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THE SUPRACHIASMATIC NUCLEUS IN STATIONARY ORGANOTYPIC CULTURE

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Abstract—Suprachiasmatic nuclei, derived from neonate rats, were maintained for several weeks in stationary organotypic culture. Hypothalamic slice explants, supported by Millicell filters and incubated in Petri dishes containing serum-based medium, flattened appreciably, yet preserved the organization of the suprachiasmatic nucleus and the surrounding hypothalamic tissue. After two to three weeks, cultures were fixed, and three neuronal sub-populations were identified as vasopressinergic, vasoactive intestinal peptide-containing, or GABA-containing. The GABAergic component of the cultured suprachiasmatic nucleus was particularly profuse, projecting extensively into the hypothalamic slice. Unilateral ablation of the nucleus in the explant dramatically reduced ipsilateral GABA-immunoreactivity in the slice. Explants in which an incision separated the bilateral suprachiasmatic nucleus from the paraventricular nucleus, deprived the latter of its fine-caliber GABA-immunoreactive input. Extra- or intra-cellular electrophysiological recordings from the suprachiasmatic nucleus were obtained in 51 of 58 cultures. The electrical properties of the long-term cultured suprachiasmatic nucleus were similar to those recorded in acute slices from adult rats. In six cultures recordings were extended for up to 10–24 h.

Within long-term stationary organotypic cultures of the suprachiasmatic nucleus, sub-populations of neurons, intrinsic to the nucleus *in vivo*, were identified immunocytochemically. Lesion studies supported the observation that the main source of the GABAergic innervation within the entire hypothalamic slice explant appeared to be the suprachiasmatic nucleus. Electrophysiological studies confirmed the viability of the long-term cultured nucleus and revealed changes in spontaneous electrical activity that may indicate circadian fluctuation.

Key words: long-term culture, neuropeptides, GABA; electrophysiology.

The hypothalamic suprachiasmatic nucleus (SCN) contains a master circadian pacemaker that regulates most, if not all, circadian rhythms relating to mammalian physiology and behavior.[25,37,38] The SCN is a small bilateral well-circumscribed nucleus, in the rat consisting of about 8000 cells on each side.[39] As aptly stated by Inouye *et al.*:[23] "This is one of the rare instances in neuroscience in which overt behavior can be safely attributable to the activity of a very limited group of cells in the brain". However, while the anatomical locus of the brain's circadian clock has been defined, the mechanism of its autonomous oscillatory activity is not yet understood. The intense current interest in the SCN includes the quest for experimental models that might facilitate analysis of rhythmicity at cellular and molecular levels.

In vivo, pacemaker activity in the SCN is normally entrained to environmental day/night cycles via retinal input. However, even when this input is interrupted surgically[22] or by continuous darkness,[25,37,38] endogenous circadian rhythmicity within the nucleus persists. *In vitro*, the SCN may be isolated as an explant, creating a simpler experimental entity deprived of retinal and cerebral inputs. Short-term explants of the SCN (one to three days) have provided valuable basic information regarding circadian rhythmicity of neuronal firing (reviewed in Ref. 17), secretion of vasopressin[15,18] and *in vitro* glucose utilization.[27] However, one of the difficulties of investigating SCN rhythmicity concerns the protracted period of the circadian cycle, requiring extended survival of the *in vitro* preparation in order to complete a controlled experiment. Long-term organotypic culture of the SCN is a more recent innovation, embodying the potential for recording rhythmogenesis over multiple cycles, establishing the capacity for sustained circadian secretion of ligands endogenous to the nucleus, and analysing how the isolated SCN adapts to deafferentiation. Hypothalamic slice explants containing the SCN have been maintained for several weeks using the roller-tube technique[36,42] and free-floating in a rocking Petri dish.[7,8]

The present report describes a novel stationary long-term organotypic culture procedure for the SCN, including some of its experimental applications. The method is based on a recent innovation by Stoppini *et al.*,[34] and incorporates aspects of the Wray

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Abbreviations: ABC, avidin biotin peroxidase complex, ACSF, artificial cerebrospinal fluid; DAB, diaminobenzidine; PVN, paraventricular nucleus; SCN, suprachiasmatic nucleus; VIP, vasoactive intestinal peptide; VP–NP, vasopressin-neurophysin.

technique for hypothalamic slice explants.[42,43] Advantages of the present procedure include simplicity of handling, high yields of viable cultures, superior structural features and ready amenability to electrophysiological recording. Brief preliminary reports have been made.[3–5,41]

EXPERIMENTAL PROCEDURES

Animals

Pregnant Sabra rats of the Hebrew University Medical School strain were housed in the animal facility under standard conditions, with food and water *ad libitum*, and a 12:12 h light/dark cycle, lights on at 6.00 a.m. Pups were used for experiments seven to nine days post-natal.

Culture procedure

Three hours after lights on pups were taken from the animal house, and kept in a warmed and padded box, until the entire litter was used over a period of about two h. The head and neck of each pup was swabbed with 70% alcohol, prior to swift decapitation, after which the brain was dissected out. Hypothalami were excised and sectioned at about 400 μm on a modified Sorvall TC2 chopper. For chopping, a block of tissue containing the hypothalamus was placed on a sterile dry Teflon stage, to which it adhered, no glue or plasma clot being required. Sections were transferred into drops of glucose-enriched (5 mg/ml) Gey's balanced salt solution (GIBCO) in a Petri dish, and those presumed to contain the SCN were selected with the aid of an inverted microscope. Landmarks were the third cerebral ventricle, the optic chiasm and the ventral twin bulges of the nucleus itself. Selected sections, trimmed dorsally at the level of the anterior commissure and laterally at the supraoptic nuclei, were kept in drops of Gey's solution at 4°C for 1–2 h. Explants were then placed on Millicell-CM filter inserts (Millipore, pore size 0.4 μm, diameter 30 mm), as recommended by Stoppini *et al.*[34] Slices adhered to the filters within a day, with no need for plasma clots or other strategies to promote adherence, as is required for roller-tube culturing. Several slices from the same brain were planted on a single filter, although only one or two slices at most were found to contain the SCN after two or three weeks in culture. Each filter insert was placed in a Petri dish (35 mm diameter), containing 1.1 ml culture medium, wetting the exposed surfaces of the explants, but not submerging them. Excessive evaporation was not encountered, and medium was replaced about twice weekly. Incubation of the cultures was stationary, at 36°C in 5% CO_2 enriched air.

The culture medium was composed of heat inactivated horse serum (25%), Eagle's basal medium (50%), Hank's or Earle's basal salt solution (25%), supplemented with 5 mg/ml glucose, 1.0 mM glutamine, 25 μg/ml penicillin, 25 μg/ml streptomycin and 50 μg/ml neomycin (all from GIBCO). Antibiotics were omitted after the first three days of culture.

Fixation

After two to three weeks in culture, explants were immersed in fixative, appropriate for both light and electron microscopy. Fixative solutions contained 2–4% buffered glutaraldehyde, or a mixture of 0.1% glutaraldehyde, 4% paraformaldehyde and 0.2% picric acid in 0.1 M cacodylate buffer, pH 6.9. Fixation lasted about 4 h at room temperature, after which tissue was stored for up to a week in dilute fixative (1:4) at 4°C until further processing.

Immunocytochemistry

Vasopressin–neurophysin (VP–NP), vasoactive intestinal peptide (VIP) and GABA were localized using monoclonal anti-VP–NP antibodies,[6] polyclonal anti-VIP antiserum (Incstar) and anti-GABA antiserum (code No. 09, courtesy of Dr Peter Somogyi). All antibodies were well-characterized, and had been used by us previously for study of the intact rodent SCN, using immunocytochemical procedures as previously described.[11,13] Briefly, for pre-embedding immunoperoxidase procedures, explants on supporting filters underwent the following 30 min steps, interspersed with appropriate buffer or distilled water rinses: 0.1% H_2O_2 in acidified methanol (for light microscopy only); 0.5% borohydride in Tris-buffered saline; and blocking solution containing 2% egg albumin, 0.5% glycine, 0.5% lysine and 0.9% sodium chloride in 0.5 M Tris buffer. Triton X-100 (0.5%) was added for light microscopy only. Primary antibody incubation was overnight (anti-VP–NP 1:10; anti-VIP 1:2,500; anti-GABA 1:25,000), followed by the appropriate biotinylated secondary antibody, then avidin–biotinylated horseradish peroxidase (Vectastain Elite ABC kit). The chromogen was 3,3′-diaminobenzidine tetrahydrochloride (DAB 5 mg/10 ml) with 6 μl 30% H_2O_2. For light microscopy cultures were lifted as whole mounts onto subbed slides, treated with dilute osmium tetroxide (0.1%), dehydrated, cleared and cover-slipped with Entellan (Merck). For ultrastructural study cultures were post-fixed in a mixture of 1% osmium tetroxide and 1.5% potassium ferrocyanide in cacodylate buffer, dehydrated in ascending concentrations of ethanol up to 70%, stained en bloc with 5% uranyl acetate in 70% ethanol at 4°C overnight, after which dehydration was completed to 100% ethanol. Individual cultures were excised together with a portion of the supporting filter, infiltrated with resin (EM-BED-812, Electron Microscopy Sciences) and flat embedded with the aid of pre-formed resin blocks made in inverted Beem capsules. The fragments of adhering filter were not an impediment to sectioning the tissue.

Ultrathin sections were collected on nickel grids for post-embedding immunogold labeling. Briefly, sections were etched in saturated aqueous sodium metaperiodate, dipped in 1% aqueous sodium borohydride, and incubated in Tris–HCl (pH 7.4), containing 0.85% NaCl, 0.2% bovine serum albumin, 0.13% NaN_3 and 0.5% Tween-20, followed by 5% normal goat serum as blocker. Incubation in primary antibodies (anti-VP–NP 1:1, anti-VIP 1:500, anti-GABA 1:5000) was for 3 h at room temperature, followed by gold probes (goat anti-mouse 10 nm gold or goat anti-rabbit 12 nm gold, Jackson Immunoresearch Labs.) diluted in Tris-HCl (pH 8.2), for 3 h at room temperature. After immunogold labeling grids were post-fixed in 2.5% glutaraldehyde (15 minutes), and then contrasted with saturated aqueous uranyl acetate and Reynold's lead citrate. Grids were viewed and photographed in JEOL-100 CX or Philips 300 electron microscopes.

Specificity of immunolabeling was verified by increasing dilutions of primary antibodies, or substitution with normal sera. Absorption controls for antisera had been carried out previously.[11–13]

Dissection of the slice explant

The purpose of this set of experiments was to establish to what extent the profuse GABAergic innervation within the entire hypothalamic slice explant originated from the SCN. A mini-scalpel was devised by inserting a minute fragment of breakable blade into a holder. Unilateral ablation of the SCN was performed on 19 slice explants prior to planting on filters. For ablation, three incisions were made: the first slightly lateral to the left SCN, the second at the mid-line between the two halves of the nucleus and the third slightly dorsal to the left SCN. The fragment of excised tissue, containing the left half of the SCN, was discarded. In another 10 explants a horizontal cut was made, separating the bilateral SCN from the more dorsal parts of the slice including the hypothalamic paraventricular nucleus (PVN). Both dorsal and ventral parts were cultured

together in normal alignment. After two to three weeks cultures were fixed and processed pre-embedding for GABA immunoreactivity, using the ABC procedure described above.

Electrophysiology

Electrical recordings were made from one to three-week-old cultures. The bilateral SCN was visually identified using a dissecting microscope, by its location at the base of third ventricle and its pale almost round shape. Millicell filter inserts, bearing cultured explants, were transferred to an oval-shaped recording chamber, about 3.5 cm wide and 5.0 cm long. Four holes, about 2 mm diameter, were bored in the walls of the filter insert with the aid of a heated needle, to insure free exchange of solutions between filter insert and chamber. Cultures were transferred to artificial cerebrospinal fluid (ACSF), an isotonic serum-free medium, to which they were acclimated for at least 2 h. The chamber was continuously perfused with (ACSF) at a rate of 2–4 ml/min, and temperature was maintained constant in the range of 34–36°C. Cultures were submerged in ACSF during recording. The composition of the ACSF (mM) was: NaCl 124; KCl 5; $MgSO_4$ 1.3; KH_2PO_4 1.2; $NaHCO_3$ 26; $CaCl_2$ 2.4; glucose 10, (pH 7.4), aerated with 95% O_2 and 5% CO_2. Test substances [tetrodotoxin (TTX) 2 μM; $NiCl_2$ 1 mM] were administered via the perfusion fluid.

Extracellular recordings were made in the current-follower mode (Axopatch-1D amplifier) using patch electrodes (6–16 MΩ) filled with ACSF. Electrical signals were stored on videotape using a Neurodata DR-484 and analysed by LabView 2.2.1 data-aquisition and programming system. The average frequency of cell firing, the reciprocal of the average interspike interval, was calculated from 200 s period of continuous recording. For detection of possible circadian rhythmicity, spontaneous activity of randomly selected cells was recorded, about three cells per hour, over periods of 10–24 h.

Brain slice preparations were derived from adult male Sabra rats kept on a 12:12 light/dark regime (light on 6.00 a.m.) for at least two weeks. Wheel-running activity was monitored by a DATAQUEST III activity monitoring system (Mini-Mitter, Oregon). Animals showing distinct circadian locomotor rhythmicity were selected for experiments. Anaesthetized rats were decapitated 2–4 h after lights on, and the brain was rapidly removed and placed in aerated (95% O_2, 5% CO_2) ice-cold ACSF for approximately 5 min. Each hypothalamus was Vibratome sliced at approximately 400 μm, and one appropriate slice was pre-incubated in ACSF for at least 1 h at room temperature prior to recording.

RESULTS

General observations

After one to three weeks in stationary culture, slice explants flattened appreciably. However, the twin bulges of the SCN remained somewhat thicker than the surrounding tissue, as has been observed also in roller-tube cultures.[36,42] Explant survival was excellent, only about 5% of the slices being discarded as unfit for experiments, due to detachment from the filter or shriveling. Immunolabeling, as an indication of viability and normal development of the cultured SCN, was invariably effective. Labeling of three neuronal phenotypes endogenous to the SCN, vasopressinergic (Fig. 1), VIP-containing (Fig. 3) and GABAergic (Fig. 4), confirmed the predicted location of the SCN within the slice explant. Moreover, the general structural features of the surrounding hypothalamic tissue resembled those of normal adult brains. Gross structural deformation of the surrounding tissue, often encountered in roller-tube cultures,[36] was never observed. Tiers of ependymal cells usually filled the space of the ventricular lumen, but unlike roller-tube cultures, the ependyma did not encroach upon the hypothalamic parenchyma.[42] Immuno-ultrastructural features of the stationary cultures (Figs 2, 3, 5) were superior to those of roller-tube cultures, which may be marred by vacuoles, lacunae and distended extracellular space. Electrical activity was readily detected in 88% of the SCN slice explants from which recordings were attempted, and there were preliminary indications of circadian changes in the rate of spontaneous electrical activity.

Vasopressinergic innervation

Numerous intensely labeled VP–NP-immunoreactive somata were evident throughout the cultured SCN, most often within the dorsomedial region (Fig. 1a–c), as is the case also in the *in vivo* SCN. Within the hypothalamic slice, labeling of vasopressinergic neurons outside the SCN was associated mainly with the PVN and with scattered periventricular magnocellular neurons. In culture the average area of vasopressinergic somata within the SCN was 112.8 μm^2 ($\pm$20.4), versus 81.4 μ^2 ($\pm$16.7) *in vivo*, which is commensurate with the overall flattening and spreading of the explants. Immunoreactive processes, profuse within the confines of the SCN, also extended beyond the nucleus into the sub-paraventricular zone (Fig. 1a). Vasopressinergic processes from the SCN sometimes spanned the space of the third ventricle or extended ventrally into the area that had been occupied by the optic chiasm, which degenerates in culture.

At the ultrastructural level ovoid VP–NP-immunoreactive somata contained well-preserved organelles typical of peptidergic neurons (Fig. 2a). Somata were postsynaptic to a variety of terminals, and double labeling with post-embedding immunogold showed that a considerable number of these terminals were GABAergic (Fig. 2c). Numerous VP–NP-immunoreactive synaptic terminals were also evident, some of these impinging on VP–NP-positive somata or dendrites (Fig. 2b), although in other cases the chemical identity of the postsynaptic element was unknown. Similar features have been described also in the adult SCN,[13] although homologous innervation was a rare finding *in vivo*, whereas in thin sections derived from cultured SCNs, many grids yielded images of homologous synapses. Since the VP–NP component of the SCN labels only sparsely in neonatal rats (not shown), from which the explants are derived, the proliferous vasopressinergic innervation of the SCN after a few weeks in culture attests to robust *in vitro* maturation, as has been reported also in roller-tube cultures.[36,42]

Abbreviations used in the figures

IIIv	third ventricle	rer	rough endoplasmic reticulum
D	primary dendrite	S	soma
G	Golgi complexes	SPV	sub-paraventricular zone
l	lysosomes	sv	synaptic vesicles
m	mitochondria	T	terminal
N	nuclei	VP	vasopressinergic soma
nt	neurotubules		

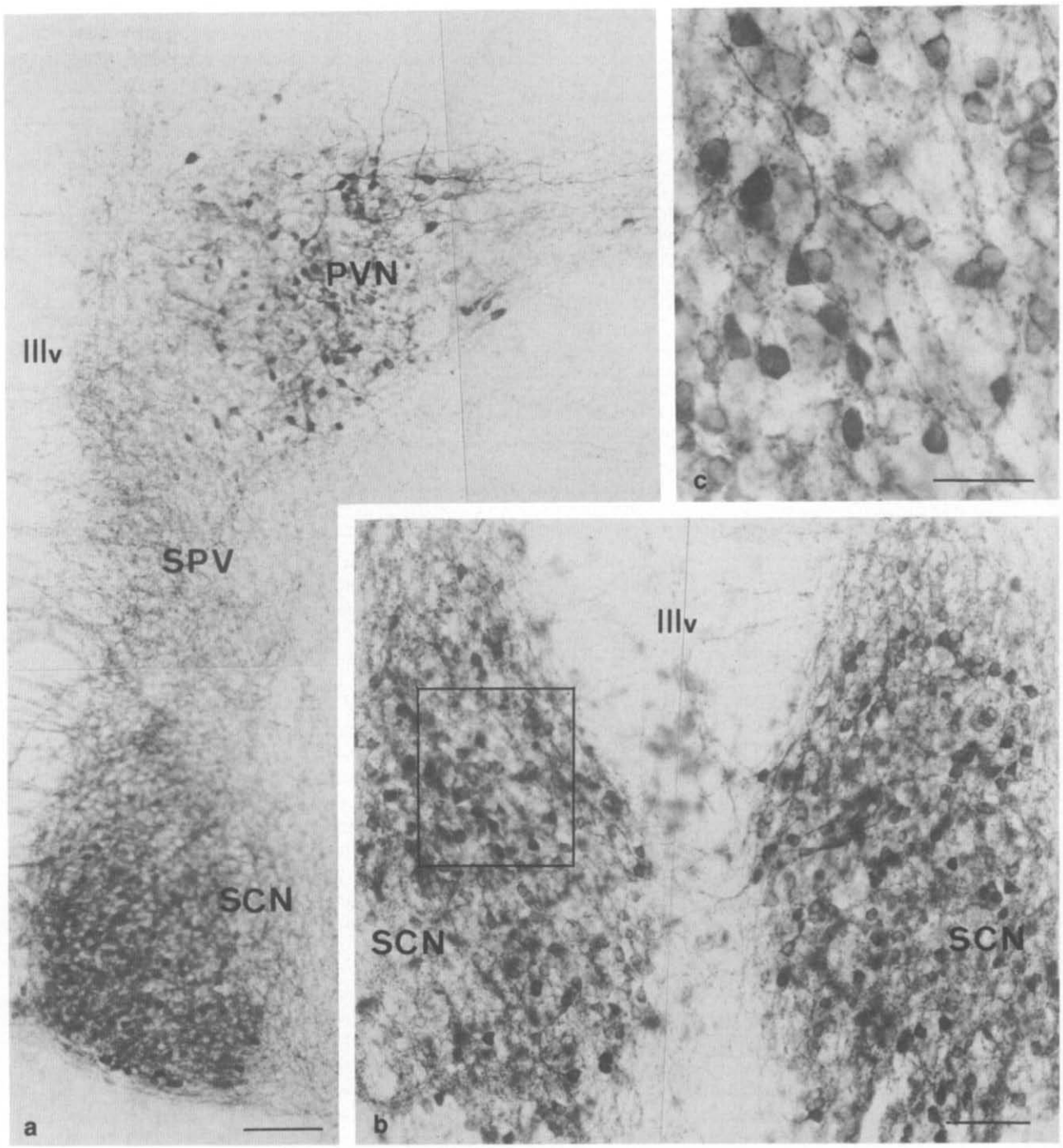

Fig. 1. Slice explant cultures containing the SCN, 20 days *in vitro*. Immunoperoxidase labeling for VP–NP. (a) Low-power micrograph showing one side of the slice explant; vasopressinergic somata and processes in the SCN and PVN. The SPV is traversed by immunoreactive processes originating from the SCN; some fibers also cross the space of the IIIv. (b) Medium-power micrograph showing bilateral SCN containing dense aggregation of immunoreactive cells and processes close to the base of the IIIv. (c) High-power micrograph of portion of (b) square showing immunoreactive somata with intensely stained cytoplasm and pale nuclei; note varicose processes. Scale bars: a, 150 μm; b, 100 μm; c, 50 μm.

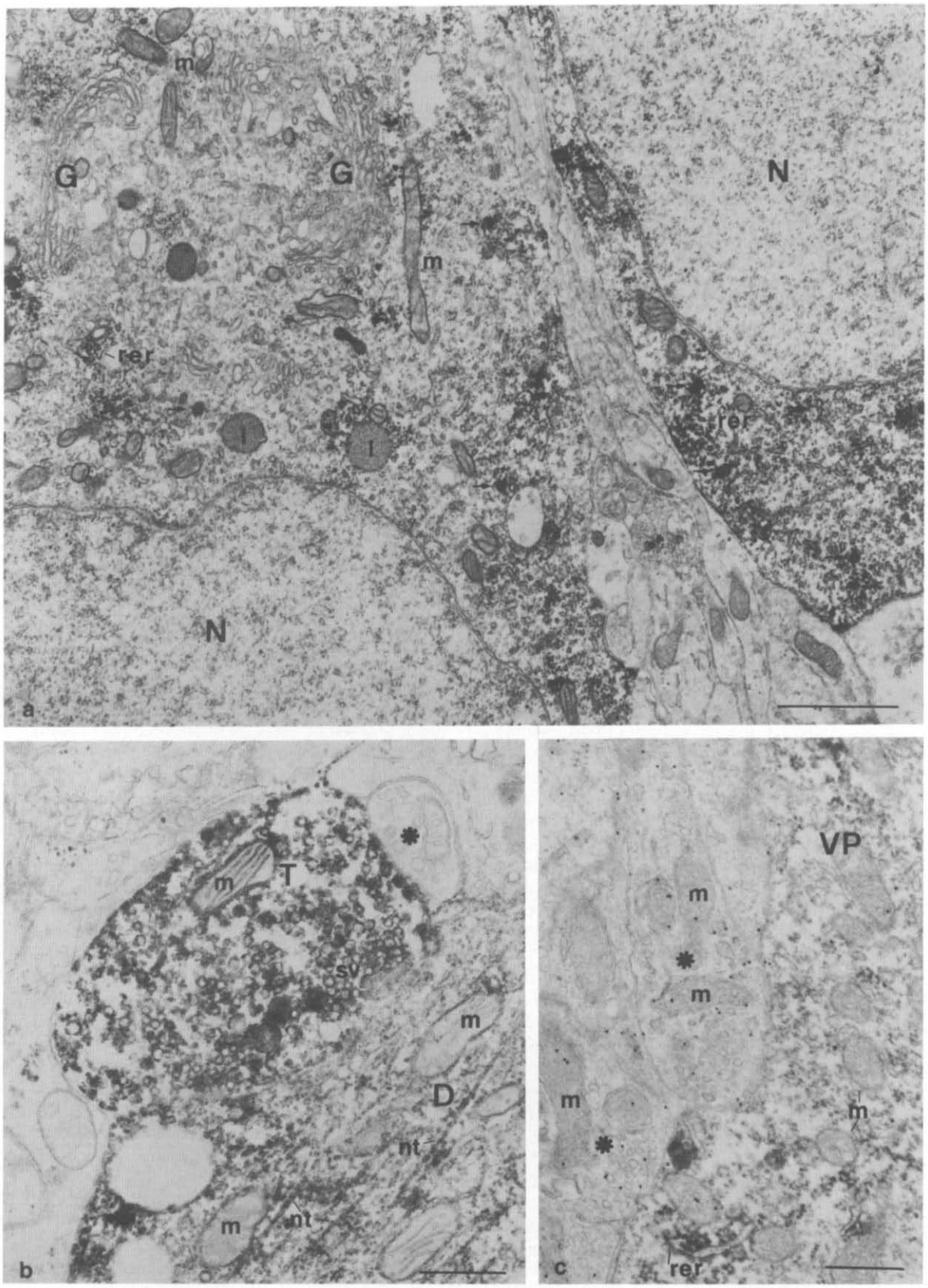

Fig. 2. Electronmicrographs showing immunoperoxidase VP–NP labeling in 20-day (a,c) and 14-day (b) SCN cultures. (a) Portions of two immunolabeled somata; reaction product is most intense over secretory granules (small arrows) and rough endoplasmic reticulum. Mitochondria, Golgi complexes, lysosomes and nuclei are unstained. (b) Immuno-labeled axon terminal impinging upon homologously-labeled primary dendrite. The terminal contains numerous electron-lucent synaptic vesicles and a few dense-core vesicles. Asterisk indicates small unlabeled terminal closely adjacent to the immunoreactive terminal. (c) Double-immunocytochemical labeling showing two immunogold-labeled GABAergic terminals (asterisks) impinging on an immunoperoxidase stained vasopressinergic soma. GABA-immunoreactivity is mainly over synaptic vesicles and mitochondria. Scale bars: a, 1 μm; b and c, 0.5 μm.

Vasoactive intestinal peptide-containing innervation

VIP-immunoreactive somata were most conspicuous in the ventrolateral SCN culture (Fig. 3a,b), as is also the case *in vivo*.[10] The average area of VIP-labeled somata in culture was 104.2 μm^2 ($\pm$25.5), compared with 76.7 μm^2 ($\pm$ 14.5) *in vivo*. Immunoreactive processes, abundant throughout the SCN, extended dorsally beyond the nucleus (Fig. 3a), albeit not as far as nor with as high a density as in the adult SCN. Adequate immunolabeling of VIP-containing elements in the cultured SCN was obtained only after low-glutaraldehyde (0.1%) paraformaldehyde–picric acid fixation, whereas in the adult SCN high-glutaraldehyde (4–5%) fixatives invariably promote rich VIP labeling (not shown).

The ultrastructure of cultured VIP-containing somata was of the regular peptidergic type, showing extensive rough endoplasmic reticulum, Golgi complexes and secretory granules (Fig. 3c). VIP-immunoreactive axonal boutons were readily apparent, terminating on both non-immunoreactive as well as on homologous elements (Figs 3d,e, 5a). Homologous VIP innervation occurs also *in vivo*, but is rare (unpublished observations) compared with that in the cultured SCN.

GABAergic innervation

Light microscopic immunoperoxidase labeling revealed extensive and well-defined GABA-immunoreactivity associated with the SCN and its projections (Figs 4a,b, 6, 7). .In contrast, roller-tube cultures of the SCN yielded only sporadic GABAergic neurons with sparse projections.[42] Similar immunoperoxidase labeling of GABAergic neurons in the SCN of the adult rat elicits a smudged and diffuse immunoreactivity lacking clarity of cellular elements (not shown). Within the hypothalamic slice explant the overwhelming majority of GABAergic somata were concentrated within the SCN (Figs 4a, 6). A small number were also found outside the SCN, notably within the PVN, although these were far fewer than those reported by Okamura *et al.*[28] in the adult rat hypothalamus. The GABAergic somata within the SCN were smaller than those outside the nucleus [103.2 μm^2 ($\pm$21.8) versus 183.2 μm^2 ($\pm$47.8)], and their varicose processes were of finer caliber (Figs 4a, 6, 7). The dense immunoreactive plexus within the nucleus appeared to be continuous with the extensive arborization of GABAergic processes in the periventricular region, the sub-paraventricular zone and the PVN (Fig. 4a). Some processes also passed between the two sides of the bilateral SCN, while others extended into the ventricular space or projected ventrally into the area that had been occupied by the optic chiasma. Profuseness of GABA-labeling, as regards both the intrinsic and the efferent components of the SCN, increased with the age of the culture.

Ultrastructurally, immunoperoxidase staining was dense within GABAergic somata, processes and terminals (Figs 4d, 5c,d). Immunogold labeling provided better resolution of GABAergic terminals, showing gold particles over synaptic vesicles and mitochondria (Fig. 2c). GABAergic terminals impinged upon a large variety of profiles, including VP–NP and VIP-containing elements; homologous associations (GABA on GABA) were also evident (Fig. 5d), a phenomenon that we have not encountered *in vivo*. GABAergic boutons also featured prominently within heterogeneous synaptic glomeruli (Fig. 5c).

Dissection of the slice explant

As described above, the SCN in stationary culture is not only exceptionally well-endowed with intrinsic GABAergic neurons, but these also project extensively into the surrounding hypothalamic tissue. Unilateral ablation of the SCN in all instances ($n = 19$) caused a dramatic ipsilateral reduction in density of fine-caliber GABAergic processes, while the contralateral density remained apparently unchanged (Fig. 6). This suggested that in the slice explant cultures, the SCN may be the main source of GABA within the surrounding hypothalamic tissue. Within the bilateral PVN, intrinsic GABA-immunoreactive neurons were characterized by large somata with thick radial projections. On the side on which the SCN had been left intact, a multitude of fine varicose fibers entered the PVN vertically, but on the side from which the SCN had been excised, these fine-caliber GABA-immunoreactive fibers were greatly reduced (Fig. 6). The intact half of the SCN may be responsible for the few GABAergic projections that grew into the lesioned side of the hypothalamic slice, since in the adult rat the two halves of the bilateral SCN are connected by reciprocal projections, some of which are GABAergic.[9] Following ablation, ectoptic or "errant" GABA-immunoreactive cells, apparently

Fig. 3. VIP-immunolabeling of 20-day SCN cultures. (a) Micrograph of unilateral SCN showing VIP-immunoreactive somata concentrated ventro-laterally, varicose processes dorsally. Space of third ventricle is lined with tiers of ependyma (staining is artefactual). (b) Higher magnification micrograph of portion (square) of 3a, showing variance in immunolabeling density of VIP-containing somata. (c) Electronmicrograph of portion of VIP-containing soma; immunoperoxidase reaction product is mainly over secretory granules (small arrows) and rough endoplasmic reticulum. (d) Immunoperoxidase-labeled VIP-containing terminal and unlabeled terminal (asterisk) impinge upon VIP-immunoreactive soma. Arrowheads indicate contact zone between homologous VIP-immunoreactive profiles. (e) Immunogold labeling of VIP-immunoreactive terminal; gold particles are primarily over secretory granules (small arrows). Asterisk indicates adjacent unlabeled terminal. Scale bars: a, 100 μm; b, 50 μm; c, 1 μm; d,e, 0.5 μm.

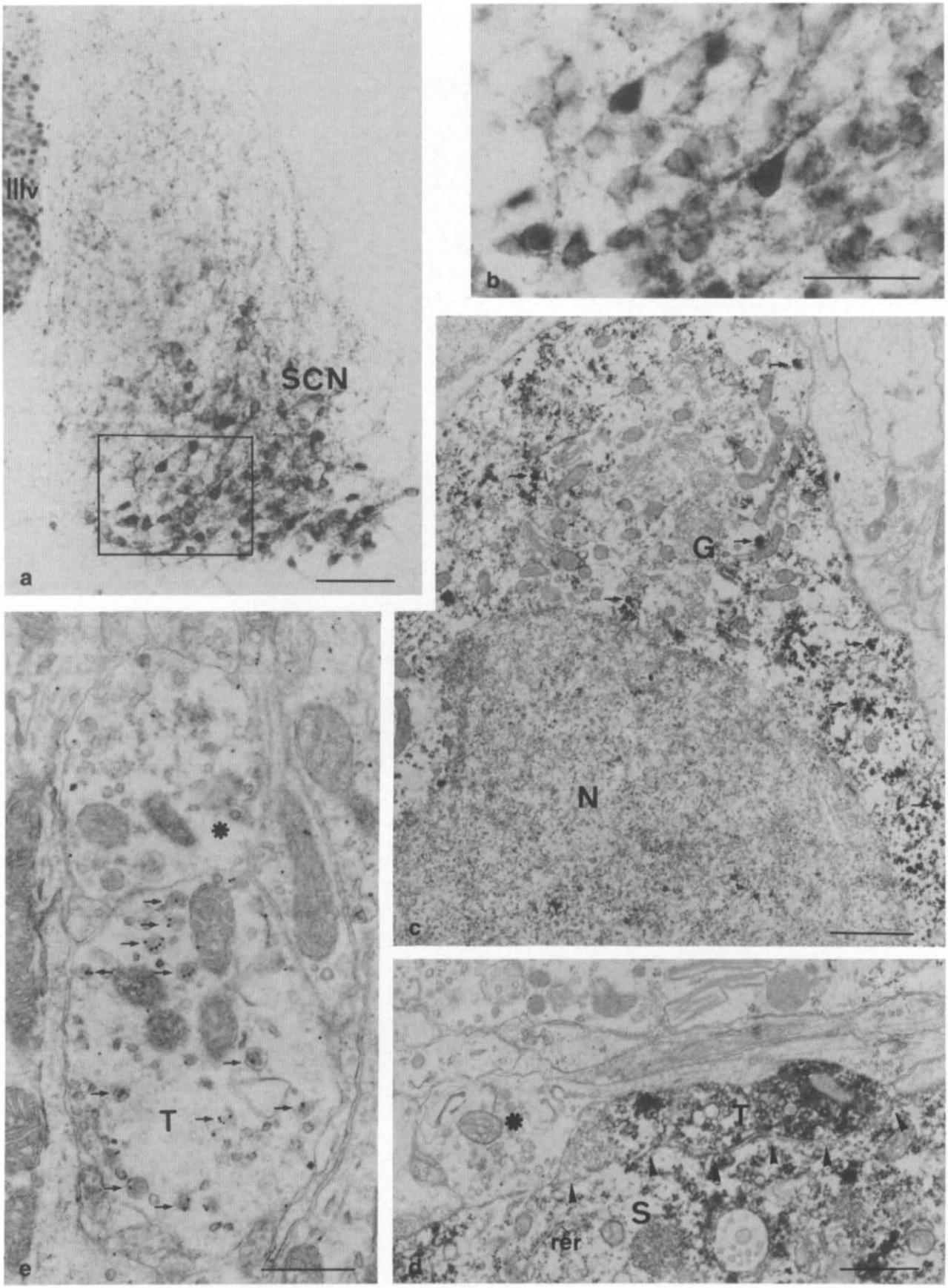

Fig. 3

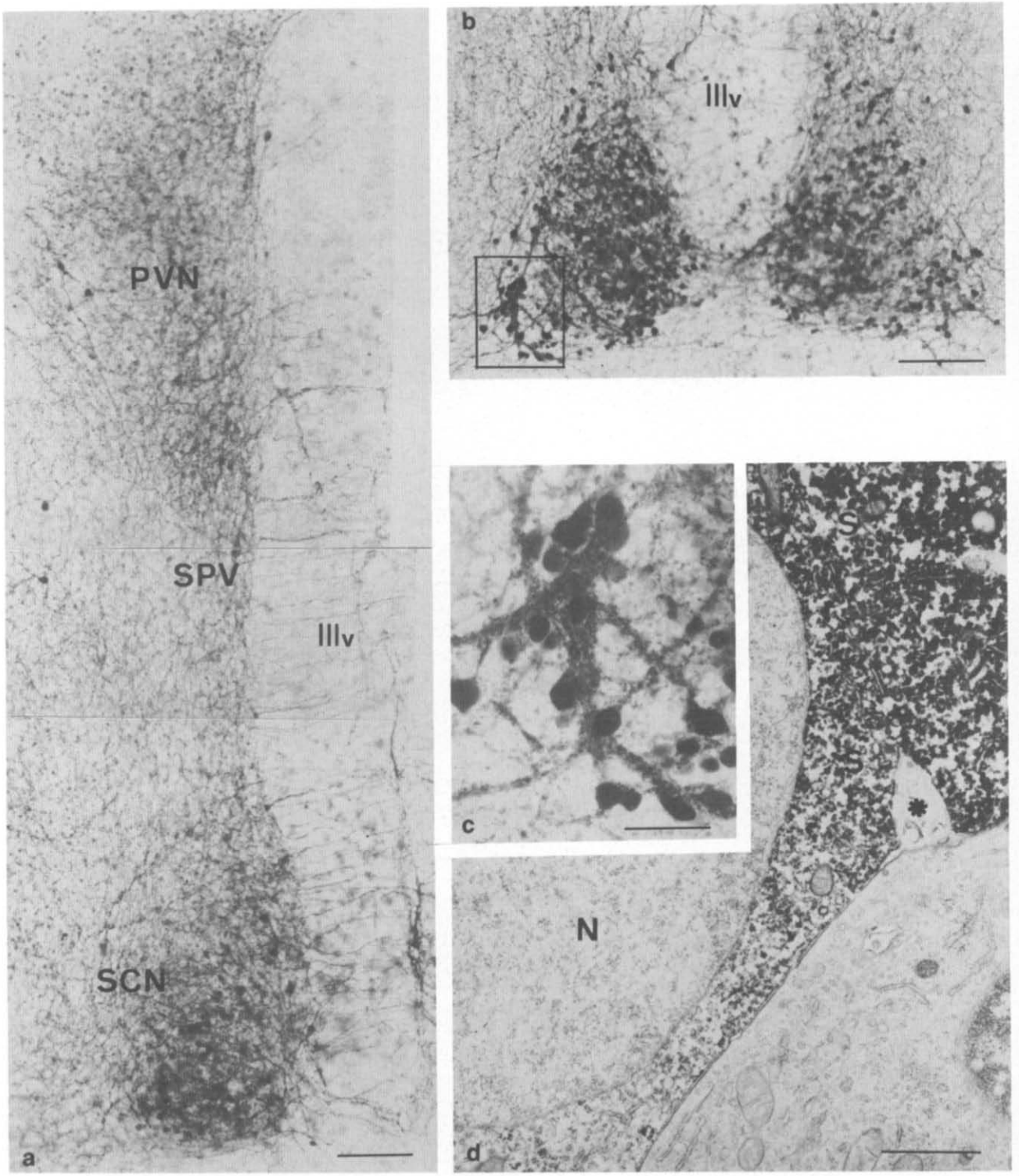

Fig. 4. GABA-immunoreactivity in slice explants, cultured for 20 days. Immunoperoxidase labeleing. (a) Unilateral micrograph of slice explant, showing distribution of GABA-containing somata and processes within the SCN and the surrounding hypothalamic tissue. The great majority of GABA-immunoreactive somata are concentrated within the SCN (see also Fig. 4b). Varicose processes course dorsalwards from the SCN into the sub-paraventricular zone and the PVN. Immunoreactive fibers also traverse the space of the third ventricle. (b) Micrograph of bilateral SCN showing GABA-immunoreactive somata distributed throughout the nucleus, in both ventral and dorsal sub-divisions. (c) Higher magnification micrograph of portion of Fig. 4b (square), showing intensely labeled GABA-ergic neurons. (d) Electronmicrograph of portions of two heavily-labeled GABA-immunoreactive somata; asterisk indicates small non-immunoreactive axonal terminal; at lower right is portion of non-immunoreactive soma. Scale bars: a, 150 μm; b, 100 μm; c, 50 μm; d, 1 μm.

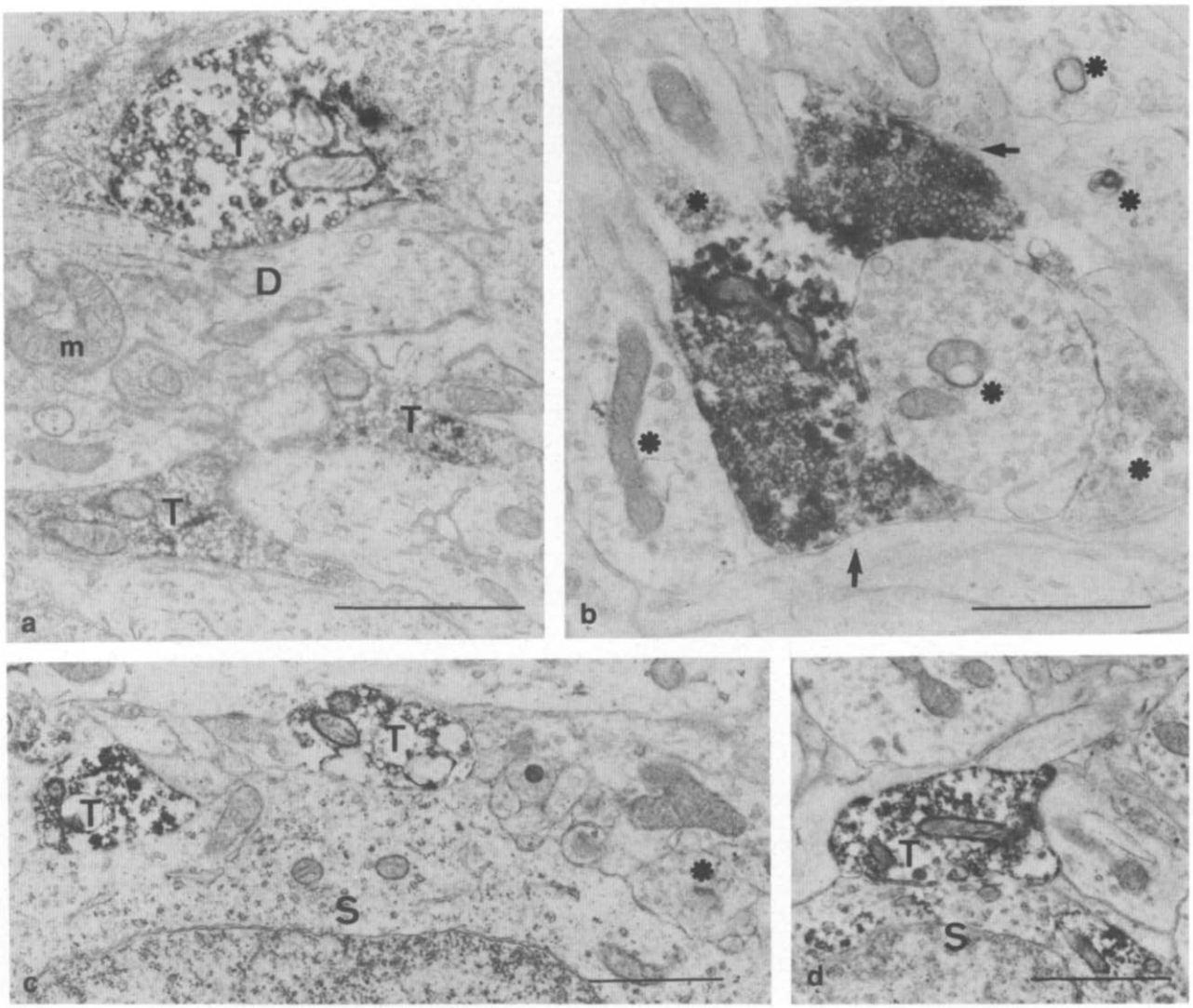

Fig. 5. Electronmicrographs showing a variety of immunoperoxidase-labeled axonal terminals in 20-day SCN cultures. (a) Profiles of three VIP-immunoreactive terminals, two of which impinge upon the same non-immunoreactive dendrite. (b) Synaptic glomerulus containing two heavily-labeled GABA-immunoreactive terminals (arrows) and several non-immunoreactive terminals (asterisks). (c) Profiles of two immunolabeled VIP-containing terminals, and unlabeled terminal (asterisk) impinge upon non-immunoreactive soma. (d) GABA-immunoreactive terminal impinging upon homologous soma. Scale bars: 1 μm.

of SCN origin, were also found within the space of the ventricular lumen (Fig. 6).

The results of 10 experiments in which a horizontal incision of the slice explant almost completely separated the SCN from the PVN were unambiguous (see Fig. 7). The ventral part of the slice, which contained the bilateral SCN, displayed rich arborization of fine-caliber axons. This distinctive GABA-immunoreactive plexus was missing from the dorsal half of the explant, which contained only the large GABAergic neurons intrinsic to the PVN (Fig. 7).

Electrophysiological recording

Despite the fact that recordings were carried out on explants submerged in aqueous medium, and not at the air/liquid interface, as they had been cultured, electrical activity was readily detected in 51 out of 58 SCN slice explants studied (88%). The spontaneous all-or-none activity was manifested in extracellular recordings as 46 ± 18 pA negative current signals (an average of 40 cells from four explants, at least 10 spikes from each cell). The four superimposed traces shown in Fig. 8a demonstrate the shape and constancy of these signals. Such relatively large signals were usually recorded near the cell membrane. At larger distances from the cell membrane, smaller bipolar signals were obtained.

The pattern of spontaneous activity varied from highly regular to random mode. The two examples shown in Fig. 8b and c represent the extremes of an

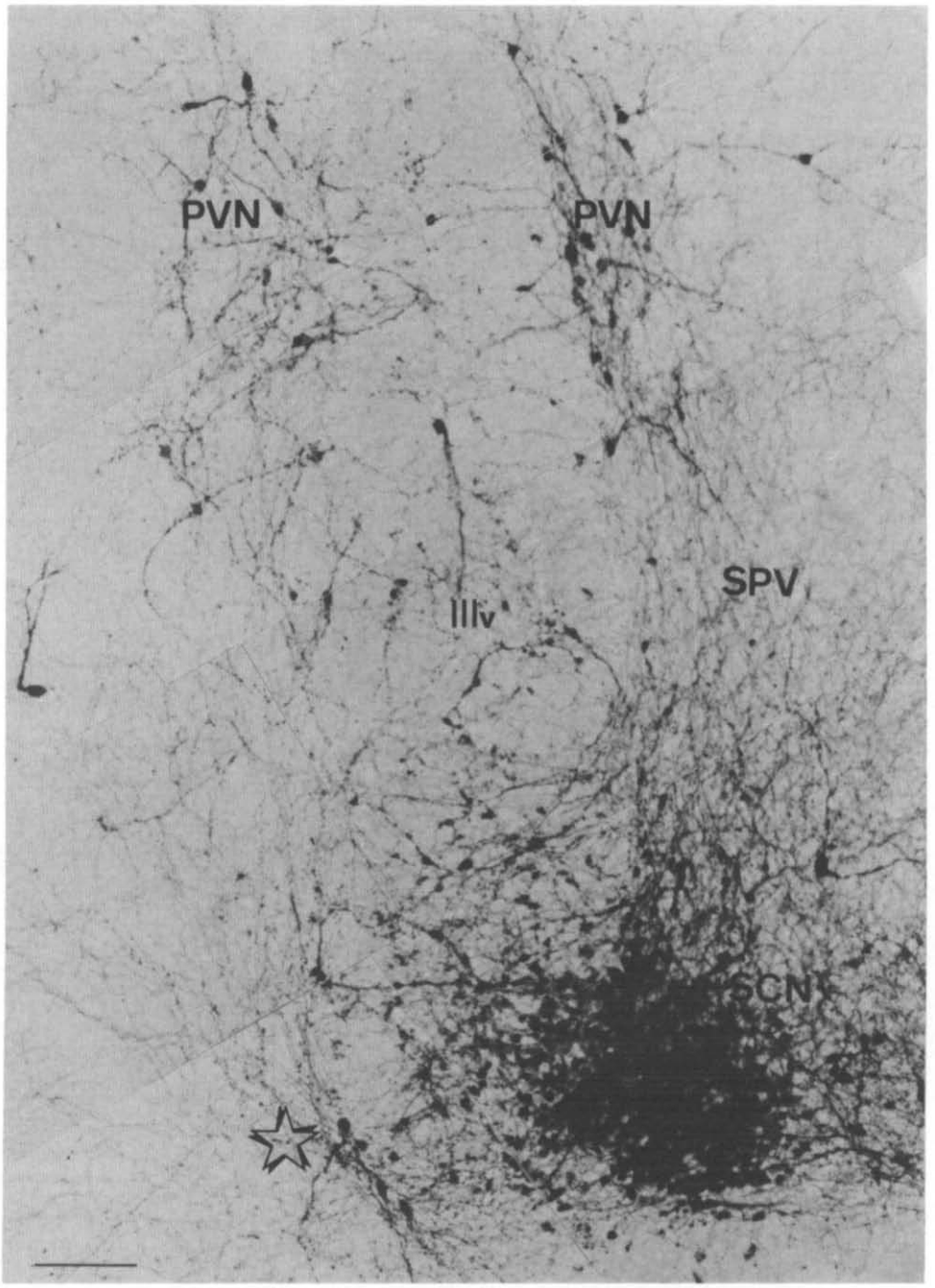

Fig. 6. GABA-immunoperoxidase labeling of slice explant, 20 days *in vitro*, demonstrating the effect of unilateral SCN ablation (before planting), on the GABA innervation of the entire slice. On the right is an intact SCN with numerous immunoreactive somata giving rise to dense immunoreactive projections coursing into the sub-paraventricular zone and the PVN. Within the PVN there are several magnocellular GABA-immunoreactive somata with sparse radial projections. Some GABA-immunoreactive processes traverse the space of the third ventricle. On the left, the star indicates the area of unilateral SCN ablation; a few "errant" GABA-containing somata and processes remain, giving rise to sparse verticle projections (compare to right side) into the sub-paraventricular zone and the PVN. In the bilateral PVN, magnocellular GABA-containing neurons are similar on both sides. The general impression is that the richest GABA-innervation of the hypothalamic slice arises from the SCN. Scale bar: 100 μm.

Fig. 7. GABA-immunoperoxidase labeling of dissected slice explant, 20 days in culture, demonstrating the effect of a horizontal incision (dotted line) between the SCN and the PVN. Although a complete incision was attempted (dotted line), on the left some reattachment of tissues may be seen. The lower half of the cut third ventricle has reformed and healed, at the level of what was originally the sub-paraventricular zone. The lower half of the dissected slice shows abundant fine-caliber GABAergic projections emanating from immunoreactive cells within the SCN. These projections are most dense around the ventricle, but are also evident in the lateral areas. The upper half of the dissected slice shows the bilateral PVN, containing a number of GABAergic magnocellular neurons with sparse projections, but the rich fine caliber GABAergic input from the SCN is conspicuously absent. Scale bar: 150 μm.

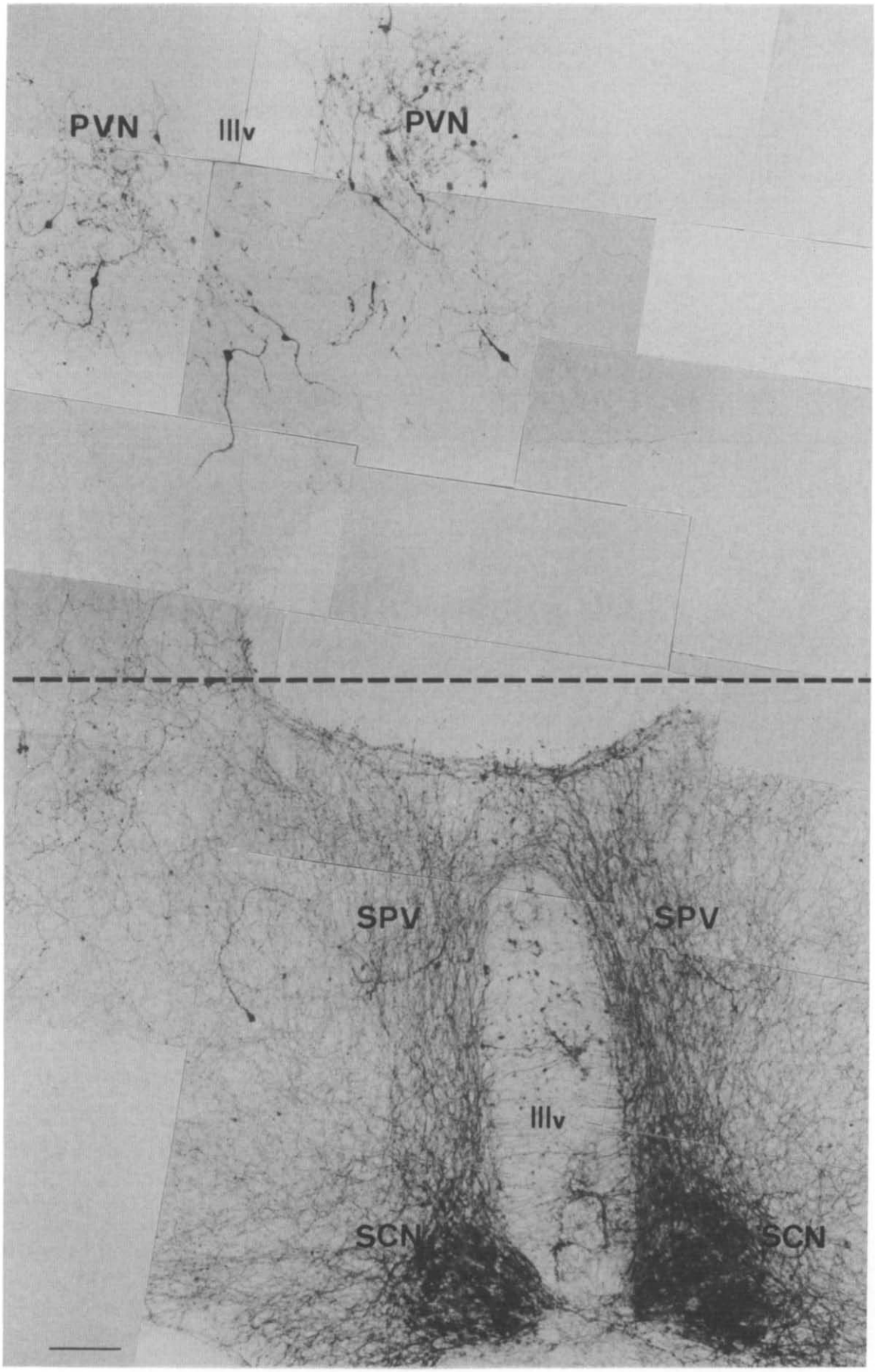

Fig. 7.

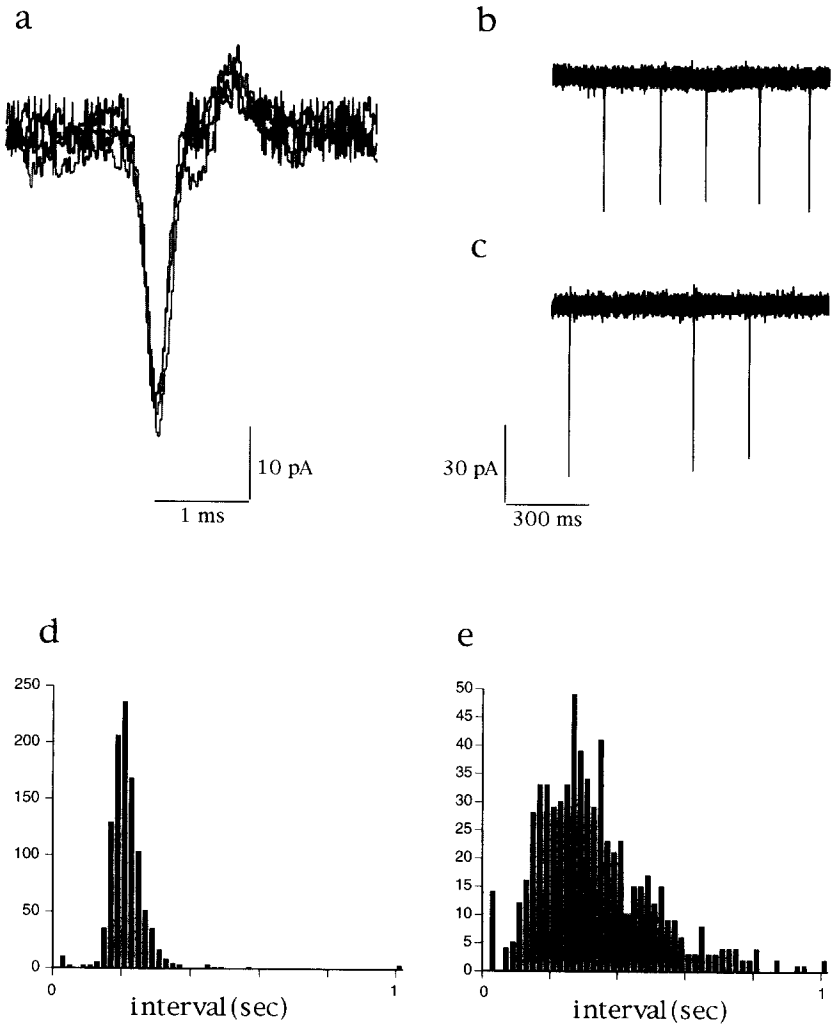

Fig. 8. Extracellular recordings of spontaneous activity in cultured SCN. (a) Four superimposed traces of all-or-none signals recorded near the membrane of a spontaneously active neuron. The traces were aligned by the rising (elevated) phase of the signals. The fast, almost monophasic, response indicates the proximity of the electrode to the cell membrane. (b,c) Records of one second long spontaneous activity, demonstrating regularly and randomly active cells, respectively. (d,e) The interspike intervals distribution of the recordings partially shown in b and c respectively. In these examples an average frequency of 5.1 and 3.2 Hz was calculated.

entire spectrum of firing patterns that were recorded. The variance of the interspike interval, reflecting the regularity of firing, was found to be in negative correlation with the firing frequency. Moreover, continuous recording from one cell for about 6 h revealed a decrease that was associated with a change in the firing pattern from regular to irregular. The histograms of the interspike intervals of the examples shown in Fig. 8b and c are demonstrated in Fig. 8d and e respectively. In these examples the average interval of the regular mode (Fig. 8d) was 197 ms, with a relatively small deviation (S.D.: 74 ms). In random mode (Fig. 8e) the average interval was 57% larger (310 ms), but the SD was 230% greater (170 ms). Spontaneous activity was abolished by TTX, and its frequency was reversibly reduced (mean reduction $57 \pm 9\%$; $n = 3$) by 1 mM $NiCl_2$, which is known to block voltage dependent Ca^{2+} conductances. Bursts of spontaneous firing, when more than two spikes appeared within an interval shorter than 30 ms, were rarely encountered.

In six explants spontaneous electrical activity was sampled for periods longer than 10 h and up to 24 h. In four cases the frequency of spontaneous activity varied within the recording session from an average of 1.7 ± 0.7 Hz to an average of 6.0 ± 1.2 Hz. This significant difference ($P < 0.005$, two-tailed unpaired t-test) indicates that in all four cases the average firing rate changes by a factor of three within 10 h of recording. Both the time course and the extent of the change are commensurate with a system that undergoes circadian variation in activity. An example is shown in Fig. 9a where the averaged frequency of spontaneous activity was plotted as a function of recording time. In this case, the average frequency increased significantly ($P = 0.03$) from 3.0 Hz at the

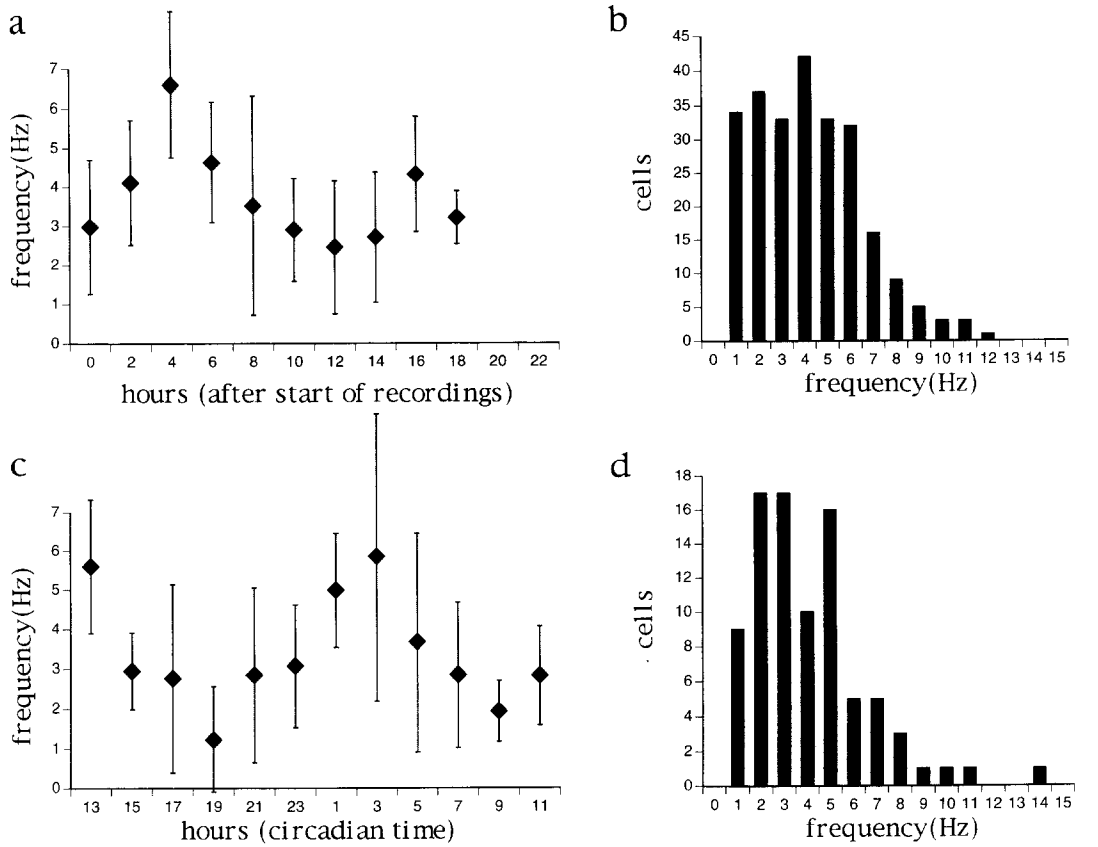

Fig. 9. Circadian fluctuation of spontaneous electrical activity in long-term cultured and freshly prepared SCN. (a,c) The average frequency of the spontaneous electrical activity as a function of time after onset of recordings in culture (a) and of circadian time in freshly prepared slice (c). The frequencies are averaged over 2-h bins. (b,d) The distributions of the mean frequencies of single cells from four cultures (b) and from one fresh slice (d). The mean $\pm$ S.D. is 3.68 ± 2.34 Hz ($n = 248$) in the cultures and 3.49 ± 2.45 Hz ($n = 86$) in the slice. The distributions are not significantly different ($P > 0.5$).

beginning of the recording session to 6.6 Hz after 6 h. This initial increase was followed by a monotonic decrease in frequency lasting for 10 h and reaching a significantly ($P = 0.004$) lower level of 2.5 Hz. The significant increase in frequency after 18 h of recording ($P = 0.035$) indicates that the preceding changes were not due to run-down of the cultured slice, but a consequence of endogenous fluctuation that may be circadian.

For purposes of comparison, circadian fluctuation of spontaneous electrical activity recorded from a freshly prepared SCN slice, derived from an adult rat, is shown in Fig. 9c. In this case, the average frequency of spontaneous activity was plotted as a function of circadian time, showing peak activity during subjective day. The similarity between the two rhythms (compare Fig. 9a and c) further supports the possibility that the cultured SCN does indeed retain its capacity to generate circadian rhythm. Similar results were observed in three additional cultures, whereas in two other cases the changes in spontaneous activity did not reveal clear rhythmicity.

The possibility that the SCN explants generate circadian rhythm is also supported by the observation that the distribution of neuronal firing frequencies recorded from freshly prepared slices is similar to that observed in neurons from long-term cultured tissue. The frequency distribution of 248 cells recorded from the four oscillating cultures is shown in Fig. 9b. The same ($P > 0.5$, two-tailed unpaired t-test) distribution was observed in 86 neurons recorded in a freshly prepared slice (Fig. 9d). It should be noted that the shape of both histograms, which cannot be fitted by Gaussian distribution, might result from rhythmic phenomena.

DISCUSSION

The present study has shown that hypothalamic slice explants containing the SCN can be maintained for several weeks in stationary organotypic culture, avoiding the structural deformations and permutations that often accompany rotating cultures. In stationary explants, both the chemoarchitecture of the main intrinsic cell populations of the SCN and the organization of the surrounding hypothalamic tissue resemble that in the adult. The stability and predictability of structures within the stationary culture

facilitated lesion experiments, which in combination with GABA immunolabeling enabled us to assess the extent to which the GABAergic innervation of the entire slice originated from the SCN. Viability of the cultures was confirmed electrophysiologically, and preliminary evidence for circadian fluctuation of electrical activity was indicated.

Suprachiasmatic nucleus culture techniques

Three different approaches have been applied to long-term organotypic culture of the SCN. Wray *et al.*,[42,43] were the first to apply Gahwiler's roller-tube procedure[16] to hypothalamic slice explants containing the SCN. This technique facilitated several studies of the *in vitro* SCN including immunocytochemical, *in situ* hybridization[36,42] and assay of neuropeptides secreted into the culture medium.[33,36] The roller-tube procedure requires adhering each slice explant to a glass coverslip with a drop of thrombin-clotted chicken plasma, insertion into a tube containing medium, and placing into a roller-drum. Rotating is thought to promote thinning of cultures to a monolayer. However, sources of suitable chicken plasma and thrombin may be inconsistent, and during continuous rotation on the roller-drum explants may detach from their coverslips, so that a considerable proportion of the slices that are planted do not survive. As for the morphological integrity of roller-tube cultures, although SCN structure may be preserved adequately, the surrounding tissue is often distorted, and permutations such as atypical and excessive glial proliferation, including ectoptic growth into the plasma clot tend to occur.[36,42]

An alternative method for long-term SCN culture involves supporting the explants on small grids of polyamide gauze, which free-float in medium contained in rocking Petri dishes. Although these explants do not thin to monolayers, they do fulfil their intended purpose for long-term electrophysiological recording.[7,8]

The stationary organotypic culture procedure of Stoppini *et al.*,[34] adapted in the present study for SCN explants, retains all the advantages of the aforementioned techniques, while avoiding the disadvantages. Slice explants readily adhered to Millicell filters with 95% survival for experimentation. Several explants from the same or different neonates may be cultured on the same filter, promoting not only economy of handling, but also facilitating co-culture experiments. Stationary culture and maintenance of explants at the interface between culture medium and air probably contributed to flattening of the tissue.

On the whole it appears that hypothalamic explants grown on porous supports such as Anocel filters,[42] polyamide gauze[7] and Millicell filters (present study) are less prone to distortion and permutation than those grown on glass coverslips.[36,42] In fact, a combination of glass coverslips and rotation may exacerbate distortion in hypothalamic slice explants in roller-tube preparations, although this does not occur in linearly organized neural tissue such as hippocampus and cerebellum, in which structural integrity is well-preserved.[16]

Expression of neuropeptides

In vivo, a number of neuropeptides, including vasopressin, VIP, gastrin releasing peptide, peptide histidine isoleucine, somatostatin, cholecystokinin and substance P have been immunolocalized within the SCN, and in some cases their mRNAs have been demonstrated (reviewed in Refs. 1, 23, 40). Endogenous circadian secretory cycles within the adult SCN have been demonstrated for vasopressin and somatostatin and their respective mRNAs,[23] and most recently for VIP mRNA.[19] There is an on going discussion about the roles of the various neuropeptides in signal transduction within the SCN. Do these peptidergic cells embody an oscillatory mechanism, or are they "merely" up-stream or down-stream to the elusive clock mechanism?[23] However, even though precise chronobiological properties of SCN cells have yet to be defined, the intrinsic neuropeptides have proved to be reliable chemo-architectural markers within the SCN. It has been demonstrated repeatedly that the somata of two of the most prevalent neuropeptides in the SCN, vasopressinergic and VIP-containing, are concentrated respectively dorso-medially and ventro-laterally within the nucleus.

In long-term organotypic cultures of the SCN, expression of vasopressin and VIP immunoreactivity and/or their respective mRNAs has been reported irrespective of the culture procedure employed (see Refs 7, 36, 42 and present study). Endogenous circadian secretory rhythmicity in long-term SCN explants, has recently been demonstrated for vasopressin[36] and for VIP[33]. The case for the latter is particularly interesting since it was previously maintained that VIP secretory cycles in the SCN depend on retinal input.[1,23]

As for VIP-immunoreactivity in long-term SCN explants, both Wray *et al.* (Ref. 42; roller-tube) and the present study (stationary culture) found that VIP labeling was noticibly less robust *in vitro* than *in vivo*. However, Tominiga *et al* (Ref. 36; roller-tube) claim the opposite: that VIP-immunoreactivity in SCNs of adult rats, even after colchicine treatment, is much weaker than that in their long-term SCN cultures. This disparity may be explained by different fixation procedures, or VIP antibodies, or strains of rats: Wistar[36] versus Sprague–Dawley[42] and Sabra (present study). Another likely possibility is that the maturity of the explants when planted may be a contributing factor to their subsequent development: explants from four-day old neonates[36] may still be fully programmed to proceed with VIP cell development during culturing, while a decline in developmental and regenerative potential may occur in those derived from seven-day[42] and nine-day old neonates (present study). It is noteworthy that gastrin releasing peptide

which is co-localized in some VIP cells in the SCN,[30] was also expressed more weakly in culture than *in vivo*.[42] Nevertheless, long-term SCN cultures derived from seven to 12-day-old neonates have yielded highly satisfactory results for a variety of other phenotypes and parameters (see Refs 7, 33, 42 and present study), which may indicate a differential in developmental capacity of VIP cells in the SCN.

Ultrastructurally, vasopressinergic and VIP-containing neurons in stationary cultures of the SCN appear to have the same characteristics as those *in vivo*, including extensive local circuit synapse formation. Interestingly, autaptic and/or homologous synapses containing vasopressin or VIP or GABA, are invariably found on ultrastructural grids derived from cultures whereas such images are rare in ultrathin sections derived from the adult SCN. One may speculate that in the slice cultures many afferent synaptic inputs degenerate, vacating post-synaptic sites for available homologous terminals.

GABA in the suprachiasmatic nucleus

It is currently claimed that GABA is the principal neurotransmitter in the SCN,[26] and that the nucleus probably uses this inhibitory transmitter to regulate its endogenous circadian cycles, as well as the rhythmicity it imposes on other regions of the brain.[9,31] In the adult SCN, total GABA content may fluctuate in circadian fashion, even when the nucleus is deprived of retinal input.[2] Both GABA and its synthesizing enzyme glutamic acid decarboxylase are claimed to be ubiquitous in somata of the SCN in adult animals.[26,28,29] However, whether or not GABA is present in all SCN somata *in vivo*[26] is a moot point, since it is difficult to elicit optimal and unambiguous immunoperoxidase labeling of GABAergic cell bodies in the SCN of adult animals (Refs 28, 29 and unpublished observations). This difficulty has been attributed to the extremely dense network of GABA-immunoreactive processes in which the somata are embedded.[28,29] Likewise, it is not clear to what extent the adult SCN may use GABA as a neurotransmitter in all its efferent projections, since only about one-third of the outputs from the nucleus were found to be GABA-immunogold labeled.[9] GABA-immunoreactive axonal terminals are particularly abundant within the adult SCN,[9,11,12,40] but *in vivo* it is difficult to establish to what extent they arise from local circuit neurons or from GABA-containing afferents.

To adequately discern GABAergic somata in the SCN *in vivo*, animals require pre-treatment with colchicine.[26,28,29] The present *in vitro* study of the SCN has demonstrated in the clearest possible fashion to date, the intrinsic GABA-immunoreactive component of the nucleus in its entirety, including its efferent projections into nearby hypothalamic areas. Why this was accomplished in stationary organotypic cultures of the SCN, but not by using similar immunoperoxidase procedures applied to the adult SCN remains a subject for speculation. It is possible that the profuse GABAergic innervation observed in culture is due to the absence of a growth limiting factor that exists in the intact brain but not in the explant. On the other hand, the fact that the SCN explant lacks all or most of its normal GABAergic inputs from the brain, may have rendered its intrinsic GABAergic component easier to visualize. It is also interesting that numerous hypothalamic GABAergic somata (outside the SCN) demonstrated in the adult rat brain,[28] are not found in the explant. This may indicate different capacities for *in vitro* survival between different categories of GABAergic neurons: those within the SCN versus those in the surrounding hypothalamus.

The stability and reproducibility of the stationary SCN culture, combined with fortuitous GABA-immunoperoxidase labeling, enabled us to demonstrate that even when deprived of its GABAergic inputs, the SCN is exceptionally well-endowed with intrinsic GABA. Moreover, with the aid of lesion experiments, the GABAergic somata of the SCN were shown to be the main source of profuse labeling within the entire hypothalamic slice *in vitro*. It may be speculated that a considerable proportion of the GABAergic terminals described within the adult hypothalamus[14] could originate from the SCN. In adult rats, Buijs *et al.*[9] have reported terminals of SCN origin in the peripheral PVN. Our studies on the hypothalamic explant underscore this finding by demonstrating the obvious reduction of the fine caliber GABAergic input from the SCN to the PVN as a result of the appropriate lesions. Needless to say, complete analogy between the GABAergic innervation that emanates from the SCN in culture and that that exists *in vivo* cannot be claimed. On the other hand, this robust GABA immunoreactivity should not be dismissed as artefact or permutation peculiar to the culture only.

Electrophysiological recording

In the present study we compared the spontaneous electrical activity recorded extracellularly in stationary SCN cultures with that in freshly prepared slices from adult rats, and found it to be similar in both experimental models. This matches results in long-term SCN explants supported by free-floating grids,[7] as well as in short-term brain slices.[20] Extracellular recordings from SCN neurons reveal two modes of firing pattern: regular and irregular.[7,24,35] This prompted the notion that the two different modes are generated by two different cell populations.[7,21,44] On the other hand, it has been shown that the same cell can fire in both regular and irregular modes, depending on its membrane potential.[24] Our findings concerning the variable firing patterns of SCN cells support the hypothesis that these patterns reflect different functional states rather than different electrophysiological properties.[24,35] It is still unclear, however, whether firing frequency is determined

by intrinsic membrane properties or by network connectivity.

In four out of six cases we found indications of circadian fluctuation in electrical activity within the culture. However, the limited duration of recordings (up to 24 h) and therefore the inability to apply spectrum analysis, call for caution in evaluating the significance of these results. Nevertheless, there were three experimental observations that support the idea of circadian oscillatory activity in the cultures. (1) The time dependent changes in mean firing frequency recorded from randomly selected cells within the cultures, resembled those observed in freshly-prepared short-term slices. In both instances the mean frequency changed by 5 Hz from peak to trough within 10 h. (2) The distribution of firing frequencies of neurons in the cultures did not differ from that in freshly prepared slices. In fact, the not-Gaussian distribution in both experimental models may reflect underlying circadian fluctuations. (3) The increase in mean firing frequency after 10 h of monotonic decrease (Fig. 9a) indicates that the decrease was not merely run-down of the tissue, but the result of a slow process that governs spontaneous electrical activity.

CONCLUSION

Stationary organotypic cultures of the rat SCN present a viable, stable and easily reproducible model with which to study basic functions in the brain's circadian clock. Cyto-architectural and electrophysiological properties reflect those of the adult animal. The clearly visualized GABA-immunoreactive innervation in the stationary SCN culture may provide new information about the extent to which this inhibitory neurotransmitter could innervate both the nucleus itself and the surrounding hypothalamic tissue.

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