR neurons are directly and/or indirectly innervated by the retinohypothalamic tract.

In summary, the outcome of the present study suggests that at least three different cell groups characterized by, respectively, PHI alone, GRP, and VIP fully or partly colocalized with PHI play a role during light-induced phase shifts. It is suggested that the PHI neurons are more prominently involved in light-induced phase delays and the GRP neurons together with the VIP/(PHI) neurons in light-induced phase advances.

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