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Ultrastructural Evidence for Intra- and Extranuclear Projections of GABAergic Neurons of the Suprachiasmatic Nucleus

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ABSTRACT

GABAergic projections of the suprachiasmatic nucleus (SCN) were demonstrated in a double-labelling ultrastructural study which visualised the efferents of the SCN by PHA-L tracing, diaminobenzidine (DAB) immunocytochemistry, and GABA with immunogold postembedding staining. The results show a strong contralateral projection of the SCN that is partly GABA-containing. In addition, ipsilateral SCN projections to the dorsomedial hypothalamus and periventricular part of the paraventricular nucleus and subparaventricular nucleus were shown to contain GABA. The present results indicate that the SCN may utilize this inhibitory neurotransmitter to regulate and organize its own circadian rhythm as well as using GABA to transmit its diurnal information to other regions of the brain.

Key words: vasopressin, paraventricular nucleus, circadian rhythm, immunocytochemistry, transplantation

The suprachiasmatic nucleus (SCN) is now accepted as the major circadian pacemaker in the mammalian brain (Moore, '83; Ralph et al., '90; Rusak and Bina, '90). The SCN has been shown to exhibit a circadian rhythm in electrical activity, neurotransmitter synthesis, and neurotransmitter release both in vivo and in vitro (Inouye and Kawamura, '79; Earnest and Sladek, '86; Gillette and Reppert, '87; Bos and Mirmiran, '90; Albers et al., '92). Most probably the SCN uses its projections to transmit its circadian information to other parts of the central nervous system (Kalsbeek and Buijs, '92). In addition to the various peptidergic transmitters that have been demonstrated in SCN neurons, the inhibitory amino acid neurotransmitter gamma aminobutyric acid (GABA) or its synthesising enzyme, glutamic acid decarboxylase (GAD) have recently been demonstrated in SCN cell bodies (Okamura et al., '89; François-Bellan et al., '90; Moore and Speh, '92). The additional demonstration of GAD mRNA in SCN cell bodies is further documentation for GABA synthesis in SCN neurons (Okamura et al., '89; Moore and Speh, '92). Apart from this anatomical localisation, numerous functional studies suggest a physiological role for GABA in the SCN in the entrainment of circadian rhythms, (e.g., Turek and Losee-Olson, '86; Ralph and Menaker, '87; Smith et al., '90). Further, electrophysiological studies indicate that nearly all SCN neurons are inhibited by the exogenous application of GABA (Mason et al., '91). It remains unclear, however, whether GABA-containing cell bodies present in the SCN contribute to the web of GABA fibers in the SCN (van den Pol, '86) or if this GABA network originates from elsewhere. Current evidence indicating the colocalisation of GABA with neuropeptide Y (NPY) suggests that part of the GABA innervation may arise from the intergeniculate leaflet neurons that project to the SCN (Card and Moore, '82; François-Bellan et al., '90). Apart from its contribution to the SCN network, it is still unclear whether the SCN may use GABA as a transmitter in its projection neurons.

This question has prompted us to investigate the possible morphological basis for the involvement of GABA in the control of circadian rhythmicity by studying the presence of this inhibitory amino acid neurotransmitter in SCN projections to the medial hypothalamus. The anterograde tracer *Phaseolus vulgaris* leucoagglutinin (PHA-L) was used in a preembedding immunocytochemical procedure to demonstrate the projections of the SCN. Subsequently, the visible PHA-L-labelled target areas of the SCN were evaluated in an ultrastructural study using postembedding staining for GABA and a protocol that allowed the demonstration of GABA in PHA-L-labelled SCN efferents (Buijs et al., '93c).

MATERIALS AND METHODS

The present study used 36 male Sprague-Dawley rats (200–250 g). Animals were kept in a humidity and tempera-

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ture-controlled environment with free access to food and water. Lights were on from 7 AM to 7 PM. Following anaesthesia with Innovarvet (0.4 mg/ml fentanyl, 0.06 ml/100 g body weight im), each animal received an iontophoretic injection from a 30–40 μm glass capillary containing 2% PHA-L (Vector Laboratories) in 0.05 M Tris/0.9% NaCl (pH 7.6), by means of a 6 μA positive current (5 seconds on, 5 seconds off) from a constant current voltage device. After recovery, animals were allowed to survive for 5–7 days. Then, under deep pentobarbiturate anaesthesia (60 mg/kg ip), animals were transcardially perfused with 500 ml 3% glutaraldehyde and 1% freshly prepared paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) preceded by 50–100 ml 0.9% NaCl.

After perfusion, the brains were carefully removed from the skull, sectioned in ±5 mm thick slices, and postfixed for another 2 hours. During postfixation a small 25 ml container holding the brain and fixative was transferred to a microwave in a 300 ml container with ice and water. The brain was exposed to microwaves at "defrost" or the low energy setting for 15 minutes. During this procedure care was taken that the container with ice and water in which the small container holding the fixative and brain, always contained ice, i.e., heating was avoided.

After fixation, transverse 50–30 μm vibratome sections were cut. The sections were treated with $NaBH_4$ (5 mg/ml in 0.05 M Tris/0.9% NaCl, pH 7.6) for 5 minutes. The sections were washed extensively until they sank again, and were allowed to stand in buffer for another hour before further incubation. The primary antibody (anti-PHA-L, Kruimel NBR) was used in a low (1:2,000–1:10,000) dilution overnight at 4°C. The incubation buffer consisted of 0.05 M Tris, 0.9% NaCl, 0.2% gelatin, pH 7.2. By means of the biotinylated goat anti-rabbit IgG in combination with the ABC elite kit (Vector Laboratories), PHA-L was visualised with diaminobenzidine (DAB) as the chromogen (5 mg/10 ml) and 10 μl 3% H_2O_2 as electron donor. Care was taken not to overdevelop this reaction and to achieve a light staining. Sections were washed twice in distilled water and postfixed in 1% OsO_4 and 1.5% potassium ferrocyanide in 0.1 M cacodylate buffer for 20–60 minutes. Subsequently, the sections were washed in distilled water, dehydrated through a graded series of ethanol, propylene oxide, and embedded in epoxy resin.

After polymerisation (24 hours at 60°C), the sections were examined with the light microscope, and the selected area was dissected out, flat-mounted on prepolymerised epon blocks with cyanolite glue, and hardened for 10 minutes at 65°C. Ultrathin sections (80–90 nm) were made of the desired area and collected on 200–300 mesh gold grids. Postembedding immunogold labelling was carried out in a drop of fluid on parafilm in a Petri dish or in the small slots of a grid box. Grids were rinsed 3 times in buffer or in distilled water in small beakers with a stir bar.

Subsequently the grids were 1) incubated for 3–4 hours in GABA antibody (1:1,000; Moortje 18-9) in incubation buffer (Tris buffered saline (TBS) supplemented with 0.8% bovine serum albumin (BSA), 0.1% fish gelatin, 2 mM sodium azide, 1% normal goat serum, and 0.5% Triton X-100) at 37°C; 2) rinsed 6 times in TBS supplemented with 0.8% BSA, 0.1% gelatin, 2 mM sodium azide (wash buffer); 3) incubated in a secondary antibody (goat anti-rabbit) conjugated to colloidal gold particles (GAR 10 nm or GAR 15 nm, Amersham, 1:30) in incubation buffer for 90 minutes at 37°C; and 4) rinsed 3 times in TBS and 3 times in double distilled water. After drying, the sections were examined with the electron microscope.

Specificity tests included the omission of the first antiserum and its replacement by another rabbit antiserum, and solid phase adsorption of the antibody on lysine covered saccharose beads to which GABA was conjugated with glutaraldehyde.

RESULTS

Of a total of 36 injected animals, 7 had injections confined to the SCN. In the remaining 29 cases, the injections were either not visible (i.e., too deep or in the ventricle) or were in areas close to the SCN.

PHA-L injections inside the SCN invariably resulted in labelling of cell bodies within the boundaries of the SCN but seldom resulted in labelled neurons outside that nucleus. On the other hand, injections outside the nucleus never labelled neurons within the SCN. For the present study only those injections were used which had no cell bodies labelled outside the SCN.

Contralateral projections

All successful injections into the SCN resulted in labelled cells in the rostral, the ventral, and the dorsal SCN, forming a dense network of PHA-L-labelled fibers not only in the ipsilateral SCN but also in the contralateral SCN. In the latter case these PHA-L-positive fibers branched extensively, forming pericellular structures suggestive of dense innervation of the contralateral SCN (Figs. 1–3). In general, a more ventral injection resulted in a more ventral labelling in the contralateral SCN (Figs. 1, 3), while both a more dorsal and a lateral injection site resulted in more dorsal and lateral labelling contralaterally (Fig. 2). The central part of the SCN mostly showed the lowest fiber density (Figs. 1–3). Injections just outside the SCN did not show extensive innervation of the ipsilateral SCN and certainly did not reach the contralateral SCN. The neurons close to the SCN can therefore not be the source of the contralateral innervation.

Ipsilateral projections

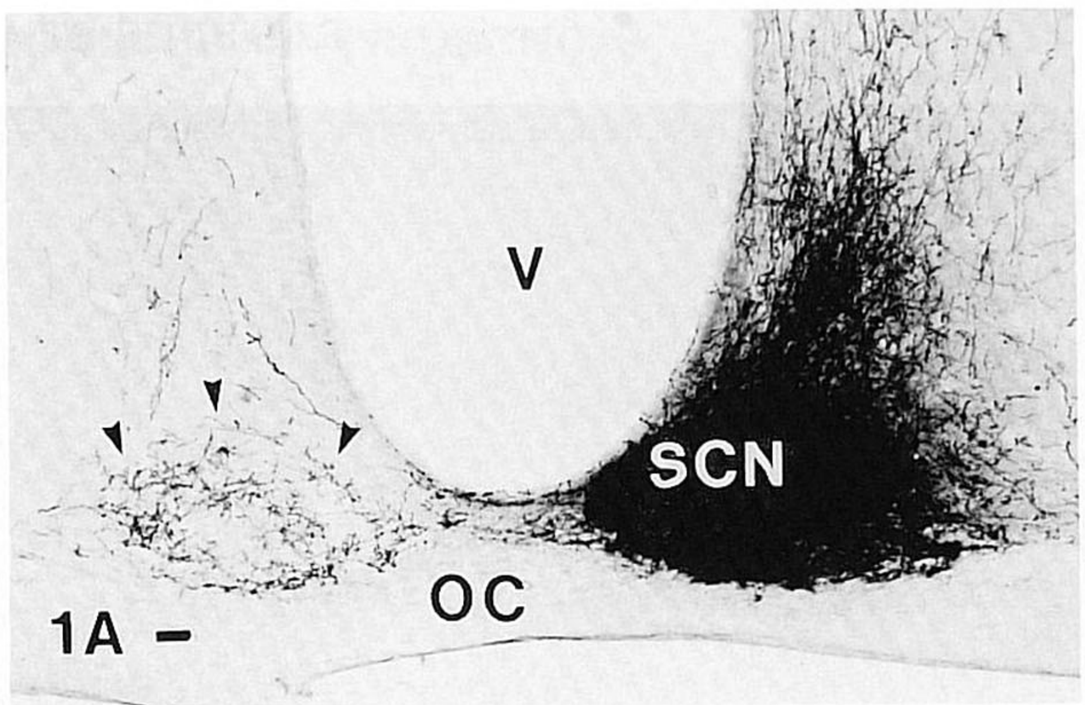
PHA-L-labelled cells in the SCN always produced ipsilateral projections parallel to the surface of the optic nerve; most probably, these fibers continued either rostrally to the

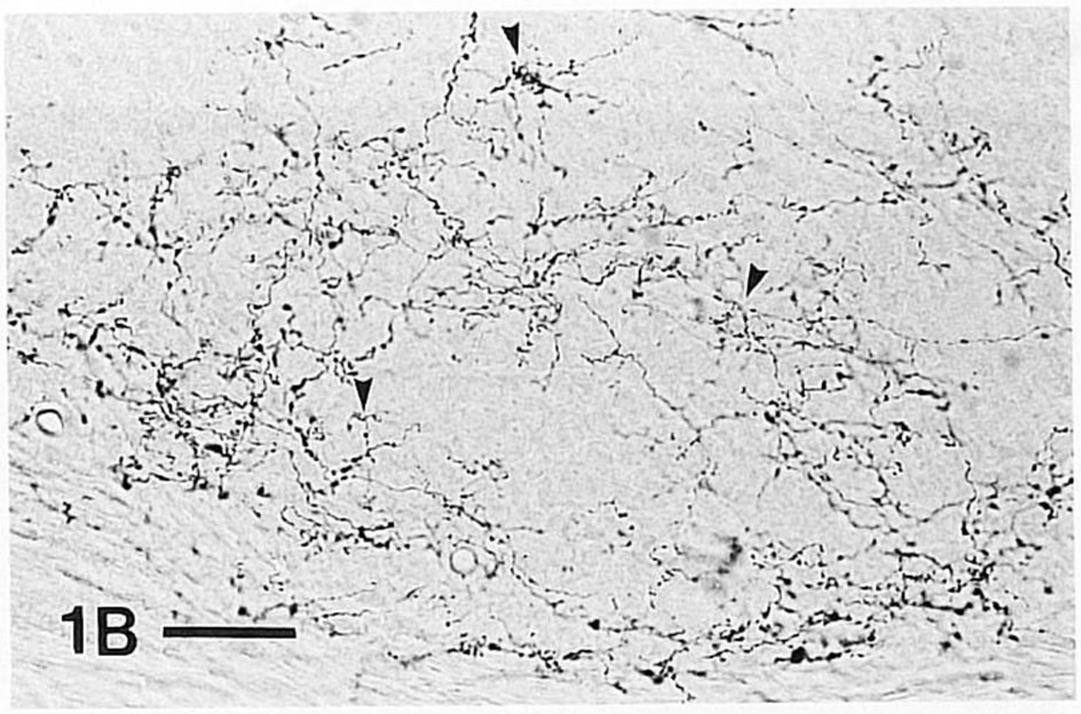
Figs. 1–3. Light micrographs of the contralateral projections of the suprachiasmatic nucleus (SCN) after an iontophoretic injection of PHA-L into the SCN. The position of the letters SCN indicates the approximate site of the center of the injection. OC, optic chiasm; V, ventricle. Scale bars = 50 μm.

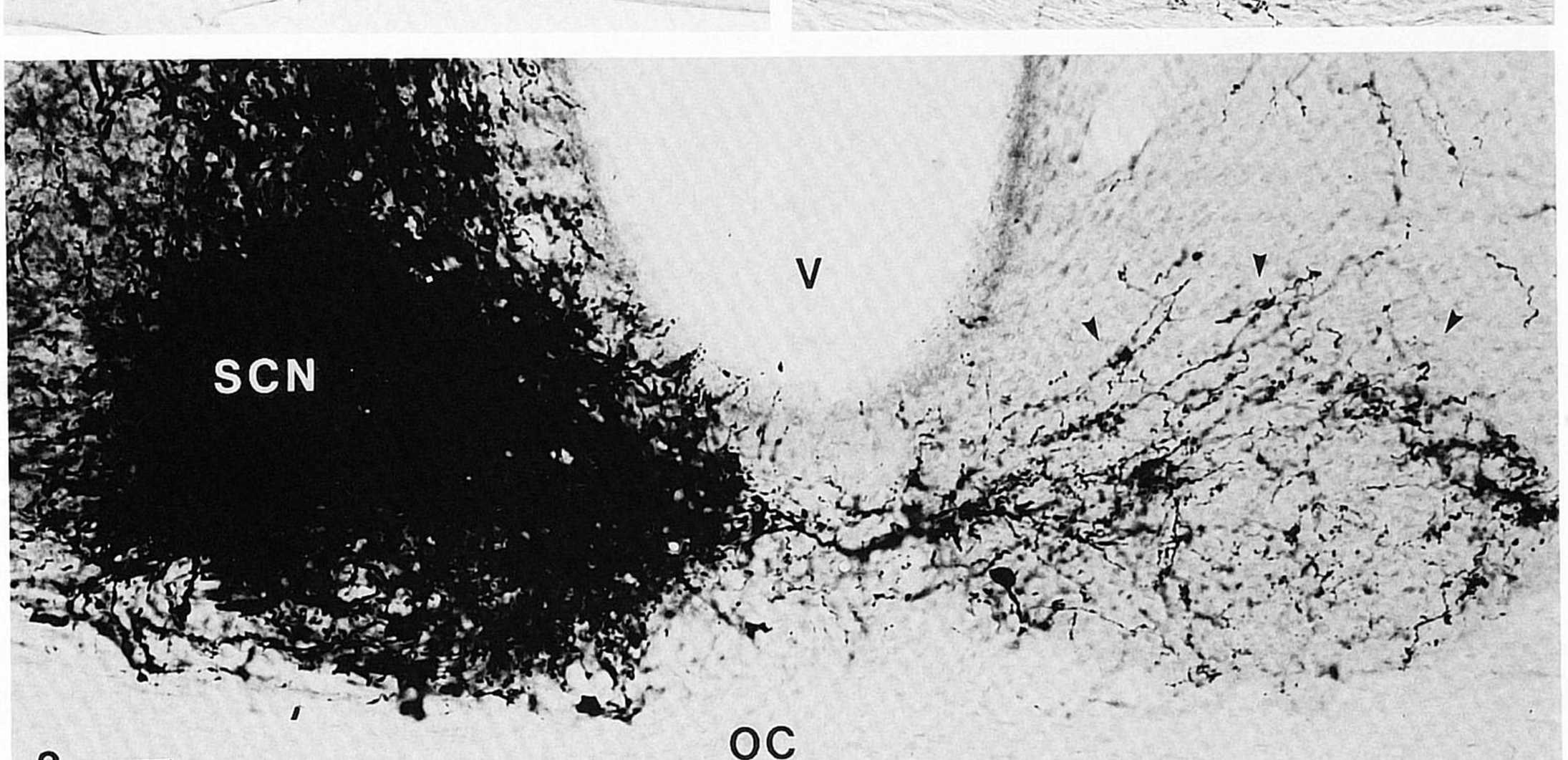
Fig. 1. **A:** Fiber concentration in the contralateral SCN (arrowheads). The PHA-L injection is concentrated in the dorsomedial part of the SCN. **B:** A higher magnification of the contralateral SCN illustrates perineuronal terminations and fiber branching (arrowheads).

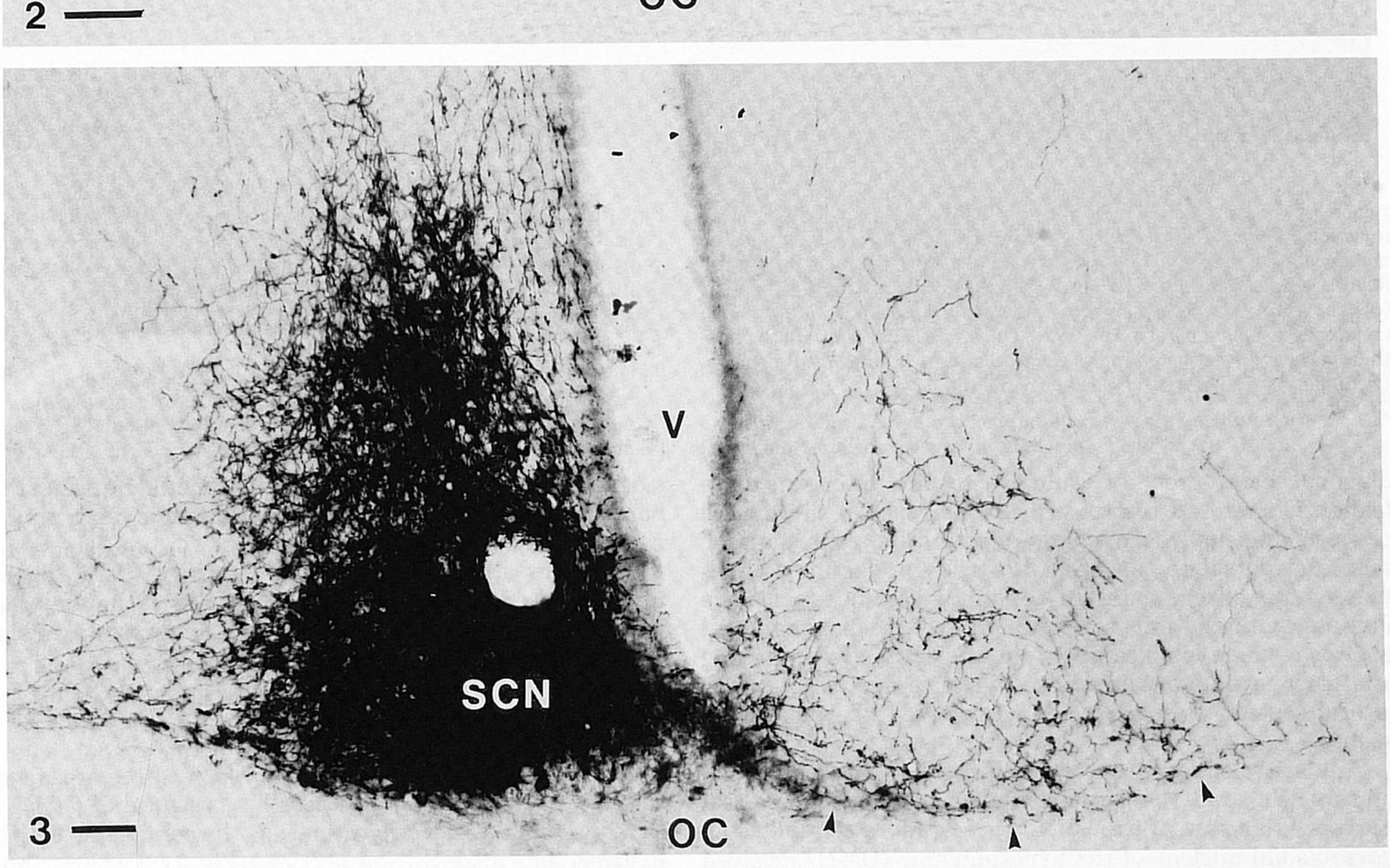
Fig. 2. PHA-L injection concentrated in the dorsolateral part of the SCN resulting in contralateral projections that seem to be concentrated more in the dorsal part of the SCN (arrowheads).

Fig. 3. PHA-L injection concentration in the ventromedial part of the SCN resulting in contralateral projections that seem to be concentrated more in the ventral part of the SCN than in the dorsal part (arrowheads).









Figures 1–3

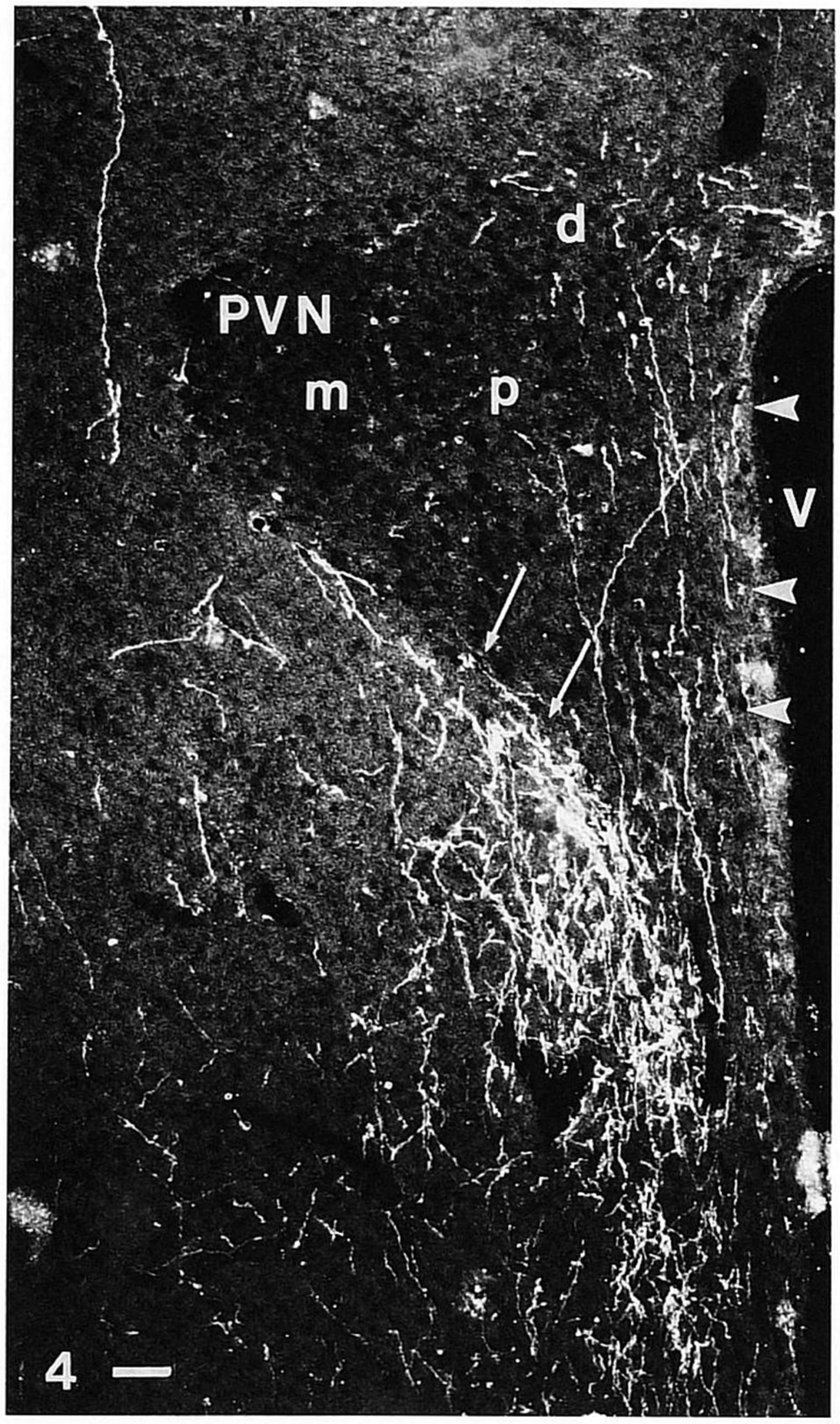


Fig. 4. Darkfield illustration of the projections of the SCN to the region of the paraventricular nucleus (PVN) after an iontophoretic injection of PHA-L into the SCN. Note the absence of fibers in the magnocellular and parvicellular part of the PVN (m,p). Only a few fibers are present in the periventricular part and dorsal part of the PVN (d). The long arrows indicate the dense innervation in the subparaventricular zone just ventral to the PVN. The arrowheads indicate the area that has been investigated in the electron microscopic study as periventricular PVN. V, ventricle. Scale bar = 50 μm.

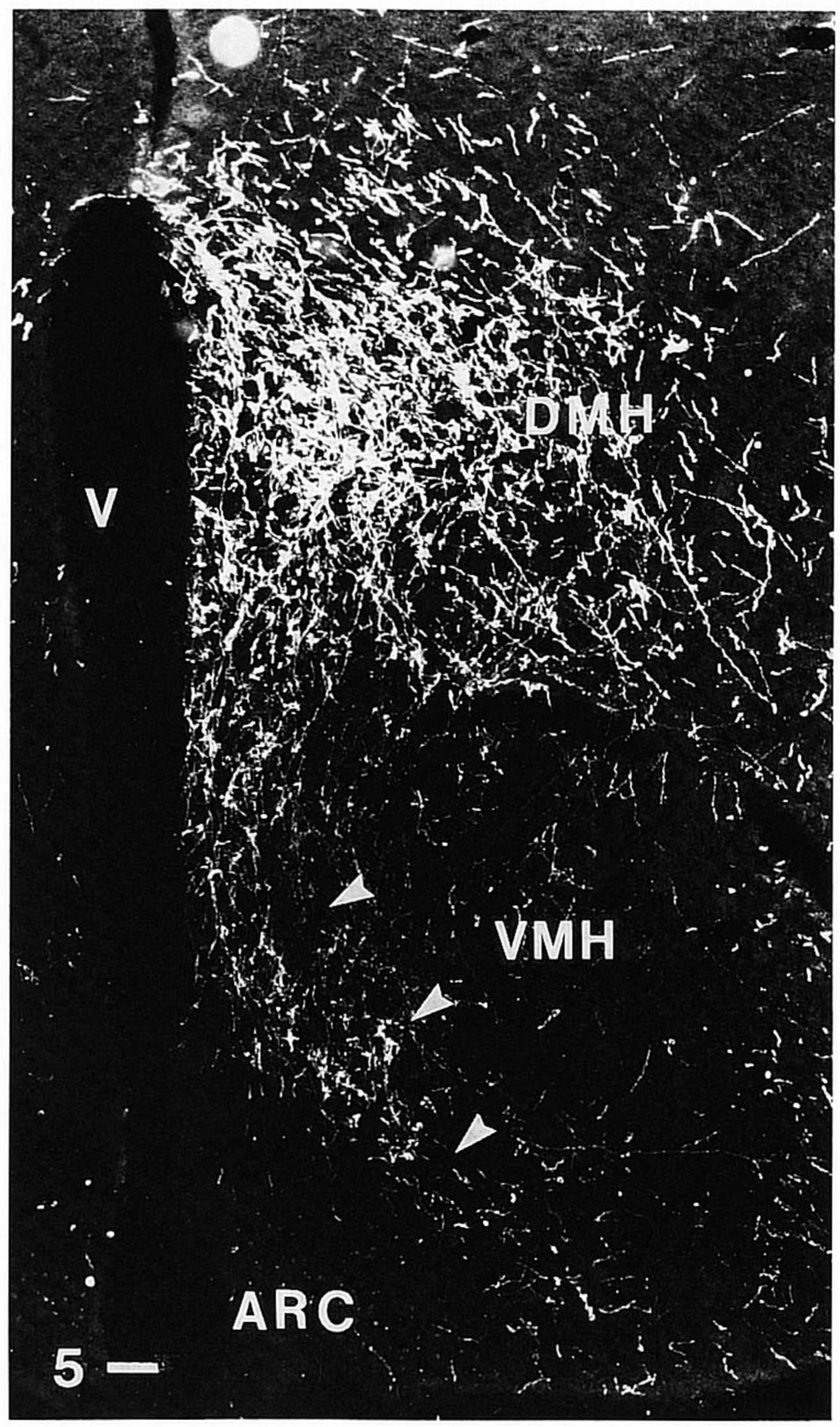


Fig. 5. Darkfield illustration of SCN projections to the dorsomedial hypothalamus (DMH), where a dense terminal field can be observed. Note the presence of a concentration of fibers in the region between the ventromedial hypothalamus (VMH) and the arcuate nucleus (ARC; arrowheads). V, ventricle. Scale bar = 50 μm.

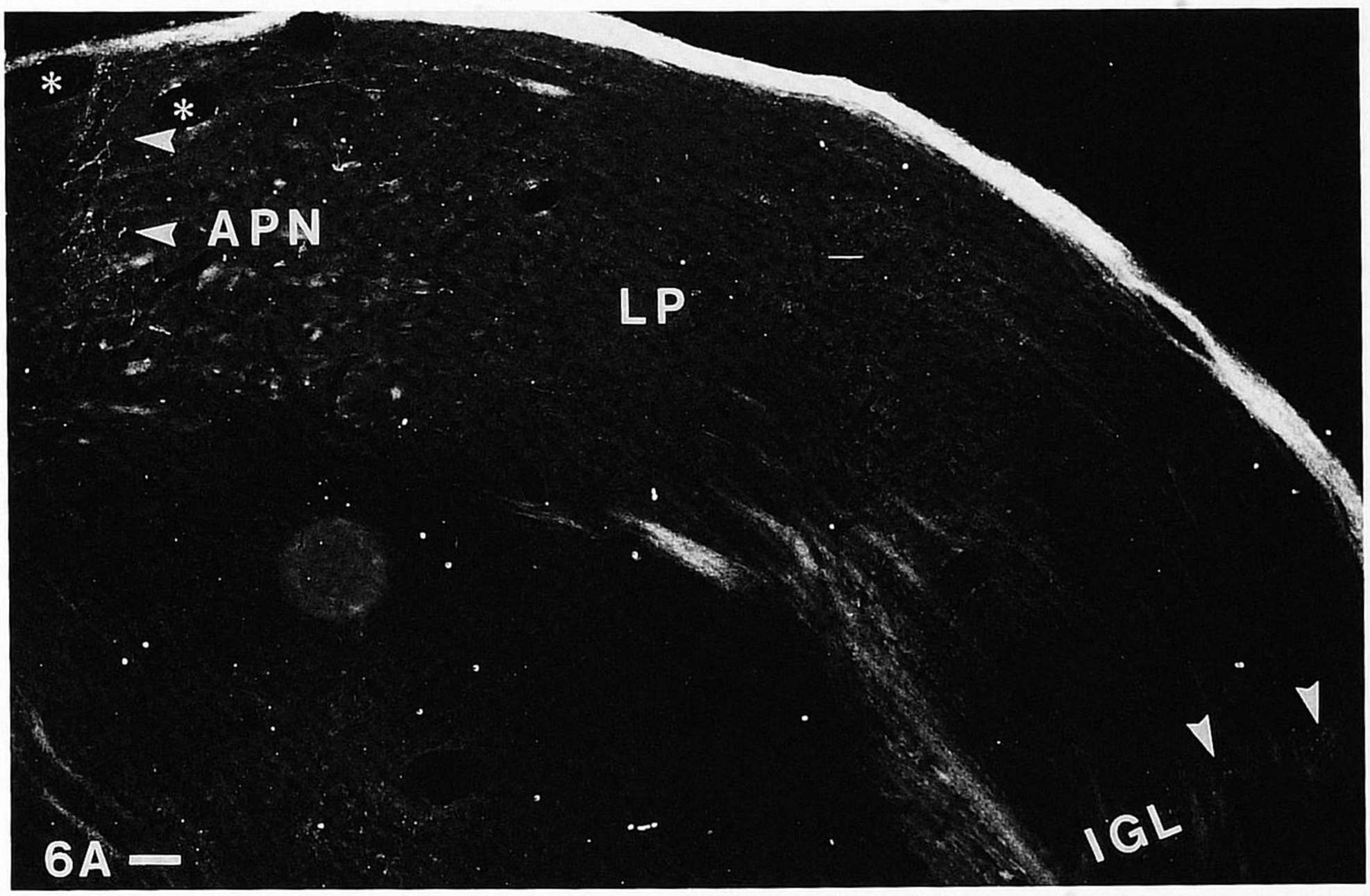
diagonal band area, or caudally to the intergeniculate leaflet. Dorsal and rostral projections reached the medial preoptic nucleus where a dense termination zone could be seen just dorsal to the SCN. Rostrally projecting PHA-L fibers were observed in the organum vasculosum of the lamina terminalis and the vertical limb of the diagonal band of Broca, where labelled fibers showed extensive branching and perineuronal organisation. In the ventral parts of the lateral septum only a few PHA-L-labelled fibers could be seen.

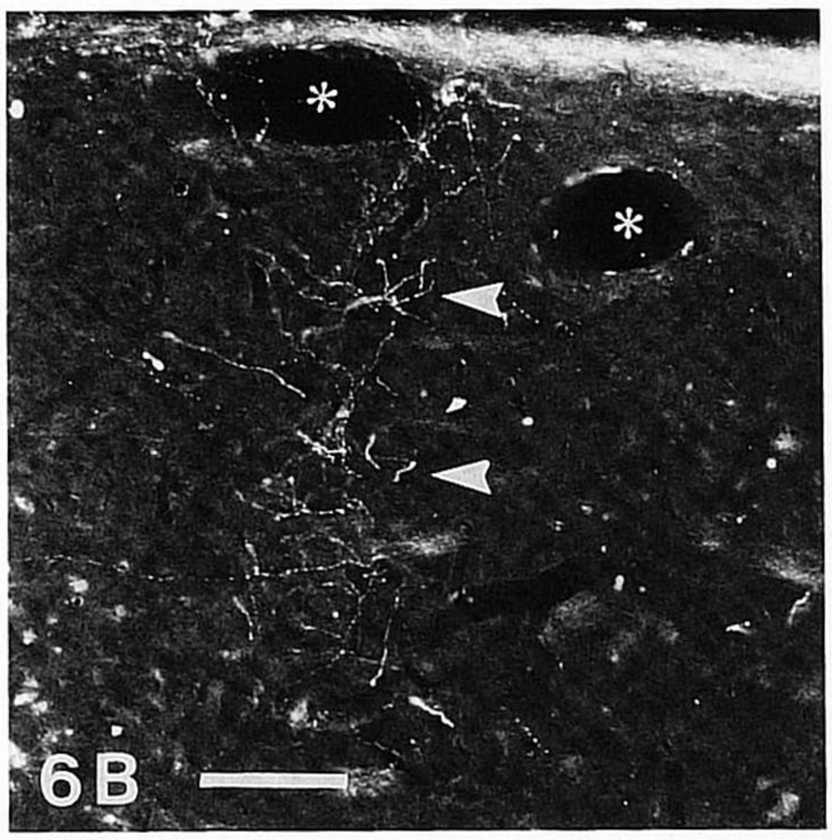
Caudal projections from the SCN reached the rostral part of the PVN and the periventricular, ventral and dorsal parvocellular part of the PVN (Fig. 4). More caudally, the dorsomedial nucleus of the hypothalamus (DMH) received a dense SCN input, while PHA-L fibers could be seen at the border of the ventromedial hypothalamus and arcuate nucleus (Fig. 5).

More dorsally and caudally, projecting PHA-L-labelled fibers reached the paraventricular nucleus of the thalamus, the intergeniculate leaflet, and the anterior pretectal nucleus (Fig. 6).

Electron microscopy

For the electron microscopic approach, three areas were selected to examine for the possible presence of GABA in PHA-L-labelled SCN projections: 1) the contralateral SCN, to investigate intra-SCN projections (Fig. 7); 2) the dorsome-





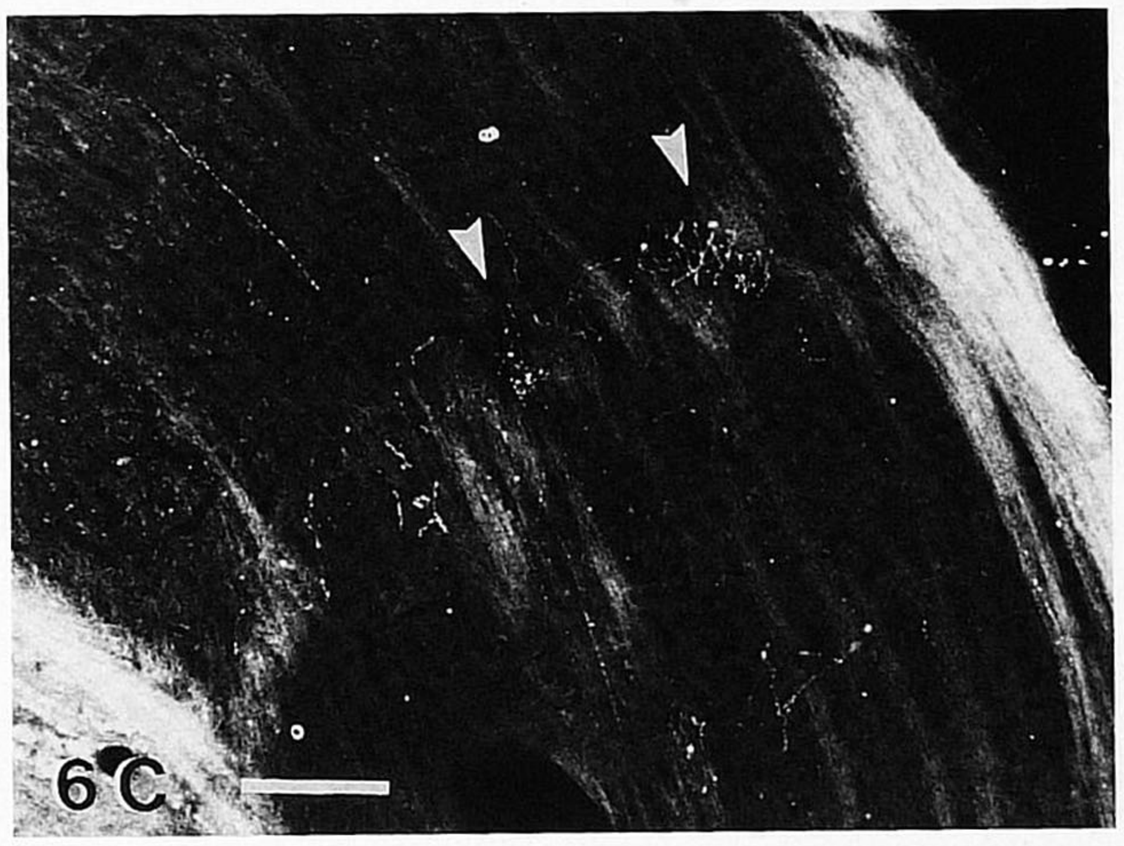


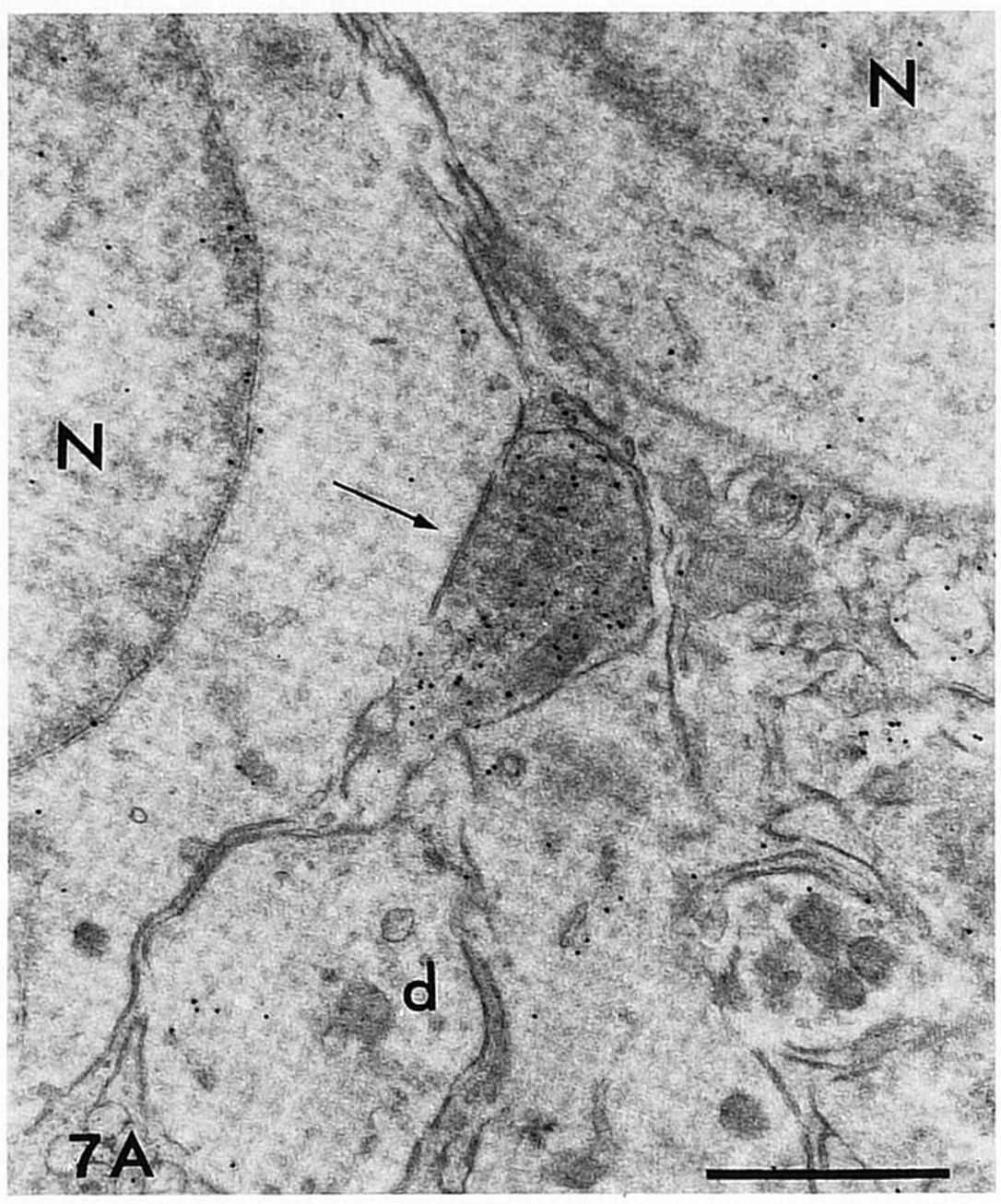
Fig. 6. **A–C:** Darkfield micrographs illustrating PHA-L-labelled fibers originating from the SCN terminating in the intergeniculate leaflet (IGL) and in the region of the anterior pretectal nucleus (APN; arrowheads). B: Higher magnification of the APN illustrating branching fibers in this area; the asterisk in blood vessels refers to A. C: Branching fibers in the region of the IGL, indicated by arrowheads; the arrowheads in C have approximately the same position as in A. LP, lateral posterior nucleus of the thalamus. Scale bars = 50 μm.

dial hypothalamus, one of the main target areas of vasopressin-containing SCN fibers (Fig. 8); and 3) the sub-PVN and periventricular PVN, the main target areas of vasoactive intestinal peptide (VIP) and vasopressin-containing SCN fibers, respectively (Figs. 9, 10).

At the electron microscopic level, the DAB-labelled PHA-L fibers were clearly distinguishable from nonlabelled fibers by their dense osmiophilic DAB precipitate (Figs. 7–10).

In order to investigate the possible GABAergic content of these PHA-L-filled terminals, the ultrathin sections were subjected to postembedding staining using antibodies to GABA. The presence of the GABA antibody was detected using a second antibody to which 10 or 15 nm colloidal gold was conjugated. This 10 or 15 nm gold labelling provided strong enough contrast with DAB to allow visualisation of GABA even in structures that were densely labelled with DAB (Fig. 8A). The densest gold labelling in GABA-labelled terminals was often observed on mitochondria, regardless of whether that terminal was DAB positive or not.

Light DAB labelling in the PHA-L filled structures allowed the determination of the presence of a postsynaptic density in the postsynaptic structure. In none of the observed profiles could such a postsynaptic density be observed, suggesting that most SCN terminals are symmetric.



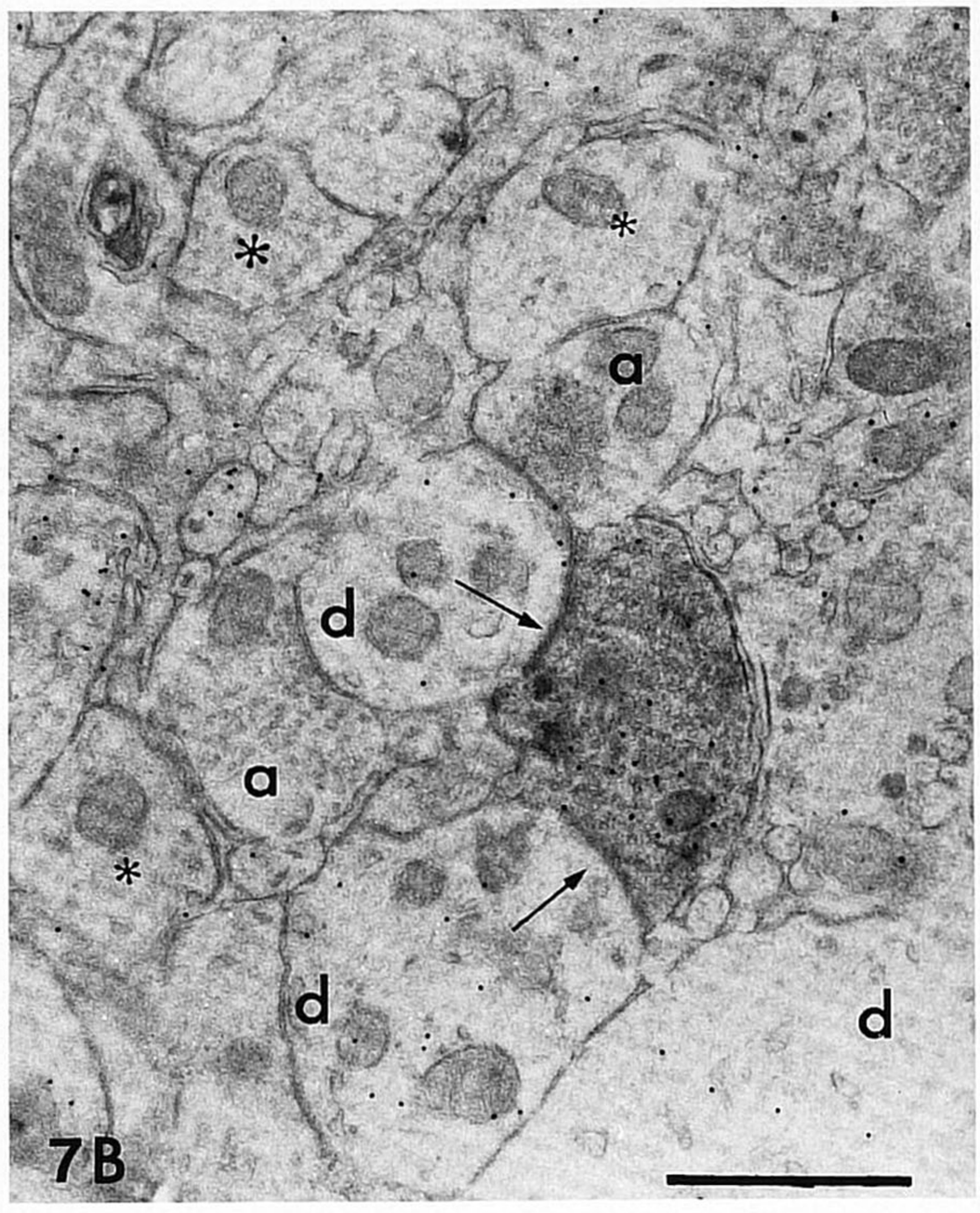


Fig. 7. **A,B:** Immunoelectron microscopic labelling in the contralateral SCN for GABA and PHA-L after a PHA-L injection in the unilateral SCN. The PHA-L is visualised by the amorphous dark diaminobenzidine (DAB) reaction product, while GABA is illustrated by the 10 nm gold deposits. Note the synaptic contact between the PHA-L–GABA-labelled terminal and a neuronal cell body (A, arrow) and a simultaneous contact with two dendrites (B, arrows). Note the presence of a light GABA labelling in some dendrites (d), while it is absent in other dendrites (asterisks) and in an unlabelled axon (a). N, nucleus. Scale bars = 1 μm.

Suprachiasmatic nucleus

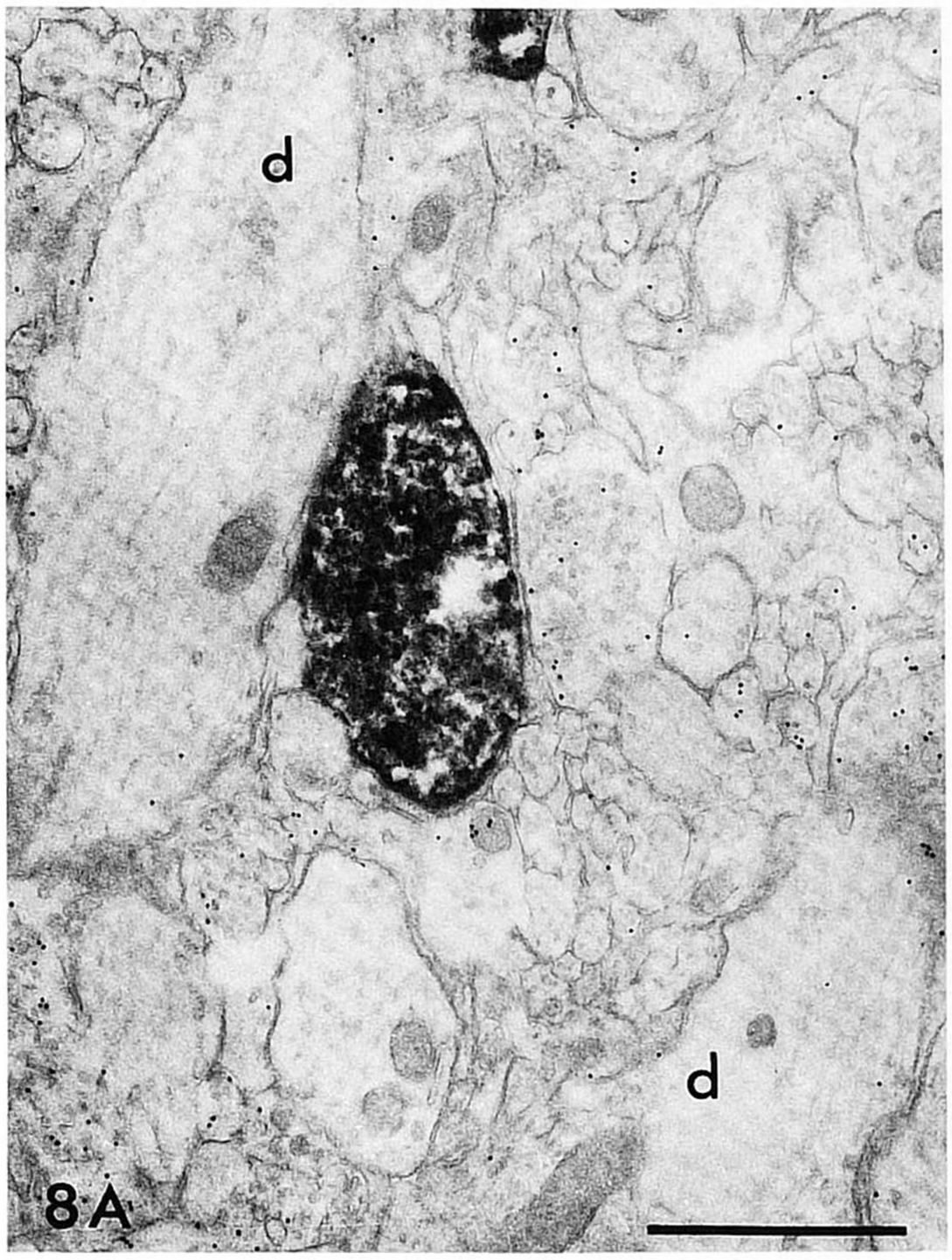
At the ultrastructural level PHA-L-labelled fibers that reached the contralateral SCN formed contacts with nonlabelled elements. These contacts were frequently observed as synaptic specialisations in which the postsynaptic element proved to be a dendritic spine, a dendrite, or a neuronal cell body (Fig. 7A,B). The labelled terminals were symmetric in appearance and usually contained one or more mitochondria and had DAB labelling varying in intensity from light to very dark.

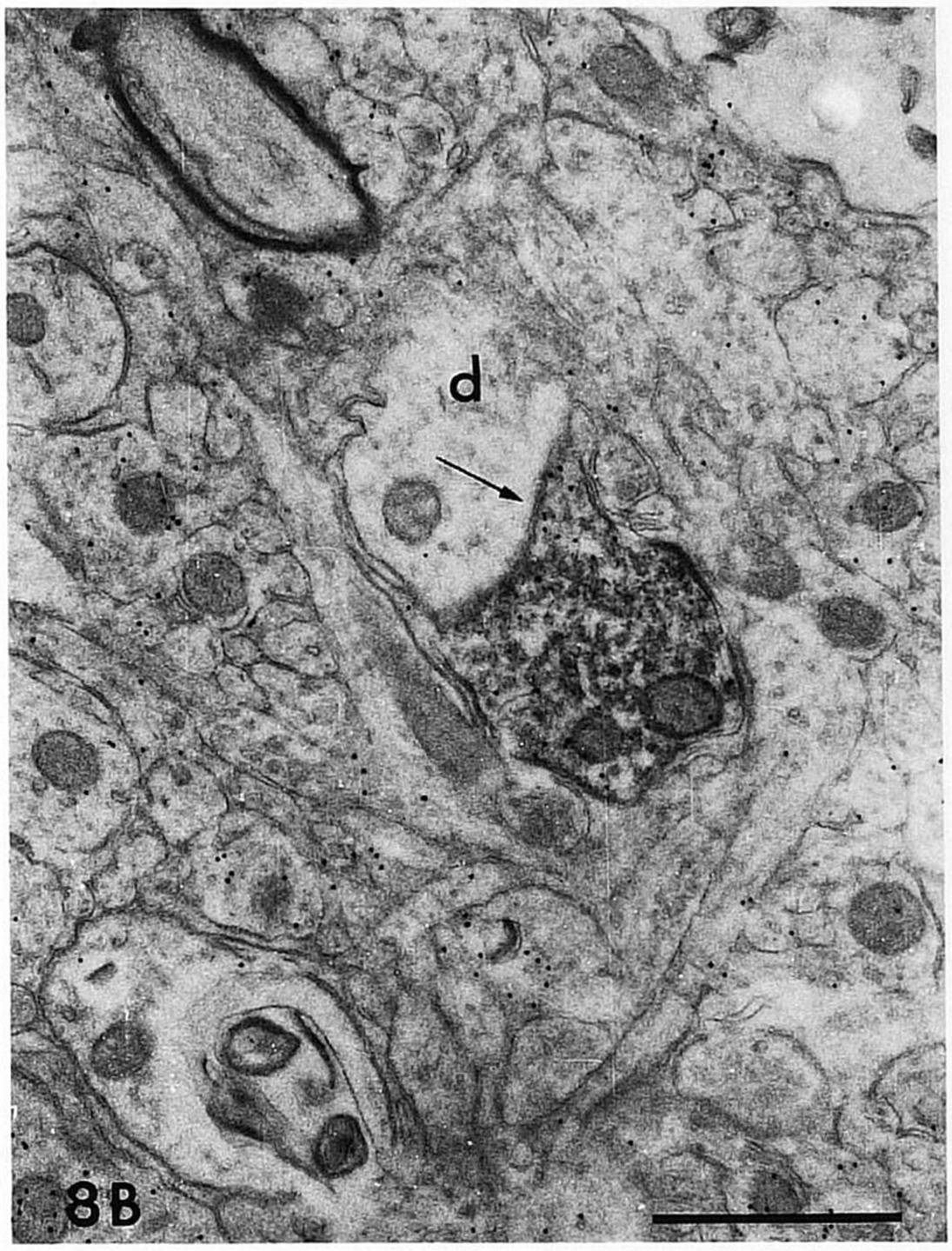
The application of postembedding immunogold labelling for GABA resulted in the visualisation of a large number of GABA-positive profiles within the SCN. Most cell bodies showed a low level of labelling with a slightly higher density of gold granules over the nucleus (Fig. 7A); most dendrites also showed light GABA staining. GABAergic axonal terminals could be distinguished by light to dense labelling, whereas non-GABAergic terminals were unlabelled (Fig. 7B). The very densely labelled GABA terminals in the SCN never showed any DAB precipitate. Terminals which contained DAB precipitate showed GABA labelling, the intensity of which varied from moderate to dense.

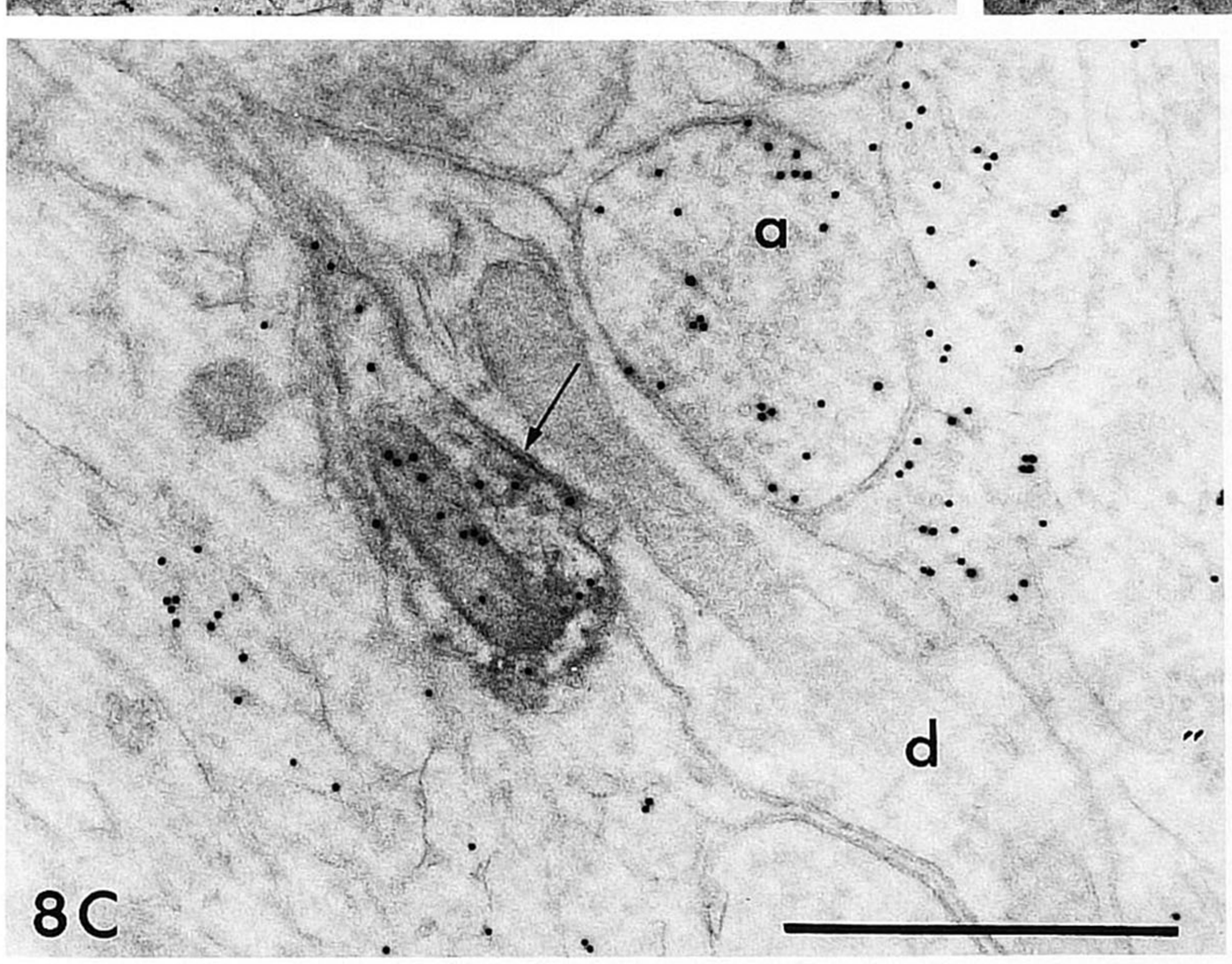
Although the gold labelling illustrating GABA was most easily distinguished in lightly DAB-stained terminals, the density of the gold labelling in dense DAB-filled terminals appeared to be no different from that in the lightly stained ones (Fig. 8A). If, depending on its size, a terminal contained less than 5 gold granules, it was considered to be negative. Most often, however, GABA-negative terminals were quite obvious just by the lack of any gold deposit. Because of the variation in injection sites in different subareas of the SCN, the proportion of DAB-labelled terminals also showing GABA-positive staining was not investigated quantitatively in all animals. However, we investigated at least 100 DAB terminals per animal (in a minimum of three sections in three animals) and established whether that terminal was DAB gold labelled or not. Thus we found that a minority of 20–30% of the PHA-L terminals were positive for GABA irrespective of the placement of the injection. Most frequently, PHA-L–GABA-positive elements contacted dendritic spines or shafts that simultaneously received synaptic input from other neurons (Fig. 7A,B). Frequently PHA-L–GABA-labelled axons contacted a dendrite that appeared to be clearly positive for GABA (Fig. 7B). Never did GABA-positive dendrites show intense gold labelling; at most only a few gold granules (five to ten) could be demonstrated on dendritic elements (Fig. 7A,B). Naturally, with such sparse labelling, it sometimes becomes very difficult to be absolutely certain whether a structure is GABA containing or not. However, most often, especially in other brain regions, dendritic elements are completely negative, devoid of any gold granules, indicating that there is hardly any background staining, thus facilitating a positive decision on the lightly labelled structures.

Dorsomedial hypothalamus

In the DMH, a mixture of PHA-L and PHA-L–GABA-labelled terminals could be visualised; most formed synap-







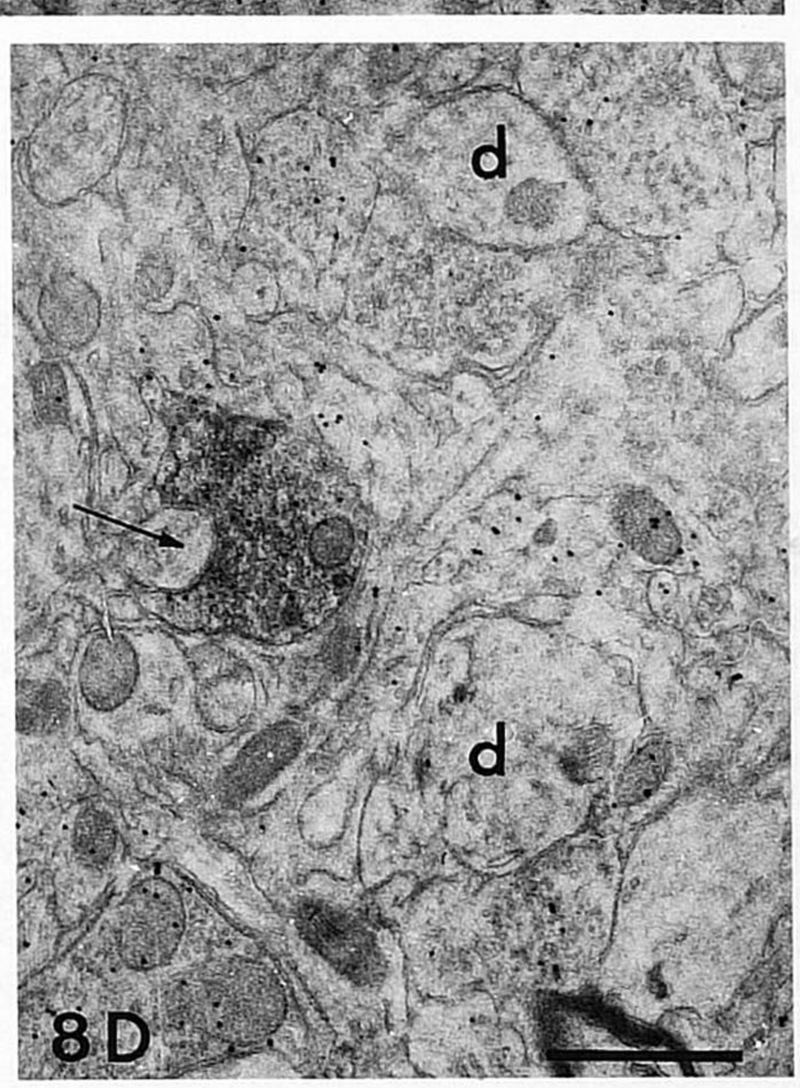


Fig. 8. PHA-L-labelled terminals with GABA labelling in the dorsomedial nucleus of the hypothalamus. **A:** PHA-L-labelled terminal with a very dense DAB precipitate. GABA labelling is evident (but not as easily seen) as 10 nm gold particles in the same terminal. **B:** PHA-L–GABA-labelled terminal (arrow) showing a synaptic specialisation with an unlabelled dendrite (d). **C:** Two GABA-labelled axons with 15 nm gold particles forming a synaptic terminal with the same unlabelled dendrite (d); one axon shows GABA labelling only (a), and the other axon is labelled for PHA-L and GABA (arrow). **D:** PHA-L–GABA-labelled terminal forming a synaptic junction (arrow) with an unlabelled dendritic spine. Note that unlike in the SCN, dendrites (d) show no GABA labelling. Scale bars = 1 μm.

tic contacts with dendritic elements (dendritic shafts and spines) (Fig. 8). Only occasionally did a PHA-L–GABA double-labelled terminal form a synaptic junction with a cell body. In contrast to intra-SCN terminals, the PHA-L- or the PHA-L–GABA-labelled terminals in the DMH did not contact other GABA-positive neuronal elements in the

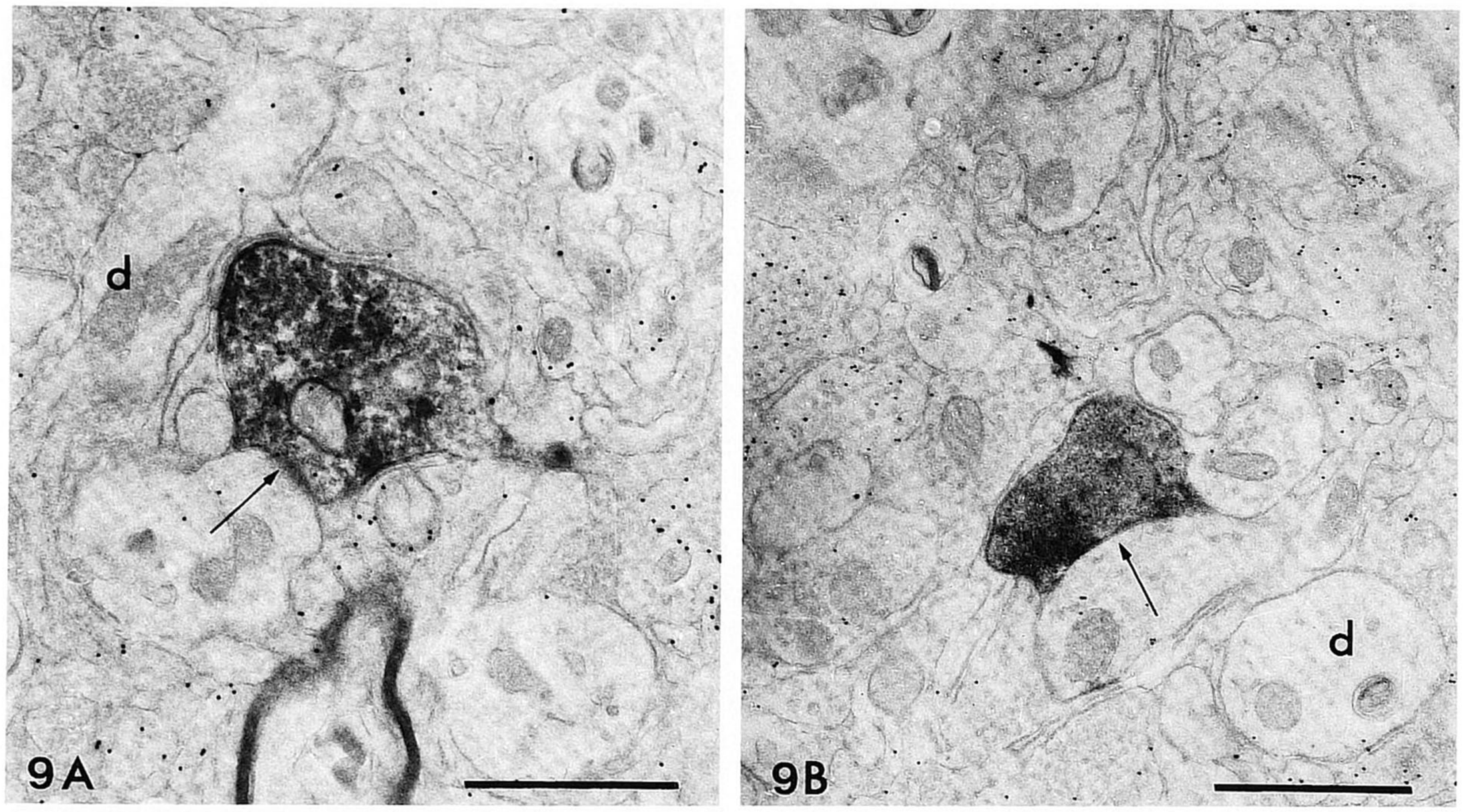


Fig. 9. **A,B:** PHA-L–GABA-labelled terminals in the periventricular part of the paraventricular nucleus forming a synaptic contact (arrows) with an unlabelled dendrite. Note the absence of staining in the other dendrites (d), while in B many other axons also show GABA labelling. Scale bars = 1 μm.

DMH. In general, few dendrites showed GABA labelling in the DMH (Fig. 8A–C). The number of PHA-L-labelled terminals that also showed GABA immunoreactivity was between 20% and 30%.

Paraventricular nucleus

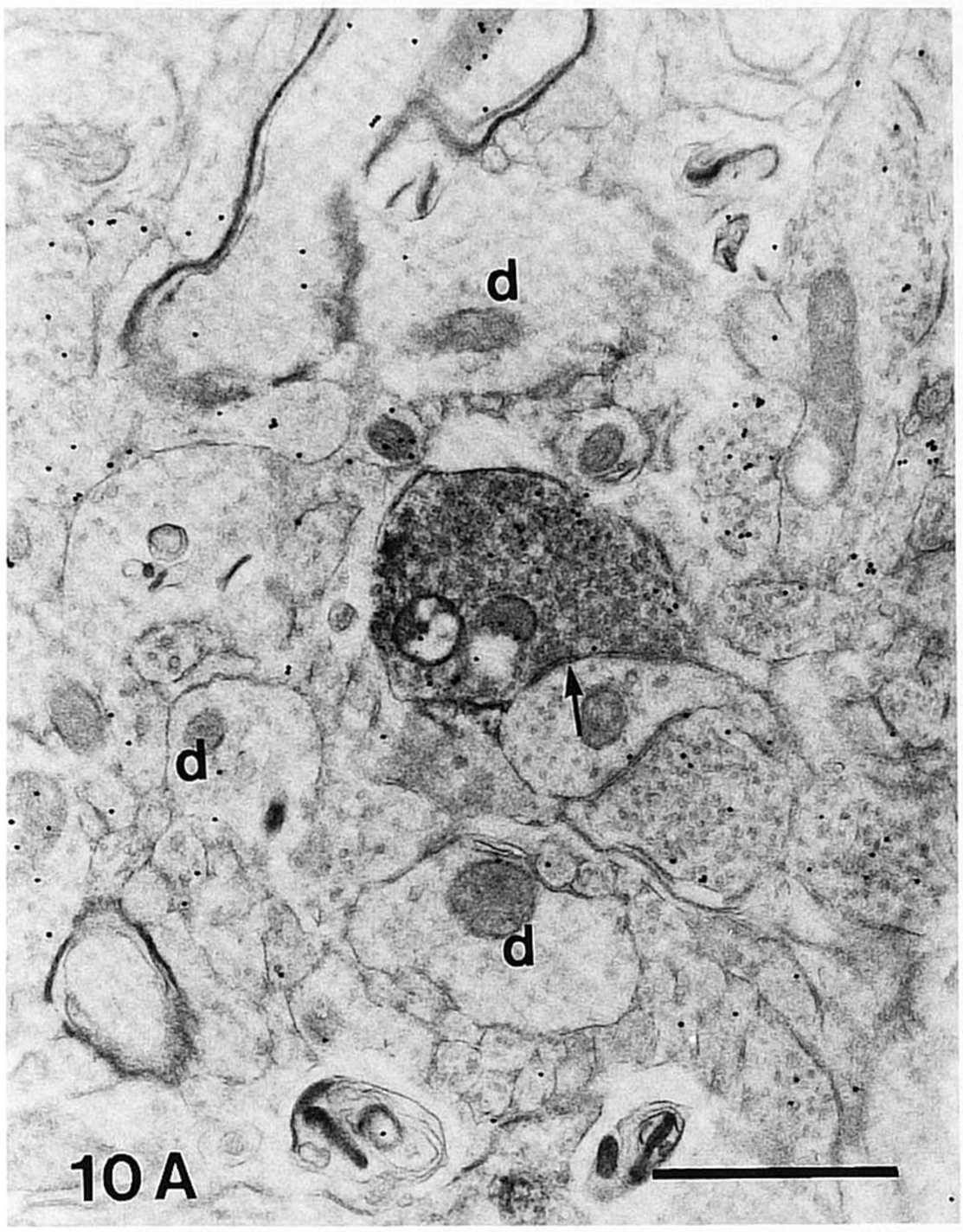
In the periventricular part of the PVN many PHA-L- and PHA-L–GABA-positive terminals could be seen to form synaptic junctions with cell bodies and dendritic shafts (Fig. 9A,B). For the electron microscope studies relating to the periventricular part of the PVN, the selected part is indicated by the arrowheads in Figure 4. Especially in the sub-PVN, most terminals could be seen on dendritic elements (Fig. 10A,B). Unlike in the SCN, the postsynaptic structures did not exhibit GABA-positive labelling. The number of PHA-L-labelled, DAB-positive terminals that also showed GABA immunoreactivity did not differ from the number observed in the contralateral SCN and in the DMH. In all three areas, approximately 20–30% of the PHA-L-labelled terminals also showed GABA immunoreactivity.

DISCUSSION

The light microscopic results of the present PHA-L tracing experiment provide additional information compared with the study of Watts et al. ('87). The present results indicate that, apart from the projection to the area just ventral of the PVN, the subparaventricular zone (sub-PVN), a substantial projection exists from the SCN to the dorsomedial nucleus of the hypothalamus which is at least equal in density to that of the sub-PVN. In addition, evidence is provided for an SCN projection to the periventricular part of the PVN. These findings are in complete agreement with earlier autoradiographic tracing experiments (Berk and Finkelstein, '81; Stephan et al., '81) or with SCN lesion studies followed by vasopressin staining (Hoorneman and Buijs, '82). Finally, the present study illustrates that both SCNs are coupled by a strong contralateral projection, which has also been reported by Pickard ('82) for the golden hamster. Apart from these light microscopic observations, the present ultrastructural study illustrates that the SCN does not only send fibers passing through the area of the PVN but that certain neurons are targeted in this nucleus. In a previous study we provided evidence that many of these neurons are involved in stress-related processes (Buijs et al., '93a). Apart from that study, no other information is available about the chemical or anatomical properties of the target neurons of the SCN.

The present results also illustrate that the rostral SCN projections extend to the preoptic area and the diagonal band of Broca. It is quite possible that this rostral projection reflects the recently demonstrated input of SCN-VIP neurons on gonadotrophin-releasing hormone (GNRH) neurons in these areas, thus forming the anatomical basis for the circadian control of GNRH release (Van der Beek et al., '93).

Our experiments demonstrate a vast network of fibers originating from the SCN in both the ipsilateral and contralateral SCN, suggesting that an important internal network of SCN fibers exists. The ultrastructural data on the strong reciprocal innervation of the SCN show that these fibers are not passing through the contralateral SCN but form synaptic contacts with specific neurons. The postembedding immunogold data show that part of this contralateral projection uses GABA as a neurotransmitter.



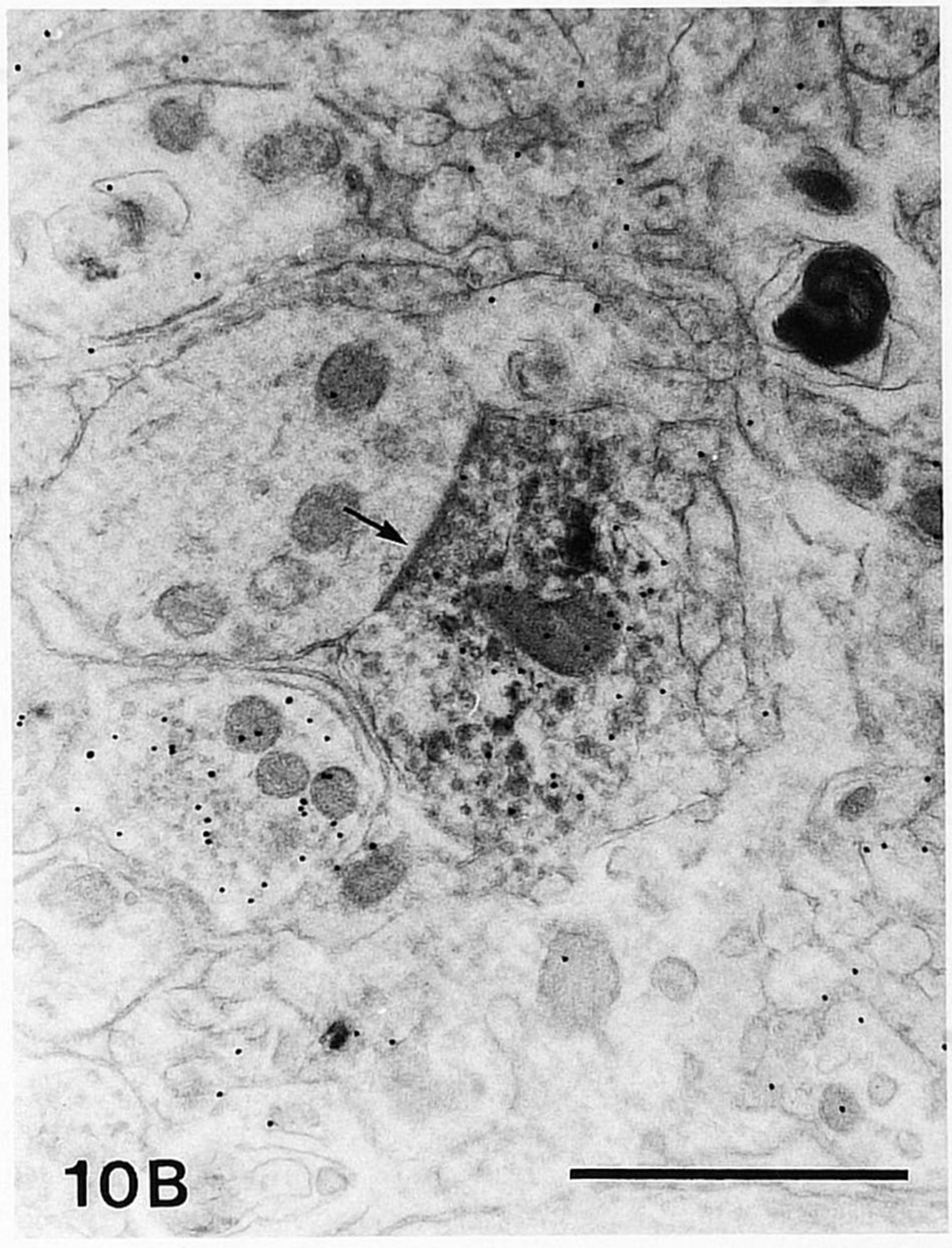


Fig. 10. **A,B:** PHA-L–GABA-labelled terminals in the sub-PVN forming synaptic contracts with unlabelled dendrites (arrows). In A the same dendrite receives a synaptic input from another terminal which is positive only for GABA. Note that other dendrites (d) are not GABA-positive. Scale bars = 1 μm.

The observation that only 20–30% of the PHA-L-labelled axons contain demonstrable quantities of GABA indicates that apart from this amino acid other transmitters are also used to carry information in this contralateral projection. This reciprocal innervation most probably serves to ensure the synchronisation of the two SCNs. It is assumed that an asynchrony of the two SCNs may be accountable for the occurrence of a split rhythm under constant conditions (Moore, '83; Rosenwasser and Adler, '86). Recent experiments aimed at proving this hypothesis using surgical transection of the connection between the two SCNs did not support this hypothesis, however (Yanovski et al., '90). Nevertheless, the present results demonstrate a strong neuronal integration between the two SCNs. Additionally, these results show that GABA most probably will play a role in this integration. This role, however, still needs to be elucidated.

Several experiments indicate the time-shifting effects of GABA agonists both in and outside the SCN (see for review, Turek and Van Reeth, '88). Recent studies aimed at specifying the site and method of action of these GABA agonists indicate that the SCN indeed might be a site of action for GABA agonists. However, these experiments also showed that GABA antagonists failed to induce time shifting (Smith et al., '90), suggesting that GABAergic neurotransmission may not normally be involved in the circadian timing system. Thus the normal physiological role for GABA in the intrinsic SCN projections is still obscure. In view of the largely inhibitory action of GABA in the SCN (Mason et al., '91) it would depend on the target neurons reached by these intrinsic GABA neurons as to whether their activation would further activate the SCN (if the target neurons are intrinsic and also GABAergic), or whether it would dampen the activity of the SCN (if the target neurons are intrinsic and contain an excitatory transmitter). Our observation, that the target neurons of GABA-containing SCN projections are frequently also GABAergic, illustrates that both options are possible. If, however, as some reports indicate (Okamura et al., '89; Moore and Speh, '93), all SCN cells are GABAergic, this must be a very powerful internal mechanism to shut down the activity of the SCN. The intrinsic and collateral projections furthermore indicate the possibilities for very fine tuning and stabilisation of the electrical activity of SCN neurons. To understand this process more precisely, a number of physiological and anatomical questions need to be addressed, as only a better knowledge of the underlying anatomical circuits and the resulting physiological responses will allow any conclusions on the functioning of the network.

Possibly all neurons in the SCN have the synthetic capacity for the enzyme GAD or contain GABA (Okamura et al., '89; Moore and Speh, '93), suggesting that in principle every neuron in the SCN synthesises GABA. Our results, which indicate that GABA can only be demonstrated in a relatively small portion of the SCN efferents, raise doubt as to whether every neuron uses this possibility to synthesise GABA; it might be that GABA is only used in part of the axonal processes. On the other hand, GABA

might be present in every terminal of the SCN but in too low a concentration to be detected using this postembedding technique. However, the possibility that GABA could be demonstrated in a large number of DAB and non-DAB-positive terminals, in confirmation of previous observations in the SCN (van den Pol, '86), indicates the validity of the present approach. Therefore the question can be raised as to whether GABA might be of physiological importance if its levels are too low to be detected using the present sensitive techniques. At the least, we illustrate that a small portion of the terminals originating from SCN contain GABA in high concentrations, while it is undetectable in the majority of terminals. This may indicate another more intriguing possibility, i.e., that GABA levels in the terminals fluctuate with the day/night cycle and with the activity in the SCN in such a way that all neurons use GABA as neurotransmitter but only during a certain period of the day/night cycle.

In agreement with previous tracing experiments which used tritiated amino acids and autoradiography as detection methods (Stephan et al., '81; Berk and Finkelstein, '82) it was demonstrated here that the DMH can be considered an important target zone of SCN fibers. SCN lesion studies in combination with vasopressin and VIP staining have indicated that primarily vasopressin fibers from the SCN reach the DMH (Hoorneman and Buijs, '82; Kalsbeek and Buijs, '92; Buijs et al., '93b). The present results indicate that apart from VP and VIP, SCN projections to the DMH and to the peripheral parts of the PVN utilise GABA as neurotransmitter. This large overlap with other known peptidergic projections of the SCN suggests that this amino acid neurotransmitter might be colocalised with one or more of the peptidergic neurotransmitters of the SCN.

The elucidation of the pathways of the SCN is of considerable interest for those who study the mechanisms used by the biological clock to enforce its rhythm onto the rest of the central nervous system. Although the question of whether the SCN is able to transmit its information by means of diffusion is not completely resolved (see, e.g., Lehman et al., '87), it seems that experiments with transplanted SCNs illustrate that the existence of defined neuronal connections is not necessary, per se, for the expression of certain behavioural rhythms. This may be concluded from the fact that successful SCN transplants that restored overt behavioural rhythms often failed to show outgrowth of the graft into the host (Lehman et al., '87; Canbeyli et al., '91). This, however, does not imply that under normal physiological conditions the transfer of SCN information is mediated by diffusion via the cerebrospinal fluid. The present results provide ultrastructural evidence that SCN neurons are able to target specific neurons in the CNS. Moreover, morphological evidence is presented at the ultrastructural level showing that the inhibitory neurotransmitter GABA is available in projections of the SCN. These results indicate that it is doubtful that the SCN is able to express its full function by reaching its target neurons by means of diffusion of its messenger molecules. This method might be feasible for peptidergic molecules, but for the highly degradable and uptake-sensitive amino acid GABA, such a messenger role by diffusion seems highly unlikely.

One of the most intriguing aspects of the present observations is the possible physiological role of this amino acid neurotransmitter in these SCN projections. As seems logical to assume from the inhibitory effects of GABA (Mason et al., '91), the SCN could use these projections to inhibit several systems in the CNS. In this regard, several primary questions need to be addressed: At what moment of the day/night cycle will GABA be released? What are the actual target neurons in- and outside the SCN reached by these GABAergic efferents?

SCN neurons in general have a higher activity rate during the light phase of the light/dark cycle (Inouye and Kawamura, '79). SCN neurons also maintain this higher activity during the subjective light phase in constant dark conditions or even in vitro (Groos and Hendricks, '82; Gillette and Reppert, '87; Bos and Mirmiran, '91). These observations may indicate that GABA is released during the light period and thus may serve to inhibit neurons in the DMH and the peripheral PVN and in other regions where SCN GABA efferents may terminate. This suggests that during the (subjective) light period, at least in rats, the SCN may use GABA to inhibit a number of processes. These processes may range from behavioural activity to corticosteroid secretion. This may be congruent with our recent observations using SCN lesions in which we concluded that the SCN inhibits corticosteroid secretion more during the light than during the dark period (Buijs et al., '93a).

Such an inhibitory function may hold for the SCN in rodents in general; the higher electrical activity of the SCN during the light period suggests that the SCN is actively involved in inhibiting several behavioral and hormonal processes. Our results indicate that GABA may be used in this function.

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