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REGULAR ARTICLE

The pineal gland of the shrew (*Blarina brevicauda* and *Blarina carolinensis*): a light and electron microscopic study of pinealocytes

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Abstract

The pineal gland structure and ultrastructure in the Northern (*Blarina brevicauda*) and Southern short-tailed shrew (*Blarina carolinensis*) are described by light and electron microscopy. Results observed were similar to other mammals of Insectivora described previously, specifically, the hedgehog (*Erinaceus europaeus*) and the Old World mole (*Talpa europea*). Two different types of pinealocytes were noticed by electron microscopy, in addition to relatively few glial cells. Granular vesicles were not noticed in abundance. The granular endoplasmic reticulum was observed and studded with vesicles. The golgi apparatus was well developed and appeared often. Synaptic ribbons were observed in several different formations consisting of ribbons and/or rods. The ciliary derivative, the rudimentary photoreceptor structures found in the pinealocytes of population I, was noticed in a 9 + 0 tubular pattern. Within these semifossorial shrews, the relationship between specific intracellular organelles and their function was discussed.

Keywords Pineal gland · Shrew · Ultrastructure · Light microscopy · Electron microscopy · Mole · Hedgehog

Introduction

Experiments in the latter half of the last century have shown that the pineal gland is a multifunctional organ of high significance. For example, Kappers (1979) has outlined more than 50 biological processes in the human pineal gland. In vertebrates, the pineal gland is one of two organs along with the retina that produces melatonin (Korf 2002). Melatonin acts as an endogenous circadian rhythm generator, increasing at night and decreasing during the day (Macchi and Bruce 2004). The pineal gland receives environmental light information from its connection via the superior cervical ganglia to the suprachiasmatic nucleus which is innervated by the retina through the retinohypothalmic tract (Stehle et al. 2011). This system forms the photoneuroendocrine system (Oksche and Hartwig 1979; Maronde and Stehle 2007) which acts as the most salient regulator of diurnal and nocturnal production of melatonin (Macchi and Bruce 2004). Despite recent gains in the understanding of pineal gland structure (Quay 1965; Kappers 1965; Bhatnagar 1992), function (Sapède and Cau 2013), and gene expression (Rath et al. 2013), their remain many questions to be investigated concerning the pineal gland and pinealocytes (Macchi and Bruce 2004). Furthermore, pinealocyte ultrastructure is described in only 2% of mammals (Bhatnagar 1992); therefore, additional investigations into the pineal gland structure and function are warranted. Lastly, within the literature, there is considerable debate on the two different populations of pinealocytes (Pévet 1977, 1978). The debate arises over whether two populations of pinealocytes exist or does one population of pinealocytes possess different structural components at different times, thus possessing different function/physiology. Pévet (1976) indicated that perhaps the two secretary processes are interrelated. These unanswered questions concerning pinealocyte structure and function warrant further studies in mammals.

The present study was conducted to investigate the shrew pineal gland. The shrew is a semi-fossorial mammal with small eyes and rudimentary visual system (Branis and Burda 1994; Barton et al. 1995). The pineal gland in animals comprising the Order Insectivora, of which the shrew belongs, has had only five species described to date: the hedgehog

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(*Erinaceus europaeus*; Pévet and Saboureau 1974; Pévet 1975, 1976), the Old World mole (Talpa europea; Pévet 1974, 1976), the golden mole (*Amblysomus hottentotus*; Pévet and Kuyper 1978), and two Japanese moles (*Mogera kobeae* and *Mogera wogura*; Kikuchi et al. 1984). Therefore, additional descriptions of the pineal gland in mammals of Insectivora are justified. To accomplish this objective, the macroscopic anatomy and ultrastructure from two species of shrews were described, the Northern short-tailed shrew (*Blarina brevicauda*) and the Southern short-tailed shrew (*Blarina carolinensis*). Due to the rudimentary visual system, small eyes, and fossorality of these two mammals, analyzing the pineal gland and pinealocytes in animals which have reduced environmental lights conditions is of high interest (Oksche and Hartwig 1979; Maronde and Stehle 2007; Stehle et al. 2011). Mammals of Insectivora, specifically hedgehogs, moles, and shrews, were the primary focus of discussion and literature review. The selected light and electron microscopic studies reviewed focused on the structure and function of the pineal gland. The present ultrastructural electron microscopy study concentrated on describing the two different populations of pinealocytes.

Materials and methods

Sample preparation

The present study and procedures were approved by the animal research ethics committees of the City University of Hong Kong, Hong Kong SAR China. Shrews were captured in Virginia, USA. The pineal gland structure and ultrastructure were assessed from two species, the Northern short-tailed (*Blarina brevicauda*) and the Sorthern short-tailed (*Blarina carolinensis*) adult shrew. A total of 8 shrews (4 *B. brevicauda* and 4 *B. carolinensis*, 4 males and 4 females) were chosen for the present study. The shrews were caught in their natural habitat during the summer months. Shrews were processed for light and electron microscopy immediately after being caught. Shrews were anesthetized with sodium pentobarbital, the thoracic cavity was opened, and a perfusion needle was inserted into the left ventricle. The right atrium was slightly clipped and the left ventricle was flushed with room temperature 1% $NaNO_2$ heparinized in 0.01 M phosphate buffer (7.4) for 30 s, followed by 100 ml of fixative containing depolymerized paraformaldehyde in 0.1 M phosphate buffer at pH 7.4. The perfusion was continued until shrews were exsanguinated, and fixative flowed from the right atrium. The skull of shrews was excoriated of skin and an incision was made cutting along bregma to lambda in the sagittal suture to softly aide separation. Tweezers or forceps were inserted between the premaxilia and maxilia with the entire skull separated at the midline between the left/right frontal and parietal skull bones. The intraparietal and occipital bones were separated from the lambdoid suture resulting in an open cranium. Skull bones were pulled away and the brain was extracted. Brain sections were trimmed to a 5-mm area circumscribing the pineal gland, washed and then immersed in fixative overnight at room temperature. Subsequently, respective light microscopy or transmission electron microscopy steps were applied.

Light microscopy

For light microscopy, brains were embedded in paraffin. Titrations of ethanol changes starting at 75% ethanol to 100% ethanol were administrated hourly, followed by xylene immersions and two paraffin wax steps at 60 °C for 2 h. Sections were trimmed further, mounted on slides, dried for 0.5 h, and then baked at 50 °C in preparation for ~ 5–10 μm sectioning. Sections were deparaffinized with three changes of xylene, 10 min each, dripped in ethanol with gelatin, cut on a EM UC7 ultramicrotome (Leica Microsystems, Wetzlar, Germany) using a diamond knife, and placed on slides (Kapelsohn 2015). Slides were rehydrated overnight with two changes of 100% ethanol for 3 min followed by 95 and 80% ethanol for 1 min each and rinsed in distilled water. Nissl staining was conducted with slides stained by cresyl violet solution for 10 min (Sigma C5042 Cresyl Violet acetate) in a warm bath (37 °C), rinsed in distilled water, and differentiated in 95% ethanol for 30 min. Subsequently, slides were dehydrated in 100% alcohol, cleared in xylene two changes for 5 min each, and mounted with Permount™ mountant (Fischer Scientific). All slides were examined with an optical light microscope (Leica DM2700 M) using a digital USB to PC connected 5 M pixel HD camera (Leica MC170 HD). Images presented in the manuscript were unaltered for contrast and brightness and analyzed using ImageJ (Rasband 2016), upsampled to 1200 × 1200 pixels/cm^2 at 8.7-cm width using a cubic interpolation (The GIMP Team 2017, v2.8.22).

Electron microscopy

For electron microscopy, sections were rinsed in three changes of buffer, dipped in alcoholic gelatin, placed on slides, dried at room temperature, dehydrated through ethanol and xylene, and mounted in Permount (Fisher). Tissue was postfixed in 1% osmium tetroxide (Sigma 201030) 0.1 M in sodium cacodylate (Sigma C0250) buffer (pH 6.5) for 60 min at 4 °C, and then dehydrated through a graded ethanol series, followed by immersion in 100% acetone. Tissue was flat-embedded between fluorocarbon-coated coverslips in a 6/4 mixture of Spurr/Epon resin (Sigma EM0300 and 45359; Hajibagheri 1999). After overnight polymerization at 70 °C, silver thin sections were poststained with aqueous uranyl acetate (Watson 1958) and lead citrate (Reynolds 1963).

Sections were viewed with a JEOL 100CX-II Scanning-Transmission Electron Microscopy (STEM; JEOL USA, Inc.). The STEM had the following specifications: accelerating voltage 100 kV with magnification range 360× to 320,000× for TEM and 10× to 200,000× for SEM with a 2 Å resolution, and vacuum pressure 10 Torr. Images were developed, digitalized, and analyzed using ImageJ (Rasband 2016). Identification of ultrastructure was based on descriptions largely from the manuscripts of Pévet (Pévet 1974, 1976, 1977, 1978, 1981; Kappers 1979; Bhatnagar 1992).

Results

No differences in the ultrastructure of the pineal gland were observed between *B. brevicauda* and *B. carolinensis*. Furthermore, no differences were noted between females and males. Individual shrews did not significantly differ in their structure or ultrastructure as viewed by light microscopy and electron microscopy, respectively. The description of the pineal gland in the shrew is divided into light and electron microscopic observations.

Light microscopy

Macroscopic observations

The pineal gland in the shrew is quadrangular in shape (see Fig. 1a, b). In the coronal plane, the pineal gland is approximately a square measuring 435 μm vertically and 440 μm horizontally (Fig. 1a). In the sagittal plane, the pineal gland measures 210 μm vertically and 425 μm horizontally. The formula for the volume of a quadrangle is V = lwh. The volume of the pineal gland from the largest sections in both the coronal and sagittal plane is (0.435 mm) * (0.440 mm) * (0.425 mm) = 0.08135 mm^3. It should be noted that this is an estimate as the pineal gland is not a perfect quadrangle and in some planes is more pyramidal. Also, taking the largest sections overestimates the actual size of the pineal gland.

Pinealocytes

The parenchyma of the pineal gland in the shrew consists mainly of pinealocytes, with few glial cells and various degrees of vascularization (Fig. 1). Pinealocytes in the shrew had an average circumference of 24.09 μm. Table 1 summarizes the data from 50 measured pinealocytes.

Electron microscopy: pinealocytes general

The use of electron microscopy enabled the characterization of two populations of pinealocytes based on ultrastructural differences between the intracellular organelles (Table 2).

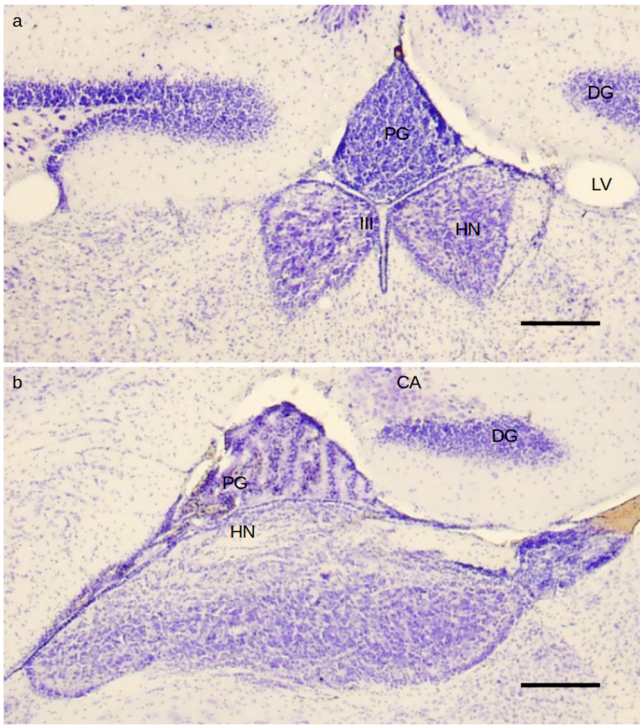

Fig. 1 Histological sections of the shrew (*Blarina brevicauda*). **a** Coronal cection and **b** sagittal section. Magnification 40×. PG-pineal gland, III-third ventricle, HN-habenular nuclei, LV-lateral ventricle, DG-dentate gyrus, CA-cornu ammonis

These were taken at different magnifications to highlight the features of interest (Figs. 2, 3, 4, 5, 6, 7, and 8). In the shrew, pinealocytes of population I (light pinealocytes, parenchymal cells, and clear pinealocytes) were characterized by the production of granular vesicles by the saccules of the golgi apparatus (GA; Figs. 2 and 3), and the pinealocytes of population II were characterized by the presence of a secretory material accumulating directly from the cisterns of the granular endoplasmic reticulum (GER; Fig. 7). We confirm finding two populations of pinealocytes, although at times difficult to differentiate. Pinealocytes of population I were observed very often, but we cannot conclude that one pinealocyte population was more predominant, because ultra-sectioning our slices was contained to the midline of the pineal gland. Nevertheless in the ultra-sections, pinealocytes of population I appeared more often than pinealocytes of population II (see Table 2).

We found subsurface cisterns (SC; Fig. 3; unique junctional complex), often very thin, but in some images containing dense material (Fig. 6: SI-Fig. 1). In the shrew, SC are numerous and found independent of the pinealocyte population (see Figs. 2 and 3 for pinealocytes of population I and Fig. 8 for pinealocytes of population II). We observed ciliary derivative (CD), which are rudimentary photoreceptor structures found in the pinealocytes of population I (see Figs. 2 and 3; Collin 1970). Figure 2 shows the cross section of the CD as it approaches a planar cross-sectional face, as observed in Fig. 3.

Table 1 Circumference of pinealocytes

Mean (μm)	Standard deviation (μm)	Standard error (μm)	Minimum (μm)	Maximum (μm)	Range (μm)
24.08773	5.565142	0.7870299	11.51376	35.36106	23.8473

The CD was characterized by a 9 + 0 tubular pattern as observed in the transverse section. Here, nine circularly dense structures encircle a less dense middle cilium (see Fig. 3). In the shrew, CD was observed very often in the pinealocytes of population I (see Figs. 2, 3, 6, and 8).

Pinealocytes of population I

The pinealocytes of population I were observed alone (Fig. 2) or in close proximity to pinealocytes of population II (Fig. 7; SI-Figs. 1 and 2). Granular vesicles (GV) produced by the sacculues of GA were not observed often and were previously deemed rare (Pévet 1977, 1978). Surprisingly, synaptic ribbons (SR) and vesicle-crowned rodlets (or lamella–VCR) were observed in abundance (see Figs. 2, 3, and 5). Synaptic ribbons (SR) are similar structures to VCR and were once thought to be distinguished by having an axodendritic orientation, being surrounded by clear vesicles, and having features of a true synaptic junction (e.g., pre- and postsynaptic thickening, free synaptic vesicles, etc.), as opposed to VCR which have a somato-somatic orientation and never have features of a synaptic junction (Pévet 1978). In the shrew, SR appear to be located diffusely, ranging from the pinealocyte process to the perikaryon. The SR were found in groups averaging five rods or ribbons per grouping. Very often, SR were found in organized rows called ribbon fields with clear vesicles between each row (see Figs. 5 and 6).

Pinealocytes of population II

The pinealocytes of population II were observed (Figs. 7 and 8) in close proximity to pinealocytes of population I (Fig. 8; SI-Figs. 1 and 2). The ependymal like secretory process (ESP) accumulates secretory material directly from the cisterns of the GER, the defining characteristic of pinealocytes of population II. A GER with abundant glycogen granules attached to the cisterns is observed next to a nerve ending (see Fig. 7). The glycogen granular material, thought to be tryptophan (Pévet 1976; tryptophan is the precursor to melatonin Stehle et al. 2011) and stimulated by fossorial life in darkness (Pévet 1974, 1977), was observed (Figs. 7, 8, SI-Fig. 1). The ESP and the GER were found in connection with the vacuolation system, which aggregates the glycogen granules into vacuoles. A pinealocyte of population II was observed next to a pinealocyte of population I, the former possessing GER and un-granulated endoplasmic reticulum or smooth endoplasmic reticulum (SER) and the later possessing GA and a CD (see Fig. 8). Glycogen granules (GG), small electron dense carbohydrate storage units, with an unknown physiological function, were found in pinealocytes of population II. The GG were found in the shrew to be associated with the GER and with clear vacuoles (SI-Fig-1; part of the vacuolar systems—VS); however, they were small in size (see Fig. 8). In the shrew, large vacuoles were not prominently observed. A congregation of small light and dark vesicles was observed with a mixture of dense and non-dense material (SI-Fig. 3).

Discussion

Macroscopic observations

Using light microscopy, Quay (1965) described the pineal gland in the shrew (*Blarina brevicauda*) as being attached to the roof of the diencephalon whereas in the hedgehog and mole, Pévet (1976) described it as being attached to the pars intercalaris near the roof of the third ventricle situated posterior to the habenular commissure. Based on Bhatnagar et al. (1986) description of bats, the pineal in the present paper was similar to the *Scotophilus donganii* and *Hipposideros commersoni* type A pineal organ (Vollrath 1981). The pineal gland in the adult hedgehog, mole, and shrew is situated in the septum interpositum which is dorsal to the roof of the third ventricle (Kappers

Table 2 Summary of pinealocyte populations

Pinealocyte	Common nomenclature	Pinealocyte abundance	Primary organelle	Primary organelle abundance
Population I	Light pinealocytes, parenchymal cells, and clear pinealocytes	+++	Golgi apparatus	+++
Population II	Dark pinealocytes, interstitial cells, pigment containing cells	+	Granular endoplasmic reticulum	++

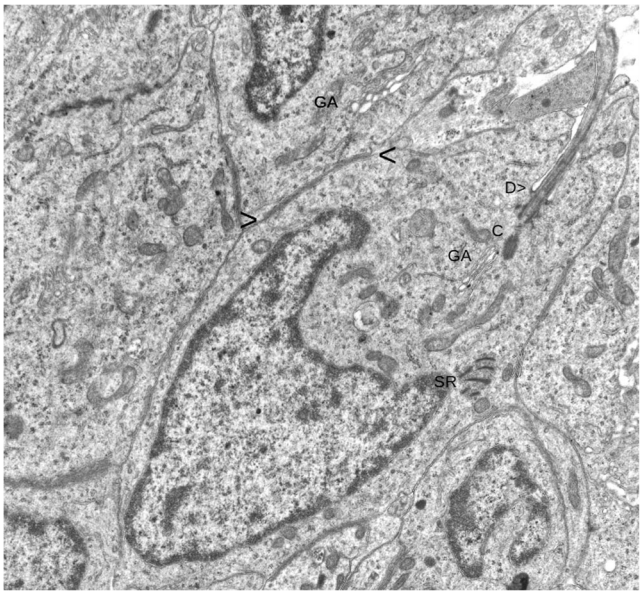

Fig. 2 Magnification 12,000×. Pinealocyte of population I. GA-golgi apparatus, SR-synaptic ribbons, D-diplosome ciliary derivative, C-centriole, > and < represent the subsurface cistern

1976). The present study found the location of the pineal gland in the shrew consistent with previous descriptions (see Fig. 1a, b). The pineal gland in the present study of the shrew was found to be more compact than previous descriptions (Quay 1965). The pineal gland of the shrew was located dorsal to the habenular nuclei situated beneath the third ventricle.

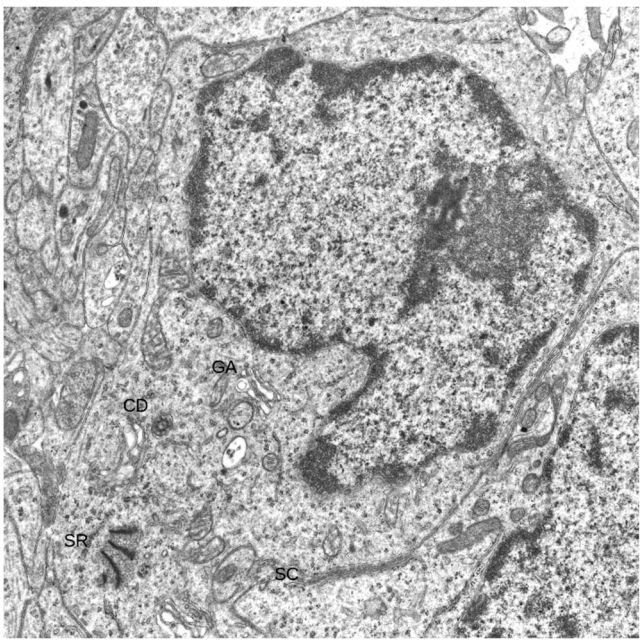

Fig. 3 Pinealocyte of population I. Magnification 14,500×, CD-ciliary derivative, SR-synaptic ribbons, SC-subsurface cistern

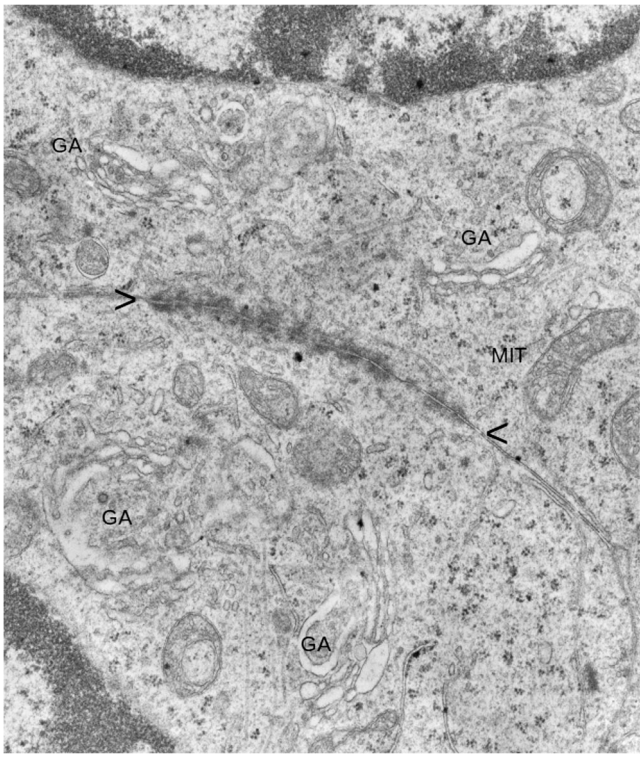

Fig. 4 Pinealocyte of population I. Magnification 39,200×. Pinealocyte of population I. GA-golgi apparatus, MIT-mitochondria, > and < represent a unique junctional complex

Pinealocytes

The parenchyma of the pineal gland in the shrew consists mainly of pinealocytes, with few glial cells and various degrees of vascularization as was previously reported in both the hedgehog and mole (Pévet 1976). Average sizes of pinealocytes in mammals of Insectivora are reported in one study to date which was conducted in Japanese moles (*Mogera kobeae* and *Mogera wogura*). The pinealocytes of the Japanese moles averaged 15 μm by 7 μm in diameter (Kikuchi et al. 1984). Pinealocytes in the shrew had an average circumference of 24.09 μm. Table 1 summarizes the data from 50 measured pinealocytes. In hedgehogs and moles, the pinealocytes are described as having a large nucleus with the entire space filled by the nucleolus. The nucleus is spherical to oval in shape and the nuclear envelop is lobulated or indented (Kappers 1976). This is consistent with the findings in the shrew. The body of the pinealocyte in hedgehogs and moles is described as possessing several cytoplasmic processes which extend some distance to other pinealocytes. Pévet (1976) characterized the pinealocyte as being a multipolar neuron. With light microscopy, dark and light gradations of pinealocytes are noticeable in hedgehogs and moles, as was found here in the shrew. However, the definite categorization of pinealocytes into dark and light gradations was not possible at this level of magnification. Recently, Rath et al. (2016) used

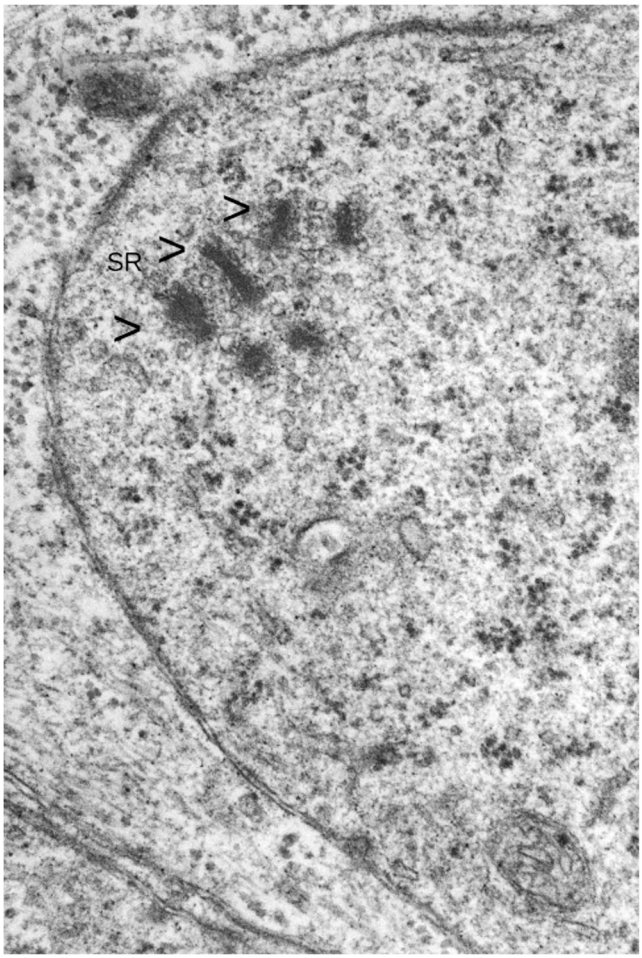

Fig. 5 Close up of SR-synaptic ribbons. Magnification 81,200×

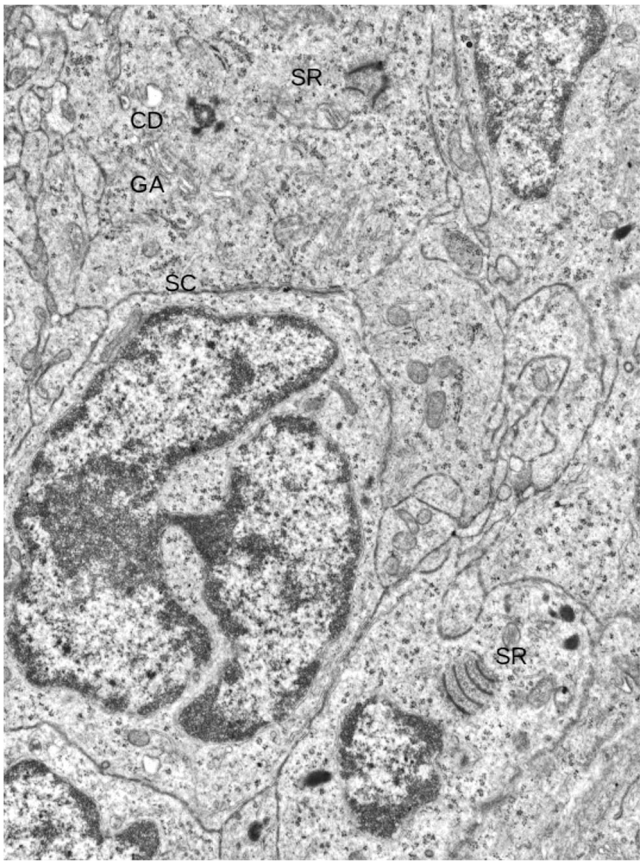

Fig. 6 Magnification 18,000×. SR-synaptic ribbons (four ribbons uniquely arranged in rows, incrementally bigger from front to back), SC-subsurface cistern, GA-golgi apparatus, CD-ciliary derivative: the CD appears blurred and is surrounded by 4 electron dense vesicles

immunohistochemistry methods in rat pineal and found acetylserotonin o-methyltransferase (ASMT) stains one pinealocyte dark, while one pinealocyte is not stained. Similarly, pinealocytes in the pineal complex were positively stained for both bovine retinal S-antigen (SAG) and ASMT, whereas others were immunopositive for only one of the proteins. Lastly, the study found colocalization of ASMT with TPH1 in some cells, whereas other cells were positive for only one of the proteins (Rath et al. 2016). Here, it is most likely that the two different pinealocyte populations are stained differently due to the different biological states (Rath et al. 2016). In the shrew, dark and light pinealocytes were noticed at 100× magnification with light microscopy under oil immersion. However, only a slight gradation of pinealocyte color was noticed. Here, some pinealocytes appeared lighter than others which often could have been attributed to a pinealocyte having more or less chromatin in the nucleolus or darker/lighter cytoplasm. In slide section, some pinealocytes appeared to have more heterochromatin while others euchromatin and thus darker/lighter cytoplasm and this could have contributed to the relative darker or lighter appearance, respectively. This is an artifact due to the plane of section through which the slide is fixed and is only relative to the particular plane of view, not truly being indicative of and a distinguishing feature of the pinealocyte. The two different populations of pinealocytes were only differentiated by electron microscopy.

Electron microscopy: pinealocytes

The use of electron microscopy enables the characterization of two populations of pinealocytes in hedgehogs and moles based on ultrastructural differences between the intracellular organelles. Pinealocytes of population I are characterized by the production of granular vesicles by the saccules of the golgi apparatus (GA) and the pinealocytes of population II are characterized by the presence of a secretory material accumulating directly from the cisterns of the granular endoplasmic reticulum (GER; Pévet 1977). The categorization of pinealocytes into either one or two populations based on ultrastructure is consistent with the review by Bhatnagar (1992). However, caution must be used in the categorization of pinealocytes as authors have reported two populations when in fact only one population of pinealocytes was present (Pévet 1976; Bhatnagar 1992). Furthermore, adding to the complication is

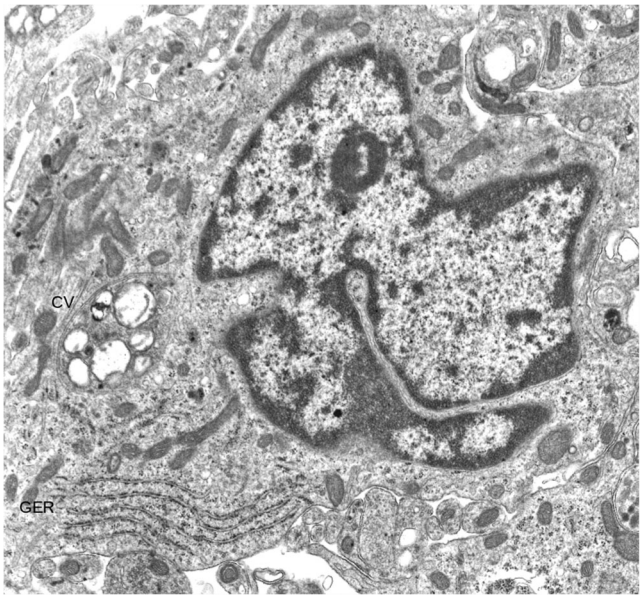

Fig. 7 Magnification 14,500×. Pinealocyte of population II. GER-granular endoplasmic reticulum, CV-clear vesicles

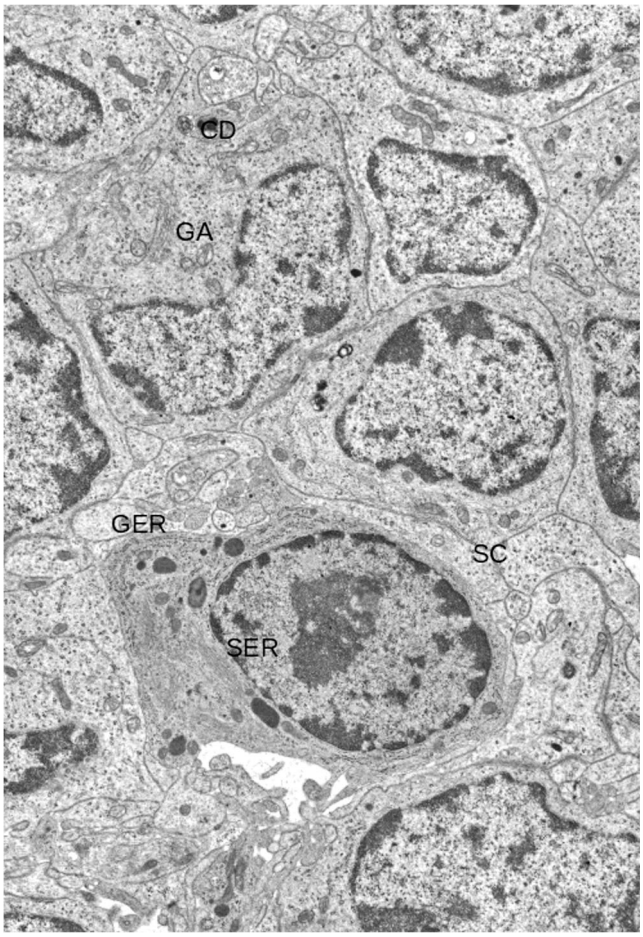

Fig. 8 Magnification 12,000×. Pinealocyte of population II. CD-ciliary derivative, GA-golgi apparatus, GER-granular endoplasmic reticulum, SER-smooth endoplasmic reticulum, SC-subsurface cistern

the fact that there are several names for each of the two populations of pinealocytes. According to Pévet (1977), the nomenclature of pinealocytes has been categorized by ultrastructure into: pinealocytes of population I (light pinealocytes, parenchymal cells, and clear pinealocytes) and pinealocytes of population II (dark pinealocytes, interstitial cells, pigment containing cells). The ultrastructural categorization is based on differences observed within pinealocytes in the intracellular organelles and can only be made definitively, "sensu stricto", by electron microscopy (Pévet 1977). Currently, considerable disagreement in the literature on pineal ultrastructure concerning the nomenclature of pinealocytes exists and whether or not two populations or one population of pinealocytes can be distinguished. Furthermore, pinealocytes in hedgehogs and moles distinguished as light and dark pinealocytes are suggested to be one pinealocyte population in different physiological secretory phases. Unfortunately, pinealocytes viewed in microscopy, due to the static nature of the slides and the preparation in vitro (Pévet 1974, 1976, 1977, 1978, 1981; Kappers 1979; Bhatnagar 1992), inhibit the ability to determine the phase difference. For hedgehogs and moles, it is unknown whether both types of secretory processes can occur in one pinealocyte population because studies have not confirmed that each pinealocyte can structurally exhibit both physiological processes (Pévet and Collin 1976; Pévet 1978). Consequently, in the most recent review by Bhatnagar (1992), hedgehogs and moles are classified as possessing two distinct populations of pinealocytes (i.e., light and dark) categorized by ultrastructural morphology. The finding in the shrew was consistent with other mammals of Insectivora where two populations of pinealocytes were noted. Here, we further delve into the review of the pinealocyte ultrastructure by dividing our observations into: (1) structures previously reported irrespective of the pinealocyte population, (2) structures in pinealocytes of population I, and (3) structures in pinealocytes of population II.

Structures previously reported irrespective of the pinealocyte population

Subsurface cisterns

Subsurface cisterns (SC) are areas of the cell membrane which face adjacent pinealocytes and are theorized to allow exchange of metabolites or ions between neighboring cells (Rosenbluth 1962). The membranes often have ribosomes on the interior; however, never have ribosomes been found within the cistern itself or on the exterior of the cell membrane (Pévet 1978). In the order Insectivora (i.e., hedgehogs and moles), SC are numerous due to their active pineal gland which supports the idea of their involvement in intracellular communication (Pévet 1974, 1976). Their function in intracellular communication is further corroborated by research in the

gerbil (*Meriones unguiculatus*) and rat (Tutter et al. 1991a, 1991b; Heinzeller and Tutter 1991; Karasek and Marek 1978). In the shrew, SC are numerous and found independent of the pinealocyte population (see Figs. 2 and 3 for pinealocytes of population I and Fig. 8 for pinealocytes of population II).

Ciliary derivative

Ciliary derivative (CD) is rudimentary photoreceptor structures found in the pinealocytes of population I (see Fig. 2; Collin 1970). The CD are characterized by a 9 + 0 tubular pattern in the transverse section whereby nine circularly dense structures encircle a less dense middle cilium (see Fig. 3). The CD have been observed in the pinealocytes of the hedgehog and the mole (Pévet 1976). The function of CD in the pinealocytes of population I, the pinealocytes of the sensory line, is unknown (Collin 1970). In the shrew, CD were observed very often in the pinealocytes of population I (see Figs. 2, 3, 6, and 8).

Pinealocytes of population I

Granular vesicles

Granular vesicles (GV) are produced by the saccules of the GA and are observed in the pinealocyte process of population I pinealocytes (Pévet 1978). In mammals of Insectivora studied to date, including one study referencing a shrew species (*Crocidura russula*—greater white-toothed shrew) (Pévet and Voute, unpublished; Dekar-Madoui et al. 2012), GV are exceedingly rare (Pévet 1977, 1978). In contrast, GV have been noted in the tree shrew (*Tupaia glis*; Hwang 1982; Kado et al. 1999), although these belong to the order Scandentia and not the order Insectivora, which comprises the shrews, moles, and hedgehogs. No GV were observed in abundance in either species of shrew; however, GA were abundant and observed quite often (see Fig. 4). The absence of GV in the shrews in this study could be related to the general rarity observed in some mammals of Insectivora (e.g., the hedgehog and mole) or it could be related to the functioning of GV. Although it is generally accepted that the GV are secreted, this has not been observed (Pévet 1978). Structurally, GV have several functional processes attributed to their presence; nevertheless, the function of GV in mammals of Insectivora is unknown. The theorized correlation of GV to the visual system is well established due to the pinealocytes of population I being characterized as cells in the sensory line derived from and showing features of rudimentary photoreceptor cells (Collin 1970; Pévet 1977). In the shrews of this study, GV were absent which is interesting in light of what is known about the visual system of these mammals (i.e., semi-fossorial).

The visual system in shrews is poorly developed and considerably less functional than other mammals of Insectivora. Greater fossoriality between different shrew species is associated with smaller visual system components (Barton et al. 1995). Shrews possess small eyes and vision is thought to play a rudimentary role for sensory information (Branis and Burda 1994). The shrew (*Blarina brevicauda*) appears to navigate in part by echolocation (Tomasi 1979). Structurally, because the pinealocytes of population I are from the sensory line, there could be an association between the particularly small visual system components and the lack of GV. This is extremely relevant because GV are absent from pinealocytes in shrews which could be caused by the lack of and smaller size of the visual system components. Hedgehogs and moles could have rare occurrences of GV, not complete absence due to their better preserved visual system components.

Synaptic ribbons and vesicle-crowned rodlets (or lamella)

Vesicle-crowned rodlets are electron dense rods surrounded by a layer of clear vesicles. VCR form groups and are located very close to the cell membrane on the inside of the cell often found near the pinealocyte process. Synaptic ribbons (SR) are similar structures to VCR and were once thought to be distinguished by having an axodendritic orientation, being surrounded by clear vesicles, and having features of a true synaptic junction (e.g., pre- and postsynaptic thickening, free synaptic vesicles, etc.), as opposed to VCR which have a somato-somatic orientation and never have features of a synaptic junction (Pévet 1978). Although this distinction was made between VCR and SR by Pévet (1978), the terminology in the review by McNulty and Fox (1992) did not differentiate the two types of organelles and termed both groups synaptic ribbons (SR) using subscripts based on the physiology, lighting, circadian, and seasonal states of the SR in the pinealocytes (Karasek 1976; Karasek et al. 1982; Karasek et al. 1988; Kurumado and Mori 1980; Kosaras Kosaras et al. 1983; Garcia et al. 1987). The VCR observed in hedgehogs and moles are usually seen during the period of sexual quiescence (Pévet 1976). The shrews in this study were caught during the summer months which is during their circannual sexual cycle, the greatest reproductive activity from March through September with a brief decline in August (Getz 1989). Therefore, the abundant SR observed (studded VCR were not abundantly observed, will be titled SR in the shrew; see Figs. 2, 3, and 5). These quantitative/qualitative descriptions for the hedgehog and mole are similar to what was observed here in the shrew and was similar to other species described (McNulty and Fox 1992). In the hedgehog, mole and in the shrew of this study, VCR and SR are found in the light pinealocytes or the pinealocytes of population I.

In the present study, SR appear to be located diffusely, ranging from the pinealocyte process to the perikaryon. The

SR were found in groups averaging five rods or ribbons per grouping. Very often, SR were found in organized rows called ribbon fields (McNulty and Fox 1992) with clear vesicles between each row (see Fig. 6). The function of SR in the pineal gland has also been shown to be related to the circadian cycle which is induced by environmental cues such as photoperiod, in particular an increase in the number of synaptic ribbons is associated with the dark/nocturnal period, independent of whether a short or long day/light cycle is used (Garcia, Soriano, and Torner Garcia et al. 1987).

Pinealocytes of population II

The ependymal-like secretory process

The ependymal like secretory process (ESP) is the name given to the development of secretory material accumulating directly from the cisterns of the GER which is characteristic of pinealocytes of population II. The ESP has been observed in the hedgehog, mole (Pévet 1976), and in the present study of the shrew. This form of synthesis has been termed ependymal secretion as it was first observed in the ependymal cells near the subcommissural organ (Pévet 1978). The ESP is exceedingly common in the fossorial blind mammals of Insectivora (Pévet 1976). A GER with abundant glycogen granules attached to the cisterns is observed next to a nerve ending (see Fig. 7).

The glycogen granular material in the hedgehog is a tryptophan compound and is thought to be stimulated by subterranean life (Pévet 1976; tryptophan is the precursor to melatonin: Stehle et al. 2011). This is substantiated by the observation in the pineal gland of the hedgehog and mole where an increase in the flocculent material of the GER is seen in animals from their natural environment as compared to those in the laboratory. Therefore, it has been hypothesized that the continuous life in darkness experienced by the fossorial mammals enhances the secretory process (Pévet 1974, 1977). Furthermore, at the beginning of the period of sexual quiescent and when sexual activity is decreased experimentally, the flocculent material is seen to accumulate in the cisterns of the GER (Pévet and Smith 1975; Pévet and Saboureau 1973, 1974). The ESP and the GER are found in connection with the vacuolation system which aggregates the glycogen granules into vacuoles. A pinealocyte of population II is observed next to a pinealocyte of population I, the former possessing GER and un-granulated endoplasmic reticulum or smooth endoplasmic reticulum (SER) and the later possessing GA and a CD (see Fig. 8).

Glycogen granules and the vacuolar systems

Glycogen granules (GG) are small electron dense carbohydrate storage units with an unknown physiological function. Their amounts range from minimal to none in both the hedgehog and mole, but they seem to be fairly numerous in the blind subterranean golden mole (*Amblysomus hottentotus*; Pévet and Kuyper 1978). The abundance of GG in one fossorial mammal, but not in another and the lack of GG correlating to the seasonal cycle, as most other organelle structures seen in hedgehogs and moles, contributes to the difficulty and confusion of interpreting results from experiments (Pévet 1976). In the golden mole, GG range in size from 40 to 350 nm and are found in abundance (Pévet and Kuyper 1978). In the hedgehog and mole, GG range in size from 80 to 200 nm (Pévet 1976). The physiologic function of GG is unknown, however several physiological processes have been described.

The GG and the GER appear to have several common physiological processes which are involved in the pinealocyte vacuolization system. During granulation of the GER, the GG involute and insert into the GER membrane wall. When present, GG are found everywhere in pinealocyte cytoplasm except for near the nucleus. It appears they are numerous up to the edge of the nucleus wall and then they form a circle where they are absent and the nuclear membrane is present (Pévet 1978). Here, GG were found in the shrew to be associated with the GER and with clear vacuoles; however, they are small in size (see Fig. 8). Small vacuoles concatenate in an aggregation to form larger vacuoles. The vacuolation of the pinealocytes could be a degenerative process, but this is contradicted by the many vacuolated pinealocytes seen in the female mole during the pre-estrus phase (Pévet 1976; Pévet and Smith 1975). The theory by Pévet (1978) attributes the vacuoles as a hormone retention or storage mechanism, but this has not been validated experimentally.

Conclusions

The objective of the present study was to determine structure and ultrasturcture in the pineal gland of the shrew. We investigated the shrew pineal gland because few animals in the order Insectivora have been studied. Further, analyzing animals living in reduced lighting conditions give clues as to pineal glands' role in the photoneuroendocrine system (Oksche and Hartwig 1979; Macchi and Bruce 2004). Although most mammals have lost the pineal gland direct connection to light responsiveness, studying fossorial or semi-fossorial mammals living in reduced light allows us to see how the pineal gland is integrated and responds to light. In the present study, we found two populations of pinealocytes (light and dark) similar to moles and hedgehogs. The pinealocytes of population I were most abundantly observed. Synaptic ribbons were found throughout each pinealocyte and observed often in different configurations. The shrew in the present manuscript was similar to the ultrastructure of hedgehogs and moles (Hedgehog, *Erinaceus europaeus*; Pévet and Saboureau 1974; Pévet 1975; Pévet 1976; Old World mole, Talpa europea; Pévet 1974; Pévet 1976; the golden mole,

Amblysomus hottentotus; Pévet and Kuyper 1978; Japanese moles, *Mogera kobeae*, and *Mogera wogura*; Kikuchi et al. 1984). Future studies should study other animals of Insectivora which live in different lighted conditions.

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Compliance with ethical standards

The present study and procedures were approved by the animal research ethics committees of the City University of Hong Kong, Hong Kong SAR, China.

Conflict of interest The authors declare that they have no conflict of interest.

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