Influence of continuous light and darkness on the secretory pinealocytes of *Heteropneustes fossilis*

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In an earlier study on *Heteropneustes fossilis*, evidence of secretory activity in the pinealocytes had been demonstrated at the electron microscopic (EM) level and it was found to exist in two phases: a secretory phase (light cells) and a storage phase (dark cells). In the present investigation, *H. fossilis* was subjected to artificial photoperiods of continuous illumination and continuous darkness for a period of ten days and the effect on the secretory pinealocytes was studied at the EM level. Marked results were observed within the short period of ten days emphasizing the role of environmental photoperiod on the secretory activity of the pinealocytes. During continuous illuminated phase, both light and dark cells were observed: the light cells showed intense secretory activity and dark cells a storage one. During the dark phase both types of cells were present but in different metabolic states and neither of the cells demonstrated synthetic nor storage activity. Light cells were metabolically active but not secretory active and dark cells showed a necrotic condition. Phagocytotic activity of the dark cells was also seen. Intense neural activity was also observed during exposure to both the artificial photoperiods. The results highlight the role of light on the secretory activities of the pinealocytes of the catfish pineal organ.

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1. Introduction

The pineal organ of lower vertebrates is believed to act as photochemical transducer mediating the effects of day length on the biology of species (Collin 1979; Ueck and Wake 1979). The process involves morphological and biochemical changes in the pinealocytes. The influence of environmental light on the anatomy and physiology of the pineal has been investigated in a number of mammalian and submammalian species (Alexander *et al* 1970; Hafeez *et al* 1978; Meissl *et al* 1978; Pevet 1979) and its influence on the metabolic activity of the pinealocytes is widely reported. McNulty (1982a) suggested that in fish pineals alteration in ultrastructure, in response to photic environment, involve not only photosensory processes but also reflect changes in their presumed secretory activities. Some ultrastructural studies have reported the influence of light and darkness on the pineal organ of teleosts (Lowenstein 1956; Omura 1975; Hafeez *et al* 1978; McNulty 1982b, c). Some of the studies are morphometric analyses of the various cellular organelles, others are biochemical analyses involving hydroxy indole-O-methyl transferase (HIOMT), acetyl serotonin methyl transferase (ASMT), free amino acids and amines under altered photoperiod conditions. In fish, the pineal organ acts as a direct photoreceptor transducing light information into neural and humoral (melatonin) signals. The pineal organ and its secretory product melatonin are often regarded as synchronizers of daily rhythms to the external light/dark (LD) cycles. In *Heteropneustes fossilis*, a nocturnal Indian catfish, ultrastructural indications for the presence of secretory activity in the pinealocytes of the pineal parenchyma were reported and two morphological cell types: light and dark cells representing different phases of the secretory process were demonstrated (Srivastava 1999). The aim of the present investigation is to study the effect of artificial photoperiods on the secretory activity of these pinealocytes.

Keywords. Artificial photoperiod; dark cell; light cell; pineal; secretory pinealocytes; synapse

2. Materials and methods

Adult catfishes of both sexes (av.wt. 25–30 g, 16–18 cm in length) were obtained from the local market in the months of April/May and maintained in natural condition in cement tanks. They were fed with goat liver on alternate days.

Two batches of five healthy fishes (av.wt. 25–30 g) were maintained under photoperiods of constant illumination (LL) and constant darkness (DD) for a period of ten days. For LL, glass aquaria provided with a fluorescent tube-light (40 W; 6500 lux intensity) which was placed above the water surface, was used for the experiment. For constant darkness the aquaria were kept in a dark room, and changing of water and feeding were done in red light. On the eleventh day of the experiment the fishes were anaesthetised with MS222, decapitated and the pineal dissected out and fixed in Karnovosky fixative. The entire procedure was carried out under the same photic environment under which the experiment was conducted i.e. fixation was done in red light in DD experiment and under the same fluorescent light as used in the LL experiment. In both the experimental condition, the fishes were sacrificed at 11 am.

Fixation in Karnovosky was done for 1 h at room temperature; washed in 0·1 M phosphate buffer and then processed by routine electron microscopic (EM) methods. Araldite resin blocks were made, 1 μm thick semithin sections were obtained and stained with Toluidine blue. Ultrathin sections were stained with uranyl acetate and lead citrate and examined under Philips CM-10 transmission electron microscope (TEM).

3. Results

In the present ultrastructural study, the effect of artificial photoperiod is remarkably evident in the secretory pinealocytes. Exposure to artificial photoperiod conditions of DD and LL, shows two very different morphology of the pineal parenchyma, compared to that observed in the normal photoperiod conditions (table 1). Since the study under normal photoperiod (Srivastava 1999) was also conducted in the same period as the present i.e. in April/May, the results of the former have been used as normal for the present experimental study. It is also observed that the fishes are highly active during the DD exposure and totally inactive during the LL phase.

3.1 *Under continuous darkness (DD)*

After exposure to photoperiods of continuous darkness for a period of ten days, both light and dark cells are observed in the pineal parenchyma (figure 1a). The light cells show a considerably enhanced state of metabolic activity. Mitochondria appear large in size (0·5 μm–1 μm in diameter)as compared to that under normal photoperiod conditions when the mitochondrial diameter was 0·03 μm–1·5 μm. They have a wide lumen and the cristae are distended. Numerous polyribosomes are seen in the cytoplasm. Few endoplasmic reticulum are observed as compared to that under normal photoperiod conditions however, several membrane bound spaces are visible in the cytoplasm, which are 0·08 μm–0·24 μm in width (figure 1c, d).

The cells also show an increased activity in their terminal processes, which contain several clear vesicles (45–135 nm in diameter) and are in close proximity to several axonic and dendritic endings. Synaptic thickenings (figures 2a, b) in the membranes are seen at several points of contact between the light cells and afferent nerve fibres. In DD conditions, there is a characteristic close interaction of pinealocyte processes and nerve processes; besides many axon-axon synapses are also seen.

The dark cells are sparsely seen and possess a different cellular constitution. The secretory granules present under normal photoperiod conditions are lacking; instead the cytoplasm shows large homogenous/crystalline accumulations which are often present as long tubular extensions (figure 2c) and the nucleus is generally reduced in size and shifted to a corner of the cell (figures 1a, 2c). The cell lack all other inclusions except for very few mitochondria. Vacuolated type of dark cells commonly seen in the normal pineals are characteristically absent. However, some secretory granules are seen scattered in the connective tissue stroma as well as in some of the endothelial cells, which also show outpushings towards the capillary lumen (figure 2d). At certain sites, the secretory granules appear spilled out into the capillary lumen (figure 2e).

Numerous microglial cells are also observed in the parenchyma (figure 2f). These cells are elongated to spindle shaped, have an irregular nucleus with heterochromatin pushed to one corner of the nucleoplasm. The cytoplasm is occupied predominantly by dense dark homogenous substance. Few microtubules are seen running through the cytoplasm. Nerve fibers are aligned close to these cells.

3.2 *Under continuous illumination (LL)*

In the light adapted pineals, the light cells show elaborate synthetic apparatus (figure 3a, b). The ultrastructural changes noted are prominent Golgi bodies, active mitochondria, extensive rough endoplasmic reticulum, numerous ribosomes and polyribosomes. The mitochondria are 0·15 μm–0·2 μm in diameter, darker in shade, with reduced lumen and numerous densely folded cristae. Rough endoplasmic reticulum occupy a major portion in most of the light cells. In some of the cells extensive endoplasmic

Table 1. Structural characteristics of the light and dark cells under natural photoperiod conditions (for details see Srivastava 1999) in *H. fossilis*.

Cell characteristics	Light cell	Dark cell
Nucleus	Large with heterochromatin patches	Large with patches of heterochromatin, occupies major part of the cytoplasm
Cytoplasm	Appears lighter in shade, possess all the inclusions	Appears dark in shade, has few inclusions
Mitochondria	0·03–1·5 μm in diameter	0·02–1·0 μm in diameter
ER	Abundant, rough type	Sparse, rough type
Ribosomes	Abundant	Few
Secretory granules	Small dense granules (80 nm–110 nm in diameter)	Large mature secretory vesicles (280 nm–420 nm) in diameter
Other inclusions	Large lipid bodies (700 nm–800 nm in diameter)	Vacuoles (140 nm–370 nm) in diameter in one subtype; myeloid bodies in necrotic cells (another subtype)
Secretory activity	Strong synthetic activity	Shows storage and release activity: 3 subtypes–(i) with mature secretory vesicles (storage phase); (ii) with vacuoles (release phase); (iii) with myeloid bodies (necrotic phase)

reticulum are present, the cisterns of which contain dense material. These cells also contain large vacuoles 3–4 μm in diameter having electron lucent material (figure 3d).

The nucleus appears active with disintegrated euchromatin and heterochromatin and wide spaces (30–60 nm) in between the inner and outer nuclear membranes. However, at many points the thinning of the nuclear membranes is observed. In some typical cell types, the nucleus is highly active with the nucleoplasm flowing out into the cytoplasm through the nuclear pores, however, the heterochromatin seems to be retained within the nucleus (figure 3c). Such types of cells also show the formation of vesicles – both dense and clear. Lamellar whorls 50–60 nm in diameter are characteristic feature of these cell types and they enclose large clear vesicles of diameter varying between 30–40 nm.

Numerous processes of the light cells are present which are closely intertwined with nerve cell processes. Club-shaped terminals of these cells (figure 3e) are observed to end freely in the extracellular spaces. They contain numerous broken fragments of rough endoplasmic reticulum of lengths 0·5 μm–1 μm, and several dense vesicles. Pinocytotic vesicles are also observed in the cell membranes of such terminal endings. Nerve endings of afferent fibres closely approximate the terminal cell processes and synaptic contacts in the form of synaptic clefts and vesicles are seen at several places (figure 3f).

Dark cells are present characteristically loaded with dense secretory granules (180 nm–200 nm in diameter) (figure 3g). These cells are usually located in the perivascular spaces close to blood capillaries. Mitochondria are the only cytoplasmic inclusion present in these cells and major part of the cytoplasm is occupied by the secretory granules.

4. Discussion

The present ultrastructural study on the secretory pinealocytes of *H. fossilis* after exposure to artificial photoperiods of continuous illumination and darkness, strongly indicates that the activities of the pinealocytes are affected by environmental photoperiod as manifested by the changes in their structural characteristics (table 2). The effects are noticeable within a short period of ten days. Moreover, an increase in the neural activity is also strongly evident under both the conditions.

In the dark-adapted pineal organs, the light cells show an increase in the metabolic activity as is evident from the enlarged mitochondria which appear swollen with distended cristae and with wide lumens, along with other features as numerous polyribosomes and distended rough endoplasmic reticulum. A significant increase in mitochondrial volume and area of smooth endoplasmic reticulum per photoreceptor cell was noted in a study on the effect of continuous DD on the morphology of *Carassius auratus* (McNulty 1982c). In a similar study examining the effect on daily changes in pineal morphology, McNulty (1982b) found an increase in the volume of endoplasmic reticulum in the photoreceptor cell. An increase in the relative volume of mitochondria, rER and Golgi complexes during night has also been observed in sheep in studies relating to day-night changes (Redondo

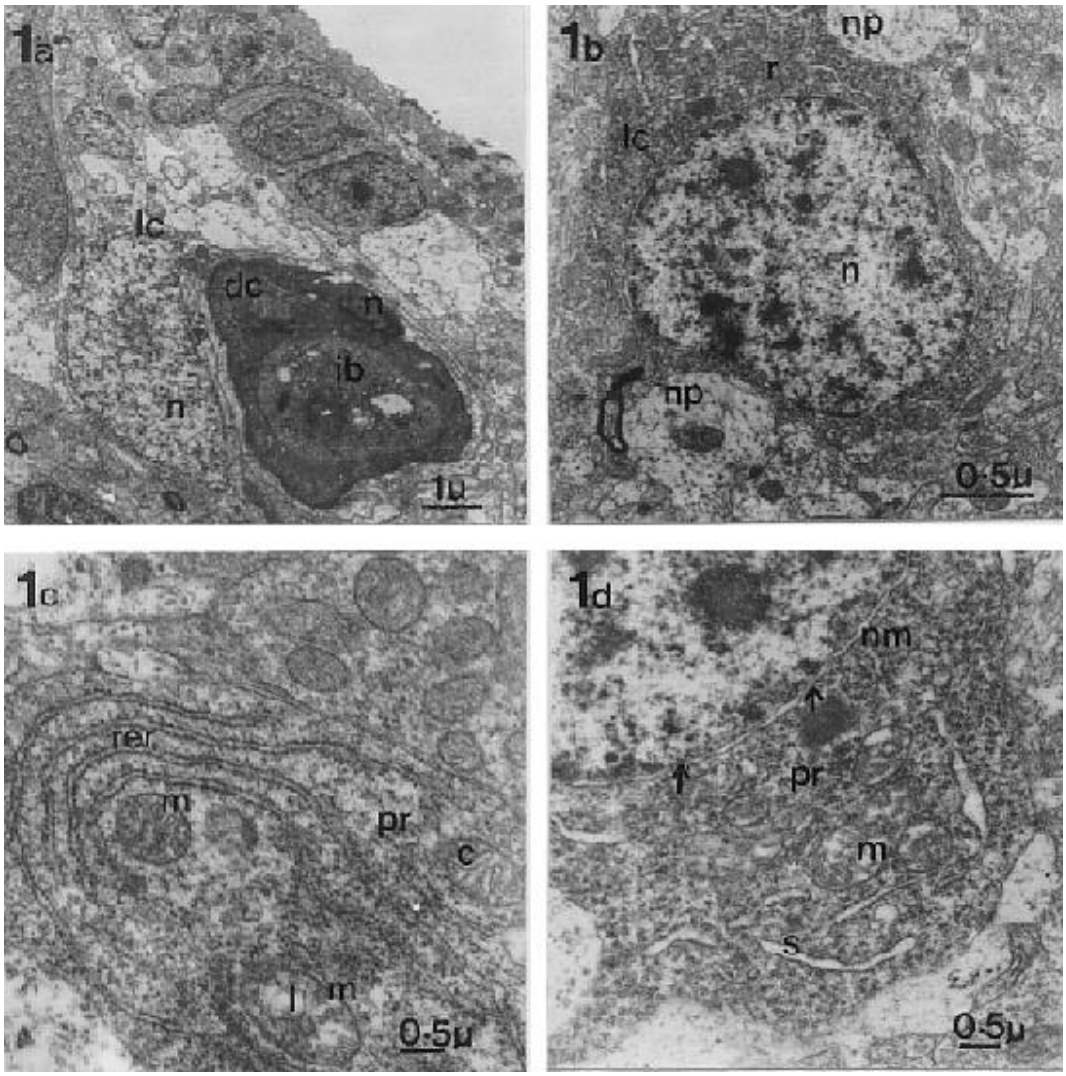

Figure 1. **(a)** Light (lc) and dark cells (dc) in the pineal parenchyma of the dark adapted *H. fossilis*. Note the difference in nuclei (n) in the two cells and the inclusion body (ib) occupying a major part of the dark cell (× 8200). **(b)** Light cell (lc) showing a large nucleus (n), cytoplasm with numerous free ribosomes (r) and flanked by nerve processes (np) (× 27000). **(c)** Numerous large sized mitochondria (m) with swollen cristae (c) and wide lumen (l), rough endoplasmic reticulum (rer) and polyribosomes (pr) in the cytoplasm of the light cell (× 12000). **(d)** Several membrane bound spaces (s), large mitochondria (m) and polyribosomes (pr) in the light cell (lc). Note the wide space in between the inner and outer nuclear membranes (nm) and thinning of the membranes at several points (↑) (× 12000).

et al 2003). In the same study, the pineal volume and the mean volume of pinealocytes were also found to be significantly greater in animals killed at night.

Increase in size of the mitochondria under DD conditions may also be considered important in view of the biochemical studies of Hori *et al* (1976) which demonstrated the existence of the enzyme tryptophan 5-hydroxylase in the mitochondrial fraction of the bovine pineal. Romjin *et al* (1977) also observed *in vitro* a spherical enlargement of mitochondria in the light pinealocytes of the rabbit after addition of an inhibitor of the enzyme. They hypothesized that hydroxylation of tryptophan to 5-hydroxytryptophan takes place in the mitochondria, while conversion of 5-hydroxytryptophan to serotonin occurs freely in the cytosol, and that the enlargement of mitochondria after treatments was a compensatory reaction to inhibition of the enzyme tryptophan 5-hydroxylase located on the mitochondrial membrane. A comparison of the results of the present and other studies points to the fact that continuous DD (absence of light) brings about some physiological change in certain organelles, viz. mitochondria and ER of the pinealocytes, leading to their increase in size.

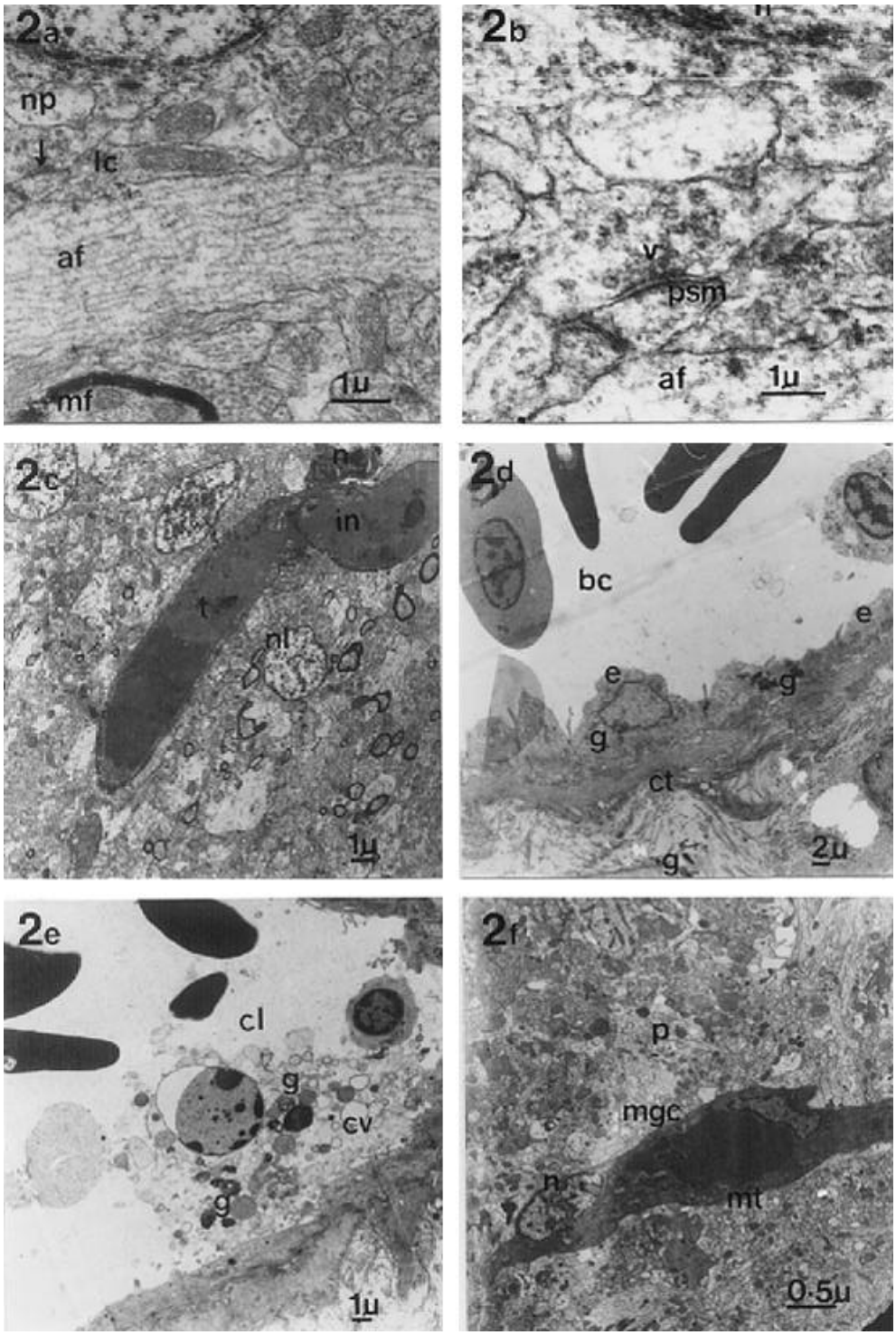

Figure 2. **(a)** Cell processes of light cells (lc) and nerve processes (np) occur intertwined with each other. Synaptic cleft is clearly seen (↓). Myelinated nerve fibres (mf) and microtubules in an axonic fibre (af) are seen in close proximity (× 11000). **(b)** Synaptic site seen with post synaptic thickening (psm) in the nerve membrane and dense vesicles (v) in the cell process; nucleus (n) of the cell and axonic fibre (af) are also seen in close proximity (× 11000). **(c)** Dark cell with dense inclusions (in) occupying the major part of the cell and also the tubular protrusion (t). Nucleus (n) is very much reduced and restricted to one corner of the cell. Nuclei of light cell (nl) seen in proximity (× 4000). **(d)** Outpushings of endothelial cells (e) into the blood capillary (bc). Note the thick connective tissue stroma (ct) and scattered secretory granules (g) in the stroma (× 1500). **(e)** Diffused secretory granules (g) and clear vesicles (cv) into the capillary lumen (cl) (× 1500). **(f)** Microglial cells (mgc) in the pineal parenchyma (p). Note the indented nucleus (n) and microtubules (mt) running its length (× 17500).

The dark cells under DD conditions are generally necrotic looking cells with dense homogenous cytoplasmic inclusions. They do not contain any secretory granules as observed in normal LD conditions and are comparable to the third and final stage of the secretory pinealocytes under the normal photoperiod – the necrotic cells. At certain sites in the perivascular areas, the scattered dense granules appear to migrate through the connective tissue stroma into the endothelial cells which bulge outwards into the capillary lumen. These dense granules finally diffuse into the capillaries (figure 2e). Collin and Oksche (1981) have advocated the diffusion mechanism (molecular dispersion) for the release of dense core vesicle and also the active role of connective tissue in the process. Since none of the loaded dark cells were observed in the dark-adapted pineal organ, it is very probable that under DD conditions the synthesized products are released. Appearance of numerous microglial cells is significant. However, their role is not clear. Pineal cell types resembling microglial cells are reported involved in phagocytotic activity particularly that of the outer segments of photoreceptor cells (Herwig 1981). In the present case, these cells may be involved in the phagocytosis of the necrotic dark cells. Inclusion bodies and myelin fibres have been reported in the pineal organ of blind cave fish exposed to continuous darkness (Omura 1975).

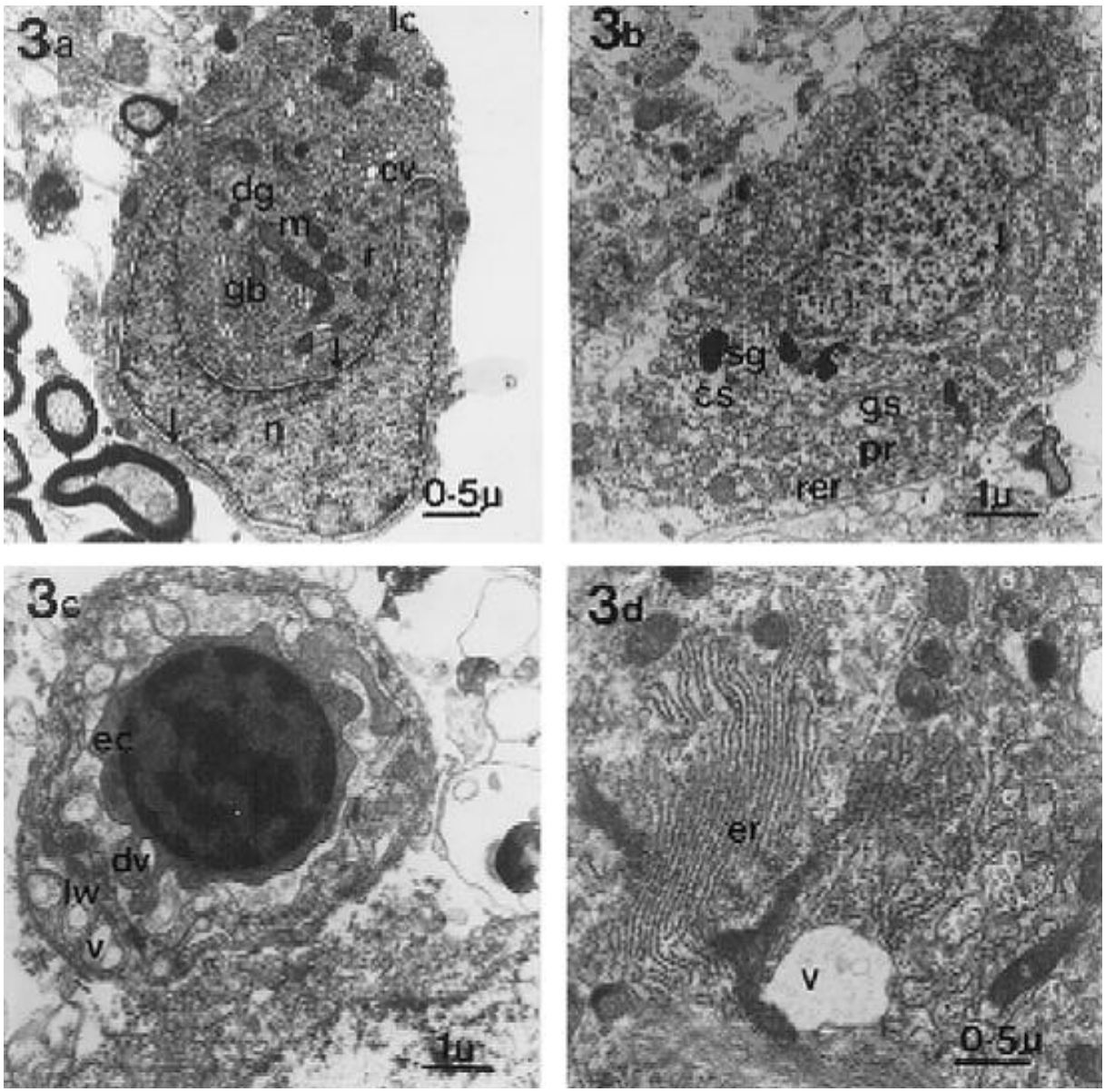

Figure 3. (a–d)

Under DD conditions, synaptic activity was observed at several points of contact between the light cells and afferent nerve cells (figure 2a, b). The proximity of the nerve cells to the secretory pinealocytes indicate that a close interaction exists between the two. The terminal processes of these pinealocytes are also in close contact with the nerve cell and their processes. It is noteworthy that the terminals of the light cells contain clear vesicles in DD. The pineal appears to be highly neurally active with the occurrence of several axon–axon synapses as well.

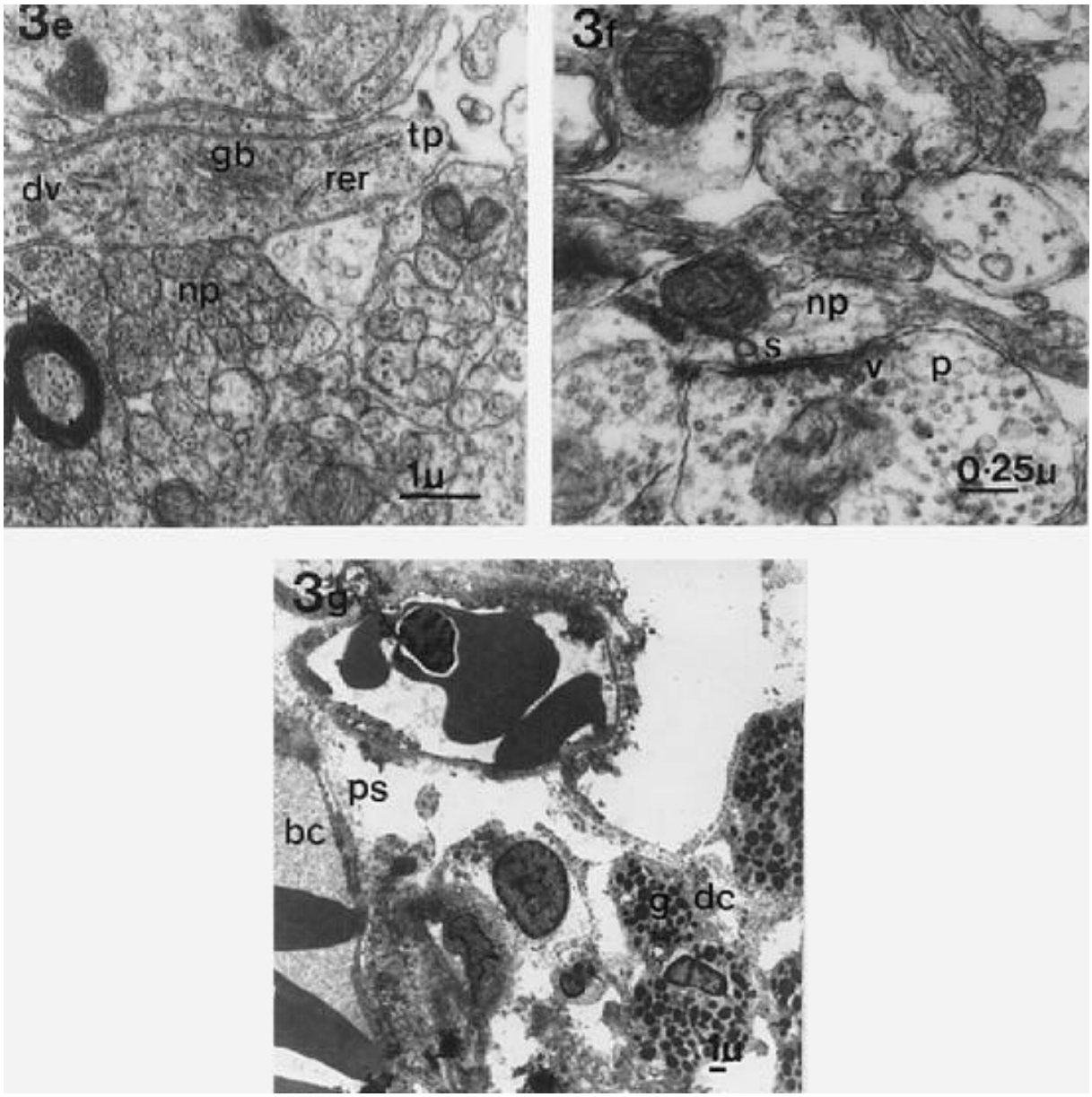

Figure 3. **(a)** Light pinealocyte (lc) in an active synthetic state in the light adapted pineal of *H. fossilis*. Note the prominent Golgi bodies (gb), small dense mitochondria (m) and numerous clear vesicles (cv) and few dense granules (dg) and ribosomes (r) in the cytoplasm. Nucleus (n) shows interruptions (↓) in the nuclear membrane (nm) and a wide gap is present between the inner and outer membranes (× 16000). **(b)** Synthetically active soma (cs) where the nuclear and cytoplasmic contents appear one. Breaks in the nuclear membrane (↓), numerous polyribosomes (pr), secretory granules (sg), Golgi saccules (gs), rough endoplasmic reticulum (rer) are characteristically seen (× 10,000). **(c)** Characteristic cell types where euchromatin (ec) flows out into the cytoplasm and forms dense vesicles (dv). Lamellar whorls (lw) are seen encircling the vesicles (v) (× 10,000). **(d)** Extensive endoplasmic reticulum (er) occupying the cytoplasm of light cell. Note the dense substance (*) within the cisterns and large vacuoles (v) with flocculent material (× 42000). **(e)** Club shaped terminal processes (tp) of light secretory pinealocytes surrounded by numerous nerve cell processes (np). Dense vesicles (dv), fragments of rough endoplasmic reticulum (rer) and Golgi bodies (gb) are also seen (× 11500). **(f)** Synaptic site (s) between the secretory pinealocyte (p) and nerve cell process (np). Note the numerous vesicles (v) in the secretory cell (× 33000). **(g)** Dark cells (dc) loaded with secretory granules (g) predominantly present in the perivascular spaces (ps); (bc) blood capillary (× 2000).

Table 2. Comparison of the structural characteristics of the light and dark cells under artificial photoperiod conditions of DD and LL in *H. fossilis*.

Cell type	Structural characteristics	Artificial photoperiod	
		DD conditions	LL conditions
Light Cell			
	Cytoplasm	Have large mitochondria, few ER, several polyribosomes	Average size mitochondria, numerous rER, ribosomes and polyribosomes
	Nucleus	Disintegrated euchromatin and heterochromatin. Wide gap between inner and outer membranes	Same as in DD
	Mitochondria	Large in size (0·5–1 μm), distended cristae, wide lumen	Average in size 0·15–0·2 μm, densely folded cristae, normal lumen
	Endoplasmic reticulum	Rough type (rER), few in No.	Extensive rER, cisterns contain dense material
	Ribosomes	Numerous ribosomes and polyribosomes	Numerous ribosomes and polyribosomes
	Vacuoles	Sparse	Large vacuoles (3–4 μm in diam.) having electronlucent material
	Cell terminals	Contain clear vesicles surrounded by several nerve processes	Contain several dense vesicles, fragments of rER, and surrounded by nerve processes
	Secretory activity	No secretory activity	Intense secretory activity
Dark Cell			
	Cytoplasm	Occupied by large homogenous/crystalline inclusions	Occupied by secretory granules
	Nucleus	Reduced in size, shifted to one corner of the cell	Normal
	Mitochondria	Sparse	Few in No.
	Endoplasmic reticulum	Absent	Absent
	Ribosomes	Absent	Absent
	Vacuoles	Lacking	Lacking
	Secretory granules	Absent	Abundantly present
	Secretory activity	Necrotic phase	Storage phase

Some of the notable morphological features manifest in the light adapted pinealocytes of *H. fossilis* are small fragments of rough endoplasmic reticulum and dense vesicles predominantly occupying the terminals of the light cells. The light cells appear highly secretory active after the light adapted phase. Highly significant are some of the cells that show the presence of lamellar whorls and the involvement of the nuclear material in the formation of dense vesicles (figure 3c). Lamellar whorls are known to be indicative of increased secretory activity (Reiter 1981). A similar concentric arrangements of membranes have also been observed in the pineal supportive cells of deep sea fish, *Nezumia liolepis* (McNulty 1976) and many mammalian pinealocytes. A relationship between the appearance of these structures and pineal antigonadotrophic activity has been suggested (Pevet 1979). The dark cells are prominently seen in the perivascular spaces, loaded with secretory granules. Evidently, a stimulation of the secretory and storage capacities of the pinealocytes is observed in the pineals exposed to continuous illumination. It is interesting that numerous nerve processes surround the pinealocyte terminals, and synaptic thickenings at many cell-nerve interface (figure 3f) are also present.

Significantly the endoplasmic reticulum appear to be highly active in the synthetic mechanism in the light adapted pineal of *H. fossilis*. Ependymal type of secretion which is a characteristic feature of nocturnal mammalian pinealocytes (Pevet 1979) is indicated in the pinealocytes of this nocturnal fish. Extended lengths of endoplasmic reticulum with wide lumens filled with dense substance and vacuoles containing flocculent material in the same cell are strong morphological indications. Ependymal type of secretory process involves granular endoplasmic reticulum in the process of secretion without the direct involvement of Golgi bodies (Pevet 1979), and the activity

of such a process is specially influenced by the activity of the gonadal axis (Reiter 1981). A functional relationship between pineal organ, photoperiod and gonadal maturation has been suggested for several fish species like gold fish (Fenwick 1970a; De Vlaming and Vodicinik 1978; Hontela and Peter 1980), killifish (Urasaki 1972, 1973) and a cyprinid golden shiner (de Vlaming 1975). In pineal physiology the ependymal type of secretion plays an important role and may be considered to be an indicator of a very active pineal gland.

The present study strongly emphasizes the significance of light and darkness on the activities of the secretory pinealocytes in *H. fossilis*. The secretory pinealocytes (light and dark cells) and their subtypes which represent the morphological correlates of a secretory cycle (Srivastava 1999) are definitely influenced by the presence or absence of light. The structural characteristics of these pinealocytes viz. mitochondria, ER, ribosomes show response to changes in the environmental photoperiod strongly emphasizing the fact that their cellular activities are sensitive to photic conditions. The study also shows that periods of prolonged light or darkness are capable of influencing the secretory process. Under artificial prolonged photoperiods of LL or DD, in each of the cell type only some stage of the secretory mechanism seems to be greatly pronounced e.g. in LL the light cells exhibit increased secretory activity only and the dark cells a storage one with no indication of any release; in DD most of the dark cells are withered and necrotic indicating the release of the vesicles while the light cells show heightened metabolic state but no trace of any secretory activity. Light appears to play an important role in the secretory physiology of these pinealocytes. It can be assumed that while the presence of continued light stimulates the synthetic phase, the discharge of secreted products takes place in the absence of light during which also no synthetic mechanism occurs. The pineal indole amine metabolism involves several steps in the synthesis of melatonin/serotonin and it would require thorough biochemical and immunocytochemical investigations to ascertain how the steps would be affected by light in this fish. An all time high levels of the circulating melatonin during night is a constant feature of the vertebrate physiology. A similar pattern is also seen in fishes where the melatonin synthesized in the pineal gland is released into the circulation and serves as a reliable hormonal indicator of the day/night cycle (i.e. high at night and low during the day).

Increased neural activity under both the LL and DD conditions is highly significant. Synaptic thickenings and clefts have been observed at several points in both the DD and LL conditions. It is interesting that clear vesicles were observed in the light cell terminals under DD and dense vesicles under LL conditions. Synaptic activity was not so frequently observed in the pineal organ under normal photoperiod conditions. The findings indicate a strong correlation between the neural and secretory activities of the pinealocytes particularly under the altered photoperiod conditions. Two important considerations can be envisaged from this study:

(i) Continuous illumination (LL) stimulates intense synthetic activity in the light cells and a storage phase in the dark cells.

(ii) Continuous darkness (DD) causes a possible release of secreted substances as indicated by the numerous necrotic dark cells and a cessation of secretory activity.

Recent literature clearly point out that a variation in pineal metabolism occurs in response to environmental factors as light and temperature. Melatonin and serotonin – the products of pineal metabolism through their rhythmic circulation in blood may serve as biological clocks as demonstrated for gold fish and pike (Iigo *et al* 1991). In teleost fish, complete melatonin rhythm generating systems are located within individual photoreceptor cells in the pineal gland and retina (Falcon 1999) and light has been demonstrated controlling the melatonin synthesis (Klein *et al* 1997; Falcon 1999). Photic regulation of melatonin secretion by exercising control over that of arylalkylamine N-acetyltransferase (AANAT) activity has been shown in the pinealocytes of teleost fish (Klein *et al* 1997) and in the European hamster (Garidou *et al* 2003).

The effects of exposure to constant illumination and darkness are both visible after 10 days in *H. fossilis* the lowest time for visible changes; it took 20 days in *Gambusia affinis holbroki* (Cheze and Lahaye 1969) and six months to an year in *Carassius auratus* (McNulty 1982a) to induce histological changes in the pineal upon exposure to constant illumination and darkness. It would be interesting to find out if the effects observed in 10 days are manifested in lesser periods of exposure also. This would be important in view of the daily fluctuating melatonin/serotonin levels. The catfish is known to exhibit light dependant diel activity patterns (Srivastava 1992, 1993). Any alteration in the external light/dark cycle would fluctuate the melatonin levels in blood thus affecting the circadian biology of the species.

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