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Subcommissural Organ-associated Neurons in Fetal and Neonatal Rabbit

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Summary. Neurons which initially lie in the basal region of the subcommissural organ (SCO) were investigated in 20 rabbit fetuses from day 15 to 30 of gestation, and in eight neonatal, 4 and 8 day old rabbits. These SCO-associated neurons, first observed on day 17 of gestation, develop into (1) a rostral mesodiencephalic nerve cell group situated in an area dorsal to the rostral-most part of the SCO and (2) a more caudal layer of single neurons extending throughout the length of the SCO.

The present findings are discussed in relation to recent histochemical studies that demonstrated AChE-positive neurons in the pineal complex and subcommissural area of frogs and to recent fluorescence microscopic studies in fetal and adult rats in which a 5-HT system is known to extend from the *nucleus raphe dorsalis* (B7) along the SCO to the pineal stalk and habenular region. The term "SCO-associated neurons" is a purely morphological way of describing the neurons in question as the neural interconnections of these neurons are still a matter of speculation.

Key words: Subcommissural organ — Neurons — Development — Rabbit.

Introduction

In a recent ultrastructural investigation (Kimble and Møllgård, 1973), nerve cells were found in close relationship to the subcommissural organ (SCO) of the adult rabbit. These neurons are spaced fairly evenly from the rostral to the caudal extremeties of the SCO and nestle down very close to the hypendyma, most often in the vicinity of a hypendymal capillary. In a previous publication we (Møllgård and Møller, 1973) have reported the discovery of a small mesodiencephalic nerve cell cluster located dorsal to the rostral area of the human fetal SCO, as well as a miniature nerve connecting this nerve cell group and the pineal gland. The existence of a similar nerve has now been established in sheep and rabbit fetuses as well (Møller *et al.*, 1975).

The purpose of the present study is to probe further into the nature of the fetal mesodiencephalic neurons, their relationship to the "pineal nerve" (see Møller *et al.*, 1975) and their correlation, if any, to the single layer of neurons associated with the hypendyma of the adult rabbit SCO. We have, therefore, examined the rabbit SCO at various stages of embryonic development in order to determine the time of appearance as well as developmental patterns characteristic of these SCO-associated neurons.

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Material and Methods

Rabbit fetuses of the following days of gestation were examined: 15, 17, 18, 20, 22, 23, 24, 25, 26, 27, 30. In addition, newborn as well as 4 and 8 day old rabbits were used to obtain post-embryonic material.

Pregnant rabbits were anesthetized by injection of 5% Nembutal (30 mg/kg body wt) in the auricular vein. The abdomen was opened and the fetuses removed from the uterus. The entire fetal brain was fixed by immersion either in Formol-calcium (pH 5.3) (Pearse,1968) for 24 hrs in the case of paraffin embedding or in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) for 8 hrs in the case of Epon embedding.

Serial paraffin sections (4–5 μm thick) were cut in sagittal or frontal planes. The sections were stained routinely by (1) toluidine blue (Kramer and Windrum, 1955), or (2) by Bock's Chromalum-Gallocyanin method (Bock, 1966) with or without pre-treatment with RNAase.

The specimens for Epon embedding were washed in buffer. They were then cut into blocks containing the pineal gland and the SCO. The blocks were post-fixed in 2% OsO_4 in 0.1 M Na-cacodylate buffer, stained *en bloc* in 0.5% aqueous uranyl acetate for 1 h and embedded in Epon after dehydration. The blocks were carefully oriented during embedding so that survey sections of the blocks would be strictly in the sagittal plane. One micron thick serial sections stained with toluidine blue were examined in the light microscope. Thin sections were cut from selected areas, and after post-staining with uranyl acetate and lead citrate viewed in a Hitachi HS8 microscope operated at 50 KV.

Results

Light Microscopy. Definitive neurons, exhibiting large, clear nuclei with distinct nucleoli and a strongly basophilic cytoplasm, are first observed in the 17 day embryo lying basal to the SCO ependyma. (In agreement with other workers in the field of developmental neurobiology—see, e.g. Jacobson, 1970—we describe cells exhibiting the classical morphology of neurons as neurons, even though they may not have expressed their full developmental capacity.) An examination of *15 day* embryos reveals similarly located *single* cells with sparse perikaryal cytoplasm; these may be immature neurons. The neurons of the 17 day embryos are evenly distributed, randomly oriented, and are of considerable number. By day 18, the even distribution along the SCO characteristic of the 17 day embryo has given way to a pattern in which a decidedly higher concentration of nerve cells is found rostrally than caudally (Fig. 1). In later stages (Fig. 2) this pattern consists of (1) a cluster of neurons located rostrally (the mesodiencephalic group of neurons) and (2) a layer of scattered neurons which extends along the length of the SCO, usually just basal to the SCO ependyma. The mesodiencephalic neurons are separated from the SCO ependyma and show no preferential orientation. The single neurons along the SCO, on the other hand, often exhibit an orientation perpendicular to the long axis of the SCO_0-ependymal cells, and are frequently seen close to the hypendymal capillary network (Fig. 2a).

The center of the mesodiencephalic group of neurons is found dorsal to the rostral-most part of the SCO (Fig. 3, 3a, and 3b). When the pineal nerve is present, nerve cells from the mesodiencephalic group can be followed dorsally toward and into the nerve. Furthermore, dendrites from the mesodiencephalic nerve cell group can sometimes be seen to extend towards the site of penetration of the pineal nerve into the CNS (Fig. 3a). Rostral to the mesodiencephalic nerve cell group, single neurons are observed both along the floor of the pineal recess

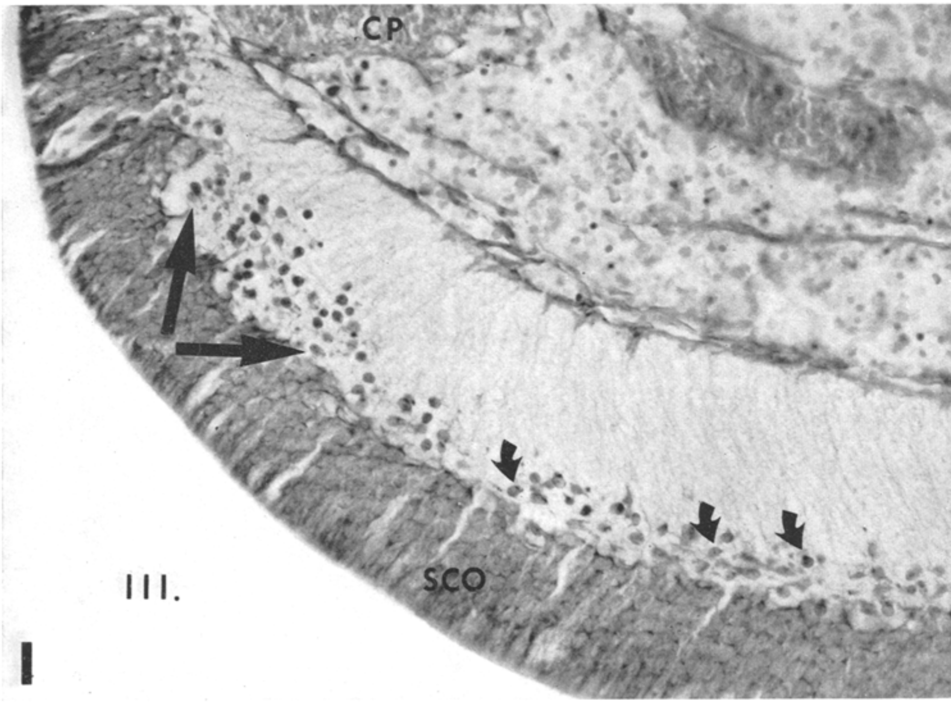


Fig. 1. Sagittal section through the *SCO* from a rabbit fetus of day 18 of gestation. Note the high concentration of nerve cells rostrally in the area encompassed by two *large arrows*. *Small arrows* point to a few of the well differentiated neurons of the more caudal group. *III* third ventricle. *CP* pineal gland. Toluidine-blue stained paraffin section. $\times 300$

and in the caudal area of the habenular region just basal to the ependyma. Caudally, along the length of most of the SCO region, there is a transition from a multiple layered arrangement of nerve cells to a single layer of neurons.

As one follows the development of the SCO-pineal complex in neonatal and young (4 and 8 day old) rabbits, the pattern of distribution of SCO-associated neurons remains the same relative to the SCO ependyma and the pineal recess. However, the pineal gland develops in such a way that the rosettes of pinealocytes which are situated dorsal to the rostral-most region of the SCO, and caudal to the mesodiencephalic group (Fig. 2) seem to fuse with the main tissue of the pineal gland. In this manner, the mesodiencephalic neurons appear to take up a central position in the proximal part of the developing pineal stalk.

Electron Microscopy. Thin sections confirm the fact that the cells which we describe light microscopically as SCO-associated neurons are, indeed, neurons (Fig. 4). These cells exhibit large nuclei with relatively loose chromatin, an electron lucent cytoplasm filled with organelles, and numerous cytoplasmic processes extending from the cell body. A few axo-somatic and axo-dendritic synapses (Fig. 5a and b) are observed on cells of the mesodiencephalic nerve cell group in 25 day embryos, and by day 27 of gestation these synapses are quite frequent.

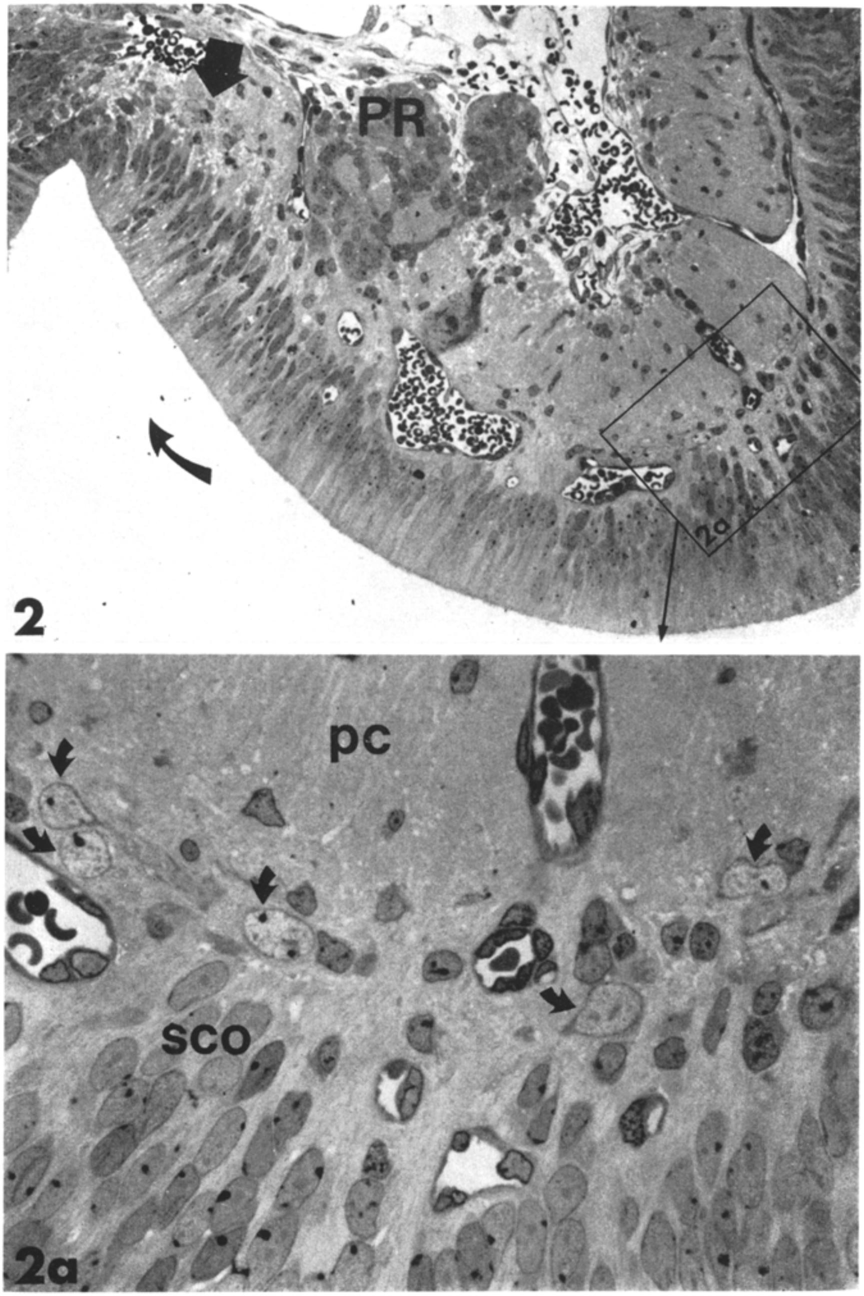


Fig. 2 and a. Sagittal section through the SCO from a rabbit fetus of day 27 of gestation. Note the mesodiencephalic nerve cell group (*thick arrow*), rosettes of pinealocytes (*PR*) located dorsal to the rostral-most part of the SCO. *Curved arrow* points to the top of the pineal recess. Toluidine-blue stained Epon section. × 145. (a) Magnification of region outlined in black in Fig. 2. Distinct neurons (*arrows*) are located in the hypendymal region between the SCO ependyma (*sco*) and the posterior commissure (*pc*). Their predominant orientation is perpendicular to the long axis of the SCO cells and parallel to the vessels. × 570

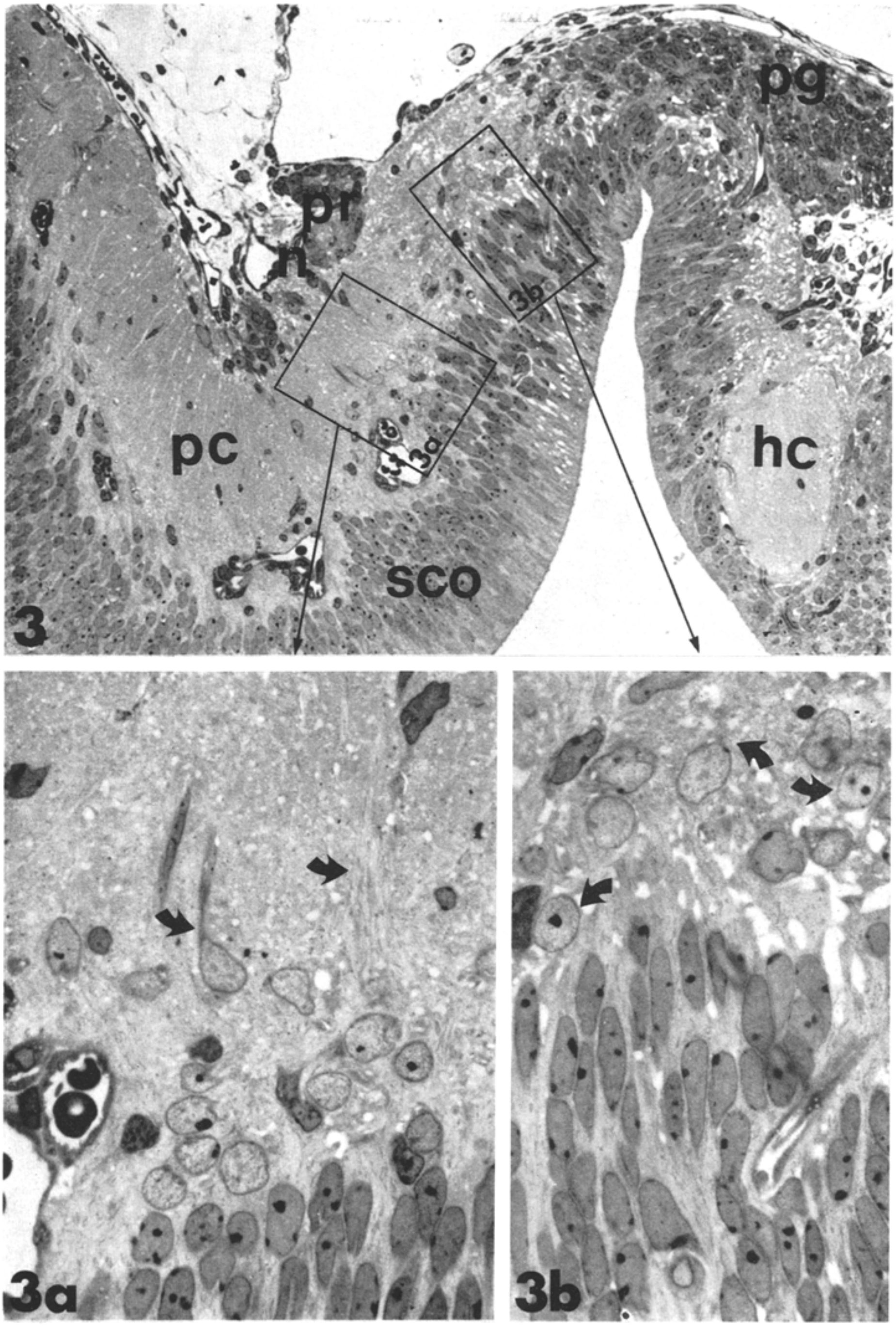


Fig. 3, a and b. Sagittal section through the SCO from a rabbit fetus of day 23 of gestation. Note the pineal gland (*pg*), habenular commissure (*hc*), posterior commissure (*pc*), SCO ependyma (*sco*), rosettes of pinealocytes (*pr*), entrance of the pineal nerve into the brain (*n*). Toluidine-blue stained Epon section. × 160. (a) Dendrites (*arrows*), distinguishable due to their size and basophilic staining, extend through the posterior commissure from mesodiencephalic neurons toward the site of penetration of the pineal nerve. × 630. (b) Additional mesodiencephalic neurons (*arrows*). Note that there is no predominant orientation with respect to the SCO ependyma. × 630

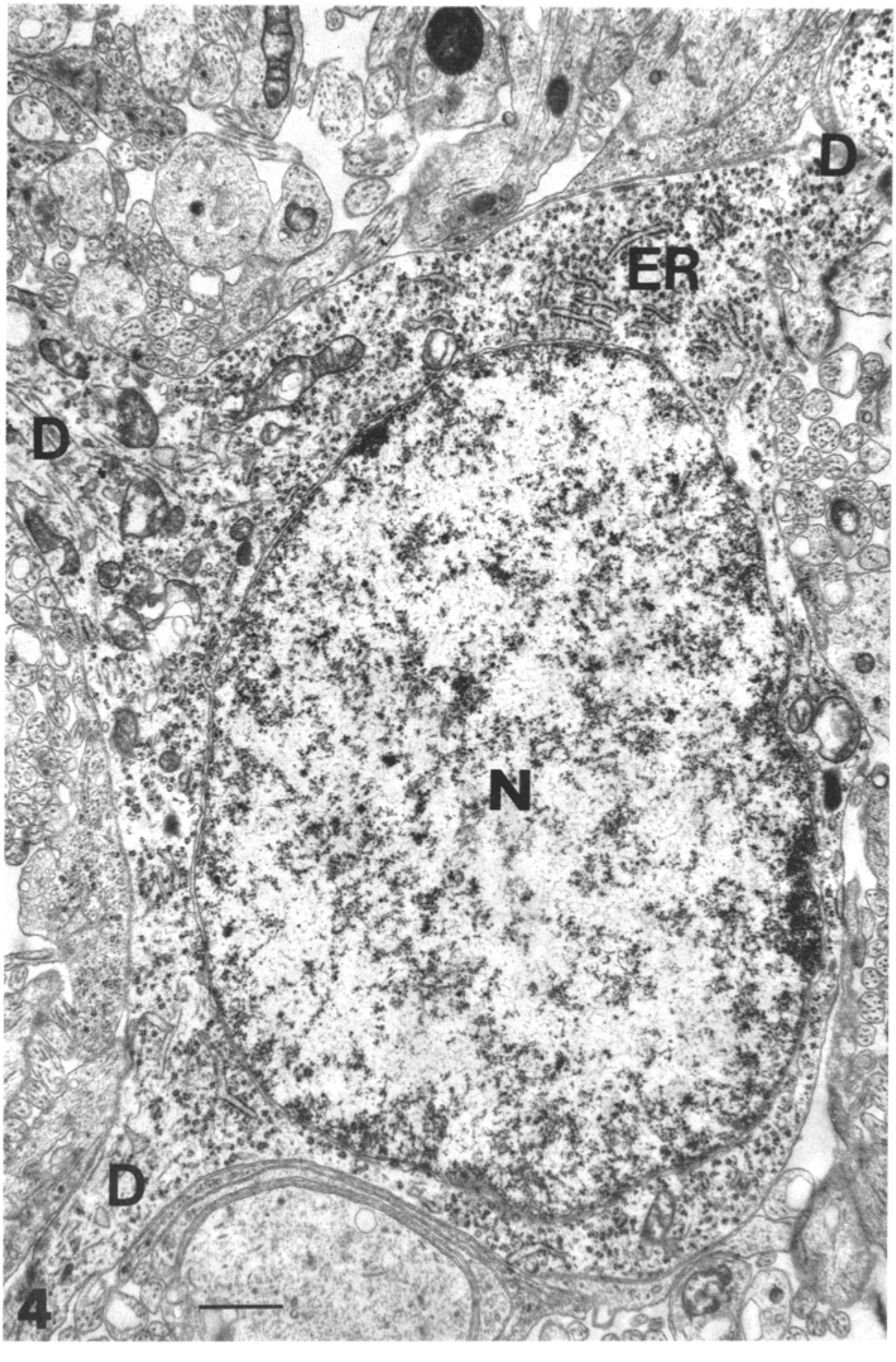
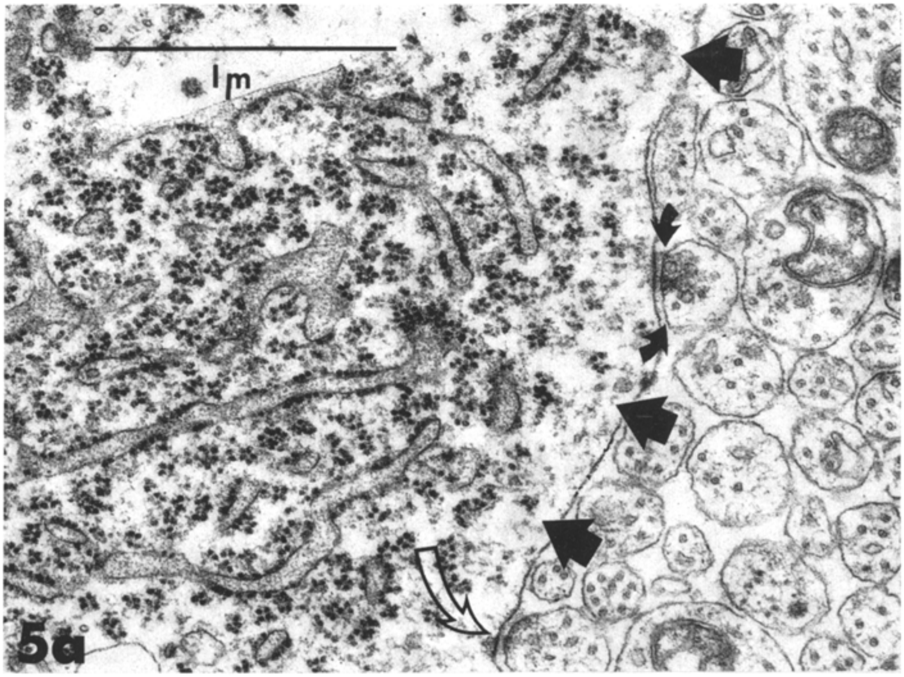


Fig. 4. A mesodiencephalic neuron. Cisternae of granular endoplasmic reticulum (*ER*) are seen above the large elongated nucleus (*N*). Three dendrites (*D*) emerge from the perikaryon in this section. Day 27 of gestation. $\times$ 11500. Bar indicates 1 μm



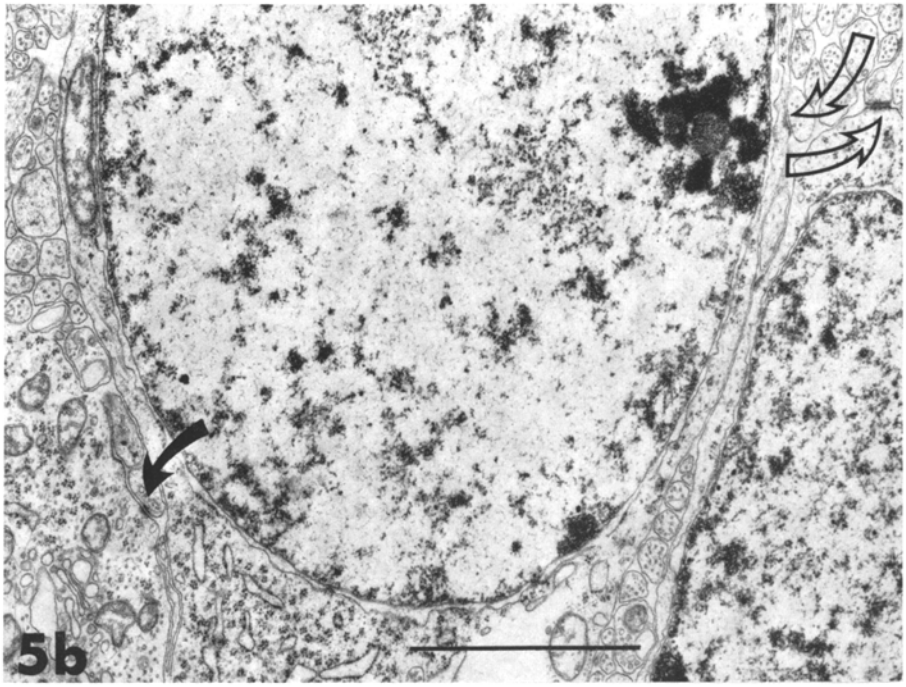


Fig. 5. (a) An axo-somatic synapse (between *curved arrows*) can be recognized on a mesodiencephalic nerve cell (*large arrows*). A few presynaptic clear vesicles are clustered adjacent to a presynaptic density. The postsynaptic density is also clearly distinguishable. *Open curved arrow* points to a developing synapse. $\times 40000$. Bar indicates 1 μm. (b) Immature synapses are recognizable already at day 25 of gestation. *Open arrows* point to axo-somatic contacts of mesodiencephalic neurons, and *black arrow* marks an axo-dendritic contact in the vicinity. $\times 14500$. Bar indicates 2 μm

Discussion

Our previous reports (Møllgard and Møller, 1973; Møller *et al.*, 1975) together with the present work demonstrate the existence of a group of SCO-associated neurons in fetal mammals. It should be pointed out that the term "SCO-associated neurons" is a purely morphological way of describing the nerve cells in question, and is not meant to imply a functional relationship to the SCO for which we have no evidence. The pattern of development in the fetal rabbit appears to be as follows. At day 15 to 17 of gestation these neurons become recognizable, and from day 18 of gestation a more rostral mesodiencephalic nerve cell group can be distinguished from a more caudal single layer of neurons extending along the length of the SCO region in close contact with the hypendyma. In addition, single neurons extend rostrally in the floor of the pineal recess and into the habenular region. At day 23 to 25 of gestation, a nerve (the "pineal nerve") connects the mesodiencephalic nerve cell group to the pineal gland (see Møller *et al.*, 1975). In neonatal and young animals, the mesodiencephalic neurons seem to take up a more central position in the proximal part of the developing pineal stalk, and the pineal nerve can no longer be seen traversing the subarachnoid space caudal to the pineal gland. A relationship of the mesodiencephalic neurons to the single layer of neurons located more caudally, to the pineal nerve dorsally, and to the neurons located in the floor of the pineal recess rostrally is suggestive from this pattern of development.

Many previous authors have described the entrance of nerve fibers from the posterior commissure into the hypendymal region of the SCO of mammals (for a review see Palkovits, 1965); however, the origin and termination of these fibers are largely unknown. A group of nerve cells ("*ganglion pericommissurale*") has been observed in a superolateral position to the SCO of the dog (Yamada *et al.*, 1957) and the monkey (Hosaka *et al.*, 1957). Since the single layer of SCO-associated neurons observed in the rabbit is located in the hypendyma just basal to the SCO ependyma, the relationship between these neurons and those of "*ganglion pericommissurale*" is unclear.

No electron microscopic studies have dealt specifically with SCO-associated neurons and nerve fibers in mammals, but certain comments can be found in various investigations on the SCO. Stanka *et al.* (1964) found a single nerve cell in the hypendyma of the adult rat, and Isomäki *et al.* (1965) noted the presence of some nerve cells in the hypendyma of the calf. More recently, Kimble and Møllgård (1973) described a single layer of neurons lying just basal to the SCO (in its hypendyma) in the adult rabbit which must belong to the same system as those described in the present study in the fetal and neonatal rabbit.

In a thorough analysis of central monoamine neurons in the fetal rat by fluorescence microscopy, Seiger and Olson (1973) described a 5-hydroxytryptamine (5-HT) axon pathway from the dorsorostral part of the *nucleus raphe dorsalis* (or B7, according to the nomenclature of Dahlström and Fuxe, 1964) ascending directly through tegmentum and tectum dorsally towards the habenular region. In addition to the 5-HT axon extension from B7, a single layer of scattered *5-HT containing cells* situated dorsally to the anterior part of the cerebral aqueduct has been observed in fetal rats (Seiger and Olson, personal communication). 5-HT containing fibers associated with the basal region of the SCO have also been described in adult rats (Fuxe, 1965; Björklund *et al.*, 1972). The presence of *5-HT containing cells* in the *lamina intercalaris*, a characteristic region in the adult rat located between the habenular and posterior commissures which includes the floor of the pineal recess and the basal-most part of the pineal stalk, was also pointed out by Björklund *et al.* (1972). In agreement with Ariëns Kappers (1960), these authors considered the cells of lamina intercalaris to be more or less well differentiated pinealocytes, but they stated that the nature of these cells is not clear. Very recently Wiklund (1975) has provided further evidence for the fact that the cells of the lamina intercalaris are of pinealocyte rather than neuronal nature in an ontogenetic study on the rat. However, in a preliminary ultrastructural investigation on the *lamina intercalaris* of the adult rat we have observed definite neurons among the very characteristic cells of that area (unpublished). Further investigations should be performed in order to rule out the possibility that at least some of the fluorescent cells observed along the SCO and in the *lamina intercalaris* of the rat are in fact neurons and thus an equivalent to the SCO-associated neurons observed in the present study.

Another and perhaps more valid hypothesis with regard to the nature of the SCO-associated neurons described in the present study can be derived from recent findings of Wake *et al.* (1974). These authors have identified a group of acetylcholinesterase-containing nerve cells in the pineal complex and subcommissural area of frogs. The AChE-positive neurons are mainly distributed in a single layer between the pineal tract and the base of the SCO-ependymal cells, and, furthermore, a ganglion-like group of nerve cells is situated at the caudal pole of the pineal organ in the region dorsal to the rostral-most area of the SCO (also cf.: Fig. 22 in Wake *et al.*, 1974).

Fluorescence histochemical investigations and acetylcholinesterase reactions should be performed on the SCO-region of fetal and neonatal rabbits in order to establish whether the SCO-associated neurons described in this study represent a rostral extension of the *nucleus raphe dorsalis*, or whether the neurons are related—phylogenetically or ontogenetically—to (1) the late developing, probably non-neuronal 5 HT-system described in rats (Wiklund, 1975), (2) the group of AChE-positive neurons described in frogs (Wake *et al.*, 1974) or (3) some, at present, unknown system.

Also the input-output relations of the fetal and neonatal SCO-associated neurons are unknown at present. We would like to emphasize the fact that the term "SCO-associated neurons" is a purely morphological way of describing the neurons in question and is not meant to imply any functional association of the neurons with the SCO. In fact, certain unpublished ultrastructural findings in the rat SCO (Møllgård *et al.*, unpublished) indicate that the SCO-associated neurons may have no connection to the SCO ependyma. Only one type of synapse seems to terminate directly on the basal extensions of the SCO-cells, and these axons probably originate from a 5 HT-neuron system in the brain stem. This implies that "local" neurons, e.g., "SCO-associated (AChE-containing?) neurons", do not innervate the SCO-ependymal or hypendymal cells.

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