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Presence of a Pineal Nerve in Sheep and Rabbit Fetuses

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Summary. The presence of a nerve located just caudal to the pineal gland in the mid-sagittal plane is demonstrated in sheep and rabbit fetuses. This nerve lies freely in the sub-arachnoid space and extends from the pineal gland to a region of the CNS located dorsal to the rostralmost part of the subcommissural organ (SCO). In rabbit fetuses the nerve is observed on days 23 and 24 of gestation; we suggest that it is an ontogenetic equivalent to the pineal nerve of anuran amphibians. The developmental fate of the mammalian fetal pineal nerve is discussed.

Key words: Pineal nerve — Sheep and rabbit fetuses — Subcommissuralorgan — Ontogeny.

Introduction

A definitive classification of the diverse nerve fibers associated with the mammalian pineal gland is still a matter for dispute. Only a sympathetic innervation via *nervus conarius*, has been well established. This nerve was first described by Kolmer and Löwy (1922) in the dog and goat pineal, and has since been observed in monkey and man (Le Gros Clark, 1940) as well as in many other mammalian species. Kappers (1960) demonstrated in the rat pineal that *nervus conarius* is present bilaterally and that it contains sympathetic fibers from the superior cervical ganglion. Many later investigations have shown the influence of this sympathetic input on pineal activity (for a review, see Wurtman *et al.*, 1968). In addition to this sympathetic input, a parasympathetic innervation of the pineal has been reported in the macaque (Kenny, 1961), ferret (David *et al.*, 1973) and rabbit (Romijn, 1973). However, no functional role of this suggested parasympathetic input has been demonstrated in relation to pineal activity.

Nerve fibers penetrating into the pineal gland from the brain, especially from the habenular and posterior commissures, have been reported in mammals by many authors (e.g. Pines, 1927; Gardner, 1953; Scharenberg and Liss, 1965). However, Kappers (1960) has demonstrated in the rat, and Romijn (1972) in the rabbit, that aberrant fibers from these commissures approach the pineal, and then turn back into the central nervous system without actually innervating the pineal parenchyma. These authors concluded that the former reports of a central nervous connection to the pineal organ were based on observations of aberrant commissural fibers. Nevertheless, a nervous connection between the habenular

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nuclei and the pineal has been recently described in the ferret (David and Herbert, 1973).

In addition to the above-mentioned nervous connections of the pineal gland, the presence of another pineal-associated nerve has recently been described by light microscopy in human fetuses (Møllgård and Møller, 1973). This nerve is found midsagittally and runs between the pineal gland and a small mesodiencephalic nerve cell group situated dorsal to the rostralmost area of the subcommissural organ (SCO). In the present investigation we show that this nerve is present in both sheep and rabbit fetuses and suggest a homology between this nerve and the pineal nerve present in the anuran amphibians.

Material and Methods

Three sheep fetuses and 6 rabbit fetuses were used. The gestational ages of the fetuses were: *Sheep;* 60 days (gestation period 150 days). *Rabbit;* 20, 22, 23, 24, 25 and 27 days (gestation period 30 days).

Anaesthesia and Fixation

Sheep. The ewes were anaesthetized with i.v. thiopentone (5% w/v in sterile water, 0.2—0.3 ml/kg body wt.) followed by chloralose (1% w/v in 0.9% saline, up to 3 mg/kg body wt., supplemented at hourly intervals by 0.3—0.6 mg/kg). One horn of the uterus was delivered through an oblique lower midline abdominal incision in the anaesthetized ewe. A canula was inserted into the umbilical vein and the umbilical artery was opened. 40 ml of 1% Karnovsky fixative was injected with a syringe over a period of 16 min. After perfusion-fixation the brain was removed, immersed for 6 hours in 1% Karnovsky fixative, and then transferrred to 0.1 M Na-cacodylate buffer at 4 °C.

Rabbit. Pregnant rabbits were anaesthetized by injecting 5% Nembutal (30 mg/kg body wt.) into the auricular vein. The abdomen was opened and the fetuses removed from the uterus. The total brain was fixed by immersion in 2.5% glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) at 4 °C for 6—10 h, and transferred to 0.1 M Na-cacodylate buffer at 4 °C.

Postfixation, Embedding and Orientation of Tissue

After 12—48 h in the buffer blocks of tissue containing the pineal and the subcommissural organ were cut from the dorsal mesodiencephalic area. These blocks were postfixed in 2% OsO_4 in 0.1 M Na-cacodylate buffer for 2 h, stained *en bloc* in 0.5% aqueous uranyl acetate for 1 h, dehydrated in increasing concentrations of ethanol, transferred to propylene oxide and embedded in Epon. During the embedding the blocks were carefully positioned so the survey sections of the blocks would be oriented strictly in the sagittal plane.

One-micron-thick serial sections stained with toluidine blue were examined by light microscopy.

Thin sections were cut from selected areas, and after post-staining with uranyl acetate and lead citrate were viewed in a Hitachi HS8 electron microscope operated at 50 kV.

Results

Sheep Fetuses

Light Microscopy. In the 60-day fetus a well defined nerve is observed lying in the midsagittal plane just caudal to the pineal gland in the subarachnoid space (Fig. 1). As the nerve is followed dorsally it becomes increasingly thinner until it reaches the apex of the pineal. Nerve fibers extend between the pineal and the nerve along its entire course. Several single nerve cells are located within the nerve (Fig. 1, inset) which is surrounded by a thin capsule of connective tissue.

As the nerve is followed towards the area dorsal to the rostralmost part of the SCO, a small nerve cell cluster is observed in the substance of the nerve just outside the external limiting membrane (ELM). The nerve penetrates the CNS at this point as a massive plug of intermingling fibers. Fibers arising from this mesh of nerve are oriented towards the SCO hypendyma and are easily distinguishable from the transversely cut commissural fibers.

Electron Microscopy. At the ultrastructural level the nervous processes are seen to include both dendrites and unmyelinated axons (Fig. 3). Investigation of the nerve cell cluster located just outside the ELM shows that these cells are surrounded by many nerve fibers. Nerve boutons with clear vesicles are found in this region, but no membrane specializations indicative of mature synapses could be detected.

Many nerve processes are located among pinealocytes in the ventro-caudal part of the pineal which lies adjacent to the site of entry of the nerve. These fibers pierce the ELM surrounding the pineal suggesting an interconnection with the nerve.

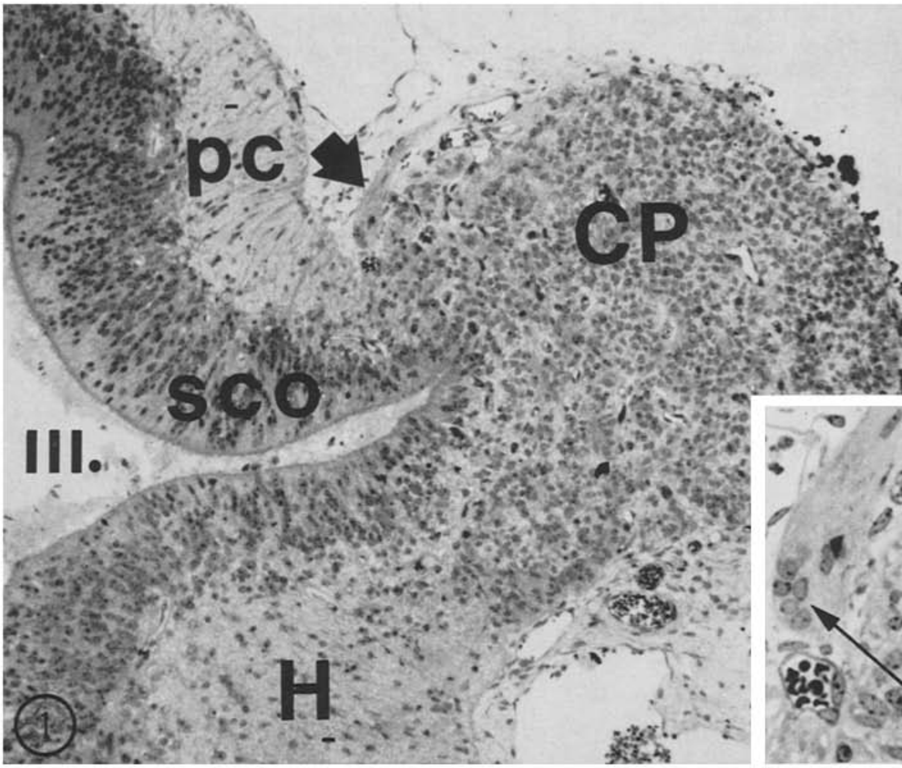
Rabbit Fetuses

Light Microscopy. Of the various stages of rabbit fetuses examined, only the 23- and 24-day fetuses exhibit clearly the presence of nerve piercing the ELM at the SCO-pineal angle. No such nerve is found in the 20- or 22-day fetuses and in the 25- and 27-day fetuses no distinct nerve seems to be present in the subarachnoid space. The following description is based, therefore, on the morphology observed at day 24 of gestation.

A single nerve, located midsagittally, penetrates the ELM dorsal to the rostralmost part of the SCO (Fig. 2). At its site of emergence, the nerve is flanked on either side by rosettes of pinealocytes and consists of a broad band of nerve fibers as well as a small group of nerve cells (Fig. 2, inset). A few single nerve cells occur in association with the nerve as it runs in the subarachnoid space along the caudal border of the pineal. As one follows the course of the nerve in the subarachnoid space, it becomes increasingly thinner until it is difficult to distinguish between the few nerve bundles left and the connective tissue fibers found in the vicinity. No nerve fibers could be seen crossing the ELM which separates the nerve from the pineal. This suggests that the fibers may be spread out on the surface of the pineal in this region.

Electron Microscopy. Ultrathin sections of the site of entry of the nerve verify that we are dealing with nerve tissue and that it is found outside the CNS (Fig. 4). The nervous processes seen include both axons and dendrites. Axo-dendritic synapses are frequent and most often contain clear vesicles (Fig. 5). Synapses with dense core vesicles are occasionally observed. Numerous collagen fibers are found intermingled among the processes. The ELM becomes very intricate at the periphery of the nerve, and can no longer be detected in the region where nerve fibers are seen to penetrate.

The rosette of pinealocytes which borders the nerve caudally contains several nerve fibers. A nerve terminal exhibiting a large accumulation of clear and a few dense core vesicles is observed surrounded by pinealocytes which is a configuration typical in the adult pineal gland.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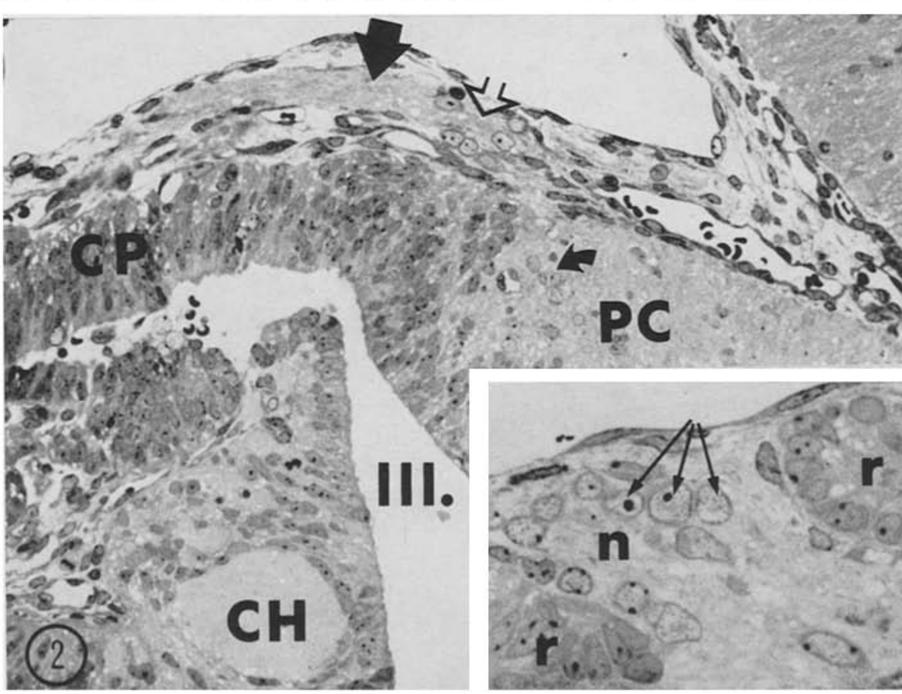
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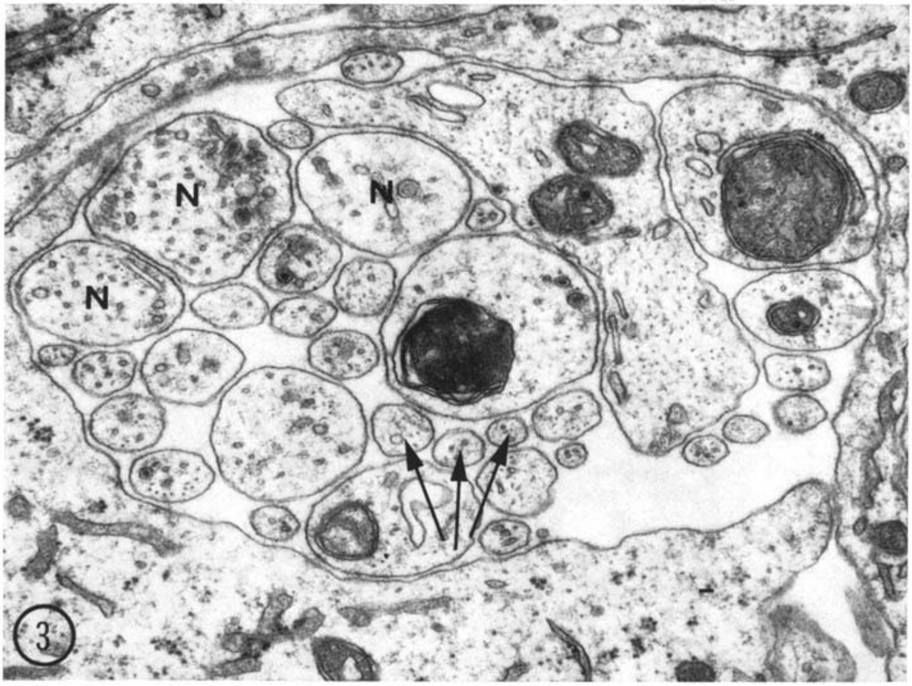


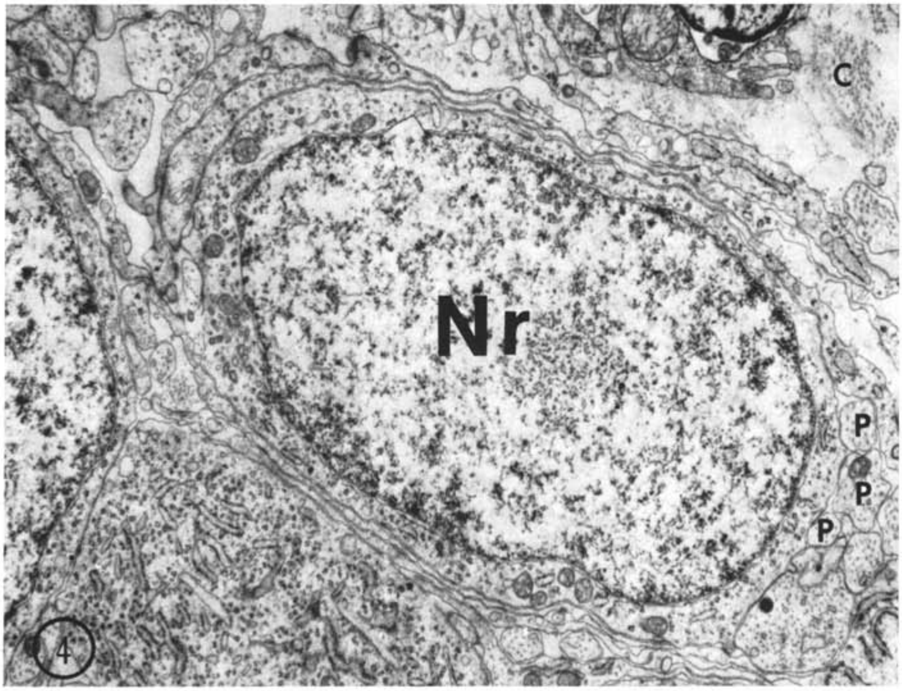
Fig. 1. Midsagittal section through the epithalamic region of a 60-day sheep fetus. The pineal gland (*CP*) is located between the habenular area (*H*) rostrally and the subcommissural organ (*SCO*) and posterior commissure (*PC*) caudally. The pineal nerve (*arrow*) is seen caudal to the pineal gland. *III* Third ventricle. 2-micron-thick Epon section, toluidine blue, ×125. *Inset:* Sagittal section through a part of the pineal nerve just before entering the CNS. A small group of nerve cells (*arrow*) is seen in the nerve. ×360

Fig. 2. Midsagittal section through the epithalamic region of a 24-day rabbit fetus. The pineal nerve (*black arrow*) is seen dorsal to the pineal gland with a small nerve cell cluster (*white arrow*) outside the external limiting membrane. The mesodiencephalic nerve cell group (*curved arrow*) is observed dorsal to the rostralmost area of the SCO. *CP* Pineal gland, *PC* posterior commissure, *CH* habenular commissure, *III* third ventricle. 1-micron-thick Epon section, toluidine blue, ×230. *Inset:* The site of entry of the nerve (*n*) into the brain. Distinct nerve cells are present (*arrows*). Pineal rosettes (*r*) border the nerve both caudally and rostrally. ×500

Fig. 3. Transverse section through a part of the pineal nerve in the 60-day sheep fetus showing thick nerve fibers (*N*) and thinner ones (*arrows*). ×30.500

Discussion

In a previous publication we reported the finding of a nervous connection between the pineal gland and the pineal-SCO angle in the human fetus. This nerve is located midsagittally, and can be followed from the dorso-caudal region of the pineal gland, through the subarachnoid space to the area dorsal to the rostralmost part of the SCO, where it penetrates the external limiting membrane (ELM) (Møllgård and Møller, 1973). A mesodiencephalic nerve cell group located just inside the site of entry of the nerve, as well as a small group of nerve cells lying outside the ELM in association with the nerve were also described.



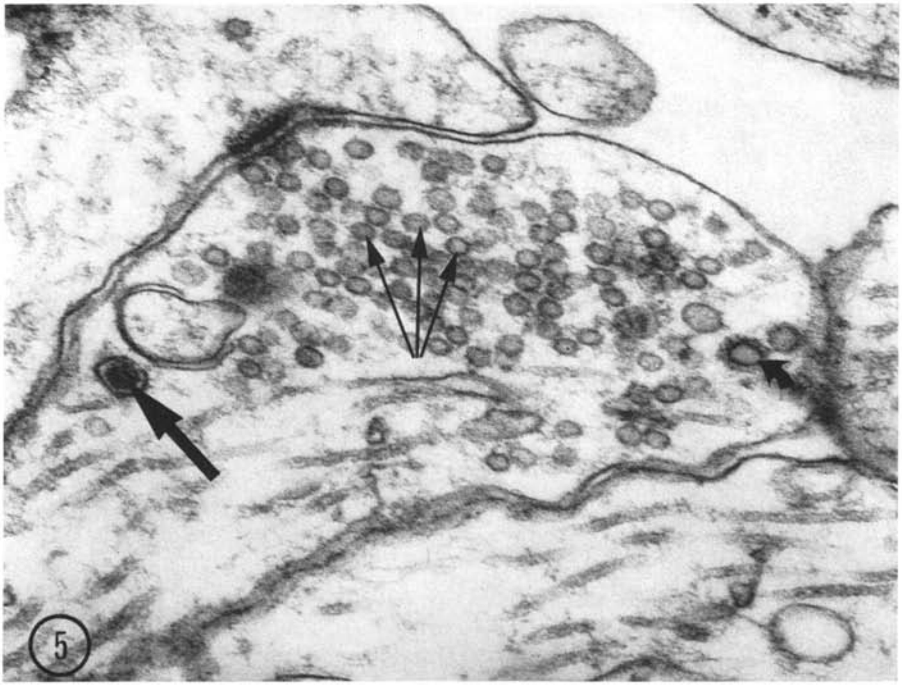


Fig. 4. Electron micrograph of the pineal nerve in a 24-day rabbit fetus. The nerve shows neuronal perikarya (*Nr*) and axons (*P*). Note that the presence of collagen fibers (*C*) proves that these neuronal elements are located outside the CNS. $\times 11.000$

Fig. 5. A typical axo-dendritic synapse within the pineal nerve of a 24-day rabbit fetus. It contains clear vesicles (*thin arrows*), dense granules (*thick arrow*) and a coated vesicle (*curved arrow*). $\times 61.000$

In the present investigation we have demonstrated that an equivalent nerve exists in the fetuses of sheep and rabbit, which represent two different mammalian orders. The existence of this nerve in these mammalian fetuses strongly indicates that it represents a general phenomenon among mammals.

In the human fetus we described (Møllgård and Møller, 1973) a collection of nerve cells in the nerve itself which appeared to be a ganglion. In the sheep and rabbit fetuses no such group of nerve cells is present in the "pineal nerve". Instead the nerve cells are located at varying intervals along the nerve, the majority assembling in the ventro-caudal part just before entering the CNS.

The anuran pineal complex proves to be one of the best documented cases of the presence of a nervous connection between the pineal and the region of the SCO. Here, fibers run from the frontal organ, a separated outgrowth from the pineal body, through the subarachnoid space as the pineal nerve to the posterior wall of the pineal body. After penetration into the pineal, the fibers continue as part of the pineal tract to the pineal-SCO angle. Recently, in the frog these fibers have been traced as far as the region of the SCO (Paul, 1971), where previous ultrastructural studies have shown synapses on the SCO cells (Diederen, 1970; Rodríguez, 1970). However, other sources of the presynaptic fiber elements cannot be excluded, and most authors assume that the pineal nerve-pineal tract system does not innervate the SCO of lower vertebrates (Diederen, 1970; Hafeez and Zerihun, 1974). Thus, in the frog, the pineal nerve fibers may terminate on some other structures of the diencephalo-mesencephalic border region (Wake *et al.*, 1974). Since the mammalian fetal nerve (in sheep and human fetuses) runs from the apex of the pineal gland through the subarachnoid space to an area of the pineal-SCO angle which exhibits pineal rosettes, and which may thus be considered a pre-pineal area, we suggest cautiously that this nerve may be homologous to the anuran pineal nerve. Moreover, apparent continuations from the mammalian fetal nerve are oriented towards the SCO region which adds some support to the suggested homology.

The mammalian fetal pineal nerve probably includes both pinealofugal (afferent) as well as pinealopetal (efferent) fibers. The presence of afferent fibers is suggested by the fact that the nerve cells and axo-dendritic synapses occur just outside the CNS in this fetal nerve entering the CNS. Moreover, we have seen a few fibers of the nerve clearly branching in the afferent direction in silver impregnated sections of human fetuses (unpublished observations). Furthermore, a study of the SCO-associated nerve cells in rabbit fetuses (Kimble and Møllgård, in preparation) has revealed that some members of the mesodiencephalic nerve cell group send large, highly basophilic dendrites towards the site of entry of the nerve. An efferent pathway along the fetal nerve is suggested by the observation in rabbit fetuses that axons, which arise from the nervous mesh located in the vicinity of the site of penetration of the nerve, terminate in boutons inside the pineal rosettes flanking the nerve. The fact that the anuran pineal nerve consists of both afferent and efferent fibers (Ueck *et al.*, 1971; Paul, 1971; Böttger and Böttger, 1972) is of considerable interest in this context.

Both the function and fate of the mammalian fetal pineal nerve are crucial and unsolved questions. An investigation in our laboratory of newborn and adult rabbits and humans has failed to show the post-embryonic presence of this nerve. It is possible that the nerve is simply a vestigial homologue of the pineal nerve and of little more than phylogenetic significance. However, considering the fetal nature of the nerve, the possibility that it may play a functional role in development of the SCO-pineal complex must not be overlooked.

A remaining possibility is that the nerve persists in the adult, having been incorporated into the pineal stalk and parenchyma. Although it has been demonstrated that many of the nerve fibers in mammalian pineal stalk are aberrant elements from the habenular and posterior commissures which never contact pineal parenchymal cells (for a review, see Kappers, 1965), some fibers (probably both dendrites and unmyelinated axons) might well connect the CNS and the pineal gland through the stalk. Difficulties in demonstrating such fibers with the available light microscopic techniques must be kept in mind. The possibility of incorporation of the nerve is now being investigated in older fetuses in our laboratory.

It is concluded that a single midsagittal nerve connecting the pineal gland and the region dorsal to the rostralmost part of the SCO is present in sheep and rabbit fetuses. The presence of a similar nerve in the human fetus suggests that it represents a general feature of the fetal mammalian brain. We believe this nerve to be ontogenetically equivalent to the pineal nerve observed in anuran amphibians. (For a review of intrinsic pineal nerve tracts in birds, see Menaker and Oksche, 1974.)

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