Afferent Innervation of the Utricular Macula in Pigeons

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Si, Xiaohong, Mridha Md. Zakir, and J. David Dickman. Afferent innervation of the utricular macula in pigeons. J Neurophysiol 89: 1660–1677, 2003; 10.1152/jn.00690.2002. Biotinylated dextran amine (BDA) was used to retrogradely label afferents innervating the utricular macula in adult pigeons. The pigeon utricular macula consists of a large rectangular-shaped neuroepithelium with a dorsally curved anterior edge and an extended medioposterior tail. The macula could be demarcated into several regions based on cytoarchitectural differences. The striola occupied 30% of the macula and contained a large density of type I hair cells with fewer type II hair cells. Medial and lateral extrastriola zones were located outside the striola and contained only type II hair cells. A six- to eight-cell-wide band of type II hair cells existed near the center of the striola. The reversal line marked by the morphological polarization of hair cells coursed throughout the epithelium, near the peripheral margin, and through the center of the type II band. Calyx afferents innervated type I hair cells with calyceal terminals that contained between 2 and 15 receptor cells. Calyx afferents were located only in the striola region, exclusive of the type II band, had small total fiber innervation areas and low innervation densities. Dimorph afferents innervated both type I and type II hair cells with calyceal and bouton terminals and were primarily located in the striola region. Dimorph afferents had smaller calyceal terminals with few type I hair cells, extended fiber branches withbouton terminals and larger innervation areas. Bouton afferents innervated only type II hair cells in the extrastriola and type II band regions. Bouton afferents innervating the type II band had smaller terminal fields with fewer bouton terminals and smaller innervation areas than fibers located in the extrastriolar zones. Bouton afferents had the most bouton terminals on the longest fibers, the largest innervation areas with the highest innervation densities of all afferents. Among all afferents, smaller terminal innervation fields were observed in the striola and large fields were located in the extrastriola. The cellular organization and innervation patterns of the utricular maculae in birds appear to represent an organ in adaptive evolution, different from that observed for amphibians or mammals.

INTRODUCTION

The vestibular otolith system functions to detect translational motion and position of the head with respect to gravity. In birds, the otolith system is extensive, with three different endorgans including the utricular, the saccular, and the lagena maculae. Since Retzius (1884), Ramon y Cajal (1909) and Lorente de No (1926) first described differences in afferent fiber size, as well as differences in each fibers terminal field in the sensory epithelia of the maculae, questions concerning the functional utility of the observed morphological variations have been posed. Anatomical descriptions have shown that receptor hair cells differ in structure and number throughout the maculae but are organized with a common plan that spans several animal classes. For example, all vertebrate utricular maculae have a specialized region: the striola, which differs in receptor cell density and otoconial formation (Werner 1933). In the approximate center of the striola lies an imaginary line, demarcated by the opposing reversal of the hair cell’s morphological polarization (Flock 1964; Lindeman 1969). The striola typically runs longitudinally through the neuroepithelium; however, its exact shape and position differs among species. In fish (Platt 1975; Popper and Northcutt 1983), amphibians (Baird and Schuff 1994), and birds (Jørgensen and Andersen 1973), the utricular striola is eccentrically placed near the lateral border of the macula, whereas in mammals (Flock 1964; Fernandez et al. 1990), it lies more medially toward the center of the macula. In chinchillas, a utricular striola was described that comprised 10% of the surface epithelium and divided the rest of the macula into a medial extrastriola (40%) and a lateral extrastriola (50%) (Fernandez et al. 1990).

In amniote vertebrates, two distinct types of vestibular receptor cells exist based on differing morphologies (Lysakowski and Goldberg 1997; Wersäll 1956), innervations (Kevetter et al. 1994; Lysakowski and Goldberg 1997), and channel properties (Correia and Lang 1990; Eatock et al. 1994; Rennie and Correia 1994; Ricci et al. 1997; Rüschi et al. 1998). Mature type I hair cells are characterized by their amphora shape and the calyceal afferent terminal that engulfs the hair cell. Type II hair cells are generally thinner, tube shaped, and are innervated by bouton terminals. In birds, a differential distribution of the two receptor types in the utricular sensory epithelium has been shown with a higher concentration of type I hair cells contained within the striolar region and more numerous type II hair cells outside the striola (Jorgensen and Andersen 1973; Rosenhall 1970). In mammals, the utricular macula can be proportioned into separate areas: the striola region with a high concentration of type I hair cells, the juxtastrial region with a mixture of type I and type II hair cells, and the extrastriolar region, which lies in the peripheral macula and also contains a mixture of type I and II hair cells (Fernandez et al. 1990). In birds, the striolar region of the utricles and lagena otolith organs are substantially different from those described for the mammalian...
Primary afferents that innervate receptor cells of the utricular neuroepithelium have generally been characterized based on their regional innervation patterns. For vertebrates that possess both type I and II cells, only the chinchilla has been thoroughly examined, and three classes of afferents were described (Fernandez et al. 1990). Calyx afferents exclusively innervated either one or multiple type I hair cells and were found only in the striola region. Dimorph afferents innervated both type I and type II hair cells and were distributed throughout the macula. Although few in number, bouton afferents innervated only type II hair cells and were found only in the extrastriola regions. In rats, both calyx and dimorph afferents have been described using serial section electron micrographs (Ross 1985). In frogs and fish, only bouton afferents exist, yet differences among hair cell type and afferent innervation in the maculae have been reported (Baird and Lewis 1986; Baird and Schuff 1994; Chang et al. 1992; Lewis and Li 1975).

To date, the afferent innervation patterns in bird vestibular organs have not been examined except for our recent companion study of the pigeon saccular macula (Zakir et al. 2003). Because the utricular macula of birds appears to contain similar morphological properties to those of both amphibians and mammals, it is of interest to characterize the afferent innervation patterns and terminal distribution in these animals. The present study utilized neural tracers, image analysis, and three-dimensional reconstruction techniques to quantify the morphological parameters of utricular afferents in pigeons. Differences in the anatomical organization, regional distribution of afferent types, and innervation profiles were determined and then compared with these same properties described for other species.

METHODS

Animals

The experiments were conducted in adult male pigeons (Columba livia) that ranged in age from 1 to 3 yr. The methods of study were approved by both the University of Mississippi Medical Center and the Central Institute for the Deaf Institutional Animal Care and Use Committees (work was performed at both locations), and all animal procedures were performed in accordance with the American Psychological Society guidelines.

Tracer injections

Biotinylated dextran amine (BDA, 10,000 MW; Molecular Probes) was injected into the vestibular nuclei of the pigeon brain stem. Each animal was initially anesthetized with pentobarbital sodium (16 mg/kg iv), then supplemented with ketamine hydrochloride (10 mg/kg im) given once every hour or as needed. Body temperature (40°C) was maintained with a thermal pad. Once anesthetized, the animal was placed in a stereotaxic device with the beak angled downward ~13° so that the horizontal semicircular canals were aligned with the stereotaxic horizontal plane. A small opening was made in the parietal bone, and a glass micropipette filled with BDA (10% in saline) was lowered into the brain using predetermined coordinates for the vestibular nuclei (about posterior, 3.5 mm; lateral, 2.5 mm; and dorsal, 2.5 mm relative to a point intersecting the interaural axis and the midline of the bird). BDA was passed into the brain using ionophoresis with positive current (6–8 μA, 50% duty cycle) over a 10-min duration. Using slightly different injection site coordinates for each animal, BDA was deposited into different regions of the vestibular nuclei. After cessation of injection current, the electrode was allowed to remain in position for at least 2 min with a small negative current (~0.04 μA) applied. The electrode was then retracted. Gelfoam was placed over the brain surface followed by bonewax, and the skin was sutured closed. A single dose of butorphanol (0.5 mg/kg im, Stadol) was given to alleviate postoperative pain, and the animal was returned to its home cage.

Histology

After 10–14 days of postinjection survival, the pigeon was anesthetized with pentobarbital sodium (20 mg/kg iv), the mastoid bone was opened, and an intralabyrinthine perfusion was performed using 1% glutaraldehyde, 1.25% paraformaldehyde, and 1% acrolein in 0.1 M phosphate buffer. Next, the chest cavity was opened, and the animal was transcardially perfused with sodium nitrite (1%) in saline (250 ml), followed by 750 ml of 1% glutaraldehyde-1.25% parafomaldehyde fixative. The membranous labyrinth was excised and dissected free into individual endorgan (3 semicircular canals, utricle, saccule and lagena). The brain was removed and along with the vestibular endorgans, placed in the aldehyde fixative solution for 24 h. The utricle was then trimmed so that the sensory neuroepithelium was visible. This tissue was treated with a protease (type XXIV, Sigma) 30 μg/ml for 10 min to remove the otolith membrane and otoconia by gentle agitation. The brain was blocked, frozen, and serially sectioned (60 μm) in the transverse plane. Both the endorgans and the brain sections were processed for BDA using a modified 3–3′ diaminobenzidine (DAB) procedure (Brandt and Apkarian 1992). Briefly, the tissue was incubated for 12 h in a solution of phosphate buffer, 1% Triton-X100, and 0.25% avidin–HRP (Vector Labs). The tissue was then reacted using a solution of DAB and 4% nickel-cobalt in 0.1 M phosphate buffer as the chromogen and 0.3% H2O2 as the initiator until a dense reaction product was visualized. The tissue was then rinsed in phosphate buffer. The brain sections were dehydrated using a series of graded alcohols and xylenes, mounted on glass slides and counterstained with neutral red.

After the reaction procedure, the whole maculae were placed into a depression slide, viewed using a Nikon light microscope, and the apical surface of the neuroepithelium was photographed. The utricular maculae were then embedded into plastic (Durcupan) and serially sectioned (10 mm thickness) using a rotary microtome. The sections were mounted on glass slides and counterstained (Richardson et al. 1996).

SEM preparation

In some animals, the utricular maculae (n = 14) were prepared for scanning electron microscopy (SEM). First, the otocystia and otolith membranes were removed by treatment with protease. The tissue was placed into a 0.5% osmium and 0.1 M phosphate buffer solution for 2 h. After six serial washings (10 min each) in distilled water, the tissue was dehydrated using a graded series of acetones (10 min each in 70, 90, and 95% acetone), followed by three washings of 15 min in 100% acetone. The tissue was then incubated in a 1:1 mixture of tetramethylsilane (TMS) and acetone for 45 min followed by an incubation of 45 min in a 3:1 mixture of TMS and acetone. The tissue was then incubated twice in 100% TMS for 1 h. Finally, the tissue was dried in the oven for 30 min, allowing the TMS to sublimate (Dye et al. 1999). The tissue was then mounted onto aluminum studs and gold coated. The maculae were scanned and photographed using either a JEOL TSM-3000 or a Hitachi S800 scanning electron microscope (15

J Neurophysiol • VOL 89 • MARCH 2003 • www.jn.org
Afferent reconstruction

The location of the BDA injection site for each animal and the sectioned tissue from each utricular macula was examined using video microscopy on a Nikon E600 microscope with DIC infinity optics and drawn using an image analysis and reconstruction program (NeuronLucida, MicroBrightfield). There was no correction for tissue shrinkage, which has generally been shown to be between 5 and 10% for aldehyde fixation and plastic embedding (Kushida 1962). Several parameters for each section were measured, including the section width, the relative location and width of the striola region, the type II hand, and the morphological reversal point. For each pigeon utricular macula, a planar map of the epithelial surface was plotted using the measured values.

For reconstruction of labeled afferents, only fibers that were darkly stained and sufficiently isolated from other afferents were traced in an effort to establish the complete morphological structure of the fiber. Afferents with partial staining (ghost fibers), or those that overlapped other afferents were discarded from analysis. In practice, many more BDA-stained fibers were observed in each utricular macula than could be quantitatively analyzed as was true for the anterior striola region shown in Fig. 3 where numerous labeled afferents overlapped and could not be discriminated with adequate certainty. The regional location of each reconstructed afferent within the utricular macula was made, using relevant distance measurements (anterior, medial, lateral edges, and reversal line). The three-dimensional reconstruction of the identified afferents was performed using ×60 (Infinity Optics, NA 0.95) magnification. A number of morphological parameters for each of the reconstructed afferents were quantified. Axonal diameter was defined as the average diameter of the last 5 μm of fiber prior to the point where the neuroepithelium was penetrated. Fiber length and volume were calculated as the total values of the all branches of the fiber within the neuroepithelium (above the stroma). Branch number indicated the total number of branches. The number of boutons was defined as the number of both terminal boutons and en passant boutons. The number of type I hair cells included all type I cells contained in calyceal terminals, as identified by visualization of an apical neck and top. The innervation area of the afferent was measured by drawing a contour around the reconstructed surface terminal field, then obtaining the area of the contour with the three-dimensional reconstruction software. The innervation density for an afferent was derived by dividing the total number of terminals (type I hair cells + bouton terminals) by the innervation area. The innervation angle was also calculated from the reconstructed dorsal view (clockwise rotation). A line was drawn through the major innervation axis of the terminal field. The innervation angle corresponded to the angle between the innervation axis and an imaginary axis line drawn through the utricular neuroepithelium from the anterior border to the posterior border. Thus 0° corresponded to a vector directed from the anterior border to the posterior border of the macula and 90° corresponded to an angle directed from lateral to medial.

Statistical analyses

The afferents were divided into three main groups based on their innervation patterns. Calyx, dimorph, and bouton afferents were distinguished as described in the following text. Comparisons between a number of morphological parameters, both within and between these groups of the traced afferents, were made using ANOVA. All post hoc comparisons were made using the Sheffe follow-up test. Proportional relationships were analyzed using Pearson product-moment correlations. All statistical comparisons were performed using Statistica (Statsoft).

RESULTS

Neuroepithelium of the utricle

The general morphology of the utricular macula was examined in 14 specimens using SEM views of the dorsal surface epithelium. In vivo, the macula is curved, with the anterior quarter of the neuroepithelium turning dorsally at nearly a right angle to the remaining macular surface. When processed for SEM, the epithelium was laid flat on the mounting stub allowing the anterior edge to be visualized but also producing stress fractures in the surface. As shown in Fig. 1, with the otoconia and otolith membrane removed, the extent of the neuroepithelium was determined by measuring the boundaries of the hair cell distribution. At lower magnifications, the macula surface was observed to be rectangular with a rounded anterior edge (Fig. 1A). The posterior edge of the utricular macula contained a pointed tail at the medial border, formed by an anterior projected indentation that contained no hair cells. At moderate magnifications (×1,000−5,000), the stereocilia of individual hair cells were distinctly visible allowing the boundary line (Fig. 1A, —) for the receptor neuroepithelium to be drawn. The size of the utricular macula in five pigeons as taken from the sectioned material (not adjusted for shrinkage) was measured across the center of the epithelium to an average of 1.51 ± 0.22 (SD) mm along the anterior-posterior axis and 1.19 ± 0.09 mm along the medial-lateral axis (hair cell borders). The location of the reversal line, as defined by the hair cell morphological polarization, was also determined from the surface SEM views using moderate magnifications (Fig. 1B). The reversal line coursed in an eccentric C-shaped manner to run from the mid posterior macula, along the epithelial margins, to the end of the medial posterior tail. The reversal line ran through the entire macula, extending to the edges of the posterior epithelium (Fig. 1, A and E). As shown in Fig. 1, B–D, two different morphological polarization patterns of opposing hair cells were observed along the reversal line. For hair cells in the lateral and anterior macula regions, polarizations were directed toward the reversal line (Fig. 1, B, D, and E). In contrast, hair cells located in the medial macula had polarizations that were directed parallel to the reversal line, with cells on either side of the line being organized in an anti-parallel manner (Fig. 1, B and C). Hair cells located near the medial border were polarized with their kinocilia directed toward the posterior tail, forming a zone ~50-μm wide (Fig. 1B). Hair cells located lateral to the anti-parallel reversal line were polarized anteriorly. The majority of the hair cells in the central macula were mostly polarized toward the lateral edge but systematically varied toward the appropriate opposing directions when located near to the reversal line (Fig. 1B).

To examine the regional variations in cellular architecture of the utricular macula, transverse sections through the medial-lateral axis of the neuroepithelium were cut in several animals that had no BDA label. Three major zones were demarcated in the macula based on the concentrations of type I and type II cells and their relationship to the reversal line. The striola region was defined by the high concentration of type I cells with a lower density of type II hair cells. In fact, type I hair cells were exclusively located in the striola region. The striola was located eccentrically in the lateral and anterior portions of the macula and varied in width between 150 and 400 μm.
(~30–40% of the epithelium). Coursing through the striola region was a narrow 30–50 μm wide band of four to eight exclusive type II cells. In the center of the type II band was the opposing polarization point that defined the reversal line. Thus the striola region actually consisted of three separate zones, including the medial and lateral type I hair cell rich areas (medial and lateral striola zones) and the type II band. Outside the striola region lay the extrastriola zone that contained only type II cells. The extrastriola formed a circumferential band around the macula, as well as occupied nearly the entire medial half of the epithelium. Together the extrastriola regions comprised ~60% of the epithelium.

Using the transverse sectioned tissue for all animals, contours were drawn of the apical and basal surfaces serially throughout the neuroepithelium using the reconstruction program. The traced sections were then rotated so that a top view of the macula was obtained for each utricle. As shown in Fig. 2 for one macula, the outline of the neuroepithelium was drawn from the top view sections. Along with the outline, the striolar

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**FIG. 1.** Pigeon utricular macula. A: scanning electron micrograph of the dorsal surface of the left utricular macula with otoconia and otolith membrane removed. The anterior quadrant of the utricle is normally turned upward out of the plane of the micrograph but has been flattened for optimal viewing producing some stress fractures in the surface. —, the hair cell border of the epithelium. · · · ·, the location of the reversal line with perpendicular and antiparallel morphological polarizations, respectively. Anterior to the left; medial is upward. Scale bar = 150 μm. B: surface map with → indicating direction of hair cell morphological polarization. C–E: Higher magnification of insets in A. · · ·, antiparallel reversal line (C). · · · ·, perpendicular reversal line (D and E). Scale bars = 10 μm.

**FIG. 2.** Composite surface map. Utricular macula is shown, with reversal line (thin black) and striolar (medium black) region indicated. Locations of 288 identified calyx (squares), dimorph (triangles), and bouton (circles) afferents are shown. Reconstructed afferents (n = 208) are indicated by filled symbols.
region, type II band, and extrastriola were identified and plotted. This drawing then served as a schematized map of the utricular surface for all later analyses.

**Injection sites**

BDA injections into nine vestibular nuclear regions (2 animals had bilateral injections) produced labeled afferents in the utricular epithelium that were utilized for quantitative analyses. Seventeen additional injections produced labeled afferents innervating other receptor organs and were included as part of additional studies. Injection sites centered in the more lateral portions of the vestibular nuclei primarily produced tracing in afferent fibers that innervated the utricular and saccular maculae, while medial injection sites primarily labeled canal afferents. Regional differences in the density of utricular labeled afferents were noted as the position of the injection site varied within the vestibular nuclear complex. Among the seven animals, utricular afferents were predominantly labeled with injections sites located in the lateral portions of the SVN (n = 4) and lateral vestibular nucleus (LVN; n = 5). For example, as shown in Fig. 3, an ipsilateral injection into the lateral portion of the rostral superior vestibular nucleus (SVN; Fig. 3A) of one animal (PN63) produced 138 labeled utricular fibers (Fig. 3B). Although labeled fibers were observed throughout the macula surface, most were located in the striola region of the right utricle with a dense concentration of fibers in the anterior quadrant. These findings are consistent with our previous anterograde tracing study in pigeons that demonstrated the central terminal fields of afferents from each individual vestibular receptor organ (Dickman and Fang 1996).

**BDA uptake and axon diameter**

As reported in our companion study of pigeon saccule innervation (Zakir et al. 2003), the degree of uptake by the BDA tracer into afferents of various size was determined by examining three branches of the vestibular nerve distal to Scarpa’s ganglion in two of the animals used for the utricle studies. As shown in Fig. 4A, the BDA tracer (dark fibers) was absorbed and transported by axons of various diameters. The internal diameter (lumens, not measuring the myelin) of all axons in the three branches were measured (×100 oil), and the BDA containing fibers were compared against the total population of afferents (Fig. 4B). In our sample of 2,351 total fibers in three branches, axons varied between 0.54 and 8.2 μm, with a mean value of 2.54 ± 1.6 μm. BDA-filled fibers (n = 533) ranged between 0.56 and 6.99 μm with a mean of 2.4 ± 1.7 μm, which was actually smaller than the mean 2.6 ± 1.6 μm for the non-BDA fibers [n = 1818; F(1,2349) = 7.4, P < 0.006]. As shown in Fig. 4B, the distribution of BDA-filled fibers and the total population of fibers were nearly equivalent, suggesting that little or no bias in BDA uptake by axons of either small or large diameter was present in our experiments. The vestibular afferent axon diameters observed in our sample agree well with those reported by Landolt et al. (1973) for pigeons, who found a mean of 2.7 μm for 8,720 fibers in all branches (exclusive of posterior canal) and a similar distribution of fiber diameters.

In addition, the diameters of all BDA-labeled fibers (n = 290 from 2 utricles; average of the last 5 μm of fiber beneath basement membrane) at the point where they entered the utricular neuroepithelia in these same animals were obtained. The distribution of the BDA-labeled afferents was plotted against that for the total fiber population in Fig. 4B. The results show that fibers increase in diameter as they approach the basement membrane, as the percentage of the 1- to 2-μm diam fibers decreased and the 2- to 3-μm diam fibers nearly doubled that observed in the distal nerve branches of the same animals (χ² = 51.2, P < 0.00001). Still, many of the smallest diameter fibers (0.5–1 μm) were also found to contain BDA at the level of the neuroepithelial crossing.
The calyx afferents were characterized by their specialized large calyceal terminal that contained several type I hair cells, as shown in Fig. 5. All of the calyx afferents (n = 82) observed were positioned within the striola region. Seventy-one calyx afferents were reconstructed for quantitative comparison and their locations are shown in Fig. 2. As represented by the four afferents in Fig. 5, a narrow continuum of innervation size and complexity was observed. For all calyx afferents, a single unbranched parent axon entered the neuroepithelium and was typically large, ranging between 1.0 and 6.6 μm (Fig. 4) with a mean diameter of 3.0 ± 1.0 μm (Table 1). On entering the neuroepithelium, >90% of the calyx afferents terminated with no branch point (Fig. 5, B, I, and K). Thus the calyx fibers exhibited the simplest arborization patterns of all afferents (Table 1). Some (7/71) calyx afferents did contain short daughter branches (Fig. 5, E and F); however, there was only a single calyceal terminal per branch. Corresponding to small arborization patterns, both the length and the volume of the calyx axons within the neuroepithelium were the smallest of all afferents (Table 1).

The size and structure of the calyx terminals varied widely, as illustrated by the photomicrographs and anatomical reconstructions of Figs. 5 and 6. The simplest calyceal terminals contained between two and five type I hair cells (Figs. 5, H–J, and 6, A–C), with no terminal being observed to contain only one hair cell. The more complex calyces contained many hair cells, with a mean value of 6.4 type I hair cells/calyceal terminal (Table 1). The majority (90%) of the calyx afferents consisted of a single calyceal terminal; however, a few (6/71) of the fibers exhibited two calyces and one afferent had three calyces (each on different branches; Fig. 6E). In total, for all calyceal terminals for a given fiber, the number of hair cells per calyx afferent varied between 2 and 15, with a mean value of 7.1 (Table 1). Although there was diversity in calyx structure, two major profiles were noted. The first profile appeared globular or “flower” shaped (Figs. 5, H–J, and 6, A–C) and contained hair cells that lay in close approximation. Occasionally, a small protrusion with several hair cells extended off the main calyceal structure (Fig. 5B). The second major profile consisted of a thin rectangular-shaped calyceal terminal that contained a linear array of hair cells (Figs. 5, Eb, K, and L, and 6D). The few afferents that had daughter branches sometimes had similar shaped calyces on each fiber branch and occasionally contained a mixture of flower and rectangular shaped calyces (Fig. 5, Ea–G). The most complex calyx afferent observed contained three short branch collaterals off the parent axon, with a single calyx on each branch containing several hair cells each (Fig. 6E). Due to the low amount of axonal branching and short fiber lengths, the area of macular innervation was also the smallest among the three groups of afferents (Tables 1 and 2). As shown in Fig. 7, the innervation areas for the calyx afferents were highly skewed toward small terminal fields, with most fibers having areas <400 μm². Because the number of terminals and innervation area for each fiber was small, the innervation density (Table 1) in calyx afferents were significantly lower than other fiber classes (Table 2). A final parameter that attempted to quantify the terminal pattern for each afferent was the innervation angle. As shown in Fig. 8, many of the calyx afferents innervated the epithelium with the major axis of their terminal fields directed either parallel to, or perpendicular to, the reversal line. The innervation angles

**Afferent innervation patterns**

In nine utricular maculae, the innervation patterns of 288 afferents were identified, with their locations in the macula being shown in Fig. 2. Of these 288 fibers, 208 met our criteria for quantitative analyses and were anatomically reconstructed. Three major classes of afferent innervation patterns were observed, including calyx, dimorph, and bouton fibers based on their terminal profile. For the majority of the reconstructed afferents, top view photomicrographs of the whole-mount macula were obtained prior to sectioning to aid in visualization of structure.

**CALYX AFFERENTS.** The calyx afferents were characterized by their specialized large calyceal terminal that contained several type I hair cells, as shown in Fig. 5. All of the calyx afferents

![Image](https://example.com/image.png)
were the most ordered for fibers innervating regions close to the reversal line, with more variance for fibers innervating central regions (Fig. 8). Approximately 44% (31/71) of the calyx afferents were oriented parallel to the reversal line, while another 41% (29/71) were oriented orthogonally. The remaining calyx afferents (11/71) were oriented at angles that acutely exceeded 30° of either being parallel or orthogonal to the reversal line.

**TABLE 1. Morphological parameters by afferent type**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Calyx (Range</th>
<th>Mean ± SD)</th>
<th>Dimorph (Range</th>
<th>Mean ± SD)</th>
<th>Bouton (Range</th>
<th>Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axon diameter, μm</td>
<td>1.0–6.6</td>
<td>3.0 ± 1.0</td>
<td>1.3–5.6</td>
<td>3.1 ± 0.91</td>
<td>0.86–4.7</td>
<td>2.4 ± 0.84</td>
</tr>
<tr>
<td>Branches/fiber</td>
<td>1.0–3.0</td>
<td>1.1 ± 0.4</td>
<td>2–119</td>
<td>23.3 ± 25.9</td>
<td>3–150</td>
<td>54.4 ± 30.9</td>
</tr>
<tr>
<td>Terminal fiber length, μm</td>
<td>2.9–106.5</td>
<td>29.3 ± 24.5</td>
<td>23.2–805.9</td>
<td>213.9 ± 199.2</td>
<td>75.3–1306.4</td>
<td>475.6 ± 274.0</td>
</tr>
<tr>
<td>Terminal fiber volume, μm²</td>
<td>3.6–1040</td>
<td>216.2 ± 194.0</td>
<td>25.9–3438.8</td>
<td>797.1 ± 797.7</td>
<td>139.4–5809</td>
<td>726.4 ± 834.9</td>
</tr>
<tr>
<td>Innervation area, μm²</td>
<td>47.5–968.9</td>
<td>294.5 ± 188.7</td>
<td>89.2–4436</td>
<td>692.6 ± 766.2</td>
<td>143.6–2915</td>
<td>945.4 ± 560.2</td>
</tr>
<tr>
<td>Innervation density, terminals/μm²</td>
<td>0.007–0.072</td>
<td>0.029 ± 0.013</td>
<td>0.012–0.094</td>
<td>0.038 ± 0.020</td>
<td>0.010–0.165</td>
<td>0.069 ± 0.03</td>
</tr>
<tr>
<td>Type I hair cells/calyx</td>
<td>1–15</td>
<td>6.4 ± 3.2</td>
<td>1–10</td>
<td>3.9 ± 2.5</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Type I hair cells/fiber</td>
<td>2–15</td>
<td>7.1 ± 3.0</td>
<td>1–16</td>
<td>4.9 ± 2.99</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Calyceal terminals/fiber</td>
<td>1.0–3.0</td>
<td>1.1 ± 0.4</td>
<td>1–5.0</td>
<td>1.4 ± 0.9</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Bouton terminals</td>
<td>—</td>
<td>—</td>
<td>1–129</td>
<td>16.9 ± 21.5</td>
<td>3–182</td>
<td>60.4 ± 37.6</td>
</tr>
</tbody>
</table>

Mean values are ± SD.
DIMORPH AFFERENTS. The dimorph afferents were characterized by their terminal structure that contained both calyceal and bouton terminals that innervated both type I and II hair cells. Eighty dimorph afferents were identified, and 70 of these were reconstructed for quantification (Fig. 2). Most (95%) of the dimorph afferents observed were located in the striola region, intermixed with the calyx afferents. A few dimorphs (5%) were observed to enter the neuroepithelium outside the striola zone, where fiber branches arborized to innervate a number of type II afferents, then continued into the striolar zone where calyceal

**TABLE 2. Statistical comparisons for all afferents**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Mean</th>
<th>Main Effect</th>
<th>Sheffé Post Hoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axon diameter</td>
<td>Calyx</td>
<td>3.0</td>
<td>F(2,205) = 8.9</td>
</tr>
<tr>
<td></td>
<td>Dimorph</td>
<td>3.1</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>Bouton</td>
<td>2.5</td>
<td>P &lt; 0.009</td>
</tr>
<tr>
<td>Number of branches</td>
<td>Calyx</td>
<td>1.1</td>
<td>F(2,205) = 85.2</td>
</tr>
<tr>
<td></td>
<td>Dimorph</td>
<td>23.3</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Bouton</td>
<td>54.4</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Fiber length</td>
<td>Calyx</td>
<td>29.3</td>
<td>F(2,205) = 91.6</td>
</tr>
<tr>
<td></td>
<td>Dimorph</td>
<td>213.9</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Bouton</td>
<td>475.6</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td>Fiber volume</td>
<td>Calyx</td>
<td>216.2</td>
<td>F(2,205) = 15.7</td>
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<tr>
<td></td>
<td>Dimorph</td>
<td>797.1</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Bouton</td>
<td>726.4</td>
<td>NS</td>
</tr>
<tr>
<td>Innervation area</td>
<td>Calyx</td>
<td>294.5</td>
<td>F(2,205) = 24.0</td>
</tr>
<tr>
<td></td>
<td>Dimorph</td>
<td>692.6</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Bouton</td>
<td>945.4</td>
<td>P &lt; 0.003</td>
</tr>
<tr>
<td>Innervation density</td>
<td>Calyx</td>
<td>0.029</td>
<td>F(2,205) = 56.9</td>
</tr>
<tr>
<td></td>
<td>Dimorph</td>
<td>0.038</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>Bouton</td>
<td>0.068</td>
<td>P &lt; 0.001</td>
</tr>
<tr>
<td>Number of type I cells/calyx</td>
<td>Calyx</td>
<td>6.4</td>
<td>F(1,165) = 30.9</td>
</tr>
<tr>
<td></td>
<td>Dimorph</td>
<td>3.9</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Bouton</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Number of type I cells/fiber</td>
<td>Calyx</td>
<td>7.1</td>
<td>F(1,139) = 18.9</td>
</tr>
<tr>
<td></td>
<td>Dimorph</td>
<td>4.9</td>
<td>P &lt; 0.0001</td>
</tr>
<tr>
<td></td>
<td>Bouton</td>
<td>1.4</td>
<td>—</td>
</tr>
<tr>
<td>Number of calyceal terminals</td>
<td>Calyx</td>
<td>1.1</td>
<td>F(1,139) = 4.2</td>
</tr>
<tr>
<td></td>
<td>Dimorph</td>
<td>16.9</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td></td>
<td>Bouton</td>
<td>61.6</td>
<td>—</td>
</tr>
<tr>
<td>Number of bouton terminals</td>
<td>Calyx</td>
<td>—</td>
<td>F(1,72) = 92.7</td>
</tr>
<tr>
<td></td>
<td>Dimorph</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Bouton</td>
<td>—</td>
<td>P &lt; 0.0001</td>
</tr>
</tbody>
</table>

NS, non-significant.
terminals developed. A few dimorph afferents also had terminal branch fields that innervated the type II band, while none were located in the peripheral extrastriolar zones.

The dimorph afferents varied greatly in innervation structure, as shown in Fig. 9. Similar to the calyx afferents, dimorph afferents typically consisted of a single axon that entered the neuroepithelium, although 5.7% (4/70) of the afferents branched once prior to penetrating the base layer. The diameters of the dimorph parent axons were similar to calyx afferents in both their range distribution (Fig. 4) and size (Tables 1 and 2). Simple dimorph afferents contained a single branch off the parent axon, while the most complex afferent quantified contained 119 branch fibers (out to the 25th order). The mean number of branches/fiber for the dimorph afferents was 23.3 (Table 1), which was significantly higher than calyx afferents (Table 2). The simplest innervation pattern observed consisted of afferents with a single calyx and one bouton terminal, while the most complex afferent quantified contained five calyces and 129 bouton terminals. One feature sometimes noted on simple dimorphs was a small fiber branch that appeared to extend off of a calyceal terminal body, instead of the parent axon or another fiber branch (Fig. 9, G–I). These small branch extensions typically contained several en passant boutons and finished with one, or occasionally a cluster of several, terminal boutons. As the dimorph innervation patterns increased in complexity, the number of calyces, their locations in the arborization field, and the number of terminal bouton branches also increased. Representative examples of the diversity of dimorph afferent structures observed are shown in the reconstructions of Fig. 6. Due to the longer parent axons and the more extensive arborizations of dimorphs, the total fiber lengths and fiber volumes were significantly larger than those of calyx afferents (Tables 1 and 2). Similar to the innervation patterns of the calyx afferents, the structure of the terminal fields observed for dimorph afferents could generally be classified into flower and linear profile types. The flower profile consisted of a central calyx, or group of calyces, with small branch fibers ending in terminal boutons extending away from the calyx core (Figs. 6, F–G, and I, and 9, A–C and G–I). For some afferents, the extending bouton terminal branches surrounded the calyceal core, yet for other afferents, the extensions were unilateral. Many of the small fiber branches extended upward away from the basement membrane, giving rise to en passant and terminal boutons that often extended to within 1 μm of the apical surface of the epithelium (Figs. 6, H and I, and 9, B, C, K, and L). Dimorph afferents with flower profiles appeared to be densely innervating hair cells that lay in close approximation to each other. Dimorph afferents with linear profiles were characterized by a long parent axon that coursed for tens of microns through the neuroepithelium with, if any, major branch fibers (Figs. 9, D–F, J, and L, and 6, H and J). In dimorphs, one to five small calyceal terminals were attached to the parent axon, and these could be located anywhere along the fiber tree but were most often positioned at either the beginning (Figs. 6H and 9, D–F) or the end (Fig. 9, J–L) of the terminal field.

As a group, dimorph afferents had significantly more calyceal terminals per fiber than calyx afferents (Table 2). 

![Figure 7](image1.png)  
**FIG. 7.** Number of calyx (■), dimorph (□), and bouton (■) afferents as a function of innervation area. Each bar represents the number of afferents per group for each of the binned innervation areas.

![Figure 8](image2.png)  
**FIG. 8.** Innervation angles of utricular afferents. The angle through the long terminal field axis for each afferent was plotted for each of the 208 reconstructed afferents. Innervation angles for calyx, dimorph, and bouton afferents are shown top, middle, and bottom, respectively. 0° corresponds to an angle directed from anterior-posterior direction. A 90° angle corresponds to an innervation field extending from lateral to medial.
though more in number, the calyces of the dimorph afferents contained significantly fewer hair cells per calyceal terminal (mean of 3.9, Table 1) and innervated fewer total type I hair cells per fiber (mean value of 4.9, Table 1) than calyx units (Table 2). To visualize the regional differences in calyceal morphology, the total number of type I hair cells per fiber were plotted as a function of macular location for all dimorph and calyx afferents as shown in Fig. 10. As can be observed directly from several of the photomicrographs (Fig. 9, D, E, J, and K) and reconstructions (6J), many of the linear profile dimorph afferents had long axonal arbors but produced few terminal boutons. Still, the larger terminal fields of dimorph afferents were also significantly more densely innervated than those of calyx afferents (Tables 1 and 2). Similar to calyx afferents, most of the innervation angles for the dimorph afferents were directed either parallel (46%), or perpendicular (36%) to, the reversal line (Fig. 8).

As noted in the preceding text, several of the dimorph afferents innervated the type II band region (8/70). Because the band consists of only type II hair cells, the innervation patterns of these afferents were distinct. As shown by the afferent illustrated in Fig. 9, M–O, the parent axon usually entered the neuroepithelium beneath the striola type II band, then gave rise to several small branch fibers as it extended toward either the medial or lateral striola regions. The smaller branch fibers often arborized and ended with bouton terminals primarily in the type II band. The calyx structure for these afferents was always located in the striolar area adjacent to the type II band.
For the dimorph afferent shown in Fig. 9, two collateral branches arose beneath the epithelium and coursed into the medial striola region where calyceal terminals were located. All of the dimorph afferents innervating the type II band had terminal fields in only one half of the band, with their extensions and calyces into only one (either the medial or lateral) striola regions. None of the afferents innervated the entire width of the type II band, nor did they give rise to branches that extended across the reversal striola line as evidenced by visualization of stereocilia polarizations. Still, it should be noted that it was not always possible to observe the stereocilia of each cell being innervated by a given fiber, thus it remains possible that some branches did innervate cells with opposite morphological polarizations.

**BOUTON AFFERENTS.** The innervation patterns of bouton afferents were characterized by en passant and bouton terminals that exclusively terminated on type II hair cells. These afferents \( (n = 126) \) were located in the extrastriolar regions and the type II band (Fig. 2). Sixty seven of the 126 identified bouton afferents were reconstructed for quantitative comparisons, and their locations are shown in Fig. 2. Bouton afferents generally entered the macula without branching, although 6% (4/67) of the fibers had one branch beneath the neuroepithelium basement membrane. The diameters of the bouton afferents were significantly smaller (Fig. 4) than those of the calyx and dimorphic afferents, with a mean value of 2.4 \( \mu m \) (Table 1). After entering the neuroepithelium, bouton afferents varied greatly in the size and complexity of their arborization patterns, as shown in Figs. 6 and 11. Similar to the other two groups of afferents, the bouton fibers could be classified into one of two innervation profile types. Flower profiles consisted primarily of radiating branched patterns extending from the parent axon entering the basement membrane (Figs. 6, K and M–O, and 11, A–H and M–P). Linear profiles were comprised of branching patterns that extended along a narrow width of macula surface (Figs. 6, L and P, and 11, I–L). The simplest afferent quantified

**FIG. 10.** Comparisons of total number of type I hair cells for calyx and dimorph afferents. The location of the calyx (green squares) and dimorph (red triangles) afferents as a function of number of type I hair cells per afferent. Dashed line, reversal line.

**FIG. 11.** Photomicrographs and anatomical reconstructions for 4 BDA-labeled bouton afferents. A–D: simple flower profile afferent with many small branch and terminal boutons. Surface view (A), transverse sections (B), and reconstruction for transverse (C) and top view (D). E–H: complex flower profile afferent with a thick parent axon. Surface view (E), transverse section (F), transverse (G) and top view (H) reconstruction. I–K: simple long linear profile afferent innervating the type II band. Small blue squares represent type II band borders. Surface view (I), transverse section (J), and reconstruction for transverse (K) and top view (L). M–P: flower profile afferent, with large branches off the parent axon. Surface view (M), transverse section (N), and reconstruction for transverse (O) and top (P) view. For each reconstruction, the thick black line represents the parent axon, thinner black lines represent branch fibers, and red dots represent terminal or en passant boutons. Surface views, unstained; transverse sections, Richardson’s stain. Scale bars = 10 \( \mu m \).
contained only three branch fibers and a few bouton terminals, whereas the most complex afferent had 150 branches out to the 24th order. With a mean number of 54.4 branches per fiber, the bouton afferents were significantly more arborous than either the dimorph or calyx afferents (Tables 1 and 2). Measures of the overall fiber length for the bouton afferents were compared against the two other classes of fibers according to regional location in the utricular macula, as shown in Fig. 12. As noted in the preceding text, the calyx units had very short fiber lengths, the dimorph afferents had larger fiber patterns, but the bouton afferents had the largest terminal structures of all (Table 2).

The innervation patterns of the bouton afferents were divergent, but several features appeared to be consistent among units. Both en passant and terminal boutons were present in most afferents. The number of terminals per fiber ranged between 3 and 182, with a mean of 60.4 terminals per fiber (Table 1). For a more detailed comparison, the number of bouton terminals per afferent was plotted as a function of utricle location for the bouton and dimorph afferents as shown in Fig. 12. It is apparent that bouton afferents contained many more bouton terminals per fiber than dimorph afferents. In fact, the mean number of boutons was nearly triple that observed for the dimorph afferents, a significant finding (Table 2). As illustrated in the photomicrographs and reconstructions of Figs. 6 and 11, the number of bouton terminals per branch fiber also varied. Some branch fibers contained only a single terminal bouton, usually at the end of the branch, and these single boutons could be quite large (Figs. 6, M and P, and 11, E–G, N, and O). Other fibers had numerous en passant boutons, particularly on branches extending upward from the basement membrane to the apical epithelial surface (Figs. 6, K and N–P, and 11, B, C, F, G, K, and N–O). Similar to some dimorph afferents, the branch fibers could extend to within two microns of the apical surface with en passant and terminal boutons clustered along the fiber (Figs. 6, K–P, and 9, F, G, K, and N). Many of the branch fibers appeared to have clusters of terminals in close approximation, suggesting that a dense innervation of relatively few hair cells existed (Figs. 6, K and N–P, and 11, E–H and M–O).

For both the linear and flower profile types of bouton afferents, the radiating terminal field could be either small to innervate a few hair cells or very large to innervate an area with many hair cells, as shown in Fig. 12. The innervation areas for the bouton afferents, with a mean value of 945.4 μm², was significantly larger (Table 2) and more symmetrically distributed across its range than that for either the calyx or dimorph afferents (Fig. 7). Similar variance was observed in the density of terminals, with some afferents exhibiting numerous terminals in a small area (Figs. 6, N and P, and 11E), whereas other units were more sparse (Figs. 6L and 11B). The mean innervation density for the bouton afferents was 0.069 (Table 1) terminals per square micrometer, a significantly higher value than either calyx or dimorph units (Table 2). Bouton afferents, similar to the other afferent types, tended to innervate the...
macula along angles that were directed either parallel (51\%) or perpendicular (40\%) to the reversal line (Fig. 8).

Along with dimorph units, the type II band region was innervated by bouton afferents ($n = 16$). Some of the type II band bouton afferents had long linear profiles (Figs. 6M and 11, I–L), although others had small profiles (Fig. 6K). The innervation specificity of these afferents was characterized by the reconstruction shown in Fig. 11L, where most of the units branch fibers were directed unilaterally away from the parent axon. The terminals appear to innervate type II hair cells on only one side of the reversal line. None of the bouton afferents observed had terminal fields that covered the entire width of the type II band. This suggests that in the pigeon, innervations of hair cells with oppositely directed polarizations are rare or nonexistent.

Regional differences in innervation patterns

To more closely examine the regional differences in morphological properties of the three classes of afferents, differences for the reconstructed afferents were analyzed according to afferent location. First, all afferents were plotted for terminal fiber length and innervation area as a function of macula location, as shown in Fig. 12. The utricular epithelium was then divided into anterior, central, and posterior thirds for comparisons. Examination of the regional distribution of calyx, dimorph, and bouton afferents shows that some differences in the patterns across the macula surface exist (Figs. 10 and 12). For example, the innervation areas were significantly larger for anterior calyx afferents (377 $\mu$m$^2$), as compared with either centrally (286 $\mu$m$^2$) or posteriorly (227 $\mu$m$^2$) located fibers [$F(2,68) = 3.6, P < 0.05$]. Conversely, many dimorph and bouton afferents with the largest innervation areas were located in the posterior regions of the macula (Fig. 12), although no overall significant difference for the three regional locations was found. For dimorph afferents, there was a moderate trend for more type I hair cells per fiber in the posterior third of the macula (6.5), as compared with either the central (4.4) or anterior (4.7) fibers [$F(2,67) = 2.98, P < 0.06$]. No other comparisons for regional location along the anterior-posterior axis were significant. Comparisons between calyx and dimorph afferents located on the medial and lateral side of the reversal line in the striola were also made, with no significant differences found. These results indicate that the morphology of the calyx and dimorph afferents are quite homogeneous throughout the striolar region.

For the bouton afferents, examination of the distribution plots (Fig. 12) suggests that fibers innervating the more central regions of the macula may differ from those innervating the more peripheral utricular regions. To test the observation, all of the bouton afferents that were located within 100 $\mu$m of the peripheral border were grouped ($n = 28$) and compared with those that were centrally located ($n = 39$) on several morphological measures. As shown in Table 3, peripherally located bouton afferents had larger diameter axons, higher branch orders, longer fibers, more bouton terminals, and larger innervation areas than central bouton afferents. Finally, it was of interest to examine the bouton afferents that innervated the type II band, as they represent a terminal field in a unique portion of the macula. In the distribution plots of Fig. 12, it appears that these afferents had relatively small terminal fields. Although only 16 type II band bouton afferents were quantified, statistical comparisons showed that these fibers had smaller fiber lengths, fewer bouton terminals and smaller innervation areas that afferents located elsewhere in the macula (Table 3).

**Efferent fibers of the utricle**

In the present study, central BDA injections into the vestibular nuclei were utilized to retrogradely label vestibular afferents. Indeed, labeled cell bodies in Scarpa’s ganglion were observed for all animals. However, with central BDA injections it is also possible that some of the labeled fibers and terminals in the utricular neuroepithelium were anterogradely labeled fibers. Vestibular efferents are known to have their cell bodies located in the brain stem, ventral and slightly lateral to the abducens nucleus in birds (Dickman and Fang 1996; Eden and Correia 1982). Thus for each animal utilized in the study, the efferent nuclei were bilaterally examined for BDA labeled cell bodies. In two birds, several efferent nuclei neurons were in fact labeled, and these animals were eliminated from further study.

As a further control, two animals were given large BDA injections into the contralateral brain stem in and surrounding the region that contains efferent somas. The contralateral nucleus was chosen since in all species studied to date, no vestibular primary afferent has ever been shown to cross the midline using either nerve branch or intracellular tracing experiments (Carleton and Carpenter 1984; Dickman and Fang 1996; Gacek 1969). For the two control animals, a number of known efferent fibers were obtained and their innervation patterns were examined. As shown in Fig. 13, the efferent fibers exhibited innervation patterns that were very different from afferent fibers and were readily distinguishable by visual inspection. For example, the efferent fibers typically branched several times beneath the neuroepithelium and had thin parent fibers.

**TABLE 3. Statistics for bouton afferent location**

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Peripheral</th>
<th>Central</th>
<th>Main Effect</th>
<th>Extrastriola</th>
<th>Type II Band</th>
</tr>
</thead>
<tbody>
<tr>
<td>Axon diameter</td>
<td>2.7 ± 0.82</td>
<td>2.3 ± 0.81</td>
<td>$F(1,65) = 5.8; P &lt; 0.02$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of branches</td>
<td>10.9 ± 4.4</td>
<td>8.4 ± 2.9</td>
<td>$F(1,65) = 7.9; P &lt; 0.007$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fiber length</td>
<td>601.9 ± 301.9</td>
<td>384.9 ± 213.2</td>
<td>$F(1,65) = 11.9; P &lt; 0.001$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Number of boutons/fiber</td>
<td>73.1 ± 39.2</td>
<td>51.3 ± 34.1</td>
<td>$F(1,65) = 5.9; P &lt; 0.02$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Innervation area</td>
<td>1160.9 ± 631.1</td>
<td>790.7 ± 450.7</td>
<td>$F(1,65) = 4.98; P &lt; 0.05$</td>
<td>65.4 ± 38.4</td>
<td>44.4 ± 24.9</td>
</tr>
<tr>
<td>Terminal fiber length</td>
<td>522.5 ± 283.8</td>
<td>325.9 ± 174.5</td>
<td>$F(1,65) = 6.82; P &lt; 0.01$</td>
<td>522.5 ± 283.8</td>
<td>325.9 ± 174.5</td>
</tr>
<tr>
<td>Innervation area</td>
<td>1030.8 ± 570.9</td>
<td>673.3 ± 436.4</td>
<td>$F(1,65) = 5.28; P &lt; 0.02$</td>
<td>1030.8 ± 570.9</td>
<td>673.3 ± 436.4</td>
</tr>
</tbody>
</table>

Values are means (SD). Comparisons among first 5 items and among last 3, $n = 28, 39, 51,$ and 16 for peripheral, central, extrastriola, and Type II band, respectively.
axons. Although only three efferent fibers were quantified, the largest axonal diameter of the three was 0.78 μm as compared with the smallest afferent diameter (from the 208 units) of 0.86 μm. On penetrating the basement membrane, the parent axon for the efferents extended several hundred microns, much longer than any of the afferents observed. The shortest axonal length of the efferent fibers was 2,474 μm as compared with 1,306 μm for the longest afferent quantified. Numerous very fine branch fibers extended into the epithelium with a high density of en passant and terminal boutons (Fig. 13), and no calyceal terminals were ever observed. The number of terminals on the smallest of the three efferents were 377 as compared with 182 terminals on the largest afferent (bouton) quantified. Although more numerous, the efferent terminals were noticeably smaller than those typically observed for utricular afferents. Consistent with the longer fiber and more numerous branches for efferents, the innervation area of 15,405 μm² for the smallest unit was nearly twice the largest afferent quantified with an innervation area of 7,085 μm². These characteristics for pigeon vestibular efferents were consistent with the findings obtained for semicircular canal efferents in gerbils (Purcell and Perachio 1997).

**DISCUSSION**

The present results describing the morphology and innervation patterns of the utricular macula in pigeons clearly show that there are a number of similarities with other amniote species and yet a number of interesting differences. Our study sought to quantitatively examine morphological properties of afferents that innervate the utricle so that a more complete understanding of afferent structure and terminal field patterns could be obtained for birds, as compared with the otolith organs in other animal classes. To that end, it is unfortunate that very few species have been thoroughly examined, with the major works regarding utricular innervation being reported only for chinchillas (Fernandez et al. 1990) and frogs (Baird and Lewis 1986; Baird and Schuff 1994).

**Morphology of the pigeon utricular macula**

The utricular receptor epithelium in adult pigeons is quite large for an animal of its size. With average dimensions of 1.5 × 1.2 mm, the pigeon utricular macula appears to be comparably larger than that of several other animals studied including frogs (Baird and Schuff 1994), turtles (Fontilla and Peterson 2000), chinchillas (Fernandez et al. 1990), guinea pigs (Werner 1933), and three species of fish (Chang et al. 1992; Platt 1975; Popper and Northcutt 1983). If one compares across animal classes, several morphological features are present in the pigeon utricular macula that may represent general adaptations refined through parallel evolutionary paths for motion detection in terrestrial/aerial environments. For example, as classically described, type I hair cells are present in reptiles, birds, and mammals (Jørgensen 1970, 1974; Vin- nikov et al. 1965; Wersäll 1956; Wersäll and Bagger-Sjöbäck 1974). In adult amniotes, type I and type II hair cells can be differentiated by a number of morphological (Kevetter et al.
morphological features have been described for cells. Sjoøergensen and Lang (1990) and Eatock et al. (1994) noted that only type II hair cells exist (Wersäll et al. 1988, 1995; Schessel et al. 1991). In terms of otolith organs, all three fiber types have also been observed in chinchillas (Fernandez et al. 1990), but only bouton afferents have been noted in frogs (Baird and Schuff 1994). The cytoarchitectural differences in the type I and type II hair cell distribution in the pigeon utricular macula determine much of the variation in location of different afferent innervation patterns. Calyx dimorph afferents, along with type I hair cells, were essentially confined to the striola region. Bouton fibers innervated the exclusive type II hair cell regions of the extrastriola and type II band. For comparison, chinchilla calyx fibers were restricted to the striola, bouton afferents were restricted to innervating the peripheral zones, and dimorph afferents were found throughout the macula (Fernandez et al. 1990). In chinchillas, both simple and complex calyx endings containing only one or multiple type I hair cells, respectively, have been described (Fernandez et al. 1990). However, in pigeons, no simple calyx afferents were ever observed because all calyx units innervated at least two hair cells. Further, the calyceal terminals were generally much larger in pigeons, with a mean number of 7.1 type I hair cells innervated by a calyx afferent as compared with the average two cells/afferent for chinchillas. The average axonal diameter of calyx fibers was comparable between pigeons and chinchillas, and both species exhibited little calyx afferent branching.

The pigeon utricular dimorph fibers had the most varied morphology of all afferents. Simple dimorph afferents were similar in structure to those reported for chinchillas (Fernandez et al. 1990) and contained only one calyx with one hair cell and one bouton terminal. The most complex pigeon utricular dimorph afferent consisted of five calyces and 129 bouton endings as compared with the chinchilla with up to seven calyces and >50 bouton terminals. The relative increase in the number of calyces and fewer number of bouton terminals in chinchillas as compared with pigeons support the idea that there may be many more type I hair cells in chinchillas; this may be an adaptive morphological trait. Correspondingly, there are fewer bouton units in chinchillas than pigeons. Regional differences in dimorph distribution between pigeons and mammals were also found in that chinchilla dimorphs were numerous throughout the macula epithelium, including both the striola and extrastriola (Fernandez et al. 1988, 1990). In addition, the average terminal field area (deduced from terminal field diameter) for chinchilla dimorph afferents was larger than that noted for pigeons. However, when only dimorphs innervating the striola region were compared between the two species, chinchilla afferents had smaller terminal fields than pigeons. Pigeon dimorph afferents had an average of 17 bouton terminals, similar to chinchilla striola dimorphs, but slightly less than extrastriola dimorphs.

Bouton afferents had the smallest axonal diameters among the three kinds of fibers but on average were larger than those of either chinchillas (Fernandez et al. 1990) or frogs (Baird and Schuff 1994). Pigeon utricular bouton afferents had innervation areas that were comparable to those of chinchillas but

Afferent innervation patterns

The classification of pigeon utricular afferents into calyx, dimorph, and bouton innervation patterns was based on the descriptions provided from previous studies of both utricle and semicircular canal afferents (Fernandez 1990; Fernandez et al. 1988, 1995; Schessel et al. 1991). In terms of otolith organs, all three fiber types have also been observed in chinchillas (Fernandez et al. 1990), but only bouton afferents have been noted in frogs (Baird and Schuff 1994). The cytoarchitectural differences in the type I and type II hair cell distribution in the pigeon utricular macula determine much of the variation in location of different afferent innervation patterns. Calyx dimorph afferents, along with type I hair cells, were essentially confined to the striola region. Bouton fibers innervated the exclusive type II hair cell regions of the extrastriola and type II band. For comparison, chinchilla calyx fibers were restricted to the striola, bouton afferents were restricted to innervating the peripheral zones, and dimorph afferents were found throughout the macula (Fernandez et al. 1990). In chinchillas, both simple and complex calyx endings containing only one or multiple type I hair cells, respectively, have been described (Fernandez et al. 1990). However, in pigeons, no simple calyx afferents were ever observed because all calyx units innervated at least two hair cells. Further, the calyceal terminals were generally much larger in pigeons, with a mean number of 7.1 type I hair cells innervated by a calyx afferent as compared with the average two cells/afferent for chinchillas. The average axonal diameter of calyx fibers was comparable between pigeons and chinchillas, and both species exhibited little calyx afferent branching.
larger than those reported for frogs. The average number of 60 bouton endings per afferent in pigeons was significantly larger than that reported for chinchillas (35/afferent) (Fernandez et al. 1990) but much less than the ~150/afferent (depending on regional location) reported for frogs (Baird and Schuff 1994). These results suggest that pigeon bouton afferents have smaller innervation densities than frog afferents, but both species have higher densities as compared with chinchilla afferents.

Regional organization of afferent innervation

In our pigeon sample, calyx afferents innervating anterior portions of the macula had larger innervation areas than afferents innervating either the central or posterior regions. In chinchillas, the dimorph afferents were compared across the striola, juxtastriola, and extrastriola zones (Fernandez et al. 1990). It was found that striola dimorphs had more compact terminal trees with fewer calyx and bouton endings than dimorph afferents innervating the peripheral extrastriola or juxtastriola regions. Dimorph afferents terminating in the chinchilla juxtastriola and extrastriola had similar numbers of calyces and a similar frequency of subepithelial bifurcations. In addition the axonal thickness for the chinchilla dimorph units were correlated with location: the striola dimorph afferents were thicker than the fibers in the rest of the macula. In our sample of pigeon dimorph afferents, only the posterior located fibers tended to contain larger calyceal terminals with more type I hair cells than either central or anterior fibers. Corresponding fiber lengths and innervation areas for the posterior dimorph afferents were also observed. No other statistically different morphological properties were identified when collapsed across the anterior, central, and posterior thirds or medial-lateral striola regions of the utricle.

Regional differences between pigeon bouton afferents located in the anterior, central, or posterior thirds were not found to differ statistically. However, examination of the distribution plots (Fig. 12) showed that bouton morphology was correlated with macula locations, where afferents located near the edges of the macula had significantly more bouton endings, more branch fibers, longer axonal lengths, and larger innervation areas than those located centrally. Comparisons between boutons in the type II band with those in the extrastriola also revealed interesting differences. Type II band fibers had fewer numbers of bouton terminals, shorter fiber lengths, and smaller innervation areas as compared with bouton afferents in the rest of the macula. An opposite pattern was noted for bouton afferents in frogs, where the striola bouton afferents had thicker parent axons, fewer subepithelium bifurcations, and larger terminal fields than the extrastriola bouton afferents (Baird and Schuff 1994). In pigeons, the type II band bouton afferents were the thinnest fibers among the bouton fibers innervating the utricular macula, which contrasts with the pattern observed in frogs.

In the present study, an ordered array of innervation angles for the utricular afferents being directed either perpendicular (similar to frogs) (Baird and Schuff 1994) or parallel to the reversal line was noted. Except for hair cells located on either side of the reversal line, the morphological polarizations of neighboring utricular hair cells all have similar directional orientations (Baird and Lewis 1986; Jorgensen and Andersen 1970; Lindeman 1969; Platt 1975; Rosenhall 1970; Wersäll et al. 1966). Thus as afferents develop terminal fields, branching fibers directed either parallel or perpendicular to the reversal line would ensure innervation of hair cells with similar morphological polarizations (Fig. 1). Of particular note, were the pigeon utricular afferents that innervated the type II band. These afferents were either dimorph or bouton, with extended branch fibers and terminals only to the hair cells within one-half of the type II band width. Because the reversal line generally lies in the center of the type II band (present results; Dye et al. 1999), the terminal field should not cross the central area if only hair cells with similar polarity directions were being innervated. Thus the results suggest that dimorph afferents innervating the type II band terminate on only hair cells with similar morphological polarization. The innervation angle could also have physiological significance because a given afferent’s directional sensitivity to motion is largely determined by the morphological polarizations of the hair cells it innervates. Directional selectivity would be reduced if afferents innervated oppositely polarized hair cells. In a previous study of pigeon utricular afferent response properties (Si et al. 1997), it was found that most afferents were one dimensional and narrowly tuned to directional motion. This suggests that pigeon otolith afferents innervate hair cells with very similar vector directions.

Functional considerations of afferent innervation patterns

A number of investigators have examined the correlation between morphological structure and functional responses of vestibular afferents. In semicircular canal afferents for a number of different species, the physiological response properties can be correlated with axonal diameter (Baird et al. 1988; Goldberg and Fernandez 1977; Honrubia et al. 1981; O’Leary et al. 1976; Schessel 1991), epithelial location (Honrubia et al. 1981; 1989; Myers and Lewis 1990; Baird et al. 1988; Schessel 1991), terminal morphology and terminal field arbor volume (Baird et al. 1988; Myers and Lewis 1990; Schessel 1991). Intracellular labeling of bullfrog crista afferents demonstrated that irregularly firing thicker afferents innervated hair cells in the central region of the crista, while regularly firing thinner afferents innervated the peripheral regions (Honrubia et al. 1981, 1989; Myers and Lewis 1990). Evidence from squirrel monkeys (Lysakowski et al. 1995) and chinchillas (Baird et al. 1988; Fernandez et al. 1988) indicates that irregularly firing higher gain dimorph and low gain calyx afferents innervated the central zones of the crista. Lower gain regularly firing bouton afferents innervated the peripheral regions. On the other hand, one paper provides contradictory results. In chicken semicircular canal afferents, it was shown that calyx units had tonic and bouton units had phasic response properties using intraaxonal injections of Lucifer yellow in isolated labyrinths (Yamashita and Ohmori 1990). In addition, these authors stated that the axonal diameters were thicker in the fibers with bouton endings than the calyx endings.

Less information is available for morphophysiological studies of otolith afferents. In frogs and chinchillas, it appears that the discharge regularity is more correlated with fiber diameter, macular location, and encoding properties than with the terminal morphology (Baird and Lewis 1986; Goldberg et al. 1990). Our previous study on pigeon otolith afferent response properties showed that most afferent fibers are very sensitive to
linear acceleration, with higher gains than those observed for most other species to date (Si et al. 1997). Pigeon utricular afferents were shown to have increasing gain as stimulus frequency increased with constant phase values that were advanced relative to acceleration. Whether these phasic responding afferents were bouton type or calyx type is unknown. How the location of afferents in the macula is a determinant for response properties is currently under study.

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