1984

A Comparative Morphometric and Biochemical Study of the Superficial and Deep Pineal Gland of the Golden Hamster

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A COMPARATIVE MORPHOMETRIC AND BIOCHEMICAL STUDY OF THE SUPERFICIAL AND DEEP PINEAL GLAND OF THE GOLDEN HAMSTER

by

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A Dissertation Submitted to the Faculty of the Graduate School of Loyola University of Chicago in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy

August

1984

ACKNOWLEDGEMENTS

I would first and foremost like to thank my advisor, John McNulty, for his unstinting help and guidance during this project. His enthusiasm and interest for this work was unfailing, even when my own was flagging. In his own research, he has shown an integrity and quality that has provided me with a model upon which to pattern my own endeavors. Most importantly, perhaps, he has permitted me to develop independence, the ultimate goal of graduate education. I thank him.

I would also like to thank my committee members for their participation. They have generously given of their time and expertise in order to insure the scientific validity of my work. I am particularly appreciative to Dr. Sturtevant for her encouragement and support and for her assistance in several grammatically difficult situations. I would also like to thank Dr. Neafsey for the use of his computer and for his technical advice.

Several people in the department also must be singled out for thanks. The Drs. O'Morchoe and Dr. Zitzlsperger have always been willing to listen and lend support when necessary. Linda Fox and Dwan Taylor have been most generous with their technical advice. I would also like to thank Dr. Castro for the use of his HPLC and lab facilities.

Finally, I would like to thank my family. My parents have been uncritically supportive during my stay in graduate school. Without their prayers and encouragement, this dissertation would not have been possible. My husband, George, in particular, has given both practical advice and technical assistance. He was always there when I needed him, and I thank him.

VITA

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PUBLICATIONS:

McNulty, J. A., T. A. Dombrowski, W. A. Spurrier 1980 A Seasonal Study of Pinealocytes in the 13-lined Ground Squirrel, Spermophilus tridecemlineatus., Reprod. Nutri. Dev. 20(3A): 665-672.

McNulty, J. A., T. A. Dombrowski 1980 Ultrastructural Evidence of Seasonal Changes in Pinealocytes of the 13-lined Ground Squirrel, Spermophilus tridecemlineatus: A Qualitative and Quantitative Study. Anat. Rec., 196: 387-400.

Dombrowski, T. A. and J. A. McNulty 1984 Morphometric Analysis of the Pineal Complex of the Golden Hamster over a 24-hour Light:Dark Cycle. I. The Superficial Pineal in Untreated and Optically Enucleated Animals. Am. J. Anat., in press.

Dombrowski, T. A. and J. A. McNulty 1984 Morphometric Analysis of the Pineal Complex of the Golden Hamster over a 24-hour Light:Dark Cycle. II. The Deep Pineal in Untreated and Optically Enucleated Animals. Am. J. Anat., in press.

ABSTRACTS:

Dombrowski, T. A., W. A. Spurrier, J. A. McNulty 1979 Seasonal Changes in the Fine Structure of Pinealocytes of the 13-lined Ground Squirrel. Anat. Rec., 193: 525.

Dombrowski, T. A., J. A. McNulty 1981 Specialization of the Endoplasmic Reticulum in the Deep Pineal of the Hamster: A Circadian Study in Normal and Blinded Animals. Anat. Rec., 199: 71A.

Dombrowski, T. A., J. A. McNulty 1982 A Morphometric Analysis of the Superficial and Deep Pineals of the Golden Hamster over a 24-hour Period. Anat. Rec., 202: 47A.

McNulty, J. A., M. M. Prechel, T. K. Audhya, D. Taylor, L. Fox, T. A. Dombrowski, W. H. Simmons 1984 Ultrastructural Correlates to the Summer Rise in Pineal Arginine Vasotocin Activity. A Morphometric Analysis of Pinealocytes in Male Rats. Society for Neurosciences.

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Chapter I

INTRODUCTION

The pineal gland is an endocrine organ located within the cranium. Because nervous pathways exist which send information on the photic environment to the gland, the metabolism of the pineal is related to the phasing of the light:dark cycle, with the secretion of at least one class of pineal compounds, the indoles, showing distinct circadian rhythmicity. The methoxyindole melatonin reaches maximal levels during the dark phase, while its precursor serotonin increases during the light period. Many of the enzymes associated with indole synthetic pathways exhibit 24-hour variation as well.

Exposure of a golden hamster to a period of prolonged darkness, or alternatively, optic enucleation, results in the stimulation of pineal gland function. In hamsters, this stimulation results in atrophy of the reproductive organs. Removal of the pineal gland reverses the gonadal atrophy.

The pineal gland in the golden hamster consists of superficial and deep glands which are connected by a stalk carrying blood vessels and nerves between the two parts. The two glands share a common embryological anlage, but it is not known whether the structure and function of the superficial and deep pineals diverge during postnatal

development, producing two functionally distinct populations of pinealocytes.

The purpose of this project was the analysis of the morphology of the superficial and deep pineals under different conditions in order to determine whether pinealocytes of the two glands represent the same cell population or are distinct from one another. The approach to this problem was divided into four steps:

1) The first stage was a comparison of the structure of the superficial and deep pineal glands at the light and electron microscopic levels. The cellular components of the gland, as well as the size and structure of the pinealocytes, were quantified. These data were used to define structural modifications reflecting possible functional differences between the two pineal regions.

2) A second experiment analyzed changes in the structure of the superficial and deep pineal glands over a 24-hour light:dark (14h:10h) cycle. By comparing the pattern of morphological variation in the two glands over a 24-hour period, we hoped to achieve a better understanding of the functional relationships of these two structures.

3) The third experiment determined structural alterations in the superficial and deep pineals following optic enucleation. This protocol permitted evaluation of the response of the two glands under conditions known to enhance the metabolic activities of both superficial and deep pineal glands. Optic enucleation was used to stimulate pineal function in preference to constant darkness for practical reasons relating to animal care.

4) Finally, melatonin and serotonin levels in the two portions of the pineal complex were analyzed over a 24-hour light:dark cycle (14h:10h) using High Performance Liquid Chromatography (HPLC). The purpose of this experiment was to determine whether morphological changes in pinealocytes correlated to daily rhythms in indole production in both the superficial and deep pineal glands.

Chapter II

REVIEW OF LITERATURE

"I believe that this gland resembling a pine cone (pineal body) and filling up the bifurcation of the large vein [v. cerebri magni (Galeni)] . . . was formed for the same usefulness as other glands that support veins as they divide."

Galen, On the Usefulness of the Parts of the Body, Book I, Section 489. c. 200 A.D.

"The reason that persuades me that the soul cannot have any other place in the whole body than this gland, where it immediately exercises its functions, is that I consider the other parts of our body are all double so that we have two eyes, two hands, two ears and, finally all the organs of our external senses are double, and that inasmuch as we have only one solitary and simple thought of one single thing during the same moment, it must necessarily be that there is some place where the two images which come from the two eyes or two other impressions which come from a single object by way of the double organs of the other senses, may unite before they reach the soul, so they do not present two objects instead of one. It can easily be conceived how these images or impressions could unite in this (pineal) gland through the mediation of the spirits that fill the cavities of the brain. There is no other place in the body where they could thus be united unless it be in this gland."

Rene Descartes, Les Passions de l'Ame, Part I, Art. 32. (from Human Brain and Spinal Cord), 1640.

"Much of the evidence reviewed suggests the function of the pineal gland is related to that of the gonads."

Kitay and Atschule, The Pineal Gland, 1954.

> "As currently envisioned, the pineal gland serves as an intermediary between the external environment, especially the photoperiod, and the organism as a whole. In effect, the pineal keeps the organism in proper synchrony with the prevailing environmental conditions."
>
> Russel J. Reiter, 1981a.

As the above quotations demonstrate, attempts at deciphering the function of the pineal gland span many centuries and encompass many ideas. Beginning with its discovery by the ancient Greeks, the pineal has been assigned roles ranging from the useless to the sublime. Research beginning in the 1950's, termed the third period of pineal research by Ariens Kappers (1979), has rapidly advanced the concept of the pineal gland as an endocrine organ, although many questions regarding pineal function have yet to be answered. In recent years, a vast amount of information has become available regarding the pineal gland in vertebrates. This review will be concerned only with that body of literature referring specifically to mammals.

GROSS ANATOMY

The first description of the gross structure of the pineal gland was recorded by Galen. He suggested the term 'pineal' because of the resemblance of the human pineal gland to a small pine cone. Since that time, detailed descriptions of gross pineal gland morphology have been published in a number of species. Among mammals, the pineal gland is conspicuous in its structural diversity. The gland is always located within the cranium, although its location can vary from a site adjacent to the third ventricle to a position on the undersurface of the cranium.

The pineal can exist as a discrete mass of glandular tissue, or it may be divided into separate parenchymal components, termed the superficial and deep pineal glands (Sheridan and Reiter, 1970a). A pineal 'complex', characterized by superficial and deep pineal glands, is found principally within rodent species, having been reported in the rat (Vollrath and Boeckman, 1978), Mongolian gerbil (Japha et al., 1974), brush mouse (Karasek et al., 1983a), white-footed mouse (Quay, 1956) and golden (Sheridan and Reiter, 1970a) and Chinese (Matushima and Morisawa, 1982) hamsters. In these species, the pineal complex consists of a superficial glandular portion located beneath the confluens of sinuses, a deep parenchymal mass lying between the habenular and posterior commissures in the third ventricle and a stalk of variable consistency connecting the two parts. The pineal complex in these species is generally classified as $\alpha\beta$C or αC, according to the system devised by Vollrath (1979). In this system, A or α represents pineal tissue adjacent to the third ventricle (deep pineal), C or γ designates a more superficially located glandular mass (superficial pineal), and B or β represents tissue in a position intermediate to A and C. Use of a Greek letter indicates a portion of the pineal complex that is greatly reduced in size. In the species mentioned above, the deep pineal gland is considerably smaller than the superficial gland. In rats, it comprises 1-2.5% of the pineal parenchymal tissue (Vollrath and Boeckman, 1978), while in the hamster, it is estimated at 3-12% (see Reiter, 1981a). In the sections that follow, the information given will pertain to pineal glands that present as a discrete glandular mass, or to the superficial

portion of the pineal complex. Special features relating specifically to deep pineal gland structure and function will be covered under the heading DEEP PINEAL.

FUNCTION

In general, the pineal gland acts as a mediator between the photic environment and the metabolism of the individual, particularly as it relates to reproduction. Reduced photoperiod, or blinding, causes atrophy of the reproductive organs in lower mammals (Reiter, 1969; Hoffman and Reiter, 1965), presumably due to stimulated production of a pineal antigonadotropic hormone. Gonadal atrophy spontaneously regresses after about 30 weeks, and the gonads remain refractory to pineal influence for another 22 weeks (Reiter, 1972). Pinealectomy prevents darkness-induced gonadal atrophy (Reiter, 1972). Because of the inverse relationship between reproductive status and the activity of the pineal, the gland has been implicated in seasonal reproductive rhythms (Reiter, 1974). In humans, the pineal gland has been postulated to control the timing of the onset of puberty (Silman et al., 1979)

INNERVATION

Pineal metabolism is at least partially controlled by the photic environment via information conveyed in sympathetic nerve fibers (Ueck, 1979; Ariens Kappers, 1965). Nerve fibers enter the pineal gland either on the surface of blood vessels, or as the nerve conarii, paired nerves that enter the gland at its dorsocaudal tip (Ueck, 1979; Ariens Kappers, 1960). The fibers are postganglionic sympathetic axons of cell bodies located in the superior cervical ganglion.

The pathway connecting the pineal gland to the external environment begins in the ganglion cell layer of the retina. Efferent fibers course via the retinohypothalamic tract to the suprachiasmatic nucleus, independent of other primary and accessory optic pathways. The suprachiasmatic nucleus acts as a circadian oscillator, although rhythms phased by that nucleus are entrained to the photic environment by the retinohypothalamic tract. From the suprachiasmatic nucleus, efferents project to the periventricular nucleus which in turn connects with the intermediolateral cell column of the thoracic cord via the medial forebrain bundle and the midbrain reticular formation. Preganglionic sympathetic fibers from the thoracic cord synapse in the superior cervical ganglion, sending postganglionic fibers to the pineal gland via the nerve conarii and on blood vessels. Photic stimulation results in activation of the pineal post-ganglionic fibers which stimulate pineal cells at the molecular level (Klein, 1979; Ueck, 1979, Moore, 1978; Moore and Klein, 1974).

The noradrenergic neurotransmitter released from nerve terminals within the pineal gland couples with beta adrenergic receptors on the surface of the pinealocyte cell membrane. Subsequent depolarization of the cell membrane results in increased adenylate cyclase activity which in turn stimulates the production of cyclic AMP. Increased cyclic AMP levels initiate the production of an enzyme, N-acetyltransferase (NAT) (Romero, 1978). NAT is a key enzyme in the synthesis of one class of pineal compounds, the indoleamines.

The pineal gland of the rat exhibits a rhythm in its content of the sympathetic neurotransmitter, noradrenaline, with the trough at the end of the dark period (Morgan et al., 1976). The hamster shows no change in noradrenaline content over a 24-hour period (Morgan et al., 1976), but does demonstrate a rhythm in neurotransmitter synthesis with maximal activity occurring during the dark period (Craft et al., 1984). Biochemical analysis of circadian changes in noradrenaline content and synthesis correlate well with 24-hour variation in the numbers of large granulated vesicles found in noradrenergic nerve terminals in the rat (Matsushima et al., 1979a).

Parasympathetic innervation as well has been found in the rabbit pineal gland (Romijn, 1973a, 1973b; see Ariens Kappers, 1965). Preganglionic fibers travel through the superior cervical ganglion and enter the pineal gland via the nerves conarii. These fibers synapse with infrapineal parasympathetic neurons to produce postganglionic axons that ramify throughout the pineal parenchyma. Romijn (1973a) suggested that stimulation of parasympathetic fibers may induce the depletion of serotonin stored within the pinealocyte.

The existence of a direct nervous connection between the brain and pineal gland has been a subject of considerable debate. Ariens Kappers (1965) reported nerve fibers within the rat pineal gland that arose from the habenular and posterior commissures, forming the habenulo-caudo-commissural epiphyseal system. He did not believe that these fibers played a role in pineal innervation but rather that they were aberrant fibers that entered and left the pineal without interaction. More

recent papers have, however, challenged this concept. Histochemical studies have detected cholinergic nerve fibers in the pineal stalk of the rat (Guerillot et al., 1979), while retrograde tracer studies have shown nerve fibers projecting from the habenular commissure to the pineal gland in the rat and the guinea pig (Moller and Korf, 1983a,; Semm et al., 1981; Scheider et al., 1981; Dafny, 1980). Fibers arising from the habenular nuclei have been suggested to alter pineal activity (Semm et al., 1981). The paraventricular nucleus of the hypothalamus also projects to the pineal organ, although the function of these fibers is not known (Korf and Wagner, 1980.)

BIOCHEMISTRY

The presumptive hormone of the pineal gland is the methoxyindole, melatonin. Rhythms in the production of this compound are under photic regulation, with peak values occurring during the dark period. Synthesis of melatonin begins with the amino acid, tryptophan. Tryptophan is taken up by the pineal gland against a concentration gradient, with a rise in glandular levels from midlight to early dark. Pineal tryptophan levels decline during the scotophase (Sugden, 1979). Tryptophan is hydroxylated to 5-hydroxytryptophan by the enzyme tryptophan hydroxylase. The enzyme displays a circadian rhythm in its activity in the rat, with peak values occurring three hours after the onset of darkness and continuing until the photophase (Shibuya et al., 1978). 5-Hydroxytryptophan is subsequently decarboxylated to 5-hydroxytryptamine (5HT or serotonin) by the enzyme L-amino acid decarboxylase (see Deguchi, 1979). Serotonin levels in the rat are

highest during the light period in the rat (Quay, 1963a) and the hamster (Steinlechner et al., 1983). Serotonin is N-acetylated to N-acetylserotonin by serotonin N-acetyltransferase (NAT), with acetyl co-enzyme A acting as the donor of the acetyl group. Acetylation of serotonin occurs during the dark phase (Deguchi, 1979). NAT in the rat shows a circadian rhythm in its activity with maximal levels found during the first three hours of darkness (Klein and Weller, 1970). NAT formation is controlled by a central rhythm synchronizer, believed to be the suprachiasmatic nucleus. Photic input conveyed in postganglionic sympathetic fibers determines the phasing of the rhythm of the enzyme (Moore and Klein, 1974). The nocturnal rise in NAT occurs as a result of norepinephrine stimulation (Binkley, 1976), acting via the cyclic AMP-adenylate cyclase mechanism (Mikuni et al., 1981; Shein and Wurtman, 1969). The enzyme levels decline prior to the light period (Binkley, 1976). The hamster shows a similar rhythm in NAT activity, although the amplitude of change in the levels of NAT at the onset of darkness are not as great as those seen in the rat (Rudeen et al., 1975). NAT levels are elevated at night in both diurnal and nocturnal species (Stephens and Binkley, 1978).

N-acetylserotonin is O-methylated to melatonin by the enzyme hydroxyindole-O-methyltransferase (HIOMT), with the methyl group derived from adenosylmethionine. The enzyme levels are believed to be under the sole influence of the superior cervical ganglion (Sugden and Klein, 1983). The rhythm in HIOMT shows two peaks, one at midday, and the other occurring about four hours into the dark phase (Balemans et al.,

1979). The rhythm of melatonin concentration exhibits maximal values during the dark phase (Yellon et al., 1982; Young and Anderson, 1982; Meyer er al., 1981; Reiter et al., 1981; Rollag et al., 1980; Arendt et al., 1977; Hedlund et al., 1977) and a decline prior to the onset of the light phase. The synthesis of melatonin from tryptophan takes approximately 36 hours, encompassing two dark phases and the intervening light phase (Balemans, 1979).

Other pineal products have been advanced as the definitive pineal hormone. Serotonin metabolism leads to the formation of other indole compounds in addition to melatonin, including 5-methoxy-tryptophan, 5-methoxytryptamine, 5-hydroxytryptophol, 5-methoxytryptophol, 5-hydroxyindoleacetic acid and 5-methoxyindoleacetic acid. The production of these compounds displays 24-hour variation. 5-Methoxytryptophol shows maximal values during the dark period (Carter et al., 1979), while 5-hydroxytryptophol and 5-hydroxyindoleacetic acid reach highest levels during the light period. The methoxyindoles, 5-methoxytryptamine and 5-methoxytryptophan, show marked effects upon the reproductive axis in several species (Pevet, 1983).

Peptides have also been isolated from the pineal gland. The majority of the peptides secreted have not yet been identified, although one of the peptides, arginine vasotocin, has been proposed to be a pineal hormone. Arginine vasotocin (AVT) is a diuretic hormone active in lower orders of vertebrates. It is superceded in modern mammals by oxytocin and vasopressin, although AVT has been detected in the pineals of the cow and rat (Fernstrom et al., 1980). AVT has been found in high

concentrations in the pineal stalk, suggestive of a possible ependymal origin of the peptide (Pavel, 1971). Some investigators feel that AVT itself is not present within the pineal, but rather that a peptide similar to AVT, with the same tripeptide-carboxyl terminal, is detected by the bioassay (Pevet et al., 1981; Dogertom et al., 1980; Benson and Ebels, 1978).

The amino acid, taurine, is synthesized by the pineal glands of rats (Ebels et al., 1980), and is found in a high concentration within the gland. Taurine is believed to stimulate NAT activity via beta adrenergic receptors (Wheler et al., 1979).

VASCULAR SUPPLY

The arteries to the pineal gland arise as branches of the posterior choroidal arteries, usually two to six per gland. The arteries divide into smaller vessels and enter the pineal gland via connective tissue trabeculae. Venous drainage occurs primarily into the great cerebral veins (see Reiter, 1981a).

HISTOLOGY

Histologically, the subarachnoidal surface of the pineal gland is covered with a thin capsule derived from the pia mater (Salisbury et al., 1981; Sheridan and Reiter, 1973; Das Gupta, 1968). The meningeal capsule consists of several layers of flattened cells (Krstic, 1979), resting on a basal lamina (Salisbury et al., 1981). Collagen fibrils, glial elements, blood vessels and nerve axons are also present in the capsule (Karasek et al., 1983a, 1982c; Sheridan and Reiter, 1973). The capsule invaginates, forming connective tissue septa that carry blood

vessels and nerves into the substance of the gland (Karasek et al., 1983a; Salisbury et al., 1981; Anderson, 1965). These septa produce various degrees of lobulation within the pineal. Complete lobules are seen in the pineals of man (Scharenberg, 1965) and kangaroos (Quay, 1966), while less distinct lobulation is found in hamsters (Das Gupta, 1968), cows and sheep (Anderson, 1965), white-footed mice (Samarasinghe et al., 1983) and orangutans (Quay, 1970).

The principal cell types of the pineal gland are the parenchymal cell proper, variously called the chief cell (Arstila, 1967), epiphyseal cell (Wolfe, 1965), pineocyte or pinealocyte, and the glial or interstitial cell. The pinealocyte is the most abundant cell type and demonstrates various degrees of organization. Rosettes, follicles and tubules are distinctly present in some species. More commonly, however, the cells are randomly arranged in clumps, cords or pseudofollicles (Quay, 1965).

PINEALOCYTE

Pinealocytes are irregularly shaped cells that send multiple processes into the surrounding parenchyma. The length of the pinealocyte process has rarely been determined, although it is known that those within the ground squirrel are five to 19 μm in length (Povilshock et al., 1975), while those in the Nutria are ten to 15 μm (Salisbury et al., 1981). The diameter is usually several μm (Povilshock et al., 1975; Sheridan and Reiter, 1973). Glycogen, mitochondria, microtubules, smooth endoplasmic reticulum, and clear and dense-cored vesicles can be found in the cell processes (Salisbury et al., 1981;

Karasek and Wyrzykowski, 1980; Matsushima et al., 1979b; Welsh and Reiter, 1978; Povilshock et al., 1975; Pevet, 1974; Sheridan and Reiter, 1973; Romijn, 1973c; Anderson, 1965). The processes generally terminate in club-shaped or bulbous endings, although those in the dog are described as pointed (Welser et al., 1968) and those in the Nutria irregularly shaped (Salisbury et al., 1981). The terminals end within the perivascular space, in the intercellular space, or in association with glial cells (Matsushima and Morisawa, 1982; Welsh and Reiter, 1978; Anderson, 1965).

The club shaped terminals principally contain mitochondria and membrane-bounded vesicles, with the clear form predominating. Lipid, synaptic ribbons and endoplasmic reticulum, particularly in the form of lamellar bodies, have also been noted (Karasek et al., 1983a; Samarasinghe et al., 1983; Matsushima and Morisawa, 1982; Karasek and Wyrzykowski, 1980; Povilshock et al., 1975; Pevet, 1974; Romijn, 1973c; Wartenberg, 1968; Arstila, 1967; Anderson, 1965).

In some mammalian species, the pinealocytes are unipolar, with only one process (Nutria, Salisbury et al., 1981; ground squirrel, Povilshock et al., 1975; Weddell seal, Cuello, 1973; cat, Wartenberg, 1968; dog, Welser et al., 1968; rat, Wolfe, 1965). The single 'polar' (Welser et al., 1968; Wolfe, 1965) process is referred to as dendritic or dendrite-like (Wartenberg, 1968). It is usually one to ten microns in diameter with a tapered necklike region referred to as the collicular segment (Povilshock et al., 1975; Wartenberg, 1968). The process frequently enters into the surrounding tissue at sharp angles before

ending in a clublike polar terminal (Wolfe, 1965). Cells exhibiting a single process have polarization of their cytoplasm, with the majority of the organelles located between the nucleus and the collicular region (Salisbury et al., 1981; Wartenberg, 1968; Welser et al., 1968).

The pinealocyte contains a single nucleus and a variety of cellular organelles. These features will be discussed in detail below.

Nucleus

The nucleus of the pinealocyte is usually centrally located, although pineal cells in the dog (Welser et al., 1968), Nutria (Salisbury et al., 1981) and orangutan (Quay, 1970) display eccentrically located nuclei. The nucleus is generally described as round or oval in outline, with irregular indentations in the form of invaginations or lobulations. These irregularities increase as the animal ages (Johnson, 1980). Nuclei are estimated to be six to nine μm in diameter (Wartenberg, 1968), with a volume of 250 to 400 μm^3 (Matushima et al., 1983; Boeckman, 1980; Welsh et al., 1979).

The pinealocyte nucleus remains in interphase (Bucana et al., 1974; Arstila, 1967), with evenly dispersed euchromatin in the nuclear matrix. When aggregates of heterochromatin are present, they tend to be located peripherally, adjacent to the nuclear envelope. The double membrane of the nuclear envelope contains numerous pores (Pevet, 1974; Sheridan and Reiter, 1973, 1968; Arstila, 1967; Anderson, 1965; Wolfe, 1965), as well as areas of membrane fusion, referred to as kary-occludentes (Anderson, 1965). The inner nuclear membrane has a smooth surface, while the outer membrane may display crinkling (Welsh

and Reiter, 1978), or blebbing (Anderson, 1965). The outer membrane may also be studded with ribonucleoprotein particles so that it resembles granular endoplasmic reticulum (Welser et al., 1968; Anderson, 1965).

Various types of nuclear inclusions may be found within the pinealocyte nucleus. The nuclei of the Chinese hamster (Matsushima and Morisawa, 1982) and aging rat (Johnson, 1980) contain filamentous inclusions consisting of numerous eight to ten nm filaments in parallel array. The function of the filaments is not known, although they may be indicative of cell aging. Other nuclear inclusions (Kernkugeln) are believed to be artifacts of fixation (Wolfe, 1965) or sectioning (Reyes, 1982).

Nucleoli

Pinealocyte nuclei are reported to possess a single, prominent nucleolus. Nucleolar volume in the rat is 1.9-2.7 μm^3 and it is composed of four major components: 1) a pars fibrosa comprising 50-65%, 2) a pars granulosa (35-45%), 3) nucleolar vacuoles (2-5%), light areas interspersed between the pars fibrosa and pars granulosa, and 4) nucleolar organizer regions. Nucleoli are attached to the nuclear membrane or folds of that membrane (Lew et al., 1982). These organelles are intimately related to metabolic activity within the pinealocyte.

Cytoplasm

The cytoplasm of pineal cells is electron lucent, and contains a varied array of organelles (Povilshock et al., 1975; Arstila, 1967; Wartenberg and Gusek, 1965). Various organelles commonly found in the pinealocyte cytoplasm will be described below.

Mitochondria

Mitochondria characteristically display great variability in their structure. Mitochondria are described as elongate, spherical or ovoid in shape, although branched (Karasek et al., 1983a, 1982c; Povilshock et al., 1975), H- and Y-shaped (Samarasinghe et al., 1983) and ring- or cup-shaped (Karasek et al., 1983a; Matsushima and Morisawa, 1982) forms have also been reported. Giant mitochondria measuring up to five um in diameter have also been found in some species (tree shrew, Hwang, 1982; pig, Karasek and Wyrzykowski, 1980; hamster, Bucana et al., 1974; Sheridan and Reiter, 1968; dog, Welser et al., 1968; rat, Quay, 1965; Wolfe, 1965).

Mitochondria are bounded by double unit membranes (Sheridan and Reiter, 1973), although both membranes are not always clearly distinguishable. Continuity has been speculated between the outer mitochondrial membrane and the membrane bounding lipid droplets in the rat (Wolfe, 1965). Mitochondrial membranes in the hamster are in continuity with double-membraned structures of unknown origin and significance, which may be derived from mitochondria (Clabough, 1971).

The arrangement of cristae within the mitochondria exhibits diverse structural forms as well. Cristae show longitudinal (Wolfe, 1965), transverse (Anderson, 1965; Wolfe, 1965), angular (Anderson, 1965) and random (Samarasinghe et al., 1983) orientations. Mitochondria have also been described that display cristae in the form of concentric arrays (Karasek et al., 1982c; Bucana et al., 1974), tubular vesicles (Clabough, 1971; Sheridan and Reiter, 1968; Arstila, 1967) and lamella

(Matsushima and Morisawa, 1982). The mitochondrial matrix is variously described as dense (Matsushima et al., 1979b) or lucid (Wartenberg, 1968; Anderson, 1965).

Numerous mitochondria are distributed throughout the cell, including the cell processes. They may be concentrated around specific cellular structures, including the nucleus and centrioles (Wartenberg, 1968; Wartenberg and Gusek, 1965), endoplasmic reticulum (Welser et al., 1968; Wolfe, 1965), lipid (Wolfe, 1965; Anderson, 1965) and pigment bodies (Anderson, 1965).

Enigmatic inclusions have been found within the mitochondria. In the rat, these inclusions take the form of highly ordered crystalline arrays (Heidbuchel, 1982; Wolfe, 1965) and microcylinders (Lin, 1965). Mitochondria of the Pipistrelle bat contain bundles of microfilaments (Pevet and Racey, 1981).

Golgi

The Golgi apparatus has a perinuclear orientation. The organelle is usually described as a single structure, although several investigators specifically report multiple Golgi bodies (Pevet and Racey, 1981; Pevet and Yadav, 1980; Pevet and Kuyper, 1978; Pevet et al., 1977a, 1976; Bucana et al., 1974; Sheridan and Reiter, 1973). The difference may be one of terminology, however, since some authors, most correctly in all probability, refer to a single Golgi complex, but multiple Golgi bodies.

The Golgi complex is composed of stacked saccules, or cisternae, surrounded by numerous vesicles. Two types of vesicles are most commonly described, one being a clear membrane-bounded vesicle ranging in size from 30-120 nm in diameter (Samarasinghe et al., 1983; Karasek et al., 1983a, 1982c; Matsushima et al., 1979b; Welsh and Reiter, 1978; Romijn, 1973c), while the other is a dense-cored vesicle 40-350 nm in diameter (Karasek et al., 1983a, 1982c; Pevet and Racey, 1981; Karasek and Wyrzykowski, 1980; Pevet and Yadav, 1980; Matsushima et al., 1979b; Welsh and Reiter, 1978; Romijn, 1973c). Both forms of vesicles are presumed to be formed in the Golgi complex and derived from or destined to the plasma membrane.

A third type of vesicle, the acanthosome or spiny vesicle, is found in close proximity to the Golgi complex in the rat (Wolfe, 1965) and rabbit (Romijn, 1973c). These 'coated' vesicles display spines which project into the cytoplasm perpendicular to the outer surface of their bounding membrane. They are 40-100 nm in diameter and are also believed to originate in the Golgi complex. Romijn (1973c) speculated that acanthosomes may be precursors of dense-cored vesicles, as intermediate forms are seen.

The extensive Golgi complex of the mammalian pinealocyte, with its associated vesicles, is indicative of intense secretory activity by the cell (Karasek et al., 1982c; Welsh and Reiter, 1978).

Lipid

Lipid droplets are infrequently encountered within pinealocytes in most species. Cells of the rat (Arstila, 1967; Wolfe, 1965), hamster (Sheridan and Reiter, 1968), human (Quay, 1957), mole-rat (Pevet et al., 1976), golden mole (Pevet and Kuyper, 1978) and ground squirrel (Povilshock et al., 1975) pineals do, however, display numerous lipid droplets. The droplets range in size from one half to four μm in diameter (Povilshock et al., 1975; Cuello, 1973; Wolfe, 1965), although they may increase in size with age (Johnson, 1980). They may be spherical, or be irregularly shaped with scalloped edges (Wolfe, 1965; Sheridan and Reiter, 1968). Lipid has been reported to be unbounded (Arstila, 1967) or, alternately, bounded by a single unit membrane (Povilshock et al., 1975). Working with the rat pineal, Wolfe (1965) stated that he was not able to determine whether the droplets were actually unbounded, or whether it was simply not possible to visualize the limiting membrane.

Contents of the lipid droplets may appear homogeneously pale, or with electron dense peripheries and lucent centers (Arstila, 1967) or vice versa (Povilshock et al., 1975). The contents of the droplets have been histochemically determined to be accumulations of triglycerides and phospholipids (Prop, 1965). Lipid droplets have been speculated to arise from mitochondria (Gusek and Santoro, 1960) and endoplasmic reticulum (Wolfe, 1965). Within the pinealocyte cytoplasm, they may be found in close association to glycogen and vacuoles (Pevet and Kuyper,

1978), mitochondria and granular endoplasmic reticulum (Arstila, 1967) or capillaries (Quay, 1965).

Lipid droplets have been linked to pineal metabolic activity. Zweens (1963) found that rat pineal lipid content varied with the stage of the estrous cycle, and concluded that pineal lipid content is influenced by circulating gonadotropins. De Martino et al. (1963) speculated that lipid droplets served as storage sites for pineal secretory products.

Dense Bodies

Lysosome-like dense bodies are characteristically found within mammalian pinealocytes. These structures are variously called dense (Pevet et al., 1976), grumose, or vermiculate bodies (Wolfe, 1965). The matrix of the dense bodies is finely granular with moderate electron density. Variations in the substance of the matrix appear as flocculent material or lamellar-like inclusions (Karasek and Wyrzykowski, 1980; Wartenberg, 1968). Dense bodies are limited by a single unit membrane (Matsushima and Morisawa, 1982; Pevet el al., 1977a, 1976; Pevet, 1974; Sheridan and Reiter, 1973; Welser et al., 1968; Anderson, 1965), and exhibit shapes that range from spherical to elongate. Their size varies from 0.1 μm to 1.6 μm in diameter (Karasek and Wyrzykowski, 1980; Sheridan and Reiter, 1968). The number of dense bodies increases in the aging rat (Johnson, 1980) but not in the sheep or cow (Anderson, 1965).

The function of the dense bodies is apparently lysosomal. Phosphatase analysis of dense body contents has determined that some of their population are primary lysosomes. Secondary lysosomes can also be detected on the basis of partially degraded material contained within the membrane of the dense body (Gomez-Dumm and Iturriza, 1979). Perrelet et al. (1968) postulated that action of the dense bodies aids in the release of indoleamine products from their storage site within the pinealocyte. A recent cytochemical study (Lu and Lin, 1979) demonstrated that the dense body population is heterogenous, with some dense bodies acting as lysosomes, while others serve as storage sites for pineal peptides.

Multivesicular Bodies

Multivesicular bodies (MVB) apparently function as heterolysosomes (Weiss, 1983). They are spherical bodies, bounded by a single unit membrane. Their matrix is electron lucent and contains several empty vesicles (Arstila, 1967). MVB may be found in association with the Golgi apparatus in the rat (Wolfe, 1965).

Centrioles and Microtubular Sheaves

Centrioles have been reported in the pinealocytes of virtually all species. These organelles can be associated with mitochondria (Romijn, 1973c), Golgi apparatuses (Samarasinghe et al., 1983; Hwang, 1982; Sheridan and Reiter, 1968; Wartenberg, 1968; Anderson, 1965), or cilia (Pevet and Racey, 1981; Pevet and Yadav, 1980; Pevet and Kuyper, 1978;

Pevet et al., 1977a). In several rodent species, including the rat (Lin, 1970; Wolfe, 1965), hamster (Bucana et al., 1974), guinea pig (Lin, 1972) and gerbil (Welsh and Reiter, 1978), structures known as microtubular sheaves are present. A microtubular sheave consists of a doublet or triplet of microtubules of indeterminate length, embedded within a dense matrix. Seven to ten microtubular sheaves frequently combine to form arrays of microtubules which are known as microtubular bouquets (Wolfe, 1965). Microtubular sheaves and bouquets are often found in close proximity to the Golgi complex in an area comparable to the centrosphere (Welsh and Reiter, 1978; Bucana et al., 1974). In the rat, Lin (1970) theorized that microtubular sheaves are derivatives of centrioles. Rat pinealocytes contain centrioles up to the 15th day postnatally, after which time centrioles are believed to transform into microtubular sheaves. Loss of the centrioles has been associated with the inability of rat pinealocytes to divide, so that few mitoses are evident in the pineal gland of the adult animal (Heidbuchel and Vollrath, 1983). Microtubular sheaves have also been implicated in the formation of another enigmatic structure, the synaptic ribbon (Karasek, 1976), although Welsh and Reiter (1978) found no evidence of proximity between the two organelles. They speculated, rather, that microtubular sheaves may play a role in microtubule formation.

Cilia

Cilia are commonly present in the pinealocytes of several species, including the mole rat (Pevet et al., 1976), mole (Pevet, 1974), noctule

bat (Pevet et al., 1977a), dog (Welser et al., 1968), golden mole (Pevet and Kuyper, 1978), Pipistrelle bat (Pevet and Racey, 1981) and opossum (McNulty and Hazlett, 1980). Cilia show a 9+0 microtubular arrangement, excepting those found in the dog, which exhibit a 9+2 internal structure. The 9+0 arrangement is typically found in sensory cilia, and may reflect the photoreceptor origins of the pinealocyte (Pevet et al., 1977b). Cilia are frequently associated at their base with a centriole acting as a basal body. Pinealocytes of the adult rat display few cilia, reflecting their lack of centrioles (Lin, 1970).

Endoplasmic Reticulum

Both granular (rough) and agranular (smooth) forms of the endoplasmic reticulum are evident in the cytoplasm of the pinealocyte. In some species, the granular form is predominant (tree shrew, Hwang, 1982; Eastern chipmunk, Karasek et al., 1982c; Pipistrelle bat, Pevet and Racey, 1981; Malaysian rat, Pevet and Yadav, 1980; noctule bat, Pevet et al., 1977a; mole rat, Pevet et al., 1976; mole, Pevet, 1974), while in other species the agranular form is more prevalent (Nutria, Salisbury et al., 1981; cotton rat, Matsushima et al., 1979b; gerbil, Welsh and Reiter, 1978; hamster, Sheridan and Reiter, 1968; cow and sheep, Anderson, 1965; rat, Quay, 1965). Pinealocytes of the pig (Karasek and Wyrzykowski, 1980), opossum (McNulty and Hazlett, 1980) and ground squirrel (Povilshock et al., 1975) contain only scarce amounts of either form of endoplasmic reticulum.

Cisternae of the rough endoplasmic reticulum (RER) are studded on their cytoplasmic surface with 15 nm ribonucleoprotein particles (Sheridan and Reiter, 1968). The cisternae are described as dilated (Hwang, 1982; Pevet et al., 1976) or flattened (Samarasinghe et al., 1983; Sheridan and Reiter, 1968). The cisternae of the RER may form parallel stacks similar in appearance to the Nissl substance of neuronal cell bodies (Samarasinghe et al., 1983; Karasek et al., 1983a, 1982c; Wolfe, 1965).

Dilated cisternae occasionally contain a dense or flocculent material (McNulty and Hazlett, 1980; Pevet at al., 1977b). These cisternae are believed to form vacuoles that contain flocculent material (Karasek et al., 1983a, 1982c; Pevet and Kuyper, 1978). Pevet (1979) speculated that these vacuoles contain pineal secretory products, probably peptides. Formation of the vacuoles has been associated with proximity of the RER to glycogen particles and lipid droplets (Pevet and Kuyper, 1978).

Endoplasmic reticulum in the rat pineal does not precisely fit the description for either the RER or SER. Wolfe (1965) referred to this form of the organelle as a mixed or intergrade endoplasmic reticulum, with a structure intermediate between the RER and SER. In other species, the smooth and rough endoplasmic reticulum are sometimes seen to be in continuity with one another (Sheridan and Reiter, 1973; Welser et al., 1968). The SER has also been reported to be in continuity with the membranes of nuclei (Poletti and Castellano, 1967) and mitochondria (Welser et al., 1968).

Smooth endoplasmic reticulum (SER) appears as isolated tubules or flat cisternae (Matsushima et al., 1979b; Pevet et al., 1977a, 1976; Povilshock et al., 1975; Pevet, 1974; Romijn, 1973c; Sheridan and Reiter, 1973, 1968; Welser at al., 1968). SER can be found in high concentration in certain areas of the cell (Matsushima and Morisawa, 1982; Sheridan and Reiter, 1973). In the rat (Arstila, 1967), a gradual transition occurs in the endoplasmic reticulum of the cell. SER is dominant in the center, while RER is more abundant at the periphery. SER has been implicated as the site of indoleamine synthesis (Romijn et al., 1977a). It has also been postulated to be the source of the clear vesicles found within the cytoplasm (Romijn, 1973c).

Variant forms of endoplasmic reticulum have been reported. One of these structures is variously called a cisternal grille or vernate body (Wolfe, 1965), myeloid body-type organelle (Samarasinghe et al., 1983) or circular lamellar structure (Arstila, 1967). Cisternal grilles are formed by two to 20 flattened cisternae lying in parallel arrays in close proximity (eight nm) to one another (Samarasinghe et al., 1983; Matsushima and Morisawa, 1982; McNulty and Hazlett, 1980; McNeill, 1978; Pevet et al., 1977a, 1976; Pevet, 1974; Sheridan and Reiter, 1973, 1968; Arstila, 1967; Wolfe, 1965). There is no evidence of the fusion of the membranes of the cisternae, and pores are not present (Samarasinghe et al., 1983). Cisternal grilles assume a variety of shapes, including concentric (McNulty and Hazlett, 1980; McNeill, 1978; Pevet, 1974), or lenticular or lozenge shapes (Samarasinghe et al., 1983; Matsushima and Morisawa, 1982; Pevet et al., 1977a, 1976; Arstila, 1967).

Concentrically shaped configurations contain a core of cytoplasm (Samarasinghe et al., 1983; McNeill, 1978). The cisternal membranes resemble those of the SER, although they contain a finely granular material on their surface. Ribosomes may also be present (Samarasinghe et al., 1983; Matsushima and Morisawa, 1982). Membranes of the cisternal grilles may be continuous with distinct profiles of the smooth or rough endoplasmic reticulum (Samarasinghe et al., 1983; Matsushima and Morisawa, 1982; Pevet et al., 1977a). The parallel stacks of cisternae do not appear to be confined to a specific area of the cytoplasm, although Arstila (1967) found them principally in the cell processes of rat pinealocytes.

The function of the cisternal grilles is not known. McNeill (1978) postulated that they may be involved in the synthesis of pineal compounds, while Pevet et al. (1976) suggested that their presence in blind or nocturnal mammals is related to the antigonadotropic activity of the pineal.

A related structure, the annulate lamella, has been described in the rat (McNeill, 1977; Freire and Cardinali, 1975; Lin, 1967), hamster (Bucana et al., 1971) and white-footed mouse (Samarasinghe et al., 1983). Annulate lamellae consist of numerous flat cisternae arranged into stacks. Regular 55 nm pores in each lamella are precisely aligned with those of adjacent lamellae so that clear cylindrical channels are formed (McNeill, 1977; Lin, 1967). The structure has been associated with lipid droplets, endoplasmic reticulum (McNeill, 1977), nuclei (Bucana et al., 1971, Lin, 1967) and the Golgi complex (Bucana et al.,

1971). The function of these structures is not known, but they have been related to the physiological state of the cell (Bucana et al., 1971) or secretory activity (McNeill, 1977).

Another specialization of the endoplasmic reticulum is the subsurface cisterna, which lies adjacent to the plasmalemma. The intracisternal space is electron lucent (Salisbury et al., 1981; Cuello, 1973). The surfaces of the cisternae adjacent to the plasmalemma are smooth, while those facing the cytoplasm have been reported in a few instances to be studded with ribosomes (Matsushima and Morisawa, 1982; Welsh and Reiter, 1978; Pevet, 1974). The subsurface cisternae are frequently found in continuity with either the rough or smooth endoplasmic reticulum (Matsushima and Morisawa, 1982; Welser et al., 1968).

The subsurface cisternae are most frequently located opposite the plasmalemma of other pinealocytes. The cisternae can be found at the point of contact in both cells (Salisbury et al., 1981; Pevet and Yadav, 1980; Pevet et al., 1977a, 1976; Pevet, 1974; Cuello, 1973; Wolfe, 1965), forming soma-somatic appositions. Subsurface cisternae can also be found in areas where the pinealocyte soma contacts a cell process (Salisbury et al., 1981; Pevet and Yadav, 1980; Pevet et al., 1976; Wolfe, 1965), nerve ending (Hwang, 1982) or glial cell (Salisbury et al., 1981). Subsurface cisternae have been postulated to be a corridor for the exchange of metabolites and ions (Pevet, 1974) or sites of intercellular communication (Salisbury et al., 1981).

Glycogen

Glycogen particles are found in the pinealocytes of the Eastern chipmunk, (Karasek et al, 1982c), tree shrew (Hwang, 1982), Nutria (Salisbury et al., 1981), Pipistrelle bat (Pevet and Racey, 1981), golden mole (Pevet and Kuyper, 1978), mouse (Kachi et al., 1971a) and mole rat (Pevet et al., 1976). The particles lie singly or in clusters, and their numbers vary from cell to cell. In the Pipistrelle bat, golden mole, Eastern chipmunk and brush mouse, clusters of glycogen particles are often associated with clear vacuoles. These vacuoles are believed to arise from the RER and to represent sites of the synthesis and/or maturation of secretory products (Pevet and Kuyper, 1978).

Microtubules and Microfilaments

Microtubules are tubular elements of indefinite length measuring 20 to 30 nm in diameter. They appear as two slender lines in longitudinal section (Theron et al., 1979; Sheridan and Reiter, 1973, 1968; Cuello, 1973, Welser et al., 1968; Arstila, 1967; Anderson, 1965; Wolfe, 1965). Microtubules are randomly distributed throughout the cytoplasm, but are especially prominent at the site of emergence of the cell process. They frequently run parallel to the long axis of the process (Samarasinghe et al., 1983; Sheridan and Reiter, 1968; Anderson, 1965) and often lie in association with the centrioles, from which they are believed to originate (Samarasinghe et al., 1983, Cuello, 1973; Romijn, 1973c). In the gerbil, they are found in proximity to microtubular sheaves and the Golgi complex (Welsh and Reiter, 1978).

Microtubules have been linked to pineal secretory processes (Karasek et al., 1982c) or to intracellular transport of pineal products (Anderson, 1965). Increases in the numbers of microtubules were noted in the pinealocytes of hibernating ground squirrels (McNulty and Dombrowski, 1980).

Microfilaments are relatively rare in the pinealoctye cytoplasm. Six to eight nm filaments have been reported in the cytoplasm of rats (Arstila, 1967), baboons (Theron et al., 1979), seals (Cuello, 1973), gophers (Sheridan and Reiter, 1973) and tree shrews (Hwang, 1982). Microfilament bundles have been reported in the mitochondria of the Pipistrelle bat (Pevet and Racey, 1981). The authors speculated that these filaments may represent a product of mitochondrial metabolism. Similar bundles of eight nm filaments are found in the nuclei of Chinese hamster (Matsushima and Morisawa, 1982) and aging rat (Johnson, 1980) pinealocytes.

Dense-cored vesicles

Dense cored vesicles (DCV) are found within pineal parenchymal cells of all species examined to date. The DCV were previously reported to be absent in cat pinealocytes (Wartenberg, 1968) but were demonstrated upon careful re-examination (Karasek and Hansen, 1982). Most species display only scattered granulated vesicles, although large numbers of vesicles are evident in the golden mole (Pevet and Kuyper, 1978), Eastern chipmunk (Karasek et al., 1982c), brush mouse (Karasek et al., 1983a), pig (Karasek and Wyrzykowski, 1980), white-footed mouse

(Samarasinghe et al., 1983), Malaysian rat (Pevet and Yadav, 1980), Weddell seal (Cuello, 1973), golden hamster (Sheridan, 1975) and mouse (Pellegrino de Iraldi, 1969).

DCV range in size from 40-300 nm in diameter. The vesicles are bounded by a single trilaminar membrane and contain an electron dense core surrounded by a 15 nm electron lucent rim (Romijn, 1973c; Arstila, 1967). The dense core varies in density (Pevet and Kuyper, 1978). DCV are scattered throughout the cytoplasm and are most frequently encountered in close proximity to the Golgi complex or within the terminal portions of the pinealocytes.

The site of origin of the DCV is believed to be the Golgi complex. Electron microscopic evidence supports this theory, as DCV have been seen budding off of the Golgi cisternae (Karasek et al., 1983a, 1982a,c; Matsushima and Morisawa, 1982; Karasek and Wyrzykowski, 1980; Romijn, 1973c). Formation of the DCV by the Golgi in vitro has been speculated to result from norepinephrine stimulation (Romijn and Gelsema, 1976).

DCV have been linked with pinealocyte secretory processes (Karasek et al., 1982a; Pevet, 1979). Morphological evidence of exocytosis of the contents of the DCV has been observed in some species (Pevet and Kuyper, 1978). Cytochemical analysis has shown that DCV contain a proteinaceous compound (Juillard, 1979) in addition to the indoleamine serotonin (Lu and Lin, 1979). These two compounds have been postulated to exist in two possible combinations within the vesicle: 1) a carrier protein linked with the indoleamine, or 2) peptidergic hormone and indoleamine (Karasek et al., 1982a). Functionally, DCV have been

postulated to contain the antigonadotropic compound secreted by the pineal (Benson and Krasovich, 1977).

A population of clear vesicles is also present within the pinealocytes. Obviously, in species in which DCV are rare, the clear vesicle form predominates. Possible origin of the clear vesicles from DCV following dissolution of the electron dense core has been discussed by several investigators (Pevet and Yadav, 1980; Pevet and Kuyper, 1978). Intermediate forms between DCV and clear vesicles have been observed (Karasek et al., 1983a; Pevet and Yadav, 1980; Pevet and Kuyper, 1978).

Synaptic ribbons

Synaptic ribbons are one of the most frequently investigated features of the pinealocyte and perhaps the most enigmatic. Synaptic ribbons are variously called vesicle-crowned rodlets or vesicle-crowned lamellae. The synaptic ribbon (SR) possesses an electron dense core in the form of a ribbon (McNeill and Whitehead, 1979; Romijn, 1973c; Wartenberg, 1968; Arstila, 1967) or rod (Kosaras et al., 1983; Vollrath et al., 1983; King and Dougherty, 1982; Hwang, 1982; Hewing, 1981; Pevet and Yadav, 1980; Welsh and Reiter, 1978) with intermediate forms noted (Karasek et al., 1983b; King and Dougherty, 1982). The length of the electron dense core is variable. Ribbons are formed by three opaque lamellae ten to 40 nm wide. Each lamella is separated from adjacent lamellae by a 20 nm electron lucent zone (King and Dougherty, 1980, Arstila, 1967). The dense core is surrounded by numerous clear vesicles

30-60 nm in diameter although occasional DCV are present (Karasek et al., 1983b; King and Dougherty, 1982; Pevet and Yadav, 1980; Hewing, 1980a; Romijn, 1973c). The vesicles do not contact the core, being separated from it by a 20-40 nm space (King and Dougherty, 1980).

SR are grouped into ribbon fields (RF). Some investigators consider a RF to be two or more SR in a constellation (Theron et al., 1981, 1979), while others feel that one or more SR may comprise a RF (Karasek et al., 1982b; Hewing, 1981, 1980a; Vollrath and Huss, 1973). RF may contain as many as 30 SR (Karasek et al., 1982b). SR in a RF are generally considered to be identical to those found singly within the cytoplasm, but Theron et al. (1981) noted several subtle differences between single SR and those lying in RF, including an association between the vesicles of SR in a RF and the membranes of the smooth endoplasmic reticulum.

The intracellular position of SR and RF has been analyzed at length. Vollrath and Huss (1973) found that SR and RF were about equally distributed between the perikarya and processes of pinealcoytes, but RF in cell processes contained only one synaptic ribbon predominantly, while RF in the perikarya contained two to three. In both perikarya and processes, the majority of the SR were centrally located, away from the plasma membranes. Ninety-eight percent of the SR or RF located adjacent to the plasma membrane were opposite another pinealocyte, and paired ribbon fields in adjacent pinealocytes could be seen. These intracellular locations are true for most species examined to date (Karasek et al., 1982b; Hewing, 1981; McNeill and Whitehead,

1979; Vollrath, 1973; Hopsu and Arstila, 1965), although SR of Malyasian rat pinealocytes that are adjacent to the plasmalemma most frequently lie next to a perivasular space (Pevet and Yadav, 1980) while those in the superficial pineal of the vole face adjacent glial cells (Hewing, 1981). SR are found in association with a variety of other cell structures including DCV, RER (King and Dougherty, 1980), SER (Theron et al., 1979), microtubules (King and Dougherty, 1980; Theron et al., 1979) and microfilaments (Theron et al., 1979).

The function of the SR is not yet known. They are similar to synaptic ribbons found in specialized neurons such as the photoreceptor cells, although they are not believed to function as true synaptic sites. Vollrath and Huss (1973) speculated that the SR act as functional links between adjacent pinealocytes, permitting synchronization of cell function within the gland. The SR have also been postulated to regulate the density of beta adrenergic receptors located on the plasmalemma, thus limiting nervous control of pineal gland function (Karasek et al., 1983b; King and Dougherty, 1982). Other authors (Pevet and Yadav, 1980; Hewing, 1981) have speculated that the SR are involved in the process of cell secretion, or in membrane turnover within the pinealocyte (King and Dougherty, 1980).

A related structure, the synaptic spherule, has been found in pinealocytes of the rat (Kosaros et al, 1983; Karasek and Vollrath, 1982) and guinea pig (Vollrath et al., 1983). Synaptic spherules consist of a spherical electron dense core 120-180 nm in diameter, surrounded by clear 30-40 nm vesicles. These structures are found

singly, in spherule fields, or in mixed fields with SR. The function of the spherules is not known, although they may serve as structural markers for a functionally distinct subpopulation of pinealocytes (Vollrath et al., 1983).

Corpora Arenacea

Pinealocyte cytoplasm in some species is characterized by electron dense concretions known as corpora arenacea or acervuli, psammoma bodies or brain sand. These structures have been found in the pineal of the gerbil (Japha et al., 1974), rat (Diehl, 1978) and man Allen et al., 1981). They vary in diameter from 0.5 to 3000 μm (Allen et al., 1981; Welsh and Reiter, 1978) and demonstrate a concentric internal structure. The inclusion consists of an organic matrix formed by carbohydrate and protein, with calcium and phosphorus present in large amounts (Allen et al., 1981; Japha et al., 1976). Concretions are apparently formed intracellularly, with subsequent breakdown of the cell and release of the concretion into the extracellular space (Welsh and Reiter, 1978). The concretions are generally believed to increase in size and number with age. Concretions are a normally occurring structure, and their presence has been related to the secretion of a pineal protein product (Lukaszyk and Reiter, 1975). Diehl (1978) considered the concretions to be coagulated intercellular organic debris and minerals, resulting from reduced tissue fluid drainage due to lack of lymphatics.

Cell junctions

Adjacent pinealocytes are separated by a distance of 15-25 nm (Romijn, 1973c; Wolfe, 1965), although the cells may contact each other via various types of cell junctions. Zonula adherens, desmosomes and tight junctions are found between adjacent cell bodies and on cell processes in the rabbit (Romijn, 1973c). In rat pinealocytes, however, only zonula and fascia adherens junctions are evident. The junctions are most abundant on the initial segments of the cell processes (Krstic, 1974; Wolfe, 1965). A more recent study (Taugner et al., 1981) reported that the cell junction joining rat pinealocytes is actually a gap junction difficult to characterize in section. Gap junctions between adjacent cells assume a ribbon-like form. Pinealocytes in the golden hamster are joined by desmosomes only (Huang et al., 1984).

In the rat, wider intercellular clefts, variously referred to as interfacial lakes (Wolfe, 1965) or canaliculi (Krstic, 1975), are also seen. These clefts range from one to 20 μm in diameter and up to 100 μm in length. Canaliculi extend to the capsule, with finer extensions running between adjacent cells.

SUBPOPULATIONS OF PINEALOCYTES

The presence of two distinct and different subpopulations of pinealocytes has been the subject of much conjecture. In many species, a homogeneous pinealocyte population has been reported (tree shrew, Hwang, 1982; brush-mouse, Karasek et al., 1983a; Eastern chipmunk, Karasek et al., 1982c; pig, Karasek and Wyrzykowski, 1980; opossum,

McNulty and Hazlett, 1980; Malaysian rat, Pevet and Yadav, 1980; white-footed mouse, Samarasinghe et al., 1980; cotton rat, Matsushima et al., 1979b; ground squirrel, Povilshock et al., 1975; cat and monkey, Wartenberg, 1968; sheep and cow, Anderson, 1965). Other species possess two distinct parenchymal cell populations in the pineal on the basis of their staining properties. These two cell types are the so-called light and dark pinealocytes (Chinese hamster, Matsushima and Morisawa, 1982; Nutria, Salisbury et al., 1981; mole-rat, Pevet and Kuyper, 1978; Mongolian gerbil, Welsh and Reiter, 1978; mole, Pevet, 1974; mouse, Pellegrino de Iraldi, 1969). The ultrastructural features of the two cell types are very similar, if not identical, with the electron lucency of the cytoplasm the only distinguishing characteristic. Staining properties of the two cell types are postulated to reflect different functional states of the same cell. However, alterations can be produced in appearance of the cell populations by altering the type of fixative and method of fixation, implying that the presence of light and dark pinealocytes may be an artifact of fixation.

Still other species (Pipistrelle bat, Pevet and Racey, 1981; mole rat, Miline, 1979; noctule bat, Pevet et al., 1977a; mole rat, Pevet et al., 1976; rabbit, Romijn et al., 1977b; pocket gopher, Sheridan and Reiter, 1973; hamster, Clabough, 1971, Sheridan and Reiter, 1968; dog, Welser et al., 1968; rat, Arstila, 1967) are reported to possess structurally distinct populations of cells. These two cell populations are referred to as Type I and Type II pinealocytes. Structural differences include a greater proportion of RER and glycogen in the

pinealocytes of Type II. Type II cells are often found in a perivascular location.

Pevet (1977a, b) has written extensively on the subject of different pineal populations. He postulated that pinealocytes of Type I are characterized by the presence of granular vesicles in their cytoplasm. These vesicles represent a secretory process within the cell that involves production of the granular vesicles by the Golgi complex. Type I cells are considered to be true pinealocytes, derived directly from the sensory photoreceptor line of lower vertebrates (Pevet et al., 1977b). Cells of Type II are characterized by a secretory process involving product originating directly in the cisternae of the rough endoplasmic reticulum. These cells are considered to be a class of pinealocytes. Their evolution from the sensory cell line is evidenced by the presence of cilia with a 9+0 microtubule arrangement. Pevet (1977a,b) speculated that in pineal glands possessing a homogeneous cell population, both forms of secretion are carried out by the the same cell.

Functional evidence supporting the concept of two cell populations in the pineal gland was found in the electrophysiological experiments of Semm and Vollrath (1980). The investigators detected pinealocytes in the pineal gland of the guinea pig which responded differently to a photic stimulus. One cell type was stimulated by light and inhibited by darkness, while the other cell population demonstrated the opposite responses. More recently, a possible marker in the form of synaptic ribbons and synaptic spherules was noted in the guinea pig pineal gland

(Vollrath et al., 1983). Synaptic ribbons are believed to be more abundant in nocturnal pinealocytes while spherules are more numerous in diurnally active cells.

STRUCTURAL CORRELATES OF SECRETION

Localization of pineal metabolic products to specific intracellular structures has not been extensively investigated. Several enzymes, including succinic dehydrogenase (Moller and Hoyer, 1979), tryptophan hydroxylase (Hori et al., 1976) and acetyl-serotonin transferase (Kerenyi et al., 1975) have been localized to the mitochondria. Levels of succinic dehydrogenase, an enzyme involved in the tricarboxylic citric acid cycle (Moller and Hoyer, 1979), and tryptophan hydroxylase (Shibuya et al, 1978) show circadian fluctuations, with maximal levels occurring during the dark period. DCV have been shown to contain both a protein product (Juillard, 1979) and serotonin (Lu and Lin, 1979), possibly bound together in a protein carrier-indoleamine complex. Protein/polypeptide compounds have also been localized to the dense bodies (Lu and Lin, 1979), cisternae of the RER (Pevet, 1974) and the corpora arenacea (Welsh and Beitz, 1981; Lukaszyk and Reiter, 1975).

The intracellular pathways involved in the production of pineal compounds have not yet been defined. The smooth endoplasmic reticulum (Romijn, 1976, 1975), lipid droplets (Gonzalez and Blaquez, 1975) and dense-cored vesicles (Lu and Lin, 1979) have all been postulated to be involved in indoleamine production in the pinealocyte. Autoradiographic

labelling studies with indole precursors, however, imply that indoleamine production takes place diffusely within the cytosol (Romijn et al., 1977a; Gershon and Ross, 1966).

Several investigators have advanced theories regarding the pathways involved in the synthesis of pineal products. Juillard and Collin (1980) postulated two separate synthetic pathways. One pathway involves the production of a proteinaceous compound on the ribosomes. The protein product is then transported to the Golgi complex, where it is packaged into dense-cored vesicles. DCV transport the protein to the terminals of the cell processes for secretion. Indoleamine production takes place diffusely within the pinealocyte cytoplasm (agranular pool), although a small portion may be found within the DCV (granular pool).

Pevet (1979) also theorized that DCV were produced by the Golgi complex. He believed that an additional pathway of secretion existed, referred to as ependymal-like secretion. Ependymal-like secretion involves the production of a proteinaceous material by the RER. This material either remains within the cisternae as a flocculent matter or buds directly off of the RER in the form of vacuoles or vesicles. These vacuoles presumably are then secreted. In animals that exhibit two distinct cell populations, each synthetic process (i.e. that involving the DCV and that involving GER) was postulated to be localized to a specific cell population. In species with only one cell population, both processes could be found within the same cell (Pevet and Ariens Kappers, 1977).

GLIAL CELLS

Supporting cells within the pineal parenchyma are generally described as being a form of neuroglia, although Arstila (1967) and Wolfe (1965) disputed this, arguing that neuroglial cells and pineal interstitial cells differed in their fine structure. The cells are described as resembling fibrous or filamentous astrocytes (Karasek et al., 1982c; Hwang, 1982; Salisbury et al., 1981; Povilshock et al., 1975; Romijn, 1973c; Das Gupta, 1968; Wartenberg, 1968; Quay, 1965; Scharenberg, 1965). Interstitial cells in the rat and human were confirmed as astrocytes by labelling them with glial fibrillary acidic (GFA) protein (Papasosomenos, 1983; Moller et al., 1978).

The interstitial cells possess an irregularly shaped (Hwang, 1982; Povilshock et al., 1975) or oval (Salisbury et al., 1981; Das Gupta, 1968) nucleus of variable electron density. The cytoplasm, which is usually described as electron lucent (Karasek et al., 1982c), contains mitochondria, dense bodies, microtubules, rough endoplasmic reticulum, Golgi bodies, glycogen, ribosomes and numerous microfilaments (Hwang, 1982; Salisbury et al., 1981; Povilshock et al., 1975; Sheridan and Reiter, 1973). Interstitial cells send out extensive processes into the pineal parenchyma or to the pineal capsule. Processes within the pineal parenchyma end either in the perivascular space or around pinealocytes (Romijn, 1973c; Quay, 1965). Processes that reach the perivascular space may lie on the basal lamina of the endothelium and partition the vessel from the surrounding parenchyma (Salisbury et al., 1981; Wartenberg, 1968). Interstitial cell processes are

distinguishable from pinealocyte processes by the presence of microfilaments four to nine nm in diameter (Wartenberg, 1968; Quay, 1965) and lack of vesicles in their terminals (Salisbury et al., 1981). The cell processes may be connected to one another by desmosomes, intermediate junctions and tight junctions, although the junctions do not appear concomitantly in the same process (Wartenberg, 1968).

Unidentified interstitial cells described in pineal glands of other species fit the description given above for the filamentous astrocyte (Welsh and Reiter, 1978; Cuello, 1973; Arstila, 1967; Anderson, 1965).

Oligodendroglial-like cells have been described in the pocket gopher (Sheridan and Reiter, 1973), human (Scharenberg, 1965), ground squirrel (Povilshock et al., 1975) and Eastern chipmunk (Karasek et al., 1982c). These cells contain a round or oval dense nucleus and electron dense cytoplasm containing rough endoplasmic reticulum, Golgi, mitochondria, liposomes and centrioles. Microfilaments are not in evidence ((Karasek et al., 1982c). These cells also send forth cytoplasmic processes, but these processes are not as extensive as those found in astrocyte-like cells.

Interstitial cells in the pineal are described as being primarily supportive or nutritive in their function (Hwang, 1982; Povilshock et al., 1975; Sheridan and Reiter, 1973). Existence of a partial or complete glial barrier between the pineal parenchyma and the blood vessels of the gland has also been postulated (Salisbury et al., 1981; Cuello, 1973; Wartenberg, 1968). Hwang (1982) speculated that the glial

cells, pinealocytes and pigment-containing cells found in the tree shrew pineal gland represented the same cell line at different functional stages. Glial cells were regarded as the intermediate stage between the secretory pinealocytes and senescent pigment cells. Glial cells were considered secretory cells. Based upon histochemical evidence (Lukaszyk and Reiter, 1975), a possible secretory function of the glial cells has also been theorized by Welsh and Reiter (1978). Pineal gland glial content has been shown to increase with age (Quay, 1965).

PIGMENT CELLS

In the pineal glands of the pocket gopher (Sheridan and Reiter, 1973) and the tree shrew (Hwang, 1982), a third cell type, termed the pigment cell, has been noted. In addition to the usual complement of organelles, this cell contains prominent pigment granules measuring one to five μm in diameter. Hwang (1982) found forms intermediate between the glial cells and the pigment cells, and felt that the two cells were part of the same cell line (see GLIAL CELLS), while Sheridan and Reiter (1973) speculated that pigment cells could be mast cells. Pevet (1977a) felt that pigment cells represented an additional population of pinealocytes.

BLOOD VESSELS

Abundant blood vessels are found within the pineal parenchyma. Capillary structure is apparently species specific, since fenestrae have been noted only in the endothelial cells of the rabbit (Romijn, 1973c), Eastern chipmunk (Karasek et al., 1982c), cotton rat (Matsushima et al., 1979b), Chinese hamster (Matsushima and Morisawa, 1982), golden hamster

(Sheridan and Walker, 1975), ground squirrrel, mouse and rat (Matsushima and Reiter, 1975). The presence or absence of fenestrae has been linked to differences in the physiology of the glands (Matsushima and Reiter, 1975), although lack of fenestrae cannot be correlated with the existence of either a blood:brain or blood:CSF barrier within the pineal (Welsh and Beitz, 1981).

Endothelial cells within the capillaries resemble those found in other tissues. The cells contain a well-developed Golgi complex, mitochondria, ribosomes, granular endoplasmic reticulum and numerous vesicles or caveolae. The cells are joined by tight junctions (Anderson, 1965). The vesicles have been implicated in the transport of substances through the capillary wall. Other possible pathways of transport are through the intercellular junctions, via fenestrae and through channels formed by the fusion of vesicles with luminal and abluminal membranes of the endothelial cells (Moller et al., 1978).

Pineal blood vessels are limited by a basal lamina which may split to contain one or two pericytes (Matsushima and Reiter, 1975; Cuello, 1973). The blood vessels are surrounded by a perivascular space which varies considerably in size, being rather extensive in rodent species (Matsushima and Reiter, 1975; Rodin and Turner, 1966), the opossum (McNulty and Hazlett, 1980), gerbil (Welsh and Reiter, 1978) and the pocket gopher (Sheridan and Reiter, 1973). Variation in the size of the perivascular space may, however, be the result of the fixation method used (Karasek et al., 1982c; Wolfe, 1965). The perivascular space is limited at its periphery by a second basal lamina upon which

pinealocytes and glial cells abut. Contents of the perivascular space include pinealocyte and glial cell processes, nerve fiber endings, collagen fibrils, fibroblasts, macrophages and mast cells (Karasek et al., 1982c; Welsh and Reiter, 1978; Povilshock et al., 1975; Romijn, 1973c; Sheridan and Reiter, 1973, 1968; Wartenberg, 1968; Rodin and Turner, 1966).

NERVE FIBERS

Myelinated and unmyelinated nerve fibers can be found throughout the pineal parenchyma, although the latter predominate. The terminals of nerve axons contain mitochondria and numerous vesicles of both the clear and dense-cored variety (Anderson, 1965). Dense-cored vesicles found in nerve fiber endings are smaller (35-80 nm in diameter) than those found within the terminals of pinealocyte processes and more uniform in appearance (Karasek et al., 1982c; Povilshock et al., 1975; Sheridan and Reiter, 1973, 1968). Nerve processes are found in the pineal capsule, in the intercellular and perivascular spaces and ramifying though the pineal parenchyma. Nerve terminals are adrenergic in nature (Pellegrino de Iraldi and Gueudet 1969; Owman, 1965), although serotonin is also believed to be stored within the dense-cored vesicles (Jaim-Etcheverry and Zicher, 1980). Synaptic contact between the nerve terminals and pinealocytes has been noted in the tree shrew only (Hwang, 1982).

Neurons and neuron-like cell bodies have been found within the pineals of non-Eutherian mammals (McNulty and Hazlett, 1980; Kenny and Scheelings, 1979), Eastern chipmunks (Karasek et al., 1982c) and ground

squirrels (Matsushima and Reiter, 1978). These cells resemble true neurons in their structure, and have synaptic contact with other nerve endings in the pineal gland. The function of these cells is not yet clear, although they may be part of an intrapineal nervous network.

STRUCTURAL CIRCADIAN RHYTHMS

Structural correlates for rhythms in pineal gland metabolic activity have been sought by numerous investigators, although the only comprehensive work to date was compiled by Welsh et al. (1979), working with the superficial pineal of the Mongolian gerbil. Other investigators have focused only upon changes in individual organelles, usually synaptic ribbons and dense-cored vesicles.

Circadian changes in the volume of pinealocytes were found in the superficial pineal gland of the gerbil (Welsh et al., 1979), with peak values at the end of the light period and at middark and trough values early in the light period. Mouse pinealocytes also exhibited 24-hour variation in size, but peak values occurred early in the light period (Kachi et al., 1971b). Changes in pinealocyte volume were not correlated with glandular metabolic activity by the authors.

Alterations in the volume of pinealocyte nuclei over a 24-hour period has been examined in a number of species although results are variable. Nuclei of gerbil (Welsh et al., 1979) and hamster (Vollrath, 1979) pinealocytes did not exhibit significant changes in their volume over a 24-hour period. Midlight peaks in nuclear volume were shown in the Chinese hamster (Matsushima et al., 1983) and the rat (Quay and Renzoni, 1966). Matsushima et al. (1983) suggested that rhythms in

nuclear volume may be related to pineal protein and ribonucleic acid (RNA) content, which showed simultaneous peaks at midlight.

Recently, several studies have been published that infer an ultradian as well as circadian rhythmicity in the pineal gland, based upon changes in nuclear volume. Becker and Vollrath (1983) and Diehl (1981) found that the rhythm in nuclear volume of rat pinealocytes located in the center of the pineal was not synchronized with that of cells in the periphery of the gland. The phase of the rhythms differed depending on the day of sacrifice. Diehl (1981) related the changes in nuclear size to the pineal's metabolic activity, while Becker and Vollrath (1983) speculated that their inconsistent results could be due to different populations of pinealocytes within the gland, some of which were active at night and others which were active during daylight hours. Variations in the rhythms from day to day may be the result of sampling different proportions of the two populations.

Circadian variation has been reported in the nucleolar volume of rats (Lew et al., 1982; Quay and Renzoni, 1966). Quay and Renzoni (1966) found peak nucleolar diameter at midlight,, while Lew et al. (1982) reported early to midlight maxima. The authors theorized that larger nucleolar volumes may precede increases in cytoplasmic protein synthesis. Welsh et al. (1979) found no nucleolar rhythm in the Mongolian gerbil.

Interspecies variation may play a role in circadian variation in the cytoplasmic volume of pinealocytes. The pinealocytes of the gerbil (Welsh et al., 1979) demonstrated maximal cytoplasmic volume at middark

and minimal values at midlight , while cells of the Chinese hamster (Matsushima et al., 1983) exhibited the inverse pattern. Twenty-four hour changes in the gerbil cytoplasm were related to intracytoplasmic localization of the synthesis of pineal indoleamines. Alterations in the cytoplasm of Chinese hamster cells was believed to be indicative of changes in the functional activity of those cells.

Conflicting reports exist on the circadian rhythmicity of pineal mitotic activity. While there is general agreement that mitotic activity of rat pineals is low, varying between nine and 22.8 mitotic figures per gland (Reuber and Vollrath, 1983; Quay and Renzoni, 1966), the reported time of maximal mitotic activity varies. Two studies (Reuber and Vollrath, 1983; Quay and Renzoni, 1966) reported peak mitotic activity during the light period, while Becker and Vollrath (1982) found highest values during the dark phase. Reuber and Vollrath (1983) speculated that the conflicting rhythms in mitotic activity may be the result of sampling error.

The intercellular spaces of the rat pineal, referred to as the pineal canaliculi, have been reported to vary over a 24-hour light:dark cycle. Canaliculi are present between adjacent pinealocytes and join with the pericapillary spaces. Evidence of circadian rhythmicity was based upon the extent to which the canaliculi could be perfused with an India ink solution. Maximal perfusion distances were obtained at the end of the dark period. Twenty-four hour alterations in canalicular perfusing capacity were theorized to result from the release of

serotonin into the canaliculi. Canaliculi are possible routes of transport of serotonin between the pinealocyte and blood vessels (Quay, 1974, 1973).

Six cytoplasmic elements found within gerbil pinealocytes were shown to exhibit circadian rhythms: free cytoplasm, smooth endoplasmic reticulum, rough endoplasmic reticulum/ribosomes, mitochondria, secretory vesicles and microtubules (Welsh et al., 1979). Free cytoplasm exhibited a biphasic rhythm, with peaks at middark and at the end of the light period. The volume density of the smooth and rough endoplasmic reticulum peaked one hour after scotophase began, and reached a nadir one hour prior to photophase. Peak values for the smooth and rough endoplasmic reticulum were equated with maximal indoleamine and protein levels, respectively. Mitochondria exhibited a marginally significant rhythm, with highest volumes at middark and lowest volumes one hour after the dark phase began. It was not known whether the increase was due to changes in the size of individual mitochondria, or in the number of mitochondria per cell. The increase in mitochondrial volume density was believed to reflect greater synthetic activity by the pinealocytes due to the presence of enzymes, such as tryptophan hydroxylase, within the mitochondria. The secretory vesicles and microtubules reached maximal values one hour prior to the dark phase, indicating a possible relationship between the functions of the two structures. It was suggested that microtubules play a role in the intracellular transport of vesicles.

Microtubules in the baboon showed peak values two hours after the dark phase began (Theron et al., 1981). The change in baboon microtubule content was speculated to result from a photic induction of their assembly. Glycogen content in the pinealocytes of mice showed significant variation over a 24-hour light:dark cycle, with lowest values at the end of the dark phase and highest levels at the end of the light phase. Glycogen levels were believed to reflect the functional activity of the pineal in response to light (Kachi et al., 1971b).

Circadian variation in the number of dense-cored vesicles has been investigated in a number of laboratory animals including the hamster (Benson and Krasovich, 1982), Mongolian gerbil (Welsh et al., 1979), mouse (Kachi, 1979; Krasovich and Benson, 1982. and rabbit (Romijn et al., 1976). The data from all species are not in agreement, but the area of pinealocyte cytoplasm sampled was not uniform in all studies. Mice maintained on a 12h:12h light:dark cycle showed a late photoperiod maximum and late scotoperiod minimum in the number of DCV, while hamsters, maintained on a similar lighting regimen, did not exhibit a significant rhythm. Hamsters maintained on a 10h:14h L:D did, however, show a rhythm similar to that seen in mice. Similar mid to late light period maxima were found in the number of DCV in the Golgi region and cell processes of the rabbit (Romijn et al., 1976) and in the whole cytoplasm and Golgi region of mouse pinealocytes (Kachi, 1979). The gerbil did not demonstrate a rhythm in pinealocyte DCV content over a 24-hour period (Welsh et al., 1979). In all studies, authors speculate that the number of DCV represent the rate of synthesis or secretion of a

product of pineal metabolism. The product is stored within the DCV, which increase in number during the course of the light period. The content of the vesicles is then released, beginning with the onset of darkness. The mechanism of release is not known. Melatonin has been postulated to stimulate the synthesis or release of the granulated vesicles (Benson and Krasovich, 1977).

Synaptic ribbons and ribbon fields exhibit marked circadian rhythmicity over a light:dark cycle. These structures show maximal dark phase values in all mammalian species examined to date (Chinese hamster, Matsushima et al., 1983; guinea pig, Vollrath et al., 1983; Vollrath, 1973; chipmunk and ground squirrel, Karasek et al., 1982b; baboon, Theron et at., 1981; rat, Kuromado and Mori, 1977). The circadian variation found in these structures has been postulated to result from increased intercellular communication within the cells of the pineal gland (Vollrath, 1973) and/or increased storage of secretory products produced during the dark phase.

Synaptic spherules have also been examined for the presence of rhythms. Spherules in the rat (Karasek and Vollrath, 1982) exhibit a rhythm identical to that shown by synaptic ribbons and ribbon fields, with peak values occurring during the dark phase, while spherules in the guinea pig reach maximal levels during the light phase (Vollrath et al., 1983). Synaptic spherules in the pinealocytes of the guinea pig were believed to be present in a population of pinealocytes active during the light phase, while synaptic ribbons were found in greater numbers in nocturnally active pinealocytes. Spherules in the Chinese hamster,

designated as Type 2 synaptic ribbons, did not exhibit circadian variation (Matsushima et al., 1983).

EFFECTS OF ALTERED LIGHTING PERIODS ON PINEALOCYTE STRUCTURE

Various experiments have been conducted to determine the effects of altered lighting regimens on pineal structure. Darkness, or reduced photoperiod, is considered to be stimulatory to pineal function, while prolonged periods of illumination inhibit pineal antigonadotropic activity.

Effect of Reduced Photoperiod

Reduction of the length of the photoperiod was shown to have no effect on the weight of the pineal gland in rats (Fiske et al., 1961). Pinealocytes of rats exposed to a 1h:23h L:D regimen demonstrated smaller nuclei, greater variability in nuclear structure and less heterochromatin. The amount of smooth endoplasmic reticulum was increased, as were the number of lipid droplets. Hypertrophy of the SER was related to increased production of a pineal antigonadotropic compound, while the decrease in nuclear volume was regarded as paradoxical in view of the other cellular changes (Barratt et al., 1977). Brush mice kept on a shortened light schedule showed more consistent changes in cell structure. Pinealocyte nuclei and cytoplasm increased in volume. Values for the Golgi complex, rough endoplasmic reticulum, mitochondria, vacuoles containing flocculent material and dense-cored vesicles were also elevated. These changes suggested greater cellular activity. Pinealocytes of brush mice kept under short

photoperiod were apparently more active than those maintained under a long photoperiod (Karasek et al., 1983a).

In hamsters, reduction of the photoperiod increased the amplitude of the rhythm in dense-cored vesicles, with peak values found at the end of the light phase (Krasovich and Benson, 1982). Counts were made of DCV in pericapillary pinealocyte processes.

The above experiments were of a chronic nature. The effect of an acute lighting change was investigated by using the synaptic ribbons of the guinea pig. Extension of the dark period inhibited the normal decrease in the number of ribbon fields. The maleable response in the synaptic ribbons was taken as a positive indication of synaptic ribbon involvement in the regulation of pineal function (Vollrath, 1976).

Effect of Total Darkness or Optic Enucleation

Blinded male hamsters showed a growth spurt following optic enucleation (Hoffman, 1983). The degree of growth varied seasonally, with maximal weights occuring during the late summer and early fall. Pinealectomy abolished the growth response in blinded animals. Hoffman (1983) speculated that the pineal is involved in the growth and development of hamsters, possibly even to the determination of the mean life span.

Constant darkness did not affect pineal wet or dry weight in the rat (Quay, 1961a) nor did pinealocytes in rabbits (Romijn, 1975) exhibit any qualitatively observable changes following exposure to constant darkness. In other species, variations in the structure of pinealocytes

indicate increased cellular activity in response to constant darkness. These structural changes are described in greater detail below.

Light microscopic analysis of the pineal glands of rats kept in constant darkness showed larger pinealocytes with a more basophilic cytoplasm (Roth et al., 1962). The nuclei of these cells demonstrated greater polymorphism in their shape and displayed prominent nucleoli. Irregularities in the shape of the nuclei made quantitative assessment of their size difficult, but there was a qualitatively visible increase in their volume. The authors concluded that the cellular changes were indicative of increased nucleoprotein and protein synthesis in the pineal gland related to increased glandular activity. This study did not include animals kept under a normal (14h:10h) light:dark cycle.

Light microscopic examination also revealed more prominent nucleoli in pinealocytes of the dark-exposed collared lemming (Quay, 1978). The presence of larger, more obvious granules in the cytoplasm of pinealocytes of the white-footed mouse was also noted and was believed indicative of increased pineal synthetic activity, possibly related to the antigonadotropic activity of the pineal glands (Quay, 1956).

Differences between light and dark pinealocytes were enhanced in dark-exposed rats (Freire and Cardinali, 1975). Pinealocytes in this species also demonstrated increased numbers of ribosomes and polyribosomes, rough endoplasmic reticulum, procentrioles and microtubules, prominent nucleoli, Golgi apparatus and annulate lamellae. NAT and HIOMT activities were also greater in dark-exposed rats. The

cellular and biochemical alterations were similar to those induced by the administration of melatonin to animals maintained under a normal lighting regimen. Freire and Cardinali (1975) concluded that both constant darkness and exogenous melatonin administration resulted in activation of the pineal gland.

Synaptic ribbons and ribbon fields in blinded rats initially declined in number for a period of three months and then returned to normal levels (Kurumado and Mori, 1980). Despite the decline in the numbers of synaptic ribbons and ribbon fields, animals blinded for one month still exhibited a normal circadian rhythm with peak values occurring during the dark phase; Six months after optic enucleation, the circadian rhythm was identical to that of control animals in both mean volume and amplitude. Maintainence of the rhythm of SR in blinded rats implies either the presence of an external stimulus in addition to the photic environment, or the existence of an internal clock regulating SR rhythms.

Long term (30 weeks) blinded rats and hamsters possessed pinealocytes that appeared to have decreased volume (Satodate et al., 1973). Nucleoli were present in less that half of the pinealocytes examined. The number of lipid droplets, mitochondria and vesicles declined in comparison to control animals. Cytological changes were indicative of exhaustion or degeneration of the pineal cells.

Light pinealocytes of optically enucleated mice exhibited a greater amount of smooth endoplasmic reticulum, Golgi, vesicles and multivesicular bodies (Benson and Satterfield, 1975). Dark pinealocytes

were not affected by blinding. The results indicated a link between pineal antigonadotropic activity and light pinealocyte function. Upson and Benson (1977) noted similar hypertrophy of the Golgi complex of cells from blinded mice but did not find any changes in the endoplasmic reticulum or multivesicular bodies. This discrepancy may be the result of a shorter time span between enucleation and sacrifice in the latter study. The authors also reported a 55% decline in the number of DCV located in pericapillary terminals as well as a decline in the number of agranular vesicles. Large, irregular vacuoles were commonly seen in process endings. Decreases in the number of vesicles found in pinealocyte terminals were postulated to result from the increased synthesis and release of pineal secretory products. Kachi et al. (1974) found that the rhythm in glycogen content in the mouse was light-dependent and was abolished after exposure to constant darkness for a period greater than seven days. Glycogen present in mouse pinealocytes was considered to represent an energy source for cellular activity, although the precise metabolic activity related to the mobilization of glycogen stores is not known.

Synaptic ribbons in the pinealocytes of guinea pigs subjected to constant darkness for periods ranging from 26-70 days showed variable responses (Vollrath and Huss, 1973). Twenty percent of the animals showed a strong increase in the number of ribbon fields, 30% demonstrated a moderate increase, 10% exhibited no change and 40% declined in number. In light of other experimentally induced changes in these organelles, the authors viewed the SR as devices providing for

intercellular communication in the pineal. The SR increased in number in animals maintained in constant light or constant dark, however, thus rendering results that are difficult to interpret. Lues (1971) found a consistent increase in the synaptic ribbons of guinea pig cells exposed to constant darkness.

Blinded hamsters, sacrificed eight weeks after blinding, (Lin et al., 1975) exhibited enlarged pinealocytes with prominent nucleoli. The cells contained a hypertrophied smooth endoplasmic reticulum with dense-cored vesicles interspersed in its meshwork. Annulate lamellae and abundant lipid droplets were also present. These changes were taken as indications of pineal gland activation after blinding. Pinealocytes of blinded hamsters appeared to be much more active than those taken from ganglionectomized animals. Based on these observations, the authors theorized that the smooth endoplasmic reticulum was involved in production of the pineal antigonadotropic product and possibly contained some of the enzymes involved in the synthesis of melatonin.

Hamsters sacrificed six weeks after optic enucleation showed increased numbers of multivesicular bodies in the perikarya and cell processes of pinealocytes (Clabough, 1971). Increased numbers of both clear and dense-cored vesicles and enlargement of the Golgi complex were also noted. The most striking difference between blinded and control animals was the presence of membraneous lamellar structures in the pinealocyte perikarya and cell processes. The lamellar structures consisted of concentric whirls of unit membranes, with vesicles, lipid droplets and glycogen-like particles in close association. These

structures measured up to five um in diameter and were postulated to arise from the mitochondria. Clabough (1971) related the lamellar structures to pineal antigonadotropic activity, either directly or as a by-product of that activity.

Synaptic ribbons (Hewing, 1980a) and dense-cored vesicles (Sheridan, 1975) increase in number in the pinealocytes of hamsters maintained in total darkness. Hewing (1980a) frequently found the two structures in close apposition to one another, speculating that the dense-cored vesicles may play a role in the formation and growth of the synaptic ribbons. Sheridan (1975) attributed the greater numbers of DCV to stimulated production of the pineal antigonadotropic compound. In contrast, Krasovich and Benson (1982) reported a decrease in the number of granulated vesicles located in pinealocyte terminals of blinded hamsters. They were not able to reconcile these changes with those of Clabough (1971) and Sheridan (1975), although they cited sampling differences and different cytoplasmic test areas as possible reasons for the discrepancy.

Dark exposed gerbil pinealocytes showed large electron dense structures in the cytoplasm of their cell processes. These structures were circular in shape, and were frequently formed by smaller circular structures. Glycogen granules also decreased in amount in comparison to control animals. (Gregorek, 1973).

Dormice showed ultrastructural changes in pinealocyte structure characterized by well developed endoplasmic reticulum and liposomes.

These two structures were correlated with protein secretions and pineal serotonin levels, respectively (Roux and Richoux, 1981).

Exposure to Constant Illumination

Exposure to constant illumination led to changes in the pineal parenchyma consistent with a decline in glandular function. A marked decrease in pineal gland weight was noted in rats exposed to constant light (Fiske et al., 1961).

Cellular changes also reflected decreased glandular function. The volume of pinealocytes declined (Kachi et al., 1971b; Quay, 1963b) and the cytoplasm became more eosinophilic and less granular (Roth et al., 1962). Nucleoli in these animals were also less prominent (Quay, 1963b; Roth et al., 1961).

Pinealocyte alterations at the ultrastructural level showed some interspecies variation. Rough endoplasmic reticulum increased in the dormouse (Roux and Richoux, 1981) and declined in the rabbit (Romijn, 1975) following constant light exposure. Golgi bodies also increased in size in the dormouse. Changes in the RER and Golgi bodies of the dormouse were attributed to the production of a progonadotropic compound by those organelles. In the rabbit, synaptic ribbons lengthened, while Golgi-associated DCV and those located in cell terminals declined in number. Changes in the rabbit were believed to indicate a decrease in pineal function resulting from a non-invasive sympatholysis produced by the constant illumination.

Light-exposed mice exhibited a decrease in the number of lipid droplets and size of the Golgi complex. Mitochondria appeared swollen,

a characteristic seen in the pinealocytes of rats exposed to constant light (Halaris and Matussek, 1969). The number of dense-cored vesicles in the polar terminals decreased by 70%. These changes were correlated with previously reported declines in pineal weight, protein synthesis and antigonadotropic activity following exposure to constant light. Decreases in the number of DCV were believed to reflect inhibition of the production of the pineal antigonadotropic product (Upson et al., 1976).

Additional changes in structure of pinealocytes of light-exposed animals included a decline in lipid content (Roux and Richoux, 1981; Quay, 1963), a transient increase in glycogen levels (Kachi et al., 1971a) and increased sensitivity of the mitochondria to fixation, possibly due to enhanced serotonin metabolism by that organelle (Halaris and Matussek, 1969).

DEEP PINEAL

As mentioned above, in many species there is a separate and distinct part of the pineal complex that borders the third ventricle and is referred to as the deep pineal gland.

DEVELOPMENT

The deep pineal arises from the separation of embryonic pineal tissue into superficial and deep portions. In the hamster, this separation occurs postnatally (Sheridan and Rollag, 1983; Sheridan and Walker, 1975). Immediately after birth, the pineal gland of the hamster consists of a single mass of tissue located in the roof of the

diencephalon. The pineal tissue at that time has a lumen, the pineal recess, which is continuous with the third ventricle. After birth, the pineal evagination loses its lumen, and its parenchyma becomes less dense. On the third postnatal day, the embryonic anlage develops a dorsal constriction at the level of the habenular and posterior commissures which produces an uneven division of the tissue into a larger distal mass and smaller proximal mass adjacent to the third ventricle. The distal component, which will become the superficial pineal, begins a dorso-caudal migration that is completed by day five of the second postnatal week. Cordlike strands of pinealocytes remain in the pineal stalk connecting the superficial and deep pineal glands. These strands of tissue are almost completely gone by the end of the third week, leaving principally blood vessels and nerves in the stalk. In gerbils, a distinct deep pineal gland is found in the fourth postnatal week (Japha et al., 1977).

Abundant blood vessels are found in the superficial pineal gland by the end of the third week postnatally, while the deep pineal gland is vascularized at a later date. Postganglionic sympathetic axons are evident in the superficial pineal gland by day 11 postnatally, particularly in the perivascular spaces. Sympathetic innervation is not evident in the deep gland until day 19.

The pinealocytes of the superficial and deep pineal glands are both derived from undifferentiated cells located in the pineal anlage. The stem cell, which is rounded with no processes, contains a nucleus with dense peripheral chromatin and a cytoplasm of variable density.

The nuclear:cytoplasmic ratio is high. Mitoses are common. Stem cell cytoplasm contains abundant smooth endoplasmic reticulum, free ribosomes and a prominent Golgi complex associated with both clear and dense-cored vesicles. Cilia are frequently present. Cellular processes appear the first postnatal day, and by day four the cells have nearly attained adult size. Light and dark cells are evident by day seven to eight. Cells of both the superficial and deep pineal glands resemble those of the mature glands by the end of the second postnatal week.

BIOCHEMISTRY

Deep pineal tissue in the adult rat contains the indoleamine serotonin (Moore, 1975; Wiklund, 1974; Bjorklund et al., 1972), although the compound does not show a circadian variation in its levels (Moore, 1975). Tissue in this area demonstrates non-rhythmic NAT activity as well. HIOMT is present at low levels (Moore, 1975). Twenty-day old hamsters possess a rhythm in melatonin content similar to that found in the superficial pineal glands of adult animals, with dark phase elevations in melatonin (Sheridan and Rollag, 1983). The level of melatonin in the deep pineal gland is about 5% of that of the superficial gland, a ratio consistent with the relative size of the two structures. The parallel rhythms were speculated to reflect either similar neuronal mechanisms regulating synthesis and secretion in the two glands or the storage of melatonin produced by the superficial pineal gland in the deep gland.

INNERVATION

The deep pineal gland is innervated by postganglionic nerve fibers that reach the deep gland from the superficial gland via the pineal stalk (Nielsen and Moller, 1978; Reiter and Hedlund, 1976). These fibers project to the superficial pineal gland from the superior cervical ganglion via the nerve conarii or on blood vessels, and hence to the deep gland. Removal of either the superior cervical ganglion or the superficial gland results in a loss of sympathetic nerve fibers to the deep gland (Reiter and Hedlund, 1976). Sympathetic fibers that enter the deep pineal gland in the rat and gerbil continue into the stria medullaris and habenular nuclei, suggesting a functional connection between the pineal complex and those structures (Nielsen and Moller, 1978; Wiklund, 1974; Bjorklund et al., 1972).

Centrally arising afferents are postulated to innervate the deep pineal gland as well (Moller and Korf, 1983a). Fibers that arise from centrally located nuclei and project to the superficial pineal gland must pass through the deep gland. These fibers have been seen in the deep pineal gland of the gerbil (Moller and Korf, 1983a). In the golden hamster, central innervation has been postulated to be responsible for the maintainence of deep pineal gland integrity following removal of the superficial gland, with consequent disruption of postganglionic sympathetic innervation to the deep gland. (Legait et al., 1979). In other species (rat, Heidbuchel and Vollrath, 1983; mouse, Glass and Lynch, 1981), removal of the superficial pineal gland resulted in atrophic changes in the structure of the deep pineal gland (i.e.

decreased volume of the deep pineal and pinealocytes), which the authors interpreted as the complete loss of deep pineal gland innervation.

HISTOLOGY

The subarachnoidal surface of the deep pineal gland is covered by the connective tissue elements of the pia mater, creating a capsule similar to that found in the superficial gland (Sheridan and Rollag, 1983; Sheridan and Walker, 1975). The surface of the deep pineal gland lying adjacent to the third ventricle is covered by ependyma continuous with that lining the ventricle. This ependyma may extend upward to cover portions of the pineal stalk in the region of the suprapineal recess. In several rodent species, ependyma over the surface of the deep gland is discontinuous in its central portion, so that pinealocytes of the deep gland directly contact the cerebrospinal fluid (Welsh, 1983; Hewing, 1982, 1980b, 1978). In the gerbil, ependyma is deficient in the area of the suprapineal recess (Welsh, 1983). Hewing (1978) suggested that deep pinealocytes in these areas could either monitor the content of, absorb substances from or secrete substances into the CSF of the third ventricle.

The microscopic structure of the deep pineal gland is qualitatively similar to that of the superficial gland, although some differences have been noted. Boeckman (1980) found that nuclear volume of deep pinealocytes of the rat were smaller (207 μm^3) than those of the superficial gland (251 μm^3). The cells contain a single nucleolus, as opposed to two in nuclei of superficial pinealocytes. Cell boundaries

of pinealocytes of the deep gland are less distinct than those of the superficial.

Ultrastructurally, the cells of the deep pineal gland resemble those of the superficial gland. The cells are irregular in shape, with many processes. The cytoplasmic content of the two cell populations is similar (Sheridan and Reiter, 1970b) and dense cored vesicles are present (mouse and gerbil, Moller, 1981; hamster, Sheridan and Reiter, 1970b). The mitotic activity of the deep gland is similar to that of the superficial (Reuber and Vollrath, 1983). Synaptic ribbons have been reported in the deep pineal gland of the vole, but they have a different intracellular distribution than is evident in the cells of the superficial gland, with the majority of the SR abutting upon glial cells. Furthermore, SR in the deep pineal gland are rarely found facing the perivascular space, concentrated instead in the CSF-contacting area adjacent to the third ventricle (Hewing, 1981). The deep pineal of the gerbil lacks corpora arenacea, although these structures are common features of the superficial gland (Japha et al., 1976). Glial cells are reported to be more abundant in the deep gland in the hamster and capillaries are non-fenestrated (Sheridan and Walker, 1975; Sheridan and Reiter, 1968).

Circadian rhythms have been examined in the nuclei of deep pinealocytes of the rat (Boeckman, 1980) and hamster (Vollrath, 1979). Nuclei of rat pinealocytes displayed ultradian rhythms on the three days sampled, with maximal size at midday in two of the experiments. These rhythms differed from ultradian rhythms reported for the nuclear volume

of superficial pinealocytes, a discrepancy that was attributed to innervation of the deep pineal by centrally located nuclei. Rhythmic variation of nuclei of superficial and deep pinealocytes of the hamster exhibit similar asynchrony, with the volume of deep pineal nuclei peaking at the middle of the light period, and no circadian variation evident in nuclei of the superficial gland (Vollrath, 1979).

The effects of various lighting regimens on the structure of deep pineal cells has focused to date upon the synaptic ribbons in the hamster. These structures were found to increase in number following exposure of the animal to constant darkness. This response is similar to that shown by the cells of the superficial pineal gland and is believed to result from stimulation of the cells of the deep pineal gland (Hewing, 1980).

Chapter III

Title: Morphometric Analysis of the Pineal Complex of the Golden Hamster over a 24-hour Light:Dark Cycle. I. The Superficial Pineal Gland in Untreated and Optically Enucleated Animals.

Abstract

Morphometric analysis of the superficial pineal gland of intact and blinded golden hamsters was conducted at both the light and electron microscopic level. The volume of the superficial gland was estimated to be 151 x 10^6 μm^3, comprising 90-94% of the total pineal parenchymal tissue. Analysis of structural rhythms in animals maintained under a 14h:10h L:D cycle showed significant 24-hour variations in values for pinealocyte nuclei, nucleoli, rough and smooth endoplasmic reticulum, Golgi bodies, dense bodies and dense-cored vesicles. Peak values for these structures generally occurred at the light:dark interphase. These results provide morphological correlates for known rhythmic variations in the synthesis of pineal gland products. Superficial pineals examined eight weeks after optic enucleation exhibited a decrease in the volume of pinealocyte nuclei and cytoplasm, while nucleolar size and the amounts of smooth and rough endoplasmic reticulum, Golgi bodies, dense bodies and dense-cored vesicles were enhanced. The latter changes are

interpreted as indications of increased synthetic activity by the superficial pineal gland in response to light deprivation.

Key Words: Morphometry, Superficial Pineal, Golden Hamster, Circadian Rhythm, Optical Enucleation

Introduction

The pineal gland of the golden hamster, Mesocricetus auratus, is divided into superficial and deep glandular portions joined by a stalk (Sheridan and Reiter, 1970a). Because the subcranial location of the superficial pineal makes it readily accessible, pineal research to date has focused upon the structure and physiology of the superficial component. Metabolism of the superficial pineal gland is under the control of the photic environment, with at least one class of compounds, the indoles, rhythmically synthesized over a 24-hour period.

Alterations in structure reflecting the circadian nature of the superficial pineal have been reported in the rat, gerbil and Chinese hamster. Pinealocyte nuclei (Matsushima et al., 1983; Becker and Vollrath, 1983; Diehl, 1981; Quay and Renzoni, 1966), nucleoli (Lew et al., 1982; Quay and Renzoni, 1966) and cytoplasm (Matsushima et al., 1983; Welsh et al., 1979) exhibit changes in volume over a 24-hour period which can be correlated with circadian fluctuations in pineal gland metabolism. Alterations are also found in subcellular structures,

although quantitative analyses to date have focused primarily upon changes in the number of dense-cored vesicles (Krasovich and Benson, 1982; Kachi, 1979; Benson and Krasovich, 1977; Romijn et al., 1976) and synaptic ribbons (Theron et al., 1981; Kurumado and Mori, 1977; Vollrath, 1973). The response of other cellular components over a light:dark cycle has been examined only in the gerbil (Welsh et al., 1979). In view of the importance of the golden hamster as an experimental model for pineal research, an investigation of circadian changes in the structure of its pineal complex was considered important. Therefore, one purpose of this study was to apply stereological techniques in order to quantify morphological changes in the pineal gland over a 24-hour period.

Structural evidence of the degree of pineal gland activity has also been sought following exposure to a variety of lighting conditions, including constant darkness or blinding. Both of these conditions are considered stimulatory to glandular metabolism and result in morphologic changes in several organelles involved in cellular secretion, including the Golgi complex (Romijn, 1975; Clabough, 1971), endoplasmic reticulum (Barratt et al., 1977; Freire and Cardinali, 1975; Lin et al., 1975) and dense-cored vesicles (Sheridan, 1975; Clabough, 1971). A second purpose of this study was a detailed quantitative evaluation of those changes that occur in pineal morphology following optic enucleation.

Materials and Methods

Seventy-six golden hamsters (*Mesocricetus auratus*) of both sexes, aged approximately two months (80-90 g), were obtained from Engle Laboratory Animals (New Jersey). The animals were maintained on a 14h:10h light:dark cycle (light = 0600 to 2000 h) for a period of at least one month. During this time, food and water were available *ad libitum* and animals were housed four to five per cage.

Volume of the Pineal Complex

Four of these hamsters (three male and one female) were used to determine the total volume of the pineal complex. Following cardiac perfusion with a 10% buffered formalin solution, the entire pineal complex was dissected and embedded in glycol methacrylate. Serial seven µm sagittal sections were cut on a JB4 microtome, mounted on slides and stained with hematoxylin and eosin.

Sections containing the pineal complex were projected by a camera lucida onto the digitizing tablet of a Zeiss Videoplan image analyzer and the profile of each part of the complex traced. The computed areas were multiplied by the section thickness (seven µm) in order to obtain the volumes of the respective parts.

Morphometric Analysis

Experimental Groups

Seventy-two hamsters were divided into two experimental groups. One group of 36 untreated (normal, NOR) hamsters, 18 male and 18 female,

was sacrificed during the light:dark cycle to which they had been entrained. The second group of 36 hamsters, 18 male and 18 female, was optically enucleated (ON) under sodium pentobarbital anesthesia. The eye socket was packed with gel foam to control bleeding and prophalactically treated with Nitrofurazone Wound Powder (Med Tech, Inc.) to prevent infection. Animals were closely observed for 24 hours post-operatively. After ON, animals were housed singly for a period of eight weeks prior to sacrifice.

In both experimental groups, six hamsters (three male and three female) were sacrificed at each of the following time points: 0100, 0500, 0700, 1300, 1900 and 2100 h. It has been established that pineal rhythms 'free-run' in photically deprived animals because the gland's endogenous rhythms are no longer synchronized to the photophase (Ralph et al., 1971). It was therefore considered important to sample ON animals at time periods identical to those used for NOR hamsters. This would yield the best estimate of the overall effects of blinding on pineal structure. Prior to sacrifice, hamsters were anesthesized with intraperitoneal injections of sodium pentobarbital. Hamsters were sacrificed during a period extending from early April to early May. Additional hamsters in both experimental groups were also sacrificed in late June and July to replace animals showing poor fixation.

Microscopy

Hamsters in the two experimental groups were perfused through the left ventricle with a flush of physiological saline followed by a

fixative solution containing 1% glutaraldehyde and 1.25% paraformaldehyde in a phosphate buffer (potassium phosphate monobasic, sodium phosphate dibasic). The buffer had previously been adjusted to 330 mOsm with glucose, and was titrated to a pH of 7.4. All perfusions that occurred during the scotophase were performed under photographic safelight (Thomas Duplex sodium vapor lamp, equipped with red and yellow filters). Upon completion of the perfusion, the pineal complex was dissected out under normal room light and the superficial and deep pineal glands separated. Pineals were then placed in a fresh 4% glutaraldehyde:buffer solution for one hour, then post-fixed in buffered 1% osmium tetroxide for an additional hour. Tissue was dehydrated in a graded series of acetones and embedded in Araldite 502. Superficial pineal glands were oriented in the blocks so that cross sections of the tissue would be obtained.

Each block of superficial pineal tissue was serially sectioned at one μm thickness, mounted on glass slides and stained with toluidine blue. Beginning caudally, every tenth section was photographed by using a Zeiss Ultraphot II microscope. These micrographs were printed at a final magnification of 1000 X.

For electron microscopy, thin sections (70-80 nm) were taken from an area of the superficial gland 20 to 50 μm anterior to the caudal tip, and stained with uranyl acetate and lead citrate. Twenty pinealocytes per gland were systematically sampled using an RCA EMU-3F microscope equipped with a 35 mm camera. The micrographs were printed at a final

magnification of 40,000 X. Calibration tests using a commercial test grid (LPI Grating Grid, Ladd Research Industries, Inc.) were routinely performed to check for the accuracy of magnification.

The reproductive organs of each animal (testes in males, uterus and ovaries in females) were dissected, blotted dry and weighed. Each specimen was cut into one to two mm square blocks, processed for microscopy and embedded in Araldite 502. One micron sections were cut, mounted on glass slides and stained with toluidine blue. Sections were qualitatively examined for evidence of gonadal atrophy in ON animals.

Stereology

Light microscopic analysis employed the Zeiss Videoplan using its standard stereology program. The perimeter of pineal structures (e.g. nuclei, nucleoli, glial cells, blood vessels) was traced onto the Videoplan's digitizing tablet, yielding the area, perimeter and maximal diameter of each structure per unit area of tissue. Data generated by the computer included estimates of the volume density ($Vv=\mu m^3/\mu m^3$), numerical density ($Nv=\#/\mu m^3$), and mean volume ($\bar{v}=\mu m^3$) of pinealocyte nuclei and nucleoli, glial cells, blood vessels and miscellaneous structures (nerve fibers and intercellular clefts). The pinealocyte cytoplasm represented the remaining volume density. Mean volume of pinealocyte cytoplasm was calculated by dividing the volume density of the cytoplasm by the numerical density of the pinealocyte nuclei ($Vv_{cyt}/Nv_{nuc}=v$). This equation assumes that the pinealocytes are mononuclear, which was found to be the case in this study.

Electron micrographs, coded so that the time of sacrifice was not known, were analyzed using a 100-point Coherent Multipurpose Grid as a transparent overlay, employing the point-counting techniques outlined by Weibel and Bolender (1972). The Multipurpose Grid contained 50 lines, 15 mm in length, each separated by a distance of 15 mm. For calculating the volume density, the ends of each line were regarded as points. Volume densities were estimated for the Golgi bodies, mitochondria, lipid droplets, multivesicular bodies and dense bodies by dividing the total number of points falling on each structure by the total number of points falling on the cytoplasm (P_o/P_c). Golgi complexes were further analyzed as to number per test area of cytoplasm, width, length, number of saccules, and number of Golgi-associated clear vesicles. Surface densities of the smooth and rough endoplasmic reticulum were estimated by counting the number of linear intersects for each organelle and utilizing the formula, $Sv_{er}=I \times 4/P_c \times z$, where I is the number of intersects, P_c is the points falling on cytoplasm, and z is the distance between lines. The number of dense-cored vesicles per unit area of test cytoplasm was also counted.

Statistical Analysis

The means and standard errors of the mean (SEM) for each of the above variables were calculated for each animal. Animals in each experimental group were pooled according to the time of sacrifice and analyzed using a one-way analysis of variance (ANOVA) for the effect of time on each variable.

Variables for each experimental group were then pooled to yield average values for a 24-hour period. This was done to account for free-running rhythms in the blinded animals. Values from NOR and ON animals were compared using a Student's t test. Sex differences within experimental groups were analyzed with a Student's t test.

Results

The pineal complex is divided into superficial and deep parts (Fig. 1). Light microscopic examination of the superficial pineal gland showed numerous pinealocytes with centrally located nuclei containing one or more prominent nucleoli (Figs. 2a and 2b). On the basis of cytoplasmic staining properties, only a single population of pinealocytes was evident in this study. Glial cells, identifiable by their intensely stained ovoid nuclei, possessed long cytoplasmic processes which extended for considerable distances between adjacent pinealocytes. Blood vessels were numerous, and bundles of myelinated nerve fibers were commonly encountered throughout the parenchyma. Intercellular clefts were particularly prominent adjacent to capillaries.

At the electron microscopic level, pinealocytes of the NOR group contained a variety of organelles including numerous Golgi bodies, mitochondria, and an abundant endoplasmic reticulum, predominantly of the smooth variety (Figs. 3a and 4a). Dense-cored vesicles were present

ABBREVIATIONS USED IN FIGURES

BV = Blood Vessel
CP = Cell Processes
DP = Deep Pineal
G = Glial Cell Nucleus
N = Pinealocyte Nucleus
Nu = Pinealocyte Nucleolus
SC = Superior Colliculus
SER = Smooth Endoplasmic Reticulum
SP = Superficial Pineal
g = Golgi Body
m = Mitochondria

PLATE I

Figure 1. A midsagittal section of the hamster brain, illustrating the entire pineal complex. A thin stalk (arrows) can be seen connecting the superficial and deep glandular portions. 11X.

Figure 2. Light micrographs taken from the superficial pineal glands of normal (a) and blinded (b) animals. The cellular components quantified at the light level are identified. Glial cell nuclei can be seen in the parenchyma (arrowheads). Pinealocyte nucleoli are indicated by arrows. 180X. Bar represents 50 µm.

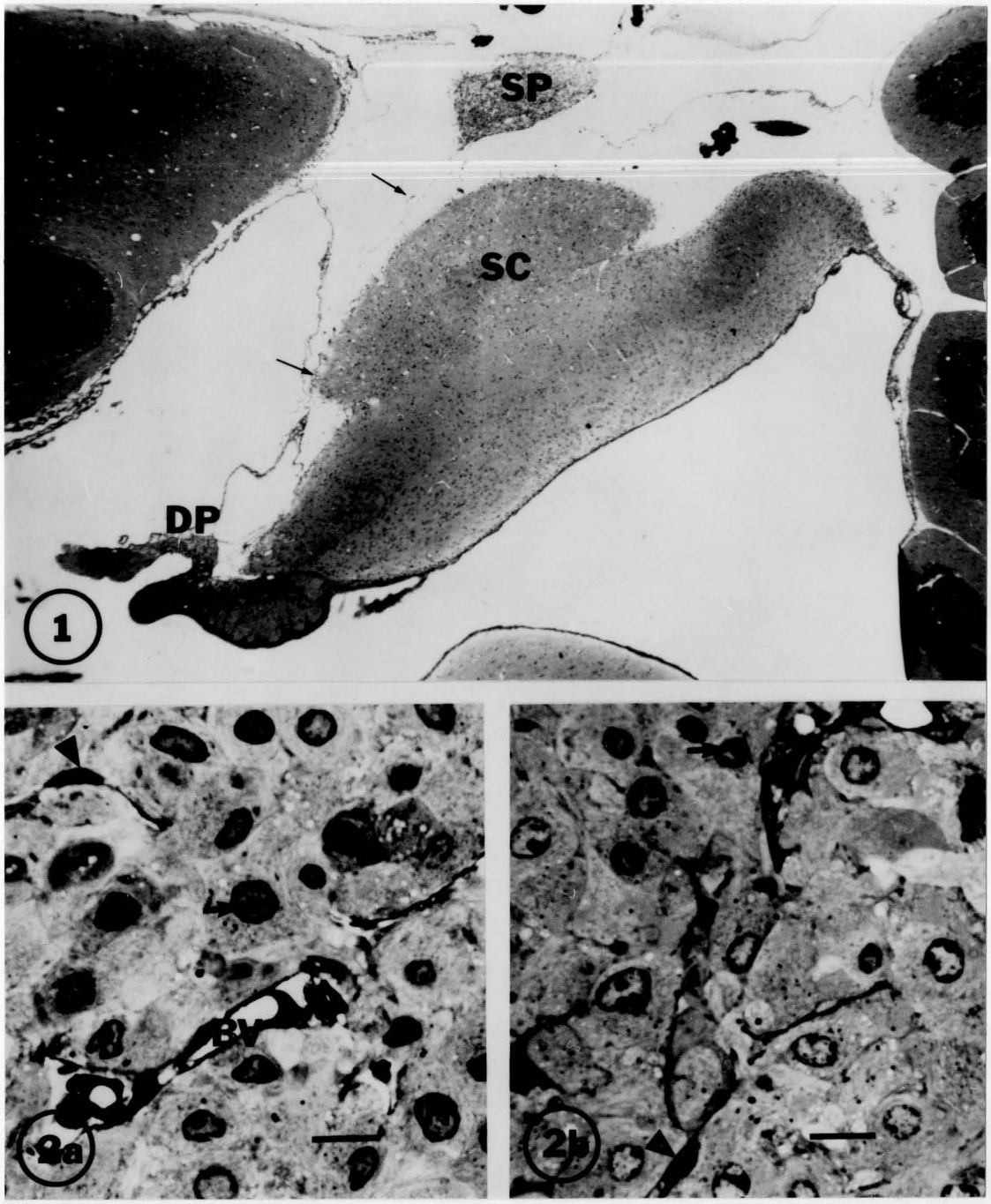

SP
SC
DP
1
BV
2a
2b

PLATE II

Figure 3. Low magnification electron micrographs depicting the pinealocytes of normal (a) and blinded (b) animals. Note large accumulation of smooth endoplasmic reticulum in the pinealocyte cell process of the normal hamster. 6900X. Bar represents one μm.

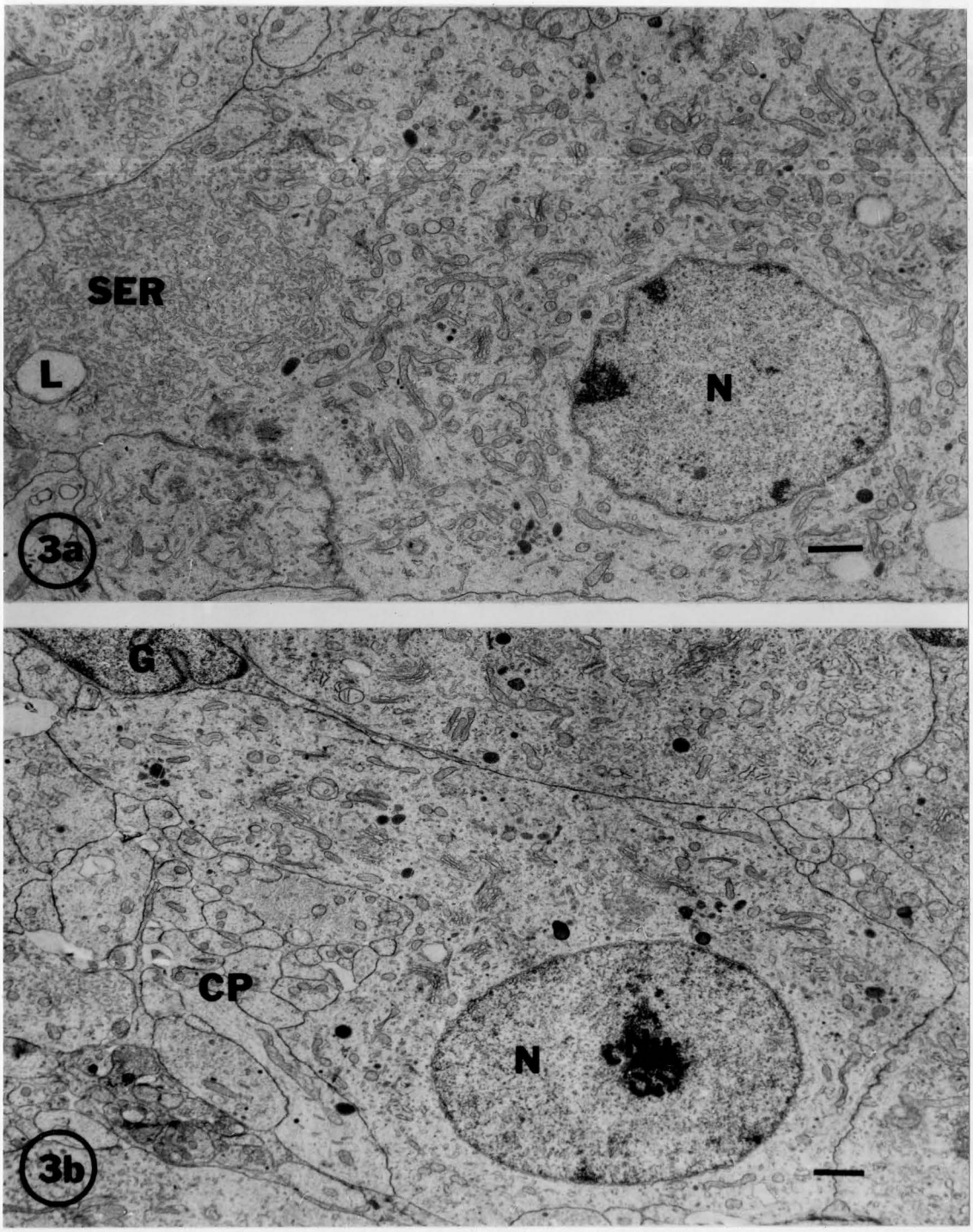
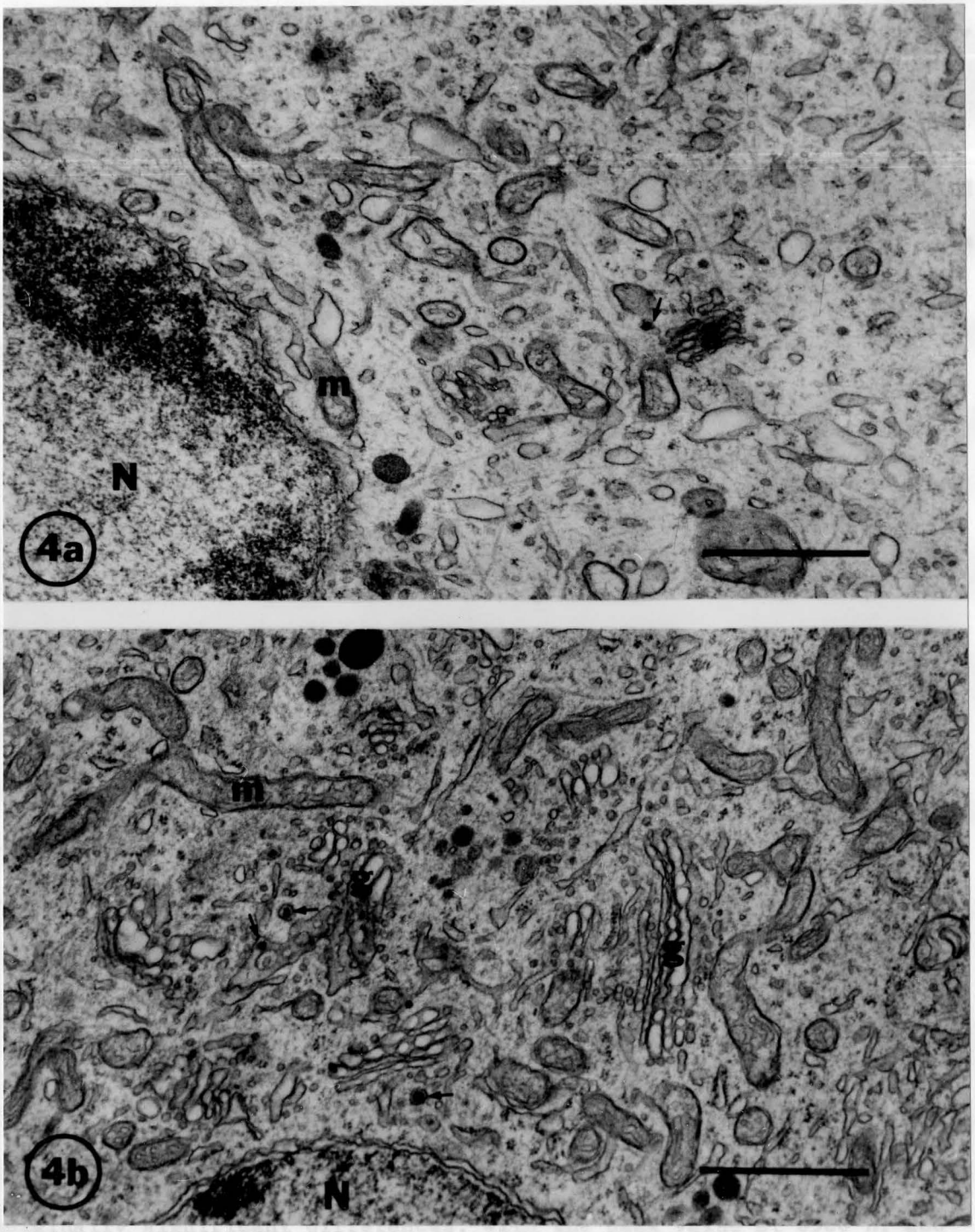

SER
L
N
3a
G
CP
N
3b

PLATE III

Figure 4. High magnification electron micrographs of the pinealocyte cytoplasm of normal (a) and blinded (b) animals. Golgi complexes are obviously more numerous in Figure 4b. Arrows indicate dense-cored vesicles. 22,800X. Bar represents one μm.


m
N
4a
m
4b
N

throughout the cytoplasm, and were sometimes closely related to the Golgi complex, from which they are presumed to originate. Dense bodies, multivesicular bodies and lipid droplets were less commonly seen. Synaptic ribbons ("vesicle-crowned rods") were usually located in the periphery of the cell. Pinealocytes of the ON group generally exhibited an expanded smooth endoplasmic reticulum, increased dense-cored vesicles and more prominent Golgi bodies (Figs. 3b and 4b).

Quantitative Analysis

The mean volume of the superficial pineal gland was estimated to be 151.5×10^6 μm^3 ($\pm 10.2 \times 10^6$ μm^3), comprising 90-94% of the total volume of the pineal complex (superficial and deep parts-Fig. 1). The volume occupied by pinealocytes (139.4×10^6 μm^3) was approximately 92% of the volume of the superficial pineal. Dividing this figure by the mean pinealocyte volume (7038 μm^3 - from Table I) gave an estimate of the total number of pinealocytes (20,000) within the superficial pineal gland. Volume fractions of the gland occupied by the remaining components were as follows: glial cells (2%), blood vessels (2%), and miscellaneous features including nerve bundles and intercellular spaces (4%).

Circadian Analysis of NOR Hamsters

Stereological measurements of pineal structure showed significant variations over a 24-hour period for both the nuclei and nucleoli of pinealocytes (Table I). The mean volume of pinealocyte nuclei exhibited marginally significant differences, with the peak value at 0500 h, and

ABBREVIATIONS USED IN TABLES

DB = Dense Bodies
DCV = Dense-cored Vesicles
MITO = Mitochondria
MVB = Multivesicular Bodies
Nv = Numerical Density (#/μm3)
RER = Rough Endoplasmic Reticulum
SER = Smooth Endoplasmic Reticulum
Sv = Surface Density (Surface Area/μm3)
Vv = Volume Density ($\mu m^3/\mu m^3$)
$\bar{v}$ = Mean Volume (μm3)

Table I. Means (± SEM) for light microscopic analysis of pinealocytes from untreated (NOR) animals over a 24-hour period. Pooled data from all sample times combined are given below for both untreated and blinded (ON) groups. Vv = $\mu m^3/\mu m^3$; Nv = $\#/\mu m^3$; $\bar{v}$ = μm^3.

TIME	PINEALOCYTE NUCLEI Vv	PINEALOCYTE NUCLEI Nv	PINEALOCYTE NUCLEI $\bar{v}$	PINEALOCYTE NUCLEOLI Vv	PINEALOCYTE NUCLEOLI Nv	PINEALOCYTE NUCLEOLI $\bar{v}$	PINEALOCYTE CYTOPLASM Vv	PINEALOCYTE CYTOPLASM $\bar{v}$
0100	0.048 ±.003	0.144 ±.010	340.17 ± 9.63	0.006 ±.000	0.806 ±.046	7.591 ±.445	0.873 ±.007	6627.10 ±538.02
0500	0.044 ±.004	0.130 ±.014	351.70 ±10.31	0.005 ±.001	0.684 ±.120	7.645 ±.376	0.889 ±.008	7685.31 ±783.36
0700	0.045 ±.002	0.145 ±.008	316.65 ± 8.76	0.005 ±.000	0.679 ±.064	7.696 ±.129	0.871 ±.009	6383.17 ±420.56
1300	0.046 ±.004	0.160 ±.017	291.20 ±13.38	0.007 ±.001	1.170 ±.123	6.860 ±.752	0.879 ±.006	6121.19 ±632.52
1900	0.043 ±.003	0.145 ±.015	310.23 ±16.17	0.005 ±.000	0.940 ±.089	5.569 ±.597	0.896 ±.004	7107.67 ±964.99
2100	0.045 ±.003	0.149 ±.015	312.77 ±16.58	0.006 ±.000	0.883 ±.112	7.199 ±.997	0.883 ±.008	6399.90 ±562.24
F value	0.30	0.53	2.91	3.59	3.62	1.74	1.90	0.73
p<	-	-	0.05	0.025	0.025	-	-	-
NOR	0.045 ±.001	0.146 ±.005	317.67 ± 6.16	0.006 ±.000	0.860 ±.046	7.093 ±.264	0.883 ±.003	6720.73 ±269.92
ON	0.045 ±.001	0.161 ±.005	296.18 ± 6.23	0.005 ±.000	0.533 ±.018	9.046 ±.252	0.863 ±.004	5878.83 ±167.47
t value	0.32	2.07	4.15	2.86	6.51	5.27	4.13	2.65
p<	-	0.05	0.001	0.01	0.001	0.001	0.001	0.01

PLATE IV

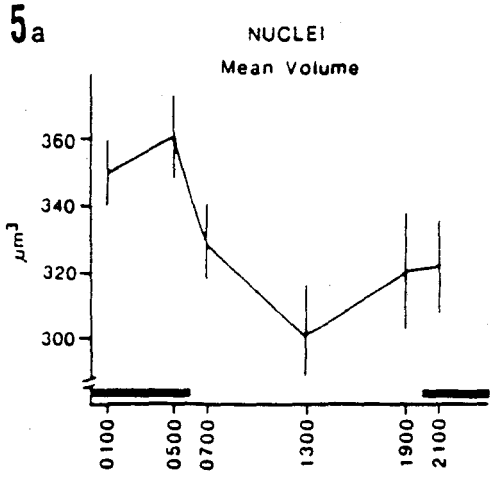
Figure 5. Morphological analysis of pinealocyte nuclei and nucleoli of normal animals over a 24-hour light:dark cycle. For all graphs, the solid bar indicates the duration of the dark period. Each point is the mean of six animals. The vertical lines represent $\pm$ the standard error of the mean. Additional information pertinent to the graphs is contained in the Results section and in Tables I, III and IV.

5a. Nuclear mean volume, one way analysis of variance, $p<0.05$.

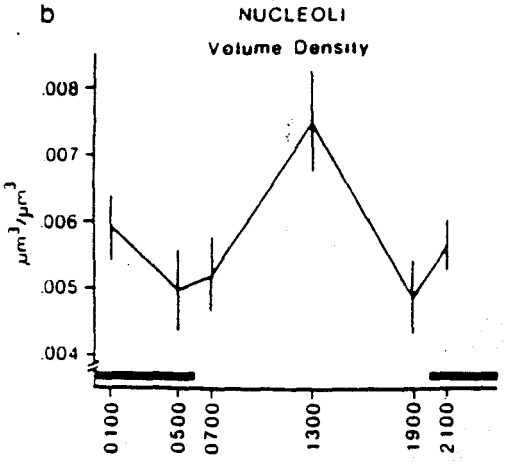
5b. Volume density of nucleoli, $p<0.025$.

5c. Numerical density of nucleoli, $p<0.025$.

5a


NUCLEI
Mean Volume
360
340
320
300
μm³
0100
0500
0700
1300
1900
2100

b


NUCLEOLI
Volume Density
.008
.007
.006
.005
.004
μm³/μm³
0100
0500
0700
1300
1900
2100

c

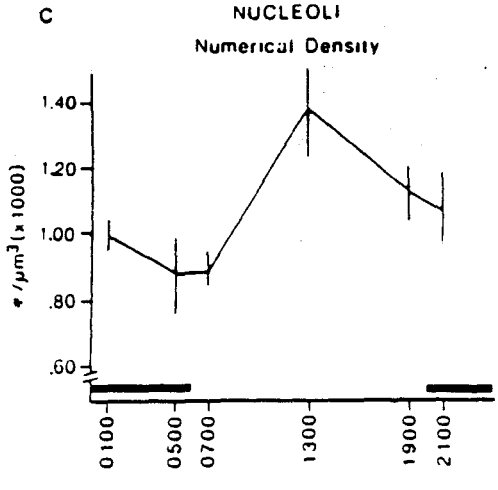

NUCLEOLI
Numerical Density
1.40
1.20
1.00
.80
.60
#/μm³(x1000)
0100
0500
0700
1300
1900
2100

Table II. Means (±SEM) for light microscopic analysis of glia and blood vessels from untreated (NOR) animals over a 24-hour period. Pooled data from all sample times combined are given below for both untreated and blinded (ON) groups. Vv = $\mu m^3/\mu m^3$; Nv = $\#/\mu m^3$; $\overline{v}$ = μm^3.

	GLIAL CELLS			BLOOD VESSELS		
TIME	Vv	Nv	$\overline{v}$	Vv	Nv	$\overline{v}$
0100	0.013 ±.002	0.112 ±.016	123.63 ± 9.20	0.012 ±.003	0.013 ±.005	1890.63 ±684.18
0500	0.011 ±.001	0.101 ±.011	113.83 ± 7.04	0.012 ±.002	0.016 ±.002	1605.76 ±541.32
0700	0.013 ±.002	0.121 ±.016	113.35 ± 2.90	0.010 ±.003	0.014 ±.002	1492.50 ±652.04
1300	0.015 ±.002	0.138 ±.018	113.96 ±10.53	0.013 ±.004	0.016 ±.004	832.37 ±305.50
1900	0.012 ±.001	0.102 ±.012	110.93 ± 3.05	0.010 ±.002	0.014 ±.003	996.61 ±301.89
2100	0.012 ±.001	0.118 ±.013	102.03 ± 4.67	0.014 ±.006	0.017 ±.003	1574.84 ±836.38
F value	.86	.90	1.01	.15	.33	.47
p<	-	-	-	-	-	-
NOR	0.013 ±.000	0.115 ±.006	112.96 ± 2.81	0.012 ±.001	0.015 ±.001	1398.80 ±230.56
ON	0.016 ±.001	0.170 ±.010	100.60 ± 3.52	0.016 ±.001	0.019 ±.001	1497.18 ±239.15
t value	3.515	4.708	2.705	2.064	2.860	.29
p<	0.001	0.001	0.01	0.05	0.01	-

the trough at midlight (Fig. 5a). Nucleoli in these cells showed a significant rise during the middle of the light phase in both volume density (Fig. 5b) and numerical density (Fig. 5c). Quantitative changes in glial cells, blood vessels, and the cytoplasm of pinealocytes were not detectable over a light:dark cycle (Table II).

At the subcellular level, pinealocytes showed significant daily changes in the surface densities of both rough and smooth endoplasmic reticulum, the volume densities of Golgi bodies and dense bodies, and number of granular vesicles per unit area of cytoplasm (Tables III and IV). There was a trend for maximal values of these organelles to occur during either the latter part of the light phase or early dark (Figs. 6b-d, 7a). An exception was the smooth endoplasmic reticulum, which peaked during midlight (Fig. 6a). Further examination of the Golgi apparatus determined that circadian fluctuations in its volume density were due, at least in part, to changes in the size of this organelle. Measurements of both the length and width of individual Golgi revealed a gradual increase in these dimensions over the course of the photophase (Figs. 7b and 7c). The number of clear vesicles associated with each Golgi apparatus followed a similar pattern over the light:dark cycle (Fig. 7d). Plotting the data revealed no differences in the values for male and female hamsters.

Circadian Analysis of ON Hamsters

Morphometric analysis of pineal structure in ON animals failed to detect any rhythms, presumably as the result of free-running rhythms

Table III. Means (±SEM) for electron microscopic analysis of organelles (excluding Golgi) from untreated (NOR) animals over a 24-hour period. Pooled data from all sample times combined are given below for both untreated and blinded (ON) groups. Vv = $\mu m^3/\mu m^3$; Sv = Surface Area/μm^3.

TIME	MITO Vv	SER Sv	RER Sv	DCV $\#/\mu m^2$	LIPID Vv	DB Vv	MVB Vv
0100	0.100 ±.006	6.106 ±.416	0.498 ±.068	0.101 ±.009	0.006 ±.001	0.008 ±.001	0.001 ±.000
0500	0.095 ±.008	5.475 ±.436	0.370 ±.062	0.102 ±.009	0.007 ±.002	0.010 ±.001	0.001 ±.000
0700	0.094 ±.004	6.765 ±.194	0.276 ±.059	0.115 ±.010	0.004 ±.002	0.009 ±.002	0.001 ±.000
1300	0.095 ±.010	7.022 ±.218	0.258 ±.069	0.117 ±.000	0.004 ±.002	0.008 ±.001	0.000 ±.000
1900	0.094 ±.004	5.728 ±.183	0.464 ±.027	0.184 ±.017	0.004 ±.001	0.011 ±.001	0.002 ±.000
2100	0.098 ±.007	4.840 ±.202	0.591 ±.042	0.140 ±.013	0.002 ±.001	0.013 ±.001	0.001 ±.000
F value	0.11	7.65	5.38	8.05	1.08	3.24	1.00
p<	-	0.001	0.005	0.001	-	0.025	-
NOR	0.096 ±.003	5.989 ±.168	0.410 ±.030	0.126 ±.006	0.004 ±.001	0.010 ±.001	0.001 ±.000
ON	0.091 ±.002	7.368 ±.190	0.543 ±.032	0.173 ±.010	0.005 ±.001	0.014 ±.001	0.001 ±.000
t value	1.643	5.346	3.021	4.065	0.714	3.615	0.352
p<	-	0.001	0.01	0.001	-	0.001	-

PLATE V

Figure 6. Morphological analysis of cytoplasmic organelles (excepting Golgi) of normal animals over a 24-hour light:dark cycle.

6a. Surface area of smooth endoplasmic reticulum, $p<0.001$.

6b. Surface area of rough endoplasmic reticulum, $p<0.005$.

6c. Number of dense-cored vesicles per μm^2 of test cytoplasm, $p<0.001$.

6d. Volume density of dense bodies, $p<0.025$.

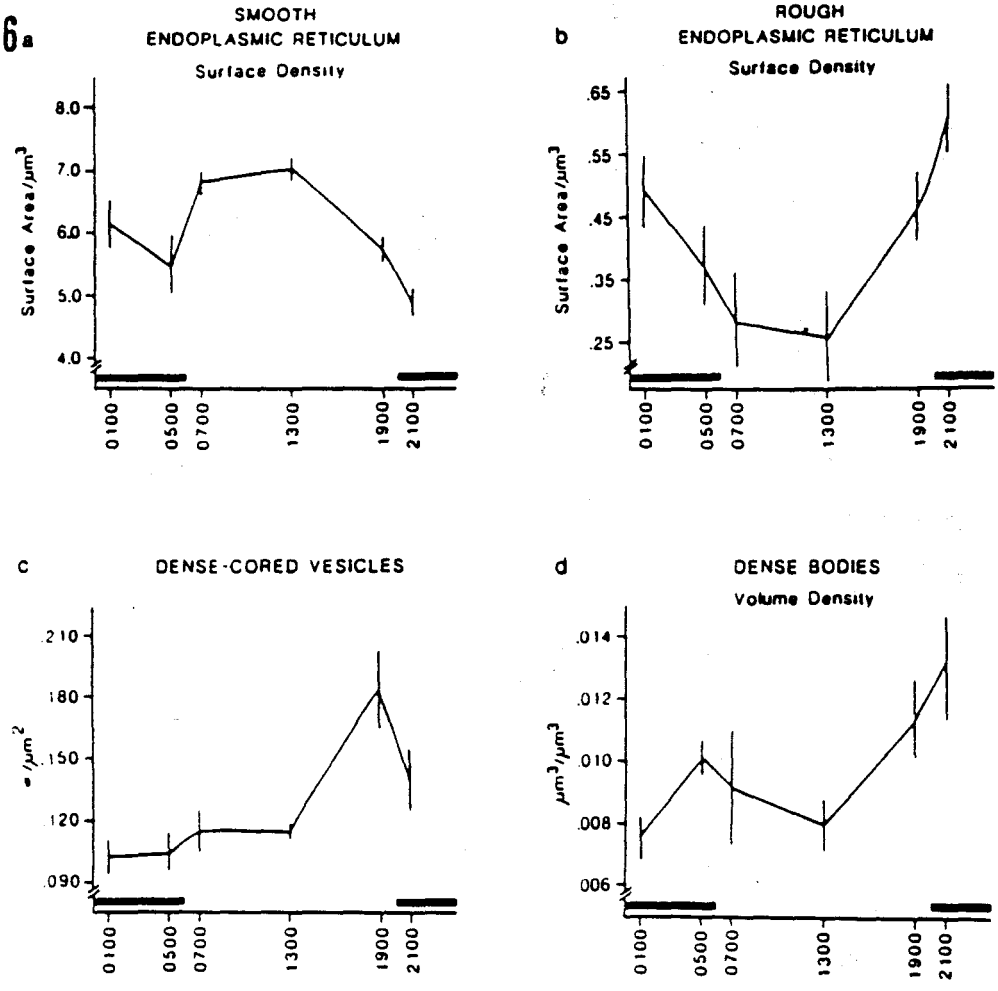

6a
SMOOTH
ENDOPLASMIC RETICULUM
Surface Density
Surface Area/μm³
8.0
7.0
6.0
5.0
4.0
b
ROUGH
ENDOPLASMIC RETICULUM
Surface Density
.65
.55
.45
.35
.25
0100
0500
0700
1300
1900
2100
c
DENSE-CORED VESICLES
.210
.180
.150
.120
.090
d
DENSE BODIES
Volume Density
μm³/μm³
.014
.012
.010
.008
.006

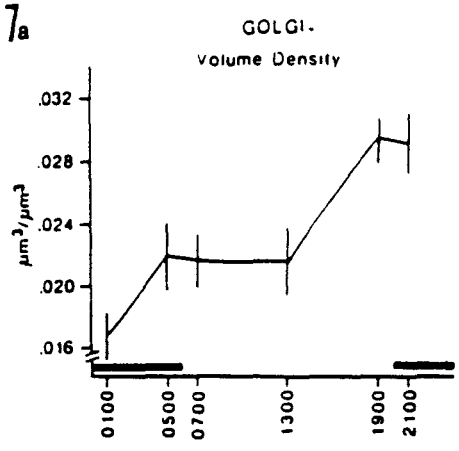
Table IV. Means (± SEM) for electron microscopic analysis of Golgi bodies from untreated (NOR) animals over a 24-hour period. Pooled data from all sample times combined are given below for both untreated and blinded (ON) groups. Vv = $\mu m^3/\mu m^3$.

TIME	Vv	#/Area	WIDTH	LENGTH	NUMBER SACCULES	NUMBER VESICLES
0100	0.014 ±.001	1.608 ±.165	0.304 ±.012	0.545 ±.021	4.353 ±.100	11.201 ±1.079
0500	0.020 ±.002	2.267 ±.162	0.282 ±.011	0.529 ±.023	4.455 ±.086	12.532 ± .752
0700	0.019 ±.002	2.208 ±.220	0.269 ±.014	0.547 ±.029	4.598 ±.057	14.005 ±1.350
1300	0.019 ±.002	1.908 ±.181	0.288 ±.011	0.626 ±.023	4.530 ±.139	15.556 ± .934
1900	0.027 ±.002	2.240 ±.086	0.332 ±.024	0.699 ±.032	4.721 ±.070	17.631 ±1.170
2100	0.027 ±.002	1.983 ±.174	0.329 ±.010	0.651 ±.026	4.382 ±.081	15.366 ± .864
F value	7.92	2.29	3.23	7.50	2.23	4.88
p<	0.001	-	0.025	0.001	-	0.005
NOR	0.021 ±.001	2.036 ±.075	0.301 ±.007	0.602 ±.014	4.506 ±.041	14.382 ± .531
ON	0.028 ±.001	2.114 ±.102	0.294 ±.004	0.652 ±.038	4.521 ±.048	16.457 ± .425
t value	4.298	6.092	0.882	1.206	0.223	3.006
p<	0.001	0.001	-	-	-	0.01

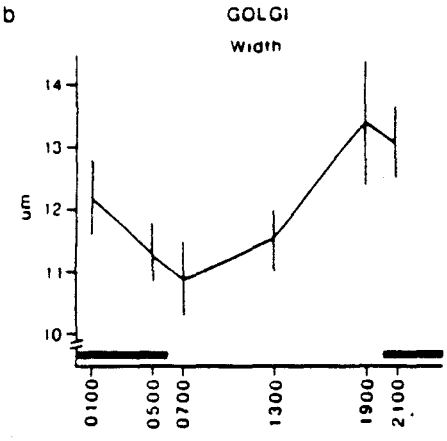
PLATE VI

Figure 7. Morphological analysis of the Golgi bodies of normal animals over a 24-hour light:dark cycle.
7a. Volume density, $p<0.001$.
7b. Width, $p<0.025$.
7c. Length, $p<0.001$.
7d. Number of associated clear vesicles per Golgi body, $p<0.005$.

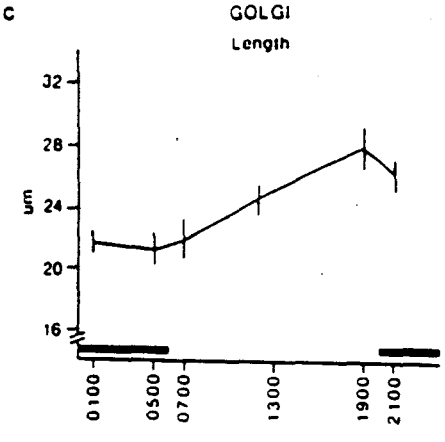
7a


GOLGI
Volume Density
.032
.028
.024
.020
.016
μm³/μm³
0100
0500
0700
1300
1900
2100

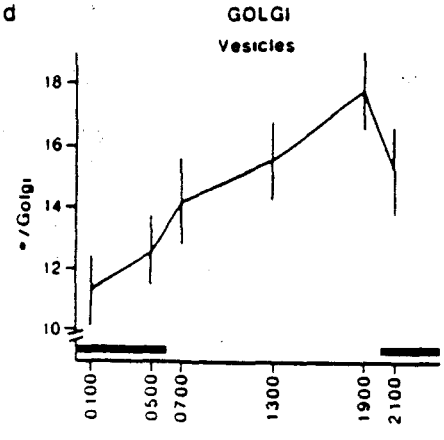
b


GOLGI
Width
14
13
12
11
10
um
0100
0500
0700
1300
1900
2100

c


GOLGI
Length
32
28
24
20
16
um
0100
0500
0700
1300
1900
2100

d


GOLGI
Vesicles
18
16
14
12
10
•/Golgi
0100
0500
0700
1300
1900
2100

within the pineal glands of the enucleated animals (Ralph et al., 1972). As described in the methods, data from all sample times were subsequently pooled for comparison with pooled values obtained from NOR animals.

Statistical Analysis of Structural Changes Following ON

Optic enucleation caused a significant decline in the volume density of nucleoli and cytoplasm, the numerical density of nucleoli and the mean volume of nuclei and cytoplasm (Table I). A higher numerical density of nuclei and larger mean volume of nucleoli resulted from blinding. This treatment also caused a significant increase in both size and number of glial cells and the volume density and numerical density of blood vessels (Table II).

Quantitative analyses at the ultrastructural level suggested that blinding had no effect on the volume densities of mitochondria, lipid inclusions and multivesicular bodies (Table III). Greater values were found for the volume densities of both Golgi bodies and dense bodies, the surface density of the smooth and rough endoplasmic reticulum and the number dense-cored vesicles per unit area of cytoplasm. The greater volume density of the Golgi bodies was apparently due to increased number rather than size of this organelle (Table IV). The number of Golgi-associated clear vesicles also demonstrated a significant increase.

Analysis of grouped data from NOR animals showed no significant differences based on sex. In contrast, when analyzed with the Student's

t test, females from the ON group had a greater volume density of pinealocyte nuclei ($p<.05$) and mitochondria ($p<.02$) compared to males.

Discussion

Disproportionate variations relative to body size are found in the volume of the pineal gland of mammals, with glandular size differing as much as 125-fold among species (Quay, 1980). Similar disparities exist in the number of pinealocytes within the gland, varing seven-fold between the collared lemming (Quay, 1978) and white-footed mouse (Quay, 1956). The estimated volume of the superficial pineal gland in the present study approximated that previously calculated for the golden hamster (Quay, 1980). However, the total number of pinealocytes in the superficial gland differed five-fold from a previously reported estimate for the collared lemming, which possesses a pineal of comparable size (Quay, 1978). This implies that the hamster pineal gland contains larger, but fewer pinealocytes. Species-specific differences have been correlated with habitat (Quay, 1980), although the relationship between the number and size of pinealocytes and the levels of metabolic activity has yet to be elucidated.

Correlation of pineal synthetic processes with changes in pinealocyte morphology over a 24-hour span, or following blinding, requires localization of these processes to specific cellular

structures. The pineal is generally acknowledged to synthesize at least two classes of compounds, indoles and peptides (see Reiter, 1981a). Few direct correlations have been drawn between specific organelles and indole synthesis, since indole production is believed to occur diffusely within the cytosol (Romijn et al., 1977a). Serotonin has, however, been demonstrated within the dense-cored vesicles (Lu and Lin, 1979), and the production of granular vesicles by the Golgi apparatus (Karasek et al., 1982a) implicates that organelle in indole production as well. Involvement of the smooth endoplasmic reticulum has also been postulated, although its role in the indole metabolic pathway has not yet been defined (Romijn, 1975). In contrast, peptide formation is believed to follow a clearly outlined pathway involving synthesis upon the ribosomes and transport of the product to the Golgi complex for packaging (Juilllard and Collin, 1980). Peptide products could thus be contained within the Golgi-associated clear vesicles and within dense-cored vesicles. Granular vesicles have been shown to contain a proteinaceous compound, possibly bound to serotonin (Juillard, 1979).

The present study demonstrated strong rhythms in pinealocyte structure reflecting the circadian nature of the gland. Important indicators of cellular activity, such as nuclei, nucleoli and Golgi bodies, exhibited significant rhythmic variation in their structure. It was possible to correlate several of those rhythms with known metabolic processes, establishing structural markers for cellular function.

Reported mean nuclear volumes for superficial pinealocytes vary between 250 μm^3 in the rat and Chinese hamster (Matsushima et al., 1983; Boeckman, 1980) and 400 μm^3 in the gerbil (Welsh et al., 1979). The values for nuclear size in this report were within this range and exhibited 24-hour fluctuations with peak values toward the end of the scotophase. The rhythm in nuclear volume was similar to the pattern of melatonin production in hamsters maintained on a 14h:10h light:dark cycle (Panke et al., 1980). Our findings of circadian variation in nuclear volume are not, however, in agreement with earlier reports. Pinealocytes in the rat (Quay and Renzoni, 1966) and Chinese hamster (Matsushima et al., 1983) showed midday peaks in nuclear volume, while nuclei in the gerbil (Welsh et al., 1979) and a hamster (Vollrath, 1979) did not exhibit variation over a light:dark cycle. Delineation of a relationship between nuclear size and the metabolic activity of pinealocytes is further complicated by recent reports of ultradian, as well as circadian, rhythms in pinealocyte nuclear size in the rat (Becker and Vollrath, 1983; Diehl, 1981).

Unlike the nuclei, nucleoli did not exhibit a circadian rhythm in their mean volume in this study, although rhythms in nucleolar volume density and numerical density were found. Since the rhythm in volume density closely followed that of numerical density, peak midday values were probably a result of an increase in number, rather than size, of the nucleoli. Midday peaks in the diameter of nucleoli of rat pinealocytes (Quay and Renzoni, 1966) were previously found to coincide

with the maximal values for pineal RNA content (Nir et al., 1971). A more recent study of the rat pineal gland also reported highest nucleolar values during the light period (Lew et al., 1982).

In contrast to other studies, the cytoplasmic volume of hamster pinealocytes in the present report lacked rhythmicity. The cytoplasmic volume of pinealocytes in the gerbil peaked at middark (Welsh et al., 1979), while in the Chinese hamster, maximal pinealocyte size occurred at midlight, corresponding to peak nuclear volumes (Matsushima et al., 1983). Alterations in the volume of the cytoplasm were related by the authors to variations in pinealocyte synthetic activity. Species-specific differences make it difficult to extrapolate a consistent relationship between pineal activity and cell size.

Considering the circadian production of pineal indoles in the golden hamster (Reiter, 1981b), it is noteworthy that the organelles commonly associated with secretory activity (i.e. endoplasmic reticulum, Golgi bodies and dense-cored vesicles) showed pronounced 24-hour rhythms in this study. The rhythm in rough endoplasmic reticulum showed maximal values during the early to middle dark period. Despite differences in the variables measured (surface area vs. volume density), the rhythm reported here for the rough endoplasmic reticulum is similar to that reported for the gerbil (Welsh et al., 1979). Since protein synthesis increases prior to melatonin production (Morrissey and Lovenberg, 1978), the peak values for the endoplasmic reticulum may reflect enhanced

protein synthesis during the dark phase, possibly as a result of increased enzyme production.

The smooth endoplasmic reticulum has been associated with the degree of pinealocyte activity (Lu and Lin, 1979) and rate of indoleamine production (Romijn, 1975). In this study, the rhythm in the smooth endoplasmic reticulum was biphasic, with troughs at the light to dark and dark to light interphases. The pattern of the rhythm is similar to the pattern of indole production by hamster pinealocytes, since melatonin and serotonin peak during the dark and light periods, respectively, with declines at the interphases. The rhythm in the smooth endoplasmic reticulum in the gerbil pineal showed maximal values during the early dark period only (Welsh et al., 1979).

Rhythms in the volume density of the Golgi complex and number of dense-cored vesicles followed similar patterns. The increased volume density of the Golgi complex at the end of the photophase was due to a greater size of this organelle. Enlargement of the Golgi body may be indicative of enhanced activity, since there was a corresponding increase in the number of dense-cored vesicles and Golgi-associated clear vesicles. On the other hand, arrested transport of substances from the Golgi may account for the greater size of this organelle and increased number of vesicles. Peak values for the dense-cored vesicles coincided with maximum levels of pineal serotonin in the hamster (Steinlechner et al., 1983). Maximal numbers of granular vesicles have been previously reported to occur middle to late in the light period in

mice (Benson and Krasovich, 1977; Kachi, 1979) and rabbits (Romijn et al., 1976). In contrast to the present study, the dense-cored vesicles of hamsters maintained on a 14:10 light:dark cycle did not show a rhythm (Krasovich and Benson, 1982). This difference may, however, be the result of evaluating the granular vesicle population in different areas of the pinealocyte. Our study focused upon the perikaryonal cytoplasm, while Krasovich and Benson (1982) examined pinealocyte processes adjacent to capillaries.

The volume density of the pinealocyte dense bodies also exhibited a strong circadian rhythm. The dense body population of pinealocytes has been postulated to be heterogenous in nature (Lu and Lin, 1979). The authors determined that some of the bodies were lysosomes on the basis of a positive acid phosphatase reaction, while others were speculated to be storage sites for proteins, polypeptides, or catecholamines. Circadian rhythms in the volume density of dense bodies may thus indicate an increase in peptidergic synthesis at the light:dark interphase.

Many of the pinealocyte structures which exhibited pronounced circadian rhythms under a 14h:10h light:dark cycle responded strongly to the stimulus of blinding. Optic enucleation frequently caused an increase in the values of most structures, although the nuclei and cytoplasm of pinealocytes showed decreased volumes after blinding.

In many cell types, nuclear volume corresponds to cellular activity (Hildebrand, 1980). Since blinding is considered stimulatory

to pineal function, an increase in nuclear volume might be expected as a result of that treatment. In this study, however, nuclear volume decreased following blinding. A similar decline in nuclear volume was reported by Barratt et al. (1977) in hamsters maintained on a 'stimulatory' lighting regime (1h:23h) for a period of eight weeks. Administration of exogenous melatonin (Barratt et al., 1977) or bovine pineal extract (Holmgren et al., 1960) also decreased nuclear volume in pinealocytes, a result attributed to a possible feedback control of the pineal by its own secretions. The decline in nuclear volume suggests decreased pineal metabolic activity which may occur when gonadal atrophy has maximized after eight weeks blindness (Reiter, 1968). It is not known whether decreased nuclear volumes are present in the weeks immediately following optic enculeation.

The increase in nucleolar mean volume after blinding was accompanied by a decline in numerical density, resulting in larger, but fewer, nucleoli. Freire and Cardinali (1975) reported that pinealocyte nucleoli in rats exposed to constant darkness were more prominent than those of control animals. Since nucleolar volume has been postulated to enlarge as a result of increased RNA production prior to protein synthesis (Nir et al., 1971), greater nucleolar volume in the present study may indicate increased synthetic activity by pinealocytes after blinding.

The hypothetical 'stimulatory' condition resulting from optic encleation is further supported by changes in the secretory organelles

as well as the dense bodies. The endoplasmic reticulum of the superficial pineal showed a greater surface density after blinding, with the most pronounced increase in the smooth portion. Hypertrophy of the smooth endoplasmic reticulum confirms findings in blinded hamsters (Lin et al., 1975) or those maintained on a short (one hour) photoperiod (Barratt et al., 1977). The amount of rough endoplasmic reticulum has been shown to increase in rats exposed to constant darkness (Freire and Cardinali, 1975).

Hypertrophy of the Golgi apparatus in response to a 'stimulatory' lighting regime (e.g. blinding or constant darkness) has been reported in a variety of species (mice: Upson and Benson, 1977; rat: Freire and Cardinali, 1975; hamster: Clabough, 1971) and is generally equated with increased glandular activity. In this study, the Golgi bodies did not show evidence of hypertrophy after blinding. The greater volume density of the Golgi complexes was due to an increase in number rather than size. The corresponding increase in the number of Golgi-associated clear vesicles and dense-cored vesicles may, however, imply enhanced Golgi activity. Increased numbers of clear and dense-cored vesicles found in hamsters after blinding were postulated to be indicative of increased production of a pineal antigonadotrophic substance (Sheridan, 1975; Clabough, 1971). Conversely, dense-cored vesicles were found to decline in number in optically enucleated hamsters, but that study focused upon granular vesicles located in the perivascular pinealocyte processes rather than within the perikaryon (Krasovich and Benson,

1982). Clear and dense-cored vesicles declined in blinded mice, a decrease attributed to enhanced release of pineal secretory product (Upson and Benson, 1977). The greater number of vesicles found in this study, when considered in conjunction with the increases in the endoplasmic reticulum and the Golgi bodies, indicates greater metabolic activity of the gland after blinding. Similarly, higher values for the dense bodies, possible storage sites for the products of pineal metabolism (Lu and Lin, 1979), may also indicate increased synthetic activity by the gland.

In summary, the superficial pineal gland of the golden hamster exhibited quantitative changes in structure that could be correlated with the circadian metabolic rhythms of the organ. Cellular components involved in synthesis and secretion showed the most pronounced rhythms over a 24-hour light:dark cycle. The observation that pinealocytes in blinded animals had significantly larger nucleoli and expanded endoplasmic reticulum, as well as increased numbers of Golgi bodies and associated vesicles, is further evidence in support of the hypothesis that synthesis and intracellular transport of pineal factors are related to these organelles.

Chapter IV

Title: Morphometric Analysis of the Pineal Complex of the Golden Hamster over a 24-hour Light:Dark Cycle. II. The Deep Pineal Gland in Untreated and Optically Enucleated Animals.

Abstract

The deep pineal gland of golden hamsters was morphometrically analyzed and quantitatively compared with the superficial pineal under 14h:10h lighting regimen and following blinding of the animals. The deep pineal comprised 6-10% of the total pineal parenchymal tissue. Pinealocytes of the deep gland were smaller than the cells of the superficial pineal, and showed a greater percent volume of Golgi bodies, rough endoplasmic reticulum and dense-cored vesicles. Twenty-four hour variations in the volume and numerical density of nucleoli and volume density and number per area of Golgi bodies were found in deep pinealocytes. These fluctuations were out of phase with comparable rhythms in the superficial pineal gland, suggesting that distinct subpopulations of pinealocytes are present within the respective parts. Blinding resulted in decreased nuclear and nucleolar mean volume, while the amount of smooth endoplasmic reticulum, Golgi bodies, dense bodies and dense-cored vesicles increased significantly. Marginal increases

were seen in the volume density of mitochondria and lipid droplets. The greater abundance of those organelles involved in synthesis and secretion suggests enhanced cellular activity after blinding. Many of the morphological responses are similar to alterations in the pinealocytes of the superficial pineal following optic enucleation. Differences in cell structure between the superficial and deep pineals may indicate distinct subpopulations of pinealocytes within the respective parts.

Key Words: Morphometry, Deep Pineal, Golden Hamster, Circadian Rhythm, Optical Enucleation

Introduction

The pineal complex of several species (vole, Hewing, 1981; rat, Boeckman, 1980; gerbil, Japha et al., 1977; golden hamster, Sheridan and Reiter, 1970a; white-footed mouse, Quay, 1956), possesses a distinct and separate deep component, located adjacent to the third ventricle and connected to the superfical gland by a stalk. The superficial and deep pineals arise from a common embryological anlage (Sheridan and Walker, 1975; Ariens Kappers, 1960) with subsequent dorsal migration of the superficial pineal to a location below the cranium. Both glands are innervated by post-ganglionic sympathetic fibers, with nerve fibers

passing via the stalk from the superficial to deep glands (Nielsen and Moller, 1978; Reiter and Hedlund, 1976; Bjorklund et al., 1972).

Due to the small size and relative inaccessibility of the deep pineal, little research to date has focused upon the function of the gland and its possible relationship to the superficial pineal. Recent results indicate, however, that the deep gland does not play a role in short-day induced testicular regression in the golden hamster (Anderson and Turek, 1983). At the biochemical level, analyses have established the presence of the indoles serotonin (Wiklund, 1974) and melatonin (Sheridan and Rollag, 1983) within the deep pineal.

Comparative anatomical studies of superficial and deep pineals at the cellular level has suggested that they are structurally similar. The deep pineal, however, contains fewer blood vessels (Boeckman, 1980) and more glial cells (Sheridan and Walker, 1975), and the pinealocytes are generally smaller than those of the superficial part (Boeckman, 1980; Vollrath, 1979; Quay, 1956). Furthermore, the intracellular distribution of pinealocyte synaptic ribbons differs between the deep and superficial glands (Hewing, 1980).

Melatonin is known to be produced by the deep pineal of the golden hamster in a circadian pattern similar to that seen in the superficial gland (Sheridan and Rollag, 1983), but structural confirmation of glandular rhythms is scarce. Rhythms in nuclear volume were seen in the deep pinealocytes of the rat (Boeckman, 1980) and hamster (species

unknown; Vollrath, 1979), but the rhythmic patterns differed between the two species. No other cellular structure has been examined for morphological rhythms.

The effect on the deep pineal of a lighting regimen that is considered stimulatory to the superficial gland has also not been fully examined. Serotonin levels increase in the area of the deep pineal in rats kept in constant darkness (Moore, 1975) and increases were seen in the smooth endoplasmic reticulum, dense-cored vesicles and synaptic ribbons of the deep pinealocytes of golden hamsters kept under similar conditions (Hewing, 1980).

The present study sought to address the following questions: 1) how do cells of the superficial and deep pineals compare in terms of their organellar composition, 2) does the deep pineal show structural evidence of a circadian rhythm, and 3) how do the cells of the deep pineal respond under experimental conditions known to be stimulatory to the function of the superficial gland. In search of answers to these questions, we undertook morphometric analyses of both the superficial and deep pineals under normal lighting conditions and following blinding. Those results on the superficial pineal gland are presented in the preceding paper. Here we report our findings on the deep pineal gland.

Materials and Methods

Additional details regarding the following methods are contained within the preceding paper (Dombrowski and McNulty, Chapter III).

Volume of the Pineal Complex

Using the Zeiss Videoplan, data were obtained from four additional animals regarding the total volume of the deep pineal, and its percent volume in the pineal complex.

Morphometric Analysis

Experimental Groups

Seventy-two golden hamsters, housed four to five per cage were entrained to a 14h:10h light:dark cycle (lights = 0600 to 2000 h). Food and water were available ad libitum. The hamsters were divided into two experimental groups. The first group of 36 untreated (normal, NOR) hamsters, (18 male and 18 female) were sacrificed over the light:dark cycle used for entrainment. The second group of 36 animals (18 male and 18 female) had been optically enucleated (ON) eight weeks prior to sacrifice. All animals were sacrificed over a 24-hour period at 0100, 0500, 0700, 1300, 1900 and 2100 h following anesthesia with sodium pentobarbital, i.p.

Microscopy

The two groups were sacrificed by cardiac perfusion with a phosphate buffer containing 1% glutaraldehyde:1.25% paraformaldehye (330 mOsm, pH 7.4). After dissection, both pineals were placed

in a 4% glutaraldehyde:buffer solution, then post-fixed in 1% osmium tetroxide. After dehydration in a graded series of acetone, deep pineals were embedded in Araldite 502 (details in Appendix A). Tissue was oriented to permit cutting of cross sections. Deep pineals were serially cut at one micron, mounted on slides and stained with toluidine blue. Beginning at the caudal tip of the gland, every tenth section was photographed with a Zeiss Ultraphot II, and printed at a final magnification of 1000 X.

Thin sections for electron microscopy (70-80 nm) were taken from an area of the gland immediately caudal to the habenular commissure, and stained with uranyl acetate and lead citrate. An area of pinealocyte cytoplasm containing a Golgi complex and part of the nucleus was photographed with an RCA EMU 3F electron microscope and printed at a final magnification of 40,000 X. Twenty micrographs per gland were used for stereological analysis.

Stereology

Variables analyzed for light microscopy included volume density, numerical density and mean volume of pinealocyte nuclei and nucleoli, glial cells, blood vessels and miscellaneous structures, and the volume density and mean volume of the pinealocyte cytoplasm.

Ultrastructurally, the volume densities of the mitochondria, lipid droplets, dense bodies, multivesicular bodies and Golgi complexes were estimated. The surface densities of the smooth and rough endoplasmic reticulum and number of dense-cored vesicles within a test area of

cytoplasm were calculated. Golgi bodies were further analyzed for the number per test area, width, length and number of saccules, and number of Golgi-associated clear vesicles.

Statistical Analysis

Variables in each experimental group were averaged to determine the mean and SEM for each animal, then grouped according to the time of sacrifice. The data were analyzed with the one-way analysis of variance to determine significant differences over a 24-hour period.

Data from each variable were then pooled according to experimental group, and values for NOR and ON animals compared using a Student's t test to detect structural alterations resulting from blinding. Sex differences within each group were analyzed with the Student's t test.

Results

The deep pineal gland of hamsters exhibited a homogeneously stained population of pinealocytes (Figs. 1a and 1b). The nuclei were spherical in shape with the nuclear envelope usually indented along one side. Nucleoli were prominent and there were frequently more than one per nucleus. Glial cells were common, and easily identified by their oval or irregularly shaped nuclei and relatively sparse cytoplasm. These cells were distributed throughout the parenchyma, although they appeared to be more numerous in the area of the gland adjacent to the habenular commissure. Blood vessels were often encountered, as were large bundles of myelinated nerve fibers.

The pinealocytes were irregularly shaped cells, with long cytoplasmic processes (Figs. 2a and 3a). The perikarya contained many mitochondria and Golgi bodies, numerous dense-cored vesicles and an extensive endoplasmic reticulum which was predominantly of the smooth variety (Fig. 3a). Multivesicular bodies and dense bodies were commonly seen. Lipid droplets were relatively rare. Synaptic ribbons were present adjacent to the cell membrane. Pinealocytes of the deep pinealalso contained a cylindrical inclusion found within a cisterna of the endoplasmic reticulum (Fig. 3a). These structures frequently appeared in clusters, closely associated with lipid droplets. Glial cells had an ultrastructure similar to that of glial cells within the superficial pineal gland, with a sparse cytoplasm containing large numbers of microfilaments. Blood vessels were of the non-fenestrated type. The nerve fiber bundles contained both myelinated and unmyelinated fibers. Qualitatively, the pinealocytes of ON animals had expanded Golgi bodies, increased amounts of smooth endoplasmic reticulum and a greater number of dense-cored vesicles (Figs. 2b and 3b).

Quantitative Analysis

The volume of the deep pineal gland averaged 12.3 X 10^6 μm^3 ($\pm$.92 X 10^6 μm^3 SEM), or 6 to 10% of the total volume of the pineal complex. Pinealocytes occupied approximately 95% (11.7 X 10^6 μm^3) of the volume of the deep pineal gland. Glial cells and blood vessels each comprised 1%, with the remaining 3% occupied by miscellaneous features (nerve fibers, intercellular clefts). It was calculated that the deep

ABBREVIATIONS USED IN FIGURES

BV = Blood Vessel
CP = Cell Processes
L = Lipid
N = Pinealocyte Nucleus
Nu = Pinealocyte Nucleolus
NF = Nerve Fiber Bundle
RER = Rough Endoplasmic Reticulum
db = Dense Body
g = Golgi Body
m = Mitochondria

PLATE I

Figure 1. Light micrographs of the deep pineal glands of normal (a) and blinded (b) animals. Glial cell nuclei (arrowhead) are distinguishable from the nuclei of neighboring pinealocytes. A nerve fiber bundle is visible in Figure 1a. A portion of the pineal capsule (PC) is present in the lower micrograph. Arrow indicates pinealocyte nucleoli. 180X. Bar represents 50 μm.

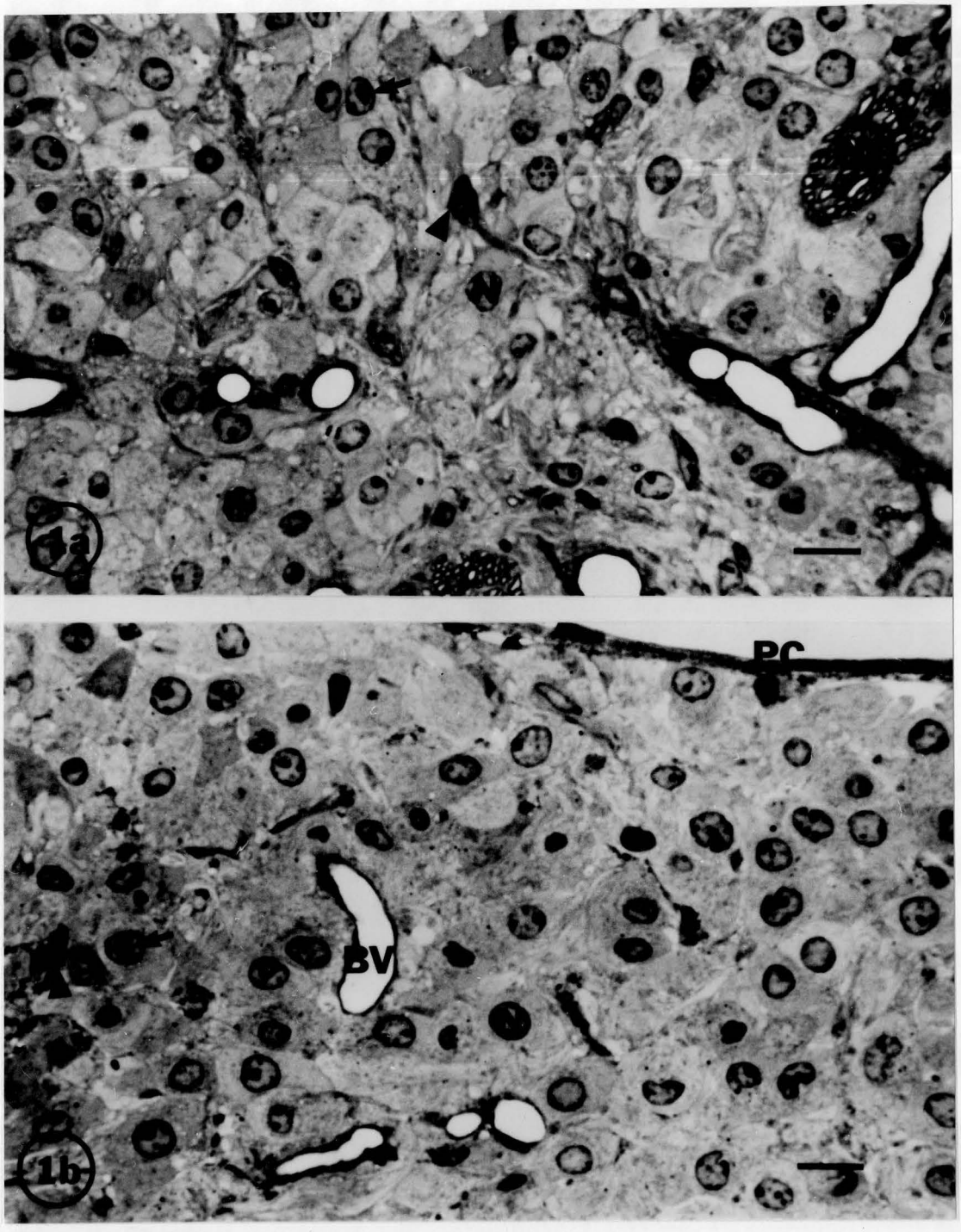
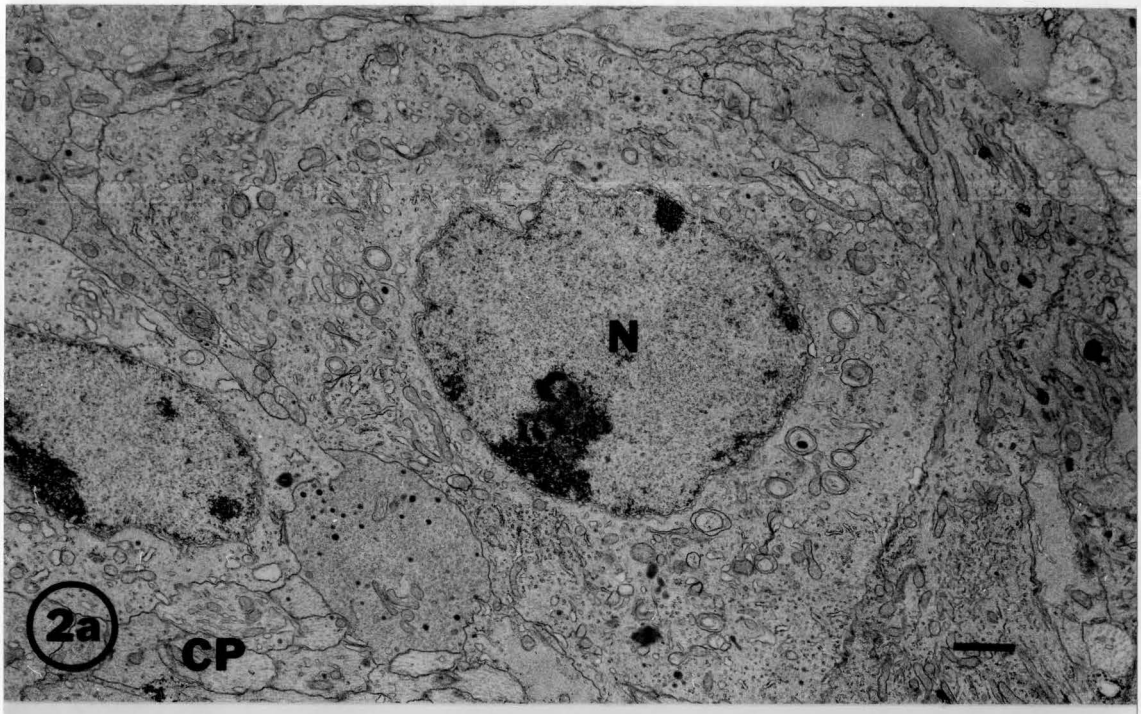

1a
PC
BV
1b

PLATE II

Figure 2. Low magnification electron micrographs of pinealocytes of normal (a) and blinded (b) animals. Abundant cell processes (CP) are found in Figure 2a. Stacks of rough endoplasmic reticulum, resembling Nissl substance, can be seen in Figure 2b. 6900X. Bar represents one μm.


N
2a
CP

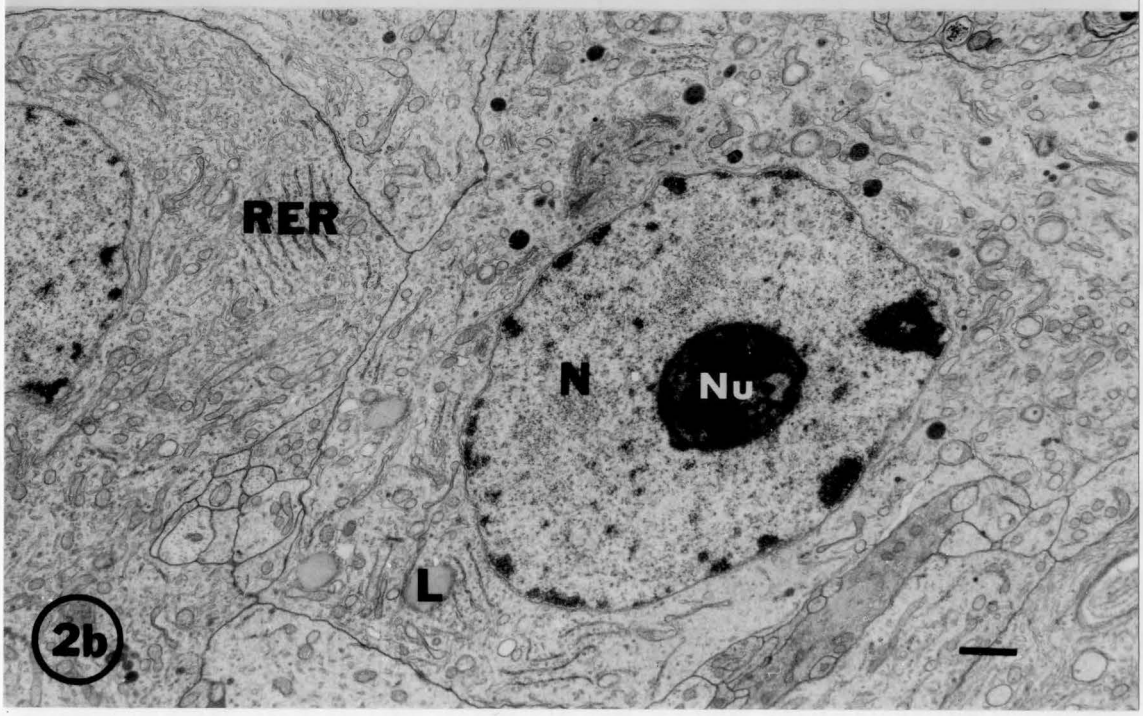
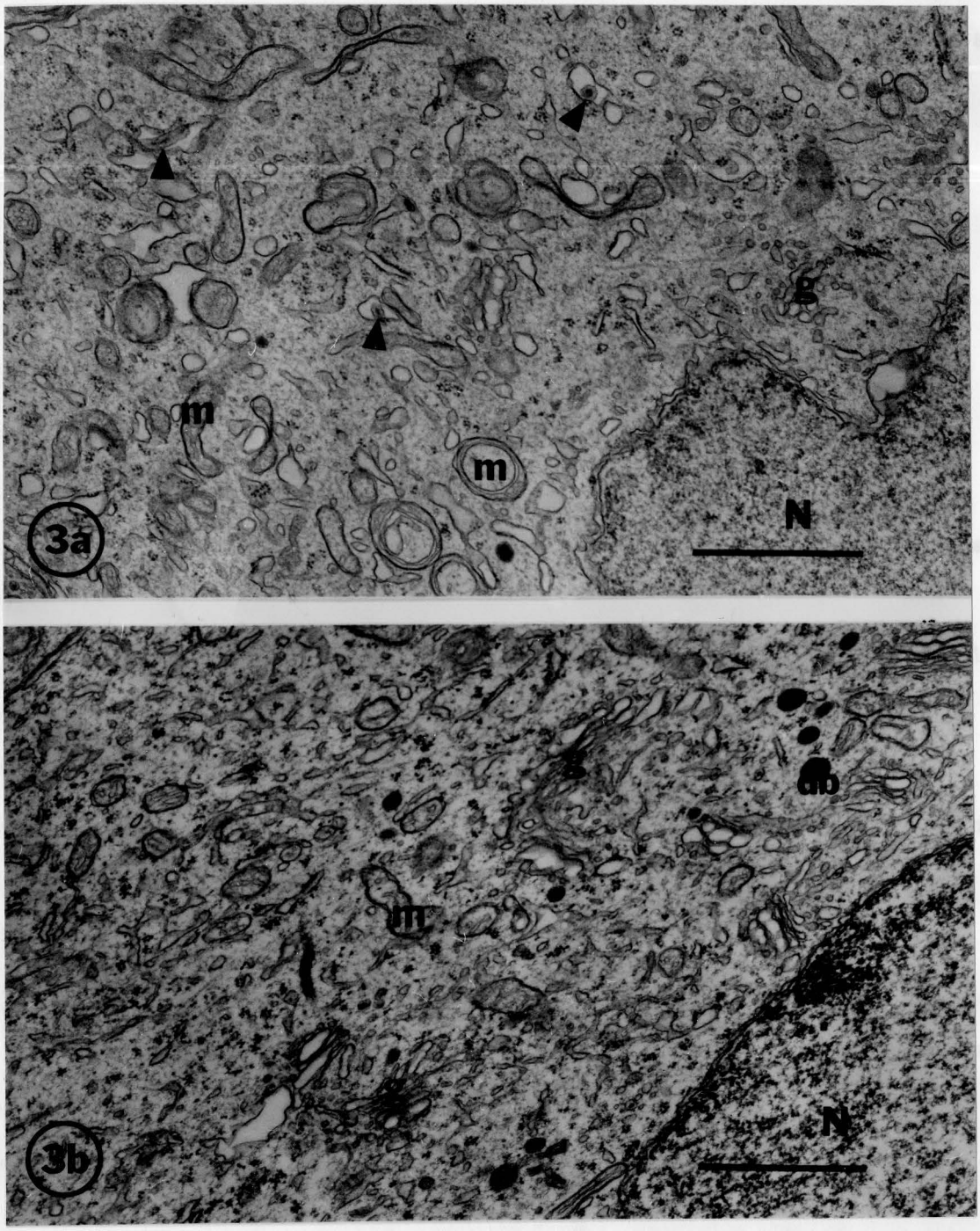

RER
N
Nu
L
2b

PLATE III

Figure 3. High magnification electron micrographs of the pinealocyte cytoplasm of normal (a) and blinded (b) hamsters. Several views of the enigmatic cellular structure mentioned in the text are evident in the upper photo (arrowheads). The pinealocyte of the normal animal (3a) also shows circular mitochondrial profiles commonly found in pinealocytes. 22,800X. Bar represents one μm.


g
m
m
N
3a
db
m
N
3b

gland contained approximately 4,700 pinealocytes. This was estimated by dividing the volume of the deep pineal gland occupied by pinealocytes by the mean volume of the pinealocyte (2499 μm^3 - from Table I).

Circadian Analysis of NOR Hamsters

Evaluation of NOR animals showed variation in the volume density and numerical density of pinealocyte nucleoli, with peak values occurring in the mid- to late-dark and minimal values occurring during the light period (Figs. 4a and 4b). There was a sharp decline in both the volume and numerical densities of nucleoli at the dark to light interphase (0500 to 0700 h). Over a light:dark cycle, quantitative changes were not detectable in the nuclei and cytoplasm of pinealocytes, glial cells and blood vessels (Tables I and II).

At the ultrastructural level, a circadian rhythm was found in only one of the organelles examined. The Golgi complex showed marginally significant changes in both volume density and number per area, with peak values at the dark-to-light interphase and a gradual decline during the light period, reaching a trough at 2100 h
(Figs. 4c and 4d).

Circadian Analysis of ON Hamsters

Statistical evaluation of deep pineals of ON hamsters detected no significant rhythms. As in the preceding paper, variables were pooled for comparison to similar values obtained from NOR animals.

ABBREVIATIONS USED IN TABLES

DB = Dense Bodies
DCV = Dense-cored Vesicles
MITO = Mitochondria
MVB = Multivesicular Bodies
Nv = Numerical Density (#/μm3)
RER = Rough Endoplasmic Reticulum
SER = Smooth Endoplasmic Reticulum
Sv = Surface Density (Surface Area/μm3)
Vv = Volume Density ($\mu m^3/\mu m^3$)
$\overline{v}$ = Mean Volume (μm3)

Table I. Means (±SEM) for light microscopic analysis of pinealocytes from untreated (NOR) animals over a 24-hour period. Pooled data from all sample times combined are given below for both untreated and blinded (ON) groups. Vv = $\mu m^3/\mu m^3$; Nv = $\#/\mu m^3$; $\bar{v}$ = μm^3.

TIME	PINEALOCYTE NUCLEI Vv	PINEALOCYTE NUCLEI Nv	PINEALOCYTE NUCLEI $\bar{v}$	PINEALOCYTE NUCLEOLI Vv	PINEALOCYTE NUCLEOLI Nv	PINEALOCYTE NUCLEOLI $\bar{v}$	PINEALOCYTE CYTOPLASM Vv	PINEALOCYTE CYTOPLASM $\bar{v}$
0100	0.088 ±.002	0.407 ±.016	219.70 ±10.82	0.011 ±.000	1.474 ±.075	7.712 ±.372	0.875 ±.004	2186.63 ±109.15
0500	0.083 ±.004	0.428 ±.032	197.44 ± 9.46	0.011 ±.001	1.347 ±.148	8.674 ±.566	0.867 ±.009	2174.39 ±159.26
0700	0.082 ±.003	0.398 ±.013	209.75 ±10.78	0.009 ±.000	1.092 ±.040	8.280 ±.231	0.861 ±.012	2243.47 ± 93.08
1300	0.080 ±.004	0.376 ±.024	216.82 ± 9.98	0.008 ±.001	1.052 ±.118	7.752 ±.394	0.879 ±.014	2471.51 ±169.94
1900	0.084 ±.005	0.405 ±.051	217.99 ±17.02	0.008 ±.001	1.040 ±.113	7.820 ±.681	0.879 ±.006	2420.76 ±331.00
2100	0.086 ±.004	0.412 ±.034	214.74 ±16.82	0.010 ±.001	1.183 ±.104	8.425 ±.988	0.864 ±.007	2221.75 ±174.54
F value	0.50	0.30	0.41	4.11	2.81	0.47	0.69	0.45
p<	-	-	-	0.01	0.05	-	-	-
NOR	0.084 ±.002	0.404 ±.012	212.74 ± 5.03	0.010 ±.000	1.198 ±.048	8.111 ±.232	0.871 ±.004	2286.42 ± 74.17
ON	0.068 ±.002	0.391 ±.010	181.09 ± 6.55	0.007 ±.000	1.009 ±.030	7.635 ±.266	0.859 ±.005	2348.11 ± 61.11
t value	6.645	0.807	3.78	4.402	3.269	1.328	1.912	0.63
p<	0.001	_	0.001	0.001	0.01	_	_	_

Table II. Mean (±SEM) for light microscopic analysis of glia and blood vessels from untreated (NOR) animals over a 24-hour period. Pooled data from all sample times combined are given below for both untreated and blinded (ON) groups. Vv = $\mu m^3/\mu m^3$; Nv = $\#/\mu m^3$; $\bar{v}$ = μm^3.

	GLIAL CELLS			BLOOD VESSELS		
TIME	Vv	Nv	$\bar{v}$	Vv	Nv	$\bar{v}$
0100	0.009 ±.001	0.113 ±.006	88.87 ±4.89	0.009 ±.001	0.017 ±.002	573.98 ±63.04
0500	0.008 ±.001	0.098 ±.008	82.54 ±4.45	0.011 ±.002	0.018 ±.003	871.23 ±276.51
0700	0.010 ±.001	0.121 ±.017	86.10 ±5.08	0.010 ±.002	0.017 ±.002	719.70 ±42.27
1300	0.010 ±.001	0.110 ±.013	87.73 ±4.48	0.014 ±.003	0.021 ±.001	727.78 ±123.57
1900	0.007 ±.001	0.088 ±.016	88.52 ±9.34	0.008 ±.001	0.018 ±.003	617.31 ±111.02
2100	0.011 ±.001	0.144 ±.029	87.54 ±8.43	0.008 ±.001	0.019 ±.003	597.64 ±88.36
F value	1.62	1.37	0.13	1.59	0.32	0.64
p<	-	-	-	-	-	-
NOR	0.009 ±.000	0.112 ±.007	82.11 ±5.39	0.010 ±.001	0.018 ±.001	686.55 ±55.44
ON	0.012 ±.001	0.128 ±.005	97.91 ±3.88	0.011 ±.001	0.015 ±.001	878.20 ±99.23
t value	3.68	1.91	2.34	0.24	2.40	1.66
p<	0.001	-	0.05	-	0.02	-

PLATE IV

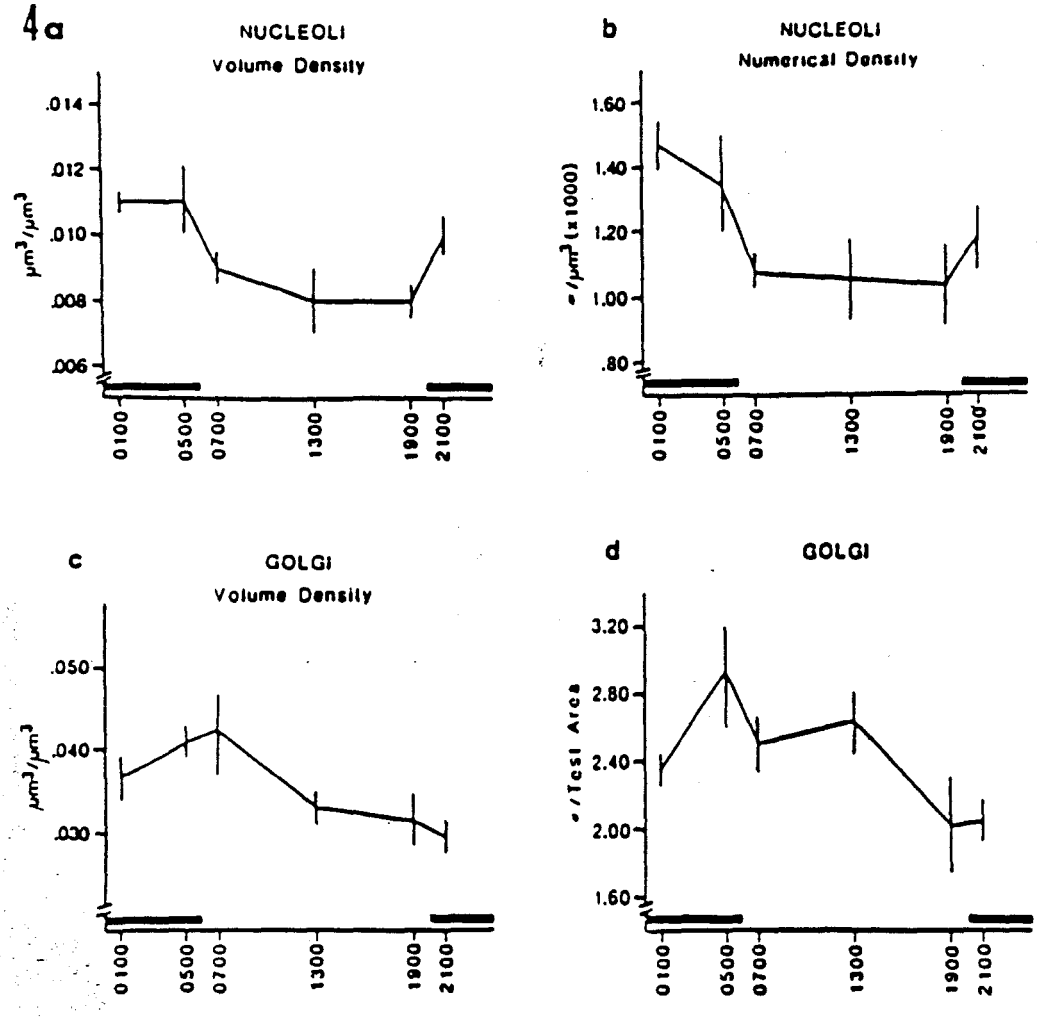
Figure 4. Morphometric analysis of the deep pineal gland of normal animals over a 24-hour light:dark cycle. For all graphs, solid bar indicates the dark period. Each point is the mean of six animals. The vertical lines represent $\pm$ the standard error of the mean. For additional data, see Results section and Tables I and IV.

4a. Volume density of pinealocyte nucleoli, one way analysis of variance, $p<0.01$.

4b. Numerical density of nucleoli, $p<0.05$.

4c. Volume density of Golgi, $p<0.05$.

4d. Number of Golgi per test area of cytoplasm, $p<0.05$.


4a
NUCLEOLI
Volume Density
.014
.012
.010
.008
.006
μm³/μm³
0100
0500
0700
1300
1900
2100
b
NUCLEOLI
Numerical Density
1.60
1.40
1.20
1.00
.80
#/μm³ (x1000)
0100
0500
0700
1300
1900
2100
c
GOLGI
Volume Density
.050
.040
.030
μm³/μm³
0100
0500
0700
1300
1900
2100
d
GOLGI
3.20
2.80
2.40
2.00
1.60
#/Test Area
0100
0500
0700
1300
1900
2100

Statistical Analysis of Structural Changes Following ON

Highly significant decreases in the volume density and mean volume of pinealocyte nuclei were found in the ON group. Both the volume density and numerical density of pinealocyte nucleoli declined, while cytoplasmic values remained unchanged (Table I). Glial cells exhibited a significantly greater volume density and mean volume following optic enucleation. The numerical density of blood vessels declined, while other variables involving vasculature did not change (Table II).

At the ultrastructural level, the volume density of mitochondria, lipid and dense bodies, surface density of the smooth endoplasmic reticulum and number of dense-cored vesicles per cubic micrometer of cytoplasm were significantly enhanced in ON animals (Table III). The volume density of the Golgi bodies also increased as a result of the greater number and increased overall length of that organelle. Finally, the number of Golgi-associated clear vesicles was also elevated (Table IV).

With regard to sex differences, t-test analysis determined that females in the NOR group contained a greater volume density of pinealocyte cytoplasm ($p<.05$) and increased numerical density of glial cells ($p<.05$) and dense-cored vesicles ($p<.01$). In ON hamsters, the volume density of the dense bodies ($p<.05$) was greater in females.

Table III. Means (+SEM) for electron microscopic analysis of organelles (excluding Golgi) from untreated (NOR) animals over a 24-hour period. Pooled data from all sample times combined are given below for both untreated and blinded (ON) groups. Vv = $\mu m^3/\mu m^3$; Sv = Surface Area/μm^3.

TIME	MITO Vv	SER Sv	RER Sv	DCV #/μm^2	LIPID Vv	DB Vv	MVB Vv
0100	0.070 ±.004	5.692 ±.441	0.837 ±.068	0.128 ±.012	0.001 ±.001	0.008 ±.001	0.000 ±.000
0500	0.069 ±.004	6.045 ±.160	0.624 ±.043	0.157 ±.012	0.002 ±.001	0.009 ±.001	0.001 ±.000
0700	0.082 ±.008	6.186 ±.412	0.667 ±.097	0.163 ±.026	0.001 ±.001	0.008 ±.002	0.001 ±.000
1300	0.075 ±.007	5.686 ±.328	0.585 ±.148	0.150 ±.019	0.000 ±.000	0.009 ±.001	0.001 ±.001
1900	0.093 ±.011	5.895 ±.226	0.534 ±.062	0.140 ±.010	0.000 ±.000	0.011 ±.001	0.001 ±.000
2100	0.073 ±.008	5.831 ±.551	0.598 ±.106	0.138 ±.007	0.000 ±.000	0.011 ±.002	0.001 ±.000
F value	1.44	0.28	1.26	0.68	2.19	0.95	0.85
p<	-	-	-	-	-	-	-
NOR	0.077 ±.003	5.889 ±.146	0.641 ±.039	0.146 ±.006	0.001 ±.000	0.009 ±.001	0.001 ±.000
ON	0.084 ±.002	8.296 ±.198	0.617 ±.033	0.183 ±.008	0.002 ±.000	0.015 ±.001	0.001 ±.000
t value	2.03	9.66	0.46	3.48	2.28	6.55	0.79
p<	0.05	0.001	-	0.001	0.05	0.001	-

Table IV. Means (±SEM) for electron microscopic analysis of Golgi bodies from untreated (NOR) animals over a 24-hour period. Pooled data from all sample times combined are given below for both untreated and blinded (ON) groups. Vv = $\mu m^3/\mu m^3$.

TIME	Vv	#/Area	WIDTH	LENGTH	NUMBER SACCULES	NUMBER VESICLES
0100	0.036 ±.003	2.358 ±.085	0.332 ±.010	0.737 ±.040	4.220 ±.068	13.187 ±1.264
0500	0.041 ±.002	2.917 ±.287	0.325 ±.015	0.693 ±.020	4.252 ±.051	13.500 ±1.656
0700	0.042 ±.005	2.508 ±.153	0.287 ±.022	0.721 ±.055	4.122 ±.168	12.332 ±1.211
1300	0.033 ±.002	2.625 ±.185	0.279 ±.012	0.683 ±.044	4.277 ±.139	14.333 ±1.556
1900	0.032 ±.003	2.083 ±.272	0.314 ±.009	0.743 ±.023	4.343 ±.111	16.432 ± .847
2100	0.030 ±.002	2.050 ±.123	0.284 ±.014	0.769 ±.049	4.174 ±.075	15.784 ± .424
F value	2.57	2.80	2.24	0.63	0.50	1.64
p<	0.05	0.05	-	-	-	-
NOR	0.036 ±.001	2.424 ±.091	0.304 ±.007	0.731 ±.017	4.231 ±.043	14.261 ± .526
ON	0.046 ±.001	2.733 ±.075	0.296 ±.005	0.799 ±.018	4.328 ±.030	18.392 ± .504
t value	5.934	2.596	1.021	2.683	1.818	5.592
p<	0.001	0.02	-	0.01	-	0.001

Discussion

The percent volume of the pineal complex occupied by the deep pineal varies widely among species, ranging from 1.5 to 3.0% in the rat (Boeckman, 1980) to 18% in the white-footed mouse (Quay, 1956). The value estimated in this study for the deep pineal (6-10%) lies within this range and is in agreement with previously reported values for the golden hamster (see Reiter, 1981a). Although relatively small in size compared to the superficial pineal, the deep pineal has direct contact with the cerebrospinal fluid (CSF) of the third ventricle (gerbil, Welsh, 1983; vole, Hewing, 1980b; hamster, Hewing, 1978). Pinealocytes in this region could therefore function to monitor the concentration of substances within the CSF, or, alternatively, to secrete substances directly into the ventricle, where they could more readily act upon the midbrain and hypothalamus, pineal target areas (Hewing, 1978). If the deep pineal does indeed secrete substances into the third ventricle, large amounts of secretory product would not be necessary, since the compounds would face less dilution in the CSF than in the greater volume of circulating blood (Reiter, 1981a).

A functional relationship between the superficial and deep pineal glands has not yet been defined. The common embryological origin (Sheridan and Walker, 1975; Ariens Kappers, 1960) and similar histological appearance (Boeckman, 1980; Sheridan and Reiter, 1970a) may indicate similar function(s), especially since the deep pineal gland,

like the superficial gland, produces the indoles serotonin (Wiklund, 1974) and melatonin (Sheridan and Rollag, 1983). Unlike the superficial gland, however, the deep gland does not aid in testicular regression produced in hamsters by short photoperiod exposure (Anderson and Turek, 1983).

Pinealocytes of the deep pineal are smaller than those of the superficial and possess nuclei with less volume (Boeckman, 1980; Vollrath, 1979; Quay, 1956) and fewer nucleoli (Boeckman, 1980). Pinealocytes in both portions of the pineal complex contain a similar complement of organelles (Sheridan and Reiter, 1970b), but morphometric analyses in this study detected differences in the proportions of several cell structures. Pinealocytes of the deep gland contained a greater percent volume of Golgi bodies, rough endoplasmic reticulum and dense-cored vesicles, organelles which are involved in peptidergic synthesis in the pineal (Juillard and Collin, 1980). Pinealocytes in the superficial part contained a larger volume density of mitochondria and lipid bodies. These differences in the organellar composition of pinealocytes supports the hypothesis that the two regions are functionally distinct.

The abundance of glial cells and blood vessels as quantified in this study were not in close agreement with previously published reports. Glial cells in the deep pineal were similar in their numerical density to those in the superficial gland, in contrast to the qualitative observations of Sheridan and Walker (1975) who concluded

that glia were more concentrated in the deep gland of the hamster. We also found blood vessels to be more abundant in the deep portion of the pineal complex in the hamster, while the converse has been reported in the rat (Boeckman, 1980). In the present study, the vessels seen in the deep pineal were smaller than those of the superficial gland.

Few data are available regarding circadian patterns of metabolism in the deep pineal. A rhythm in melatonin content matching that of the superficial gland was detected in the deep pineal of weanling hamsters (Sheridan and Rollag, 1983). Serotonin levels in the deep pineal of the rat, however, did not vary over a 24-hour period (Moore, 1975). Morphometric analyses in the present study found little evidence of rhythms in the structure of the deep pineal, with only nucleoli and Golgi bodies exhibiting significant variation. Since indole synthesis has been postulated to take place diffusely within the cytosol of the superficial pineal (Romijn et al., 1977a), rhythms in the production of these compounds may not necessarily be reflected in the organelles examined.

Contrary to findings in our study, rhythms in the nuclear volume of pinealocytes of the deep pineal have been demonstrated in the rat (Boeckman, 1980) and the hamster (Vollrath, 1979). Nuclei of rat pinealocytes showed ultradian, as well as circadian rhythms with no consistent pattern, while nuclear volume in the hamster exhibited peak values during the light period. The rhythm in the latter species (Vollrath, 1979) was similar to that reported for nuclear variations

seen in superficial pinealocytes of the rat (Quay and Renzoni, 1966) and the Chinese hamster (Matsushima et al., 1983). The present study did demonstrate that nucleoli of deep pinealocytes underwent a significant 24-hour rhythm. Changes in the volume density of nucleoli were paralleled by rhythmic alterations in numerical density, implying a change in nucleolar number rather than size over the L:D cycle. Nucleolar rhythms peaked during the dark period, the opposite of rhythms in the nucleoli of the superficial gland, which have been reported to reach maximal levels during the light period (Dombrowski and McNulty, Chapter III; Lew et al., 1982; Quay and Renzoni, 1966). The lack of synchrony in the nucleolar rhythms in both parts suggests important functional differences in terms of protein synthesis.

The other cellular structure which exhibited a significant rhythm, the Golgi complex, also showed variations that were out of phase with circadian changes in that organelle in the superficial pineal. Maximal values in the volume density and numerical density of the Golgi bodies occurred at the dark to light interphase in the deep pineal, while significant variations in the Golgi bodies of the superficial pineal were found at the light to dark interphase. This asynchrony of structural rhythms of the superficial and deep pineal glands was unexpected since both parts receive innervation via sympathetic post-ganglionic afferent fibers arising in the superior cervical ganglion (Reiter and Hedlund, 1976), and their production of melatonin is in phase (Sheridan and Rollag, 1983). Several factors may explain

these observations. First, the deep pineal appears to receive additional input from afferent nerve fibers arising from the central nervous system. Legait et al. (1979) found that removal of the superficial pineal, with consequent disruption of the sympathetic innervation to the deep gland, did not result in atrophy or involution of the deep pineal. The authors speculated that an additional innervation, arising from centrally located nuclei, maintained the integrity of the deep pineal. Furthermore, the pineal complex of the Mongolian gerbil has been shown to receive pinealopetal afferent fibers from the medial and lateral habenular nuclei and nucleus of the posterior commissure, which enter the deep pineal and travel to the superficial gland via the stalk (Moller and Korf, 1983a,b). Centrally arising fibers could thus modulate function in the deep pineal, and result in rhythms in the superficial and deep pineals that are not in phase. Second, there may exist functionally distinct populations of pinealocytes as proposed above. Asynchrony of the circadian rhythms in the nucleoli and Golgi of both populations may reflect the production of compounds (peptides?) other than melatonin.

Deep pinealocytes of blinded hamsters showed decreases in the volume densities and mean volumes of nuclei and nucleoli. A decrease in nuclear mean volume was also seen in the superficial gland after optic enucleation (Dombrowski and McNulty, Chapter III), and in the pinealocytes of hamsters maintained on a short (1h:23h) light:dark cycle (Barratt et al., 1977). This decrease in nuclear size is considered

indicative of a decline in cellular activity (Hildebrand, 1980). The decline in nucleolar volume density was apparently due to the simultaneous decrease in numerical density.

At the ultrastructural level, highly significant changes were seen in several organelles, including the smooth endoplasmic reticulum, Golgi bodies, dense-cored vesicles and dense bodies. Enhancement of the smooth endoplasmic reticulum may be related to increased indole synthesis (Romijn, 1975). Serotonin levels in the medial habenular region of the rat, which is adjacent to the deep pineal, increased following three weeks exposure to constant darkness (Moore, 1975). Hewing (1978) also reported hypertrophy of the smooth endoplasmic reticulum in the deep pineal of hamsters after exposure to constant darkness. The Golgi apparatus gave evidence of enhanced secretory activity with hypertrophy of that organelle and increased numerical density. The Golgi-associated clear vesicles and the dense-cored vesicles showed highly signficant increases in number, also indicating greater Golgi activity. Greater numbers of dense-cored vesicles have been reported in the superficial pineals of blinded hamsters (Sheridan, 1975; Clabough, 1971) and in the deep pineals of hamsters exposed to constant darkness (Hewing, 1978).

In summary, although qualitatively similar to parenchymal cells of the superficial pineal, pinealocytes of the deep gland show significant quantitative differences. The higher levels of rough endoplasmic reticulum, Golgi bodies and dense-cored vesicles in the deep

pinealocytes of normal (14h:10h) animals suggest that a greater proportion of the cellular metabolism is engaged in the production of peptidergic compounds. Another indication that the superficial and deep pineals are functionally not closely linked was the differing responses of the respective pinealocytes over a light:dark cycle and in response to the stimulatory condition of optic enucleation.

Chapter V

Title: Analysis of a Membrane-Bounded Structure Found in Deep Pinealocytes of the Golden Hamster. Circadian Variation and the Effects of Optic Enucleation.

Abstract

The presence of a previously unreported inclusion was noted in the cytoplasm of deep pinealocytes in the golden hamster. Reconstruction from serial sections showed that the structure was cylindrical, measuring approximately 100 nm in width and up to 400 nm in length, and contained a dense core. The inclusion was enclosed by a cisterna of the endoplasmic reticulum (ER). Clusters of inclusions could occasionally be seen. A flocculent material was sometimes present between adjacent cisternae within the cluster, and lipid droplets were frequently found in the vicinity. The number of ER cisternae that contained inclusions showed significant variation over a 24-hour span in animals maintained under a 14h:10h light:dark cycle, with peak values at 0500 h. This rhythm was damped in blinded animals. Circadian rhythms were not detectable under either regimen when the data were expressed as the total number of inclusion profiles per unit area of cytoplasm.

Key Words: Deep Pineal, Endoplasmic Reticulum, Optic Enucleation, Circadian Rhythm

Introduction

The pineal gland of the golden hamster (Mesocricetus auratus) is divided into superficial and deep glandular components (Sheridan and Reiter, 1970a). Although both portions develop from a common anlage (Sheridan and Walker, 1975), they separate during post-natal development and may differentiate into two distinct populations of cells. Quantitative morphological analyses have detected several dissimilarities between pinealocytes of the two glands (Dombrowski and McNulty, Chapter IV; Boeckman, 1980). Physiological evidence also indicates that the superficial and deep pineal glands may not subserve the same function, since ablation of the deep gland does not prevent short-day induced testicular regression in the golden hamster (Anderson and Turek, 1983).

A recent investigation of the pineal complex of the golden hamster revealed the presence of an as yet unidentified inclusion in the cytoplasm of the pinealocytes of the deep pineal (Dombrowski and McNulty, Chapter IV). Located within an intracisternal space of the endoplasmic reticulum, the inclusion consisted of an electron dense core limited by a single membrane. This communication describes the

ultrastructure and topographical relationships of this inclusion. In order to determine whether this structure was functionally related to the metabolic processes of pinealocytes, inclusions were quantified in the deep pinealocytes of normal and blinded animals.

Materials and Methods

Two experimental groups of 36 golden hamsters each, aged approximately two months, (18 males and 18 females) were utilized in this study. The first group was entrained to a 14h:10h light:dark cycle for a period of one month (lights = 0600 to 2000 h). The second group was optically enucleated eight weeks prior to sacrifice. Six animals from each group (three male and three female) were sacrificed by cardiac perfusion with a buffered glutaraldehyde:paraformaldehyde fixative at the following time points: 0100, 0500, 0700, 1300, 1900 and 2100 h. All dark phase perfusions were performed under sodium vapor safelight. Sacrifices spanned a period from early April to early May. Additional hamsters in both experimental groups were sacrificed in late June and July to replace poorly fixed tissue. Superficial and deep pineals were dissected from each animal and fixed for an additional hour in a 4% glutaraldehyde:buffer solution. Following post-fixation in a 1% osmium tetroxide:buffer solution for one hour, tissues were dehydrated in a graded series of acetones and embedded in Araldite 502 (details in Appendix A). Superficial and deep pineals were sectioned for electron microscopy at 70-90 nm and stained with uranyl acetate and lead citrate.

Twenty to 29 pinealocytes per gland were photographed in a systematic fashion using an RCA EMU-3 electron microscope. Micrographs were printed at a final magnification of 40,000X and coded. Individuals unfamilar with the codes counted the inclusions using two independent methods. The first individual counted the total number of inclusions per micrograph. This value was divided by the cytoplasmic area as estimated by point-counting stereology. The second individual counted the number of cisternae of the ER that contained one or more inclusions. This value was divided by the cytoplasmic area as measured with a Zeiss Videoplan Image analyzer. The latter method corrected for the possibility that some inclusions were folded and therefore exhibited several profiles in any one plane of section. Mean values for individual animals were grouped according to time of sacrifice and experimental group. A two-way analysis of variance test was used to test the effects of treatment and time of sacrifice.

Results

The inclusion was primarily observed in pinealocytes of the deep gland. It consisted of an electron-dense core surrounded by a trilaminar membrane. The entire structure was enclosed within a dilated

cisterna of either the smooth or rough endoplasmic reticulum (Fig. 1) and occasionally the nuclear envelope. Serial sections through the inclusion showed that it was cylindrical, with cytoplasmic continuity at either end (Fig. 2). It measured 100 nm in diameter and up to 400 nm in length.

Large networks of smooth endoplasmic reticulum were sometimes associated with numerous inclusions (Fig. 3). A homogeneous matrix lay between adjacent cisternae (arrows). Lipid droplets were commonly found in close proximity.

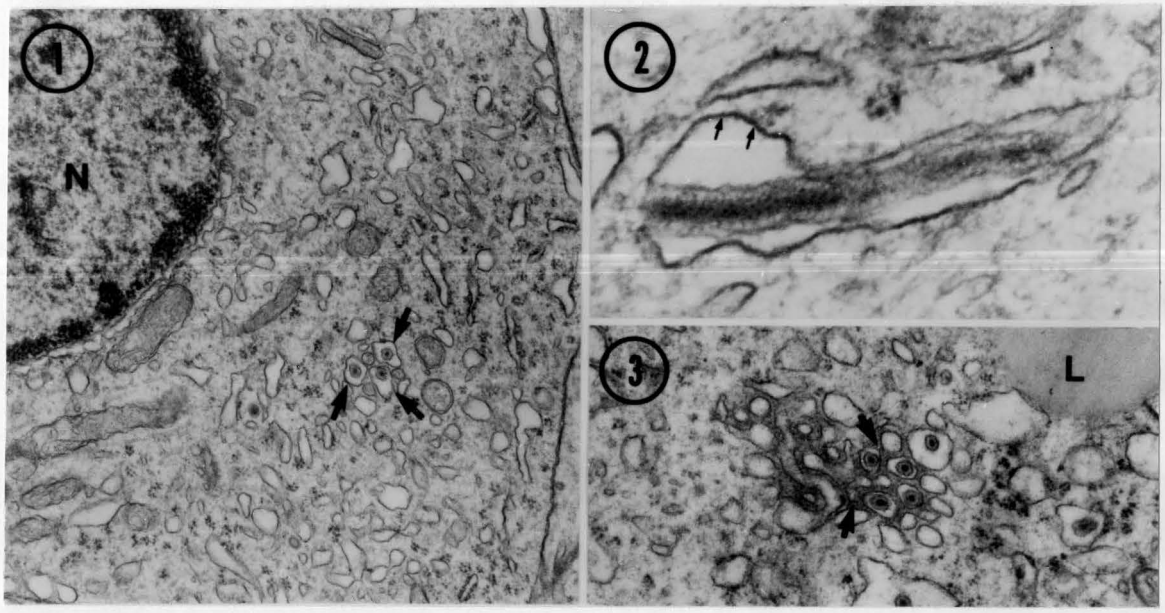
The number of cisternae per cytoplasmic area containing inclusions varied over a light:dark cycle, with peak values occurring during the latter part of the scotophase. There was a sharp decline at the dark to light interphase and the numbers remained low during the photophase (Fig. 4). The F test for the effect of blinding was significant when the data were expressed as the number of cisternae containing one or more inclusions (Table 1). This effect was due to damping of the 24-hour rhythm in the blinded group. When the number of profiles of inclusions were counted, blinding did not result in a significant difference, ($F=3.83$: $0.10<p>0.05$), although the pattern over a 24-hour period was similar to that above.

Figure 1. Cytoplasm of a deep pinealocyte containing three inclusions (arrows). Each is confined by a cisterna of the endoplasmic reticulum. N=nucleus 20,666X.

Figure 2. High magnification micrograph of an inclusion sectioned longitudinally. Its continuity with the cytoplasm is evident. Arrows indicate areas where the trilaminar membrane of the ER is noticeable. 105,600X.

Figure 3. Large complex formed by cisternae of the endoplasmic reticulum and inclusions. A single inclusion enclosed by ER can be seen to the right. The flocculent material between the cisternae is indicated by arrows. A lipid droplet (L) can be seen in close association. 23,040X.

Figure 4. Number of cisternae containing one or more inclusions over a 24-hour period. Each value represents the mean for that time point. Vertical lines indicate the standard error of the mean (SEM).


1
N
2
3
L

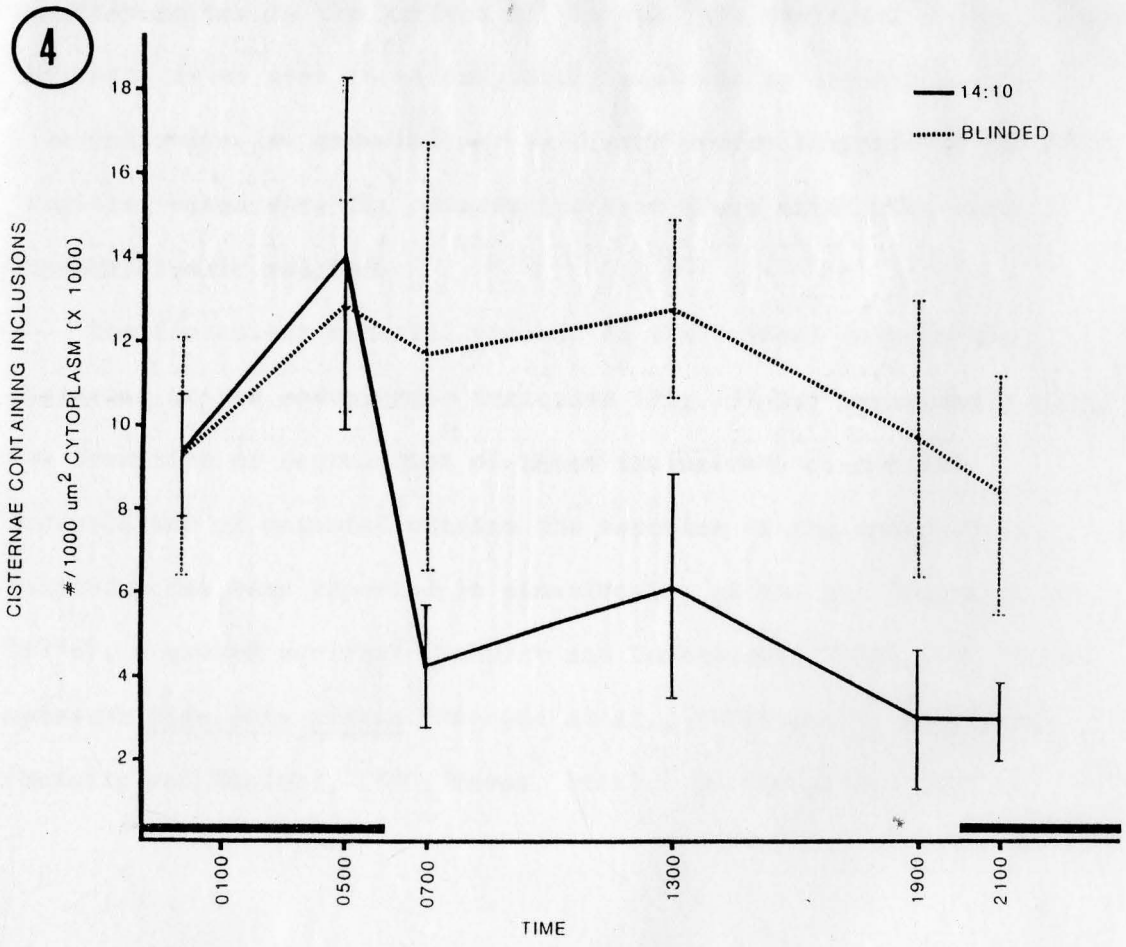

4
14:10
BLINDED
CISTERNAE CONTAINING INCLUSIONS
#/1000 um2 CYTOPLASM (X 1000)
18
16
14
12
10
8
6
4
2
0100
0500
0700
1300
1900
2100
TIME

Table 1. Results of the two-way analysis of variance test. Rows represent 14h:10h versus blinding, while columns represent the time of sacrifice. D.F.=degree(s) of freedom.

ANOVA

Source	D.F.	F	p
Group(Row)	1	6.48	$p<0.025$
Time(Column)	5	1.98	$p>0.10$
Group-Time Interaction	5	0.89	$p>0.10$

Discussion

Synthetic/secretory processes of pinealocytes generally involve two different groups of compounds (indoleamines and peptides), both of which have been associated with the endoplasmic reticulum (see Pevet, 1979). Close association of the inclusion with the endoplasmic reticulum suggests that it is synthesized there and presumably is composed at least partly of a peptide/protein material. Fluctuations in the number of inclusions implies that they are secreted or are metabolized within the cell. If the inclusions are secreted, it is not likely that they are transported to the surface of the cell via membrane transport, since they were never seen to be completely enclosed by a membrane. Alternatively, the material may be stored and/or transported in lipid droplets, based upon the observation that these structures were topographically related.

The flocculent material present in the cytosol between the cisternae of the endoplasmic reticulum (Fig. 3) may represent a stage in the formation or degradation of these inclusions. A similar accumulation of material outside the saccules of the endoplasmic reticulum has been reported in pinealocytes of the bat (Pevet et al., 1977a), a ground squirrel (McNulty and Dombrowski, 1980), and in the opossums Didelphis axarae (Machado et al., 1978) and D. virginiana (McNulty and Hazlett, 1980; Pevet, 1981). In the ground squirrel, this

specialization of the endoplasmic reticulum was observed predominantly in pinealocytes of animals sacrificed during winter months (McNulty and Dombrowski, 1980). Seasonal effects on the morphology of the deep pineal in this species have been described recently (Hewing, 1984) and may explain why this structure has not been previously reported.

The effects of light deprivation on the frequency with which these structures occurred in cisternae of the endoplasmic reticulum over a 24-hour period suggests a functional role in the synthetic/secretory processes of deep pinealocytes. Numerical changes over a light:dark cycle tended to be synchronized with circadian rhythms in the nucleoli and Golgi bodies of these cells (Dombrowski and McNulty, Chapter IV). The response of these structures to blinding should be interpreted with caution, since statistical inferences were dependent upon the method used to count the structures, and the effects were not detectable in a preliminary study based upon a smaller sample size (Dombrowski and McNulty, 1981).

Chapter VI

DISCUSSION

The precise function of the deep pineal gland remains an enigma. Arstila (1967) regarded the deep gland as a vestigial remnant of the postnatal migration of the superficial pineal. Hewing (1981, 1980a) believed the deep pineal to be metabolically active. Its function was related to its location adjacent to the third ventricle, and involved interaction of the gland with the cerebrospinal fluid. This interaction was postulated to involve secretion of pineal products into the third ventricle, absorption of substances from the CSF, and the monitoring of the contents of the CSF. Experimental manipulation of the deep pineal has shown that the deep gland has no effect upon short-day induced testicular regression in the golden hamster, indicating that the function of the deep pineal is different from that of the superficial gland (Anderson and Turek, 1983). The present study extends our knowledge of the structure and function of the deep pineal gland, particularly in relation to the superficial pineal gland.

The deep pineal gland is considerably smaller than the superficial gland and contains pinealocytes of lesser size. Nevertheless,

pinealocytes of both parts are morphologically similar and contain a characteristic complement of organelles. This observation, together with the fact that the indoles serotonin and melatonin are present in both the superficial and deep pineal glands has caused investigators to postulate that the cell populations of the two glands have identical functions. The results of the present quantitative study do not support this hypothesis. Deep pinealocytes are characterized by greater amounts of rough endoplasmic reticulum, Golgi bodies and dense-cored vesicles. These are the organelles postulated by Pevet (1977) to synthesize the protein product of Type I pinealocytes. Accordingly, it is possible that cellular processes related to protein/peptide synthesis and secretion are of greater importance in the deep pinealocytes.

Comparison of the morphological evidence of circadian rhythmicity in the superficial and deep glands lends support to the theory that the two glands contain different populations of pinealocytes. Pinealocytes of the superficial pineal underwent clear 24-hour variations in cell structure, a result which is not unexpected in view of the well-documented circadian nature of the gland. It is noteworthy that the strongest rhythms involved the secretory organelles. The fact that the peaks and troughs of those rhythms tended to occur at the light:dark interphases points to the importance of the photic environment to the metabolism of the superficial pineal.

Common innervation of the superficial and deep pineals by postganglionic sympathetic fibers has been documented in the golden

hamster (Reiter and Hedlund, 1975). On this basis, the cells of the superficial and deep pineals might be expected to display similar structural rhythms over a 24-hour period. This was not the case, however. Relatively few rhythms were present in deep pinealocytes, and those that occurred were only marginally significant. Furthermore, the rhythms were out of phase with those of the superficial gland. The paucity of structures that exhibited circadian variation in deep pinealocytes could have resulted from reduced sensitivity of these cells to stimulation by sympathetic fibers. Semm and Vollrath (1980) described three populations of pinealocytes within the guinea pig pineal gland, each responding differently to a photic stimulus. One subclass of pinealocytes was stimulated by light, another was inhibited and a third unaffected by light. It is possible that pinealocytes of the golden hamster are segregated into specific locations, e.g. the superficial and deep pineals. A second explanation of the disparities between the structural rhythms of the two glands is the effect of centrally arising fibers on the deep pineal gland. Sympathetic fibers which enter the deep pineal continue into the adjacent habenular nuclei and stria terminalis in the rat (Wiklund, 1974), and fibers from those centrally located structures enter the deep pineal and travel to the superficial gland (Moller and Korf, 1983a, 1983b; Semm et al., 1981). These centrally arising fibers could modify the influence of sympathetic nerve stimulation on deep pinealocytes, or alternatively, may serve as its sole source of innervation.

The variability in the structural responses of the superficial and deep pinealocytes to blinding is further evidence for functional differences between the two populations of cells. Alterations in superficial pinealocytes, with increases in the amount of both smooth and rough endoplasmic reticulum, Golgi, DCV and dense bodies (Figs. 1-3), imply an increase in the protein/peptide synthesizing ability of the cell, while changes in deep pinealocytes, with elevated levels of mitochondria, lipid, Golgi, smooth endoplasmic reticulum, dense bodies and DCV (Fig. 4.), may indicate increased production of a non-peptide product.

Data obtained in this study indicate that deep pinealocytes are structurally distinct from those of the superficial gland and demonstrate different circadian rhythmicity. Deep pinealocytes also respond differently to the stimulus of blinding. These results suggest that the population of parenchymal cells within the deep pineal gland represents a subpopulation of pinealocytes distinct from that of the superficial gland, subserving a separate and distinct function.

PLATE I

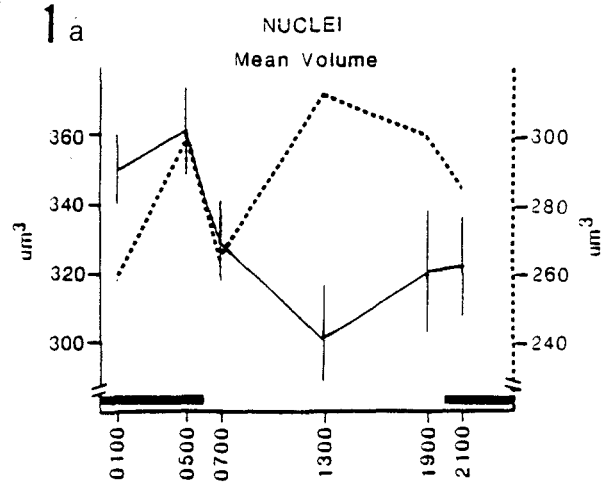
Figure 1. Morphological analysis of nuclei and nucleoli of superficial pinealocytes of normal and blinded animals over a 24-hour light:dark cycle. For all graphs, the solid line indicates values for normal animals. The broken line represents blinded hamsters. Each point is the mean of six animals. The vertical lines represent $\pm$ the standard error of the mean for normal animals. Additional information is contained in Chapter III.

1a. Nuclear mean volume

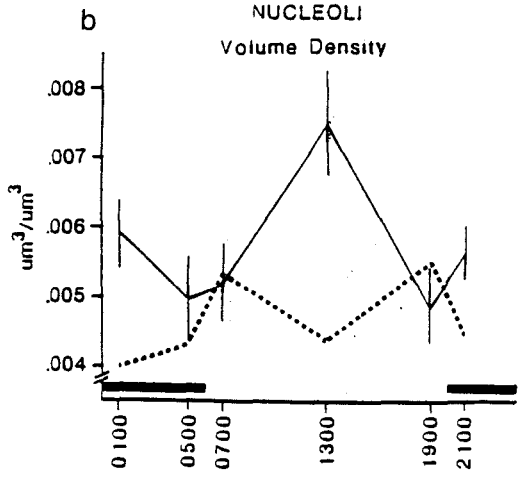
1b. Volume density of nucleoli

1c. Numerical density of nucleoli

1 a


NUCLEI
Mean Volume
360
340
320
300
um3
300
280
260
240
um3
0100
0500
0700
1300
1900
2100

b


NUCLEOLI
Volume Density
.008
.007
.006
.005
.004
um3/um3
0100
0500
0700
1300
1900
2100

c

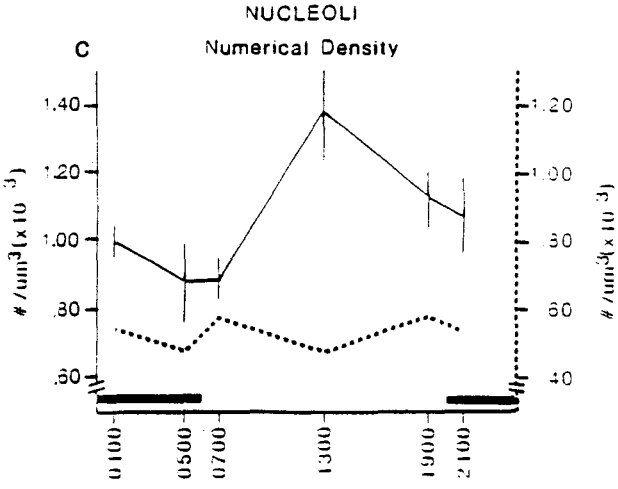

NUCLEOLI
Numerical Density
1.40
1.20
1.00
.80
.60
1.20
1.00
80
60
40
0100
0500
0700
1300
1900
2100

PLATE II

Figure 2. Morphological analysis of cytoplasmic organelles (excepting Golgi) of superficial pinealocytes of of normal and blinded animals over a 24-hour light:dark cycle.

2a. Surface area of smooth endoplasmic reticulum

2b. Surface area of rough endoplasmic reticulum

2c. Number of dense-cored vesicles per um^2 of test cytoplasm

2d. Volume density of dense bodies

2a

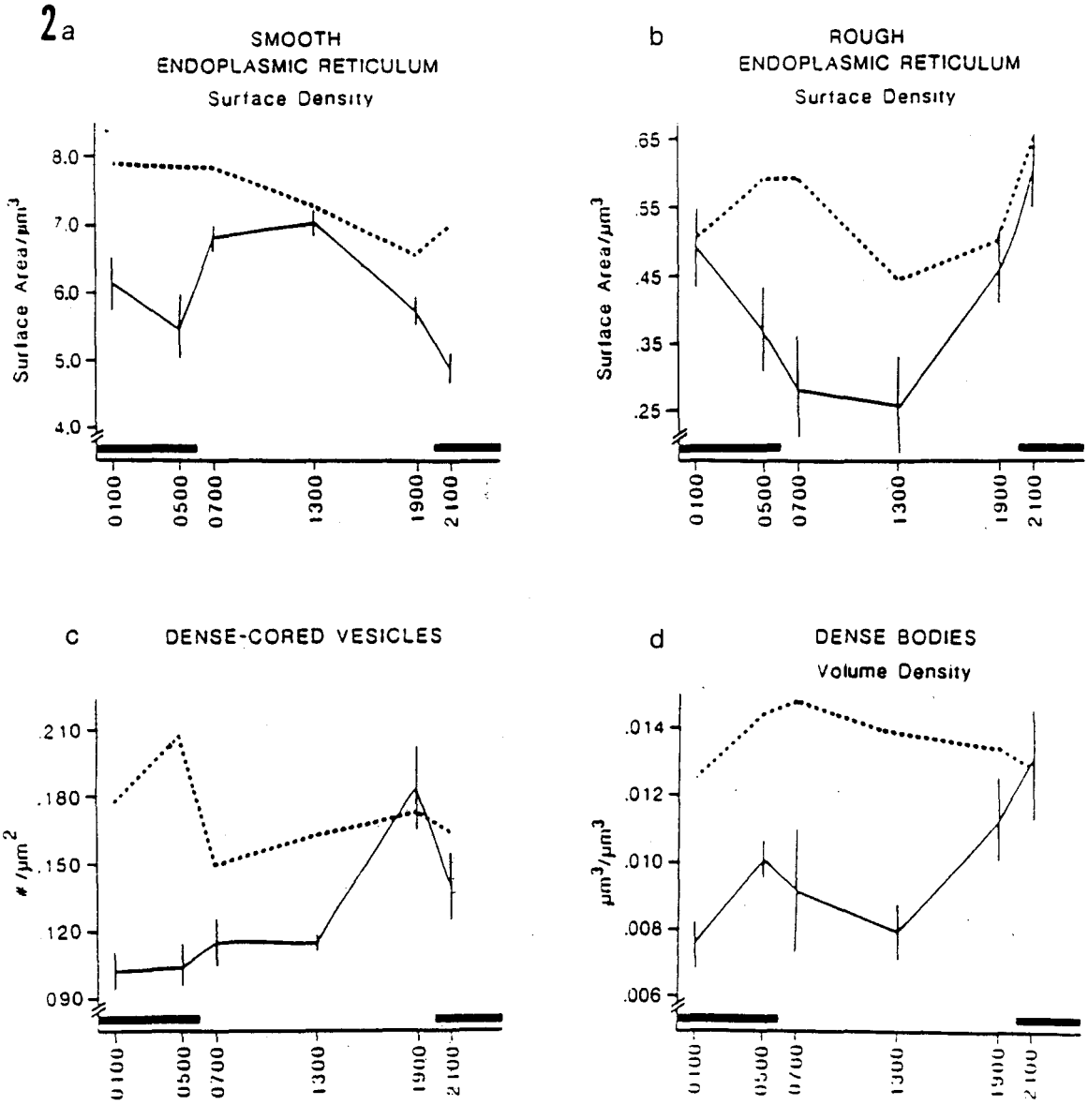

a
SMOOTH
ENDOPLASMIC RETICULUM
Surface Density
Surface Area/μm³
8.0
7.0
6.0
5.0
4.0
0100
0500
0700
1300
1900
2100
b
ROUGH
ENDOPLASMIC RETICULUM
Surface Density
Surface Area/μm³
.65
.55
.45
.35
.25
0100
0500
0700
1300
1900
2100
c
DENSE-CORED VESICLES
/μm²
.210
.180
.150
120
090
0100
0500
0700
1300
1900
2100
d
DENSE BODIES
Volume Density
μm³/μm³
.014
.012
.010
.008
.006
0100
0500
0700
1300
1900
2100

PLATE III

Figure 3. Morphological analysis of the Golgi bodies of superficial pinealocytes of normal and blinded animals over a 24-hour light:dark cycle.

3a. Volume density
3b. Width
3c. Length
3d. Number of associated clear vesicles per Golgi body

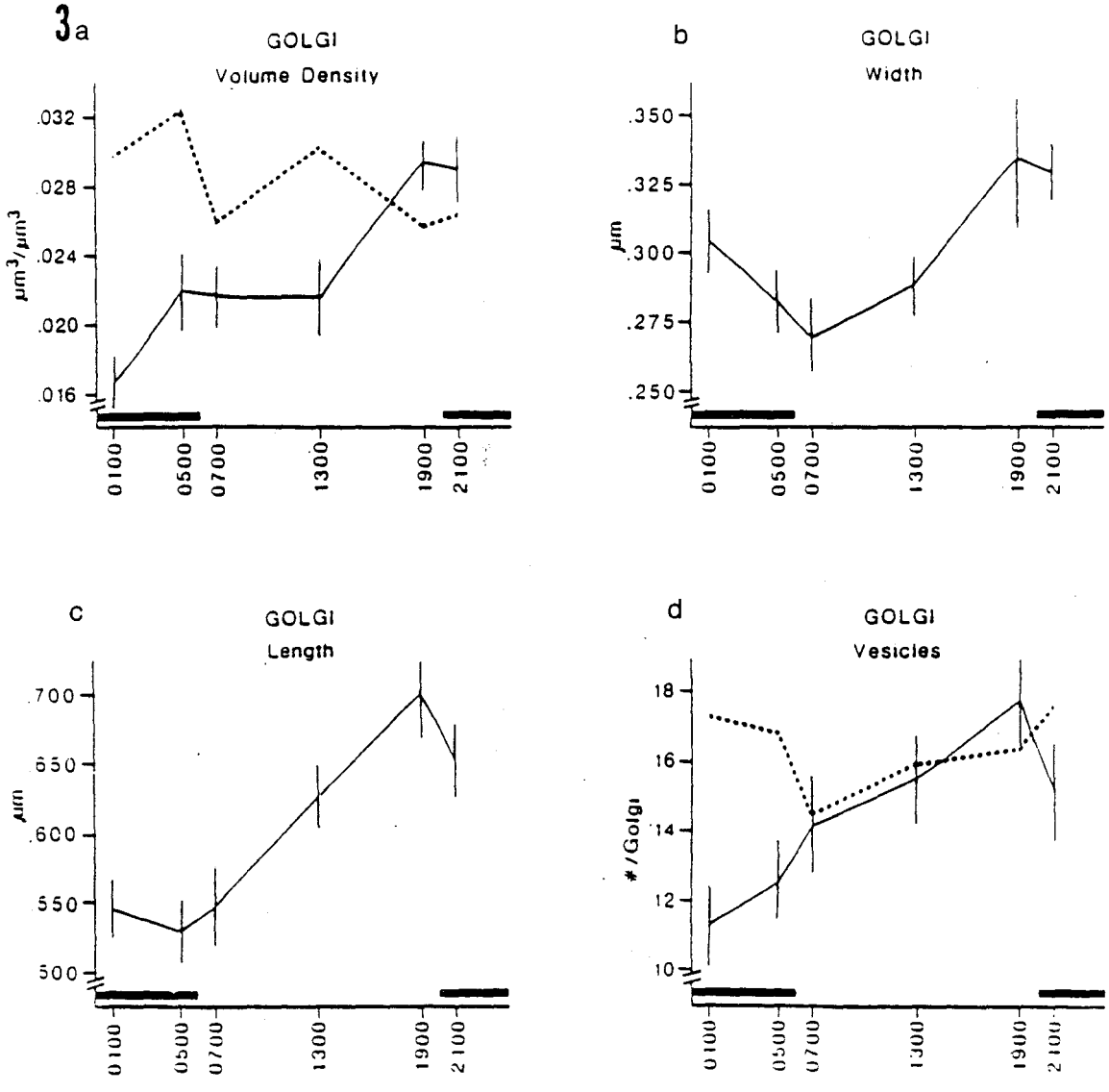
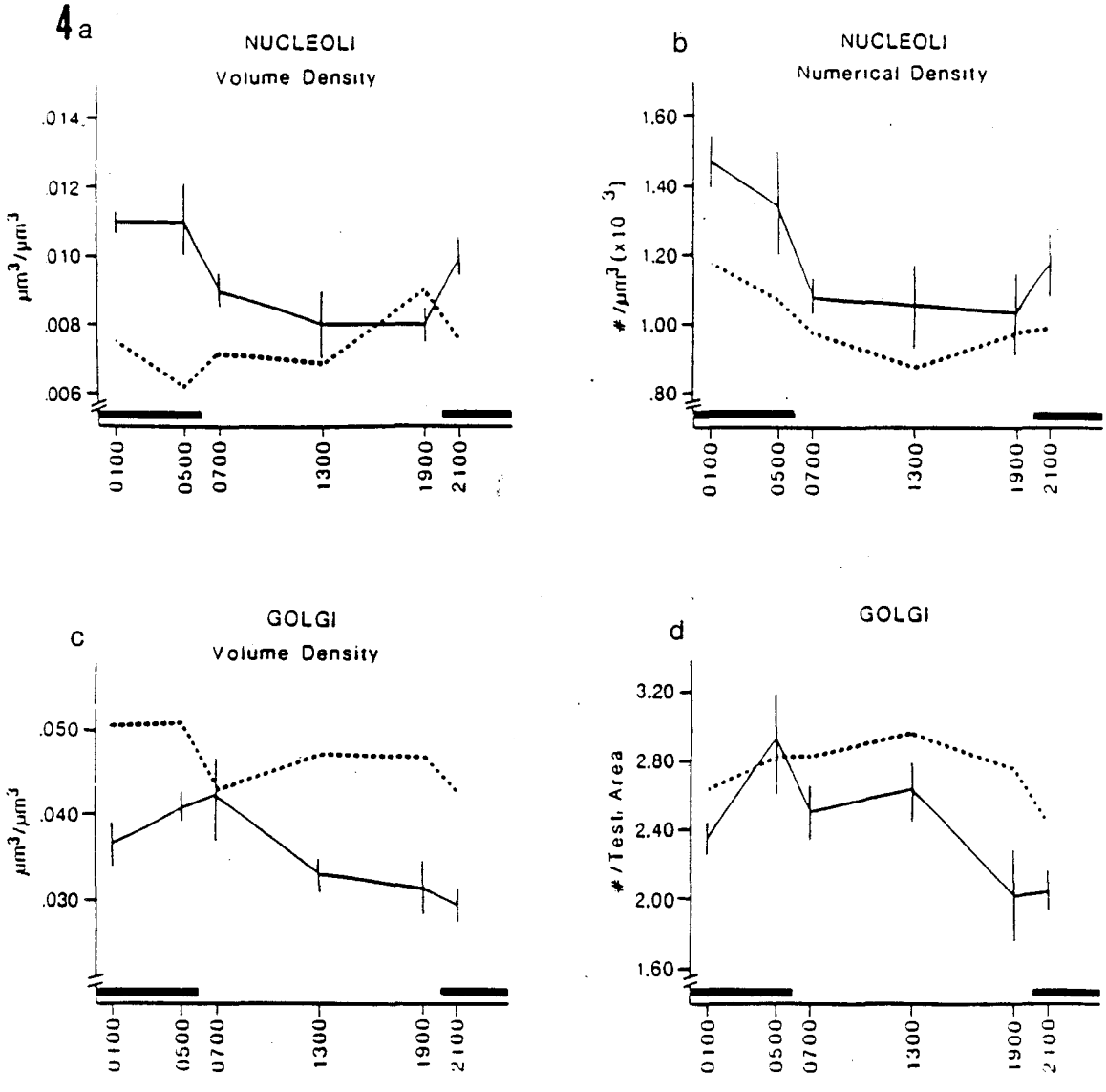

3a
GOLGI
Volume Density
.032
.028
.024
.020
.016
μm³/μm³
0100
0500
0700
1300
1900
2100
b
GOLGI
Width
.350
.325
.300
.275
.250
μm
c
GOLGI
Length
.700
.650
.600
.550
.500
μm
d
GOLGI
Vesicles
18
16
14
12
10
#/Golgi

PLATE IV

Figure 4. Morphometric analysis of the deep pineal gland of normal and blinded animals over a 24-hour light:dark cycle. For all graphs, solid line indicates values for normal animals, broken line represents blinded hamsters. Each point is the mean of six animals. The vertical lines represent $\pm$ the standard error of the mean for normal hamsters. For additional data, see Chapter IV.

4a. Volume density of pinealocyte nucleoli
4b. Numerical density of nucleoli
4c. Volume density of Golgi
4d. Number of Golgi per test area of cytoplasm


4a
NUCLEOLI
Volume Density
μm³/μm³
.014
.012
.010
.008
.006
0100
0500
0700
1300
1900
2100
b
NUCLEOLI
Numerical Density
#/μm³ (x10⁻³)
1.60
1.40
1.20
1.00
.80
c
GOLGI
Volume Density
μm³/μm³
.050
.040
.030
d
GOLGI
#/Test. Area
3.20
2.80
2.40
2.00
1.60

Chapter VII

SUMMARY

1. The pinealocytes of the superficial and deep pineal glands are quantitatively different. Deep pinealocytes are smaller in size and possess smaller nuclei with fewer nucleoli. The cytoplasm of the deep pinealocytes contains proportionally less mitochondria and lipid but more rough endoplasmic reticulum, Golgi and dense-cored vesicles. These results indicate that cells of the deep gland represent a structurally distinct population of pinealocytes. Deep pineal glands also contain smaller glial cells and more blood vessels.

2. Pinealocytes of superficial and deep pineals show morphological rhythms that are not in phase. Pinealocytes of the superficial gland demonstrate strong circadian rhythmicity with significant 24-hour variation in their nuclei, nucleoli, rough and smooth endoplasmic reticulum, dense-cored vesicles, Golgi and dense bodies. In deep pinealocytes, significant rhythms are found only in nucleolar volume and numerical density and in Golgi volume and number.

3. Cells of both superficial and deep pineal glands appear to be stimulated by blinding, with increased cytoplasmic proportions of some of their organelles. The responses in superficial and deep pinealocytes were not identical, however. Superficial pinealocytes showed decreased nuclear and cytoplasmic volumes and increased values for nucleoli, Golgi, dense bodies, smooth and rough endoplasmic reticulum and dense-cored vesicles. Deep pinealocytes demonstrated a decline in nuclear and cytoplasmic volume, as well as increases in their cellular content of mitochondria, lipid, Golgi, dense bodies, smooth endoplasmic reticulum and dense-cored vesicles.

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APPENDIX A

McNULTY-HAZLETT MODIFICATION OF KARNOFSKY FIXATION METHOD

To make 1500 ml of fixative (1% paraformaldehyde-1.25% glutaraldehyde) pH = 7.4

A. Add 15 grams paraformaldehyde to 750 ml of distilled water. Cover and keep in a 60° C oven overnight.

B. While still warm, stir and slowly add eight drops of 1N sodium hydroxide.

C. Add 75 ml of 25% glutaraldehyde.

D. Add 700 ml of Hazlett's buffer (see below). Adjust to 330 mOsm with glucose.

E. Stock solutions for Hazlett's buffer:

1. Solution A: KH_2PO_4 (monobasic) 13.62 g/1000 ml water

2. Solution B: Na_2HPO_4 (dibasic) 14.15 g/1000 ml water

To get 800 mls of buffer, add 156.8 ml of Solution A to 643.2 ml of Solution B.

F. Perfuse animal with approximately 150 ml of fixative/100 g body weight.

TISSUE PROCESSING TECHNIQUE

1. After perfusion, place tissue in 4% glutaraldehyde:Hazlett's buffer solution for one hour.

2. Rinse tissue with Hazlett's buffer.

3. Post-fix tissue in 1% osmium tetroxide:Hazlett's buffer solution for one hour.

4. Rinse tissue with Hazlett's buffer for three minutes (twice).

5. Dehydrate tissue with graded series of acetones:

   50% acetone - five minutes
   75% acetone - five minutes
   80% acetone - five minutes
   95% acetone - five minutes (three times)
   100% acetone - five minutes (three times)

6. Infiltrate tissue with 1:1 Araldite 502:100% acetone overnight.

7. Infiltrate tissue with 3:1 Araldite 502:100% acetone for approximately 12 hours.

8. Infiltrate tissue with pure Araldite 502 overnight in an unheated vacumn oven or bell jar.

9. Embed tissue in Araldite 502. Place in 60 C oven overnight to polymerize.

10. To make 100 mls of Araldite 502:

Araldite 502	- 62.0 g
DDSA*	- 48.4 g
DMP-30**	- 1.6 ml

* = Dodecenylsuccinic anhydride
** = 2,4,6-Tri(dimethylaminomethyl)phenol

APPENDIX B

STEREOLOGICAL FORMULAE

LIGHT MICROSCOPY

Abbreviations

A = Sum of particle section areas in AT
AT = Sum of reference areas
NA = Number of particles in AT
U = Sum of particle section perimeter in AT

Formulae

Volume Density (Vv) = A/AT

Numerical Density (Nv) = 2/3 x NA x U/AT x A

Mean Volume ($\bar{v}$) = 3 /2 x A^2/NA x U

ELECTRON MICROSCOPY

Abbreviations

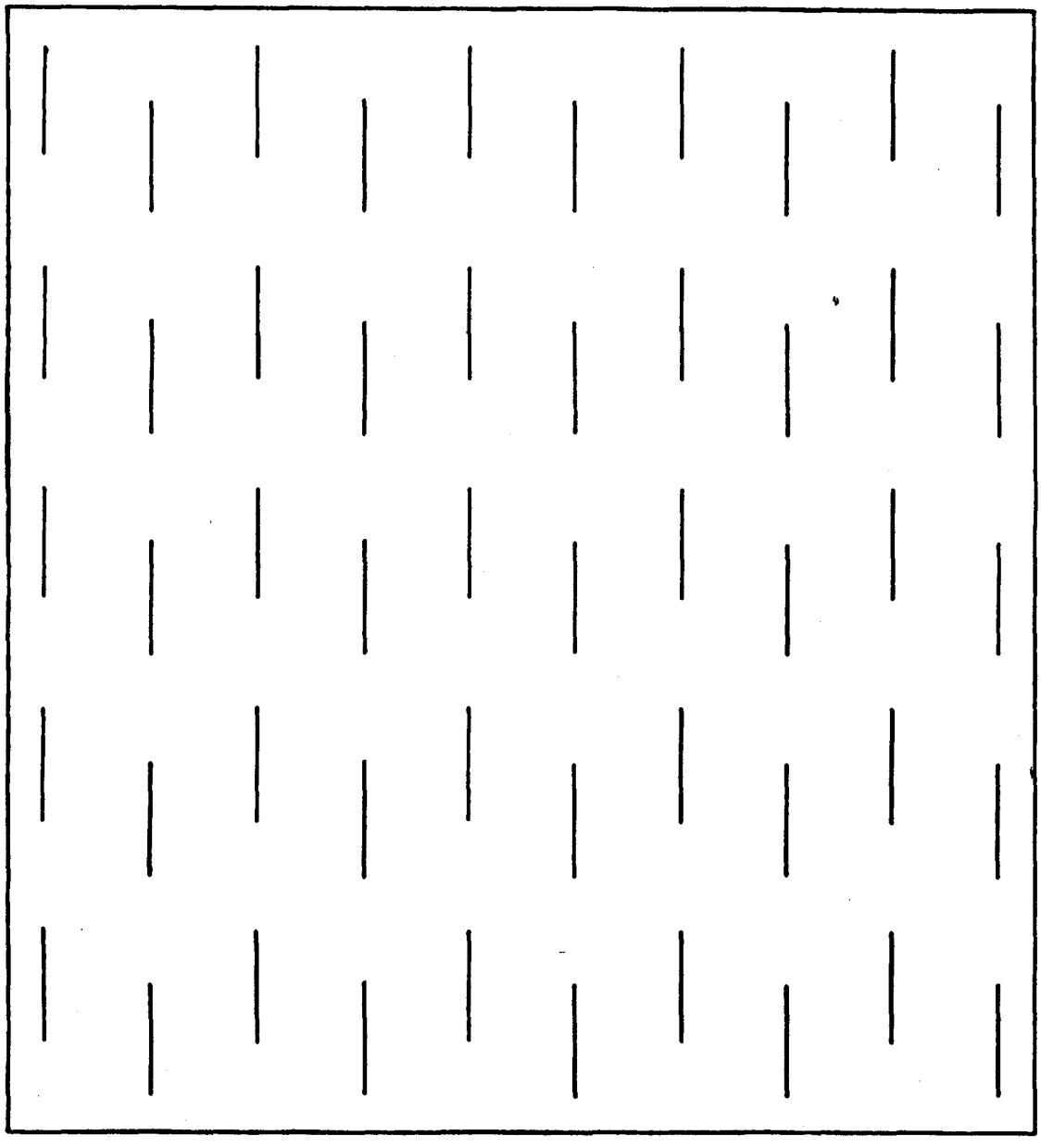
I = Intersects with the lines
P_c = Points on cytoplasm
P_o = Points on organelle
z^o = Distance between lines

Formulae

Volume Density (Vv) = P_o/P_c

Surface Density (Sv) = I x $4/P_c$ x z

Stereological point-counting grid used for electron microscopic analysis of pinealocyte cytoplasm. For this study, outer dimensions of the grid were 145 x 155 mm. Lines measured 15 mm. All distances between lines were 15 mm. For volume density estimation, ends of lines were regarded as points.



APPENDIX C

Title: Comparison of Serotonin and Melatonin Levels in the Superficial and Deep Pineals over a Light:Dark Cycle. Use of High Peformance Liquid Chromatography (HPLC).

Note: This paper outlines a preliminary study concerning indoleamine analysis in the superficial and deep pineal glands. Although the results of this study were not of publishable quality, the paper may provide valuable guidelines for other investigators wishing to use this technique.

Abstract

Indole compounds in the pineal complex of golden hamsters were separated by HPLC and detected electrochemically. In the superficial gland, melatonin content showed significant circadian variation over a 14h:10h light:dark cycle, with a trend toward maximal values occurring at 0500 h. Serotonin levels in the superficial gland did not vary over the 24-hour period. Melatonin was not detected in the deep gland. Serotonin content of deep pineal and associated habenular tissue showed a significant rhythm, with maximal levels during the photophase. These results suggest that serotonin production in these tissues is controlled by post-ganglionic sympathetic nervous input.

Key Words: Deep Pineal, HPLC, Serotonin, Melatonin, Circadian Rhythm

Introduction

The circadian rhythmicity of the superficial pineal gland of the golden hamster has been well established at both the biochemical (Steinlechner et al., 1983; Rollag et al., 1980) and morphological (Dombrowski and McNulty, this Dissertation; Reiter, 1981a) levels. The deep pineal gland also displays morphological variation over a 24-hour light:dark cycle, although the rhythms are not in phase with those found in the superficial gland (Dombrowski and McNulty, Chapter IV). The deep pineal produces the methoxyindole melatonin in a circadian pattern similar to that seen in the superficial pineal, although levels of the compound are much lower in the deep gland (Sheridan and Rollag, 1983). Serotonin content of the deep pineal gland reportedly lacks rhythmic variation, as do the enzymes hydroxyindole-O-methyltransferase (HIOMT) and N-acetyltransferase (NAT) (Moore, 1975).

Recently, several investigators have employed high performance liquid chromatography (HPLC) with electrochemical detection for the analysis of pineal indole content. These studies have, to date, focused upon the superficial pineal gland of the rat (Anderson et al., 1982; Mefford, 1981; Mefford and Barchas, 1980) and hamster (Steinlechner et al., 1984, 1983). Using HPLC we assessed serotonin and melatonin levels in the superficial and deep pineal gland of the hamster. Both glands were sampled over a 24-hour span to determine possible circadian changes in indole content.

Materials and Methods

Thirty-six golden hamsters (18 male and 18 female) approximately two months in age, were entrained to a 14h:10h light:dark cycle (lights from 0600-2000h) for a period of six weeks. Groups of six hamsters (three male and three female) were decapitated at each of the following time points: 0100, 0500, 0700, 1300, 1900 and 2100 h. Hamsters were lightly anesthesized with ether prior to sacrifice. Immediately following sacrifice, the superficial and deep pineals were dissected out, frozen on solid carbon dioxide, and stored at -80° C. Some adjacent tissue from the region of the habenular nuclei was dissected along with the deep pineal gland.

Tissue preparation

Deep pineal tissue from either the three males or three females of each group was weighed while thawing, pooled and hand homogenized in 200 µl of 0.1 M perchloric acid, with 50 µl of indoleacetic acid (IAA) added as an internal standard. Homogenate was centrifuged for one minute in a microfilter tube equipped with a 0.45 µm membrane filter (Ultipor$^{R\ TM}$). The filtrate was frozen at -80° C. Deep pineals of three animals were pooled in order to increase melatonin concentration per sample.

Superficial pineals were weighed, pooled and hand-homogenized in 200 µl of 0.1 M perchloric acid with 50 µl of IAA added as an internal standard. Homogenate was filtered as described above, then refrozen at -80° C.

Chromatographic system

The HPLC system consisted of an Altex 110A Solvent Metering Pump equipped with a pulse dampener, Rheodyne 7125 Inject Valve, Bioanalytical Systems LC-4B Amperometric detector and Linear 1210 recorder. The chromatograph was equipped with a Brownlee MPLC RP-18 Spheri-5 (five μm) column (4.6 x 220 mm) with a Brownlee MPLC RP-18 Spheri-5 (five μm) guard column. The amperometric detector was fitted with a glassy carbon electrode housed in a thin layer single electrode cube (TL 5A), spaced with a five μm gasket. The reference compartment contained a Ag/AgCl reference electrode. Potential of the working (glassy carbon) electrode was set at +0.7 to +0.9 V. The recorder was set at 1V full scale deflection.

Solutions

The buffer consisted of 0.01 M sodium acetate (HPLC grade - Baker Analyzed Reagent) in glass-distilled water. pH was adjusted to 4.5 with glacial acetic acid. The mobile phase was made up of the appropriate volume of buffer in combination with methanol (HPLC grade - Baker Analyzed Reagent). Mobile phase was filtered under vacumn using a 0.45 μm millipore filter (Alpha 450, Metricel Membrane Filter, Gelman Sci.), and degassed for 30 minutes under vacumn.

Indole standards were made up in distilled water (serotonin) or 1:9 distilled water:methanol (melatonin, indoleacetic acid) at a concentration of one mg/ml, and stored at 4° C for several weeks.

Appropriate dilutions were prepared daily using distilled water. All standards were obtained from the Sigma Chemical Company.

Melatonin Analysis

For melatonin determination, the mobile phase consisted of 65% buffer (pH 4.25) and 35% methanol (v:v). The working electrode was set at +0.9 V versus the reference electrode. The flow rate was 0.7 ml/min. Range of sensitivity for the detector was 0.1 nA for the deep pineal and 0.2 nA for the superficial pineal samples. The limit of detection for melatonin was 20 pg. Fifty µl of deep pineal and 75 µl of superficial pineal filtrate were injected. Percent recovery was estimated by comparison of IAA levels with those of previously injected standards, allowing adjustment for variations in detector sensitivity and sample volume.

Serotonin Analysis

Mobile phase consisted of 88% buffer (pH 4.25) and 12% methanol (v:v). Working electrode was +0.7 V versus reference. Flow rate was 1.2 ml/min. Range of sensitivity was 2 nA for deep pineal and 1 nA for superficial pineal tissue. Twenty µl of deep pineal and 50 µl of superficial pineal filtrate were injected. Percent recovery was based upon within-day coefficients of variation for serotonin, which ranged from 2-12 %.

Data Analysis

Chromatographs from HPLC analysis were analyzed for within peak area using the Zeiss Videoplan Image Analyzer. Data were expressed as

the mean $\pm$ the standard error of the mean. Twenty-four hour variation in the indole levels was determined using a one-way analysis of variance test.

Results

Melatonin

Retention time of melatonin was 22 minutes, with a limit of detection of 20 pg (Fig. 1a). No melatonin was detected in the deep pineal tissue. In the superficial pineal, melatonin levels showed a trend for higher levels at 0500 h (Fig. 2). Melatonin levels were low throughout the rest of the light:dark cycle.

Serotonin

Retention time for serotonin was 12 minutes. The limit of detection was 35 pg (Fig. 1b). Deep pineal tissue showed circadian rhythmicity in serotonin levels. Maximal levels occurred at 1900 h (Fig. 3a), while low values were found during the scotophase (Fig. 3b). Serotonin levels were elevated throughout the photophase (Fig. 5). Serotonin content of the superficial pineal gland did not exhibit circadian rhythmicity (Fig. 4).

Plate 1

Figure 1. a) Chromatograph of melatonin standard. Peak represents 50 pg of melatonin. Mobile phase, 35% methanol:65% sodium acetate, pH 4.5. Range .2 nA.

b) Chromatograph of serotonin standard. Peak represents 1 ng of serotonin. Mobile phase, 12% methanol:88% sodium acetate, pH 4.5. Range 2 nA.

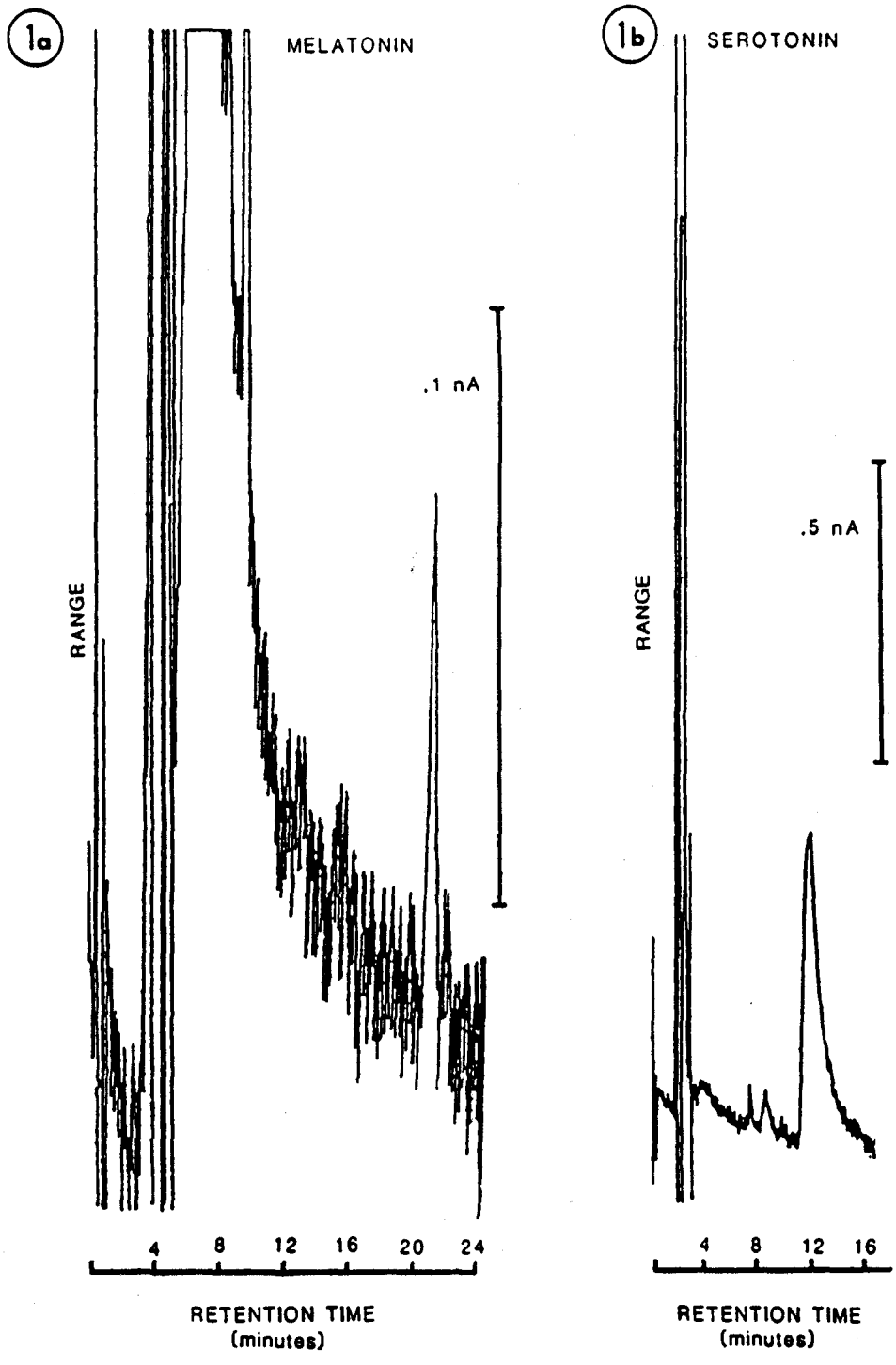

1a
MELATONIN
.1 nA
RANGE
4
8
12
16
20
24
RETENTION TIME
(minutes)
1b
SEROTONIN
.5 nA
RANGE
4
8
12
16
RETENTION TIME
(minutes)

Plate 2

Figure 2. Melatonin content of the superficial pineal gland over a 24-hour period, expressed as pg/gland.

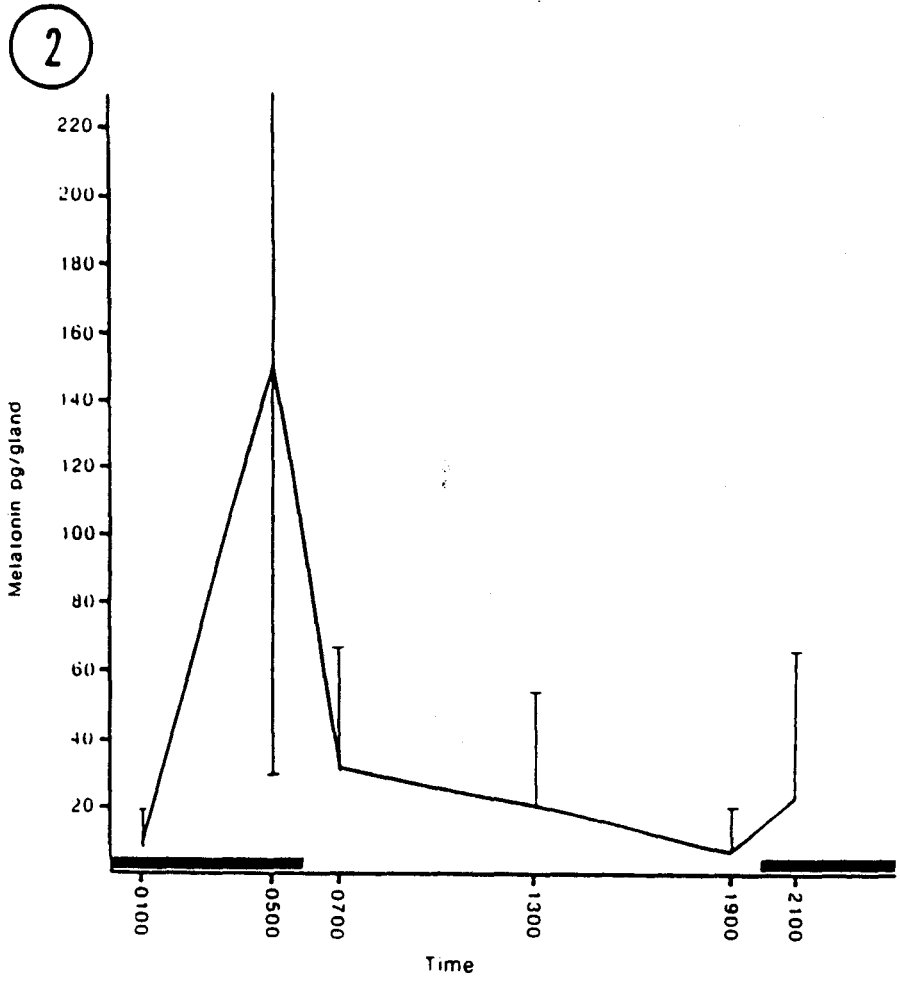

2
220
200
180
160
140
120
100
80
60
40
20
Melatonin pg/gland
0100
0500
0700
1300
1900
2100
Time

Plate 3

Figure 3. a) Chromatograph of deep pineal tissue from an animal sacrificed at 1900 h. 1=unidentified compound, 2=serotonin.

b) Chromatograph of deep pineal tissue from an animal sacrificed at 0500 h. 1=unidentified compound, 2=serotonin.

SEROTONIN LEVELS IN DEEP PINEAL TISSUE

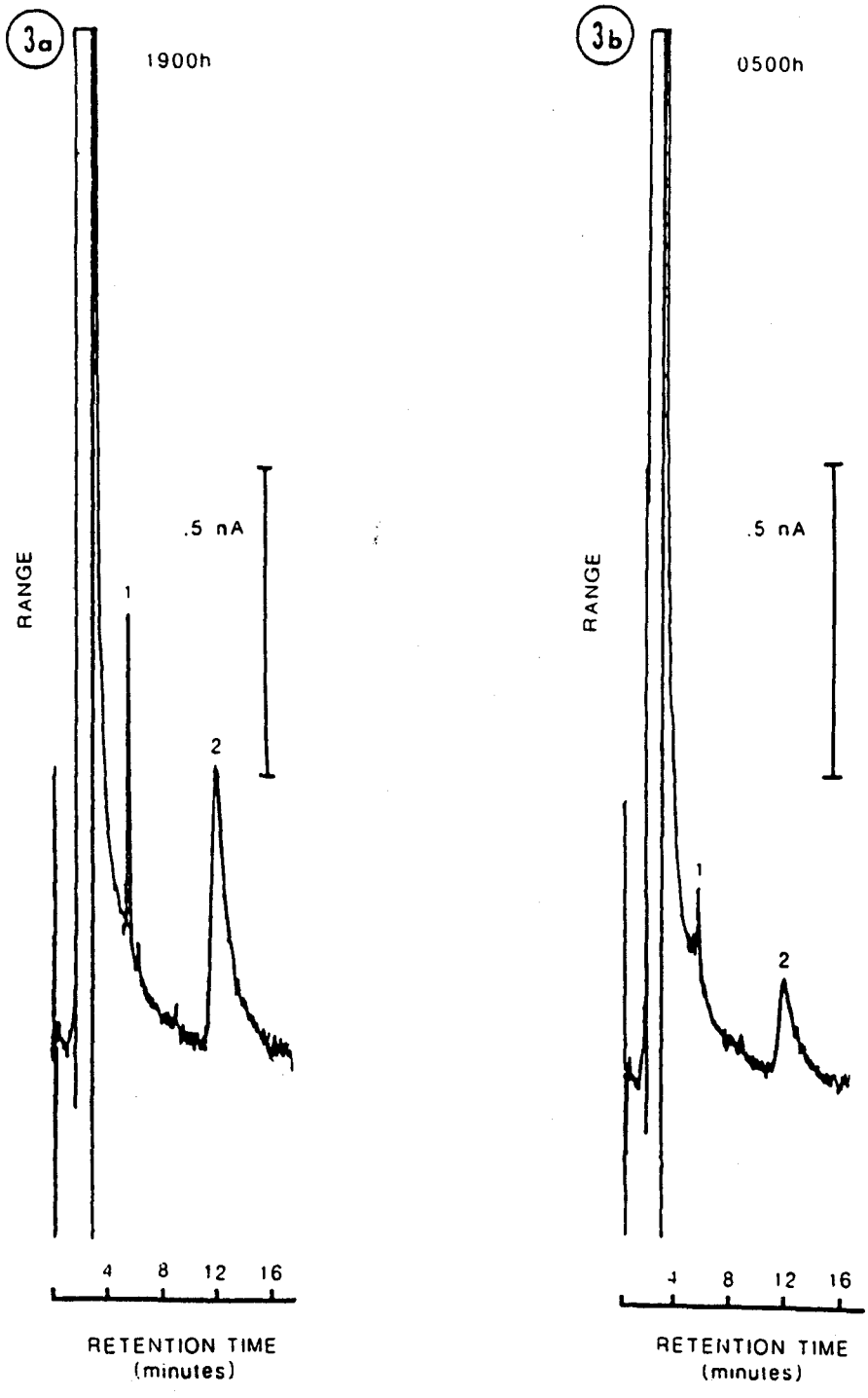
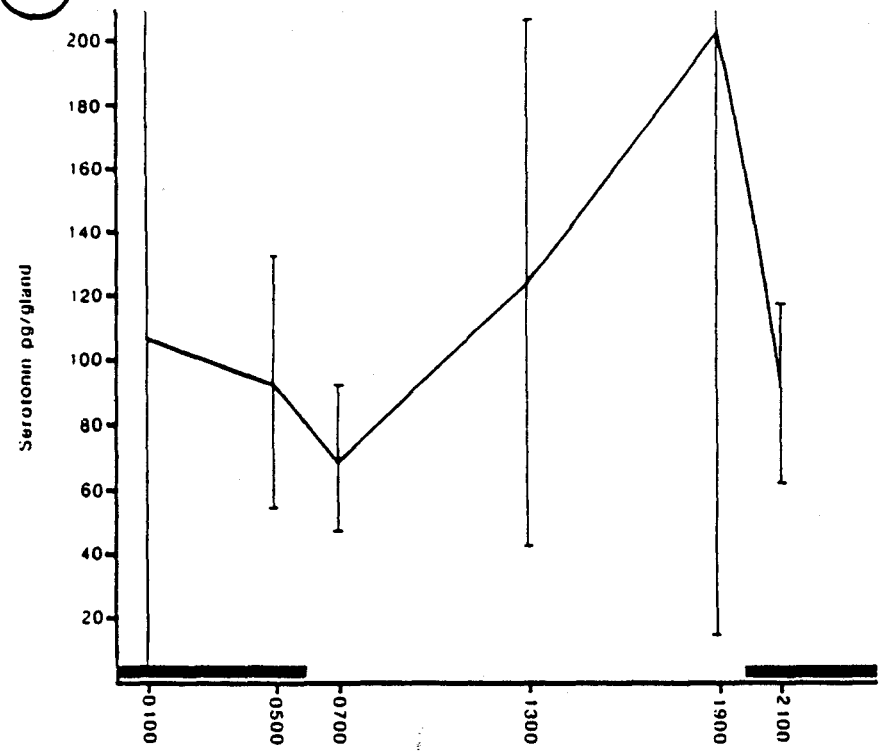


Plate 4

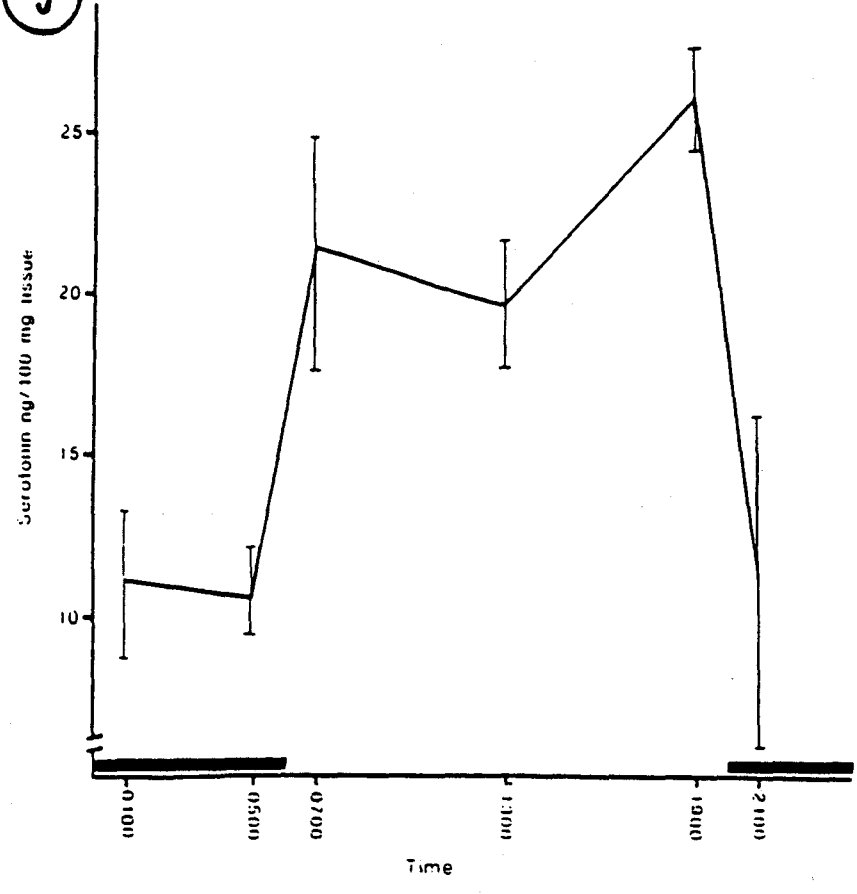
Figure 4. Serotonin content of the superficial pineal gland over a 24-hour period.

Figure 5. Serotonin content of the deep pineal gland over a 24-hour period. $p<0.025$.

4


Serotonin pg/gland
200
180
160
140
120
100
80
60
40
20
0100
0500
0700
1300
1900
2100

5


Serotonin ng/100 mg tissue
25
20
15
10
0100
0500
0700
1300
1900
2100
Time

Discussion

Melatonin

The melatonin content of the deep pineals of prepubertal hamsters was only 5% of that found in the superficial gland, ranging from five to 20 pg (Sheridan and Rollag, 1983). Melatonin was not detected in the deep pineal tissue examined in this study, possibly due to its degradation while the tissue was frozen. Because melatonin levels in the deep pineal are initially small, any significant loss of the methoxyindole content of the gland would render it undetectable.

Circadian changes in superficial pineal melatonin content in this study displayed a trend toward maximal values similar to that previously reported in the golden hamster, with peak values occurring at the end of the dark period (Rollag et al., 1980). The superficial pineal content of the methoxyindole was diminished, however, with amount of melatonin present at any given time point decreased by up to eight fold. Decreased melatonin levels could have resulted from a variety of factors: 1) the pineal filtrate may have been stored at -80° C for too prolonged a period (c. ten months), 2) temperature increases during the process of homogenizing, filtering and freezing the pineal filtrate could have allowed sufficient enzyme activation for breakdown of the melatonin contained in the gland, 3) the chromatographic system outlined in the Materials and Methods section may not have provided optimal

conditions for the detection of melatonin contained within the tissue, or 4) the use of ether anesthetic prior to sacrifice may have affected pineal melatonin levels.

Serotonin

The deep pineal gland has been reported to lack rhythmicity in its content of serotonin (Wiklund, 1974; Moore, 1975). We, however, found a significant rhythm in serotonin levels in the deep pineal gland,, with maximal levels at the end of the light period. This rhythm is similar to 24-hour changes in superficial pineal serotonin content as reported by Steinlechner et al. (1983) but not confirmed in the present study.

Tissue examined in this study of the deep pineal included not only the deep pineal gland but also portions of the habenular and posterior commissures and the habenular nuclei. The serotonin rhythm seen in the deep pineal tissue in this study may reflect circadian changes in the indoleamine content of these structures, either independently or in concert with similar changes in the deep pineal. Tissue in the region of the habenular nuclei has been shown to receive postganglionic sympathetic fibers continuous with those innervating the superficial pineal gland (Nielsen and Moller, 1978, Wiklund, 1974; Bjorklund et al., 1972). Common innervation of the superficial pineal and the habenular nuclei could result in synchronization of the metabolic processes of the two areas. The contribution of this common innervation to the serotonin rhythm found in the deep pineal in this study cannot be discounted. Involvement of the habenular nuclei in indole production may also

explain the high levels of serotonin found in the deep pineal tissue which are out of proportion with the known size of the deep pineal gland.

Lack of a rhythm in serotonin content of the superficial pineal was unexpected. Distinct 24-hour variation has been reported in the superficial pineal gland by using spectrofluoremetric analysis (Quay, 1963a) and HPLC (Steinlechner et al., 1983; Anderson et al., 1982). Serotonin levels in the hamster ranged from 20 to 70 ng per gland, with maximal levels at the end of the light period (Steinlechner et al., 1983). Serotonin levels in the present study were significantly less than those reported by Steinlechner et al. (1983). The decline in the serotonin levels in this study, in addition to the absence of significant 24-hour changes, suggests a loss of the serotonin content of the superficial pineal gland, presumably due to technical difficulties.

APPROVAL SHEET

The dissertation submitted by Teresa D. Niiro has been read and approved by the following committee:

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Dr. Nick Emannuele
Department of Internal Medicine, Hines V. A.

The final copies have been examined by the director of the dissertation and the signature which appears below verifies the fact that any necessary changes have been incorporated and that the dissertation is now given final approval by the Committee with reference to content and form.

The dissertation is therefore accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Anatomy.

19 Sep 84
Date

Director's Signature