

Morphology of Primary Axosomatic Endings in the Anteroventral Cochlear Nucleus of the Cat: A Study of the Endbulbs of Held

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ABSTRACT

The central axons of Type I spiral ganglion neurons travel in the auditory nerve and terminate in the cochlear nucleus. The ascending branches of these axons innervate the anteroventral cochlear nucleus and give rise to large axosomatic endings, called the endbulbs of Held, and smaller boutons. This paper reports a study of the endbulbs of Held, stained by horseradish peroxidase and variants of the Golgi method in kittens 2, 5, 10, 20, and 45 days postnatal and adult cats. Endbulbs tend to fall into two extreme groups with some endbulbs having an intermediate appearance; consequently, we have defined three descriptive stages of endbulbs that are conceived of as representing a developmental sequence. One group of endbulbs is found mostly in kittens younger than 10 days postnatal and is similar to the classic description of endbulbs by Ramón y Cajal ('09). The other extreme group of endbulbs is found mostly in adult cats. In these cases, the parent axonal trunk divides into several thick, gnarled branches that in turn branch again, sometimes repeatedly. These branches display irregular varicosities and form a cup-shaped arborization into which the postsynaptic cell body nestles. A chronology of postnatal endbulb development has been inferred from the relative proportions of the different endbulb stages at various ages. Maturation transforms the endbulb of Held from a large, spoon-shaped swelling having many filipodia into an elaborate tree with broad trunks and many smaller branches. Some implications of the proposed developmental sequence are discussed.

The mammalian auditory nerve is composed of axons whose cell bodies reside in the spiral ganglion of the cochlea. The peripheral extensions of these spiral ganglion neurons terminate on acoustic receptors and the central projections terminate in the cochlear nucleus. By virtue of this arrangement, the recoding and subsequent distribution of primary auditory information to the central nervous system depends to some extent on the anatomical relationships between auditory nerve fibers and cochlear nucleus neurons (Kiang, '75). Concepts of stimulus coding (Kiang, '65; Kiang et al., '65a) have been founded on the electrophysiological response characteristics of single units in the auditory nerve (Kiang et al., '65b) and the cochlear nucleus (Bourk, '76; Godfrey et al., '75a,b; Pfeiffer, '66a; Rose et al., '59) of the adult cat. There is now evidence that immature auditory nerve fibers (Pujol and Hilding, '73; Romand, '79) and cochlear nucleus neurons (Brugge

et al., '78; Pujol, '72; Romand and Marty, '75) exhibit different response characteristics from those of the adult. Such age-dependent changes in electrophysiological response properties imply corresponding morphological changes among the synaptically related neuronal elements. Because most of the literature concerning the central endings of primary auditory neurons is based on Golgi studies of young animals (Brawer and Morest, '75; Feldman and Harrison, '69; Held, 1893; Cohen, '72; Lorente de Nó, '33, '76, '79, '81; Morest, '68; Ramón y Cajal, '09; Tricomi-Allegra, '04; Vincenzi, '01), we decided to reinvestigate these neurons by using methods that are applicable to animals of all ages and by paying particular attention to adults.

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The present study is based on observations made on a series of age-graded kittens and adult cats, whose cochlear nuclei were prepared by using variants of the Golgi method and horseradish peroxidase (HRP) marking techniques. In this paper, we shall report on the largest endings in the cochlear nucleus, the so-called endbulbs of Held, which are found mainly in the anteroventral cochlear nucleus (AVCN), especially its anterior division (AVCNa).

MATERIALS AND METHODS

Subjects

Domestic cats weighing more than 2.0 kg, and hence presumed to be adults, and kittens 2, 5, 10, 20, and 45 days postnatal were used in this study. In cases of small litters (three or fewer kittens) the entire litter was histologically prepared at the same time in an attempt to assess the reliability of the methods and estimate the variability within each age group. For litters of more than three kittens, we distributed the kittens among the age groups so that as far as possible there was one kitten per age group.

Standard histology

Nissl- and protargol-stained sections were prepared for light microscopic examination as follows: The animal was anesthetized with nembutal, ventilated with a mixture of 95% O₂-5% CO₂, and perfused through the heart with 0.12 M phosphate-buffered fixative, pH 7.4, containing 2% glutaraldehyde, 2% paraformaldehyde, and 0.008% calcium chloride. The head was removed and kept in the same fixative overnight at 4°C. The next day, the brain was removed from the skull. The cochlear nucleus was measured for length (anterior tip to posterior pole) to get an additional parameter of relative maturity, and paraffin embedding procedures were initiated. Two sets of serial, alternate sections were prepared in each of the three standard planes; one set was stained with cresyl violet and the other with protargol. Tissue from 2- and 5-day-old kittens was cut at 15- μ m intervals and all the others were cut at 20 μ m.

Golgi preparations

Golgi-stained material was prepared using several variations in techniques. In the "rapid" Golgi procedure, brains were fixed by immersion in a solution of 0.25% osmium tetroxide and 3% potassium dichromate. After 4 days in the fixative, the cochlear nucleus was measured for length and the tissue placed in 0.75% AgNO₃ for 3 days; it was then placed back into fresh fixative for 3 days and finally into 0.75% AgNO₃ for 4 days. Other brains were impregnated by the Golgi-Cox procedure (Van der Loos, '56) and variants of the Golgi-Kopsch method (Adams, '79; Colonnier, '64). Usually, these brains were dehydrated, embedded in parlodion, and sectioned at 100 μ m thickness. In some instances, Golgi-stained tissue was osmicated, embedded in Epon, and cut into 1- μ m thick sections with glass knives.

Horseradish peroxidase marking

Adult cats and kittens of 5, 10, and 45 days of age were anesthetized with I.P. injections of dial-urethane, nembutal, or ketamine; body temperature was monitored with a rectal thermometer and maintained at 37°C with a circulating warm water pad. After cannulation of the trachea, the animal was placed in a headholder; the posterior

fossa was surgically opened from a dorsal approach, and the cerebellum was retracted toward the midline to expose the auditory nerve. A glass micropipette filled with HRP (Sigma type VI) was inserted into the nerve under direct visual control with the aid of an operating microscope. HRP (20–40% w/v) was delivered electrophoretically through the micropipette tip (20 μ m, O.D.). After about 24 hours, each animal was given a lethal dose of nembutal and perfused through the heart with 50 cc of isotonic saline (37°C) with 0.1% NaNO₂ followed by a warm fixative containing 1.25% paraformaldehyde and 2.5% glutaraldehyde (freshly purified) in a 0.12 M phosphate buffer containing 0.008% calcium chloride. Following perfusion and decapitation, the head was immersed in cold fixative (6–18 hours, 4°C) with just enough bone removed to expose the brainstem to the fixative. The brain was dissected from the skull and the cochlear nucleus sectioned coronally or sagittally on a vibratome into 100- μ m-thick sections. These sections were reacted with diaminobenzidine (DAB) according to the procedure of Adams ('77). In most cases, the tissue was counterstained with cresyl violet and coverslipped with Permount for light microscopic analysis. In other cases, sections containing labeled auditory nerve fibers and terminals were postfixed with 2% osmium tetroxide in 0.12M phosphate buffer, stained *en bloc* with 2% uranyl acetate, dehydrated, infiltrated with Epon, and embedded in flat dishes. HRP-filled endings were located, isolated within smaller Epon blocks by razor cuts, and mounted with epoxy onto blank Epon chucks. Thin sections were collected, stained with lead citrate and uranyl acetate, and examined on a JEOL 100S electron microscope.

Data analysis

One brain from each age group, stained by cresyl violet and protargol, was used to prepare a developmental series for the AVCN. Percentage intervals of coronal sections through the cochlear nucleus were calculated by dividing each section number by the total number of sections required to traverse the nucleus. The most anterior section is 100% and the most posterior section is 0%. Subdivisional boundaries of AVCN were determined for each age group according to previously established criteria (Brawer et al., '74; Brawer and Morest, '75) and traced onto the atlas. This allowed the position of each ending to be plotted with respect to subdivisional boundaries on the standard atlas. We have included positional information only for those endings illustrated in Figures 4, 5, 7, 9, 11, and 13. All endings in AVCNa were examined at \times 1,000 magnification (\times 100, oil-immersion, long working-distance, Neofluar objective) and classified either as endbulbs or boutons (see Results).

RESULTS

Ninety percent of the total population of axons in the auditory nerve of adult cats are thick (3–4 μ m diameter) and myelinated and arise from Type I spiral ganglion neurons (Arnesen and Osen, '78; Kiang et al., '82). We have concentrated our analytic efforts on endings from these thick axons and will specifically refer to them as auditory nerve fibers. The remainder of the population are thin, unmyelinated axons plus a few thin but heavily myelinated axons. This smaller population is thought to be composed of Type II spiral ganglion cell axons, postganglionic autonomic fibers, or aberrant efferent fibers (Arnesen and Osen, '78). When HRP is injected into the auditory nerve,

only fibers passing through the injection site are stained; among these labeled fibers, auditory nerve fibers are easily distinguished from the less frequent, fine-caliber axons. Thus, by using serial sections, it is possible to reconstruct individual auditory nerve fibers (and their endings) as they emerge from the auditory nerve root without the confusion of any other labeled neurons. Even in cases where the parent axon was not traced back to the nerve root, axon caliber was sufficient for identification as a primary fiber.

The tracing of auditory nerve fibers in Golgi preparations is much more difficult since many other processes are also impregnated. A particularly vexing problem was differentiating primary axons and their endings from intrinsic axons arising within the cochlear nucleus and axons descending from higher brain centers. In kittens 10 days postnatal and younger, when it was possible to trace individual ascending branches from their point of origin in the auditory nerve root, we could determine that the endbulbs arose without exception from auditory nerve fibers. Myelinated axons failed to impregnate in older animals; thus the Golgi method was inapplicable for fiber tracings. In these cases, we were guided by our findings in HRP material and younger Golgi material for identifying Golgi-impregnated endbulbs. Both methods appear to completely fill the intracellular spaces and give comparable appearances in other situations (Kitai and Bishop, '81; Palay and Chan-Palay, '75).

The AVCN has been defined by Brawer et al. ('74) as the region containing the zone of bifurcation and ascending branches of auditory nerve fibers. When auditory nerve fibers are stained by Golgi (Fig. 1A) or HRP (Fig. 1B) methods, the ascending branches give rise to endings that are either boutons or endbulbs depending on their sizes and shapes. We have defined small endings that measured less than 4 μm along their longest dimension as boutons. Terminal boutons arose from fine collaterals (less than 1 μm in diameter when measured within 5 μm of the ending) and were characterized by a round, oblong, or teardrop-shaped profile with a smooth surface. In animals younger than 10 days postnatal, boutons generally gave rise to one or two threadlike projections. These projections, which are absent in the adult, were less than 10 μm in length and 0.5 μm in diameter. Endings classified as boutons will not be considered further in this paper.

The larger, axosomatic endings called endbulbs of Held were initially identified on the basis of their size. A camera lucida drawing of each endbulb produced a two-dimensional silhouette; a profile of this silhouette was obtained by outlining all parts of the terminal that conformed to the shape of the postsynaptic cell body. A long and a perpendicular short axis, one of which passed through the profiles' center, could then be defined and measured. The short axis of each endbulb always measured greater than 5 μm ; the long axis measured up to 30 μm . A total of 1,303 endbulbs of Held have been examined in this study. Of all the endings, less than 1% had an appearance that could be interpreted as complicated boutons or as small endbulbs; because they were so small and found mainly in the posterior divisions of AVCN (AVCNp), will not consider them as true endbulbs.

Although all endbulbs share the common feature of being large, axosomatic endings, they can vary considerably in shape. Endbulbs tend to fall into two extreme groups with some endbulbs having an intermediate appearance; con-

sequently, we have created three categories of endbulbs. Since the characteristics used for defining endbulb categories show graded rather than abrupt changes in distribution, the boundaries between adjacent categories are not meant to be absolute. Furthermore, the two-dimensional camera lucida drawings and photomicrographs were by themselves inadequate for an accurate portrayal of the three-dimensional shape of endbulbs. Consequently, all staging was determined directly with the aid of the light microscope.

The first group of endbulbs is found in kittens but never in adult cats. These endings consist of a shallow bowl-shaped central core of cytoplasm, called the swelling, and filipodial appendages formed by thin, membranous cytoplasmic extensions from the swelling (Fig. 1A). The other extreme form of endbulb is predominantly found in adult cats. In these cases, the parent axon forms a main trunk from which several additional branches arise. These branches display irregular varicosities and ramify further, forming a cup-shaped arborization that cradles the postsynaptic cell body (Fig. 1B). The terminal branches can be fused together forming a true reticulum. It may be that in adult cats, nonreticulated endbulbs and truly reticulated endbulbs actually represent two distinct classes of endings; nevertheless, we will refer to both types of endbulbs as reticulated endings. Although reticulated endbulbs do not completely enclose the cell body, this arrangement does allow for an extensive area of contact. These adult HRP-stained endbulbs were qualitatively similar in size and shape throughout AVCNa (Fig. 2), but did not correspond in shape to our own (Fig. 1A) or previously published illustrations of the clublike Golgi-stained endbulbs of kittens (Brawer and Morest, '75; Held, 1893; Lorente de N6, '33, '76, '79, '81; Ram6n y Cajal, '09; Vincenzi, '01). Could the discrepancy in endbulb shapes be accounted for entirely by differences in age or could there be some as yet unknown difference in the way that neurons stain with HRP and Golgi methods?

In order to address the possibility of technical differences, we first needed to determine whether the HRP reaction product actually filled the entire terminal ending. Seven adult HRP-stained endbulbs were examined with electron microscopy. Multiple, random sections through each of the endings revealed that the HRP reaction product was contained by the plasma membrane and distributed diffusely throughout the cytoplasm of terminal branches and varicosities, even into very fine (less than 0.07 μm diameter) ramifications (Fig. 3). HRP reaction product was not found inside mitochondria or synaptic vesicles. In no instance where the plasma membrane was clearly defined did we observe cytoplasmic continuity between labeled and unlabeled elements. These electron microscopic observations are consistent with our light microscopic observations of HRP-stained endbulbs in the adult cat.

Endbulb morphology as a function of age

In order to address the problem of how endbulb morphology might change with maturation, we have examined endbulbs from a series of age-graded kittens and adult cats. It is difficult to use the HRP technique on kittens because their survival following surgery is poor. Therefore, we have concentrated our efforts on the analysis of Golgi-impregnated endbulbs. Wherever possible, however, we have supplemented our Golgi observations with HRP data.

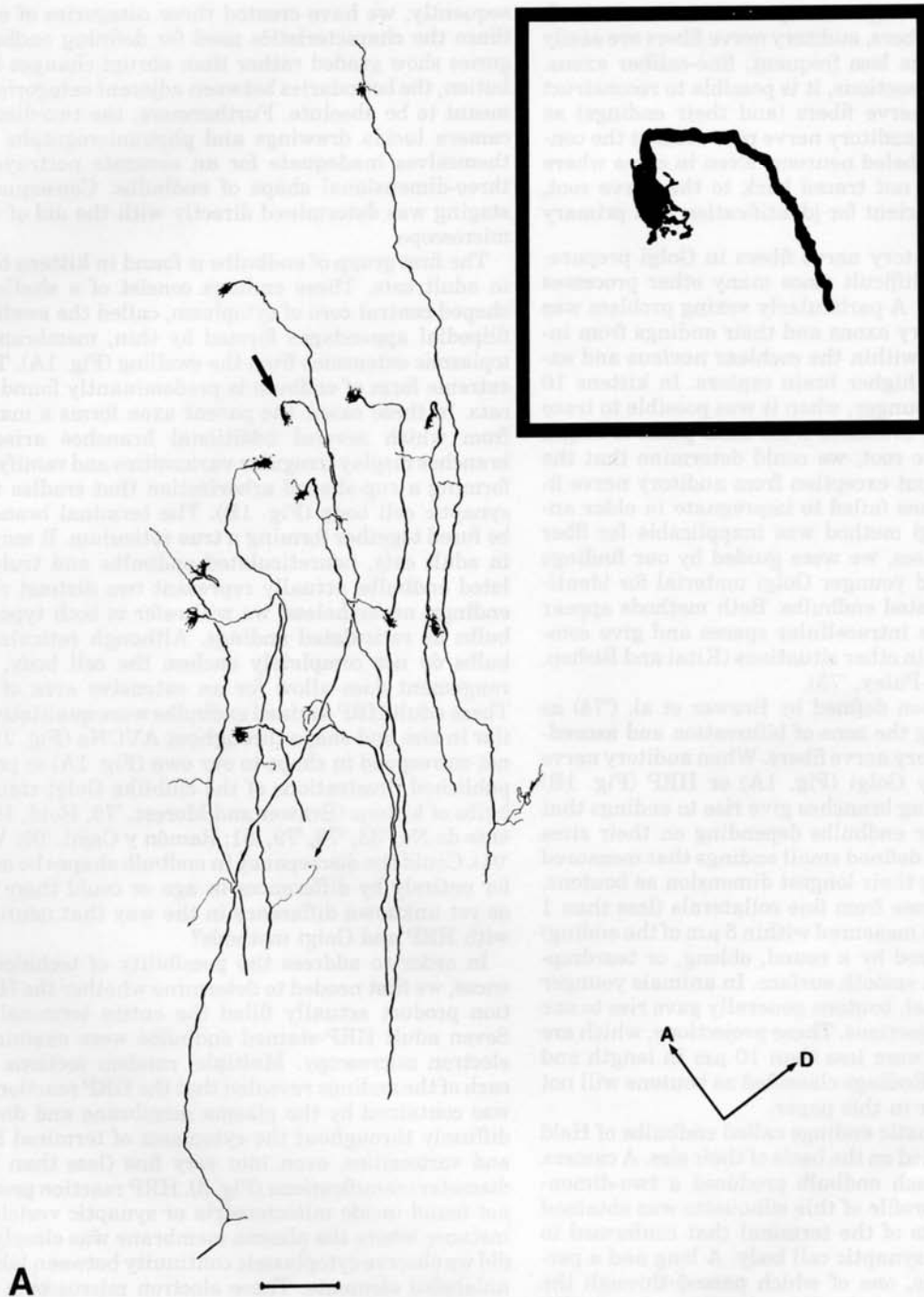


Fig. 1. A. Camera lucida drawing of ascending branches of auditory-nerve fibers in the cochlear nucleus of a 5-day old kitten. Each fiber has been traced from its origin in the nerve root or zone of bifurcation. The 12 large endbulbs of Held can be distinguished from the smaller terminal boutons. A typical endbulb (thick arrow) and bouton (thin arrow) are indicated; the endbulb is also shown at a higher magnification in the inset. Inset: In three dimensions, it is apparent that this endbulb consists of a thin cytoplasmic swelling curved against the postsynaptic cell body and many filipodia emanating from the swelling. It is a Stage I endbulb (see text), characteristic of those found in kittens younger than 10 days of age. Calibration bar equals 100 μm for the main panel and 20 μm for the inset.

A, anterior; D, dorsal. Rapid Golgi procedure. B. Camera lucida drawing of ascending branches of auditory-nerve fibers in the cochlear nucleus of an adult cat. Each fiber has been reconstructed in serial sections from its origin in the nerve root. The fiber on the left gives rise to two endbulbs; the fiber on the right gives rise to three endbulbs. A typical adult endbulb (thick arrow) and bouton (thin arrow) are indicated; the endbulb is also shown at a higher magnification in the inset. Inset: In three dimensions, this endbulb has a cup-shaped terminal arborization that arises from the axonal trunk. It is what we have called a Stage III endbulb (see text). Calibration bar equals 100 μm for the main panel and 20 μm for the inset. Orientation is the same as in Figure 1A. HRP technique.

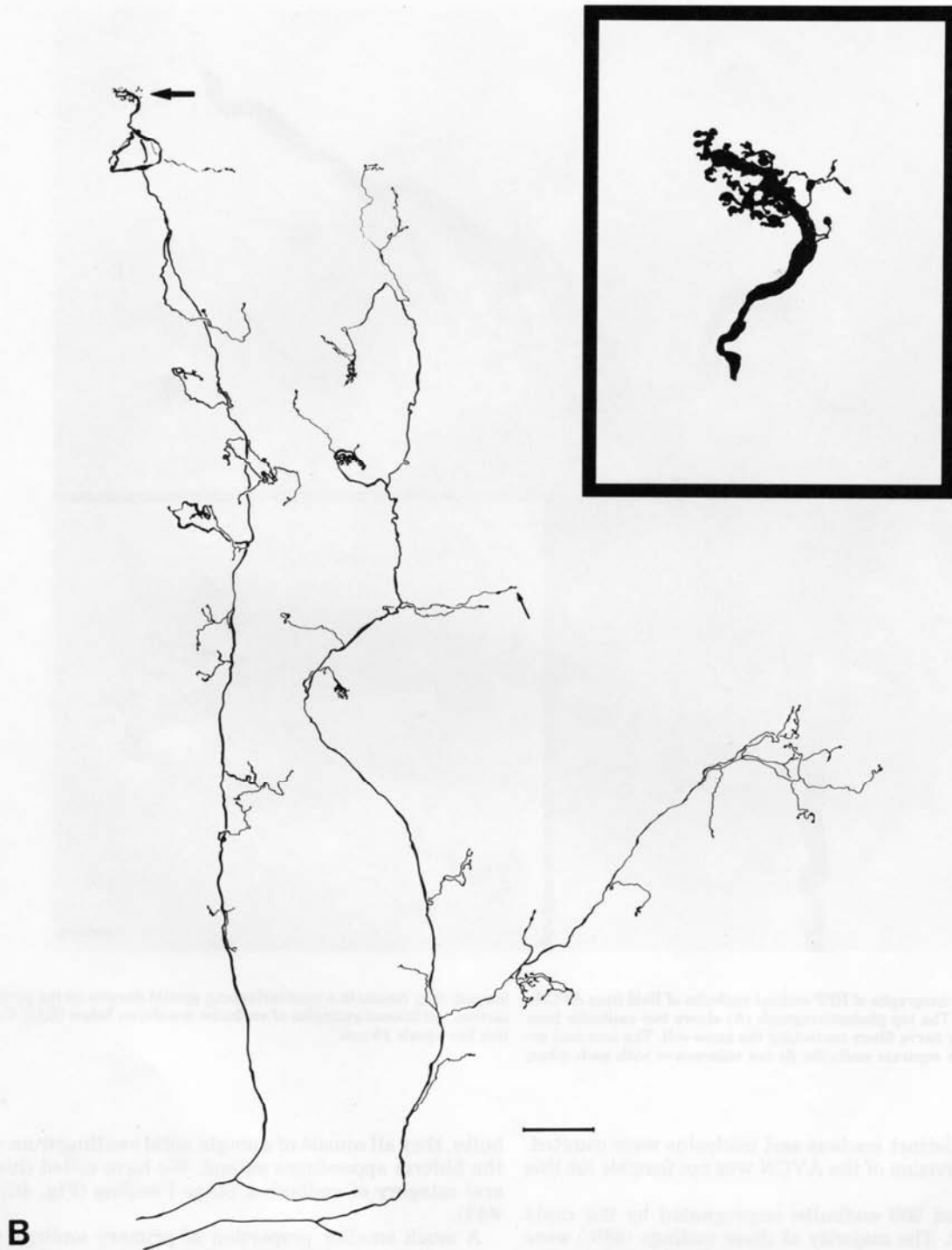


Figure 1 continued

2-day kittens

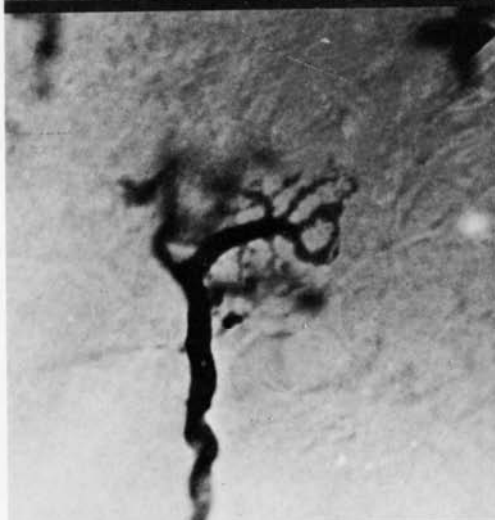
At this age, the cochlear nucleus has a mean longitudinal length of $2.64 \pm .09$ mm ($n = 7$), approximately 50% of the adult length (Fig. 4A). In Nissl preparations, cells of the ventral cochlear nucleus (VCN) are closely packed together; only globular cells and octopus cells have begun to assume their adult characteristics as described by Osen ('69). Nevertheless, based on cell packing density and the

concentration of globular and octopus cells within their presumptive adult positions, we were still able to identify the AVCN-PVCN boundary. The anterior division of the AVCN (AVCNa) was clearly distinct from the posterior division (AVCNp, Fig. 4B); AVCNa had a mean cell packing density of $1,359.4 \pm 152.8$ neurons/mm² while AVCNp had a mean packing density of 855.9 ± 215.3 neurons/mm² and contained identifiable globular cells. Only neu-

A



B



C



Fig. 2. Photomicrographs of HRP-stained endbulbs of Held from AVCN of the adult cat. The top photomicrograph (A) shows two endbulbs from different auditory nerve fibers contacting the same cell. The terminal arborizations of the separate endbulbs do not interweave with each other;

instead, they maintain a nonoverlapping spatial domain on the perikaryal surface. Additional examples of endbulbs are shown below (B,C). Calibration bar equals 10 μm .

rons with a distinct nucleus and nucleolus were counted. Further subdivision of the AVCN was not feasible for this age group.

We analyzed 209 endbulbs impregnated by the rapid Golgi method. The majority of these endings (88%) were similar to the classic description of endbulbs of Held (Ramón y Cajal, '09). They are irregular, spoon-shaped swellings with a variable number of filamentous processes. These processes appear as thin, wavy threads (less than 0.5 μm in diameter), some of which end in a small rounded bead, or as lumpy, undulating projections that spread over the surface of the postsynaptic cell. These latter processes (0.5–1.5 μm in diameter) normally appear as short (less than 10 μm in length) sprouts, although some longer ones trail off to associate with neighboring cells. While there is considerable variability in appearance of these end-

bulbs, they all consist of a single, solid swelling from which the filiform appendages extend. We have called this general category of endbulb a Stage I ending (Fig. 4C, #10, #11).

A much smaller proportion of primary endings (12%) are qualitatively different in their appearance from Stage I endings. In these cases, the terminal swelling is no longer intact; one or several fenestrations and/or fissures are apparent. The fenestrations give the swelling a "perforated spoon" appearance, while the fissures convert the ending into the general shape of a floral calyx, consisting of a variable number of concave, foliate expansions that clasp the postsynaptic cell body. The filamentous processes can still be observed in these endings, but are much reduced in both number and length. We have called this category of endbulb a Stage II ending (Fig. 4C, #9).

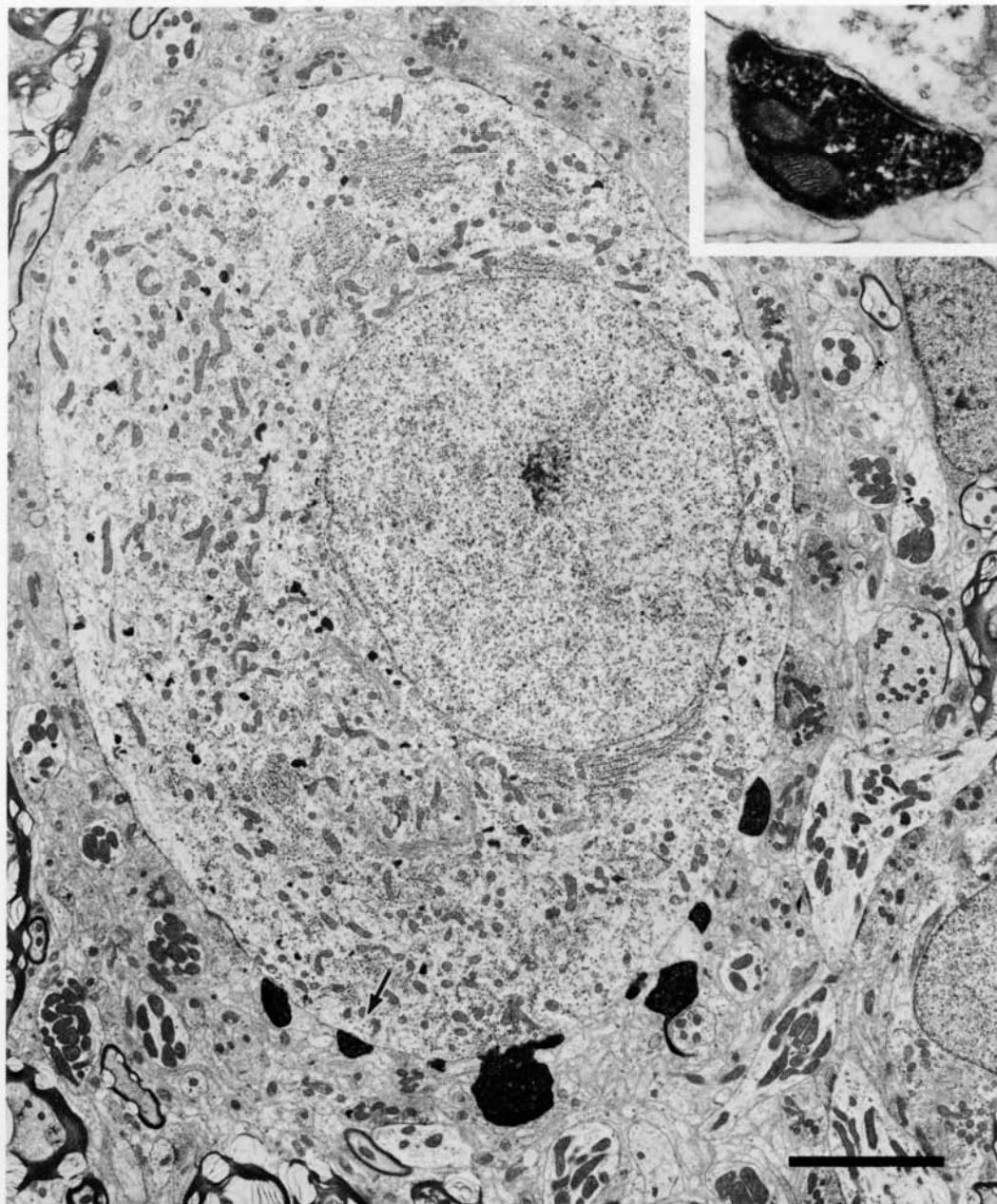


Fig. 3. Electronmicrograph of HRP-stained endbulb of Held in AVCNa of an adult cat. Note how the HRP is diffusely distributed throughout the axoplasm and contained by the plasma membrane. Analysis of random sections through this and other HRP-stained endbulbs suggests complete filling of these endings by the HRP reaction product. The postsynaptic cell body is probably that of a bushy cell according to the criteria of Cant and

Morest ('79). The arrow indicates a boutonlike varicosity which is shown at a higher magnification in the inset. Inset: this terminal appears to be making two synaptic contacts with the cell body. Postsynaptic densities, a narrowing of the intercellular cleft, and the slight convexity of the postsynaptic plasma membrane are characteristic of primary afferent synapses in AVCNa. Calibration bar equals 5 μ m.

5-day kittens

The cochlear nucleus has changed slightly in its gross anatomical appearance from that seen at day 2 (Fig. 5A); its mean longitudinal length is now $3.0 \pm .19$ mm ($n = 7$). In Nissl preparations, the adult subdivisions of AVCNa are now recognizable (Fig. 5B). The anterior part (AA) may be distinguished from the posterior part (AP) by a

distinct transition in cell spacing and cell diameter, where AA has the larger and more closely packed cells. The posterodorsal part (APD) is recognizable by its cell density and cell diameter characteristics which reflect a zone of gradation from AA to AP.

We analyzed 338 endbulbs impregnated by the rapid Golgi and Golgi-Cox techniques (Table 1). As with the 2-

2-DAY

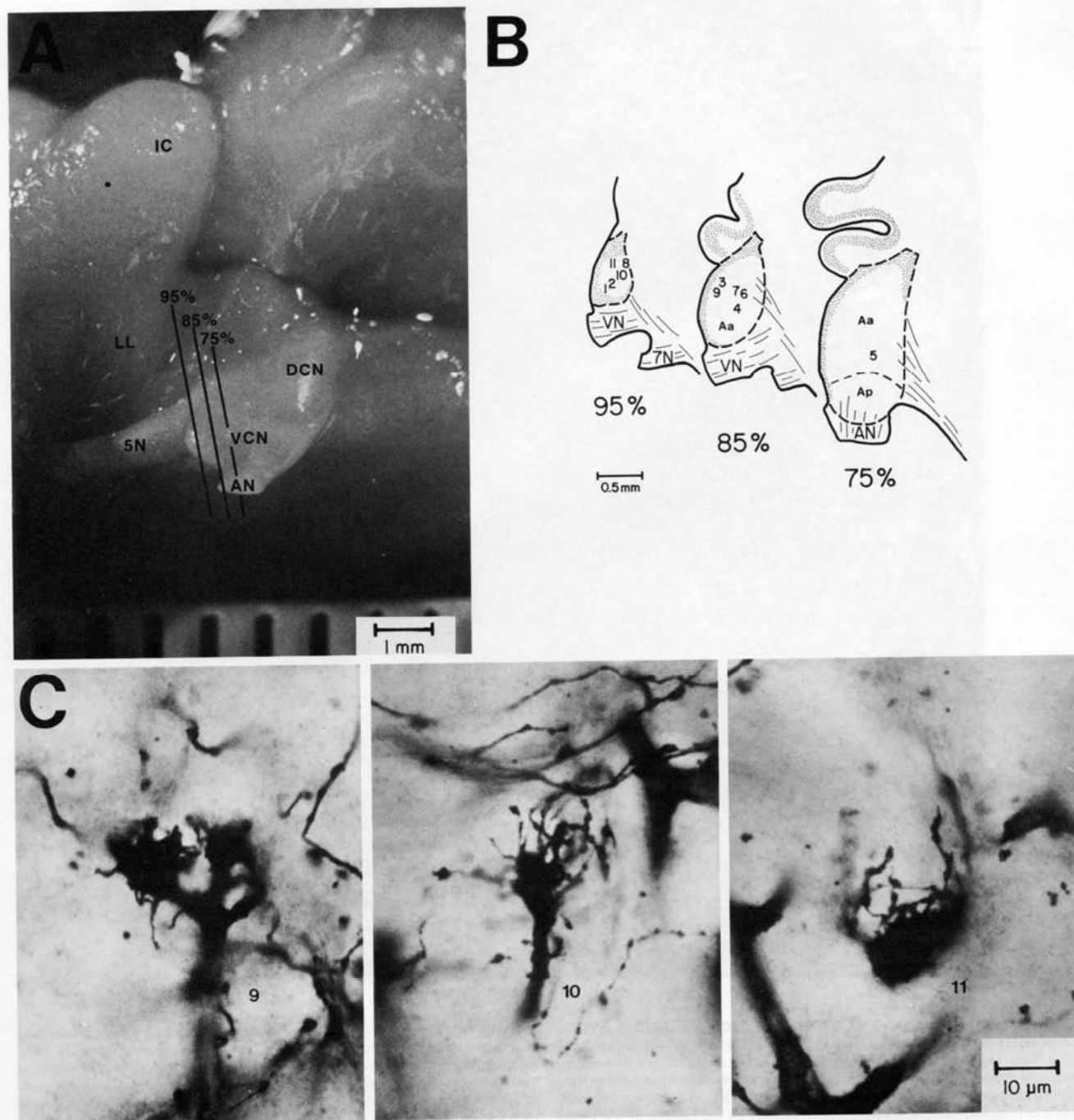


Fig. 4. Endbulbs of Held and their location in 2-day kittens. A. Lateral view of the left cochlear nucleus after part of the overlying cerebellum has been removed. The percentages and vertical lines refer to the corresponding coronal atlas sections presented in B. Anterior is to the left. Abbreviations: AN, auditory nerve; DCN, dorsal cochlear nucleus; IC, inferior colliculus; LL, lateral lemniscus; 5N, trigeminal nerve; VCN, ventral cochlear nucleus. B. Coronal atlas through AVCN indicating subdivisive bounda-

ries and endbulb location. Numbers correspond to the endbulbs in Part C and to the 2-day endbulbs of Figure 13. Abbreviations: Aa, anterior division of AVCN; Ap, posterior division of AVCN; AN, auditory nerve; VN, vestibular nerve; 7N, facial nerve. C. Photomicrographs of Golgi-stained endbulbs whose positions are plotted in B. Endbulbs #10 and #11 are classified as Stage I endings, while endbulb #9 is classified as a Stage II ending.

5-DAY

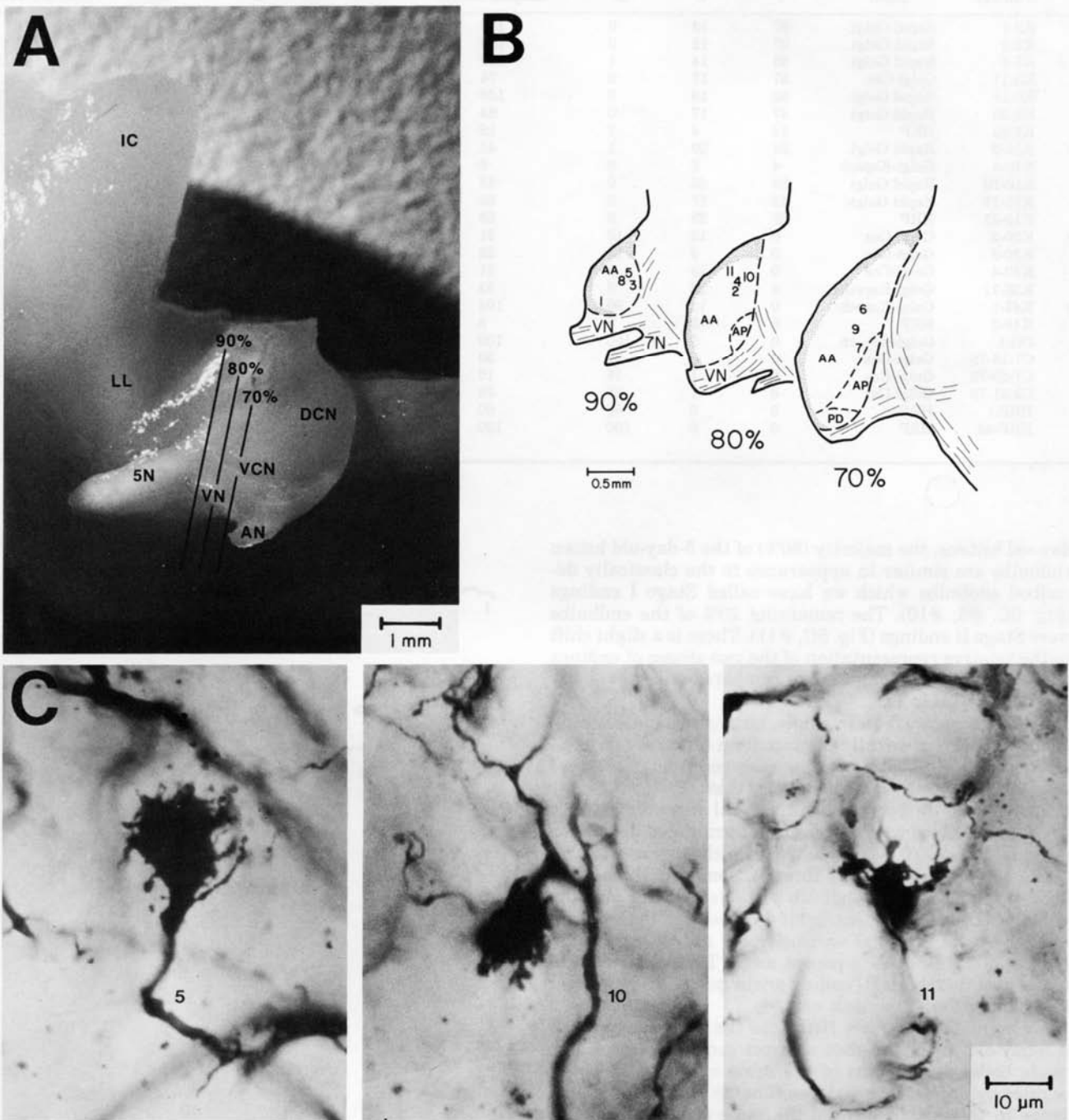


Fig. 5. Endbulbs of Held and their location in 5-day kittens. A. Lateral view of the cochlear nucleus after removal of the cerebellum. The percentages and vertical lines refer to the corresponding atlas sections presented in B. Anterior is to the left. Abbreviations are the same as in Figure 4A. B. Coronal atlas through AVCNa indicating subdivisional boundaries and endbulb number and location. Numbers correspond to the endbulbs in

Part C and to the 5-day endbulbs of Figure 13. Abbreviations: AA, anterior part of anterior division of AVCN; AP, posterior part of anterior division of AVCN; PD, dorsal part of posterior division of AVCN; VN, vestibular nerve; FN, facial nerve. C. Photomicrographs of Golgi-stained endbulbs whose locations are plotted in B. Endbulbs #5 and #10 are Stage I endings while endbulb #11 is a Stage II ending.

TABLE 1. Summary of Endbulb Staging

Age	Animal	Stain	Number of endbulbs at each stage			Number of endbulbs sampled/animal	Percentage of endbulbs at each stage			Total number of endbulbs sampled
			I	II	III		I	II	III	
2-day	K2-1	Rapid Golgi	87	13	0	100				209
	K2-2	Rapid Golgi	97	12	0	109	88%	12%	0%	
5-day	K5-4	Rapid Golgi	85	14	1	100				357
	K5-11	Golgi-Cox	57	17	0	74				
	K5-16	Rapid Golgi	82	18	0	100				
	K5-25	Rapid Golgi	47	17	0	64	80%	20%	<1%	
	K5-33	HRP	14	4	1	19				
10-day	K10-2	Rapid Golgi	24	20	1	45				186
	K10-4	Golgi-Kopsch	4	2	0	6				
	K10-10	Rapid Golgi	28	25	0	53				
	K10-12	Rapid Golgi	12	17	0	29	52%	48%	<1%	
	K10-21	HRP	28	25	0	53				
20-day	K20-2	Golgi-Cox	0	12	19	31				107
	K20-3	Golgi-Cox	0	9	13	22				
	K20-4	Golgi-Cox	0	13	8	21	4%	52%	44%	
	K20-11	Golgi-Kopsch	4	22	7	33				
	K20-11	Golgi-Kopsch	0	14	90	104				
45-day	K45-1	Golgi-Kopsch	0	0	8	8	0%	13%	87%	112
	K45-3	HRP	0	0	100	100				
Adult	CG-1	Golgi-Kopsch	0	0	30	30				332
	C7-18-78	Golgi-Cox	0	1	15	16				
	C7-20-78	Golgi-Cox	0	1	25	26	0%	1%	99%	
	C9-21-78	Golgi-Cox	0	0	60	60				
	HRP-1	HRP	0	0	100	100				
	HRP-48	HRP	0	0	100	100				
										1,303

day-old kittens, the majority (80%) of the 5-day-old kitten endbulbs are similar in appearance to the classically described endbulbs which we have called Stage I endings (Fig. 5C, #5, #10). The remaining 20% of the endbulbs were Stage II endings (Fig. 5C, #11). There is a slight shift in the relative representation of the two stages of endings within the 5-day population as compared with the 2-day population (Table 1).

In one kitten, we found a single, large axosomatic ending that was morphologically distinct from either the Stage I or Stage II endings; this ending emerged from the parent fiber and quickly divided into several thick (2–3 μ m), lumpy branches that in turn divided several more times. The branches, studded with varicosities, terminated in boutons of variable sizes (0.5–3 μ m). The branches were often interconnected by very fine threads, some of which were beaded (Fig. 13, 5-day endbulb #9). We call this endbulb a Stage III ending, identifiable by the fact that the diameter of any terminal varicosity did not exceed the diameter of the endbulb's parent axon. The morphological features of this Stage III ending are in general very similar to endbulbs found in adult cats.

We were able to inject HRP into the auditory nerve of a 5-day-old kitten, so that a direct comparison could be made between endbulbs of the same age that had been stained by different procedures. The 19 HRP-stained endbulbs in this kitten were of the same size and shape as those endbulbs impregnated by Golgi methods (Fig. 6); furthermore, their distribution across stages is similar to that defined by Golgi methods (Table 1).

10-day kittens

The mean length of the cochlear nucleus has increased to $3.56 \pm .15$ mm ($n = 7$, Fig. 7A). It was more difficult to impregnate endbulbs at this age than in younger kittens. Endbulbs were found in fewer kittens prepared by



Fig. 6. Camera lucida drawings of HRP-stained endbulbs from a 5-day kitten. Although these endbulbs were only faintly stained, their morphology is similar to the Golgi-stained endbulbs from kittens of the same age (compare with Fig. 5). Endbulbs #1, 2, 7, 9–15 are Stage I endings; endbulbs #3–6 are Stage II endings; endbulb #8 is a Stage III ending.

10-DAY

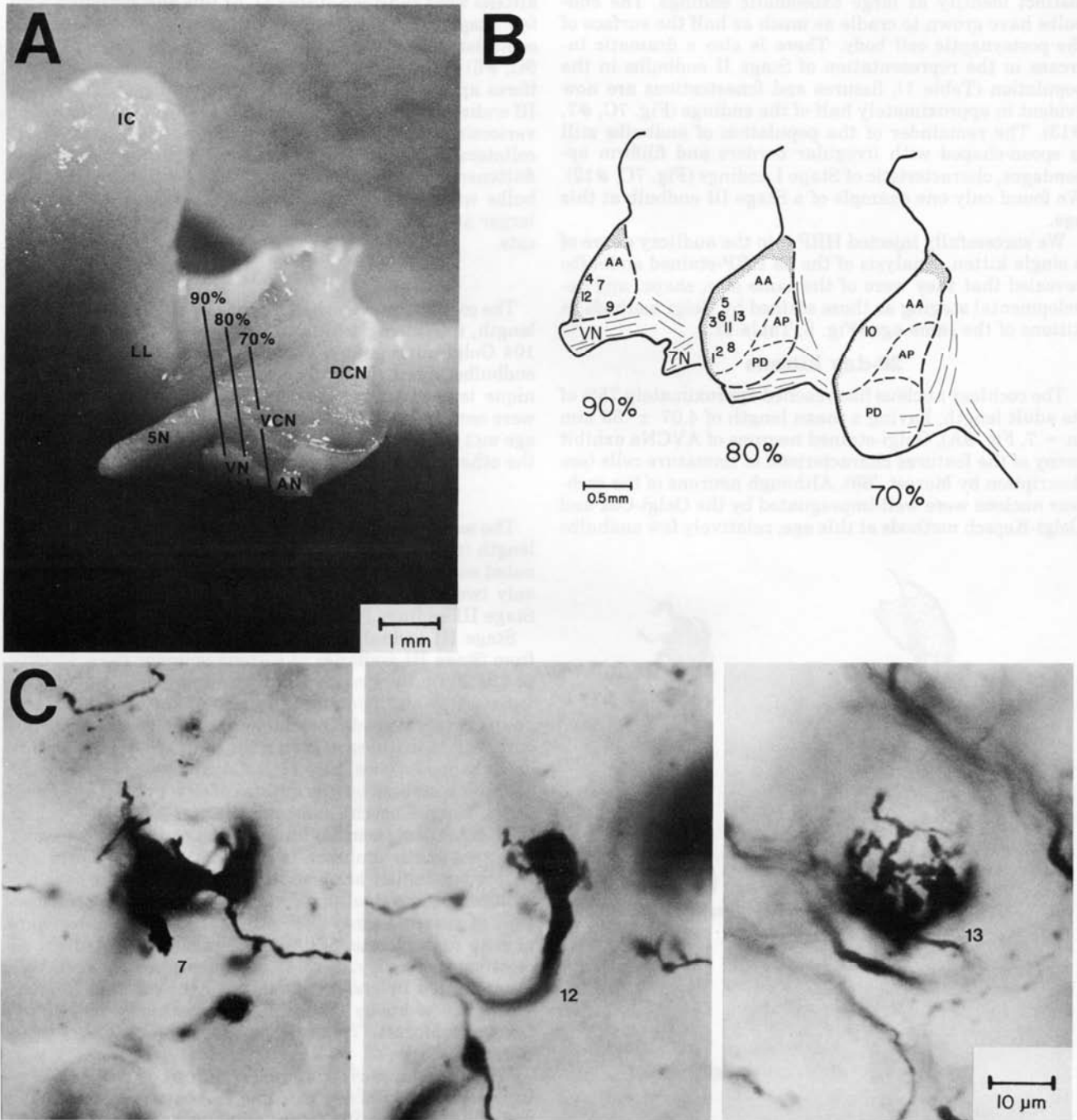


Fig. 7. Endbulbs of Held and their location in 10-day kittens. A. Lateral view of cochlear nucleus after removal of the cerebellum. The percentages and vertical lines refer to the corresponding atlas sections presented in B. Anterior is to the left, abbreviations are the same as in Figure 4A. B. Coronal atlas through AVCNa indicating subdivisional boundaries and

endbulb location. Numbers correspond to the endbulbs in Part C and to 10-day endbulbs in Figure 13. Abbreviations are the same as in Figure 5B. C. Photomicrographs of Golgi-stained endbulbs whose locations are plotted in B. Endbulb #12 is a Stage I ending while endbulbs #7 and #13 are Stage II endings.

Golgi methods, and in those kittens where endbulbs could be found, they were not plentiful. We analyzed 133 Golgi-impregnated endbulbs; the morphological diversity in endbulb form continued to be most impressive, despite their distinct identity as large axosomatic endings. The endbulbs have grown to cradle as much as half the surface of the postsynaptic cell body. There is also a dramatic increase in the representation of Stage II endbulbs in the population (Table 1); fissures and fenestrations are now evident in approximately half of the endings (Fig. 7C, #7, #13). The remainder of the population of endbulbs still is spoon-shaped with irregular borders and filiform appendages, characteristic of Stage I endings (Fig. 7C, #12). We found only one example of a Stage III endbulb at this age.

We successfully injected HRP into the auditory nerve of a single kitten. Analysis of the 53 HRP-stained endbulbs revealed that they were of the same size, shape, and developmental staging as those stained by Golgi methods in kittens of the same age (Fig. 8, Table 1).

20-day kittens

The cochlear nucleus has reached approximately 75% of its adult length, having a mean length of $4.07 \pm .33$ mm ($n = 7$, Fig. 9A). Golgi-stained neurons of AVCNa exhibit many of the features characteristic of immature cells (see description by Morest, '69). Although neurons of the cochlear nucleus were well-impregnated by the Golgi-Cox and Golgi-Kopsch methods at this age, relatively few endbulbs



Fig. 8. Camera lucida drawings of HRP-stained endbulbs from a 10-day kitten. Notice the morphological similarity of these endings to that of Golgi-stained endbulbs from kittens of the same age (compare with Fig. 7). All endbulbs are Stage I except for the two bottom endbulbs in the right column of the figure; they are Stage II endings. Calibration bar equals 20 μ m.

were stained. We were unsuccessful in staining endbulbs with HRP techniques due to the failure of the kittens at this age to survive the procedure.

One hundred and seven endbulbs from four different kittens were analyzed (Table 1). At this age, we found very few Stage I endings; there is approximately equal representation of Stage II (Fig. 9C, #1, #4) and Stage III (Fig. 9C, #6) endings. There is also a distinct reduction of filiform appendages in endbulbs of both stages. The Stage III endings exhibit several main branches with prominent varicosities (2–5 μ m in diameter) and often emit short collaterals terminating in boutons. The varicosities are flattened against the postsynaptic cells and endow the endbulbs with a "leafy" appearance. These varicosities are larger and fewer in number than those observed in adult cats.

45-day kittens

The cochlear nucleus has reached nearly 85% of its adult length, measuring $4.57 \pm .12$ mm ($n = 3$). We analyzed 104 Golgi-impregnated endbulbs and eight HRP-stained endbulbs; again, endbulb appearance with either technique is essentially identical (Fig. 10). Stage I endings were not observed. Nearly all (87%) of the endbulbs at this age met the criteria for classification as Stage III endings; the others were classified as Stage II endings (Table 1).

Adult cats

The adult cochlear nucleus measures $5.20 \pm .25$ mm in length ($n = 7$, Fig. 11A). We analyzed 172 Golgi-impregnated endbulbs and 160 HRP-stained endbulbs. We found only two examples of Stage II endbulbs; all others were Stage III endings (Table 1).

Stage III endbulbs of adult cats may be distinguished from Stage III endbulbs of kittens younger than 45 days postnatal by the presence of more numerous and smaller varicosities and boutons. The size of the varicosities in adults rarely exceeds the diameter of the parent branches of the endbulb, thereby giving the mature endbulb a more delicate appearance (Fig. 11C). Regardless of whether the endbulb is stained by precipitates of silver chromate (rapid Golgi, Golgi-Kopsch), mercuric chromate (Golgi-Cox), or HRP-DAB, the essential image of the endbulb is constant: The axosomatic embrace is formed by several main and numerous smaller branches that are often linked together by a complex reticulum of very fine strands and varicosities of assorted sizes (Fig. 12). In many cases, we were able to identify the postsynaptic neurons with light or electron microscopy. In all of these instances, the cell bodies contacted by endbulbs exhibited the cytological characteristics of bushy (or large spherical) cells (Osen, '69; Cant and Morest, '79), and they could be oval as well as round in shape.

We have summarized our observations on endbulb staging as a function of age in Table 1. At every age sampled, endbulbs representing more than one stage may be found in the population. There is, however, a progressive and predictable shift in the morphological appearance of the endbulb population with age which we have depicted in Figure 13.

DISCUSSION

On the basis of Golgi and HRP data from cats, we have been able to relate the size, location, and morphology of endbulbs of Held with age. The size or stage of an endbulb's

20-DAY

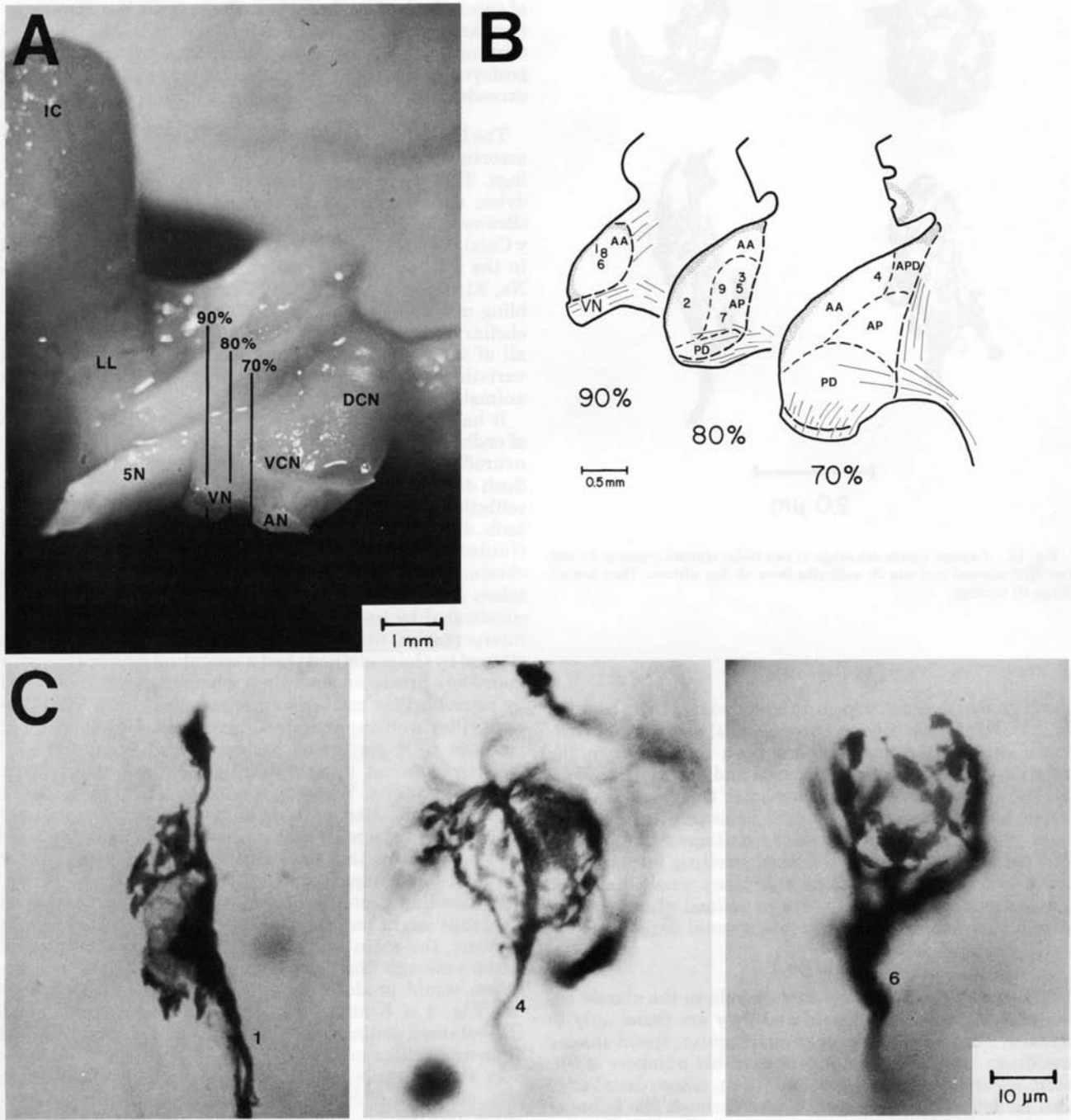


Fig. 9. Endbulbs of Held and their location in 20-day kittens. A. Lateral view of the cochlear nucleus after removal of the cerebellum. The percentages and vertical lines refer to the corresponding atlas sections presented in B. Abbreviations are the same as in Figure 4A. Anterior is to the left. B. Coronal atlas through AVCNa indicating subdivisual boundaries and endbulb location. Numbers correspond to the endbulbs in Part

C and to 20-day endbulbs in Figure 13. Abbreviations: APD, posterodorsal part of anterior division of AVCN; others are the same as in Figure 5B. C. Photomicrographs of Golgi-stained endbulbs whose positions are plotted in B. Endbulb #6 is a Stage III ending while endbulbs #1 and #4 are Stage II endings.

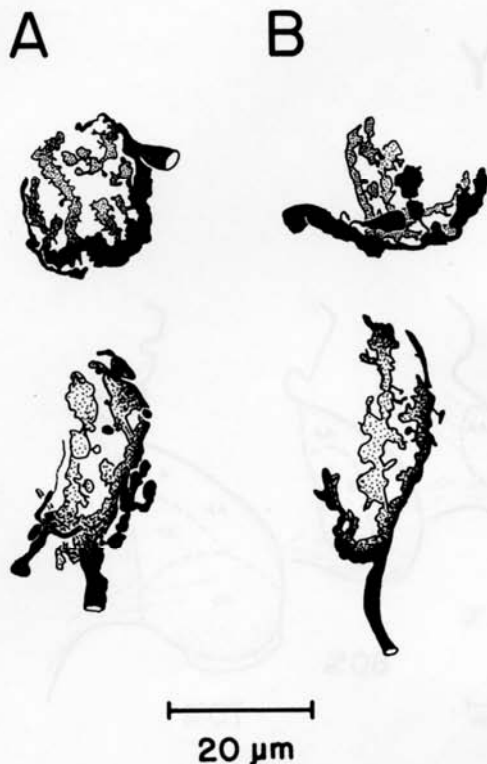


Fig. 10. Camera lucida drawings of two Golgi-stained (column A) and two HRP-stained (column B) endbulbs from 45-day kittens. They are all Stage III endings.

development does not appear to be correlated to its location in AVCNa. In addition, a chronological sequence of post-natal endbulb development has been inferred from the relative proportions of the different endbulb stages of various ages. A dynamic interpretation of this sequence for a single endbulb is proposed on the basis of the population data (Fig. 14). Maturation has transformed the endbulb of Held from a large spoon-shaped swelling into an elaborate tree with broad trunks and many small branches. Three descriptive categories can be defined which are conceived of as representing a developmental sequence.

Stage I

This category of endings corresponds to the classic description of endbulbs of Held and they are found only in kittens. These endings appear as irregular, spoon-shaped swellings from which may arise variable numbers of filiform appendages. The typical swelling, whose distal edge can be defined by extending a line through the bases of the filiform projections, is free of fenestrations or fissures.

Stage II

This category of ending is considered a transitional form between Stage I and Stage III, since it shares some morphological features with each. These endings each exhibit a terminal swelling with either fenestrations, fissures, or both.

Stage III

This category of ending is found mostly in adult cats and is characterized by a highly branched terminal arborization. The axonal trunk of each endbulb divides into several major branches which in turn generate a number of successively finer branches. These branches display irregular varicosities and are frequently linked together forming a delicate reticulum which appears to enclose the postsynaptic cell body. The width of the varicosities rarely exceeds the diameter of the major branches of the endbulb.

The literature on the endbulbs of Held presents a motley assortment of descriptions for these large, axosomatic endings. They have been variously described as bulbs, condyles, or irregular clubs bearing numerous appendices (Brawer and Morest, '75; Held, 1893; Morest, '68; Ramón y Cajal, '09), pericellular capsules like the chalices of Held in the medial nucleus of the trapezoid body (Lorente de Nó, '81; Tricomi-Allegra, '04; Vincenzi, '01), and as resembling motor end plates, cerebellar basket endings, or cerebellar mossy fibers (Held, 1893). On the basis of our data, all of these descriptions may well be accurate with the variations attributable to differences in the ages of the animals examined.

It has been known for some time that the appearance of endbulbs varies greatly depending on whether Golgi or neurofibrillar methods are used in their demonstration. Such differences were reconciled by interpreting the neurofibrils as constituting an internal skeleton of the endbulb, distinct from the surrounding cytoplasm (Gray and Guillery, '66). Consequently, comparison of the pictures obtained with the Golgi and neurofibrillar methods was taken as proof that the principal mass of the endbulbs is constituted by neuroplasm and not by neurofibrils. This interpretation fits with the observation that endbulbs stained by Golgi methods had a spoonlike appearance with many fine processes emerging, whereas endbulbs stained by neurofibrillar methods appeared like claws whose interfibrillar webbing remained unstained. However, Golgi methods were performed exclusively on immature animals, in contrast to neurofibrillar methods which were applied to adults. We were unable to visualize endbulbs using neurofibrillar methods on tissue from cats younger than 10 days of age. If such stains operate by condensing fibrillar proteins into neurofibrils that are visible under the light microscope, then the presumed diffusely distributed fibrillar proteins of the immature, spoon-shaped swellings might not coalesce into a recognizable form. In contrast, the main branches of the adult endbulb would contain enough fibrillar proteins such that their condensation would produce the familiar clawlike appearance (see Fig. 2 of Kiang et al., '65a). In this manner, adult HRP-stained endings can resemble adult endings stained by neurofibrillar methods.

In electron microscopic material prepared from adult cats, endbulbs of Held form multiple asymmetrical synapses with bushy cells of AVCN (Cant and Morest, '79; Ibata and Pappas, '76; Lenn and Reese, '66). Almost certainly, the anatomically defined bushy cells correspond to the electrophysiologically defined primary-like units in AVCN as determined by extracellular recording techniques (Bourk, '76). Primary-like units exhibit a complex wave form that is characterized by an initial positive potential (termed the prepotential) attributable to the endbulb, followed by a larger, negative potential representing

ADULT

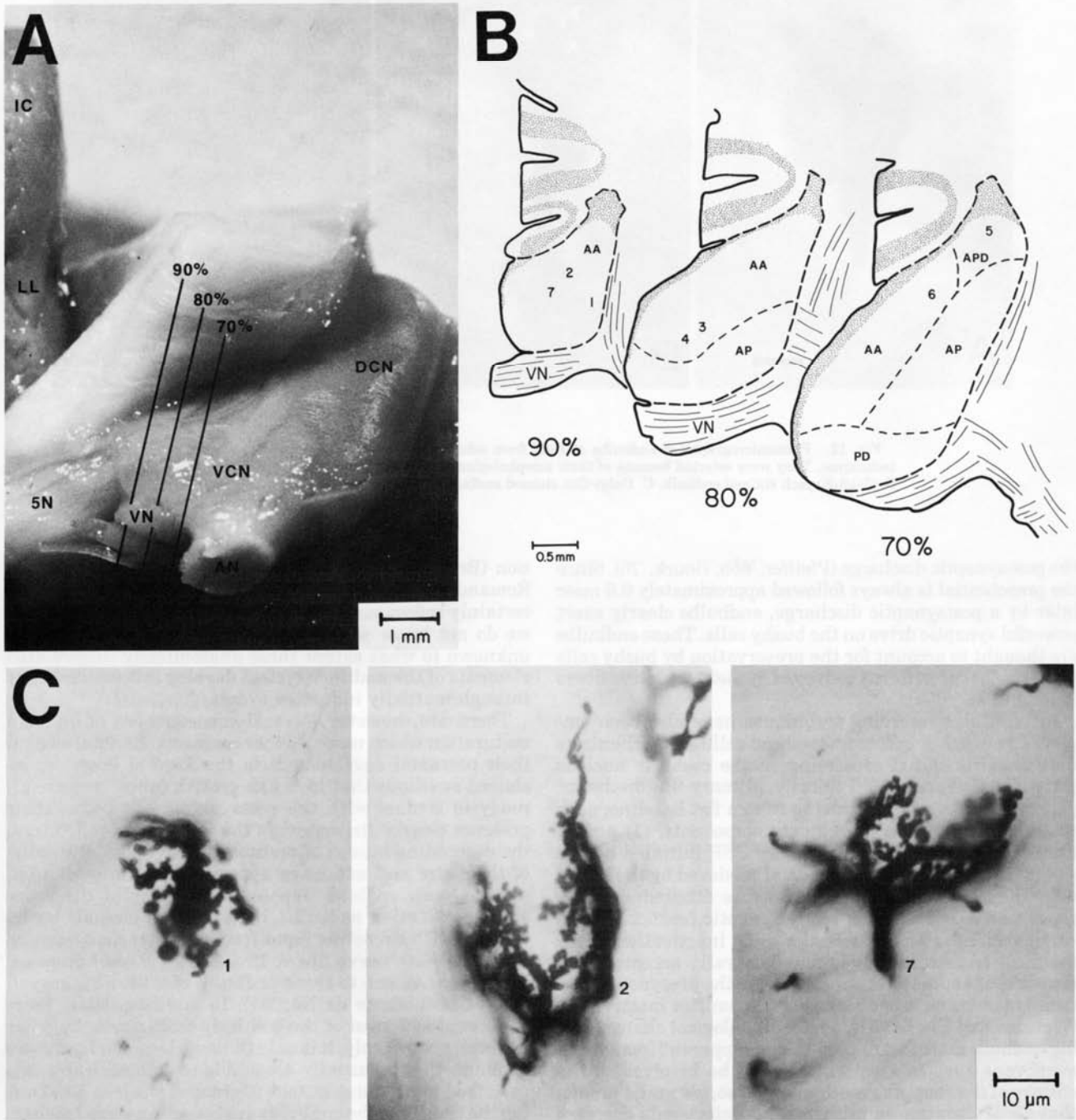


Fig. 11. Endbulbs of Held and their location in the adult cat. A. Lateral view of the cochlear nucleus after removal of the cerebellum. The percentages and vertical lines refer to the corresponding atlas sections presented in B. Anterior is to the left; abbreviations are the same as in Figure 4A. B. Coronal atlas through AVCNa indicating subdivisional boundaries and endbulb number and location. These sections correspond approxi-

mately to T-60 (90%), T-54 (80%), and T-47 (70%) of the block model for the cat cochlear nucleus (Kiang et al., '75). Numbers refer to the endbulbs in Part C and to the adult endbulbs in Figure 13. Abbreviations are the same as in Figure 9B. C. Photomicrographs of Golgi-stained endbulbs whose positions are plotted in B. These are stage III endings.

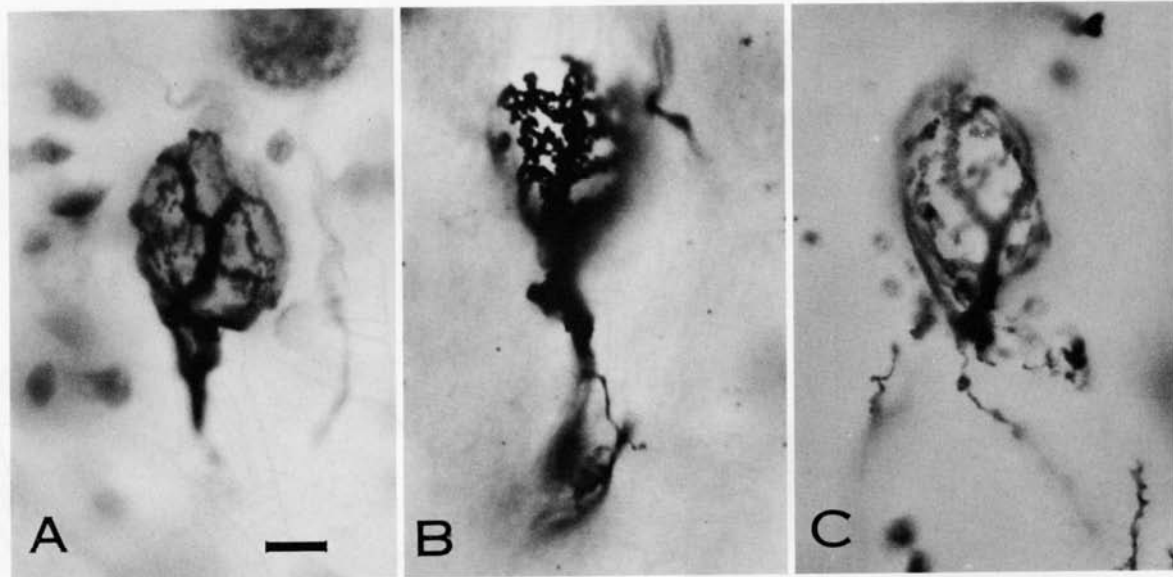


Fig. 12. Photomicrographs of endbulbs of Held from adult cats prepared by different staining techniques. They were selected because of their morphological similarity. A. HRP-stained endbulb. B. Golgi-Kopsch stained endbulb. C. Golgi-Cox stained endbulb. Scale bar equals 10 μ m.

the postsynaptic discharge (Pfeiffer, '66b; Bourk, '76). Since the prepotential is always followed approximately 0.6 msec later by a postsynaptic discharge, endbulbs clearly exert powerful synaptic drive on the bushy cells. These endbulbs are thought to account for the preservation by bushy cells of the discharge patterns conveyed by auditory nerve fibers (Kiang et al., '65a).

Intracellular recording techniques have also been employed in order to better understand cellular mechanisms that underlie signal processing in the cochlear nucleus (Britt, '76; Romand, '78). Typically, primary-like discharge patterns consist of spikes rising from a flat baseline; each spike consists of two prominent components: (1) a very brief (1–2 msec) and rather large EPSP initiated by the endbulb and (2) an action potential produced by the bushy cell. The brevity of the EPSP may be attributed to the synchronous activation of many synaptic contacts from a single endbulb coupled with the rapid inactivation of the chemical transmitter. It is now generally accepted that reuptake of neuroactive substances by the presynaptic terminal membrane is one means of transmitter inactivation (Watkins and Evans, '81). The morphological change during endbulb maturation produces an apparent increase in membrane surface area which could be involved in the efficacy of the reuptake mechanism. If so, we would predict that EPSP duration in primary-like units would decrease significantly during maturation.

During the first postnatal weeks of a cat's life, dramatic maturational changes have been observed in the morphology of the external ear (Villablanca and Olmstead, '79), middle ear (see discussion, Brugge et al., '78), organ of Corti (Pujol and Marty, '70; Pujol et al., '78, '79), and spiral ganglion and auditory nerve (Romand et al., '76, '80; Romand and Romand, '82). The concurrent manifestation of electrophysiological indices of auditory maturation

(Brugge et al., '78; Carlier et al., '79; Romand, '79; Romand and Dauzat, '81; Romand and Marty, '75) must certainly reflect some of these morphological changes, but we do not know which ones or why. Furthermore, it is unknown to what extent these anatomically interrelated elements of the auditory system develop independently or through mutually inductive events.

There are, however, several consequences of endbulb maturation which merit further comment. Endbulbs begin their postnatal development in the form of large spoon-shaped swellings that look like growth cones, and are already in contact with the postsynaptic cell body. Their presence clearly distinguishes the ascending branch from the descending branch of auditory nerve fibers. By virtue of their size and extensive apposition to the perikaryal surface, each endbulb imposes limitations of the space available to other endbulbs, insuring that a single bushy cell in AVCNa receives input from only a restricted number of auditory nerve fibers. In fact, it has been proposed that as few as one to three endbulbs contact a single cell in AVCN (Lorente de N6, '81). In addition, these large endings shield most of the cell body from access by other endings; presumably, it is only the zone between immature endbulbs that is initially accessible to noncochlear axons described in neonatal kittens (Cant and Morest, '78). During the time that the endbulbs are becoming more treelike, we would expect that inputs to bushy cells from nonprimary neurons are also developing; such endings could then reach the bushy cells through the reticulations.

Endbulbs in the adult cat typically appear as perisomatic arborizations. There appears to be spatial segregation of primary inputs onto bushy cells in that we never observed interweaving of endbulb processes from different fibers. Presumably, this segregation is a reflection of the nonoverlapping contacts initially made by the spoon-shaped

SEQUENTIAL STAGES OF POSTNATAL ENDBULB DEVELOPMENT

I

Solid ending with filipodia

II

Fenestrated ending with at least one broad, solid region

III

Reticulated ending

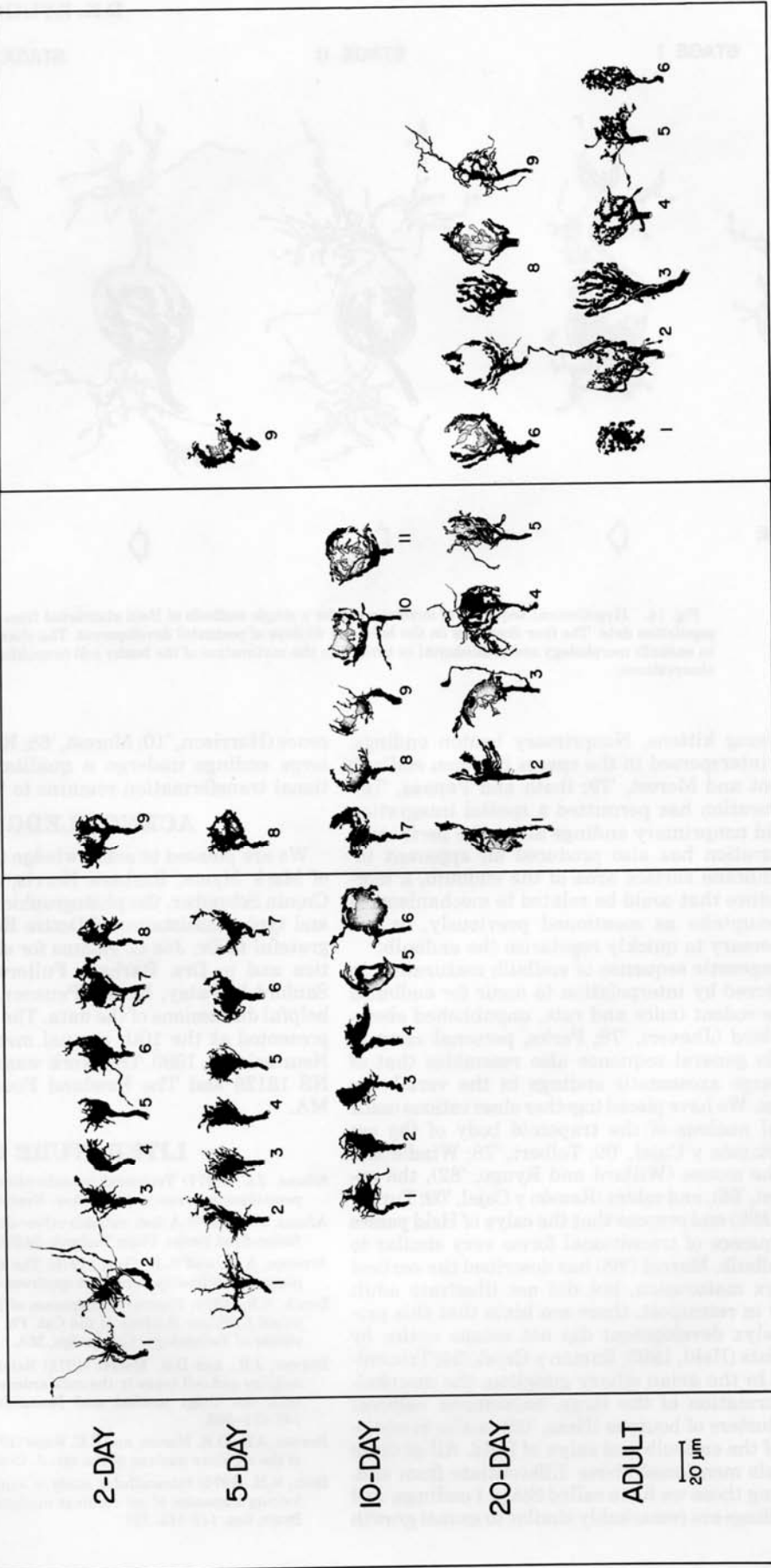


Fig. 13. Summary diagram for the proposed sequential stages of postnatal endbulb development in cats. The endbulbs are illustrated by camera lucida drawings from kittens (2, 5, 10 and 20 days of age) and adult cats. In each horizontal row, endbulbs are presented according to their proportional representation within the population and arranged according to our interpretation of their relative maturity. Except for the adult cat, the "immature" endbulbs appear on the left of each row while the progressively more "mature" endbulbs appear on the right. Endbulbs have been chosen to illustrate the range of morphological variation both within and across ages. The number under each endbulb has been plotted on their respective atlases (Part B, Figs. 4, 5, 7, 9, and 11). Endbulb #9 of the 5-day kitten is the only observation of a Stage III ending at this age. Endbulb #8 of the 20-day kitten is shown in two separate planes of focus to emphasize the complexity of some Stage III endings.

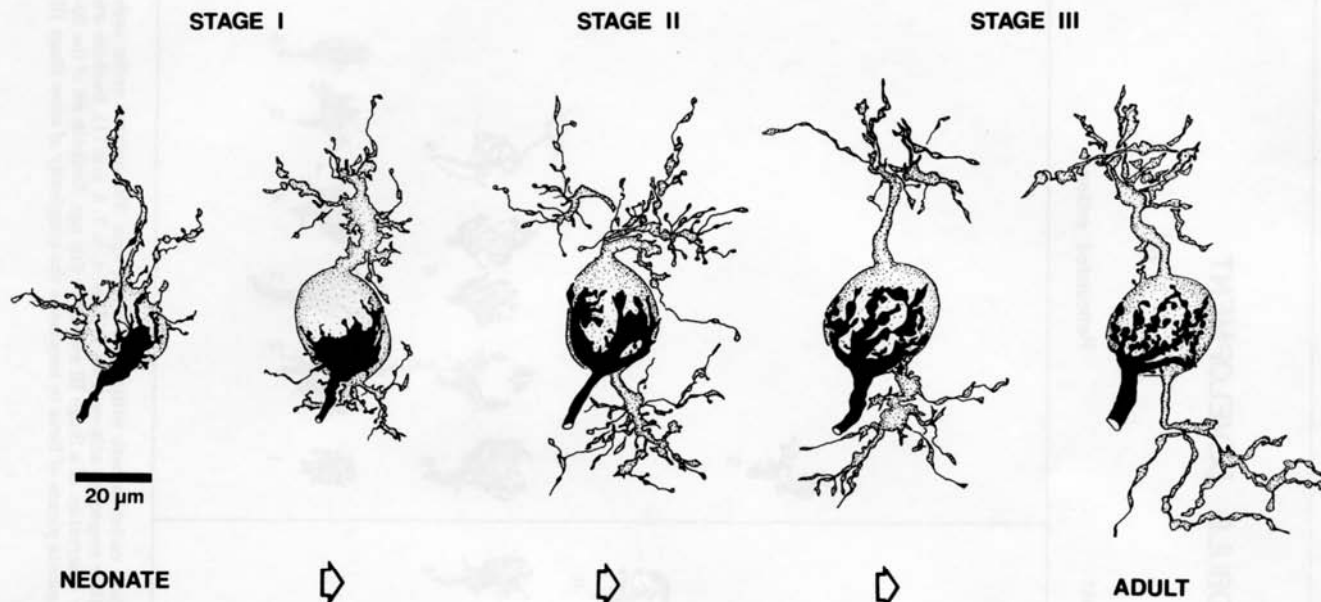


Fig. 14. Hypothetical sequence of development for a single endbulb of Held abstracted from the population data. The four drawings on the left span 45 days of postnatal development. The changes in endbulb morphology are coincidental in time with the maturation of the bushy cell (unpublished observations).

endings in young kittens. Nonprimary bouton endings, however, are interspersed in the spaces between endbulb branches (Cant and Morest, '79; Ibata and Pappas, '76). Endbulb maturation has permitted a spatial integration of primary and nonprimary endings along the perikaryal surface. Maturation has also produced an apparent increase in membrane surface area of the endbulb, a morphological feature that could be related to mechanisms of transmitter reuptake as mentioned previously, or ion transport necessary to quickly repolarize the endbulb.

The morphogenetic sequence of endbulb maturation in the cat is inferred by interpolation to occur for endbulbs of Held in the rodent (mice and rats, unpublished observations) and bird (Jhaveri, '78; Parks, personal communication). This general sequence also resembles that of some other large axosomatic endings in the vertebrate nervous system. We have pieced together observations made on the medial nucleus of the trapezoid body of the cat (Held, 1893; Ramón y Cajal, '09; Tolbert, '78; Windle and Clark, '28), the mouse (Willard and Ryugo, '82), the opossum (Morest, '68), and rabbit (Ramón y Cajal, '09; Turner and Hunter, 1899) and propose that the calyx of Held passes through a sequence of transitional forms very similar to that of the endbulb. Morest ('68) has described the earliest stages of calyx maturation, but did not illustrate adult endings. Now in retrospect, there are hints that this progression of calyx development did not escape notice by early anatomists (Held, 1893; Ramón y Cajal, '54; Tricomi-Allegra, '04). In the avian ciliary ganglion, the morphological transformation of the large, axosomatic calyceal ending into clusters of boutons (Hess, '65) is also reminiscent to that of the endbulb and calyx of Held. All of these large terminals mentioned above differentiate from endings resembling those we have called Stage I endings, and the Stage I endings are remarkably similar to axonal growth

cones (Harrison, '10; Morest, '68; Ramón y Cajal, '60). Why large endings undergo a qualitatively similar maturational transformation remains to be determined.

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