Projections of the Suprachiasmatic Nucleus to Stress-Related Areas in the Rat Hypothalamus: A Light and Electron Microscopic Study

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ABSTRACT

The purpose of the present study was to investigate the sites in the hypothalamus where the suprachiasmatic nucleus (SCN) may influence corticosteroid secretion. In spite of the well established, SCN-mediated, daily rhythms in adrenocorticotrophic hormone (ACTH) and corticosteroid secretion, previous studies determining the projections of the suprachiasmatic nucleus failed to illustrate direct connections with corticotrophin-releasing hormone neurons (CRH).

In order to identify where in the central nervous system the SCN may influence corticosteroid secretion, areas were selected that contained SCN efferents contacting neurons involved in the stress response. To achieve this in the present study, SCN efferents were visualized by Pha-L tract-tracing, together with the neurons involved in the stress response by immunocytochemical staining for c-fos protein.

The sites where these efferents contacted c-fos-positive neurons were established by light microscopic double staining and electron microscopic immunocytochemical studies. It appeared that apart from the medial parvocellular area of the paraventricular nucleus (PVN) of the hypothalamus, many more regions showed fos-positive neurons. Sites where SCN efferents contacted such neurons are limited only to areas immediately adjacent to these putative CRH neurons but are not concentrated on these neurons themselves. These areas consist of the periventricular and rostral PVN together with the dorsomedial hypothalamus: all three regions are known to project into the PVN. Therefore, it is concluded that the SCN transmits its information related to corticosteroid secretion via interneurons in and around the PVN to the CRH-containing neurons, rather than by a direct interaction with these neurons themselves.

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A daily pattern for corticosteroid and adrenocorticotrophic hormone (ACTH) release in mammals has been reported in a large number of separate studies. This involves, in the rat, diurnal differences, both in basal levels and in the response to stressful stimuli (Dunn et al., '72; Kaneko et al., '81; Kant et al., '86; Cascio et al., '87; Bradbury et al., '91). The structure responsible for the control of ACTH and corticosteroid release is the paraventricular nucleus of the hypothalamus (PVN), where the neurons that contain corticotrophin-releasing factor (CRH) together with vasopressin (VP) are located; when released into the portal system of the median eminence, these peptides liberate

ACTH from the adenohypophysis (Swanson et al., '83; Whitnall et al., '87). The suprachiasmatic nucleus (SCN) has been shown to be the major, if not the sole, source for all diurnal rhythms in mammals, including ACTH and corticosterone rhythms. In previous studies, lesioning the SCN resulted in the disappearance of the diurnal rhythm of ACTH and corticosterone secretion (Moore and Eichler, '72; Cascio et al., '87). Moreover, we recently provided evi-

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dence that the SCN inhibits basal and stress-induced corticosterone secretion, especially during the light period (Kalsbeek et al., '92; Buijs et al., '92). Therefore, it was thought that connections between the SCN and the PVN might explain the SCN influence on ACTH and corticosterone secretion. However, previous anatomical studies investigating the projections of the SCN could not provide evidence for such direct pathways and even ruled out the existence of a substantial SCN-PVN innervation that might explain the observed diurnal patterns in corticoid and ACTH secretion (Berk and Finkelstein, '81; Stephan et al., '81; Watts et al., '87). Therefore, the present study is aimed at investigating the anatomical basis for the control of the SCN on ACTH and corticosteroid secretion with emphasis on detecting other possible areas in the target zone of the SCN that may be involved in the control of ACTH/corticosteroid secretion. To achieve this aim, Pha-L tract-tracings from the SCN were undertaken to identify sites of possible interaction between SCN efferents and neurons involved in the control of the stress response. For that purpose, the latter neurons were labelled by fos immunocytochemistry after a stressful stimulus (Sagar et al., '88; Morgan and Curran, '91). In addition, to obtain insight into other possible SCN efferents, this fos labelling was combined with stains for VP and vasoactive intestinal peptide (VIP), two major SCN neurotransmitters. Using light and electron microscopic investigations, it was established where SCN efferents contacted fos-labelled neurons.

MATERIALS AND METHODS

Twenty male adult Wistar rats weighing 250–275 g were used. Animals were housed under 7–7 light-dark conditions with food and water available *ad libitum*. All animals received (under 0.09 mg/100 g intramuscular Fentanyl anaesthesia) an iontophoretic deposit of a 2.5% Pha-L (Vector Labs) solution from a 20–30 μ m micropipet using a 6 microamp positive current at 30–60 V with a 5 seconds on, 5 seconds off current over 5–10 minutes. The glass capillary (20–30 μ m tip) was aimed at the SCN, with the animal in the stereotactic apparatus (David Kopf) with Tandbar at +5 and from bregma, 0.16 anterior; 0.05 lateral and 0.86 ventral from the dura, under an angle of 2°. Five to ten days after the Pha-L deposit, the animals were restrained for 30 minutes in a small plastic restrainer and perfused under 35 mg/kg sodium pentobarbital anaesthesia 60 minutes after the beginning of the restraint, by a preperfusion with $\pm$ 50 ml 0.9% saline followed by 500 ml of 1% paraformaldehyde and 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4. Brains were dissected and postfixed for 15 minutes in the same fixative in the microwave at low setting in a small container placed in a beaker filled with ice cubes and water, followed by an additional 2 hour postfixation at room temperature. Transverse 30 μ m sections were cut on a vibratome and incubated in sheep anti-fos (Cambridge) 1:10,000 and rabbit anti-Pha-L (Kruimel) 1:4,000 or rabbit anti-VP (Truus 19-09) 1:4,000 or rabbit anti-VIP (Viper 19-09) 1:4,000. The anti-fos was demonstrated first with biotinylated goat anti-sheep IgG 1:400 and the ABC Elite detection method (Vector) and diaminobenzidine (DAB) as the chromogen or nickel-intensified DAB for light microscopy, followed by the detection of either Pha-L, VP, or VIP, using the same method (Buijs et al., '89). Part of the sections were processed for light microscopic investigation while the rest were processed for electron microscopy (EM).

For light microscopy all incubation buffers consisted of 0.05 M Tris, 0.9% NaCl, 0.2% gelatin and 0.5% Triton-X 100. For electron microscopy, instead of the 0.5% Triton-X 100 a lower concentration of 0.1% Triton-X 100 was used. Also for EM, the DAB was silver-gold-intensified using a modified method of Gallyas et al. ('86; Buijs et al., '90). The sections were postfixed for 40–60 minutes in 1% osmium with 1.5% potassium ferricyanide in 0.1 M sodium cacodylate buffer (pH 7.4), dehydrated through an ethanol series and flat-embedded in epon. Ultrathin sections were not further contrasted but collected on 200 mesh copper grids and examined under the EM (Philips). Control experiments included fos staining in animals that did not receive the restraint stress and incubation with antisera cross-adsorbed with homologous and heterologous antigens coupled (using glutaraldehyde) to a protein matrix. Further controls included fos staining only to investigate at the EM level whether the DAB-gold precipitate was only restricted to the nucleus and not visible in terminals.

RESULTS

Fos

The restraint stress used in the present procedure invariably led to a high density of fos nuclei in several parts of the central nervous system such as the hippocampus, the cortex, etc. For the purpose of this study, special attention was paid only to those areas with a putative overlap with SCN projections. This special interest was focused on the location of a high density of fos-stained neurons in the dorsal part of the hypothalamus, i.e., from the rostral PVN to the caudal dorsomedial hypothalamus (DMH). In the hypothalamus of unrestrained rats, only single fos-positive neurons were visible, mainly in the ventrolateral parts and not in the PVN-DMH area. After stress, the highest concentration of fos nuclei was visible in the parvicellular PVN and in the DMH. In other projection areas of the SCN such as the OVL and the medial preoptic area, just a few scattered fos neurons were visible after the restraint stress. In contrast, in another projection area of the SCN, the paraventricular nucleus of the thalamus, a high density of fos-labelled neurons was visible; these neurons, however, were already labelled in unrestrained control rats. Thus, for the light and electron microscopic evaluation of SCN projections to neurons that are involved in the stress response, special emphasis was put upon the area of the PVN and DMH where fos neurons appeared only after stress. Electron microscopic investigation of sections stained only for fos showed that the DAB-gold chloride precipitate was confined to nuclei and not deposited in other parts of the cell body or in axonal processes.

Fos and Pha-L

All animals that received an injection of Pha-L into the SCN showed intensely labelled neurons in the SCN with hardly any labelled cells outside it. If the injection site was outside the SCN, usually not a single cell within the SCN was labelled. So it appeared that a border of sorts exists between the SCN and the surrounding tissue, preventing the spread of Pha-L into the SCN even when the tip of the electrode was just outside the nucleus or preventing the spread of the tracer outside the SCN when the tip of the electrode was inside the SCN. One injection reached the ventral SCN, one the dorsal, and two filled it more or less completely (Fig. 1). The projections of the ventral injection

Fig. 1. **A,B:** Light microscopic illustrations of one of the Pha-L injections that filled nearly the whole suprachiasmatic nucleus (SCN). Single arrow in A indicates SCN projections towards the dorsal thalamus. Black (A) and white (B) double arrows indicate the most rostral part of the paraventricular nucleus where a concentration of

Fos-positive neurons can be visualized. B is an enlargement of that area, illustrating that Pha-L-labelled fibers fan out in this region and seem to contact Fos-labelled neurons. F, fornix; III V, third ventricle. Scale bars = 50 μ m.

showed a slightly different pattern as compared to the dorsal injection. The ventral injection resulted in labelled fibers that concentrated in the utmost ventral tip of the PVN and in the ventrolateral border of the PVN; the sub-PVN as defined by Watts et al. ('87). Most fibers tended to avoid the body of the PVN; some fibers passed the periventricular PVN and seemed to branch in the most dorsal cap of the PVN (Figs. 2, 3). Fibers arising from the ventral injection had a low proportion of projections to the DMH where many fewer fibers were visible than after a more dorsal or a whole injection (Fig. 4). The dorsal injection resulted in projections more concentrated in the region of the PVN although the areas that were reached were limited to the periphery of the nucleus, i.e., the sub-PVN, the ventral tip, the periventricular part and the most rostral and caudal parts of the PVN (Figs. 1–3). The two large injections showed more dense projections with a mixture of the dorsal and the ventral patterns and extensive projections into the dorsal hypothalamus. In general, the ventral injection most closely followed the description of SCN projections of Watts et al. ('87). The other three injections followed a more extensive projection pattern, and agreed more with the description of Berk and Finkelstein ('81).

In the rostral part of the PVN, Pha-L-labelled fibers coursing along the third ventricle appear to fan out in the area where a concentration of Fos-labelled neurons is present (Fig. 1). This pattern was prominent especially in those injections that label the dorsal or whole SCN. Injections that reach the ventral SCN do not show such extensive arborisation in the rostral PVN.

Slightly more caudal, in the midpart of the PVN, many Fos-labelled neurons were seen in all parvicellular parts of the PVN (Fig. 2). Pha-L-labelled fibers appear to contact only those neurons in the ventral parts and in the periventricular area, but not those in the medial PVN, at least not extensively (Figs. 2, 3). The periventricular innervation seems to be present mostly in the injections that fill the whole SCN or the dorsal part of it. In the more caudal PVN, the number of Fos-labelled neurons is already lower; here the Pha-L projections seem to contact only the periventricularly located Fos neurons. In the caudal hypothalamus, large numbers of Fos neurons are present in the DMH itself, and laterally in the ventromedial hypothalamus. Pha-L-labelled fibers are concentrated in the DMH and the concentration of Fos-positive neurons coincides with the concentration of Pha-L labelling (Fig. 4).

Fig. 2. **A,B:** Light microscopic illustrations of the Pha-L projections arising from the same spot as in Figure 1. The area depicted in Figure 2 is the midpart of the paraventricular nucleus. A is a low power magnification of this area illustrating a concentration of Pha-L fibers running along the border of the third ventricle in the periventricular part of the paraventricular nucleus (double arrow). The two p's illustrate the concentration of fos nuclei in the parvicellular parts of the paraventricular nucleus. In the magnocellular part (m), the density of fos nuclei is much lower. Just under the magnocellular part, Pha-L fibers can be seen in the subparaventricular nucleus, where no fos nuclei are visible

(single arrows). B is an enlargement of part of A (use the two blood vessels and the two p's for orientation). The upper p indicates the dorsal group of fos-positive nuclei where supposedly the largest number of corticotrophin-releasing hormone (CRH) neurons are concentrated (note the presence of some scarce Pha-L-labelled fibers). The lower p illustrates a ventral group of fos-positive nuclei; the Pha-L-labelled fibers seem to concentrate in the most ventral and lateral part (arrow). The two arrows point to fos-labelled neurons in the periventricular part of the paraventricular nucleus that are reached by Pha-L-labelled fibers. Scale bars = 50 μ m.

VP and VIP staining

VP and VIP fiber pattern staining could be correlated well with the Pha-L projections from the SCN. The ventral injection followed the VIP staining pattern more closely while the more dorsal or whole injections matched more with the VP staining. This can be observed, e.g., in the rostral PVN where the VP innervation is quite intense in the part where fos-labelled neurons are present; this pattern can also be seen with the dorsal and whole SCN injection. In contrast, VIP staining in contrast is hardly present in this area (Fig. 5). (See for comparison the large Pha-L injection of Fig. 1.)

It appeared that the VP fiber staining pattern in the main body of the PVN followed more closely the visible Pha-L-SCN projections into the PVN, especially where it concerned the periventricular PVN (Fig. 6). In the more medial parts of the PVN, it became logically impossible to make a fair judgment as to whether the observed VP fibers may be derived from the SCN because of the other VP-containing

neurons in that area (Fig. 6D). However, studies of SCN lesions conducted elsewhere indicate that the thin fibers in the PVN disappear together with the SCN (Kalsbeek and Buijs, '92). The VIP fiber projections were largely seen outside the PVN and were confined to the ventrolateral part just outside the PVN, the sub-PVN. Small numbers of VIP or VP-containing fibers may terminate in the parvicellular dorsal cap of the PVN; however, no fos neurons could be visualized in this region (Fig. 6AB).

VIP fibers reach the most caudal part of the PVN; thereafter the fiber density decreases rapidly until in the DMH the innervation is sparse (Fig. 7A). Vasopressin fibers, in contrast, continue from the caudal PVN into the DMH (Fig. 7B). VP fibers seem to contact fos-labelled neurons primarily in the rostral and periventricular PVN and in the DMH. The VIP fibers appear to contact fos neurons largely in the ventral PVN and in the DMH although less extensively as compared to VP fibers. In the sub-PVN, no concentration of fos-neurons was observed.

Fig. 3. **A,B:** Fos-labelled nuclei and Pha-L-labelled fibers arising from a dorsal SCN injection. The third ventricle is on the left side. A demonstrates a concentration of Pha-L-labelled fibers in the most ventral tip of the paraventricular nucleus (single arrow) and in the periventricular part of the same nucleus (double arrow). Note the absence

of Pha-L-labelled fibers in the parvicellular (p) and magnocellular (m) part of the paraventricular nucleus. B is an enlargement of the same parvicellular area as in A (use the asterisk in the blood vessel for orientation). The arrow points to a single Pha-L-labelled fiber visible in this region. Scale bars = 50 μ m.

Electron microscopy (EM)

The purpose of the EM study was to establish whether Pha-L-labelled fibers indeed terminated upon fos-labelled neurons. Thus, three different areas were selected as containing a large number of fos-labelled neurons together with many Pha-L-labelled fibers: (1) the rostral PVN; (2) the periventricular midpart of the PVN; and (3) the DMH.

The silver-gold intensification of the DAB precipitate resulted in precipitates of high contrast on cell nuclei and within terminals. Particularly when the DAB and silver-gold precipitate was small, it resulted in the labelled terminals having a clear ultrastructure not obscured by DAB or the gold precipitate. The location of fos and Pha-L in different cellular compartments allowed the use of the silver-gold intensification step for both. The gold labelling within the terminals varied from intense, obscuring most structures (Fig. 8), to sparse, leaving ample room for morphological detail (Figs. 9, 10). The intense labelling was always observed at the surface of the vibratome section, decreasing rapidly deeper in the section. This was especially true for the fos-stained nuclei that only exhibited intense fos staining at the very surface of the section. In addition to the heavy labelling, the ultrastructure was much worse at

the surface of the section. From each of the chosen areas, the rostral and periventricular PVN and DMH, at least two different samples were taken per animal and investigated for contacts between neurons with a fos-labelled nucleus and Pha-L-labelled terminals. In all samples of the rostral and periventricular PVN and of the DMH, synaptic contacts between Pha-L-labelled terminals and neurons with a fos-labelled nucleus could be visualized (Fig. 10). In addition, from the two animals with the largest SCN injection, the medial parvicellular part of the PVN was investigated. In spite of the high incidence of fos-positive nuclei, no Pha-L terminal on such a neuron could be identified.

The labelled terminals invariably had clear electron-lucent vesicles and evinced symmetric synaptic terminals with cell bodies and dendrites in the chosen areas. Logically, only when a terminal contacted a cell body that contained a nucleus, could it be established whether or not this was a fos-labelled neuron. In order to obtain an impression of whether Pha-L-labelled terminals may preferentially contact fos-labelled neurons, in three different animals ultrathin sections from the DMH and rostral PVH that demonstrated DAB-gold-labelled terminals and nuclei were chosen. The number of neurons that contained a

Fig. 4. **A,B:** Concentration of fos-labelled nuclei and Pha-L-labelled fibers from a whole SCN injection in the dorsomedial nucleus of the hypothalamus (DMH). The third ventricle is on the right side. B is a

magnification of the same area as in A, indicated by the three asterisks in the blood vessels. Arrows point to fos-labelled neurons reached by Pha-L labelled fibers. Scale bars = 50 μ m.

Fig. 5. **A,B:** Alternating transverse sections demonstrate vasoactive intestinal peptide (A)- or vasopressin (B)-containing fibers arising from the SCN coursing along the wall of the third ventricle (V) to the rostral part of the PVN where many fos-positive neurons can be seen (arrows).

Only the vasopressin-containing fibers seem to be abundant in this region (B). Note the similarity between this pattern of innervation and the one in Figure 1 with Pha-L tracing. F, fornix. Scale bars = 100 μ m.

Fig. 6. **A–D:** Alternating transverse sections in the region of the paraventricular nucleus demonstrate vasoactive intestinal peptide-containing (VIP) fibers (A) concentrating in the most ventral tip of the paraventricular nucleus and in the sub-paraventricular zone (arrows). Note the absence of VIP fibers in the paraventricular part of the PVN (p), as can also be seen in the higher magnification (C). Arrow points to single VIP fibers in the periventricular part of the PVN; use the asterisk

in the blood vessel for comparison with A. In the dorsal cap of the PVN, a few more fibers can be observed. The vasopressin-containing fibers (B) follow a more medial course to the ventral tip of the PVN (arrows) and seem to course partly through the periventricular part of the PVN, as can be seen in the high magnification (D). The arrows point to thin vasopressin fibers arising from the SCN; use the asterisk in the blood vessel for comparison with B. Scale bars = 50 μ m.

Fig. 7. **A,B:** Two consecutive transverse sections in the region of the dorsomedial nucleus of the hypothalamus (DMH). **A** demonstrates the paucity of vasoactive intestinal peptide-containing fibers in this region. The arrows point to single VIP fibers. In contrast, **B** illustrates the

density of vasopressin-containing fibers in the DMH. Note the concentration of vasopressin fibers together with many fos positive neurons (arrows). V, ventricle. Scale bars = 50 μ m.

nucleus were counted only in the grid squares that showed both fos-labelled nuclei and labelled terminals over the whole square. Subsequently, it was established if the nucleus was labelled and whether that neuron received a DAB-gold-labelled Pha-L terminal. From each animal, 100 neurons were counted. Of 300 neurons in the DMH, 90 were c-fos-positive, 69 of them (77%) receiving a labelled terminal. Of the remaining 210 neurons, only 48 (23%) received a labelled terminal. In the rostral PVN the number of c-fos-positive neurons (71) was slightly lower; 53 (74%) of them received a labelled terminal. In the rostral PVN of the remaining 229 unlabelled neurons, 96 (41%) received a labelled terminal. Variations between the animals were small.

DISCUSSION

In the present literature, no support could be found for any extensive direct SCN-PVN interaction that may explain the diurnal difference in basal- and stress-induced corticosteroid secretion. An input to the ventral, periventricular, and dorsal parvocellular sections of the PVN is described only in the paper of Berk and Finkelstein ('81), who employed tract-tracing with tritiated aminoacids and autoradiography, but they reported no projections to the medial

parvocellular PVN where the bulk of the CRH neurons are located. This observation was supported later by Watts et al. ('87) using Pha-L tracing. In spite of these negative anatomical data, all evidence indicates the involvement of the SCN in the regulation of basal- and stress-mediated ACTH and corticosteroid release (Dunn et al., '72; Kant et al., '86; Cascio et al., '87; Buijs et al., '92). Since stress is a powerful stimulus for ACTH/corticosterone secretion, the approach of the present study was to locate those neurons involved in the stress response using fos immunocytochemistry and to establish which of these neurons receive an SCN input. Of course many more neurons than only those directly involved in CRH/VP and thus ACTH/corticosterone secretion are activated by the present stress protocol, and therefore the observed fos immunoreactivity in the hypothalamus may be unrelated to the direct control of CRH secretion. The pattern of fos staining that we observed, however, was quite similar to that described by Jacobson et al. ('90) after adrenalectomy, suggesting that the majority of the neurons demonstrated by fos in the present study are involved in some way in the control of corticosterone secretion. As adrenalectomy also influences other systems, e.g., by disturbing the mineral balance, and, in our hands, did not result in consistent inducible fos staining in the parvocellular PVN, we discontinued experi-

Fig. 8. An electron micrograph of a heavily labelled Pha-L terminal (arrows) contacting a fos-labelled nucleus. Note that the density of the labelling in the terminal prevents the detection of the type of interaction. The fos labelling in the nucleus is much lighter (asterisks), illustrating the rapid decrease in staining in the nuclei from the surface to the center of the section. Also note that the structure of the tissue taken at the surface of the section is much worse than it would be in deeper tissue; compare with Figures 9 and 10. Scale bar = 0.5 μ m.

ments on fos after adrenalectomy. In addition, the present stress protocol produces a staining pattern in the hypothalamus comparable to the pattern described by Ceccatelli et al. ('89) after various stressors were employed, including restraint. The present protocol, using induction of fos labelling in combination with Pha-L tracing from the SCN, allowed us to indicate those areas where the SCN may influence stress-sensitive processes.

CRH-containing neurons projecting to the median eminence are preferentially located in the medial parvicellular PVN (Swanson et al., '83). This region, which was clearly marked by a cluster of fos-positive neurons, did not receive a clear input from Pha-L-labelled fibers arising from the SCN. The possibility cannot be excluded, of course, that some of the scattered input to fos neurons in the more ventral and periventricular PVN may also reach some CRH neurons located in these areas. However, it becomes clear from the present results that the SCN provides no major input to the medial parvicellular PVN, thereby excluding an important direct SCN input to CRH-containing neurons in the PVN. This result is in complete agreement with the previous observations on SCN efferents using tritiated aminoacid or Pha-L tract-tracing (Berk and Finkelstein, '81; Stephan et al., '81; Watts et al., '87). In addition to the CRH neurons in the PVN, the area immediately adjacent to

these neurons seems to be of crucial importance for SCN regulation of corticosteroid and ACTH secretion, since these areas showed many fos-positive neurons as well as a dense SCN input. From our light microscopic observations, three areas were pin-pointed because of the coincidence of fos nuclei and Pha-L labelling: the rostral PVN, the periventricular PVN, and the DMH. All three of them are excellent candidates as an intermediate station for SCN-PVN interactions. Anatomical observations, indicating that areas immediately surrounding the PVN project directly into the medial parvicellular PVN, support this idea (Ter Horst and Luiten, '86; Swanson, '87).

From the Pha-L tract-tracing of the SCN combined with the information on VP and VIP staining, it became possible to infer some conclusions as to SCN projections to the dorsal hypothalamus with respect to VP and VIP. Of course, the possible mixture of VP and VIP fibers derived from other sources than those of the SCN, makes it impossible to draw definite conclusions from the peptidergic staining alone. However, in combination with previous lesion studies (Hoorneman and Buijs, '82; Kalsbeek and Buijs, '92) and with the Pha-L tract-tracing from different sites in the SCN, it became clear that innervation of the dorsal hypothalamus is not balanced between VIP and VP but certain parts are more heavily innervated by VIP fibers, and other regions more by VP fibers, and vice versa. Thus, the projection of the ventral SCN and VIP innervation seems to be to the ventral zone of the PVN and sub-PVN and the caudal part of the PVN/rostral part of the DMH. The input from the dorsal SCN or VP is directed to the rostral, periventricular, and caudal PVN and to the whole DMH. In the midperiventricular part of the PVN, very fine VP-containing fibers can be visualized, which disappear after SCN lesions (Kalsbeek and Buijs, '92). Their close proximity to VP fibers originating from the PVN makes it impossible to determine whether there is a direct VP input from the SCN to CRH-containing neurons. Also, the present Pha-L study could not provide evidence for a direct SCN/CRH neuron interaction. The possibility cannot be excluded, however, that especially in the ventral part of the PVN, SCN neurons may directly interact with CRH-containing ones. Only further elaborate and careful tracing studies, in combination with CRH staining, may solve this problem. In the dorsal cap of the PVN where neurons are located that project to the brainstem and spinal cord, no fos-labelled neurons could be visualized. This area seems to receive a direct input from the SCN, which has also been reported by Berk and Finkelstein ('81). However, the direct input of SCN efferents onto neurons projecting to the spinal cord has not been demonstrated by double tracing techniques. Yet the existence of such connections may explain diurnal rhythms in autonomic processes such as blood pressure regulation or insulin and glucagon release (Yamamoto et al., '84) or the diurnal change in the sensitivity of the adrenal to ACTH (Dallman et al., '78; see also Buijs et al., '93).

The observation that with dorsal injections into the SCN (and with VP staining), a dense terminal field was found in the DMH and less so in the sub-PVN, and with a more ventral SCN injection (and with VIP staining) the reverse is seen, is support for the previous observation of Watts et al. ('87) who suggested that the sub-PVN may receive the majority of the SCN input. The ventral placement of the injections in the study of Watts et al. ('87) may explain this.

Fig. 9. **A–D:** Photomontage of electron micrographs from the dorsomedial nucleus of the hypothalamus illustrating a fos-labelled neuron, as can be seen from the diaminobenzidine (DAB) precipitate with gold chloride deposit on top of it (asterisks). The fos-labelled neuron is contacted simultaneously by three Pha-L-labelled terminals (arrows), as can be seen from the DAB-gold chloride (B,D) or gold chloride (C) deposit. The upper terminal can be seen in the magnification (B) to contact the cell body (solid arrow), probably forming a

synaptic contact at a further distance, but at this level of sectioning, forming a synaptic contact with a dendrite (open arrow). The left-hand labelled terminal (double arrow) in A is magnified in C, illustrating the gold chloride deposit and the simultaneous formation of two synaptic contacts with the cell body (arrows). The right-hand lower terminal in A is magnified in D, and forms a contact with the cell body (arrow); gold chloride deposit can be seen on the DAB precipitate demonstrative of Pha-L. Scale bars = 1 μ m in A; 0.2 μ m in B–D.

Fig. 10. **A,B:** Two different neurons of the rostral (A) and periventricular (B) part of the paraventricular nucleus of the hypothalamus with DAB-gold chloride (asterisk) deposit on the nucleus demonstrating

fos labelling. These neurons are contacted by a DAB-gold chloride-labelled terminal, illustrative of Pha-L labelling, forming a synaptic contact (arrow) with the cell body. Scale bars = 0.5 μ m.

For the EM observations, we chose to intensify the DAB precipitate using the silver-gold intensification procedure since that precipitate is much easier to recognize than DAB alone, especially over nuclei. The presence of fos and Pha-L immunoreactivity in two completely separate compart-

ments allowed us to use the gold-silver intensification after the final DAB staining, allowing visualization of much more anatomical detail in the terminals. We decided to focus our attention for the EM studies on the three areas in and around the PVN where SCN efferents seem to contact

fos-labelled neurons. The ultrastructural observations in the rostral and periventricular PVN and DMH showed that in these three regions, Pha-L-labelled terminals synaptically contacted neurons with both labelled and unlabelled nuclei. The presence of clear-cut synaptic interactions between the SCN projections and neurons in these PVN/DMH areas, clearly illustrates that the SCN is influencing these regions directly rather than sending information through fibers of passage. Recently, we provided evidence that the PVN/DMH area is the target region where VP from the SCN may inhibit corticosterone secretion (Kalsbeek et al., '92). The present results indicate that a direct interaction of SCN/VP fibers in the caudal PVN/DMH may account for the inhibitory mechanisms.

To investigate whether Pha-L terminals may preferentially contact fos-labelled neurons, we initially selected the DMH area since this area has been shown to innervate the parvocellular PVN quite extensively (Ter Horst and Luiten, '86). The result of counting Pha-L terminals from three different animals showed that although the fos-positive neurons are in the minority ($\pm 30\%$), they receive the majority of the Pha-L input ($\pm 70\%$ of the neurons that receive an SCN input are fos-positive). We realize that such an immunocytochemical approach with all its inherent penetration problems hardly allows an accurate quantitative analysis. In the present study, we therefore intended to get an impression of the magnitude of the selectivity of the input in several animals and chose to count only until we reached 100 nuclei. Nevertheless, since we took care that labelled terminals and nuclei were visible in the whole area covered by the grid square, we are confident that this distribution is relatively accurate. Of course, one cannot be absolutely sure that these fos-positive neurons of the DMH are the ones that project into the parvocellular CRH complex of the PVN. However, the preferential input of the SCN to stress-activated neurons in the DMH complex suggests that this area may be of crucial importance for the expression of the circadian rhythm in stress response and hence most likely is important in corticosterone secretion as well. Moreover, the fact that the DMH has an extensive input to the parvocellular PVN indicates that this region may serve as an important relay station between the SCN and the PVN. The observations that lesions of the DMH disrupt circadian corticosterone rhythms and diurnal feeding (Bellinger et al., '76, '85) support this hypothesis. Furthermore, the DMH as a major target is interesting because, except for its connections with the parvocellular neurons of the PVN which may account for the control of ACTH secretion, its connection to the ventromedial hypothalamus (Ter Horst and Luiten, '86), which is a regulatory center for food intake, may form the basis for the control of the SCN over food intake and other overt diurnally organized behavioral patterns (Bernardis, '73; Yamamoto et al., '84). The data on the Pha-L/c-fos interaction in the rostral PVN more or less show the same result, indicating that the same is true for the rostral and periventricular PVN receiving a substantial SCN input to fos-positive neurons. Although nothing specific with respect to target regions is known about intra-PVN connections, it has been shown that the PVN has extensive intranuclear connections (Van den Pol, '82); these intranuclear connections may serve to pass on the information from the SCN to the CRH neurons.

The present observations emphasize, as has also been noted by Watts et al. ('87) and Swanson ('87), that the SCN, rather than projecting extensively to neuroendocrine motor

neurons, projects to neurons or groups of neurons that may serve as relay stations in transmitting the received SCN information to a great variety of structures. The present results indicate that the neurons that mediate the SCN information concerning the release of ACTH and corticosterone may reside in the PVN/DMH complex but are not the CRH/VP-containing neurons themselves. Functionally, the use of such an interneuron system by the SCN seems much more efficient than SCN projections to motoneurons alone, since the interneurons may transmit the SCN information to many more structures than such neuroendocrine motor neurons could do. The extensive connection of the sub-PVN and the DMH with a large number of hypothalamic nuclei [sub-PVN with lateral septum, bed nucleus of the stria terminalis (Watts et al., '87); DMH with PVN, ventromedial hypothalamus, and brainstem (Ter Horst and Luiten, '86)] indicates that by reaching these areas the SCN very extensively multiplies its impact on the CNS.

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