VOLUMETRIC AND CYTOLOGIC VARIATION IN THE PINEAL BODY OF *PEROMYSCUS LEUCOPUS* (RODENTIA) WITH RESPECT TO SEX, CAPTIVITY AND DAY-LENGTH [1]

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THIRTEEN FIGURES

INTRODUCTION

The functional significance of the mammalian pineal organ has remained obscure, in spite of the efforts of many investigators. Nevertheless, in recent years increasing evidence suggests that the pineal has an antigonadal or antigonadotrophic activity (Simonnet, Thieblot, Melik and Segal, '54; Kitay and Altschule, '54; and others). Since the pineal of certain lower vertebrates is a light receptive organ, and since reproduction in some vertebrates including mammals is affected by day-length, the significance of the pineal in regard to photoperiodic responses should be examined.

The white-footed mouse *(Peromyscus leucopus),* a feral, nocturnal, polyestrous species with an annual winter anestrum, has been shown to be affected in its reproductive activity by changes in day-length (Whitaker, '40). Whitaker found that in light of varying daily duration according to an annual schedule the anestrum occurred in the short day-season, and that blinded mice did not exhibit cyclic reproductive activity. Due to the availability of the white-footed mouse and the knowledge of its photoperiodic reproductive behavior, this

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species was chosen for the present investigation. It is believed that cytological data presented here on the pineal in relation to modifications of day-length may provide evidence on the mechanism of photoperiodic behavior as well as on pineal function.

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MATERIAL AND METHODS

Thirty-two adult white-footed mice *(Peromyscus leucopus)* obtained from the same woodland tract in Washtenaw County, Michigan, were used in this study. Throughout their period of captivity the mice were kept in separate cages. The bottoms of the cages, solid and covered with wood shavings and shredded paper, measured 10×15 inches; the height of the cages was 8 inches. Cages exposed to artificial light in the laboratory were constructed entirely of wire mesh, except for the bottom. Food and tap water were available to the animals at all times. The food consisted of Dice's ('34) Peromyscus ration without wheat germ, hemp seed, millet seed, and iodized salt. Lettuce and apple slices were supplied once a week. Artificial illumination of the animals was supplied by ordinary household 60–100 watt electric light bulbs delivering a measured 40–100 foot-candles to the closest portions of the interiors of the cages. Within each cage there was sufficient bedding so that the mice could be in nearly complete darkness when they desired. The artificial illumination of the animals was controlled by an automatic timer and consisted of a single period of light per day timed to occur near the middle of the natural day. While in the laboratory, the mice were at a temperature of 19°–26°C. Although the mice at no time came into contact with each other, the mice within each experimental group could hear and see the animals in neighboring cages.

The experimental mice have been arranged into 7 groups according to their month of capture and the nature of their

subsequent treatment. The description of these experimental groups is summarized in table 1. For all mice except no. 32 the date of capture refers to the date of capture in the outdoor habitat. All subsequent treatment, except the first treatment period for no. 1, were in the laboratory. Animals given 14–18 hours of light per day were given a day-length which gradually increased from 14 to 18 hours during the first two

TABLE 1

Treatment and history of experimental animals

GROUP	NOS.	NUMBER AND SEX	DATE OF CAPTURE	EXPERIMENTAL TREATMENT (hours of light per day; length of period)			
1	1	1 ♀	Oct.	natural light [1] 6 wks. →	12 hrs. 69 days	→	14–18 hrs. 22 days
2	2–9	4 ♀, 4 ♂	Oct.	natural light 3–6 wks.	→	0 hrs. 91 days	
3	10–17	2 ♀, 2 ♂	Oct.	natural light 3–6 wks. →	0 hrs. 65 days	→	14–18 hrs. 25 days
4	20–21, 26–31	4 ♀, 4 ♂	Jan.	0 hrs. 26–36 days			
5	22–25	2 ♀, 2 ♂	Jan.	0 hrs. 2–8 days →		14–18 hrs. 25 days	
6	32	1 ♂	Jan.[2]	0 hrs. 26 days →		14–18 hrs. 25 days	
7	33–34	2 ♀	Jan.	14–18 hrs. 23–24 days			

[1] Outdoors 6 weeks.

[2] Captured in laboratory.

weeks and which was maintained at 18 hours for the remainder of the experiment.

The mice were sacrificed by means of ether anesthesia to death. Anesthesia of all mice was performed between 2:00 and 5:30 P.M., and with minimal disturbance by placing each caged mouse into a large covered can containing ether. The mice were not handled until unconscious. They were then weighed, measured and perfused via the heart with Bouin's

fluid (75 cm^3 sat. aq. picric acid, 25 cm^3 formalin, 5 cm^3 gl. acetic acid). Sixteen hours later each brain with attached hypophysis, pineal and meninges was removed and placed in Bouin's fluid for approximately two days. The central area of each brain with attached hypophysis and pineal was then washed in several changes of 70% ethanol, dehydrated, cleared and embedded in paraffin. Serial cross sections 7 μ in thickness were affixed in order to glass slides, 10 sections per slide. Alternate slides were stained with chrome hematoxylin and phloxine (Gomori, '41) and paraldehyde-fuchsin with Orange G, Light Green SF, and chromatrope 2R (Halmi, '52). The hematoxylin portion of the latter technique was omitted.

RESULTS

The anatomy of the pineal body. The pineal body of *Peromyscus leucopus* consists fundamentally of two parts. The more anterior and basal of these surrounds a dorsal evagination of the third ventricle (fig. 1) and is closely associated basally with the subcommissural organ and the habenular commissure. The more posterior and dorsal part of the pineal lies near the surface, between the cerebral hemispheres and over the anterior part of the cerebellum. Between the two parts of the pineal there is a continuous strand of pineal tissue in 31% of the animals, an interrupted strand of pineal tissue in 44%, scattered islets of pineal tissue in 22%, and no intermediate pineal tissue in 3%. There is no correlation between sex and abundance or arrangement of intermediate pineal tissue.

The anterior pineal is characterized by closely spaced cells with little circumnuclear cytoplasm and many phloxinophilic fibers. The posterior pineal is characterized by less closely spaced cells with more circumnuclear cytoplasm and few phloxinophilic fibers. Since the tissue of the connecting strand or islets resembles that of the posterior part of the pineal more closely than that of the anterior part of the pineal, the intermediate strands or islets are classed with the posterior pineal proper. The junction between the anterior and pos-

terior pineal types of tissue always occurs close to the anterior pineal mass. The intermediate strand of posterior pineal tissue sometimes overlaps the posterior part of the anterior pineal and, although in contact with it, can be histologically distinguished from it. However, the junction of the two types of pineal tissue is sometimes indefinite and for convenience the boundary is placed at the center of the first marked constriction at the posterior tip of the anterior pineal.

From the foregoing description it can be concluded that complete pinealectomy or complete posterior pinealectomy would be difficult in this species. This is in contrast with the laboratory rat *(Rattus)* in which there is little pineal tissue near the basal attachment next to the third ventricle.

Volumetric variation. The volumes of the anterior and posterior pineals were computed from outline camera lucida drawings of every fifth section in the following manner. The area of each outline drawing was determined by planimetry. The area of the sections between the drawn and measured ones were arithmetically graded between the values obtained for the measured sections. The areas of parts of the pineal were obtained by: (1) adding the computed areas of the drawn sections and those lying between drawn sections, (2) multiplying by the thickness of the section and (3) dividing by the square of the magnification of the drawn sections. There are numerous sources of error possible with this method of determining volume, but these possible errors appeared to be slight and insufficient to affect greatly the variation of the resulting figures. The magnitude of the probable errors in the volumes varied greatly from specimen to specimen. Errors due to: tissue compression during sectioning, optical distortion, variation in drawing technique, variation in planimetry, and variation in the areas of the sections between the sections which were actually measured were found to be slight. Of greater concern were errors due to variation in section thickness and creation of abnormal spaces by excessive perfusion pressure. Errors due to occasional sections of different thickness were detected by their difference in

staining and were corrected by comparing the cell content with that of adjacent sections of normal (7 μ) thickness. Evidences of perfusion artifact were seen in only a few specimens, and were slight in extent. They were not calculated or corrected. However, computation of the content of cells in the pineal removes the possible error due to perfusion artifact. Calculations were made of the number of cells in the pineal, including neuroglial and parenchymal cells but excluding connective tissue cells (fibroblasts, mast cells, phagocytes, lymphoid cells), cells associated with blood vessels (endothelial cells and smooth muscle), ependymal cells and subcommissural organ-type cells. For counting the number of cells in the pineal a cover glass with a square etched on one surface was placed at a suitable level in the ocular of the microscope. The area of this square, in terms of the tissue section being observed through it, was determined with a stage micrometer and planimetry of a camera lucida outline drawing of the circumscribed area. By counting the number of pineal nuclei lying within this square a computation could be made of the number of cells in this known area, since binucleate or multinucleate pineal cells were believed to be absent or extremely rare. Cell counts were made in this way in every 10th section of the posterior pineal at measured intervals varying in magnitude according to the size of the section. Generally over a hundred cells (nuclei) were counted in each such section. In very small sections, all of the cells were counted. In the anterior pineal, cell counts were made in the same way but only in the section at or very near the posterior tip of the pineal recess of the third ventricle. In the posterior pineal the cell density is usually much greater proximally and peripherally. This necessitates the making of cell counts at definite intervals and in sections at all levels.

The pineal volumes and cell contents of the specimens are presented in table 2. It should be noted first that there is great individual variation in these figures. When the data are grouped and compared in terms of sex, length of time in captivity, and exposure to darkness or increased day-length,

TABLE 2

Volumes of the anterior (Ant.) and posterior (Post.) parts of the pineal organ in mm³ and in estimated cell content. $\bar{x}$ *= mean. Se = standard error of the mean. C = coefficient of variation.* $\bar{x}♂$ *= mean of males.* $\bar{x}♀$ *= mean of females.*

SPECIMEN NUMBER AND SEX		HEAD AND BODY LENGTH	TOTAL BODY WGT.	PINEAL VOLUME			PINEAL CELL CONTENT		
				Ant.	Post.	Sum	Ant.	Post.	Sum
		mm	*gm*		*mm³*			*× 1/1000*	
1	♀	98	19.4	.0200	.1617	.1817	256	982	1238
2	♀	91	16.2	.0176	.0896	.1072	284	500	784
3	♀	97	30.3	.0202	.0740	.0942	302	445	747
4	♀	99	27.0	.0246	.1316	.1562	297	836	1133
5	♀	93	17.9	.0208	.1588	.1796	309	942	1251
6	♂	92	24.6	.0223	.0789	.1012	322	524	846
7	♂	99	30.0	.0169	.0570	.0739	248	477	725
8	♂	93	26.5	.0144	.0396	.0540	233	424	657
9	♂	90	18.6	.0220	.1379	.1599	286	1067	1353
10	♀	94	21.3	.0130	.0657	.0787	270	1048	1318
11	♂	93	18.7	.0180	.0342	.0522	313	420	733
12	♂	95	28.1	.0129	.0477	.0606	228	...	...
13	♂	95	23.9	.0210	.0936	.1146	245	668	913
14	♂	97	30.3	.0171	.0840	.1011	...	...	...
15	♂	93	26.2	.0257	.0917	.1174	246	611	857
16	♀	92	18.3	.0251	.0825	.1076	308	766	1074
17	♂	92	21.4	.0210	....	....	233	...	...
20	♂	92	26.9	.0267	.1210	.1477	171	899	1070
21	♂	95	28.3	.0139	.0755	.0894	209	912	1121
22	♀	90	21.9	.0217	.1330	.1547	525	1318	1843
23	♀	93	22.1	.0223	.0996	.1219	305	790	1095
24	♂	90	26.3	.0199	.0623	.0822	198	602	800
25	♂	92	26.0	.0232	.1716	.1948	373	1325	1698
26	♀	86	18.2	.0193	.0957	.1150	270	1048	1318
27	♀	97	24.7	.0208	.1175	.1383	303	1009	1312
28	♀	86	19.4	.0197	.0801	.0998	351	804	1155
29	♀	85	19.7	.0188	.0720	.0908	264	618	882
30	♂	90	27.5	.0188	.0510	.0698	253	509	762
31	♂	97	33.7	.0169	.0618	.0787	232	551	783
32	♂	90	19.3	.0155	.0543	.0698	205	388	593
33	♀	93	22.6	.0250	.1120	.1370	200	684	884
34	♀	89	19.7	.0163	.0340	.0503	213	337	550
$\bar{x}$		93	23.6	.0197	.0894	.1090	273	742	1017
Se				.0021	.0068	.0072	11.97	51.08	58.12
C				.5907	.4209	.3669	.2444	.3710	.3077
$\bar{x}♂$		93	25.7	.0192	.0789	.0980	250	670	922
$\bar{x}♀$		92	21.2	.0203	.1005	.1209	297	808	1106

it is found that there is a sexual difference but no differences correlated with captivity or day-length. As the mean values in table 2 indicate, the average volume and cell content of the female pineal, particularly the posterior part, exceeds those averages of the male. However, the ranges of the figures for the two sexes broadly overlap and the amount of individual variation reduces further the probable significance of this sexual difference.

Cytologic variation. Cytologic variation was examined in pineal cells in three categories: (1) anterior pineal cells (excluding stromal, ependymal, and subcommissural-type cells), (2) posterior pineal cells, and (3) chrome hematoxylin positive cells. In addition, the phloxinophilic processes and fibers in the anterior pineal, and the aldehyde fuchsin-staining material in both parts of the pineal were studied.

The staining techniques which were used in this study, failed to demonstrate the cytoplasmic contents in the great majority of pineal cells and the outlines of the cells were often obscure. However, the volume of the pineal cells showed marked individual variation. Since the boundaries of many of the cells were vague, an indirect method of estimating average pineal cell volume was used. From the data on the volume and cell content of the pineal, computations were made of the number of pineal cells per mm^3. Since the pineal stroma and intercellular spaces appeared to be minimal and of approximately the same relative volume in the animals, pineal cell density per mm^3 was used as an index of mean pineal cell volume. The figures for number of pineal cells per mm^3 were computed for the anterior pineal in the section at or near the tip of the pineal recess of the third ventricle. The figures for the number of pineal cells per mm^3. were computed for the posterior pineal from data on all sections in which cell counts and pineal areas were measured. Analysis of the resulting figures for pineal cells per mm^3 failed to reveal any significant correlation with sex, length of captivity, or day-length treatment. But the difference between anterior and posterior pineals in cell density was found to be significant

(table 3). There also appeared to be some, if perhaps slight, positive correlation between cell densities in anterior and posterior pineal of the same animals.

Visible cytologic variations, other than cell volume, were limited to nuclear structures in most of the pineal cells. A study of nuclear structural variation was made in both anterior and posterior pineal cells to ascertain whether sub-

TABLE 3

Comparative cytology of the anterior and posterior parts of the pineal. Cells × 10^6 per mm^3 = number of pineal (parenchymal + neuroglial) cells per cubic millimeter. Nuclear diameter = mean nuclear diameter of pineal cells (50 nuclei were measured in each part of the pineal in each specimen). Diameter largest granule = mean diameter of largest phloxine-staining nuclear granule per pineal cell (in 50 nuclei in each part of the pineal in each specimen). Total granule volume = mean total volume per nucleus of phloxine-staining granules of .55 μ diameter or larger (in 50 nuclei in each part of the pineal in each specimen). Se = standard error of the mean. Range = observed range. N = number of animals.

	ANTERIOR PINEAL				POSTERIOR PINEAL			
	Mean	Se	Range	N	Mean	Se	Range	N
Cells × 10^6 per mm^3	14.20	± .49	9.55–24.15	31	8.39	± .34	5.59–12.27	29
Nuclear diameter (μ)	6.13	± .06	5.79– 6.53	17	6.49	± .08	5.96– 7.19	16
Diameter largest granule (μ)	1.41	± .04	1.18– 1.63	17	1.32	± .03	1.01– 1.49	16
Total granule volume (μ^3)	3.51	± .26	1.81– 5.81	17	2.75	± .19	1.15– 3.64	16

types of cells might be distinguished by means of nuclear morphology and whether certain structural variations might be correlated with sex, length of captivity or modification of day-length. Sections stained with chrome hematoxylin and phloxine were found to be most suitable for studies of nuclear morphology and were therefore used for this purpose. Nuclear morphology of anterior pineal cells was studied in one section at or near the tip of the pineal recess of the third ventricle and

nuclear morphology of posterior pineal cells was studied in a section at or near the part of the posterior pineal of greatest cross-sectional area of each animal. In each section, camera lucida drawings were made of fifty nuclei whose maximum dimensions were in the optical plane at mid-depth in the section and which were thus as nearly entirely within the section as posible. The nuclei were drawn from small areas defined by a reticle and of an equal distance from each other and covering the entire sectional area of the pineal. The outline of each nucleus was drawn from the optical plane revealing the maximal nuclear dimensions. And within each nucleus the phloxine-staining granules were drawn, each from the optical plane revealing the maximal true dimensions.

The outline drawings of pineal nuclei and their phloxinophilic granules were analyzed first for half of the animals to determine which nuclear characteristics might be bi-or polymodal and which offered possibly significant correlations with sex or history of the animals. From the nuclear drawings, measurements were made of maximum and minimum nuclear diameters, mean diameter of all phloxinophilic granules equal to or over .55μ in mean diameter, and the number of these phloxinophilic granules equal to or greater than .55 μ in mean diameter per nucleus. From these figures, were computed mean nuclear diameters and the total volumes per nucleus of the phloxinophilic granules. Frequency diagrams of these characteristics in several animals with extreme or mean values demonstrated no significant bi-or polymodality, but they did show marked individual variation in the ranges, variances and means. The data were then analyzed to determine whether cytologic correlations with sex, length of captivity or modification of day-length were evident. The mean nuclear diameter and the mean diameter of the largest phloxinophilic granule per nucleus in anterior and posterior pineal cells showed no such correlations. But there was evidence of correlations between increased day-length and lower mean number of phloxine-staining granules ($=$ or $>$ .55μ mean diameter) per nucleus and lower total volume of these granules per nucleus

in both anterior and posterior pineals. Calculation of the total volumes of the granules was extremely laborious and the results were so variable that it appeared unlikely that calculation of granule volumes for the remaining animals would contribute much to the significance of possible correlations. The mean number of phloxine-staining granules in pineal cells was determined for all animals which could be used in comparisons of the effects of darkness and increased day-length of the pineal (table 4). The difference between the mean number of these nuclear granules in animals in the two groups was found to reach a statistical significance at least in the posterior part of the pineal.

TABLE 4

Comparison of the mean number of phloxine-staining granules (= or > .55 μ in mean diameter) per pineal cell in anterior and posterior pineals, in animals in darkness in comparison with those in 14–18 hours of light per day. Animals in groups 1 and 6 (table 1) were omitted, since their history or treatment differed. Se = standard error of the mean. R = observed range. N = number of animals.

	ANTERIOR PINEAL				POSTERIOR PINEAL			
	Mean	Se	Range	N	Mean	Se	Range	N
Darkness	5.26	± .33	2.62–7.06	16	5.26	± .22	3.48–7.54	16
Light	4.40	± .40	2.74–8.28	13	4.16	± .26	2.82–6.08	12
	*P = > 0.1 and < 0.2				P = > 0.01 and < 0.02			

* Student-Fisher *t* test, significance of differences between means.

Ten to ninety per cent of the pineal cell nuclei are deeply incised or lobed, in the posterior part of the pineal. Differential counts of incised and not incised nuclei in the cross-sections of greatest area in each posterior pineal for half of the animals failed to reveal any significant correlation with sex, captivity or day-length.

Cells containing cytoplasmic granules staining black with chrome hematoxylin were discovered in the anterior pineal and anterior part of the posterior pineal in most animals (figs. 2, 3 and 6). These cells could not be distinguished from pineal parenchymal cells by their nuclear morpholgy. Thus their detection and enumeration had to be based upon the

presence of cytoplasmic granules. The number of granules within each cell varied from few to so many that the cells appeared completely filled by them. The cytoplasmic granules frequently could be traced out into long cytoplasmic processes leading usually posterior-ward into adjacent areas of pineal tissue. In slides stained with aldehyde fuchsin, few of the cytoplasmic granules of these cells were stained (fig. 5). In cells with many cytoplasmic granules usually at least some of the granules were stained dark blue by the aldehyde fuchsin. The characteristics of these cells which distinguish them from other granular cells in the pineal should be pointed out (see plate 2). Subcommissural organ cells, mast cells, and phagocytes are the only other heavily granular cell-types that occurred in the pineal stained by the techniques used here and which might be confused with the specific cell type described above. In the basal part of the anterior pineal and often distant from both the ependymal layer and the subcommissural organ, cells identical with subcommissural organ cells were sometimes seen (figs. 11, 12). These differed from the above described "granule cells" chiefly in having (1) a smaller nucleus containing more small granules, thus appearing more ependyma-like, and (2) cytoplasmic granules smaller in size and staining less intensely with chrome hematoxylin. The staining of subcommissural organ cells in the rat with the two techniques used here has been described by Wislocki and Leduc ('54).

Mast cells are common in the periphery of the pineal and are sometimes present in the interior of either the anterior or posterior parts of the organ. Mast cells differed from the above-described "granule cells" chiefly in having: (1) a smaller nucleus containing more large granules, and (2) cytoplasmic granules of larger size and less intense staining reaction with chrome hematoxylin (figs. 8, 9, and 10).

In the pineal connective tissue, phagocytes were seen in a few specimens. The cytoplasmic inclusions of these cells

were variable in size and appearance, and did not stain intensely with chrome hematoxylin (fig. 7).

Between the pineal cells variable amounts of aldehyde-fuchsin staining material were seen in most specimens, especially in the posterior pineal. This material is in the form of a delicate intercellular network along which are distributed irregular masses of material (figs. 4 and 13). The fine fibers of the network cannot be distinguished from elastic fibers, which stain similarly with aldehyde fuchsin. But the irregular masses of material definitely associated with the fine fibers, are not readily attributed to elastic fibers or other stromal products. And they usually are located between and in close contact with pineal parenchymal cells rather than in association with blood vessels or strands of connective tissue. The network is stained pale to dark gray by chrome hematoxylin, while the associated masses of material are stained to a lesser extent. The origin of the network and the associated irregular masses is unknown and direct continuity between this material and the "granule cells" was not observed. However, the fibrous extensions of the "granule cells" and the similarity of the staining reactions of their granules to the staining reactions of the intercellular network and masses, suggested a possible connection. The cytoplasmic extensions of the granule cells" extended primarily in a posterior direction and toward the part of the pineal where the intercellular material was most abundant. The fact that the granules of "granule cells" are more intensely stained with chrome hematoxylin while the intercellular material stains more intensely with aldehyde fuchsin might be interpreted as indicating a progressive chemical change in material of the same origin. Quantitative appraisals of the relative amounts of intercellular aldehyde fuchsin-staining material and numbers of "granule cells" in the pineals were made (table 5). The resulting figures demonstrated a negative correlation ($r = -.452$) between the number of granulated "granule cells" and the amount of aldehyde

TABLE 5

Comparison of relative amounts of aldehyde fuchsin-staining material and number of chrome hematoxylin-staining "granule cells" in the anterior and posterior pineals. Specimens nos. 6 and 14 were omitted due to the fact that some critical sections were missing in one and staining was unsatisfactory in the other. The amount of aldehyde fuchsin-staining material in each part of the pineal was graded from 0 to +++, likewise the extent of granulation of each granule cell was graded from + to +++. The estimated total amount of aldehyde fuchsin-staining material was computed by doubling the number of +s recorded for the posterior pineal and adding the number recorded for the anterior pineal. No. = specimen number (history of each is given in table 1).

NO.	AMOUNT OF ALDEHYDE FUCHSIN-STAINING MATERIAL			NUMBER OF "GRANULE CELLS"								
				Posterior pineal				Anterior pineal				Total
	Posterior pineal	Anterior pineal	Estimated total	+	++	+++	Total	+	++	+++	Total	
1	+	+	3	3	10	7	20	1			1	21
2	+++	++	8			2	2		3		3	5
3	++	0	4				0	21	12	2	35	35
4	++	+	5				0	4	4		8	8
5	++	++	6	1			1	5	2	1	8	9
7	+	0	2	2	18	31	51	30	41	4	75	126
8	+++	+	7	2			2				0	2
9	0	0	0	1			1	1	21		22	23
10	++	++	6				0				0	0
11	+++	++	8				0				0	0
12	0	0	0	1			1				0	1
13	+++	+	7	2	4		6	2			2	8
15	++	++	6				0	3	9	15	27	27
16	++	+	5		2		2	5			5	7
17	++	0	4				0				0	0
20	+++	+	7	9	7	36	52	32	8	1	41	93
21	+	0	2	1		13	14	2	8	49	59	73
22	+	0	2	12	25	35	72	25	32		57	129
23	0	+	1	1			1	3	36	28	67	68
24	++	++	6				0				0	0
25	++	++	6	1	1	1	3				0	3
26	+	0	2		5	1	6	2	7	18	27	33
27	+++	+	7	2	4	14	20	1		2	3	23
28	0	0	0	8	41	51	100	18	9		27	127
29	+	+	3	6	10	1	17	5	1		6	23
30	++	+	5				0				0	0
31	++	0	4				0				0	0
32	++	+	5			1	1	4	6		10	11
33	+	+	3	5	4		9	3	2	2	7	16
34	+	+	3				0		2		2	2

fuchsin-staining material in the pineals. This correlation coefficient lies between the 2% and 1% levels of significance. The hypothesis that the "granule cells" are responsible for the deposits of aldehyde fuchsin-staining material in the pineal might be supported by this negative correlation, since storage of granules in "granule cells" is shown to be concomitant with little intercellular aldehyde fuchsin-positive material and depletion of granules is concomitant with abundant intercellular material. In specimens in which few "granule cells" were observed (table 5) it is believed that many were undetected or nearly depleted of granules since frequently clusters of only a few granules could not be readily associated with any particular cell and were not counted.

Attempts to demonstrate relationships between either the number of granulated "granule cells" or the amount of intercellular aldehyde fuchsin-staining material with length of captivity, sex, day-length, body weight, weight to head and body length ratio, and cytology of pineal parenchymal cells, were unsuccessful.

There are numerous phloxinophilic neuroglial and neural fibers in the anterior pineals of all of the animals. Some of these fibers show variation in diameter and estimates of average fiber thickness were made for all animals. This fiber variation did not appear to be related to sex, treatment of the animals, or other measured variations in the pineal.

Masses of lymphoid cells of uncertain significance were associated with the pineals of a few animals and were not included in the volumetric or cytometric analyses.

Occasional neurons were seen in a couple of the pineals, but they were too few to yield data of significance. Neither pigment cells nor calcareous deposits were seen in any of the pineals.

DISCUSSION

The results described above raise two problems which require further discussion: (1) the identification, relationships and significance of the "granule cells" and the aldehyde fuchsin-positive intercellular networks and deposits, and (2)

the meaning of the cytological difference between pineals of animals in darkness and those in an increasing day-length.

In the human pineal del Rio Hortega ('29, '32), using the silver carbonate method found granular gliocytes of a similar structure, relative number, and distribution as the chrome hematoxylin-staining granule cells described here. He considered these to be secretory cells localized in the interstitial neuroglia. However, neither he nor other authors, apparently, have described intercellular deposits of material strictly comparable to those described here staining with aldehyde fuchsin. Typical reticular and elastic fibers are usually most prevailent near vessels and are not accompanied by irregular masses of similarly staining material. But the curled or twisted fibers ("Fibras ensortijadas") of Achúcarro and Sacristán ('12) (also described by del Río Hortega, '32; Amprino, '35; Bargmann, '43) are similar to parts of the intercellular network described here.

Three circumstances described in the results above suggest a connection between the granules of the granule cells and at least part of the intercellular aldehyde fuchsin-positive material: (1) similar staining reactions, (2) posterior-ward trend of granule cell processes to areas of intercellular networks, and (3) inverse correlation between degree of granulation of the granule cells and amount of intercellular aldehyde fuchsin-staining material. An attractive hypothesis to explain these circumstances would be that the granular gliocytes are secreting material over their fibrous processes or that they are storing and then transporting this material to the immediate vicinity of the parenchymal cells. During passage the transported material gradually loses its granular character, becomes less readily stained with chrome alum hematoxylin and more intensely stained with aldehyde fuchsin. The secreted material might conceivably have a stimulative or nutritive function with regard to the pineal parenchymal cells. It may be significant, however, that neither the extent of gliocyte granulation nor the amount of the intercellular material appear to be correlated with cytological differences

in the parenchymal cells. And it is conceivable that the granular gliocytes are phagocytic cells or that they serve to transport materials away from the parenchymal cells. However, the structure of the intercellular material, and that of the cytoplasmic granulation, are more typical of a secretory process than of an ingestive one. Preliminary experiments to test these ideas have been undertaken.

Pineal parenchymal cells have been shown to be engaged in an active nucleoprotein synthesis (Wislocki and Dempsey, '48) and investigators have long been concerned with nuclear secretory activity in the pineal (see Bargmann, '43). Certainly the amount of demonstrable nuclear material, or the number and size of the nucleoli, has a bearing on nuclear synthesis and activity. In the parenchymal cell nuclei of animals subjected to an increasing day-length there were fewer nuclear granules than in those of animals remaining in continuous darkness. This might be interpreted either as an indication of increased nuclear secretory activity or of reduced nuclear protein synthesis. On the basis of the cytology of other kinds of protein-producing cells (Caspersson, '50) the latter seems more likely. Therefore, it might be suggested that the larger number of nuclear granules in animals in continuous darkness indicates a greater rate of protein synthesis. This would be in line with the hypothesis that the pineal has an antigonadotrophic activity, since Whitaker ('40) found that white-footed mice kept in continuous darkness have a lowered as well as a non-cylic reproductive activity, and Fiske ('41) found that female rats kept in the dark experience prolonged periods of metoestrus, apparently because of a decline in FSH secretion.

Investigators concerned with the mechanism of increased gonadotrophic activity in warm-blooded animals exposed to increasing day-length have been faced with the question of how the gonadotrophin secreting cells of the anterior pituitary are stimulated (for a recent review see Amoroso and Matthews, '55). A nerve pathway from the optic system seems a likely element in this mechanism. However, attempts to demonstrate

significant direct innervation of the cells of the anterior pituitary have been equivocal or unsuccessful (Vázquez-López and Williams, '52; Green, '52; Harris, '52; Zuckerman, '52; and others) and neither the integrity of the visual cortex nor of the mid-brain is essential to the photoperiodic response in ferrets, although integrity of the optic nerve and retinal stimulation are necessary (Clark, McKeown, and Zuckerman, '39; Zuckerman, '52). The following evidence suggests the possibility of the pineal being an endocrine factor in the control of photoperiodic pituitary gonadotrophic activity: (1) the primitive pineal functions as a light receptive organ (Bargmann, '43), (2) recent evidence suggests that in mammals the pineal has an antigonadotrophic or antigonadal activity (Kitay and Altschule, '54), (3) nerve fibers from the habenular and posterior commissures end on the pinal parenchymal cells and these endings degenerate completely when these fibers are cut (Gardner, '53), (4) the major portion of the nerve fibers of the pineal gland derived from the habenular commissure probably arises mainly in the habenular nuclei (Gardner, '53), and (5) habenulo-tectal and habenulo-diencephalic interconnections exist or are probable in mammals (Kappers, Huber, and Crosby, '36). Further experimentation, however, is necessary before a decision can be reached on the role of the pineal in photoperiodic antigonadotrophic activity.

SUMMARY

Analysis of the volume and cytology of the pineal organ in thirty-two, adult, white-footed mice with respect to sex, length of time in captivity ($3\frac{3}{4}$–$4\frac{1}{2}$ mos. versus $\frac{3}{4}$–1 mo.) and modification of day-length (darkness versus increasing day-length) by means of serial sections stained with chrome alum hematoxylin and phloxine or with aldehyde fuchsin demonstrates that:

1. The pineal in this species consists of an anterior and basal part around a recess of the third ventricle and a more

posterior part lying between the cerebral hemispheres and over the anterior end of the cerebellum. Pineal tissue between these parts varies in amount from none to a complete interconnecting strand.

2. The volumes of the parts of the pineal are (means ± standard errors) in mm^3 and cell content (parenchyma + neuroglia): *Anterior:* .0197 ± .0021 mm^3, 273,000 ± 11,970 cells; *Posterior:* .0894 ± .0068, 742,000 ± 51,080.

3. The posterior pineal differs histologically from the anterior chiefly in that it has few phloxinophilic fibers and it has parenchymal cells of a greater size and mean nuclear diameter.

4. Large nuclear phloxine-staining granules in the parenchymal cells average more in number per nucleus in animals maintained in darkness in contrast with those in an increasing day. This may indicate a greater protein synthetic activity in the former, and possibly is concerned with the postulated antigonadotrophic activity of the pineal.

5. In the anterior pineal and in the basal part of the posterior pineal, granular cells staining intensely with chrome alum hematoxylin and less so with aldehyde fuchsin are described and are tentatively identified as granular gliocytes.

6. Heavily granulated cells of this type counted in alternate slides numbered from 0 to 129 (estimated total = 2 × that, or = about 250) per pineal.

7. The number of heavily granulated cells of this type in the pineals is inversely proportional to the amount of a similarly staining intercellular material, which is most abundant in the posterior pineal.

8. This material is associated with the parenchymal cells and is in part morphologically distinct from typical elastic fibers or other previously described elements.

9. Significant volumetric or cytologic differences correlated with sex or length of time in captivity are not found.

10. Evidence suggesting the possibility that the pineal is a factor in photoperiodic gonadotrophic control is summarized.

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PLATE 1

EXPLANATION OF FIGURES

1 *Cross-section of the anterior pineal at mid-level.* A *recess of the third* ventricle is enclosed by the anterior pineal and another ventricular recess lies dorsal to the anterior pineal. × 159.

2 Large "granule cell," showing intensity of staining of the cytoplasmic granules. × 1860.

3 Cross-section of strand of pineal tissue between the anterior pineal and the main mass of the posterior pineal. Four "granule cells" are shown. × 390.

4 Small area in posterior pineal, showing large masses of aldehyde fuchsin-staining material. × 506.

Figures 1, 2 and 3 are from preparations stained with chrome alum hematoxylin and phloxine. Figure 4 is from a preparation stained with aldehyde fuchsin, orange G, light green SF, and chromotrope 2R. All figures are unretouched photographs.

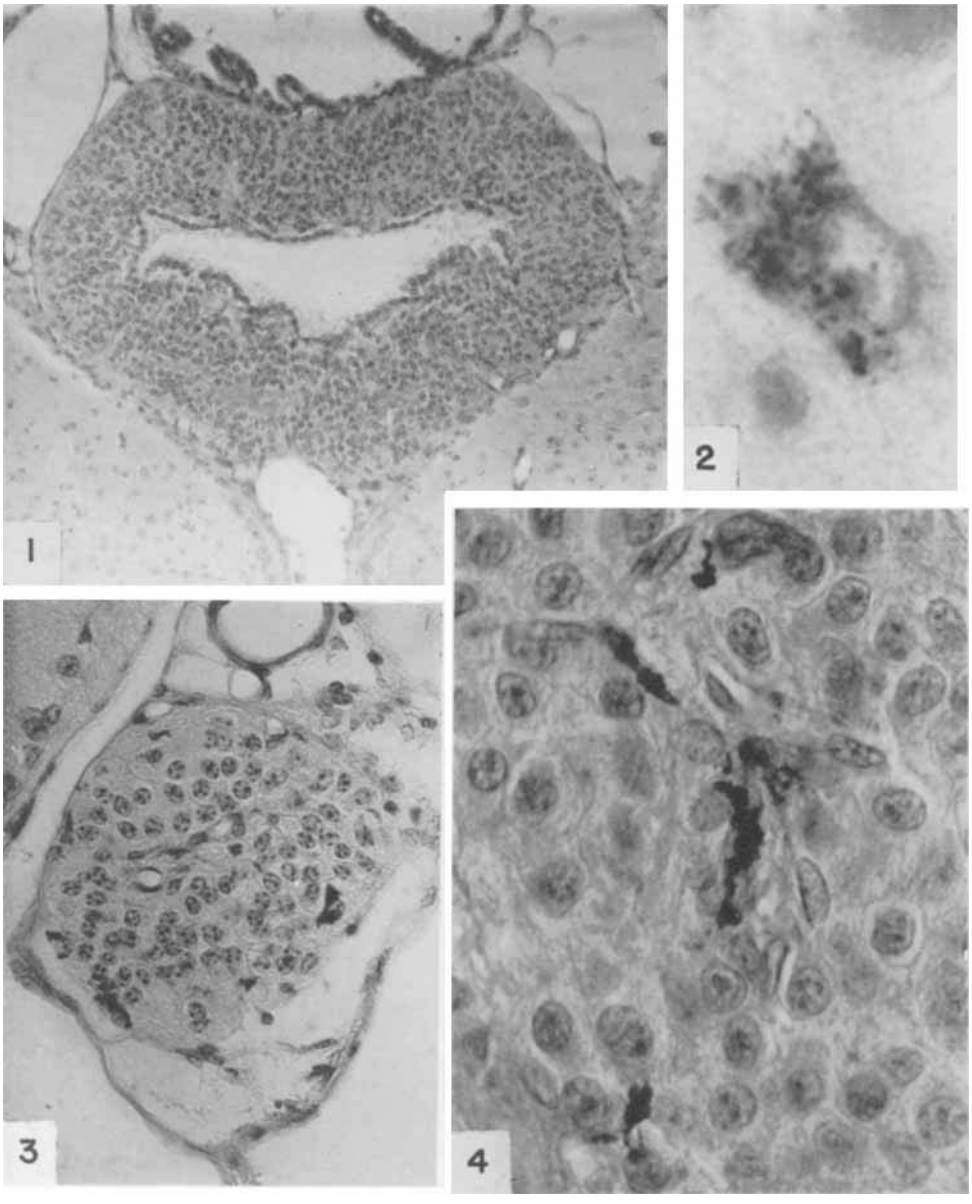
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PLATE 2

EXPLANATION OF FIGURES

5 Large "granule cell" from the anterior part of the posterior pineal. Note the incomplete staining of the cytoplasmic granules by aldehyde fuchsin.

6 "Granule cell" of average size and structure. Note intense staining of nearly all granules by chrome alum hematoxylin.

7 Phagocyte from stroma of posterior pineal.

8 Mast cells with large cytoplasmic granules, from connective tissue near the pineal.

9 and 10 Mast cells representing extremes in nuclear size and number of cytoplasmic granules.

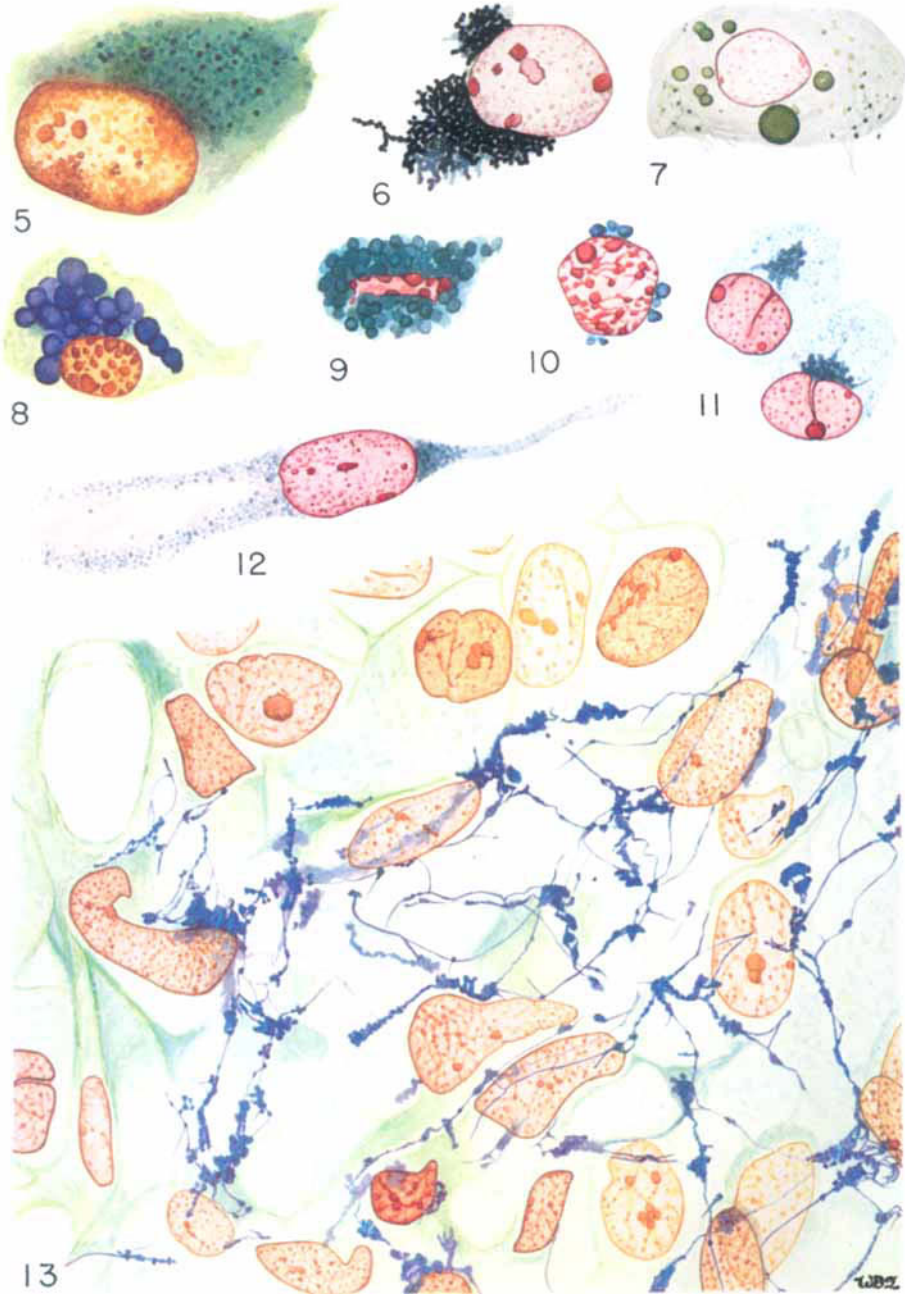
11 Isolated pair of subcommissural organ-type cells in the anterior pineal.

12 Subcommissural organ-type cell from the posterior commissure adjacent to the ventral margin of the anterior pineal.

13 Small area in the posterior pineal showing a well-developed intercellular network and associated masses of material staining dark blue with aldehyde fuchsin. Paler blue fibers and masses lie in a deeper plane in the section. Obliquely sectioned capillaries appear at upper and lower left. Majority of nuclei are of parenchymal and neuroglial cells.

Figures 5, 8 and 13 are from preparations stained with aldehyde fuchsin, orange G, light green SF, and chromotrope 2R.

Figures 6, 7, 9–12 are from preparations stained with chrome alum hematoxylin and phloxine. All × 2000.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