QUANTITATIVE RELATIONSHIPS OF CELL POPULATIONS IN OLFACTORY BULBS OF MAMMALS

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ABSTRACT

QUANTITATIVE RELATIONSHIPS OF CELL POPULATIONS
IN OLFACTORY BULBS OF MAMMALS

By

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Qualitative comparisons of olfactory bulb structure in many different mammals indicated a need to define and describe characteristics of cell populations and corresponding layers quantitatively. Four species of the carnivorous family of marsupials, the Dasyuridae, were studied because of their prominence and good definition of particular features and the large size range of their bodies and olfactory bulbs.

Selected sections from brains stained for Nissl substance were projected and olfactory bulb layers traced. A compensating polar planimeter was used to determine the areas of the traced layers. Layer volumes were calculated from areas plotted according to section position. Planimetry was performed on plots of numbers of cells versus section number to obtain the total number of cell types.

Results indicate that each olfactory bulb layer volume remains a constant function of increasing bulb volume as do the numbers of each cell type. The density of each cell type diminishes with increasing bulb volume, while more numerous cell types increase with increasing bulb volume at a faster rate than do less numerous cell types. Internal granule cells were found to be collected into groups hereafter designated as "discoids".

The discoids were found to exhibit at least two size populations. The smaller population of large discoids lies in the external region of the layer and the large population of small discoids occupy the inner part of the layer.

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Robert C. Switzer III

A THESIS

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To my family

ACKNOWLEDGMENTS

I wish to thank my parents for giving me the means to obtain my undergraduate degree which is the basis from which I launch my career, and to their unending support and encouragement. My thanks to Jean Toth Allen who introduced me to the Biophysics laboratory; to Dr. Barnett Rosenberg who fanned my spark of interest and gave me my start in Biophysics; and to Dr. John I. Johnson who, in a firm and tacit way, has guided me in the ways of producing meaningful scientific work of high excellence. A very special thanks to my wife, Julie, who, besides helping with the cell counts and typing of this manuscript, has supplied a home life conducive to productive work in the laboratory.

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Detailed studies on the olfactory bulb have been confined mostly to rodents and rabbits (Cajal, '11-'55; Valverde, '65; Loman and Mentink, '69; Allison and Warwick, '49; Nicoll, '70; Price and Powell, '70).

The work of Crosby and Humphrey ('39) covers several animals among reptiles, birds and mammals. Their work has been overlooked, probably because their descriptions considered more the populations of cells rather than nerve fiber connectivity.

References to Cajal attend more to his Golgi pictures than to his complete pictures; the populations of cells, and some other features, in the olfactory bulb described by Cajal who looked at other than Golgi material ('11-'52, '11-'55) are often ignored. This and the confusion of some aspects of his description have led to misinterpretation in some studies.

For example, Valverde's ('65) labeling of the internal plexiform layer is confused with Cajal's two separate layers, the internal plexiform layer and the internal granule cell layer (see Fig. 2). This appears to be due to the young age of Valverde's animals. Andres ('70) seems to have made the same indistinction of these layers in the part of his description dealing with mammalian olfactory bulbs.

Nicoll's ('70) interpretation of electrophysiological data involving tufted cells makes no distinction of the three populations of tufted cells which can be classified by their graded size and position. These classes would seem to be important for Nicoll's statements regarding cell size and action potential conduction velocities.

The study of Rall and Shepherd ('68) on field potentials in the rabbit olfactory bulb bases relative resistance values and densities of internal granule cells on anatomical assumptions derived from Golgi stain studies. No acknowledgment is made of the distinctive clustering of these cells (see Fig. 2) and what effect this may have on current flow.

To resurrect, stabilize and add to Cajal's description of the olfactory bulb (Cajal, '11-'52, p.651, Fig. 411; '11-'55, p.4, Fig. 1) Part I of this study, Qualitative Observations is presented.

Part I. Qualitative Observations

Methods

Subjects for the qualitative observations are listed in Table 1. They comprise a group of animals which exhibit a large range of variations of anatomical features of olfactory bulbs. These animals were also chosen to include those more commonly observed (i.e. rat, mouse, and cat), and representatives from diverse taxa (i.e. shrew, baboon, and opossum.)

Brains of all animals had already been fixed in 10% formalin and embedded in celloidin, sectioned at 22, 25 or 30um and stained, and were ready for examination when this study began. Every other section was stained for Nissl substance with thionin, the others stained for myelin with iron hematoxylin, using a Weil or Sanides-Heidenhain method.

Results

Olfactory Bulb Anatomy

The outermost layer is the fibrous layer of afferent olfactory cell axons which arrive at the bulb through the cribriform plate (Fig. 1). These axons terminate in the globular, cell-scarce glomeruli (Fig. 2). There the axons intermingle and synapse with the primary dendrites of the mitral and tufted cells and the axons and dendrites of the short axon periglomerular cells. The peripheral processes of the internal granule (IG) cells and extrabulbal axon afferents also penetrate to the glomerular layer. The glomeruli themselves are embedded in a dense matrix of granule, periglomerular and tufted cells. These cells form the external granule layer (EGL). The tufted cells of the EGL are typically small and were labeled by Cajal as peripheral or interstitial tufted cells.

The tufted cell layer (TCL) internal to the glomerular layer, has been included by many workers as part of the external plexiform layer. A fuller discussion of why this is probably so will be given in the Discussion. The tufted cells of the TCL are termed the middle tufted cells. How sharply defined this layer is depends on the number of middle tufted cells present. The tufted cells in this layer are distributed with the largest cells internal and the smallest external, nearest the glomerular layer. The Nissl substance of the tufted cell bodies has an

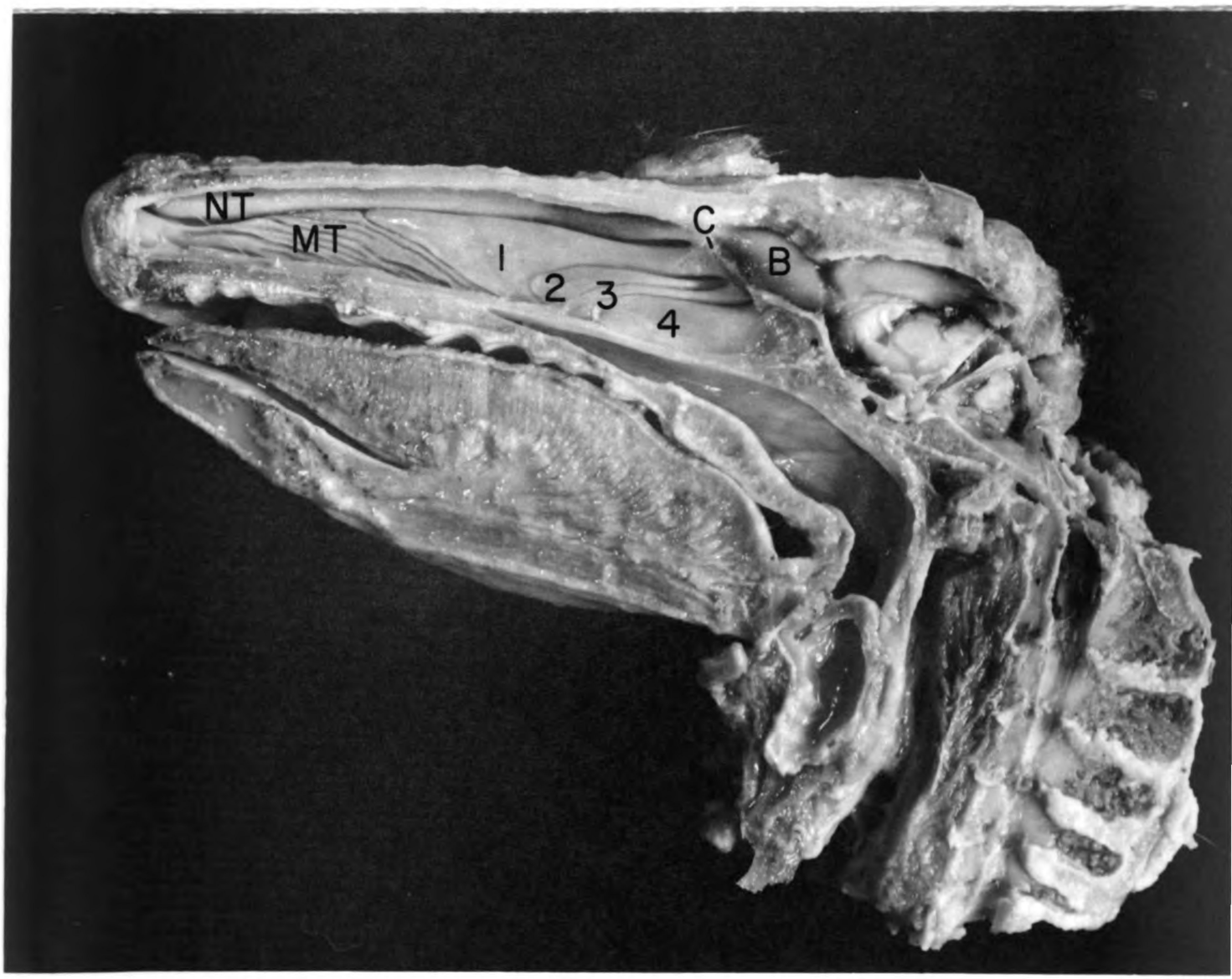

Figure 1. This photograph of a cross section of an opossum head (from which the cerebellum has been removed for another experiment) reveals, among other things, the following: B, the olfactory bulb; C, the cribriform plate, MT, the maxilloturbinal; NT, the nasoturbinal and the ethmoturbinals 1, 2, 3 and 4. It is on ethmoturbinals and the posterior half of the nasoturbinal that the olfactory epithelium is found; easily identified by the light brown-yellow color of the epithelium.

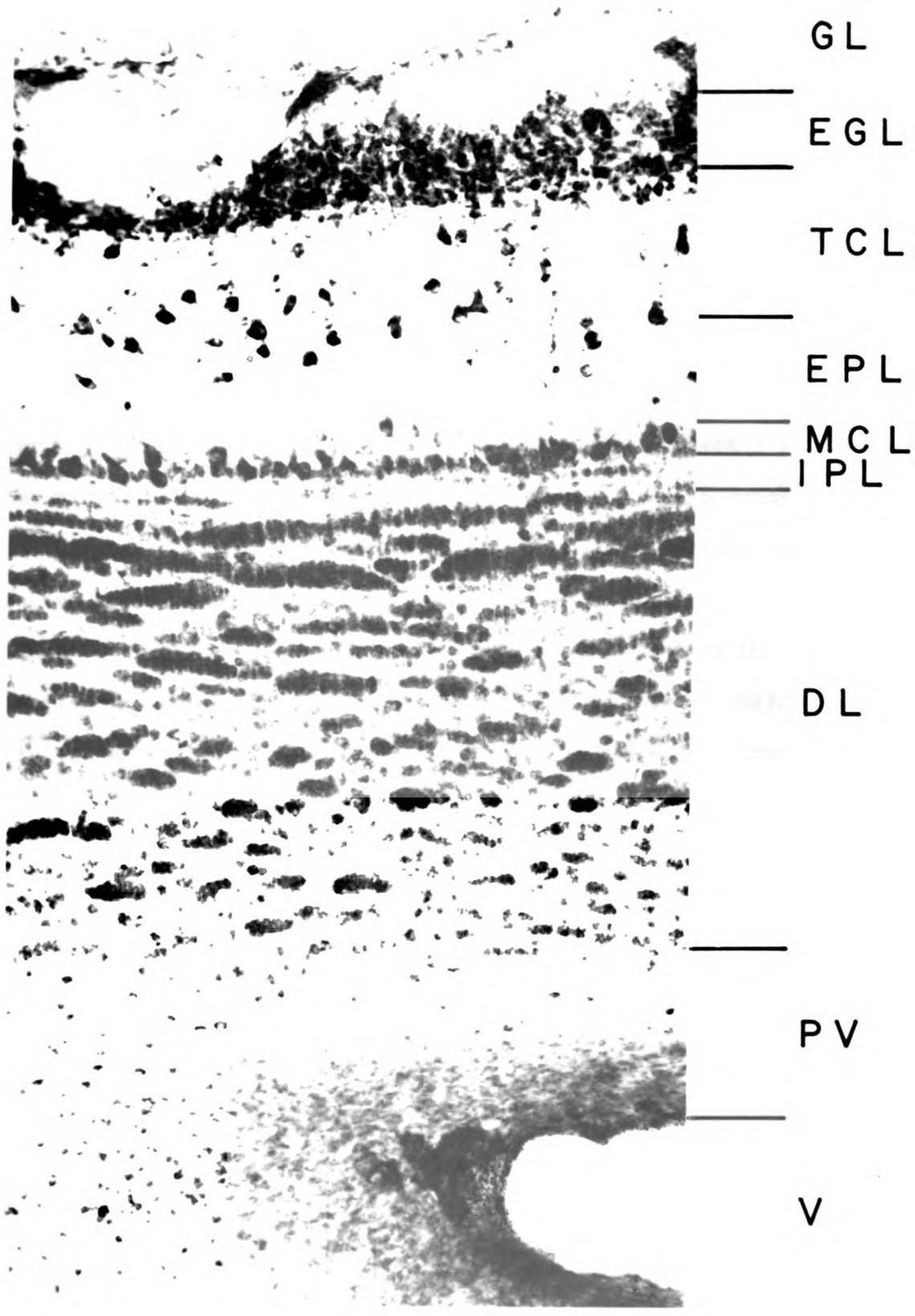

Figure 2. Layers of the olfactory bulb. This is a photomicrograph of a segment of a coronal section stained for Nissl substance with thionin from Dasyurus. Bulb layer boundaries are labelled and indicated along the right hand edge of the segment.

obvious tufted appearance where it extends into the primary dendrite and axon from the soma. Also quite evident in the larger cells of the middle tufted cell layer is a cruciform appearance of the Nissl substance extending into the horizontal dendrites.

The external plexiform layer (EPL) is located between the layer of middle tufted cells and the mitral cell layer (MCL). The EPL has received much attention in recent years following the discovery of the reciprocal synapse between the horizontal (accessory or basal) dendrites of the mitral cells and the peripheral processes of the internal granule cells (Hirata, '64; Price and Powell, '70; Rall and Shepherd, '68). These two types of fibers comprise the bulk of the EPL.

Most of the internal tufted cells occupy the inner region of the EPL while some are scattered in the outer EPL. The tufted cells nearest and in the mitral cell layer are easily confused with the mitral cells.

The mitral cell layer is made up of three cell types: mitral, tufted and granule cells. The arrangement of mitral cells varies ranging from appearing to be placed one cell deep on the surface of a basement layer of granule cells to being more than one deep and embedded in a granule cell matrix. The mitral cells, so named because of their likeness to a bishop's mitre, are the largest cells in the olfactory bulb. Tufted cells are interspersed among the mitral cells along the outer edge of the layer. There is also a range of sizes of mitral cells, some actually miniature and smaller than the larger tufted cells in some animals (e.g. opossum, Dasyurids). In these animals some of the middle

and internal tufted cells appear larger than any mitral cells. If true, then it could be expected that the two populations would exhibit appreciable overlap in their size distribution curves.

The internal plexiform layer (IPL) is a cell-scarce layer containing fibers running radially and others running parallel to the MCL. This layer is said by Cajal ('11-'52, p. 665; '11-'55, p.11) to be the richest in nonmyelinated fibers in the bulb. With the Weil and Heidenhain stains, however, the IPL shows a darker color than layers on either side implying the presence of myelin. This may not be unreasonable since afferent axons course through this layer without making contacts until terminating in the MCL or EPL. The work by Price and Powell ('70) reveals very few spines on the peripheral processes of the IG cells where they pass through the IPL, implying very little synaptic activity on them and in this layer. The IPL appears to be a layer for transmission and not for terminal contacts.

Tufted cell axons emit collaterals to the IPL. Valverde includes mitral axon collaterals in this layer but as was pointed out above, his IPL included the IG layer. Cajal specifically states that there are no mitral cell axon collaterals in the strictly defined IPL ('11-'52, p. 664, '11-'55, p.13).

Afferent fibers from the anterior commissure are represented in the IPL with extensive bifurcation of fibers in the IPL region before penetrating to the EPL and to the MCL to form retes amongst those cells (Cajal '11-'52, p.667; '11-'55, p.13). The size of the IPL appears to range from barely recognizeable to something with quite respectable dimensions among different animal types.

The next layer inward has been called the internal granule cell layer,but because of the character of the organization of the internal granule cells this layer will be referred to as the discoid layer (DL). The IG cells have nearly round somas and are mostly nuclear showing only a bare rim of cytoplasm. Cajal referred to the aggregation of IG cells as clusters or islands, ovoid in shape. Examination of these ovoids in different planes in sections cutting perpendicularly (see Fig. 2) to the olfactory bulb surface and in sections tangential to the olfactory bulb surface (Fig. 3), reveals that, three dimensionally, they are discoid in shape, that is, oblate spheroids. The term discoid will be used hereafter to refer to these IG cell aggregations as well as the layer in which they are found. Among different types of animals there is a wide range of variations in the following characteristics of discoids: density, thickness, (which can be expressed in number of cells thick) and radial gradients of length and thickness (Fig. 4 and 5).

Sections from different planes reveal some degree of regional differences of these characteristics of IG discoids. Particularly in regions of high curvature of the bulb and in the lateral-anterior regions, fiber bundles perforate the DL resulting in distortion of discoid shape.

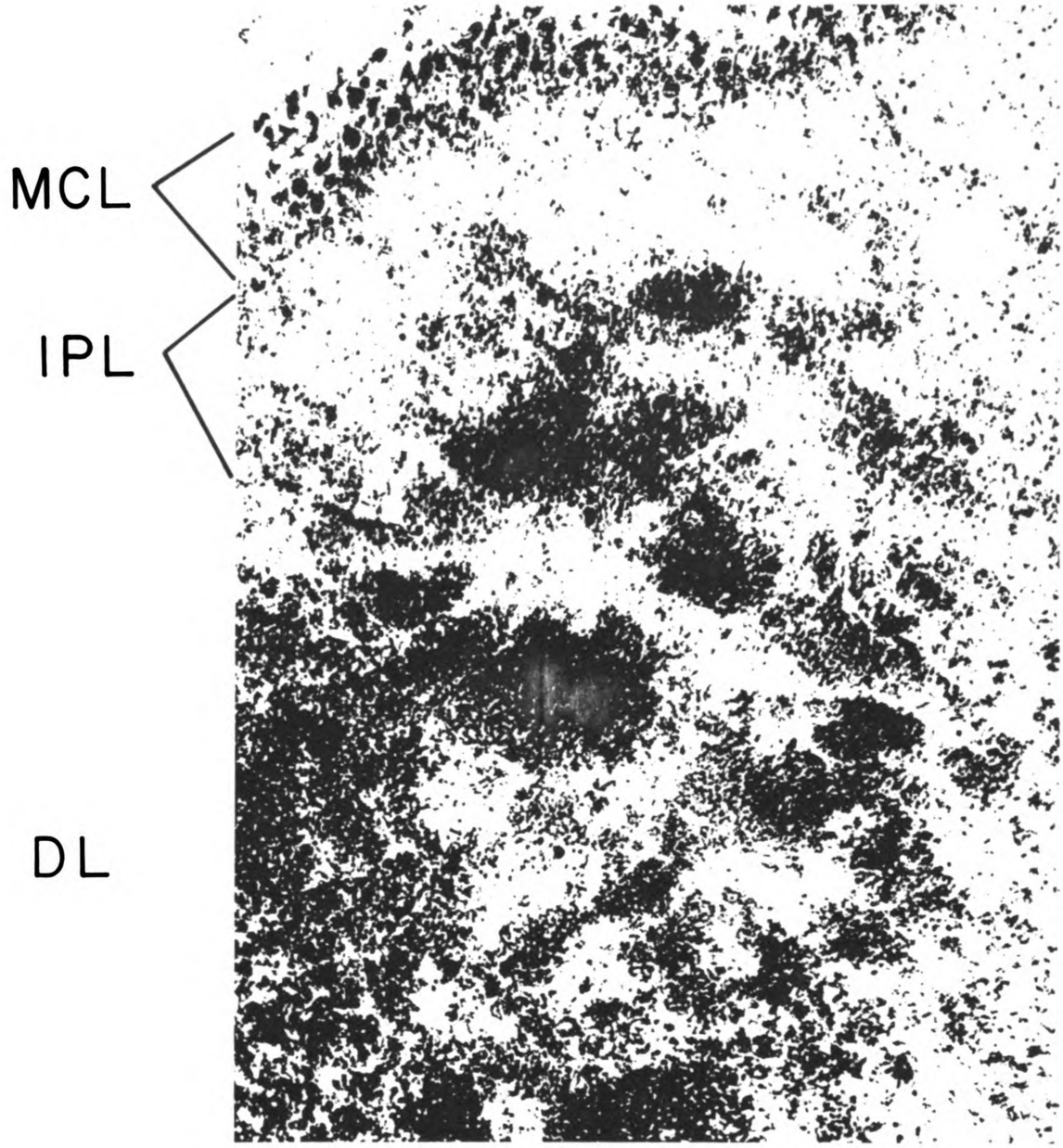

Figure 3. Tangential view of discoids of internal granule cells. Stain: thionin.

The longest, thickest and most dense discoids are typically external to the smaller ones. In some animals, however, a few smaller discoids lie most externally. In the largest discoids a columnar stacking of cells is evident in varying degrees (Fig. 6). This feature was briefly noted by Cajal ('11-'52, p. 667; '11-'55, p.13)

> ". . . . these branchlets (of thick centrifugal fibers) prefer the vicinity of the granules and the intergranular septa or columns . . . "

Where the discoids are most dense they seem to conform their shape in accordance with the presence of neighboring discoids. It is as if a "membrane" enveloped each discoid. Fibers coursing across the surfaces and in between the ends of the discoids also show a curious feature. The fibers, as revealed in myelin stains, on the ventricle side of the discoids leave vacant a space closest to the discoids while on the external surface of the discoids the fibers flow close to the discoid surface. This is evident throughout most of the DL with less prominence near the inner DL boundary (Fig. 7).

Table 1. Qualitative Comparisons of Selected Features of Olfactory Bulbs from Animals of Diverse Taxa

This table is a compilation of qualitative descriptions of some features of the olfactory bulbs of several different animals. A section from the olfactory bulb of each animal is shown in Figure 4. This table and Figure 5 show that certain features of olfactory bulb architecture are more accentuated in some animals than in others, eg. the tufted cell layer. The term "50% to the MCL" means that the inner edge of the TCL is about half way between the glomerular layer and the mitral cell layer (MCL). "Thin" is defined as three cells or less in thickness while "thick" as greater than three cells in thickness.

Table 1

	Tufted Cell Density	Tufted Cell Sizes	IPL Breadth	Mitral Cell Density	Discoid Character
Rat *Rattus norvegicus* Rodentia	Moderate, TCL-EPL line less than 50% to MCL	Obvious heterogeneity range	Wide	High	Long, thin, closely spread
Deer mouse *Peromyscus californicus* Rodentia	High, well defined, TCL-EPL line about 50% to MCL	Medium heterogeneity not obvious	Wide	High	Thick, graded size, but longest, thickest and most dense near IPL
Cat *Felis catus* Carnivora	Low, indefinite, TCL-EPL line less than 50% to MCL, barely evident.	Medium, almost homogeneous within TCL, large near TCL	Wide	High	Gradations in size, variations in thickness moderately spaced.
Shrew *Blarina brevicauda* Insectivora	High, well defined, TCL-EPL line about 50% to MCL	Even heterogeneity, largest nearly the size of mitrals	Narrow	High	Gradations in size, tightly packed, thick
Chimpanzee *Pan troglodytes* Primates	Very low, layer not well defined,mostly near glomerular layer	Medium to small but not well defined	Wide	Low clumps	Diffuse, barely evident
Baboon *Chaeropithecus papio* Primates	Low, TCL-EPL line not defineable	Medium low dispersion, almost homogeneous	Medium-wide	Medium clumping thin layer of granule cells	Thick, large, low number of smaller size small gradation in size
Opossum *Didelphis marsupialis virginiana* Marsupialia	High, very distinct, TCL-EPL line greater than 50% to MCL	Many large, heterogeneous, large range	Very wide	High	Even size gradations, long, thin
Sugar Glider *Petaurus breviceps* Marsupialia	Moderate, TCL-EPL line about 50% to MCL	Obvious heterogeneity, large cells mostly near TCL-EPL line.	Medium	High	Thickness and length graded, moderately spaced;longest and thickest in a band nearest IPL.

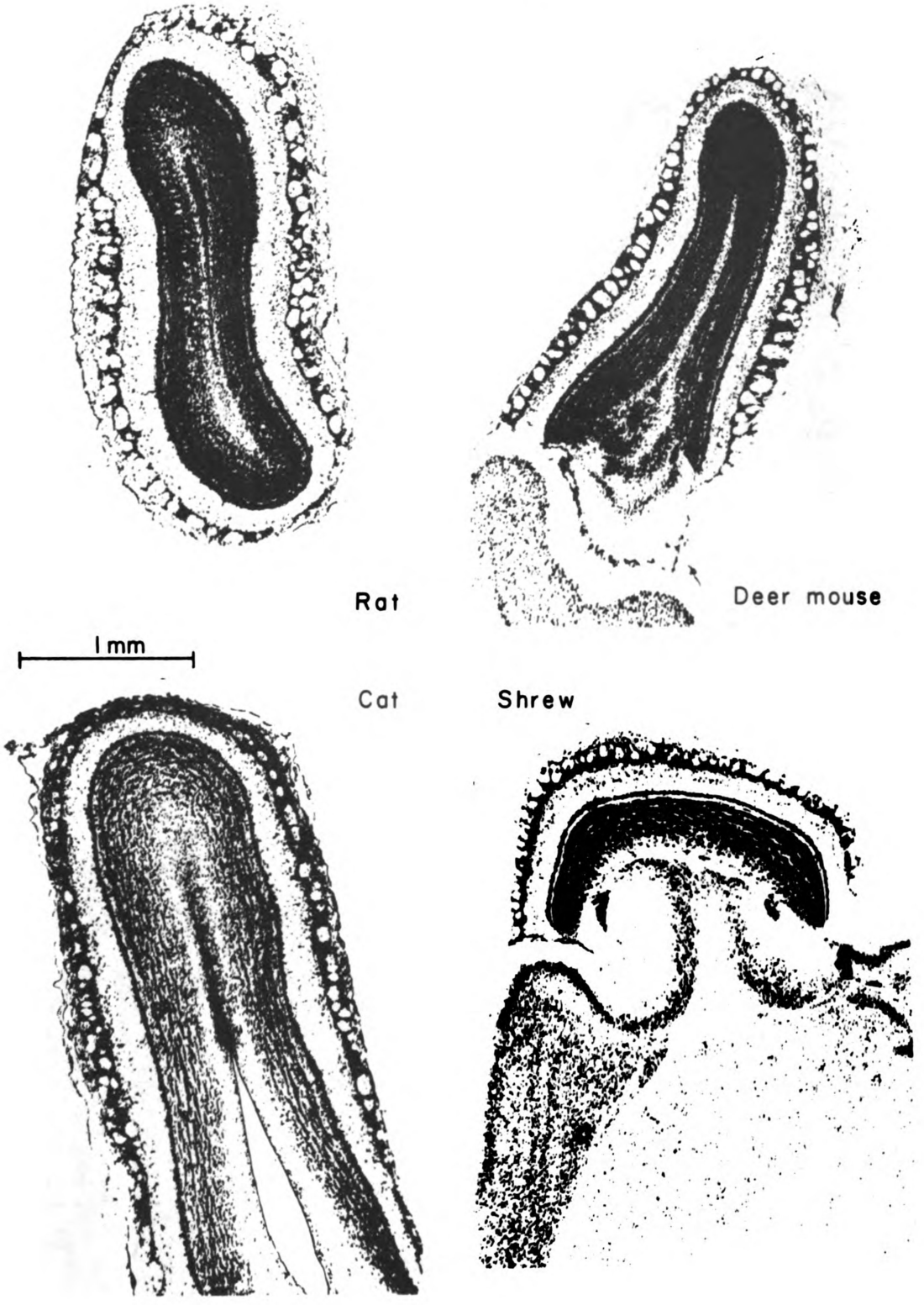

Figure 4. Sections of olfactory bulbs from animals of diverse taxa shown in these photomicrographs are those compared qualitatively in Table 1. Take particular note of: tufted cell density, breadth of the IPL, mitral cell density, and length, thickness and distribution of discoids.

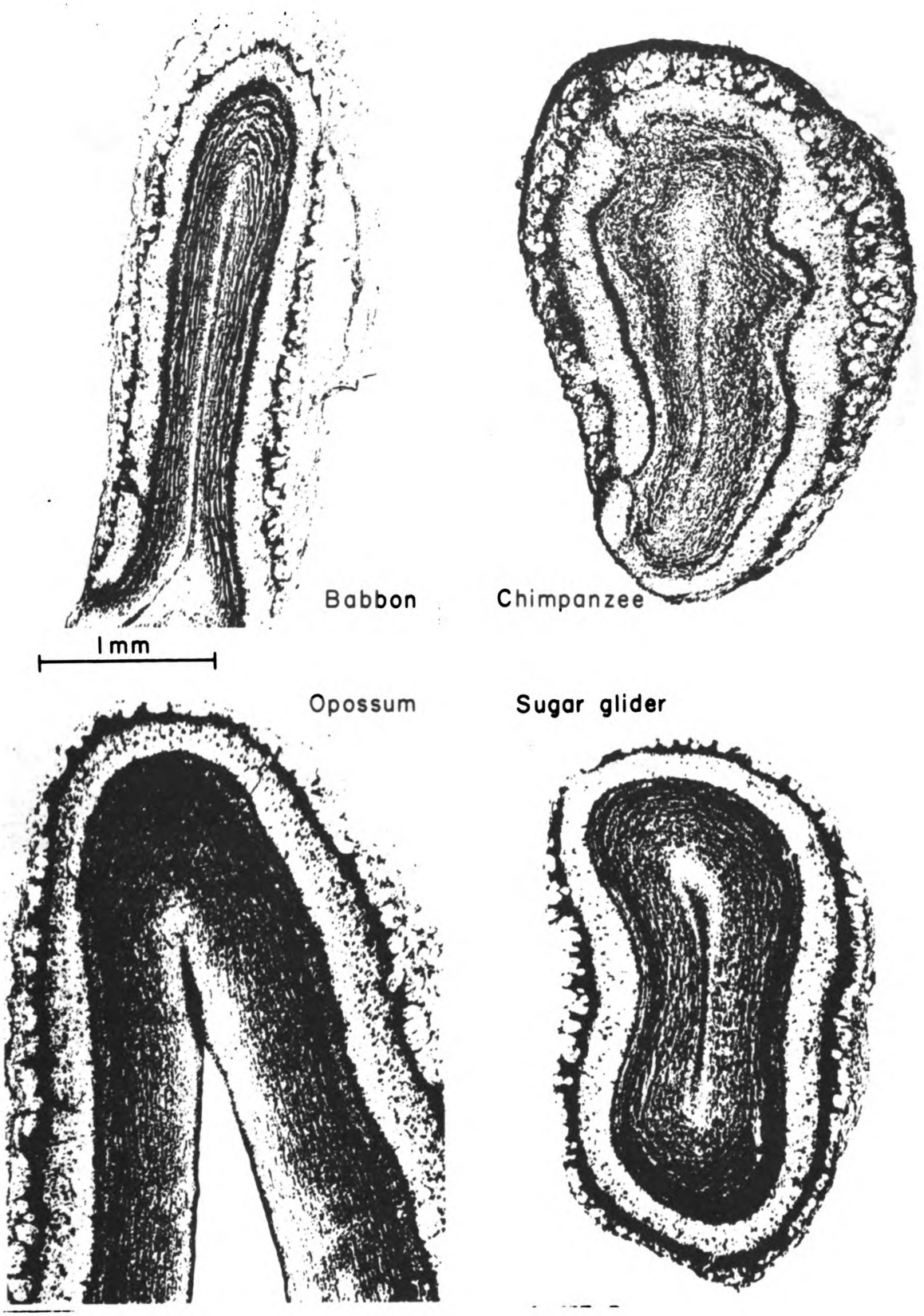

Figure 4b.

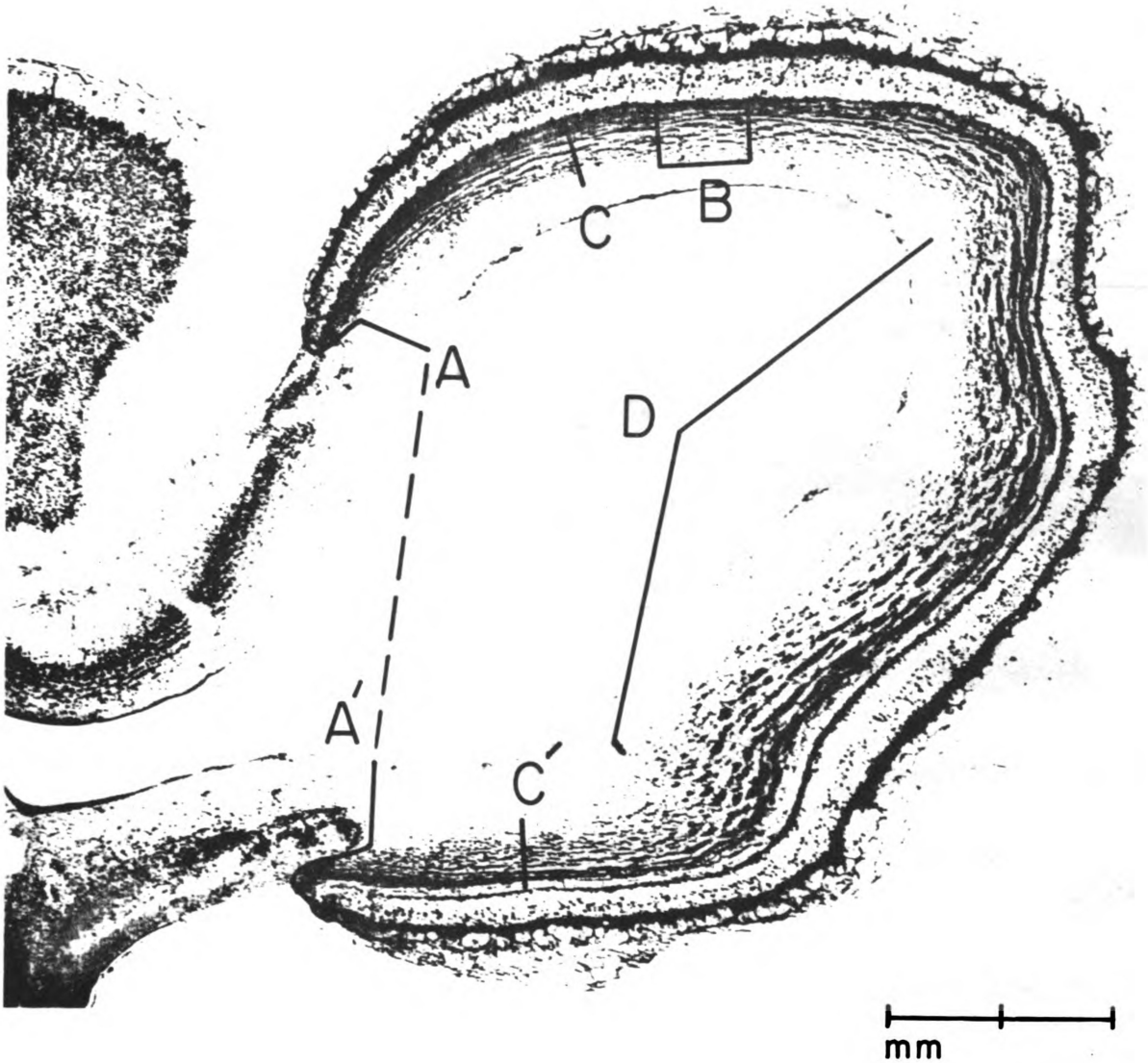

Figure 5. A micrograph of a parasagittal section from the olfactory bulb of Sarcophilus demonstrates some of the conventions for: A - A^1, the caudal boundary for parasagittal and horizontal sections of the olfactory bulb: B, the sample area for point count volumetry to determine the fractional volume of the discoids in the DL; C&C', radial sample lines for discoid size sampling and radial position of the discoids; D - an example of a region of a section where the discoids were not sectioned parallel to their minor axes. Such a region was not used in point count volumetry or radial line sampling. Such criteria rendered the very small olfactory bulb of Sminthopsis very difficult to extract discoid data from. A section from Sarcophilus, the cells were stained with thionin.

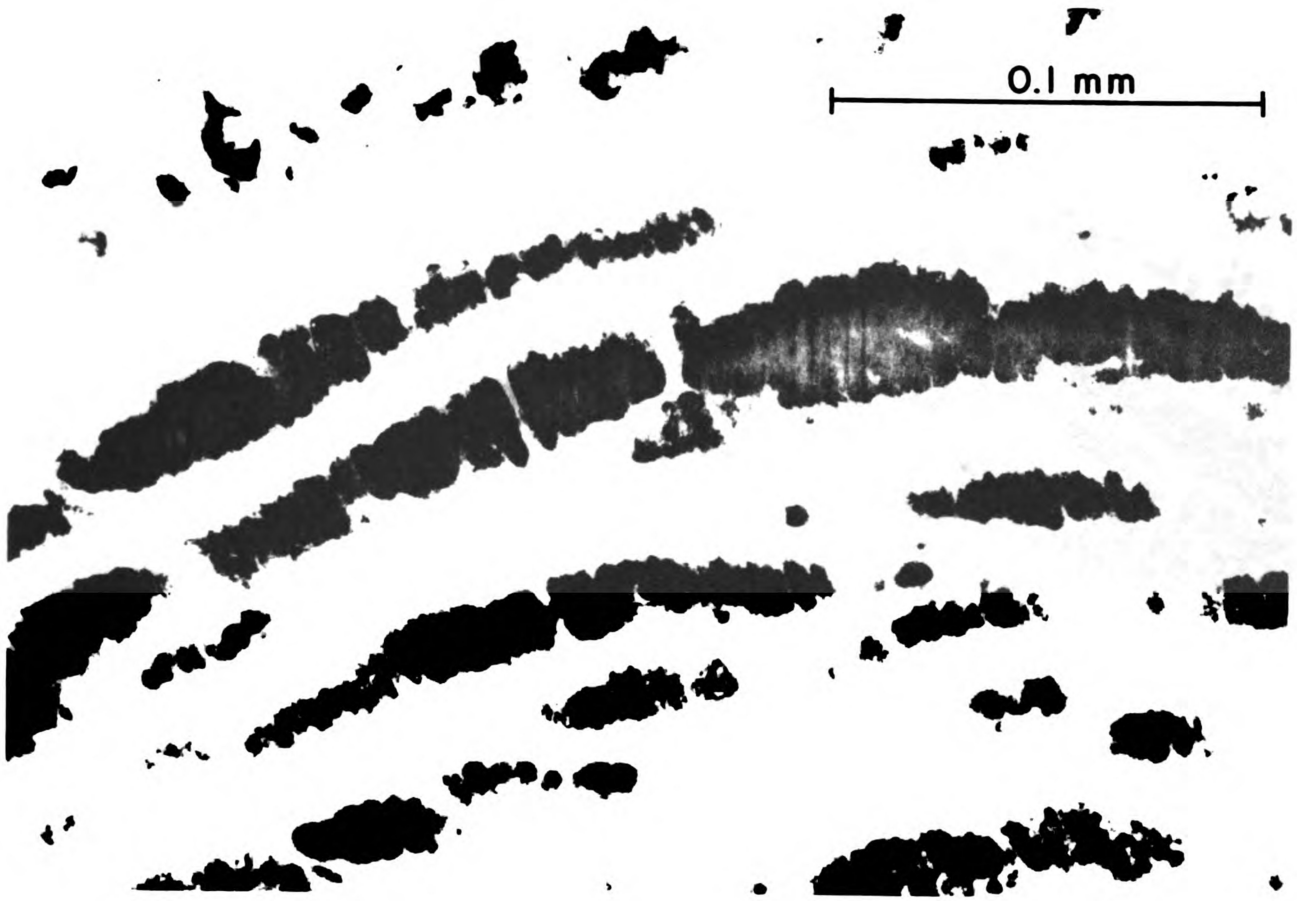

Figure 6. The columnar stacking of internal granule cells within discoids is seen in this photomicrograph from Antechinus. Section stained with thionin.

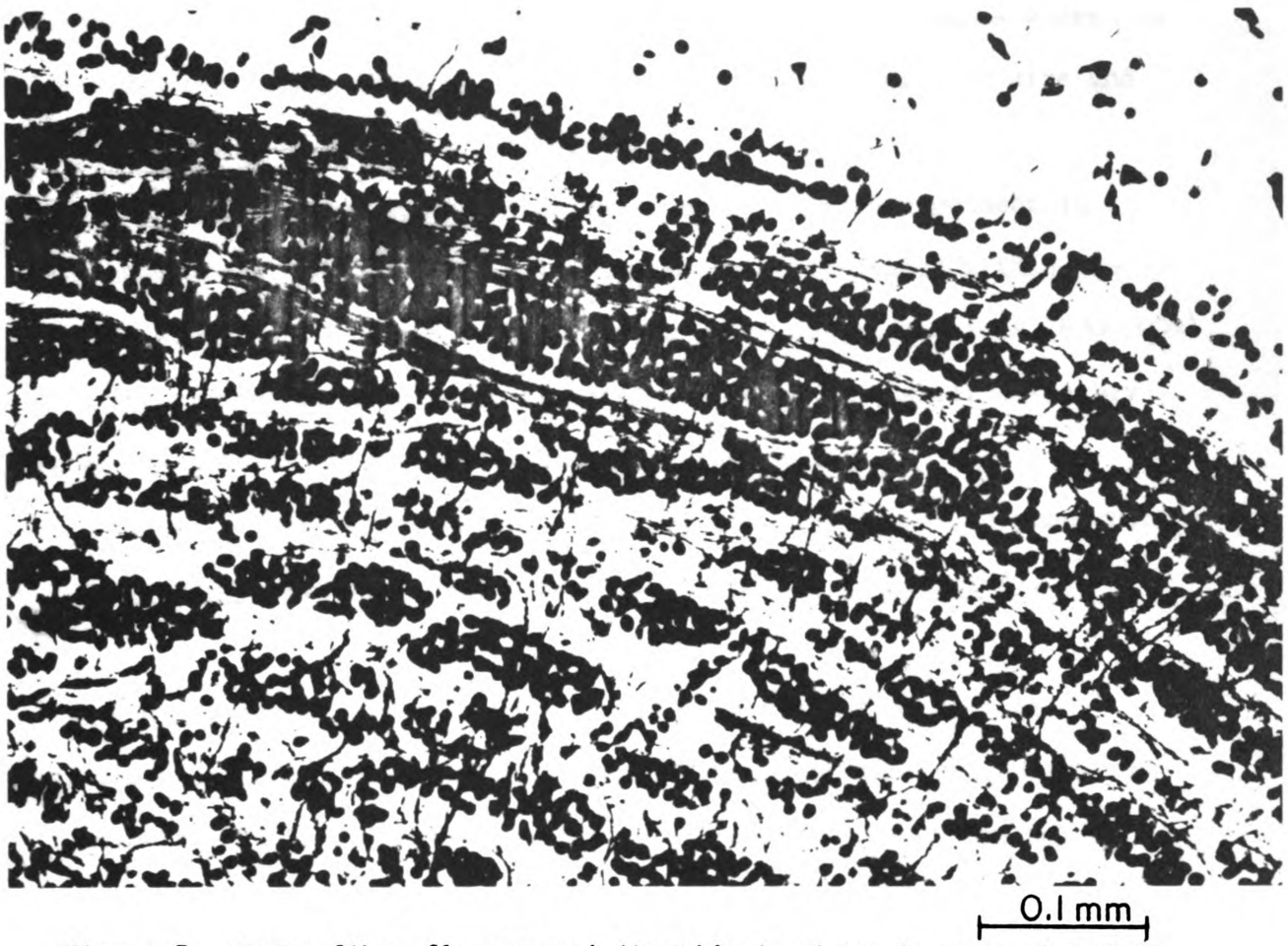

Figure 7. Nerve fiber flow around discoids is shown in a section from Petaurus breviceps stained for myelin. Note the interesting feature of fibers leaving vacant the region immediately below the larger discoids. Section stain: Sanides-Heidenhain.

Sections stained for myelin exhibit a gradation of staining density in the periventricular layer. The density of stain diminishes as the discoid layer is approached. It seems that myelination begins where the discoid layer ends. That is, the radial gradients of discoid size and degree of myelination are oppositely directed.

The various short axon cells described by Cajal are evident in varying degrees. Often these cells are somewhat concealed within the dense discoids and are apparent only with higher magnifications (600-800x).

The periventricular layer is barely existent in smaller olfactory bulbs but attains appreciable volume in larger bulbs.

The ventricle varies greatly in size; it is cavernous in some species (e.g. Sarcophilus) and barely evident in others (e.g. Petaurus).

Comparisons

Figs. 4 and 5 show sections of olfactory bulbs from various animals which point up similarities and differences seen over a number of animals. Table 1 compiles qualitative descriptions of some of the morphological features of these olfactory bulbs.

The distinctness of the tufted cell layer can be seen to vary considerably. Density of cells would be the best measure to quantify this apparent variation. The line of demarcation between TCL and EPL varies from being closer to the glomerular layer to being closer to the MCL. The degree to which the middle tufted cells follow the size gradation described by Cajal ('11-'52, p. 659; '11-'55, p. 7) ranges from barely evident to very obvious. Measurements of cell body areas plotted into histograms should resolve this apparent degree of size dispersion.

The density of mitral cells is quite high in the smaller animals and seemingly quite low in the larger animals. Chimpanzee and baboon have small olfactory bulbs but are low in mitral cell density. Determination of the MCL area and the actual number of mitral cells would put these variations in better perspective.

The IPL in cat seems to be larger than that seen in the shrew. A volume determination of the IPL instead of a comparison by its breadth would be a better basis for comparison.

The discoids in rat are relatively long, thin, and closely spaced. In contrast to chimpanzee, the discoids in baboon are very well developed. The difference between the discoids of rat and baboon is characterized by the discoids being thicker, more widely spaced and of lower size dispersion in baboon.

In all of the animals observed, differences in size distributions, density and thickness of the discoids are quite evident. To determine whether or not some of these features are due to bulb size and/or shape the discoids must be characterized quantitatively.

It is rather evident that the olfactory bulb of the rat is not the best for studies of olfactory bulb anatomy. Other mammals, such as shrew, mouse and many marsupials, show accentuation of the discoid formations and a distinct layer of middle tufted cells.

Part II. Quantitative Observations

Few quantitative studies have been done on the anatomy of the olfactory bulb. Allison and Warwick ('49) determined values for the number of olfactory receptor cells, glomeruli, tufted cells and mitral cells. Which population of tufted cells was counted is not evident.

The IG cells were ignored. The earlier studies by Holt ('17) and Smith ('28) were concerned with the number of mitral and IG cells only.

A quantitative study involving more than one animal type was done by Mann ('63) on several species of Chiroptera and on the shrew (Blarina brevicauda). No cell counts were made but volumes were determined for certain layers within the olfactory bulbs from macroslices of bulb tissue.

To maintain a truly synergistic relationship between structure and function it is appropriate to expand and reinforce the knowledge of olfactory bulb structure at not just the fiber and synaptic level but in terms of cell populations. This is important in functional considerations since confidence in interpretations of electrophysiological data is highly dependent on the knowledge of structure.

Study of olfactory bulbs of differing size and shape on a qualitative level make it quite evident that meaningful comparisons can only be obtained by quantifying the features in question.

The animals chosen for this study were from a single family which showed large variations in bulb size (Fig. 8). These were four species from the carnivorus family of Australian marsupials, the Dasyuridae: Sarcophilus harrisii (Tasmanian devil), Dasyurus viverrinus (native cat or quoll), Antechinus flavipes (common dunnart), and Sminthopsis murina (marsupial mouse). The olfactory bulbs of these animals were outstanding with respect to the accentuation of particular features, especially distinct layers of tufted cells and size gradation of IG cell discoids (Fig. 9).

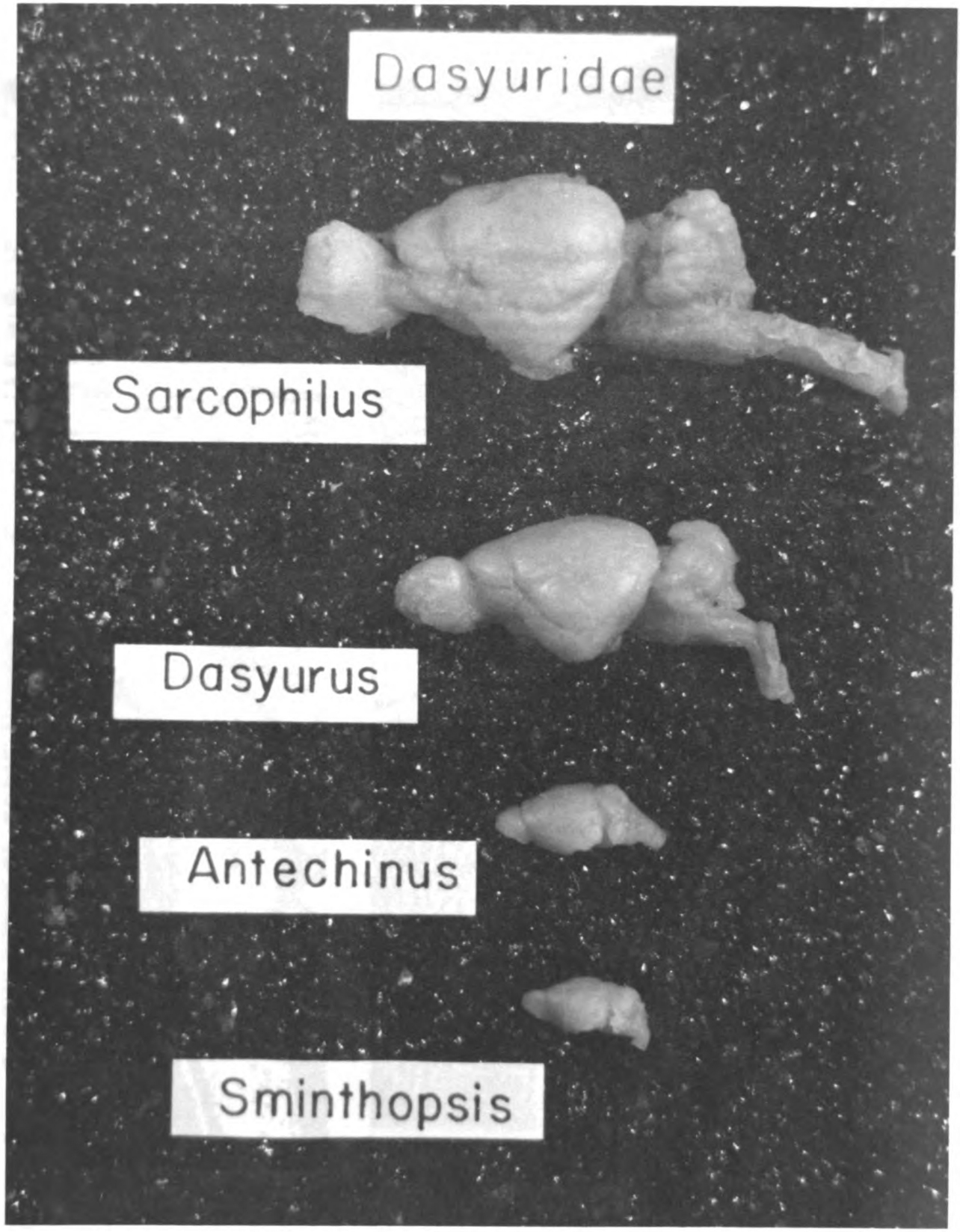

Figure 8. Brains of the Dasyuridae are shown in this photograph of casts of the four species used in the quantitative part of this study.

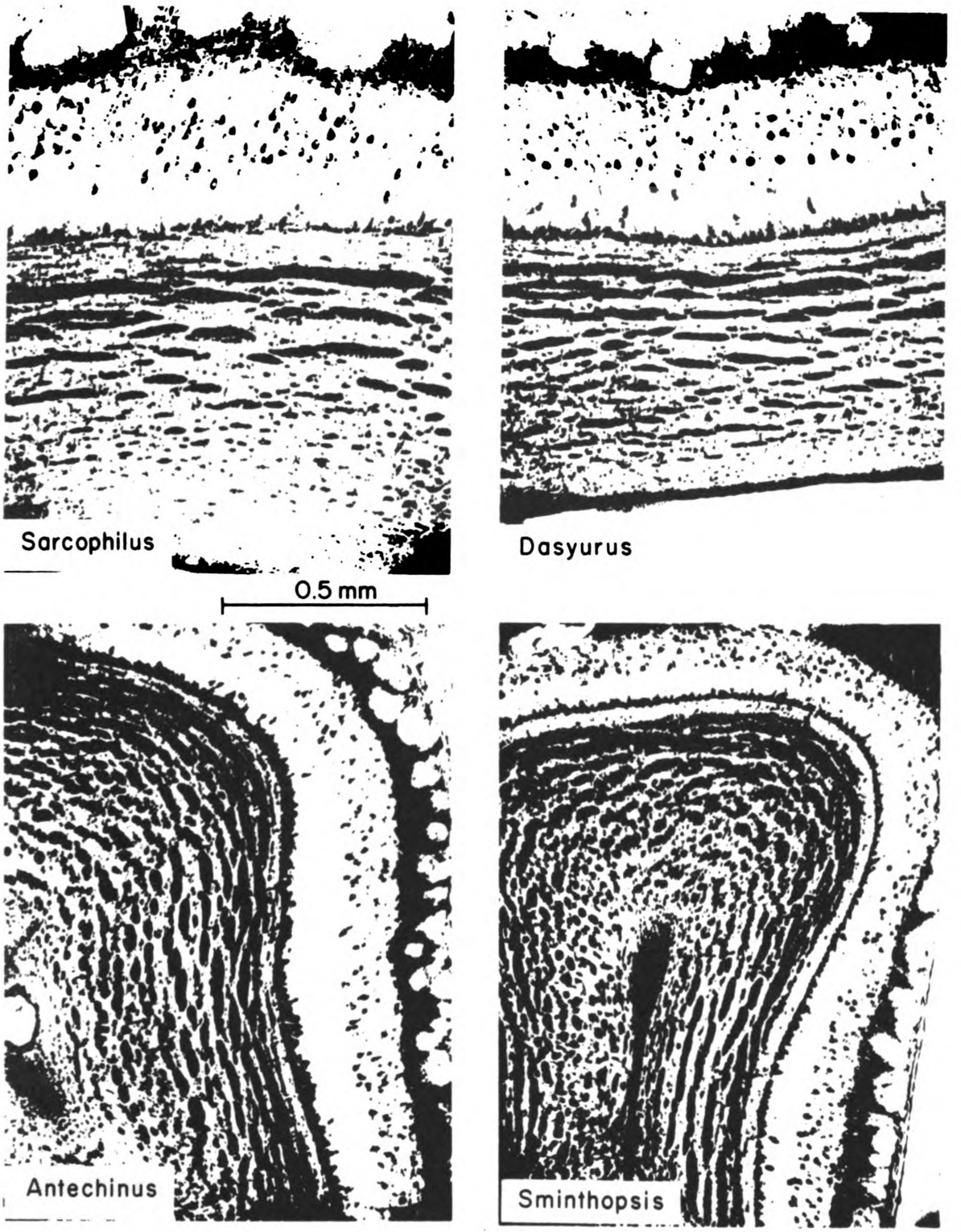

Figure 9. Sections from the olfactory bulbs of each of the four species of Dasyurids used in the quantitative part of this study are shown in this photomicrograph. Note the accentuated layer of middle tufted cells with the largest of the tufted cell perikarya predominantly occupying the inner boundary of the TCL. The discoids exhibited by the Dasyurids are large and well defined. Thionin stained sections.

There were two specimens of Sarcophilus; in one the olfactory bulbs were sectioned coronally, in the other the right bulb was sectioned parasagittally and the left bulb horizontally. Of three specimens of Dasyurus, the brains including olfactory bulbs were sectioned in different planes, one in coronal, one in parasagittal, and one in horizontal plane. The three specimens of Antechinus were sectioned as for Dasyurus. The single specimen of Sminthopsis had the left olfactory bulb sectioned parasagittally and the right coronally. For this study one bulb from each plane of section available for each species was used.

For this study answers to the following questions were sought.

-Does a given cell layer remain the same function of the bulb volume in animals of different sizes?

- How does the number of a given cell type vary with bulb size?

- Are the ratios between cell types dependent on bulb size?

-Is the density of a given cell type a function of bulb size?

- How are the discoids of internal granule cells organized?

Methods

Histological procedures were as described in Part I.

Volume Determinations

Selected thionin sections were projected at magnifications of X30, 50 or 70. Outlines were traced of the olfactory bulb perimeter including the accessory bulb and the various layers to determine volumes.

The external boundary of the bulb was taken as the outer outline of the glomeruli; this excluded olfactory nerve fibers. Any ventricle was excluded from the bulb volume.

The cell layer boundaries were defined as follows (see Fig. 2):

external granule layer (EGL)

Outer: the inner outline of the glomeruli.

Inner: a line skirting the inner edge of the cell dense region internal to the glomeruli.

Tufted cell layer (TCL)

Outer: internal outline of the EGL.

Inner: a line skirting the innermost extension of the middle tufted cells (very obvious in these animals).

external plexiform layer (EPL)

Outer: the inner boundary of the TCL.

Inner: the mitral cell layer.

mitral cell layer (MCL)

Considered as a surface populated with cells.

internal plexiform layer (IPL)

Outer: mitral cell layer.

Inner: the internal granule cell layer.

discoid layer (DL)

Outer: the inner edge of the IPL.

Inner: a line within which no discoid-like groupings of IG cells are seen.

periventricular layer

Outer: inner DL.

Inner: the ventricle or if none there is no inner boundary.

In coronal sections boundary definition was quite evident but some conventions had to be adopted in the horizontal and sagittal planes. The posterior aspect of the bulb in some sections ended adjacent to a heavy nucleus (AON) or even caudal to it. Whatever the case a line was drawn skirting the edge of the innermost extension of the olfactory bulb cells including the accessory bulb, if present, then internally along the caudal aspect of the DL. At this juncture a line was dropped to the surface of the ventricle such that it was perpendicular to the ventricular boundary (Fig. 5). When no ventricle was present a smooth line was drawn to the "corresponding" opposite point of the bulb section. The AON was always excluded.

With a set of about 12 tracings per bulb, approximately evenly distributed through the bulb, areas of the traced figures were determined with a compensating polar planimeter. Each area was traced three times and the average taken. These data yield each section's volume when multiplied by the section thickness and divided by the areal magnification due to projection (Dornfield, et al '42).

Each of these section areas were plotted on a graph where section area showed the ordinate and the abscissa depicted the distance between sections. With the sections containing the beginning and termination

points of each olfactory bulb and bulb layers plotted as the zero points, a smooth line was drawn connecting all the plotted points. By planimetry the underlying area was measured from which bulb and layer volumes were determined. The mitral cell surface area was determined similarly except that the perimeter of the layer in each section was measured with a plan measure (Keuffel and Esser #62-0310, which measures line length). These perimeters were then plotted against section distance, the area beneath the resulting curve yielding the layer's surface area.

Cell Counts

Mitral cells were counted in every other section for which an area had been determined. Plotting the obtained numbers versus section number and using the same zero points for beginning and end, as in the volume determinations, the smooth line joining these points was planimetered, yielding the total number of cells.

That this was a reasonable means of sampling was determined by selecting two sets of sections to be counted in the coronally sectioned olfactory bulb of Dasyurus. The two sets together comprised the group of the sections in which volumes had been determined, each set containing every other section of the group. Each set was plotted and a line drawn for each set of points. The number of cells determined from the planimetry of the curve was compared. The two counts were within 3% of their average.

All counts were made in collaboration with another person. Counts were compared and accepted if they were within 10% of each other for counts greater than 100 and within 10 for counts less than 100.

The internal tufted cells fall close to and mingle with the mitral cells, therefore leading to confusion as to which are which. Some of the tufted cells are clearly not in the mitral cell layer while others are close. The convention adopted in counting the mitral cells was to include all cells having their nucleoli on or within an imaginary line skirting the external edge of the bulk of the mitral cell bodies. Even so, it is felt that the estimates of numbers of mitral cells may be too high, as was judged by Allison and Warwick ('49) in their counts of mitral cells. There is probably further inaccuracy in counts in sections cut obliquely to the MCL since the external edge of the MCL was at an angle and the imaginary boundary was more difficult to determine.

The middle tufted cells lie within the boundaries described above for the tufted cell layer volume determinations. Counting the larger tufted cells near the layer's inner edge was no problem. But the smaller tufted cells approach the size of the granule-like cells in this layer and unless they obviously bore the "tufted" appearance they were not counted. The tufted cells falling close to the glomerular layer were counted only if their nucleoli fell inside an imaginary line skirting the edge of the dense region of periglomerular cells, the EGL.

Upon finishing the same procedure for the tufted cells in the coronal sections for each animal it was found that a single section sampling could be employed. The section in which counts were to be made was selected by referring to the volume curves and choosing a section from a region of the curve where the volume was changing slowly, i.e. a point where the volume profile line has a nearly zero slope. This point was usually near the center of the bulb.

This single-section sampling was tested in the coronally sectioned bulbs by selecting the section in which counts had already been made and which fit the above criteria. The cell density for that section was then determined by dividing the number of cells by the layer volume. This density was then multiplied by the total TCL volume for that bulb. The resulting cell number was compared with the cell number determined graphically and was found to be within 6%. This amount of variation was deemed satisfactory. This sampling method could not be employed for mitral cells since the values obtained had too great a variation from those values obtained graphically. Consequently, all of the mitral cell totals were determined graphically.

Number of IG Cells

Determination of the number of IG cells required different methods than the above since the cells were so numerous and dense within discoids that they were uncountable. Therefore the following scheme was employed:

1. Determine the fractional volume of the discoids in the discoid layer: V_{DL}
2. Determine the fractional volume of the cells within discoids, i.e. packing density: V_P
3. Determine average cell diameter, then calculate average cell volume: V_c
4. Volume of the discoid layer: V_L

Fitting into the equation:

$$\text{IG cells} = \frac{V_L \times V_{DL} \times V_P}{V_c}$$

The fractional volume of the discoid profile was determined with modified point count volumetry. Selected sections meeting the same criteria set forth above for tufted cell counts were projected directly through a Zeiss microscope equipped with a photo changer, projection tube and deflecting prism onto a rectangular grid of dots. The dot spacing was 2.87mm or 12 dots per cm^2. The projected image on the grid of dots was 238X.

The method for volume fraction analysis was that set forth by Hilliard and Cahn ('61) using a dense grid of dots. A less dense grid of dots can be employed only if the particles are nearly spherical, randomly oriented and distributed and of low dispersion in size. Since the discoids did not meet any of these criteria, a dense grid of dots was required.

The concentric symmetry of the discoid layer and anisotropic distribution of size of discoids called for a sampling area that spanned the region between the external and internal DL boundaries (see Fig. 5). The width of this radial sector was arbitrary.

The sample area was drawn with the radially directed sides parallel so that the total number of dots in the area could be easily calculated. Sample areas were taken in regions such that equal representation of all regions of each section would be obtained. For each olfactory bulb the discoid volume fraction was an average of all sample areas.

The packing density of cells within discoid structures was estimated as 52% based on the following observations. The cells are not closely

packed, i.e. not hexagonally packed. This is revealed by tangential sections through discoids at different levels. The largest discoids exhibit columnar stacking of cells such that the cells are in close contact but the columns themselves(Fig. 6) are not in close or frequent contact. In smaller discoids where columnar stacking is not evident the cells are in contact but "loosely packed" using the term of Dallavalle ('43), i.e. every element is not in contact with all its neighbors. In analyses of porosity in geological considerations the porosity is 48% for a loosely packed state or a 52% occupation of total volume by the packed particles.

Cell size was sampled within discoids along a given radial line through the DL. The average cell size was used to compute the cell volume. Determining the number of IG cells was then just a matter of substituting values into the equation.

Discoid Parameters

The discoids when viewed along a line parallel to their minor axis have a nearly circular profile. Slicing the discoids along this same line results in only one cut that is along the diameter while the rest are chord lengths. Viewed from the side or perpendicular to the minor axis, each of the sections of the discoid have an elliptical profile. The major axis of each of the discoid section profiles is identical to a chord length of the discoid when viewed along a line parallel to the minor axis, i.e. perpendicular to the major axis. In the rest of this text the major axis of the elliptically shaped discoid section profiles will be termed "discoid chord lengths."

The discoid section profiles were counted by using a rectangular grid reticle in the eyepiece of the microscope and scanning across rows of the grid. In the course of the scan only the leading ends of discoid profiles which appeared in each square of the grid were counted.

For the discoids a sampling procedure similar to that used for the tufted cells was found practicable except that two sections in each bulb were counted to obtain an average discoid profile density between them. From this density the total number of discoid section profiles were determined. Two sections rather than one were counted since regions of slow section to section volume change in the DL were not as extensive as for other subvolumes: DL volumes varied more from section to section: the section-to-section volume slopes were higher for the DL.

Radial Density Gradients

Since there is a high degree of concentric symmetry to the arrangement of the discoids, sampling lines placed radially on a given section (perpendicular to the major axes of the discoid profiles) were used to ascertain radial density gradients and length versus radial position of the discoids.

Placement of the radial sample line was accomplished by a line reticle placed on the top of the radiant field diaphragm. This could be brought into the same focus as the section with the stage condensor and still have the section properly illuminated. Criteria were set for beginning and end points along the radial line, and in what sections and their regions were to be measured as follows (see Fig. 5):

--Beginning or zero point: innermost part of the mitral cell layer, i.e. boundary between IPL and MCL.

--End Point: the internal boundary of the discoid layer.

--The length, zero to end point, was established as the normal length. The position of discoids along this line as converted into percentages of this normal length.

--Radial lines were placed to give as equal representation as possible of anterior, ventral, dorsal, lateral and medial regions. Only those sections or regions within a section where the discoids had been cut nearly perpendicularly were sampled. Otherwise the position and dimensions of a discoid could not be easily determined.

Discoid Size

The discoid size class analysis was carried out by first compiling a histogram of discoid profile lengths measured in the radial line sampling. Since these profiles are from discoids cut parallel to their minor axes these profile lengths are chord lengths of the discoids of which only a small percentage are diametric lengths. Because the discoids' minor axes are radially aligned and in the same plane as the sections chosen for measurement, Hennig's graphical method (Elias and Hennig, '67) can be employed for extraction of diameter size distributions from chord size distributions. The chords are from sections of discoids whose diameters fall into a larger size class. Naturally, the largest size class in a histogram of chord sizes is comprised only of diameters. The numbers of diameters in smaller size classes are obtained by progressively subtracting the number of chords that are from discoids whose diameters have already been counted.

Number of Discoids

To extract the number of discoids from the number of discoid section profiles the following formula was employed:

Define: D - as the number of whole discoids per olfactory bulb.

P - as the total number of discoid section profiles per olfactory bulb.

p - as the number of discoid section profiles in the sample.

d - as the number of discoid profiles in the sample whose major axes were actually diameters of discoids rather than chords.

Then for each animal:

$$D = \frac{d}{p} \times P$$

Results

Layer Volumes

Layer volumes, numbers of cells and ratios between them are all referenced to the olfactory bulb volume. A plot of layer volumes is shown in Fig. 10. In a log-log plot the data points for each layer can be joined by a straight line. This indicates that the volume of each layer is a constant function of the bulb volume. Each layer has a rate of increase in volume nearly that of the bulb itself indicated by the nearness of 1.0 of the slopes.

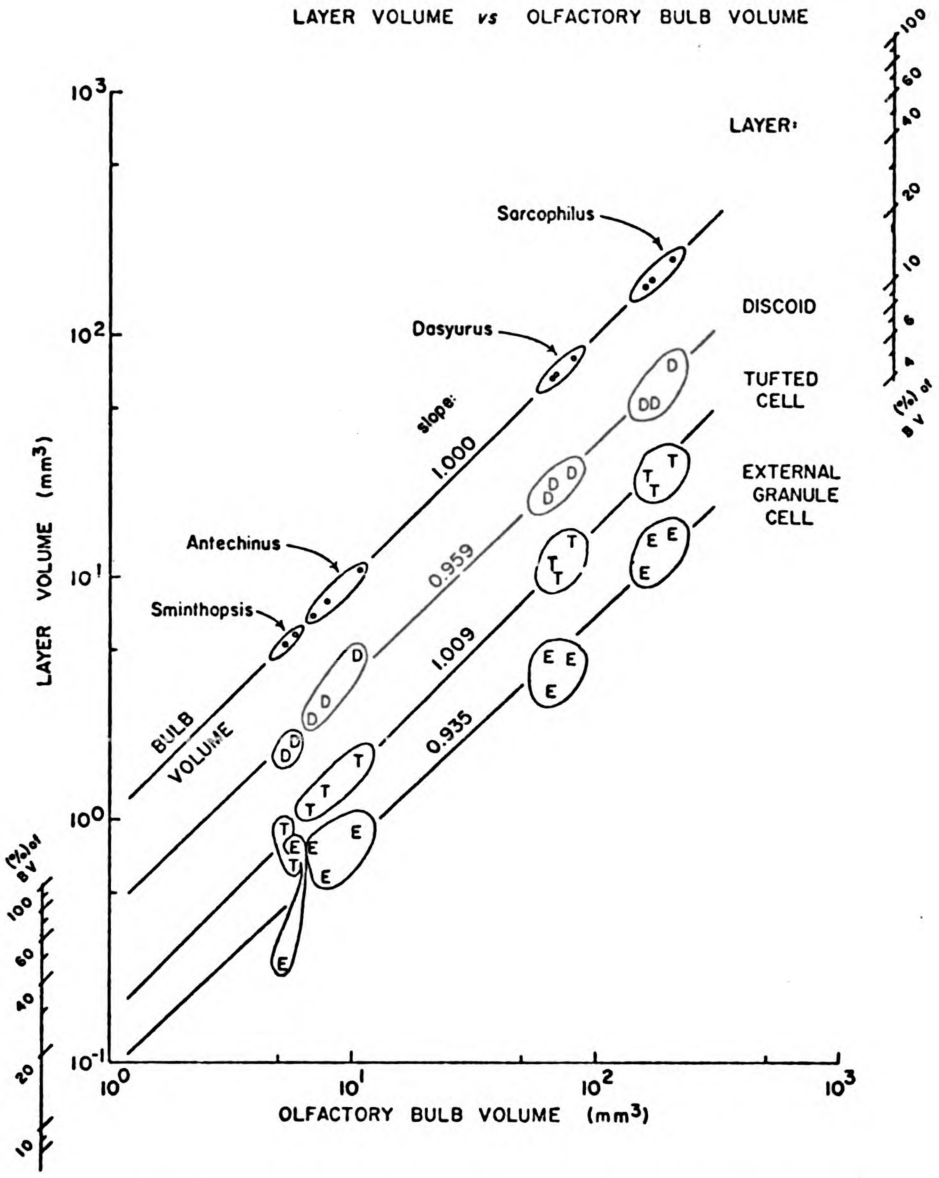

Figure 10. Layer volume plots. A log-log plot of each layer of the olfactory bulbs of the Dasyurids against the total volume of the olfactory bulbs is shown in graphs (a) and (b). Grapha (a) is a plot of the cell layer and (b) a plot of fiber layers. The data points for the olfactory bulbs of any given species is enclosed by a line. Individual data points are indicated by a letter appropriate to the layer. The olfactory bulb volume is plotted against itself to serve as a reference line for a slope of 1.0 and as an aid in determining what fraction a particular point is of the olfactory bult volume of interst. The slope of each line is given near the center of each line. The straight line character of each set of data points indicates that the particular layer remains a constant function of the bulb volume with respect to size. If the slopes were exactly 1.0 then the layer would also be a constant fraction of the olfactory bulb volume as the total volume changed. Note that in these graphs and those to follow which are log-log plots the equations of the straight lines are of the form log y=m log x + log b.

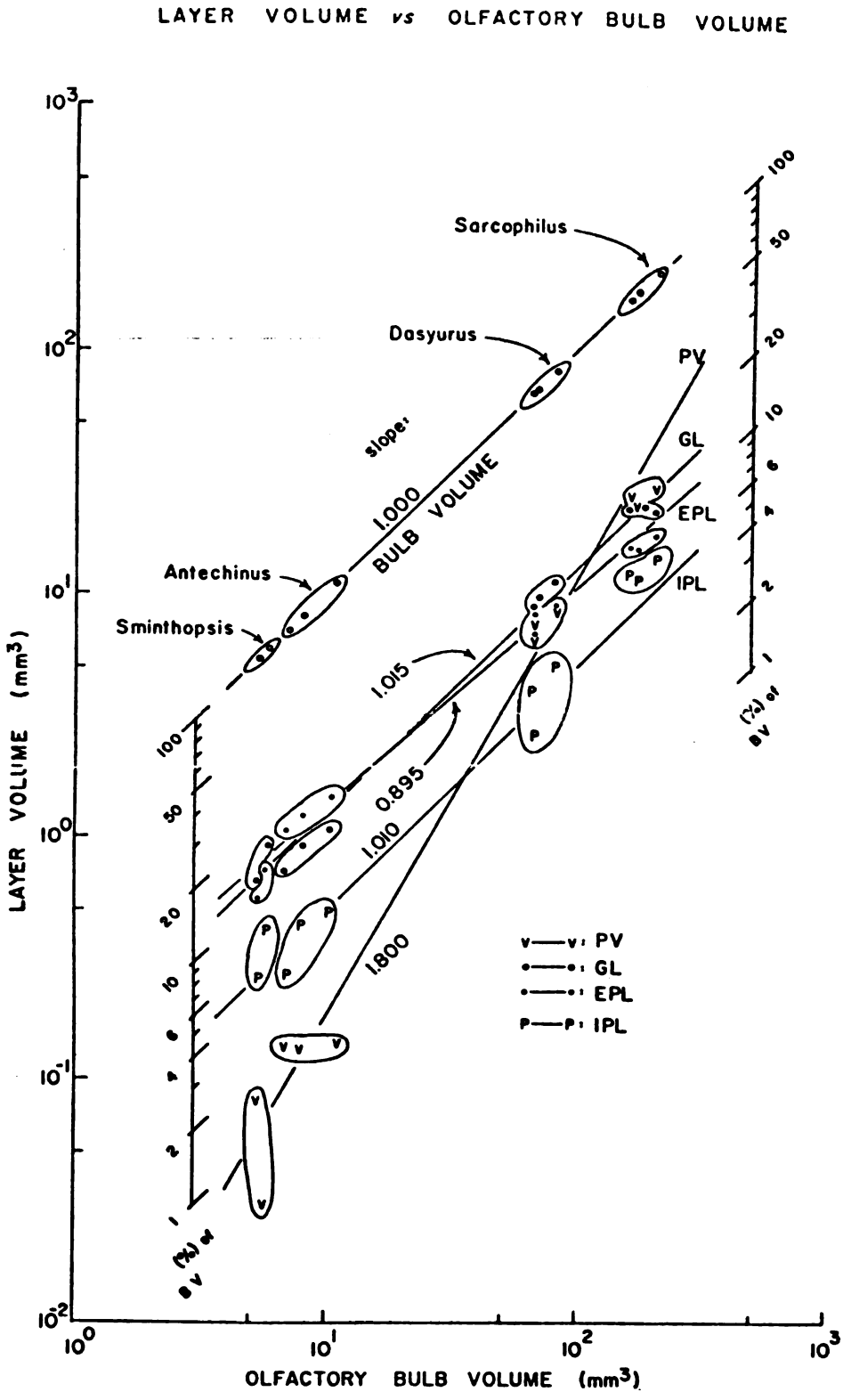
LAYER VOLUME vs OLFACTORY BULB VOLUME
LAYER VOLUME (mm3)
OLFACTORY BULB VOLUME (mm3)
Sarcophilus
Dasyurus
Antechinus
Sminthopsis
slope:
1.000
BULB VOLUME
1.015
0.895
1.010
1.800
PV
GL
EPL
IPL
v—v: PV
•—•: GL
•—•: EPL
P—P: IPL

Figure 10(b).

Number of Cells

In a similar plot the numbers of the three cells types, middle tufted, mitral and internal granule are shown in Fig. 11. The lines do not have slopes as close to 1.0 nor are they as parallel as those in the plots of layer volumes. Since the lines connecting the data points are straight the number of each cell type remains a constant function of bulb volume. The lines do not have nearly identical slopes nor are any as close to 1.0 as was the case with the plots of layer volumes. Evidently in a given layer the layer volume increases at a faster rate than the number of cells. This is reflected in a plot of cell densities against bulb volume shown in Fig. 12.

Cell Densities

The density for each cell type declines with increasing bulb volume. From Sminthopsis to Sarcophilus the IG cells decrease in density by about one half, the tufted cell density decreases by a factor of about one third and the density of mitral cells decreases by one fifth. (The mitral cell layer is here considered as a surface so that cell density is a surface density.) This trend of decreasing nerve cell density

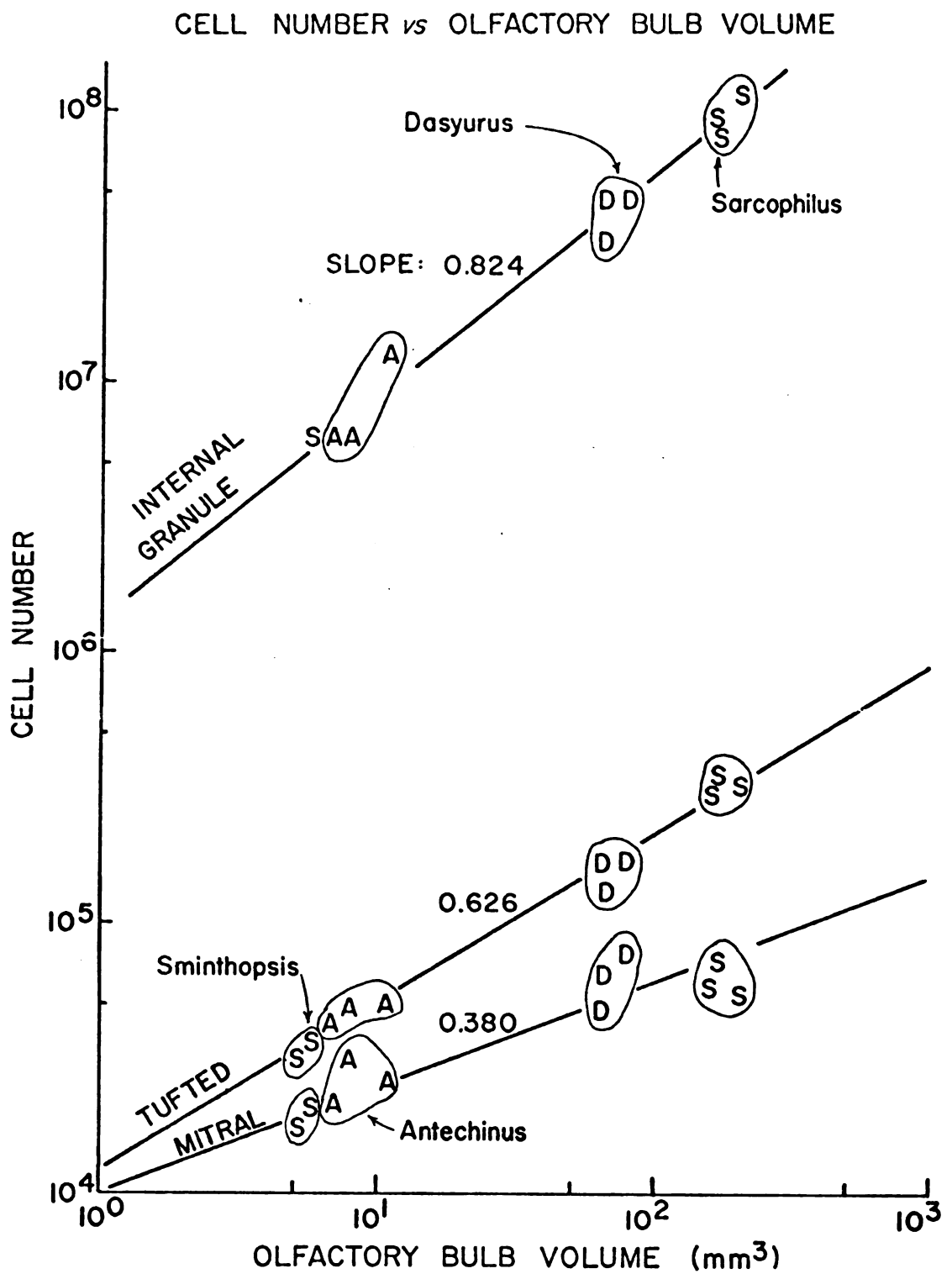

Figure 11. Number of cells. Log-log plots of each of the three cell types, internal granule, tufted (middle) and mitral cells against olfactory bulb volume are shown. The straight lines through the data points indicate that the number of each cell type remains a constant function of the total bulb volume regardless of size. (Only one data point exists for the number of IG cells in Sminthopsis since the volume fraction of the discoids used in the cell number computation could not be determined in the olfactory bulb sectioned parasagittally.)

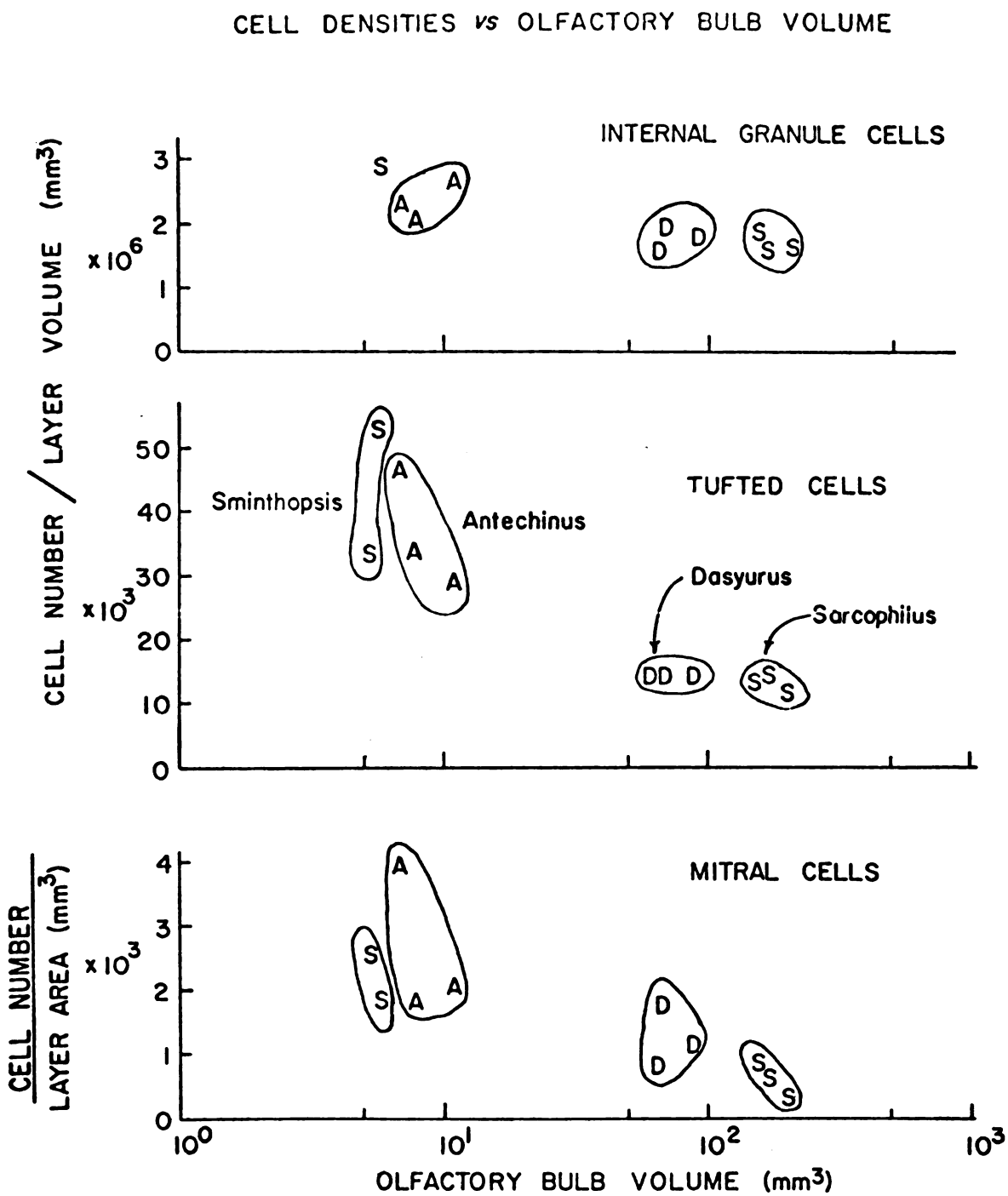

Figure 12. Graph of cell densities. The number of each cell type divided by the volume of the layer each occupies yields the cell density. Plotted against the volume of the olfactory bulb the trend of the cell density is to decrease with increasing bulb volume. Note that the mitral cell density is a surface density and not a volume density since the mitral cell layer is more of a surface populated with cells that a volume containing cells.

with increasing brain size follows that of cortical nerve cell density over the range of brain size of mouse to whale (Tower '54).

Ratio of Cell Types

The trends between the numbers of each cell type are shown in the plots of ratio of cell types against bulb volume (Fig. 13). In each case, the more numerous cell type was made the numerator. All of these ratios increase with increasing bulb volume, IG/mitrals the most and mitrals/tufted, the least. Allison and Warwick's ('49) ratio for tufted to mitral (assuming they counted only the middle tufted cells) was 2.5 which is close to the ratios exhibited by Dasyurus. The ratios ranged from 1.8:1 for Sminthopsis to 5.9:1 for Sarcophilus.

In general it is observed that while the numbers of cells increase with bulb volume and the densities of each type decrease, the ratios between cell types increase: the more numerous cells increase with bulb volume relatively more than do the less numerous cells.

Discoid Parameters

The histograms of discoid diameters in Fig. 14 reveal at least two peaks of size classes of discoids in all four animals. The primary peak is comprised of small discoids. Each animal exhibits a secondary peak of large discoids indicated by the arrows.

RATIOS OF CELL TYPES *vs* OLFACTORY BULB VOLUME

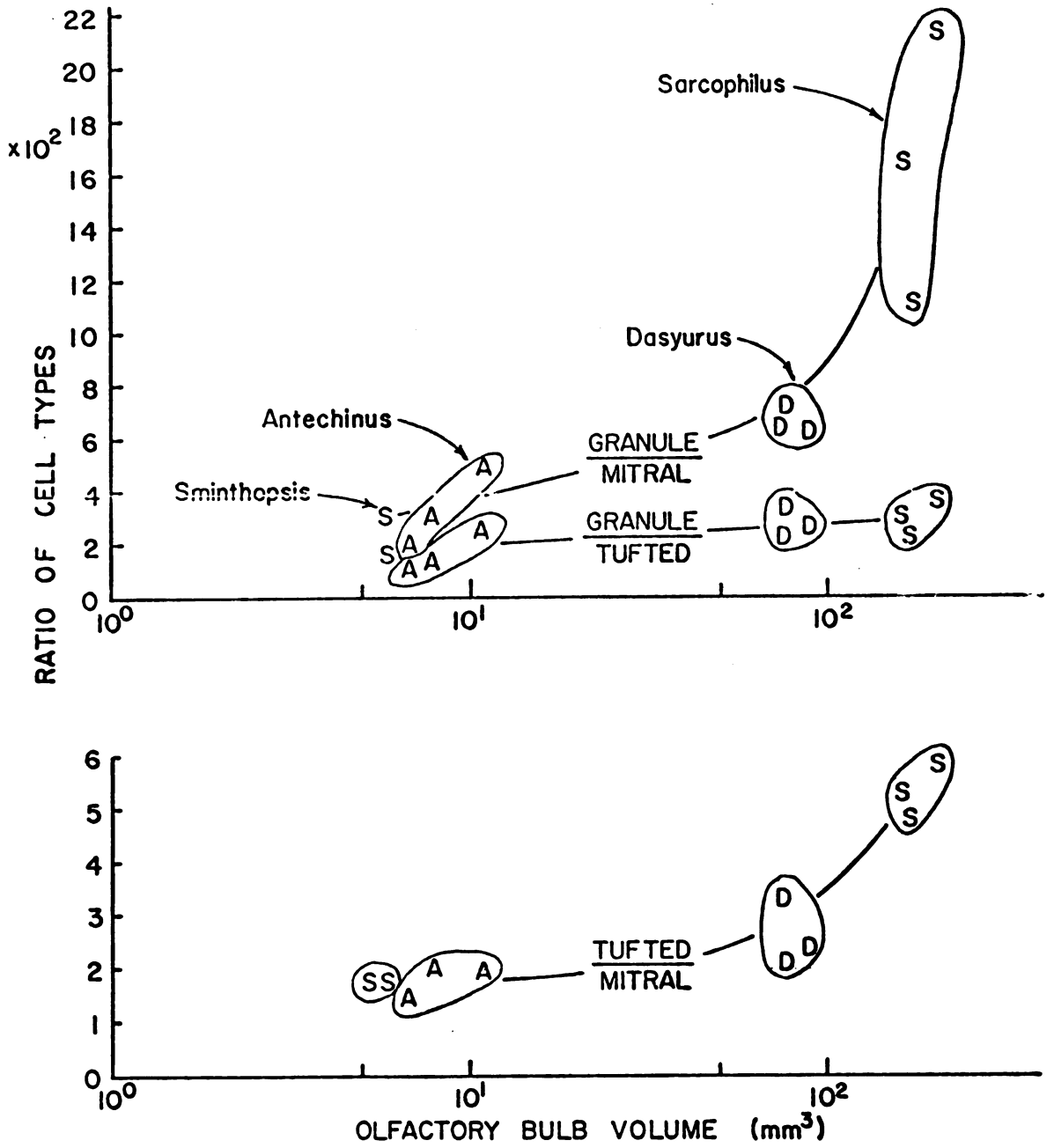

Figure 13. Graph of cell ratios. These plots show the ratios between cell types with the more numerous cell type of each possible pair as the numerator.

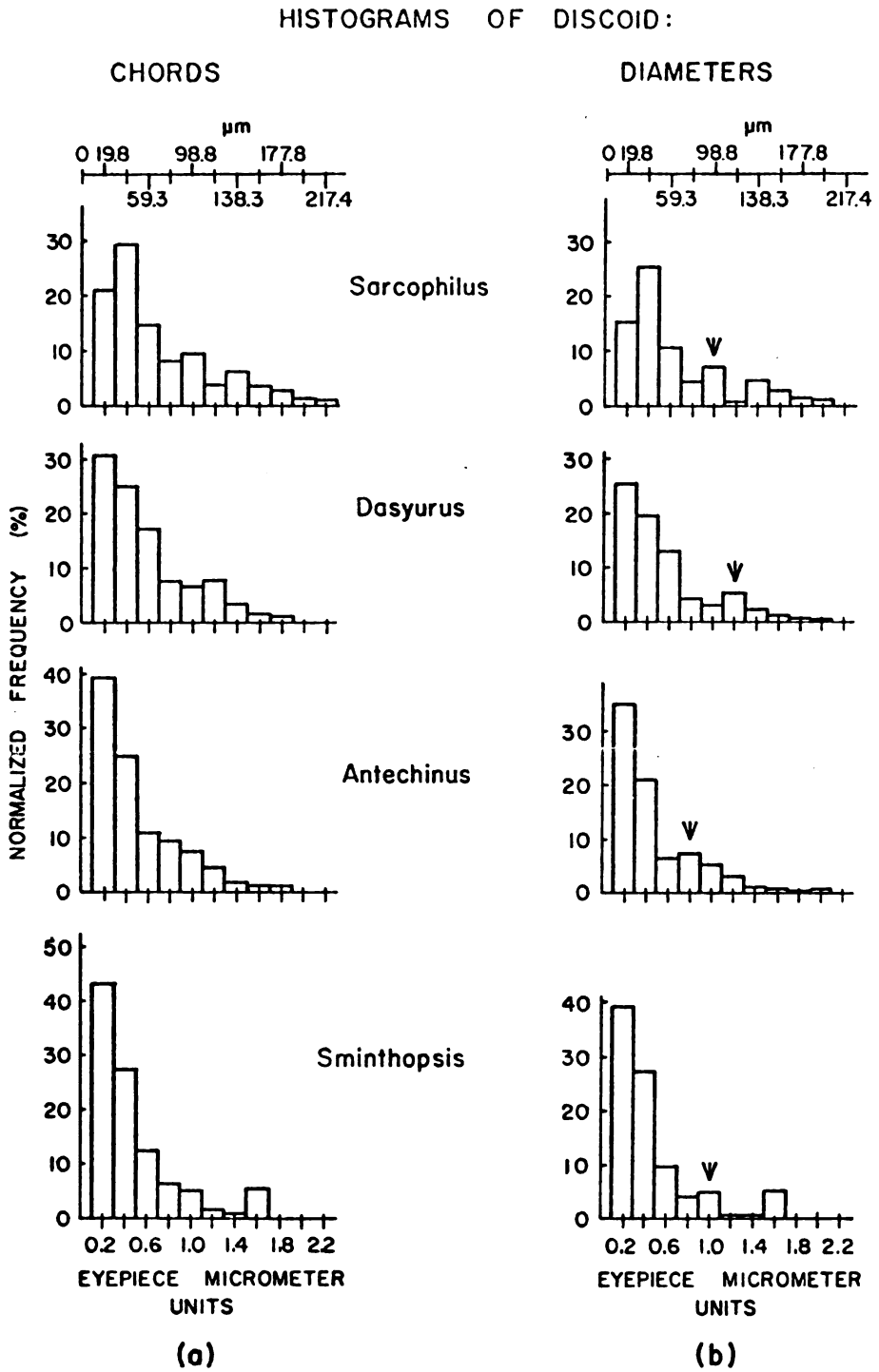

Figure 14. Histograms of the chord lengths of discoids are shown in (a). From these histograms are extracted the histograms for diameters of discoids, (b),by the method of Hennig (see text). Since the small discoids dominate the histogram profiles the overall profile of the chord histograms is not altered greatly in obtaining the diameter histograms except that secondary peaks (arrows) not present or evident in the histograms of chords appear in the diameter histograms.

In Sarcophilus and Sminthopsis, the largest and smallest animals of the group, this second peak is the 100um range. The secondary peak in Dasyurus is the next largest size class (118um) and in Antechinus it is the next lowest (79um).

From largest to smallest olfactory bulbs, the major peak frequency of small discoids increases in proportion to the whole population of discoids, while the large discoids in the secondary peak follow no trend and remain near 6%. The size class centered about 79um (0.8 units) stays very constant, deviating only in Antechinus where this is also the size class exhibiting the secondary peak.

To demonstrate that the large discoids are predominantly found in the outer DL and the small discoids are preferentially located in the inner DL, the data for the discoid chord length histograms was divided so that histograms could be drawn showing the chord size distribution for the outer and inner half of the DL. Fig. 15 shows a pair of histograms for each of the four species. If "large" discoids are defined as any profile greater than 0.7 units than it is quite evident that relatively few discoids of this profile size range occupy the inner half of the DL.

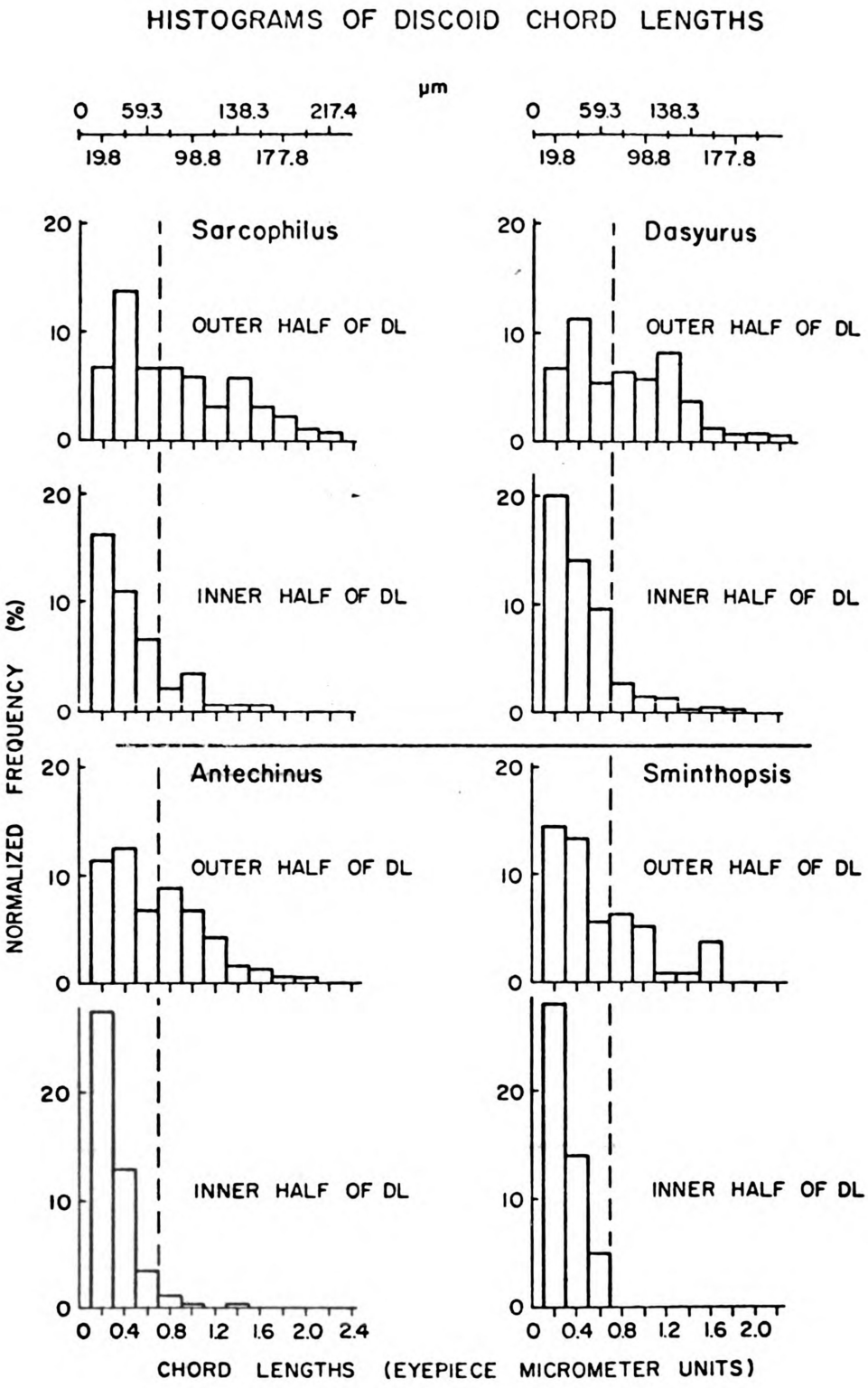

Figure 15. Histograms of chord lengths of discoids, reduced to show radial regional differences in the discoid layer, are shown here. For each species of the Dasyurids, a pair of histograms shows the chord lengths of discoids found in the outer and inner half of the discoid layer. These histograms reveal that the large discoids (discoids greater than 0.7 units designated by the dotted line) are preferentially located in the outer half of the discoid layer.

Note that since these are discoid chord histograms and not discoid diameters histogram , some of the small profiles found in the outer half of the DL are due to chord sections of the larger discoids.

The densities of IG cells within discoids as a function of bulb volume are shown in Fig. 16A. The increase in density with increasing bulb size can be attributed to the increasing proportion of large discoids in bulbs of greater size(See Fig. 14).

The number of discoids per bulb increases with increasing bulb volume as shown in Fig. 16B. The rate of increase is not as great as that for cells; the number of discoids per bulb is closer to being proportional to the square root (0.526 = m) of the bulb volume.

Another way of analyzing the discoid data is to determine the average discoid chord length as a function of radial position. This is done in Fig. 17. The most striking feature of these histograms is that the maximum average chord length of the discoids is very nearly the same for each species: regardless of olfactory bulb size the maximum average chord length of the discoids is almost the same. This could be interpreted in terms of there being some functional importance to the size of the discoids.

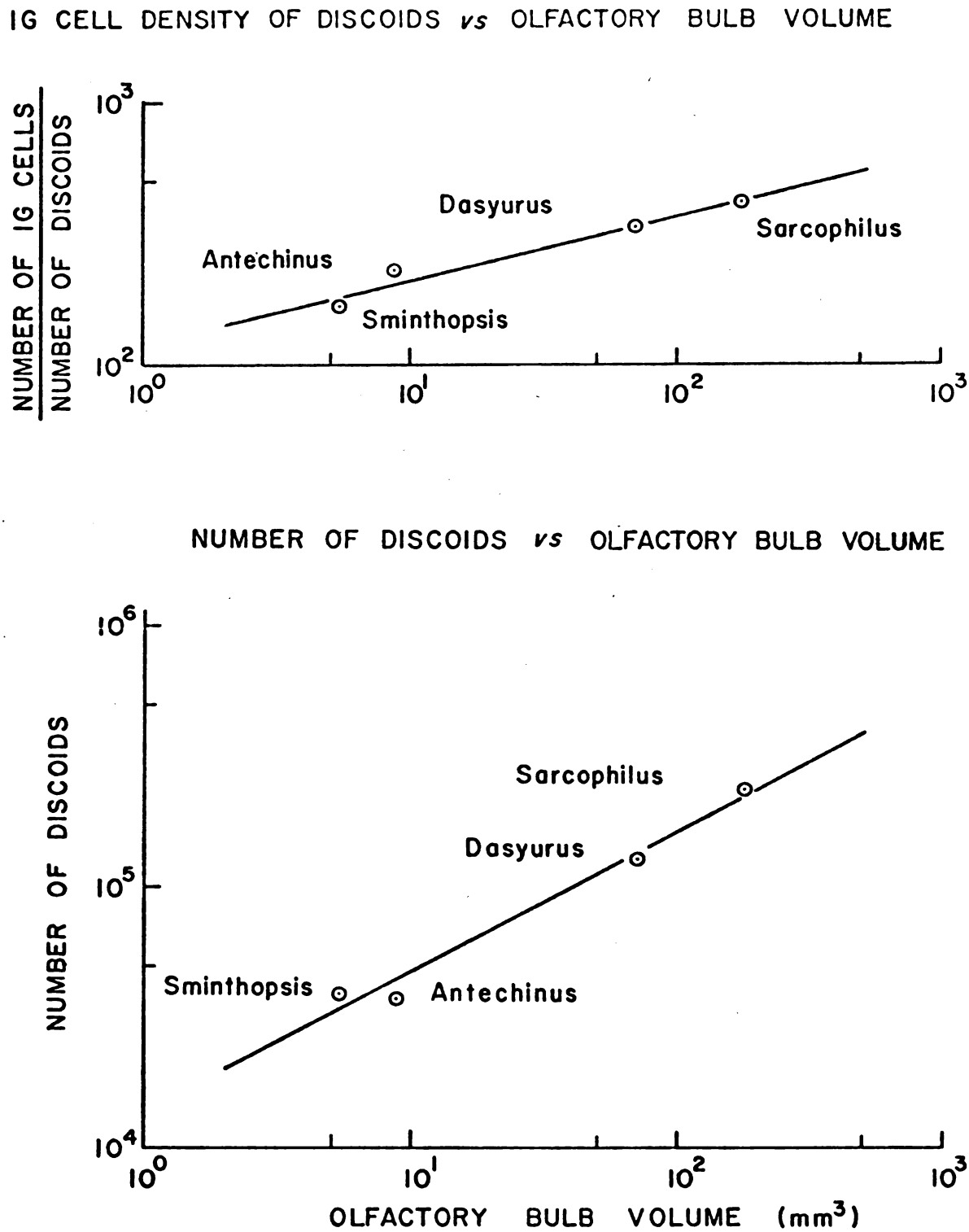

Figure 16. The density of IG cells within discoids (a) (top) and the number of discoids (b) (bottom) are plotted against olfactory bulb volume in these graphs. For each species only one data point is shown in contrast to preceeding plots since the derivation of the number of discoids came from an average from the specimens of each species.

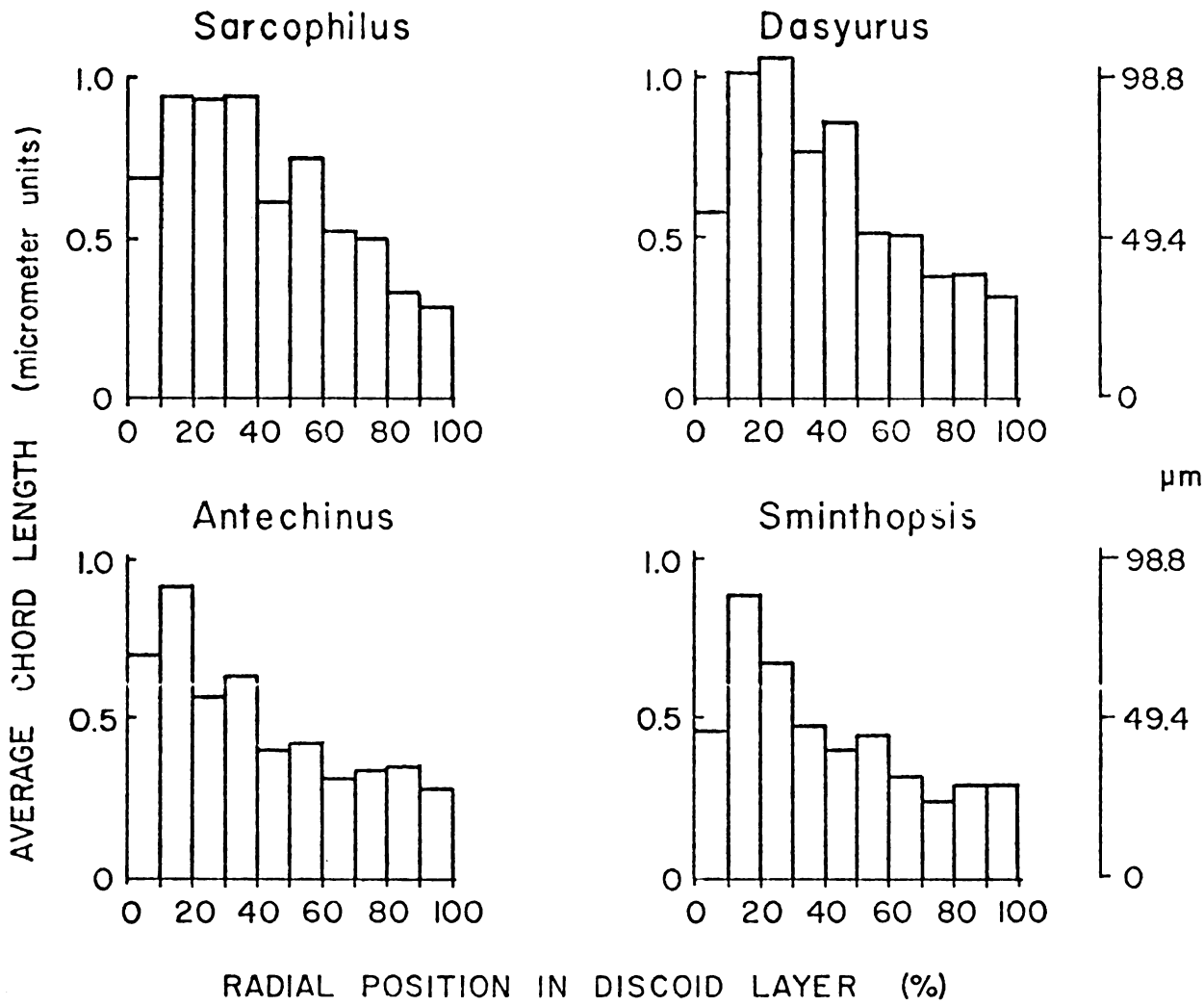

Figure 17. The average discoid chord length as a function of radial position is shown in these histograms. Of particular note is that the maximum average discoid chord length for each species is nearly the same: 1.06 units for Dasyurus; 0.94 units for Sarcophilus; 0.90 units for Antechinus and 0.88 units for Sminthopsis.

DISCUSSION

Discoids of IG Cells

The internal granule cells are organized populations of cells as manifested by their discrete grouping in the discoids. Where the internal granule cells have previously been treated as single interneurons, the possibility must now be considered that the discoids are discrete populations of interacting internal granule cells.

The close juxtaposition of cells within the discoids invites consideration of possibilities for weak electrical coupling. Price and Powell ('70) observed that where the cell membranes of internal granule cells oppose each other thickenings on the membrance exist. Although the extracellular space is maintained with no indication of a tight junction, the thickenings of the cell membrances where cells are juxtaposed may be some structural specialization related to electrical coupling between the cells. With such a situation excitation of a few granule cells in a given discoid could influence the whole population of cells within that discoid which could bring about synchronous activity.

What has been revealed quantitatively about the population of discoids was not at all expected from qualitative observations. Regardless of olfactory bulb size the distribution of size classes of discoids exhibits two peaks. Furthermore, each of those peak size classes is preferentially distributed in the radial dimension. Small discoids are congregated internally, large discoids are restricted to external locations.

A possible significance of the two size populations being differentially distributed spatially may be found in interpreting the findings of Price and Powell ('70). They revealed that axons afferent to the olfactory

bulb of rat, from the cerebral hemisphere, terminate on the individual internal granule cells in a laminar fashion. For instance, terminals from the anterior commissure terminated on the internal part of the IG cells.

In rat the discoids are smaller and more densely spaced and their size distribution less evident than in larger animals. One may then ask the questions:

1. Does the compactness of the discoid layer in the rat olfactory bulb prevent one from determining whether the termination of axons on the internal granule cells is laminar with the respect to the individual IG cells or to the whole layer?
2. In larger olfactory bulbs do the deep dendrites of the outermost IG cells extend all the way to the inner fringe of the discoid layer?

If the axons afferent to the bulb terminate in a laminar way with respect to the discoid layer and the deep dendrites of the IG cells whose perikarya are situated externally toward the IPL do not extend the full length of the layer, then the population of small discoids would be receiving fibers from the anterior commissure and the population of larger discoids would not.

Phylogeny of Discoids

Examination of the IG cells in olfactory bulbs of animals from a wide range of taxa suggests that the organization of IG cells into discoids is a specialization of structure. The degree of development of the IG cells into discoids across several taxa of animals is schematized in Fig. 18. Varying degrees of development exist not only among taxa but within. Amphibians exhibiting rudiments of discoids are observed in some

CHARACTER OF DISCOIDS ACROSS SEVERAL TAXA

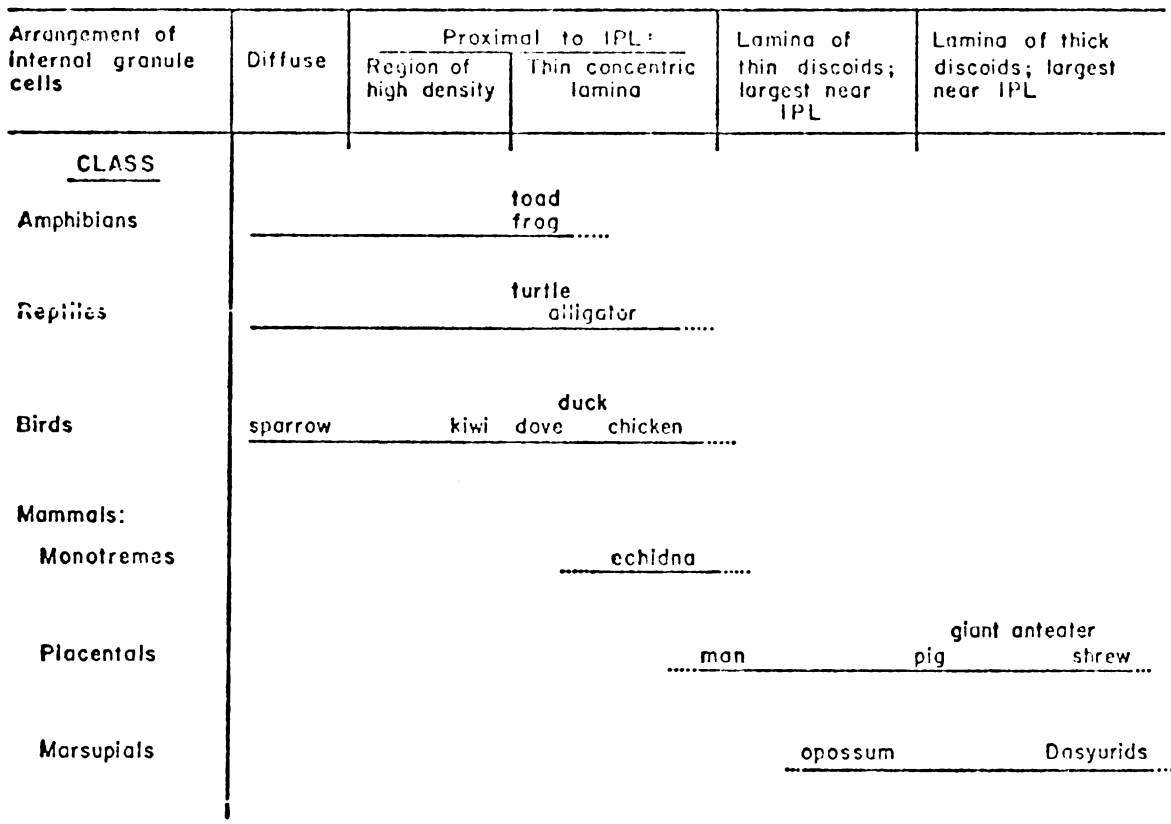

Figure 18. Phylogeny of discoids. The character of discoids across several taxa is depicted here with respect to the degree of aggregation of internal granule cells in terms of distinctness and size. "Thin" discoids are defined as discoids three or less cells in thickness and "thick" discoids are discoids greater than 3 cells in thickness. Descriptions of the layer containing the internal granule cells for some of the animals was obtained from the literature as follows: toad and frog, Hoffman '63; alligator, Crosby '17, '39; turtle, Crosby and Humphrey '39; sparrow, dove, duck and chicken, Huber and Crosby '29, kiwi, Craigie '30. Specimens of echidna and giant anteater were made available by W. I. Welker and shrew by W. L. Weller.

toads (Bufo marinus) and frogs (Rana pipiens) (Hoffman '63). The alligator (Alligator mississipiensis) (Crosby, '17, '39) seems to exhibit the most well developed discoid layer among reptiles. Alligator surpasses most birds in terms of discoid development. The sparrow (Passer domesticus) is well down this scale as is kiwi. Kiwi (Apteryx australis, Craigie, '30) seemingly demonstrates an intermediate stage in the development of discoids. No discoids are apparent in kiwi but the distribution of IG cells nearest the IPL is much denser than those toward the ventricle. From the description of Huber and Crosby ('29), dove (Columba domestica) and duck (Anas domestica) and chicken (Gallus domesticus) show, in order, increasingly more developed discoid layers of birds. Man seems to be better than alligator and more like chicken. Opossum extends the lower range of the marsupials to overlap the placentals so that shrew and pig exhibit more well developed discoid structure according to the headings on the chart in Fig. 18.

The general pattern among the placentals is the for orders, Insectivora, Dermoptera,Chiroptera, Primates, Edentata, Lagomorpha and Rodentia to have more distinctly developed discoids than the orders of Carnivora, Hyracoidea, Perissodactyla and Artiodactyla.

Ontogeny of Discoids

Schultze-Westrum ('69) studied the increasing ability of pouch young of sugar gliders to discriminate among community members by smell. In opossum pouch young, (which spend about the same time in the pouch as sugar gliders), the time course of discoid development appears to parallel the stages of greater discriminatory powers of olfaction in young sugar gliders. This parallel would be well worth detailed study.

Tufted Cells

It is evident from the layer volume determinations that the region occupied by the middle tufted cells is a well defined layer remaining a constant function of the whole bulb volume from Sminthopsis to Sarcophilus. This layer is very distinct among the Dasyurids but not so evident in many other animals. Although very poorly defined in the rat, Valverde ('65) recognized and labeled the region occupied by the middle tufted cells as the layer of tufted cells. The region between the mitral cell layer and the glomerular layer, previously called the external plexiform layer, is divided into two layers: the tufted cell layer and, internal to it, the external plexiform layer.

In some of the animals considered in this study, the middle tufted cells populate their layer so distinctly that previous notions (Crosby and Humphrey, '39; Valverde, '65) that these cells are displaced cells of any type are difficult to maintain.

The work of Price and Powell ('70) on synaptology of olfactory bulb cells indicates that the deep half of what they define as the external plexiform layer is a region of prime synaptic activity between horizontal dendrites of mitral and internal tufted cells and the peripheral processes of the IG cells. The outer half of these external plexiform layer does not get as much mention, undoubtedly because it is not part of the external plexiform layer but the (middle) tufted cell layer. Hence, the external plexiform layer should be defined as the layer where the peripheral processes of the internal granule cells synapse with the horizontal dendrites of the mitral and internal tufted cells.

This definition of the TCL raises the question about where the horizontal dendrites of the middle tufted cells synapse with the peripheral processes of the internal granule cells. Do these dendrites ramain confined to the TCL or do they mingle with the horizontal dendrites of the internal tufted cells and mitral cells? Possibly the horizontal dendrites of the large middle tufted cells do mingle while the smaller middle tufted cells confine their horizontal dendrites to the tufted cell layer. Such an arrangement implies a division of function of the tufted cells, possibly related to the projections of the axons of the various tufted cells.

Lohman and Mentink ('69) found that axons of some of the tufted cells projected to the lateral olfactory tract while others did not. The idea that the large cells either of the internal or middle tufted cell population, or both, may project to the lateral olfactory tract receives support from the elecrophysiological experiments of Nicoll ('70).

Most of the tufted cells from which Nicoll recorded after antidromic stimulation in the lateral olfactory tract of rabbit were within a region about 200 um external to the mitral cell layer. This places these cells within the internal tufted cell population zone or external plexiform layer as redefined above. He also indicates that similar responses were obtained from a few cells nearer the glomerular layer. These probably were the large cells of the middle tufted cell population. Both studies of Nicoll and Lohman were done in rabbits which do not have a well developed middle tufted cell population. This also means there are not likely to be many large cells of that population. It would seem that Nicoll recorded from the large internal tufted cells and, more peripherally, from some of the few large cells of the middle tufted cell population.

One would certainly expect to find more clear-cut results regarding tufted cell size and axon projections if studies similar to those of Nicoll, and Lohman and Mentink were carried out on animals with denser tufted cell layers such as opossum or any of the Dasyurids.

Conclusion

One of the most important things to be gleaned from the quantitative results is that within this family of animals, which covers a large range of olfactory bulb size, certain relationships between olfactory bulb components remain constant. For animals of other families these relationships may not be the same. As was pointed out before, the tufted cell layer seems to vary greatly in the volume it contributes to the bulb. The internal plexiform layer is also seen to vary considerably among other animals, being quite small in some to very wide in others.

The establishment of constancy in the character of structural features permits questions to be posed and hypotheses made about their functional significance. By observing in other groups of animals these same features, in quantified terms, variations or similarities in structure can be established which may reflect differences in function. Not only does knowing the structure enable the electrophysiological data to be interpreted but it enables one to formulate the questions necessary to design the experiments which are to probe the functioning structure.

Some of the questions which have arisen as a result of this study and can form the basis for future work are:

--Do the individual discoids project some sort of discrete inhibitory field to the mitral and tufted cells?

--Given that a discoid inhibitory field exists what is the relationship of the axon collaterals of the mitral and tufted cells to it?

--Are the closely packed internal granule cells of the discoids weakly coupled so as to affect the excitability of neighboring cells?

--Do the short axon cells in the discoid layer have inhibitory control over the cell population within a discoid? and do those short axon cells communicate between or couple discoids?

--Is there a unique area to which the tufted cell axons project and is this area accentuated in size or in definition in animals with high densities of middle tufted cells?

It is evident from this study that future experiments on the olfactory bulb can benefit by using animals in which features of interest in the olfactory bulbs are accentuated, as is the case in the marsupials studied here.

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