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Serotoninergic synapses on ependymal and hypendymal cells of the rat subcommissural organ*

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Summary

The nervous input to the subcommissural organ (SCO) of the rat has been investigated with Falck–Hillarp fluorescence histochemistry and electron microscopical techniques. Previous fluorescence histochemical observations of a dense plexus of serotoninergic nerve fibres in relation to the basal SCO were confirmed. Electron microscopically, unmyelinated fine varicose axons ranging in size from 0.1–0.6 μm were observed to penetrate into the SCO hypendyma. Boutons and presynaptic varicosities filled with a diversity of round and elongated clear vesicles, and occasional large dense cored vesicles establish asymmetric (Gray's type I) synaptic contacts with the basal processes and somata of the SCO ependymal and hypendymal cells. A typical varicosity in synaptic contact with an SCO cell contains a population of approximately 85% clear, elongated vesicles 45 x 60 nm in diameter, 15% clear, round vesicles 50 nm in diameter, and 1–2% large dense cored vesicles with a vesicle diameter of about 85 nm and a dense core diameter of 50–55 nm. The mean length of the postsynaptic membrane specialization was found to be 0.5 μm.

Experiments with specific neurotoxic drugs revealed that the nerve terminals in synaptic contact with the SCO cells are identical to the fibres of the serotoninergic plexus identified fluorescence histochemically. Thus, an intraventricular injection of either 5,6-dihydroxytryptamine or 5,7-dihydroxytryptamine induced typical degenerative changes in most of the boutons in synaptic contact with the SCO cells, and also a disappearance of the yellow fluorescent nerve plexus. It is concluded that the SCO of the rat receives a dense plexus of serotonin-containing nerve fibres which form typical synaptic contacts with the specialized ependymal cells of the SCO and that these fibres may constitute the only direct nervous input to the organ.

The degeneration of the serotoninergic synapses elicited a long-lasting, pronounced increase in the secretory activity of the SCO. Despite long survival times after the treatment with neurotoxic drugs, we found no evidence of regenerative restitution of the serotoninergic innervation nor normalization of the secretory activity of the SCO. The observed inverse relationship between secretory activity and serotoninergic innervation is in line with previous observations which indicate that the 5-hydroxytryptamine input to the SCO ependymal and hypendymal cells exerts a powerful inhibition on their protein synthetic machinery.

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Introduction

The subcommissural organ (SCO) is included in the group of specialized areas of ependyma, which are collectively called circumventricular organs. It is attached to the anterior and inferior surfaces of the posterior commissure, where it forms the lining of the roof of the cerebral aqueduct at its junction with the third ventricle. The mammalian SCO consists of two parts: a pseudostratified layer of ependymal cells, and a hypendyma (Krabbe, 1925), which consists of basal processes of SCO ependymal cells, hypendymal cells, glial cells, nerve fibres and vascular elements. Recently, a group of nerve cells – the SCO-associated neurons – was described as an additional characteristic element of the hypendyma (Kimble and Møllgård, 1975). The function of the SCO remains obscure, but the pronounced secretory activity of the organ is well established (for references, see Møllgård *et al.*, 1978).

The possible innervation of the mammalian SCO has been the subject of much dispute, which at least in part is the result of marked species differences. A rich serotoninergic (5-HT) innervation of the basal parts of the rat SCO has been described by fluorescence histochemistry (Fuxe *et al.*, 1968; Björklund *et al.*, 1972; Wiklund, 1974; Bouchaud and Arluison, 1977), which presumably is identical to the plexus of zinc iodide-positive nerve fibres observed previously in the same region by Stanka (1964). Apart from rat and Mongolian gerbil, however, no other mammalian species has so far been found to possess a 5-HT-innervated SCO (Wiklund *et al.*, 1977).

The ultrastructure of various components of the rat SCO has been described in a number of publications (Wetzstein *et al.*, 1963; Stanka *et al.*, 1964; Schwink and Wetzstein, 1966; Lin and Chen, 1969; Krstić, 1973), but these investigations did not focus on the nervous input to the region. A few publications more specifically concerned with the innervation of the mammalian SCO have suggested inputs by cholinergic (Leonieni and Rechardt, 1972), catecholaminergic and peptidergic fibres (Miline *et al.*, 1969; Miline, 1974), but these hypotheses were based on rather ambiguous experimental observations. The first conclusive ultrastructural evidence of the presence and nature of the innervation of the rat SCO has been provided by a recent study of Bouchaud and Arluison (1977) and our own work (Møllgård *et al.*, 1978). These studies demonstrated the presence of 5-HT nerve terminals in true synaptic contact with the SCO cells. The French authors speculated that this 5-HT innervation might exert a stimulatory influence on the activity of the organ, whereas we found that the 5-HT input inhibits both protein and glycoprotein synthesis, as well as the secretory activity of the rat SCO (Møllgård *et al.*, 1978).

The purpose of this study is to give a detailed description of the innervation of the rat SCO. For ultrastructural identification of 5-HT nerve elements, we have used the specific neurotoxic drugs 5,6-dihydroxytryptamine (5,6-DHT) and 5,7-dihydroxytryptamine (5,7-DHT), which are known to induce degeneration of serotoninergic axons with a very high degree of selectivity (for review, see Baumgarten *et al.*, 1977). Our experiments showed that the serotoninergic synapses constitute the

major, if not the sole, innervation of the specialized ependymal cells of the rat SCO. Removal of the serotoninergic innervation caused a sustained increase in secretory activity, which was presumably associated with a failure of the serotoninergic neurons to re-establish their connections with the SCO.

Materials and methods

About seventy female Sprague–Dawley rats weighing 180–220 g were used.

Administration of neurotoxic drugs

Under barbiturate anaesthesia (Brietel®, Lilly, 40 mg/kg) rats were stereotaxically injected in the anterior part of the lateral ventricle with 50 or 75 μg of 5,6-dihydroxytryptamine (5,6-DHT) (Regis), or 150 μg of 5,7-dihydroxytryptamine (5,7-DHT) (Regis) (all calculated as the free base). All neurotoxic drugs were administered in a volume of 20 μl saline containing 0.2 mg/ml L-ascorbic acid. Normal untreated rats and rats injected in the lateral ventricle with 20 μl of saline served as controls. Control and experimental rats were sacrificed after survival periods of 1, 2 or 3 days, 1–2 weeks or after 1, 3, 8 or 15 months.

Fluorescence histochemistry

All animals used for fluorescence histochemical detection of monoamines were pharmacologically pretreated to increase intraneuronal serotonin levels (Aghajanian *et al.*, 1973). Chloral hydrate (300 or 400 mg/kg, i.p.) was followed 10 min later by 300 mg/kg of nialamide (niamid, Pfizer) (i.p.) and, after another 15 min, by L-tryptophan (100 mg/kg, i.p.). The rats were killed by decapitation under light ether anaesthesia 1–3 h after the last injection. The brains were rapidly dissected out and blocks comprising the SCO quickly frozen in liquid propane, cooled by liquid nitrogen, and subsequently freeze-dried and processed for fluorescence histochemical detection of monoamines according to the Falck–Hillarp technique (see Björklund *et al.*, 1972). To achieve high fluorescence yield of serotonin, a two-step formaldehyde gas treatment was used (Fuxe and Jonsson, 1967). The specimens were first treated for 1 h at 80° C with paraformaldehyde equilibrated at 50% relative humidity, followed by another treatment at 80° C for 1 h with paraformaldehyde equilibrated at 70% relative humidity. The specimens were then paraffin embedded and sectioned for fluorescence microscopy (Björklund *et al.*, 1972).

Electron microscopy

Twenty-two control rats and 14 experimental rats were fixed by perfusing a solution containing 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M cacodylate buffer (pH 7.4) at a constant pressure of 140 mm Hg through the left ventricle. Following 15–20 min of perfusion fixation the brains were removed and placed in fresh fixative for a total of 6 h. Tissue blocks containing the SCO were isolated and postfixed for 2 h in 2% OsO_4 in 0.1 M cacodylate buffer. Specimens were block-stained with uranyl acetate for 1 h, quickly dehydrated in increasing concentrations of ethanol and embedded in Epon. The blocks were carefully oriented during embedding so that survey sections would be strictly in the sagittal or the frontal plane. One micrometer thick sections stained with toluidine blue were examined in the light microscope. Silver-to-grey thin sections were cut from selected areas and stained with uranyl acetate and lead citrate. From the SCO of two control and two experimental rats, blocks were oriented in the frontal plane and thin sections were cut every 10 μm from the rostral to the caudal end of the organ. Altogether more than 30 well-defined SCO areas were investigated in these rats.

Results

The different cellular components of the mammalian SCO have already been described in detail in previous investigations (see for example, Krabbe, 1925; Olsson, 1958; Wetzstein *et al.*, 1963; Stanka *et al.*, 1964; Palkovits, 1965; Schwink and Wetzstein, 1966; Sterba, 1969; Herrlinger, 1970; Krstić, 1973). An appreciation of certain ultrastructural features of the SCO is, however, of importance in the description and discussion of the innervation of the organ.

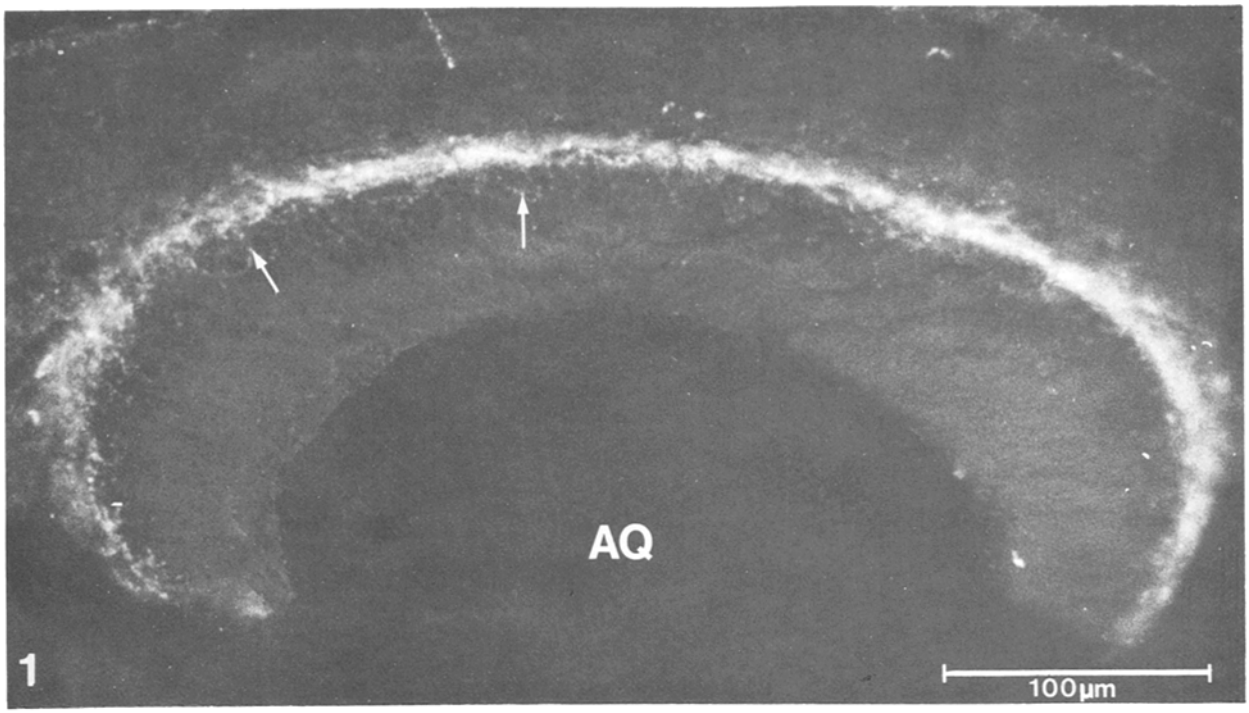
The SCO stands out as a thick layer of modified ependymal cells arranged in a pseudostratified manner under the posterior commissure. The SCO cells are seen both as elongated ependymal cells, which are in contact apically with the cerebrospinal fluid, and as basal or hypendymal cells, which are detached from the ventricular surface. In addition to SCO cells, astrocytes and nerve cells contribute to the hypendyma. The ependymal part of the SCO consists of the apical processes and perikarya of the SCO ependymal cells. The SCO hypendyma comprises a superficial and a deep portion. The superficial hypendyma contains basal processes of SCO ependymal cells intermingled with perikarya and processes of the hypendymal cells, while the deep portion of the hypendyma adjacent to the posterior commissure, contains a vascular plexus, glial cells and neurons in a dense neuropil.

The nuclei of SCO cells are often very irregular in shape and demonstrate distinct condensation of chromatin beneath the nuclear envelope. Perikarya and apical processes of the SCO ependymal cells contain dense granules of varying shape as well as many cisternae of endoplasmic reticulum filled with characteristic flocculent material. These organelles reflect the secretory activity of the SCO, which results in the production of the so-called Reissner's fibre of the ventricular system (cf. Olsson, 1958; Palkovits, 1965; Sterba, 1969). In the rat, processes of SCO hypendymal cells and the basal processes of SCO ependymal cells contain many polysomes and mitochondria, but are almost devoid of secretory elements. This is in contrast to the situation in the SCO of the rabbit (Schmidt and D'Agostino, 1966; Kimble and Møllgård, 1973) or the dog (Oksche, 1969), where a basal secretion to the blood vessels of the deep hypendyma is likely to occur.

Numerous nerve fibres and synapses are present in the neuropil of the deep

Fig. 1. Fluorescence micrograph of a frontal section through the rostral SCO of a rat. Note the very dense plexus of fluorescent serotonin-containing nerve fibres in the basal SCO. From this basal plexus individual fibres and thin bundles (arrows) extend in the apical direction, but do not reach beyond the nuclear level of the SCO ependyma. AQ, cerebral aqueduct.

Fig. 2. Two weeks after administration of 75 μg 5,6-DHT all serotoninergic fibres in relation to the SCO have degenerated and disappeared. The arrow indicates a catecholaminergic varicose fibre in the posterior commissure just dorsal to the SCO. Several thin catecholaminergic fibres can be seen in the posterior commissure and pretectal areas lateral to the SCO. The aggregation of brightly-fluorescent cells constitutes the so-called lamina intercalaris (LI), which contains many indoleamine-producing pinealocyte-like cells (cf. Wiklund, 1974). HC, habenular commissure; AQ, cerebral aqueduct.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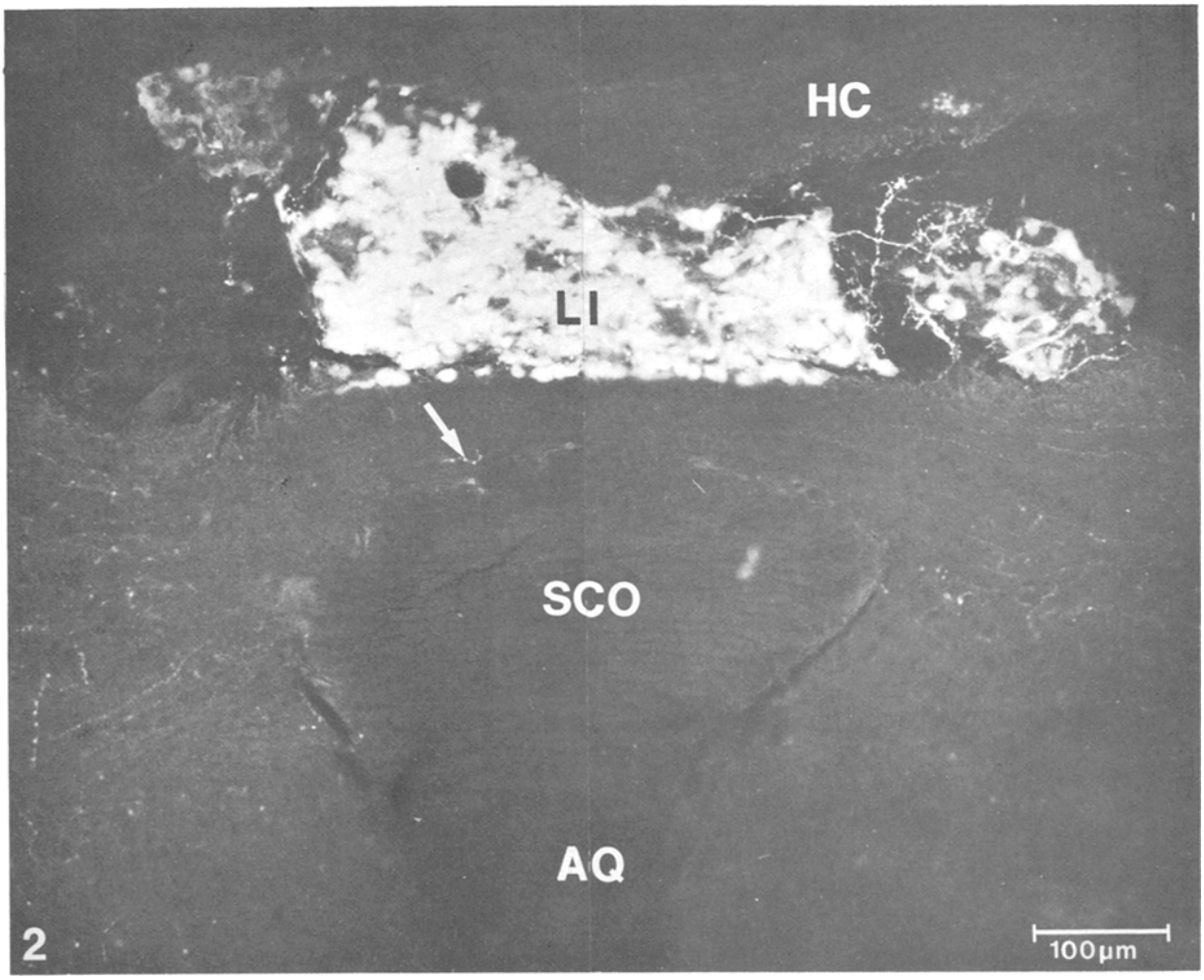
HC
LI
SCO
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hypendyma. The majority of these nerve endings form synapses with dendrites and perikarya of the SCO-associated neurons (Kimble and Møllgård, 1975). The synaptic input to this neuronal system, which is not the subject of the present study, is presumably non-monoaminergic. In our opinion, all serotonin-containing axons in the region are destined for the SCO ependymal and hypendymal cells.

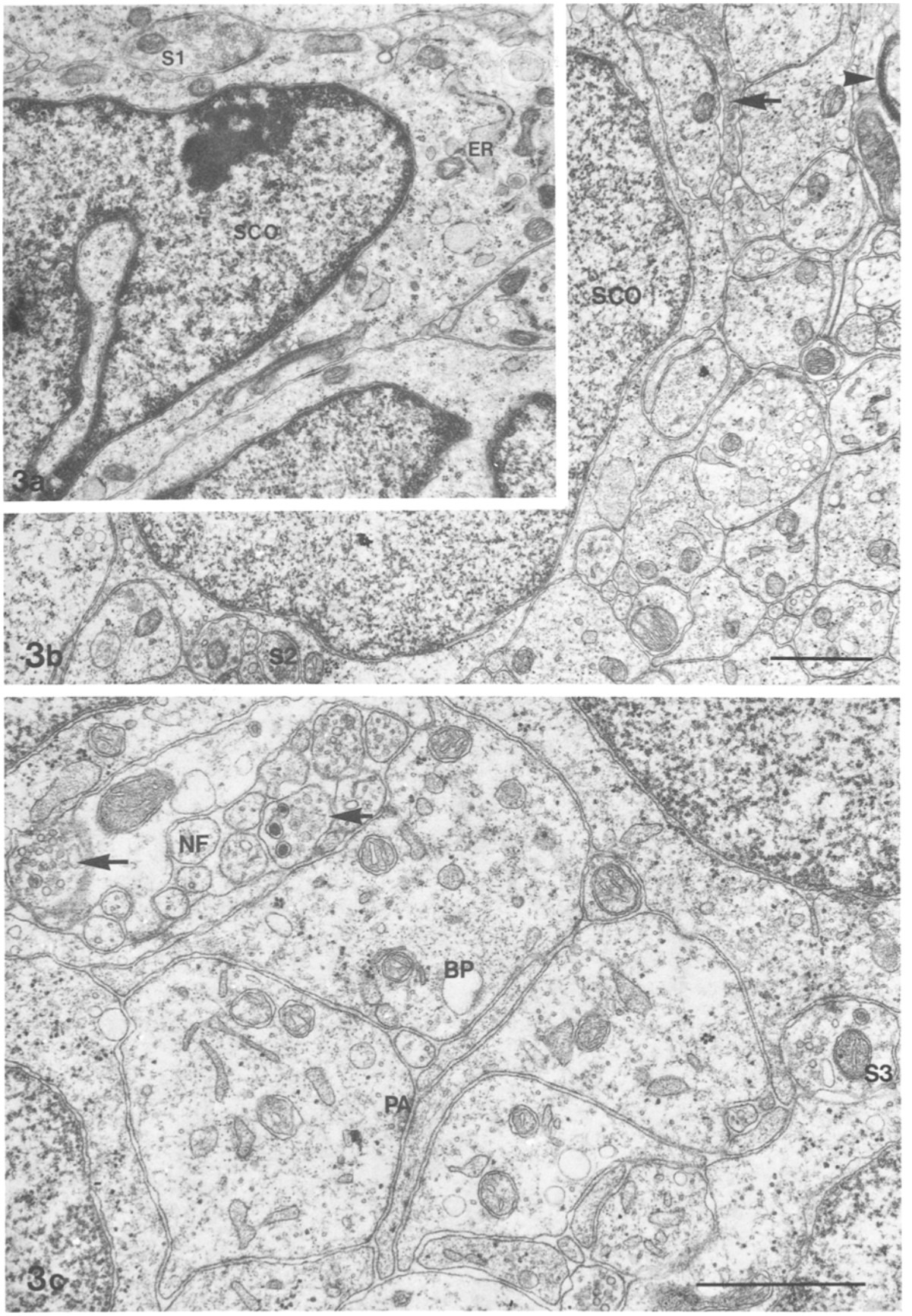
Fluorescence histochemical observations

Fluorescence histochemically the SCO of the rat is marked by a rich innervation of serotoninergic nerve fibres. In close relation to the basal parts of the pseudostratified ependymal cells of the SCO, an extremely dense plexus of brightly yellow fluorescent nerve fibres is found (Fig. 1). From this very dense basal plexus, thin varicose 5-HT-containing fibres extend apically between the SCO hypendymal cells to reach the nuclear level of the ependymal layer. No fluorescent fibres were, however, found to penetrate beyond the nuclear level into the apical two-thirds of the SCO ependyma. The ventricular surface of the SCO was always devoid of serotoninergic nerve fibres, in contrast to the rich supply of supraependymal 5-HT-containing nerve terminals of nearby single layered ependyma (cf. Lorez and Richards, 1973). The fluorescent nerve fibres that innervate the basal SCO showed the typical yellow colour and rapid fading indicative of serotonin content. No evidence was found of catecholaminergic terminals in relation to the SCO, but many catecholaminergic fibres could be seen crossing among the myelinated bundles of the posterior commissure dorsal to the organ.

Intraventricular injection of 5,6-DHT or 5,7-DHT at all doses used, caused total destruction and disappearance in most rats of the yellow fluorescent fibres innervating the SCO (Fig. 2) by one week after injection.

Since serotoninergic neurons exhibit strong regenerative capacities after lesioning with neurotoxic drugs and an ability to reinnervate even distant target areas (see Discussion), the SCO was studied in detail for signs of reinnervation by serotoninergic nerve fibres at survival periods up to 15 months after administration of the drug (usually 5,6-DHT). Unexpectedly and contrary to previous reports (Björklund *et al.*, 1973a), no unequivocal signs of regeneration of the serotoninergic innervation could be found. In most animals, even after the longest survival periods, the SCO remained devoid of serotoninergic nerve terminals. Some animals sacrificed after varying survival times showed, however, a few serotoninergic fibres in relation to the basal SCO, but neither the number nor occurrence of such fibres seemed to increase with

Figs. 3a–c. SCO cells are identified by their characteristic nuclei (SCO) and by the configuration and floccular content of the ER cisternae (ER). Abundant nerve fibres (NF) some of which exhibit large granular vesicles (arrows) are in close contact with the basal processes (BP) and perikarya. Single myelinated fibres (arrowhead on Fig. 3b) are also found in the basal SCO. Characteristic presynaptic elements (S_1, S_2 and S_3) establish synaptic contacts with SCO cells. These synapses are shown in higher magnification on Figs. 4e, f and g. Puncta adherentia-like junctions (PA) between adjacent SCO cell processes are clearly different from synaptic junctions. Scale bars: 1 μm.


S1
ER
SCO
3a
SCO
3b
S2
NF
BP
PA
S3
3c

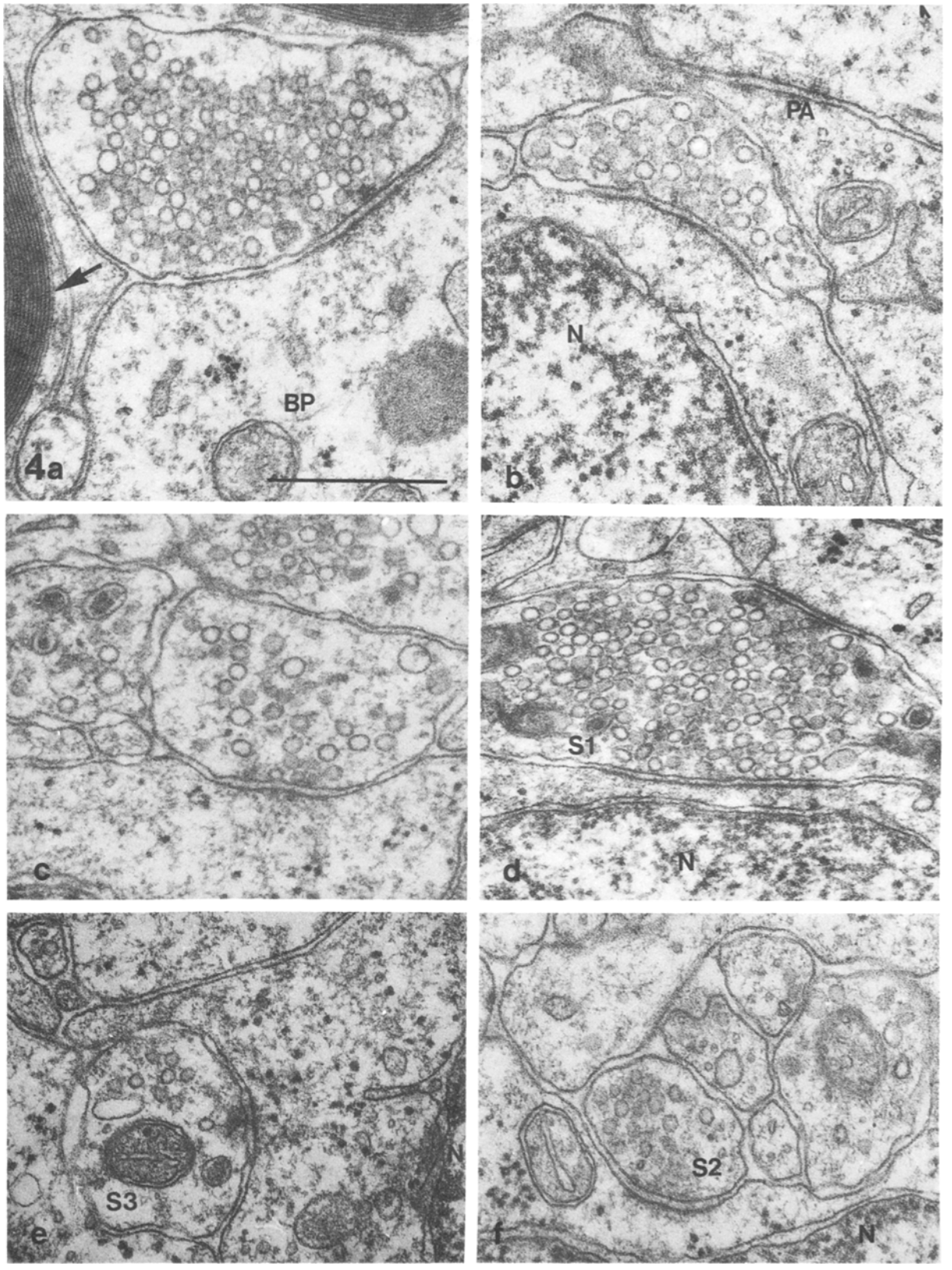
longer survival times. Thus, it could not be ruled out that these fibres possibly constituted remaining elements of the original innervation, which had been left intact due to a failure of the neurotoxic drug to induce complete destruction of the serotoninergic innervation. It was, therefore, concluded that, in most animals, the serotoninergic innervation of the SCO is completely destroyed by the intraventricular administration of 5,6-DHT or 5,7-DHT, and that the serotoninergic neurons, despite their well-documented regenerative capacities, fail to re-establish the innervation of the SCO. The plastic nervous changes elicited by chemical destruction of the serotoninergic innervation of the SCO is the subject of an accompanying paper (Wiklund and Møllgård, 1979).

Ultrastructure of the nervous input to the rat SCO

Small bundles of fine varicose unmyelinated axons penetrate from the neuropil of the deep hypendyma into the basal part of the SCO, where they establish numerous synapses with hypendymal cells and basal processes of ependymal cells (Fig. 3). Single axons or bundles containing up to four axons pass apically between hypendymal cells and basal processes of ependymal cells to reach the nuclear level of the ependyma (Fig. 3), where they terminate by forming abundant synapses located very close to the nuclei of the SCO ependymal cells (see below). Despite the diversity of postsynaptic sites – hypendymal cells, basal processes and perikarya of ependymal cells – the large majority of the synapses seem to belong to a single class, as reflected by common vesicle morphology, width of synaptic cleft and configuration of pre- and postsynaptic membrane specializations.

Many presynaptic elements (axon terminals and varicosities) were analysed in serial sections, which revealed that the boutons are spindle-shaped or spherical with an average diameter of 0.5 μm (range 0.3–0.9 μm), while the intervaricose segments of the axons averaged 0.25 μm in diameter (range 0.1–0.6 μm). Favourable longitudinal sections of axons demonstrated varicosities separated by approximately 1.5 μm. The boutons contained a characteristic population of round and elongated (elliptical) clear vesicles, which often appeared to fill them almost completely (Figs. 4a and d). However, in some boutons, few scattered vesicles were either aggregated towards the active zone (Fig. 4c) or dispersed in the axoplasm (Figs. 4e

Figs. 4a–e. High magnification of six different synapses in the SCO. In (a) a vesicle-filled varicosity establishes an asymmetric synaptic contact with a basal process (BP) of an SCO cell. Note the myelin sheath (arrow) which is characteristic of the basal SCO region. (b)–(e) all demonstrate the short distance from synapses to SCO cell nuclei. S_{1-3} are higher magnifications of the synapses shown in Figs. 3a–c, although S_1 on (d) is taken from a serial section through the same varicosity. In contrast to the section shown in Fig. 3a the section in (d) demonstrates that the varicosity is presynaptic to two SCO cells; furthermore, it exhibits three (possibly four) large dense cored vesicles. Note the small exocytotic (?) profiles in the presynaptic membranes in (b) and (c). PA, punctum adherens-like contact; N, SCO cell nucleus. Same magnification, scale bar: 0.5 μm.


BP
4a
PA
N
b
c
S1
N
d
S3
N
e
S2
N
f

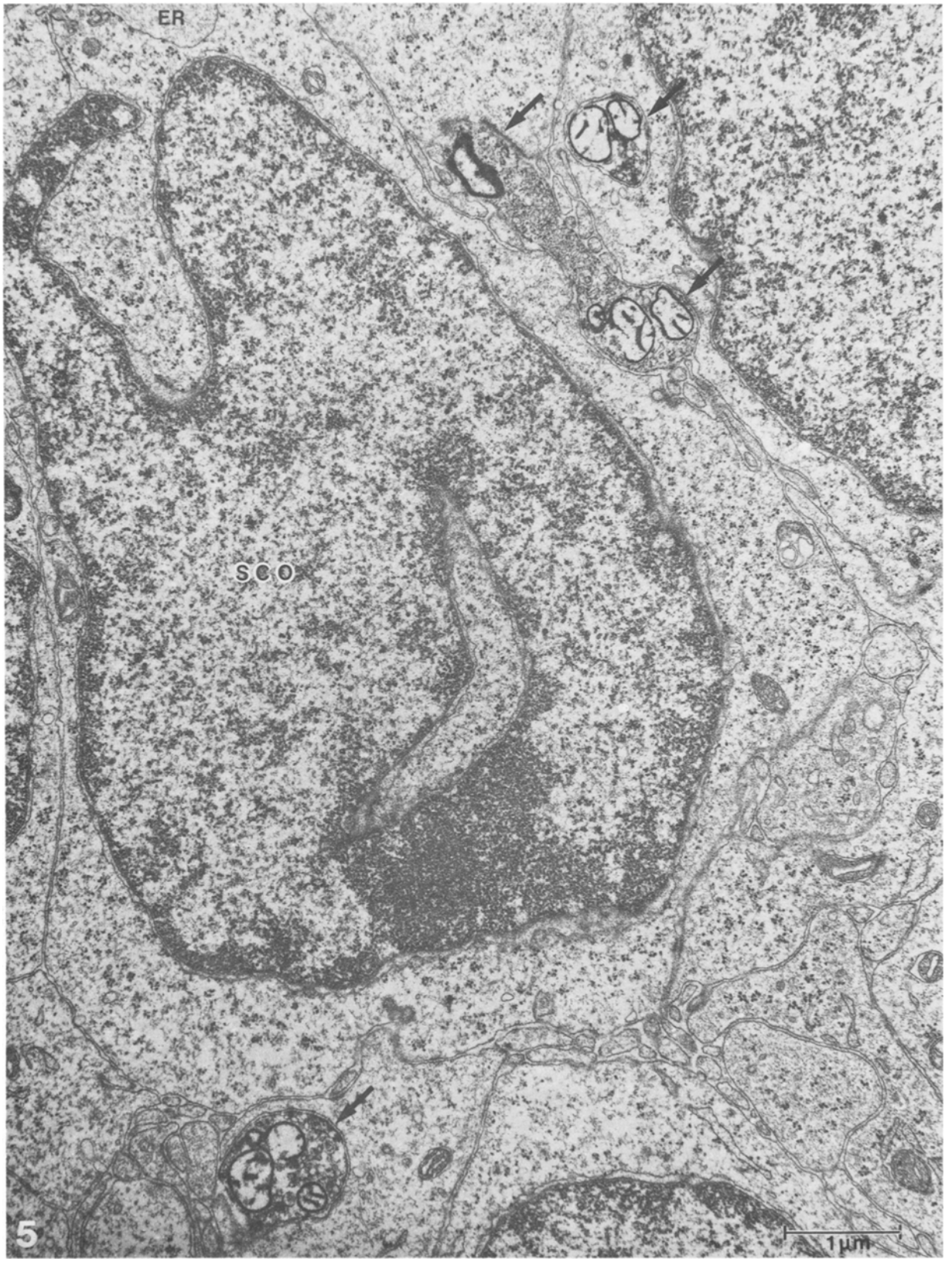
and f). In up to 30% of the sections through the presynaptic elements one or more large dense cored vesicles were visible. Clear vesicles or vacuoles of a size similar to the large dense cored vesicles were occasionally observed (Fig. 4c). Mitochondria and irregular profiles of smooth endoplasmic reticulum were usually present in the presynaptic boutons (Fig. 4e), whereas coated vesicles were infrequent.

Typical 'synaptic' membrane specializations were regularly found. The synaptic cleft was about 20 nm wide and contained an electron-dense material. The postsynaptic elements consisted most frequently of the somata of SCO ependymal and hypendymal cells, but basal processes of SCO ependymal cells and various processes of SCO hypendymal cells were also identified as sites of contact. Distinct cytoplasmic densities of variable thickness (up to 20 nm) adhered to the postsynaptic membrane, and gave the junctions the appearance of Gray's type I synapses (Figs. 4a and f). Furthermore, the examination of serial sections indicated that all boutons engage in synaptic junctions, and that all SCO ependymal cells are in synaptic contact with nerve endings. The serial sections also showed that individual axonal varicosities often engage in synaptic contacts with more than one SCO cell, and boutons in synaptic contact with at least three different SCO cells were sometimes found. The presynaptic elements in the SCO were characteristically devoid of astroglial covering.

Quantitative aspects of the structure of SCO synapses

The ultrastructural features of 30 characteristic axonal boutons in synaptic relationship with SCO cells were analysed and quantified in detail on high power electron micrographs (x 60 000). More than 2000 small clear vesicles were measured and classified as round or, if the longer diameter was at least 15% greater than the shorter diameter, as elliptical (elongated) vesicles. Though the axonal boutons were selected from different animals and from different subregions of the SCO, no characteristic variations were observed in vesicular composition or junctional morphology. This suggests that they belong to the same morphological class. A typical varicosity contained approximately 85% clear, elliptical vesicles, 45 x 60 nm in diameter, 15% clear round vesicles of 50 nm in diameter, and 1–2% large dense cored vesicles with a vesicle diameter of 85 nm and a dense core diameter of 50–55 nm. The synaptic cleft was about 20 nm and the mean length of the postsynaptic membrane specialization was 0.5 μm. The mean distance from the synapses on SCO cell perikarya to the nucleus of the cell was 0.5 μm.

Fig. 5. An SCO cell surrounded by degenerating terminals (arrows). Some postsynaptic membrane specializations can still be recognized. The axoplasm is heavily impregnated with fine granular electron-dense material and the mitochondria appear swollen and empty with an increase in electron density in the intermembranous compartment. This rat received an intraventricular injection of 5,6-DHT 24 h before the sacrifice.


ER
SCO
5
1 µm

Effects of neurotoxic drugs on the SCO innervation – ultrastructural observations
For the electron microscopical investigation short survival times after administration of the neurotoxic drug were chosen in order to use the initial stages of degeneration to identify the 5-HT innervation.

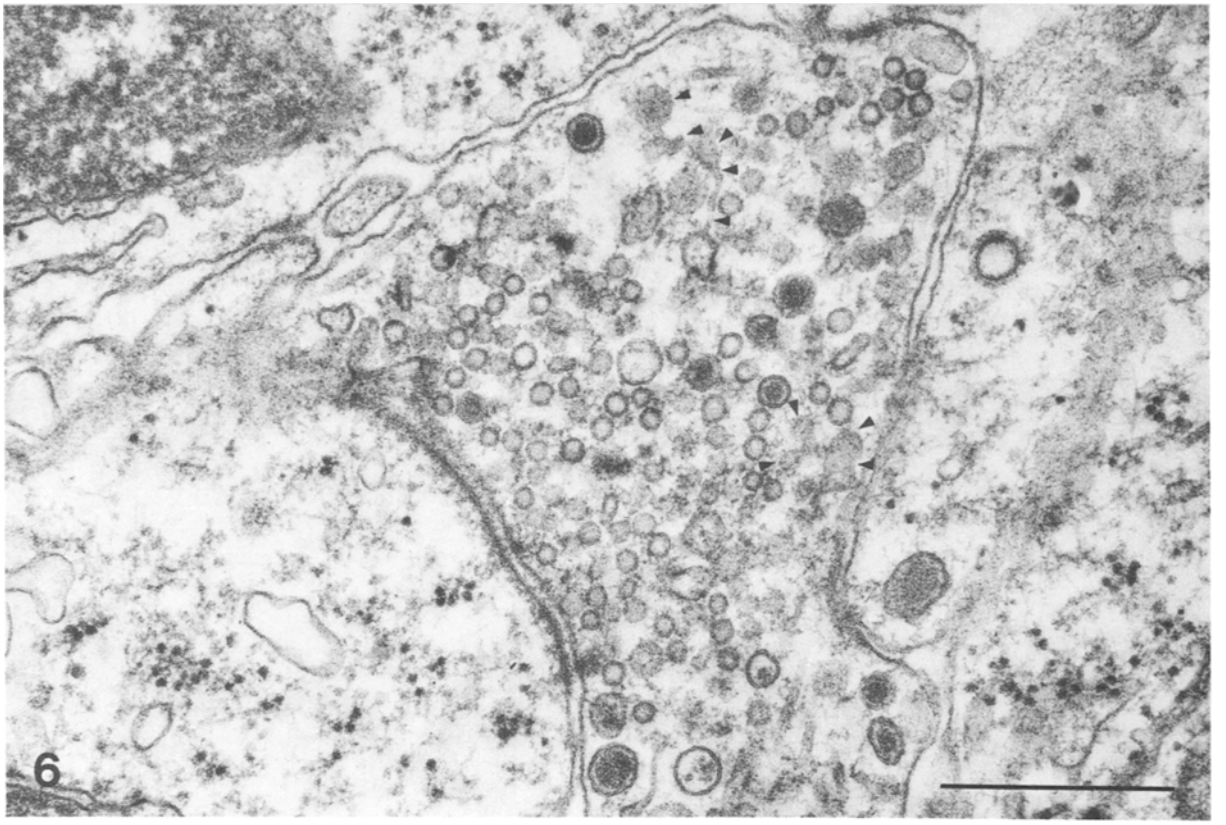
One, two and three days after administration of 5,6- or 5,7-DHT synaptic boutons in different stages of degeneration were found throughout the SCO. Most frequently, the affected terminals demonstrated the so-called electron-dense ('dark') degeneration (cf. Raisman and Matthews, 1972). The terminals undergoing this mode of degeneration exhibited a conspicuous darkening of the axoplasm and changes in mitochondrial configuration (Fig. 5). Clumping of the synaptic vesicles was also seen (Fig. 5) and this abnormal aggregation of the vesicles was further accentuated by a concomittant general shrinkage of the bouton (Fig. 7). Despite these conspicuous signs of degeneration, most boutons remained in recognizable synaptic contact with their target cells. In early stages, some boutons followed an alternative electron-lucent degeneration process characterized by general swelling and changes in vesicle morphology (Fig. 6). These terminals also remain to a large extent in contact with their targets.

The persistence of the synaptic contacts throughout the earlier stages of degeneration makes recognition of the postsynaptic target possible. Careful study revealed that the large majority of boutons in synaptic contact with SCO cells had been affected by the drug. In contrast, the synapses on perikarya and dendrites of SCO-associated neurons located in the deep hypendyma were unaffected. This indicates that most, if not all of the axons in synaptic contact with SCO cells correspond to the serotoninergic innervation, which can be visualized fluorescence histochemically. In a small number of boutons, however, no unequivocal signs of injury could be ascertained, and it remains to be established whether these terminals represent a minor non-monoaminergic innervation of the SCO, or whether they are 5-HT-containing terminals, which have either escaped the neurotoxic lesion or followed a delayed process of degeneration (see Discussion).

Fig. 6. Rat SCO 24 h after intraventricular injection of 5,7-DHT. Early stage of degeneration of a varicosity in synaptic contact with the perikaryon of an SCO cell. The discrete changes include enlargement of synaptic vesicles, and an increase in the number of smooth ER profiles (arrowheads) and large granular vesicles some of which demonstrate rarefaction of the granular content. Scale bar: 0.5 μm.

Fig. 7. Rat SCO 48 h after intraventricular injection of 5,6-DHT. The axoplasm in the two terminals has attained a uniformly increased electron opacity and the intermembranous compartment of the mitochondria (M) also exhibits the characteristic electron dense appearance. Note the shrinkage of the two terminals (arrows). Scale bar: 1 μm.

Fig. 8. Rat SCO 1 month after treatment with 5,6-DHT. Even basal processes which are normally more or less devoid of secretory elements exhibit characteristic and persistent ultrastructural changes. Compare the enlarged ER cisternae which are packed with flocculent material one month after treatment with ER cisternae of basal processes from control rats (for example, Fig. 3a). Note the moderate distension of the extracellular space. Scale bar: 1 μm.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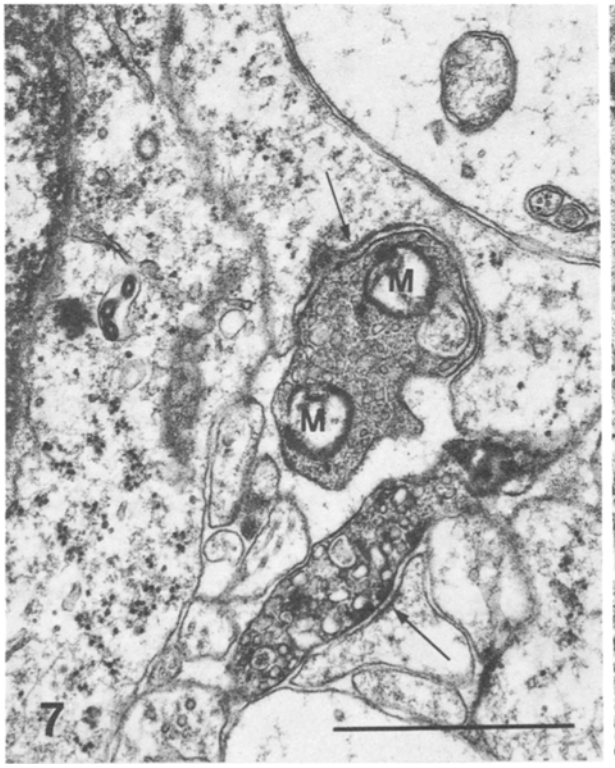
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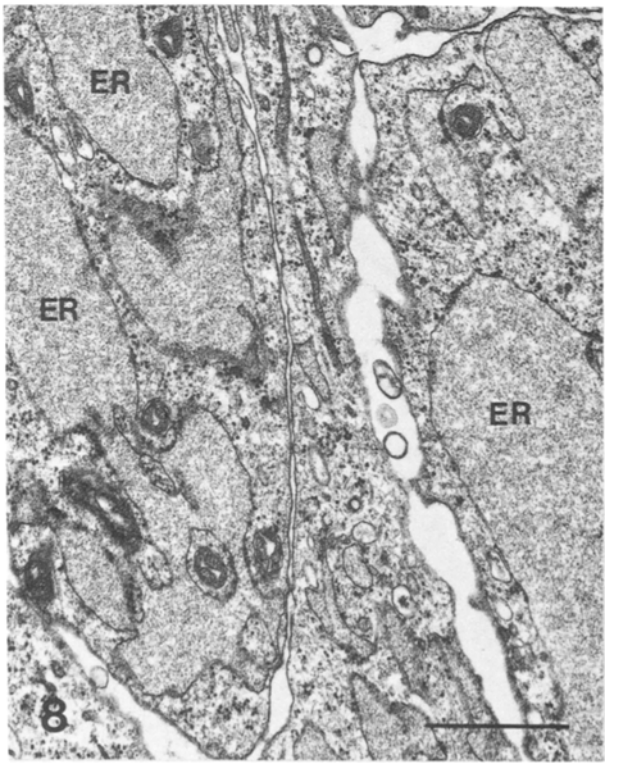
ER
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8

In the later stages investigated phagocytotic removal of the degenerated axon terminals was obvious. Thus, on the third day after treatment many of the degenerated boutons were engulfed by the SCO cells which they formerly innervated. In some cases, the postsynaptic specialization of the synapse was still visible as a part of the membrane enclosing the phagocytosed degenerated bouton (Fig. 7). Many ependymal and hypendymal cells had accumulated considerable amounts of debris in this process of phagocytosis. In the hypendyma, astrocytes were commonly engaged in the resorption of degenerating axons.

As reported earlier (Møllgård *et al.*, 1978), destruction of the serotoninergic innervation leads to a greatly increased synthetic and secretory activity in the SCO. This accelerated activity of the ependymal and hypendymal cells is reflected ultrastructurally by an enormous distension of the endoplasmic reticulum, a great increase in the number of secretory granules in both apical and basal processes, and evidence of an increased release of secretory material into the C.S.F. (apical secretion) as well as into the perivascular spaces of the deep hypendyma (basal secretion). As a possible effect of the increased secretory activity, a pronounced increase in the intercellular spaces was also seen. In the present study, the survival times after neurotoxic treatment were extended from three days to several months and, surprisingly, even after survival periods of eight months, the ultrastructural changes were still obvious. This indicates the presence of a sustained elevated secretory activity (Fig. 8). Indeed, no evidence was found to indicate a decrease in, or a normalization of the activity of the denervated cells.

Discussion

The present study provides the first detailed morphological description of the innervation of the rat SCO. Previous histochemical and ultrastructural findings (see Introduction) are confirmed and extended and several new observations are presented. Our experiments with specific neurotoxic drugs indicate that the axons in synaptic relationship with the SCO ependymal and hypendymal cells correspond to the dense serotoninergic plexus observed fluorescence histochemically.

Serotoninergic projections to the SCO

The *cell bodies* of serotoninergic neurons are mainly found in the raphé nuclei of the brain stem (Dahlström and Fuxe, 1964). From these nuclei the 5-HT neurons project to vast areas of the C.N.S. (see for example, Fuxe, 1965; Bobillier *et al.*, 1976). The origin of the SCO innervation was not investigated in the present study, but its location is suggested by recent results of Bobillier and co-workers (personal communication). Using radioautographic amino acid tracing technique they examined the efferent projections of the nucleus raphé medianus of the rat (B8 group of Dahlström and Fuxe, 1964). These experiments established the existence of a heavy projection to the basal SCO, which terminates in an arrangement identical to the fluorescence histochemically visualized serotoninergic plexus. This means that at

least a substantial part of the SCO innervation originates in the median raphé nucleus.

The organization of *ascending serotoninergic fibre bundles* has been described by Björklund *et al.* (1973c), who demonstrated that the fibres from the mesencephalic and pontine raphé nuclei, via two major ascending bundles, join in the ventral mesodiencephalic junction and contribute to the formation of the rostrally directed components of the medial forebrain bundle. Smaller bundles of indoleaminergic fibres were shown to leave the main fibre bundles at different levels to reach various target areas. The study of Björklund and co-workers did not, however, provide information about the serotoninergic route to the SCO. In preliminary investigations on the projection of serotoninergic nerve fibres to the epithalamic–pretectal area, we have distinguished three possible pathways: (1) a periventricular system of fibres running along the cerebral aqueduct; (2) fibres which in the ventral mesencephalic–diencephalic junction turn dorsally, and surrounding the fasciculus retroflexus, reach the habenular region; and (3) fibres leaving the rostral medial forebrain bundle, to run dorsally and caudally in the stria medullaris. It remains to be established, however, along which of these routes the fibres from the median raphé nucleus run to the SCO.

Serotonin neurotoxins and the SCO

The specific neurotoxic actions of 5,6-DHT and 5,7-DHT on central indoleaminergic systems have been well documented by fluorescence histochemical and biochemical investigations (see Björklund *et al.*, 1974; Baumgarten *et al.*, 1977). A few studies describing the ultrastructural features of degeneration induced by these neurotoxins have also appeared (Baumgarten and Lachenmayer, 1972; Baumgarten *et al.*, 1972). Both drugs are dihydroxylated analogues of serotonin, and their neurotoxic specificity is thought to depend on this structural similarity, which leads to preferential accumulation in serotoninergic neurons by the specific uptake mechanisms these cells have for their endogenous transmitter. Intracellularly, however, the drugs are supposed to exert a non-specific cytotoxic effect to which not only indoleaminergic neurons are susceptible (Baumgarten *et al.*, 1977).

The effects of 5,6-DHT and 5,7-DHT on the innervation of the SCO were investigated fluorescence histochemically after periods of one to two weeks, that is, at a time when the lesioned terminals can be expected to have disappeared (Björklund *et al.*, 1973b; Nobin *et al.*, 1973). This investigation showed that both neurotoxic drugs caused a virtually total destruction of the serotoninergic SCO innervation, although in some animals single yellow fluorescent nerve fibres seemed to have escaped the lesioning effect of the drug. Indoleaminergic neurons are known to possess pronounced regenerative capacities after axotomy induced by neurotoxic drugs (Björklund *et al.*, 1973a; Nobin *et al.*, 1973) and have in certain areas of the C.N.S. been observed to reinnervate even distant target areas (Nygren *et al.*, 1974; Wiklund *et al.*, 1978). However, despite detailed fluorescence histochemical investigations of the SCO after survival times of up to 15 months, we could not establish

any sign of restitution of the 5-HT innervation. Ultrastructural analysis revealed, however, that the SCO cells had been reinnervated by non-monoaminergic nerve fibres, which through a process of collateral sprouting had replaced the lost serotoninergic synapses (see the accompanying paper – Wiklund and Møllgård, 1979).

Specific degeneration induced by neurotoxic drugs can be used in ultrastructural identification of monoaminergic terminals (cf. Bloom *et al.*, 1971; Lorez and Richards, 1976; Baumgarten and Lachenmayer, 1974), but for this purpose shorter survival times are preferred (cf. Baumgarten *et al.*, 1972). In our experiments we chose survival periods of 1, 2 or 3 days after administration of 5,6-DHT or 5,7-DHT in doses shown fluorescence histochemically to produce an efficient denervation of the SCO. The large majority of the thin varicose unmyelinated axons, which established synaptic contact with SCO cells, were undergoing degeneration and it was, therefore, concluded that they correspond to the serotoninergic innervation observed fluorescence histochemically. However, a small number of boutons contacting SCO cells showed no evidence of degenerative changes. The nature of these boutons remains to be established, but several explanations can be offered for their occurrence. They could belong to an additional, minor non-monoaminergic system, which innervates the SCO. But since we found fluorescence histochemically that single yellow fluorescent fibres sometimes did remain in the SCO after 5,6-DHT or 5,7-DHT, it is also possible that they represent 5-HT fibres, which have escaped neurotoxic destruction. Furthermore, it cannot be excluded that the boutons failing to show degenerative changes a short time after administration of the neurotoxic drug, could be in a delayed process of degeneration. Indeed, the interpretation that the ultrastructurally intact boutons represent serotoninergic terminals spared from neurotoxic injury or in delayed degeneration seems to be supported by the fact that they do not differ from intact serotoninergic boutons in vesicular morphology or other ultrastructural characteristics. A final answer to the question of whether the rat SCO is exclusively innervated by serotoninergic fibres or whether a minor non-monoaminergic component exists will, however, have to await further investigations. We hope that our recently-initiated radioautographic experiments will provide the answer.

Despite many investigations, the function of the SCO still remains obscure. It is well established that the organ produces the so-called Reissner's fibre, which runs through the ventricular system from the aqueduct to the lumbar cistern (Olsson, 1958; Sterba *et al.*, 1967). However, although several suggestions have appeared in the literature, no irrefutable evidence has been presented indicating the function of this acellular structure. The function of the basally directed secretion from processes of SCO cells into the perivascular spaces of the rich hypendymal capillary plexus is even less well understood, but the secretion might be involved in some form of regulation of salt and/or water metabolism. (For a recent review of the SCO and its function, see Ziegels, 1976.)

We have previously reported that denervation of the SCO with neurotoxic drugs rapidly leads to a dramatic increase in the synthetic and secretory activities of the

organ (Møllgård *et al.*, 1978). In the present study, even after eight months no reversal in these signs of increased cellular activity was observed. Hypothetically, the cellular changes could either be a denervation effect or depend on some direct effect of the neurotoxic drugs on the SCO cells. In our previous study, we investigated these possibilities by application of [^{14}C] labelled 5,6-DHT, but the ensuing autoradiographic analysis revealed no accumulation into SCO cells, which presumably precludes the possibility of direct cytotoxic effects on the SCO cells. Furthermore, since no significant receptor effects have been reported for 5,6-DHT or 5,7-DHT, we concluded that the observed cellular changes were responses to removal of the serotoninergic innervation (Møllgård *et al.*, 1978). This implies that the serotoninergic innervation exerts a powerful tonic inhibitory control of the SCO, and that denervation removes this inhibition from the SCO cells, which respond with increased synthetic and secretory activity. Furthermore, because the serotoninergic neurons fail to reinnervate the SCO, the increase in cellular activity becomes permanent. Since the nerve terminals innervating the SCO contain large amounts of serotonin, the inhibition of the SCO activity is presumably mediated by release of this transmitter and interaction with serotoninergic receptors on the SCO cells. This conclusion needs, however, to be corroborated by more direct evidence, since it recently has been claimed that serotoninergic neurons may contain putative peptide transmitters in addition to serotonin (Chan-Palay *et al.*, 1978; Hökfelt *et al.*, 1978).

The discovery of the serotoninergic SCO innervation and the suggested powerful inhibitory regulation of the secretory activity opens the possibility of modulating the activity of the organ. Thus, pharmacological manipulations of the secretory activity with serotoninergic agonists and antagonists could prove a very valuable tool in the future exploration of SCO physiology.

Serotonin and ependyma

The 5-HT synapses on the modified ependymal cells of the SCO (also cf. Bouchaud and Arluison, 1977; Møllgård *et al.*, 1978) is as far as we know the first and at present the only example of ultrastructurally 'classical' synapses on ependymal cells in the mammalian C.N.S. Previously, 'synaptoid' junctions have been described between nerve terminals and tanycytes in the median eminence (Güldner and Wolff, 1973), but these differ from the SCO innervation by failing to display postsynaptic membrane specializations. The demonstration of the serotoninergic synapses in the SCO raises the question of the status of the SCO cells. They should perhaps not exclusively be regarded as glial cells or tanycytes, since they also show the presumed 'neuronal' characteristic of engaging in synaptic junctions.

An extensive plexus of serotoninergic nerve fibres has been found on the ventricular surface of the ordinary ependyma covering most ventricular surfaces (Lorez and Richards, 1973, 1975). These supraependymal serotoninergic fibres contain small clear synaptic vesicles and occasional large granular vesicles (Richards *et al.*, 1973). This organelle content is similar to that which we found in the boutons innervating the SCO, although the latter seem to contain a much larger population

of vesicles per bouton than the supraependymal fibres. However, there are several distinct differences between the supraependymal nerve plexus and the basal SCO innervation. The most important of these is the fact that the supraependymal nerve fibres simply overlie the ventricular surface and do not form junctional specializations with the ependymal cells. Therefore, it is possible that they are concerned in the release of material into the ventricular fluid rather than in regulation of the activities of ependymal cells. So far no conclusive evidence has been reported which indicates the function of the supraependymal serotoninergic plexus. Perhaps the present study which has shown a profound inhibitory effect exerted by the serotoninergic innervation on the secretory activity of the SCO, can give the impetus to similar functional studies on the supraependymal plexus.

Ultrastructural identification of 5-HT terminals

Specific ultrastructural identification of serotoninergic nerve terminals in several areas of the brain have been possible by recent developments in radioautographic and immunocytochemical techniques. The radioautographic technique is based on the detection of exogenously-administered tritiated serotonin, which is preferentially accumulated by and retained in serotoninergic neurons (cf. Descarries, 1975). The immunocytochemical method visualizes with specific antibodies the enzyme tryptophan hydroxylase, which catalyses the first step of the biosynthesis of serotonin (Pickel *et al.*, 1976). These techniques have the advantage of demonstrating intact nerve terminals, which the method of identification by specific neurotoxic degeneration does not offer. On the other hand they have the disadvantage of not allowing as high a resolution as conventional electron microscopy. The impaired resolution of ultrastructural details is especially evident when using the immunocytochemical techniques, where deposits of electron-dense reaction product cover labelled terminals and organelles. However, in areas of the C.N.S. where the serotoninergic boutons constitute but a small fraction of the innervation, the radioautographic and immunocytochemical techniques are clearly more advantageous for identification than chemically induced degeneration. These techniques have in recent years provided a growing body of information on the ultrastructure of serotoninergic systems.

The serotoninergic terminals in the rat neocortex were investigated by Descarries *et al.* (1975) with radioautographic technique. The neocortical terminals were found to contain both small clear and large dense cored vesicles, which were similar to those of the boutons innervating the SCO. However, in contrast to the SCO innervation, remarkably few of the neocortical serotoninergic boutons formed synaptic contacts. Instead, the majority of the visualized neocortical 5-HT axons seemed to terminate as 'free arborizations' with evenly spaced varicosities, regularly apposed to dendritic profiles, but in most cases without obvious pre- and postsynaptic membrane specializations. Ultrastructural investigations of the serotoninergic innervation of the cat caudate nucleus (Calas *et al.*, 1976 – radioautography) and the rat locus coeruleus (Pickel *et al.*, 1977 – immunocytochemistry; Léger and

Descarries, 1978 – radioautography) revealed that in these regions too, the serotoninergic nerve fibres rarely form synaptic junctions. In both regions, however, the 5-HT boutons exhibited a vesicle morphology quite different from what has been found in the neocortical and SCO-contacting 5-HT endings. The presynaptic elements in the cat caudate nucleus were characterized by numerous round and/or elongated large granular vesicles (80–120 nm in diameter) and a few clear synaptic vesicles (40–60 nm) (Calas *et al.*, 1976), and the terminals in the locus coeruleus contained unusual microvesicles and microcanaliculi (15–25 nm in diameter) and a few large granular vesicles, but no ordinary small synaptic vesicles (Léger and Descarries, 1978). Serotoninergic terminals with many synaptic junctions have been observed in the cerebellum, where Chan-Palay (1975) in a radioautographic study described the occurrence of serotonin-accumulating mossy fibre endings. These mossy fibre rosettes, and 'a small number of small synaptic boutons' (group 1b according to the classification of Chan-Palay) were, however, the only labelled boutons in the cerebellum which terminated in a predominantly 'synaptic' fashion. The majority of serotonin-labelled terminals in the cerebellum belonged to other, ultrastructurally-different classes of boutons, which rarely engaged in synaptic junctions (Chan-Palay, 1975). Therefore, also the results from the investigation of cerebellum seem to support the general impression that serotoninergic neurons most commonly terminate as free axonal varicose arborizations.

The functional implications of the observed diversity of serotoninergic terminals remain to be clarified. The variation in vesicular content presumably indicates differences in storage and possibly release mechanisms of the transmitter. The occurrence of 'synaptic' serotoninergic terminals in the SCO and the cerebellum implies a restricted effect on particular postsynaptic elements, while the relative absence of junctional specializations in, for example, the neocortical system may suggest a more generalized mode of action on a large neuronal population (cf. Descarries, 1977).

Conclusion

The serotoninergic innervation of the SCO, where the serotoninergic boutons form the major, if not the only, innervation of the target cells represents an extraordinarily simple system in the mammalian C.N.S. In other areas of the brain the serotoninergic terminals represent only a small portion of the innervation of a given region, which means that functional exploration of the serotoninergic input is complicated by the interaction of other systems. Our denervation experiments (this study and Møllgård *et al.*, 1978) show that the potent serotoninergic regulation of SCO synthetic and secretory activity is readily accessible by histochemical, ultrastructural and karyometric methods. Taken together this indicates that the SCO innervation represents a unique model in pharmacological and physiological investigations of serotoninergic innervation. It can be argued that the control of serotoninergic activities in the SCO is not representative of the serotoninergic systems, which

innervate electrically active neurons. However, the observed serotoninergic influence on tyrosine hydroxylase activity in the noradrenergic neurons of the locus coeruleus (Renaud *et al.*, 1975; Lewis *et al.*, 1976) may indicate that regulation of biosynthetic activities could be a common property of serotoninergic innervation. We, therefore, believe that further exploration of the serotoninergic innervation of the rat SCO will yield important contributions to our understanding of serotoninergic systems in general.

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