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Pineal neurons projecting to the brain of the rainbow trout, *Salmo gairdneri* Richardson (Teleostei)

In-vitro retrograde filling with horseradish peroxidase

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Summary. The morphology of intrapineal neurons that give rise to the pineal tract and project to the brain in the rainbow trout was visualized by the use of neuronal backfilling with horseradish peroxidase (HRP). The tracing was performed on excised pineal organs under in-vitro conditions at 4° C, with filling times ranging from 6 to 24 h. Large multipolar, bipolar and unipolar neurons were visualized in the rostral tip of the pineal organ ("pineal ganglion"). These neurons possessed extended dendritic trees participating in the formation of a circumscribed neuropil-like area. Throughout the pineal organ small bipolar elements were the most ubiquitous type of neuron, however, with markedly smaller numbers in the proximal portion of the pineal end-vesicle. In the pineal stalk, some bipolar neurons were observed to contact the pineal lumen, which is continuous with the third ventricle, via dendritic processes of various types. It could not be established whether any of these CSF-contacting processes were identical with photoreceptor outer segments. The basal processes of the bipolar neurons sometimes possessed distally projecting collaterals. In conclusion, it has been shown that (i) different types of neurons displaying varied patterns of regional distribution contribute to the pineal tract, and (ii) certain CSF-contacting neurons in the pineal organ send axonal processes directly toward the brain.

Key words: Pineal organ – Neuronal types – Neuronal projections – In-vitro studies – Horseradish peroxidase technique – Teleostei

The teleostean pineal body is a photosensory organ, endowed with photoreceptor cells and neurons capable of transmission of information concerning environmental lighting conditions to the brain (for references, see Dodt 1973; Meissl and Dodt 1981; Vollrath 1981). The pineal neurons possess axons that form the pineal tract and innervate a number of brain areas, which have been identified by means of cobalt filling (Hafeez and Zerihun 1974) or filling with horseradish peroxidase (HRP) (Ekström and van Veen 1983, 1984; Ekström 1984). In contrast, the intrapineal neuronal circuitry is only partly clarified. Pineal neurons of teleosts have been demonstrated by use of intravital methylene-blue staining (Holmgren 1917, 1918) and acetylcholine-esterase histochemistry (Wake 1973; Korf 1974; Falcon and Mocquard 1979; Omura 1980; Matsuura and Herwig 1981; Vigh-Teichmann et al. 1982), and classified as (pseudo-)unipolar, bipolar and multipolar elements. Three basic questions remain open to discussion: (i) Which type (or types) of neurons gives (give) rise to the pineal tract? (ii) Do (at least some) photoreceptors synapse directly on "ganglion cells"? (iii) Is an integrating network of interneurons an obligatory prerequisite of pineal sense organs? In this study, we have analyzed the first of these questions.

The wealth of electrophysiological data on the pineal organ of the rainbow trout, *Salmo gairdneri* (Dodt 1963; Morita 1966; Hanyu et al. 1969; Hanyu and Niwa 1970; Tabata et al. 1975; Tabata 1982a, b) makes it a suitable animal for the study of the intrapineal neural circuitry in teleost fishes. Acetylcholine-esterase histochemistry (Korf 1974) provides a basis for neuronal classification, and the existence of a centrally projecting pineal tract is already established (Hafeez and Zerihun 1974). In the present study, our attempt was to visualize the centrally projecting intrapineal neurons in greater detail by means of retrograde HRP filling under in vitro conditions (Ekström 1985).

Materials and methods

Twelve young rainbow trout (*Salmo gairdneri* Richardson) of 10–20 cm length were obtained from a local hatchery, and kept in running tapwater (ca. 8° C) in a room with an artificial L12:D12 photoperiod until used.

The fish were decapitated, and the roof of the skull was quickly removed to expose the pineal organ. The pineal stalk was cut with microscissors, and a few crystals of HRP (grade VI; Sigma) were applied to the transected surfaces. After 1–2 min exposure to HRP, the pineal organ was quickly dissected out, and transferred to either oxygenated Dulbecco's Modified Eagle Medium (DMEM; Serva Biochemicals) containing 20 mM HEPES buffer (Serva), or

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oxygenated retinal Ringer solution, a modification of the recipe given by Sarthy et al. (1982).

Care was taken not to touch the pineal parenchyma at other sites than at the point of transection of the stalk; the pineal organ was handled by lifting the surrounding dorsal-sac tissue. The pineal organs were incubated in the dark at 4° C (at this temperature no pinocytosis – and thus no unspecific labeling – occurs; see discussion in Ekström 1985), for 18 or 24 h in DMEM/HEPES, or 6 or 24 h in retinal Ringer.

After incubation, the pineal organs were immediately fixed in cold 2.5% glutaraldehyde in 0.1 M Sørensen's phosphate buffer (pH 7.2) for 1–3 h. They were then washed in several changes of phosphate buffer (1–3 h), and transferred to phosphate buffer containing 25% sucrose for cryoprotection. We found the shortest processing schedule the most reliable: 1 h fixation, 1 h buffer washes, and 1 h sucrose infiltration.

The pineal organs were mounted in Tissue Tek cryoprotection medium and rapidly frozen. 20- or 25-µm thick serial frozen sections were cut at −23° C on a Leitz cryostat, thaw-mounted on chrome alum-gelatine-coated slides, and air-dried for 15–60 min.

Frozen sections were reacted for HRP as follows: buffer wash (5 min), 50 mg 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma)/100 ml buffer (10 min), 50 mg DAB and 50 µl H_2O_2/100 ml buffer (20 min), buffer wash (5 min), rapid dehydration in graded alcohol series, clearing in xylene, and mounting in dammar resin. 0.1 M TRIS buffer (Sigma), pH 7.2, was used throughout.

Sections were viewed and photographed with a Leitz Orthoplan microscope. A special interference filter (Møller et al. 1984) was used to enhance the contrast of the brown HRP-DAB reaction product. Camera lucida drawings were made with 63× oil-immersion plan apochromat lenses.

Results

HRP-filled perikarya were visualized in the pineal stalk and pineal end-vesicle of the rainbow trout. All perikarya were massively labeled and resembled Golgi-impregnated neurons. Unipolar, bipolar and multipolar neurons were identified; they displayed a varied distribution in the proximal and distal subdivisions of the pineal end-vesicle and the pineal stalk. Small bipolar neurons were observed throughout the pineal organ, although being in greatest numbers in the pineal stalk. Axons constituting the pineal tract collect in a main dorsomedial fascicle and several smaller – lateral and ventral – fascicles (cf. Korf 1974; Omura 1979).

Pineal stalk

Large numbers of HRP-filled neuronal somata were observed in the pineal stalk. They were located among the axons of the pineal tract, or between the fascicles of the pineal tract and the pineal lumen (Fig. 1), and were only seldomly seen in direct apposition to the basal lamina. These somata were round or oval, with radially oriented long axes, and with diameters of approximately 5–10 µm. The somata displayed apical extensions of various shapes, which sometimes appeared to reach the pineal lumen. These processes were arbitrarily divided in four categories: (1) small, asymmetrically (laterally) situated, rounded protrusions; (2) small, symmetrically (apically) situated, rounded

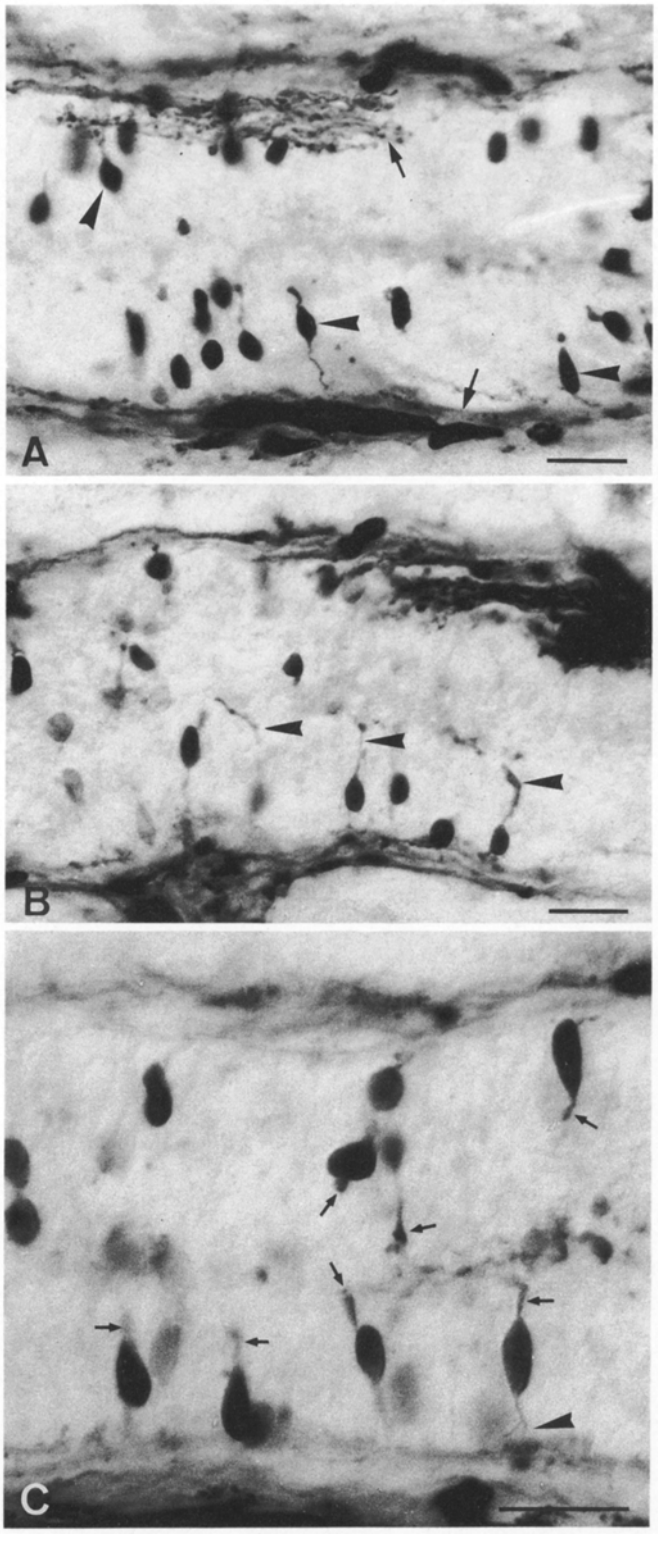

Fig. 1 A–C. Photomicrographs of HRP-filled neurons in the pineal stalk. Horizontal sections, 25 µm. Distal is to the left. Scale markers = 20 µm. **A** Different types of bipolar neurons (*arrowheads*) give rise to the pineal tract (*arrows*); cf. Fig. 2B. **B** Bipolar neurons with long apical processes (*arrowheads*); cf. Fig. 2A. **C** Bipolar neurons with apical processes in contact with the pineal lumen. Note the branching basal processes (*arrowhead*) of one neuron, and the different types of apical processes (*small arrows*)

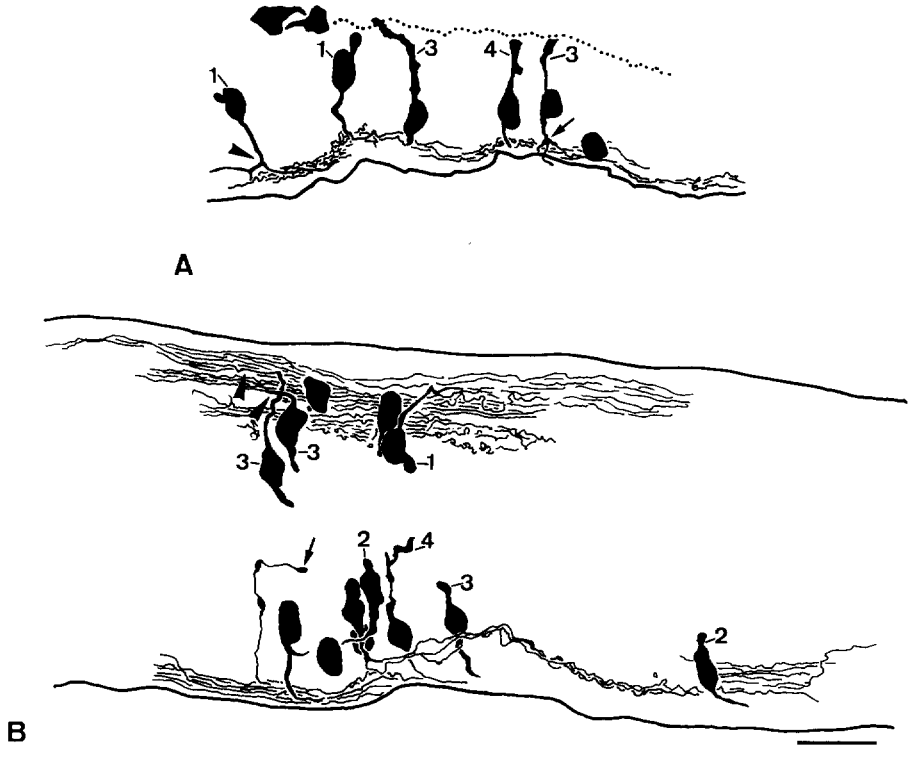

Fig. 2A, B. Camera lucida drawings from horizontal sections through the pineal stalk, demonstrating different morphological features of the bipolar neurons. Types *-1*, *-2*, *-3*, *-4*, *BL* basal lamina. Distal is to the left. For further explanations, see text. Scale marker = 20 μm. **A** Type-1 (*arrowhead*) and type-2 (*arrow*) bipolar neurons with branching basal processes. Lining of pineal lumen (*dotted line*). **B** Two type-3 bipolar neurons with major axon collaterals projecting distally (*arrowheads*). Note the varicose axon (*arrow*) obviously terminating in the photoreceptor-cell layer

protrusions; (3) elongated, unbranched dendritic extensions; and (4) elongated, branched dendritic extensions (Figs. 1, 2). The above-mentioned somata gave rise to a peripherally directed axon, which merged with the pineal tract. These axons predominantly coursed toward the brain, but were often seen to emit shorter and longer collateral processes. In some cases, longer collaterals could be traced toward the pineal end-vesicle (Figs. 1, 2). These varying types of neurons had the appearance of bipolar neurons of apparently sensory character. It could not be proven at the light-microscopic level that they might be identical with pineal photoreceptor cells. Electron-microscopic studies are in progress to clarify the nature of these small, bipolar neurons.

Axonal profiles with preterminal telodendria and terminal varicosities were occasionally observed in the vicinity of the branching axons of the bipolar neurons, and also close to the pineal lumen. It was, however, not possible to determine whether these profiles represented pinealopetally projecting axons of central origin, or axonal collaterals ascending from bipolar neurons located in the pineal stalk.

Pineal end-vesicle

In the rainbow trout, there is no distinct border between the pineal stalk and the pineal end-vesicle. Rather, the pineal epithelium thickens, and the outer diameter of the pineal stalk increases gradually toward the proximal portion of the end-vesicle. With the exception of its distal (rostral) tip, the pineal end-vesicle consists of a relatively thick, convoluted epithelium (cf. Korf 1974; Omura 1979). We call this thick, convoluted epithelium the 'proximal end-vesicle,' to distinguish it from the thin epithelium of the rostral tip, i.e., the 'distal end-vesicle.'

Massively labeled pineal-tract axons were observed in a dorsomedial and two lateral fascicles, which could be traced back to the rostral tip of the pineal end-vesicle.

An abrupt decrease in number of HRP-labeled neurons per unit area is apparent in the proximal portion of the end-vesicle. Only small numbers of bipolar neurons, of the types observed in the pineal stalk, and occasional small multipolar neurons could be demonstrated within this part of the pineal parenchyma, most of them rather remote from the basal lamina. The convoluted pineal epithelium becomes thinner in the distal portion of the end-vesicle. In this region, clusters of closely apposed neurons were massively filled with HRP. These neurons had larger somata than those encountered elsewhere in the pineal organ, with diameters of approximately 15–25 μm and longitudinal axes up to 30 μm; they belonged to unipolar, bipolar and multipolar types.

The most prominent group of neurons was situated in a medial position within the distal end-vesicle. It consisted of bipolar neurons with dendrites arborizing laterally in the horizontal plane (Figs. 3A, C, F, 4A–C) and multipolar neurons, the dendrites of which intertwined with those of the bipolar neurons (Figs. 3B, C, 4B–D). Large unipolar neurons were located lateral to the bipolar and multipolar neurons (Figs. 3A, 4A, D, E). Their proximal dendritic segments were dorsoventrally oriented (Figs. 3E, 4E). At varying distances from the soma, the dendrite turned mediad at right angle (Fig. 4A, E), or branched emitting a dorsoventral branch and another turning medially (Fig. 4E). In all cases, the medially coursing dendritic extensions were oriented in the horizontal plane; they intermingled with the dendrites of the bipolar and multipolar neurons. Thus, a large number of dendrites participated in the formation of a circumscribed neuropil-like area in the distal portion of the pineal end-vesicle. Also, the neuronal somata were densely clustered in this region, sometimes in a manner resem-

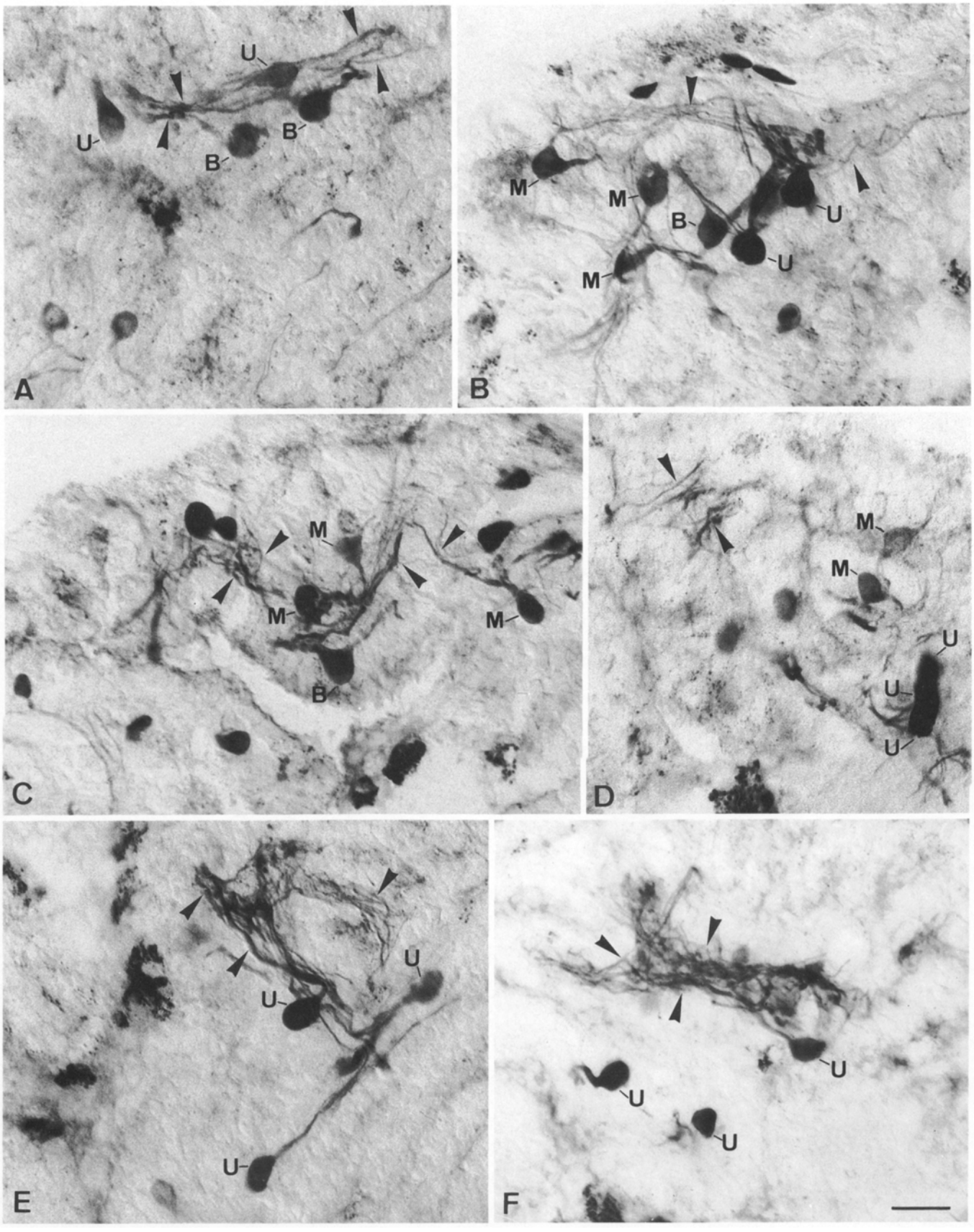

Fig. 3A–F. Photomicrographs of HRP-filled neurons in the distal tip of the pineal end-vesicle. Series of frontal 25-μm thick sections, proceeding from rostral (**A**) to caudal (**F**): A – 25 μm – B – C – D – 25 μm – E – 25 μm – F. Bipolar (*B*), multipolar (*M*), and unipolar (*U*) neurons. Note the intertwining dendrites in a circumscribed area (*arrowheads*). Scale marker = 20 μm

Fig. 4A–F. Camera lucida drawings of HRP-filled neurons in the distal end-vesicle. Abbreviations as in Fig. 3. Scale marker = 20 μm. **A–E** is a series of frontal 25-μm sections (see Fig. 3): A – 25 μm – B – C – 25 μm – D – E. **A** The dendrites of bipolar and unipolar neurons intertwine in a dense neuropil area (*arrowhead*); see Fig. 3A. Note two unipolar neurons (*arrows*) that are not directly connected to the rostromedial neuronal cluster ("pineal ganglion"). **B** Multipolar, bipolar and unipolar neurons; see Fig. 3B. **C** Large multipolar and bipolar neurons; see Fig. 3C. **D** Laterally situated, large unipolar neurons arranged in close apposition. **E** Dendrites of laterally situated, large unipolar neurons turn mediad (*arrowheads*) into the dense neuropil; see Fig. 3E. **F** Large bipolar neuron, located caudal and lateral to the rostromedial cluster (see **A–E**); axon (*arrow*)

bling a somato-somatic apposition (Fig. 3D). The dendrites of the laterally situated, so-called unipolar neurons sometimes exhibited a complex branching pattern (Fig. 4C), leaving this classification open to discussion.

Smaller, not so dense groups of neurons were located lateral to this rostromedial group. They contained predominantly unipolar (Figs. 3A, C, E, 4A, C) and bipolar neurons (Fig. 4F). Apparently, these cells did not establish direct contacts with the neurons in the rostromedial group.

The clustering of the neurons and the intertwining of their dendrites prevented a clear-cut identification and morphological analysis of single neuronal elements of the ros-

tromedial group. Unfortunately, it was not possible to obtain a complete labeling of a limited number of pinealofugal neurons.

Discussion

In a detailed study of acetylcholine-esterase (AChE)-positive neurons in the pineal and frontal organs of two species of frogs, *Rana ridibunda* and *R. esculenta*, Wake et al. (1974) put forth a model of a bineuronal intrapineal circuitry. Neurons homologous to retinal bipolars and horizontal cells were assumed to be lacking, but amacrine-like multipolar cells were proposed to represent interneurons between the photoreceptors and the (pseudo)unipolar neurons. The latter were assumed to be the sole source of the pinealofugal axons in the pineal tract. This bineuronal model, mainly based on findings in the frontal organ, has remained virtually unchallenged, although data obtained in other anamniote species and/or with different experimental techniques have indicated other possible interpretations. Previously, with the use of intravital methylene-blue staining, Paul et al. (1971) had shown that, in addition to (pseudo)unipolar neurons, multipolar neurons contribute axons to the pineal tract of *Rana temporaria* and *R. esculenta*. Later, Eldred and Nolte (1981) have convincingly demonstrated by means of tracer techniques, in yet another raniid frog (*R. pipiens*), that unipolar, bipolar and multipolar neurons of the frontal organ contribute to the pineal nerve, in apparent contradiction to the model proposed by Wake et al. (1974).

While the AChE reaction produced a detailed picture of neuronal morphology and interconnections in some raniid frog species (Wake et al. 1974), it has not been so successful with teleost pineal tissue. Briefly, it permitted only a rough classification of multipolar, bipolar and (pseudo)unipolar neurons. Generally, the largest number of neurons were found in the pineal stalk. Furthermore, the most ubiquitous type of neuron seemed to be the small unipolar neuron, while only limited numbers of multipolar (and sometimes bipolar) neurons were observed. Multipolar neurons were more frequently encountered in the pineal end-vesicle, sometimes clustered in more or less densely packed groups in the rostral pineal epithelium (Wake 1973; Korf 1974; Falcon and Mocquard 1979; Omura 1980; Matsuura and Herwig 1981; Vigh-Teichmann et al. 1982). The model of Wake et al. (1974) has not been seriously challenged by these results, although large multipolar neurons were observed to send axons into the pineal tract in the goldfish, *Carassius auratus* (Wake 1973), and the minnow, *Phoxinus phoxinus* (Vigh-Teichmann et al. 1982).

The present results, obtained with HRP backfilling of pineal neurons via their transected axons, clearly demonstrate (i) that different neuronal types contribute to the pineal tract, i.e., unipolar, bipolar and multipolar neurons, and (ii) that these neuronal types show varied patterns of distribution within the pineal organ.

In the distal portion of the pineal end-vesicle of the rainbow trout, a cluster of large unipolar, bipolar and multipolar neurons gave rise to axons converging to the pineal tract. These neurons shared common dendritic fields; their dendrites intermingled and contributed to a circumscribed neuropil-like area. This neuronal cluster appears identical to the intrapineal ganglion identified by means of AChE histochemistry (Korf 1974). The reason that only multipolar neurons were observed with the AChE reaction may be either the difficulty in classifying neuronal types with the use of this method, or that exclusively the multipolar neurons within the cluster belong to AChE-positive elements. The first alternative appears the more likely. However, the possibility that two rostral clusters exist, in analogy to the two AChE-positive neuronal clusters in the frontal organ of some frogs (one composed of (pseudo)unipolar neurons, the other of multipolar elements; cf. Wake et al. 1974), may also be considered.

In the pineal stalk, and in lower numbers throughout the end-vesicle, small bipolar neurons (5–10 μm in diameter) gave rise to axons joining the pineal tract; these may be identical with the small AChE-positive neurons (Korf 1974). In the present study, these neurons had the appearance of sensory neurons such as pineal photoreceptor cells, as judged by light microscopy. However, the existence of photoreceptor cells projecting directly into the brain seems improbable from a physiological point of view; such photoreceptors would lack a signal amplification system, and the signals would have to be transmitted to the brain as impulse activity. The true identity of these cells must be proven at the electron-microscopic level.

Vigh-Teichmann et al. (1982) described larger neurons (15–20 μm in diameter) in the pineal stalk of the European minnow. These elements were located close to the basal lamina and possessed dendritic processes directed toward the photoreceptor layer (i.e., toward the pineal lumen). Since they occur together with smaller neurons, which contribute to the pineal tract (Vigh-Teichmann et al. 1982), it is doubtful that they are identical with the bipolar cells shown to occur in the pineal stalk of the rainbow trout in the present study. However, in the light of the present evidence, it must be considered that certain receptor elements in the pineal stalk of the rainbow trout might project directly to the brain (for a discussion of this problem in the classical pineal literature, see Bargmann 1943). If this holds true, the sensory modalities conveyed by this system remain to be experimentally demonstrated; to date we have no evidence that these cells can be regarded as photosensory elements. The similarities among pineal photoreceptor cells, mammalian pinealocytes and cerebrospinal fluid (CSF)-contacting neurons have, however, been discussed (Vigh and Vigh-Teichmann 1973; Vigh-Teichmann et al. 1980). The presence of a certain type of CSF-contacting neuron in the pineal stalk, displaying direct connections with the brain, may offer new lines of research concerning pineal function in teleost fishes.

Since we cannot ascertain that all centrally projecting neurons are retrogradely labeled with HRP, detailed cell counts for comparisons with the number of AChE-positive neurons in the pineal organ of the rainbow trout (Korf 1974) are not presented here. Korf (1974) counted 1325 to 1725 AChE-positive neurons in the pineal organ of this teleost species, numbers which were consistently lower than the number of axons in the pineal tract. Our estimate is that the number of backfilled neurons in this study is smaller than that of AChE-positive neurons. This is especially evident in the proximal portion of the pineal end-vesicle, were numerous AChE-positive neurons were found (Korf 1974). The larger number of axons in the pineal tract can nevertheless be explained by the existence of axon collaterals from bipolar neurons located in the pineal stalk.

The presence of a rostral intrapineal ganglion appears

to be a specialization in some teleosts (Korf 1974; Vigh-Teichmann et al. 1982). Furthermore, regional differences in the pineal organ with respect to neuronal types appear to be characteristic of most teleost species studied to date (see above). The markedly smaller numbers of HRP-filled neurons in the proximal division of the end-vesicle may reflect a functional zonation in the pineal organ of the rainbow trout. However, this zonation apparently does not correspond to that demonstrated in the pike, *Esox lucius* (Falcon 1979a, b; Falcon and Mocquard 1979; Falcon and Meissl 1981; Falcon et al. 1980, 1981). In this latter species, the distal and proximal (stalk) zones of the pineal organ contain photoreceptor cells with regular outer segments (Falcon 1979a, b) and AChE-positive neurons (Falcon and Mocquard 1979), whereas the middle zone (corresponding to the proximal end-vesicle in the present study) displays photoreceptors with degenerate outer segments and basal processes lacking synaptic ribbons, and is practically devoid of AChE-positive neurons. Moreover, maintained spontaneous activity of single neurons and nerve fibers, as recorded from the distal and proximal (stalk) zones, were never found in the middle zone (Falcon and Meissl 1981).

In the rainbow trout, however, regular opsin-immunoreactive photoreceptor outer segments are evenly distributed along the lumen of the entire pineal organ (Vigh-Teichmann et al. 1983), and AChE-positive neurons are present also in the proximal portion of the end-vesicle (Korf 1974). The higher numbers of AChE-positive neurons compared to HRP-filled neurons might indicate that, in this area, the AChE-positive elements are interneurons, or even second-order neurons in a trineuronal chain. It is also of some interest to compare the rostral neuronal cluster in the rainbow trout with the neurons in the anuran frontal organ, as visualized by the use of AChE histochemistry (Wake et al. 1974) or HRP backfilling (Eldred and Nolte 1981). A specialization of the rostral portion of the pineal complex for processing of sensory information has been postulated by Falcon (1979a, b) in the pike. This pattern seems also to hold true for the pineal organ of the rainbow trout, and it might be compared to the specialized rostral portion of the pineal complex, i.e., the frontal organ, in certain anurans (for developmental aspects, see Vollrath 1981).

In conclusion, we have shown that, in the rainbow trout, different types of intrapineal neurons contribute to the formation of the pineal tract. Furthermore, a regional specialization in this pineal organ has been shown. The existence of pineal interneurons in the rainbow trout might only be hypothesized with reference to the fact that, in this species, the numbers of AChE-positive neurons (Korf 1974) exceed those shown by retrograde labeling (present study). According to Eldred and Nolte (1981), in the frontal organ of the frog photoreceptor cells contact neurons, similar to those visualized in the rainbow trout, with ribbon-type synapses. However, this finding certainly does not disprove the possible existence of pineal interneurons. Interneurons have been indicated by the presence of individual conventional synapses in the intrapineal neuropil formations (Bayrhuber 1972; Flight 1973; Korf 1976; Korf et al. 1981); finally, they are one of the possible explanations of the chromatic response occurring in the frontal organ of anurans and the parietal eye of lacertilians (Meissl and Dodt 1981). Pineal interneurons remain to be analyzed in further experimental work with intracellular recordings and subsequent labeling.

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