Afferent Innervation Patterns of the Saccule in Pigeons

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Zakir, M., D. Huss, and J. D. Dickman. Afferent innervation patterns of the saccule in pigeons. J Neurophysiol 89: 534–550, 2003; 10.1152/jn.00817.2001. The innervation patterns of vestibular saccular afferents were quantitatively investigated in pigeons using biotinylated dextran amine as a neural tracer and three-dimensional computer reconstruction. Type I hair cells were found throughout a large portion of the macula, with the highest density observed in the striola. Type II hair cells were located throughout the macula, with the highest density in the extrastriola. Three classes of afferent innervation patterns were observed, including calyx, dimorph, and bouton units, with 137 afferents being anatomically reconstructed and used for quantitative comparisons. Calyx afferents were located primarily in the striola, innervated a number of type I hair cells, and had small innervation areas. Most calyx afferent terminal fields were oriented parallel to the anterior-posterior axis and the morphological polarization reversal line. Dimorph afferents were located throughout the macula, contained fewer type I hair cells in a calyceal terminal than calyx afferents and had medium sized innervation areas. Bouton afferents were restricted to the extrastriola, with multi-branching fibers and large innervation areas. Most of the dimorph and bouton afferents had innervation fields that were oriented dorso-ventrally but were parallel to the neighboring reversal line. The organizational morphology of the saccule was found to be distinctly different from that of the avian utricle or lagena otolith organs and appears to represent a receptor organ undergoing evolutionary adaptation toward sensing linear motion in terrestrial and aerial species.

INTRODUCTION

It can be generally stated that in amniote vertebrates the saccule functions to detect tilts relative to gravity and vertical linear accelerations of the head (Fernández and Goldberg 1976; Fernández et al. 1972). Saccular afferents project to the vestibular nuclei as well as the cerebellum (Dickman and Fang 1996; Gacek 1969; Kevetter and Perachio 1986; Lorente de Nó 1933), and brain stem neuron responses to stimulation of the saccule have been widely reported (Hwang and Poon 1975; Ono et al. 2000; Wilson et al. 1978). In addition, accumbal-mediated responses are known to directly contribute to vestibular compensatory neuromotor behaviors, such as the vestibulo-ocular, vestibulo-colic, and vestibulo-sympathetic reflexes (Curthoys 1987; Flur and Mellström 1970, 1971; Uchino et al. 1997a; Zakir et al., 2000). However, in other animal classes, the saccule may have a different function or serve additional roles. For example, in fish the saccule responds to auditory stimuli at high frequencies and is directionally tuned to whole-body accelerations across a lower frequency bandwidth (Edds-Walton and Fay 1995; Fay 1995). In amphibians, saccular afferents are sensitive to substrate-borne vibrations as well as linear head motion (Baird and Lewis 1984; Lewis et al. 1982). In birds, which possess three otolith organs including the utricle, the saccule, and the lagena, differentiation between function for the differing otolith receptors remains to be determined. Vertical linear accelerations of the head have been shown in several bird species to elicit vestibular evoked potentials, which appear to be in part due to saccular activation (Jones 1992; Jones et al. 1998). Other investigations of avian otolith function to date have only examined neural responses from utricle afferents (Si et al. 1997) and linear vestibulo-ocular responses in pigeons (Dickman and Angelaki 1999).

In terms of structure, more information regarding the saccule has been provided. The saccule lies in an approximately parasagittal plane on the medial wall of the vestibule. The saccular macula is elongated through the anterior-posterior dimension. The neuroepithelium is densely populated with receptor hair cells, each having a distinct morphological polarization (Lowenstein and Wersäll 1959). Polarizations of the hair cells differ across the macular surface, being oriented away from an S-shaped curve that defines the reversal line for the entire epithelium (Platt 1975; Rosenhall 1970). Following Wersäll’s (1956) classification of two hair cell types based on morphology, the saccular macula in birds (as in all amniotic species) has been shown to contain both type I and type II hair cells (Jørgensen and Anderson 1973; Rosenhall 1970). Differences exist in the regional distribution of the two cell types in the saccular macula, with a high-density (nearly exclusive) of type I cells in the central region and type II cells being predominant in the periphery of the macula.

The primary afferents that innervate vestibular otolith receptors have been known to differ in their structure and termination patterns since first described by the pioneer anatomical works of Retzius (1884), Cajal (1908, 1909), and Lorente de Nó (1926). More recently, axonal labeling techniques have provided insight into afferent innervation patterns. In frogs, afferents innervating the utricle and lagena have been described, with fibers of varying thickness exhibiting en passant and terminal boutons (Baird and Lewis 1986; Baird and Schuff 1994). Afferents that innervated hair cells in the striola region generally had thick axons and large terminal fields, while
extrastriola fibers were thin and had smaller terminal fields. For vertebrates that possess both type I and II hair cells in their otolith organs, only the innervation patterns of utricle afferents in chinchillas (Fernández et al. 1990) and pigeons (Si et al. 2003) have been thoroughly examined. In these animals, three classes of utricle afferents have been identified including calyx, dimorph, and bouton afferents. In chinchillas, calyx afferents were only located in the striola region, had thick axons, and had a calyceal terminal that enclosed only type I hair cells. Bouton units were only found in the extrastriola, had thin axons that ramified into fine branches with en passant and terminal boutons, and innervated exclusively type II hair cells. Dimorph afferents were found throughout the macula, varied in size, and contained both calyceal and bouton terminals to innervate both types of hair cells. In pigeon utricles, both calyx and dimorph afferents were confined to the striola region that flanked a narrow band of type II cells running along the reversal line. Bouton afferents were primarily located in the large extrastriola regions, as well as the type II band. For the sacculae, Ross (1986) described both calyx and dimorph afferents reconstructed from ultrastructural serial images in a small portion of the macula in rats. In fish, aspects of the ultrastructural features for saccular afferents have also been described (Chang et al. 1992; Popper and Saïdel 1990), as well as bouton afferent innervation correlating morphological polarization with response spatial tuning (Lu and Popper 2001). However, to the best of our knowledge, prior to this paper, no inclusive studies of afferents innervating the saccular macula for any amniote species have been performed. In addition, there appears to be no information regarding afferent innervation of any of the avian vestibular receptors, except for the recent report describing utricular afferents in pigeons (Si et al. 2003). The current investigation constitutes part of a larger work studying the morphology and innervation of vestibular receptors in birds. Some of the results have been presented in abstract form (Zakir et al. 2001).

METHO?S

Neural tracer application

The experiments were performed in adult pigeons (Columba livia), that ranged in age between 1 and 3 yr. The methods were conducted in accordance with the guidelines established by the National Institutes of Health and by the approval of the Institutional Animal Care and Use Committee. For the surgical application of neural tracers, animals were anesthetized and the bony labyrinths exposed. Both the horizontal and posterior canals were opened, and an intralabyrinthine perfusion was performed with a 5 ml volume of 2% glutaraldehyde, 1.5% paraformaldehyde, and 1% Acrolein solution. The animal was subsequently perfused transcardially with a 2% glutaraldehyde–1.5% paraformaldehyde solution, and the whole head was placed in the aldehyde fixative for 24 h. The membranous labyrinth was then excised and the saccular maculae were dissected free. In most samples, the otocochial membrane and otocilia were removed using protease (Sigma type XXIV, for 10 min) and gentle retraction. The brain was blocked, frozen, and serially sectioned (50 μm) in the transverse plane.

The saccular maculae and the brain sections were processed for BDA using a modified dianaminobenzidine (DAB) procedure (Brandt and A?parjian 1992). The tissue was incubated for 12 h in a solution of phosphate buffer, 1% Triton-X100, and 0.25% avidin-D–HRP (Vector A-2004). The tissue was then rinsed and reacted using the chromogen DAB with a 1% nickel ammonium sulf?te-cobalt chloride solution. The reaction was initiated by addition of 0.3% H2O2 until a dense reaction product was visualized. The tissue was then rinsed in phosphate buffer. The brain sections were dehydrated and cleared using a series of graded alcohols and xylenes, mounted on glass slides, and the dorsal neuroepithelium was photographed using a Nikon light microscope. The reacted maculae were first photographed in-block from the apical epithelial surface. Next, the saccular maculae were embedded in plastic (Durcupan) and serially sectioned (10 μm thickness) using a rotary microtome. The sections were mounted on glass slides and counterstained (Richardson et al. 1960). In several animals, the saccular maculae (n = 11) were prepared for examination by scanning electron microscopy (SEM). The tissue was rinsed in distilled water, and then dehydrated using a series of graded acetone washes. Next, two final washes in 100% acetone were performed, followed by rinses in increasing concentrations of tetramethylsilane (TMS). The tissue was twice placed in 100% TMS for 15 min and allowed to desiccate at 60°C in an open container. The dried maculae were mounted on aluminum studs and gold coated.

Reconstruction of saccular afferents

The location of the BDA injection site was determined in the brain stem for each animal. In two animals, some vestibular efferent somas were filled with BDA and these animals were eliminated from further analyses. Only animals with no labeled vestibular efferent neurons were included for quantitative study, so that only the terminal innervation patterns of primary vestibular afferent fibers would be present. The sectioned tissue from each saccular macula was examined using video microscopy on a Nikon E600 microscope with DIC infinity optics and drawn using an image analysis and reconstruction program (Neurolucida, 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 distribution bands for hair cell types, the location,
and the hair cell morphological polarization reversal line. The section drawings were used for each animal to locate the reconstructed afferent fibers.

For reconstruction of labeled afferents, only fibers that were darkly stained and sufficiently isolated from other afferents so as not to misidentify terminal processes were traced. Afferents with partial staining (ghost fills), or those that overlapped other afferents and could not be assuredly distinguished were not quantitatively analyzed. Some of these identifiable afferents were included in the overall distribution map when sufficient characterization of fiber type was possible. The regional location of each reconstructed afferent relative to the epithelial borders and the reversal line was obtained. All three-dimensional (3D) reconstructions of identified afferents were performed using X80 magnification (dry lens, 0.95 NA). A number of morphological parameters for each of the reconstructed afferents were quantified, including terminal fiber length, axonal diameter, branch number and diameter, branch order, number of bouton endings, number of type I hair cells contained in calyces, calyx volume, innervation area, and fiber volume. Terminal fiber length was calculated as the sum of all branch fibers after entering the receptor epithelium. Innervation area was calculated by first rotating the reconstructed fiber image in 3D space until an apical view was obtained. Next, the perimeter of the image was drawn in the plane of the macular surface and the area of the drawn contour obtained. The calyx volume was calculated as the product of contour areas by the depth (thickness).

To determine the degree of uptake by the BDA tracer into afferents of various size, three branches of the vestibular nerve distal to Scarpa’s ganglion were examined in two animals. As shown in Fig. 1, the BDA tracer was absorbed and transported by axons of varying diameters. The lumens of all axons in the three branches were measured (100X oil), and the BDA containing fibers were compared against the total population of afferents (Fig. 1B). In our sample of 2,351 total fibers in three branches, axons varied between 0.54 and 8.2 \( \mu \text{m} \), with a mean value of 2.54 ± 1.6 (SD) \( \mu \text{m} \). BDA filled fibers (n = 533) ranged between 0.56 and 6.99 \( \mu \text{m} \), with a mean of 2.4 ± 1.7 \( \mu \text{m} \), which was significantly smaller than non-BDA fibers (n = 1,818), with a mean of 2.6 ± 1.6 \( \mu \text{m} \) \([F(1,2349) = 7.4, P < 0.006]\). As shown in Fig. 1B, the distribution of BDA-filled and the total population of fibers were equivalent, suggesting that little or no bias in BDA uptake by axons of small or large diameter was observed 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 \( \mu \text{m} \) for 8,720 fibers in all branches and a similar distribution of fiber diameters.

All statistical comparison analyses were performed using a commercial package (STATISTICA, Statsoft, Tulsa, OK) on a microcomputer. Proportional relationships were analyzed using Pearson product-moment correlations. Group comparisons were performed using ANOVA and the Scheffé post hoc test.

RESULTS

General morphology of the sacculus macula

Scanning electron microscopy was used to examine the macula surface and characterize the regional density of receptor cells, the peripheral borders, and the reversal line in seven saccules. The macula of the sacculus in adult pigeons is generally triangular shaped, with the larger receptor populated region lying anteriorly and the narrower point posteriorly. At the ventro-anterior surface, an indentation of nonreceptor epithelium exists, forming a larger receptor populated peninsula dorsally and a smaller receptor tail ventrally, as shown in Fig. 2. The reversal line characterized by the opposing morphological polarization of hair cell stereocilia was generally S-shaped as it coursed parallel to the dorsal epithelial border for most of the macula, and then ran ventrally near the posterior pole (Fig. 2). The hair cells were morphologically polarized such that the eccentrically placed kinocilia were directed away from the reversal line in the central macular region (Fig. 2, B and C), as has been classically described (Jørgensen and Andersen 1973; Lindeman 1969; Rosenhall 1970). As the reversal line turned ventrally in the posterior macula, hair cells on opposite sides were polarized in an orthogonal manner (Fig. 2B). In contrast to previous reports, we observed a continuation of the reversal line that extended along the ventral epithelial border to the end of the ventral anterior tail (dashed lines in Fig. 2B). All hair cells located ventral to the continuation line, a band ranging between 10 and 20 cells wide, had stereocilia polarizations directed posteriorly (Fig. 2, B and D). At the extreme dorsal anterior pole, the stereocilia were polarized in an anti-parallel fashion. In addition, the reversal line did not appear to be precisely directed through the macula surface, but rather coursed in a jagged manner, as has been described in several other species (Lindeman 1969). Occasionally, hair cells lying near the reversal line were observed to have the “wrong”
polarization orientation (Fig. 2C, arrow) compared with neighboring cells. Although not quantitatively examined, the length of the kinocilia was observed to vary for different hair cells and regions within the macula. On average, the kinocilium for each hair cell was two to three times greater in length than the tallest stereocilia. Many hair cells in the striola had short kinocilia (Fig. 2C), while cells in the more peripheral regions of the macula often had kinocilia that were 5–10 times longer.

In addition to the SEM tissue, cross-sections of 11 maculae containing afferents with neural BDA tracing were examined. Nine of the 11 saccular maculae were cut in the transverse plane and 2 were cut in the longitudinal plane. The macula can be characterized by the general distribution of type I and type II hair cells. As shown in Fig. 3, both hair cell types were observed through most of the epithelium; however, the largest concentration of type I hair cells was found in the central region of the saccular macula. In the peripheral regions, type II hair cells were more numerous. At the extreme peripheral edges of the epithelium, only type II hair cells were observed. However, in the dorsal macula, type I hair cells often could be observed close to the edge, within three to five cells of the border, as shown in Fig. 3, B and C (left side). Along the reversal line, there was no adjacent band of type II hair cells, as is true for the pigeon utricle and lagena (Jørgensen and Anderson 1973; Rosenhall 1970; Si et al. 2003). Instead, the region adjacent to the reversal line in the striola was densely populated by type I hair cells. For most of the sections, stereocilia were visualized and the reversal line was easily demarcated (Fig. 3A). The borders of the neuroepithelium were measured from the cross-section images. The outline of each section was traced and the 3D reconstruction of the macula surface diagrammed, as shown in Fig. 4. When pooled, the mean length (A-P distance) of the 11 measured saccular maculae was 0.96 ± 0.15 mm and the mean width was 0.55 ± 0.09 mm along the central axes of the macula.

**Afferent types and regional location**

BDA label was observed in over 250 afferents in 11 saccular maculae. Of these, 197 afferents were conclusively identifiable based on terminal profile. There were three major classes of afferent innervation patterns observed, including calyx, dimorph, and bouton fibers, as shown in Fig. 3. The calyx afferents were characterized by their large calyceal terminal that contained type I hair cells. Dimorph afferents had calyces enclosing type I hair cells as well as en passant and terminal boutons innervating type II hair cells. Bouton afferents exclusively innervated type II hair cells with en passant and bouton terminals. The regional location of each identified afferent within the neuroepithelium was determined by measuring the distance from the cell to the dorsal, ventral, anterior, and posterior borders as well as the distance to the reversal line. Each of these five measures were normalized to a proportional value of the total distance for the individual macula. One of the
saccular maculae was chosen at random to serve as a composite surface map, on which the locations of all identified afferents from the 11 saccules could be plotted. The composite map was produced by serial reconstruction of the complete saccular macula. The proportional distance values for each of the 197 identified afferents were then used to position the units on the composite map, as shown in Fig. 4. As apparent from the composite diagram, calyx units \( \text{n}/\text{H11005} \) were found throughout much of the macula, but were primarily located in the central region. Calyx afferents were also observed along the peripheral edge of the dorso-anterior epithelium (Figs. 3, B and C, and 4). However, no calyx afferents were observed in the posterior 20% of the macula nor in the ventral periphery. The distribution of the dimorph afferents \( \text{n} = 53 \) spread throughout the macula, except that only a few dimorphs were found near the reversal line (Fig. 4). In addition, dimorph units were often observed in the peripheral regions of the neuroepithelium. In contrast, bouton afferents \( \text{n} = 58 \) were exclusively located in the peripheral regions along the entire perimeter of the macula.

Based on the distribution of type I and type II hair cells and on the distribution of afferent fiber type, it appears that only two major regional zones exist in the saccular macula. A central region was demarcated using the relative density of type I and type II hair cells, as well as the locations of the calyx afferents, as shown in Figs. 3 and 4. The large central, or striolar, region of the macula contained a high-density of calyx afferents and a much lower density of dimorph afferents. No bouton afferents were observed in the striola region. In fact, 92% (79/86) of the calyx afferents, 47% (25/53) of the dimorphs, and 0% of the bouton units were located in the central striola region (Fig. 4). The extrastriola occupied the remainder of the macula and contained the bouton afferents, a number of equally distributed dimorph afferents, and a few calyx afferents in the dorsal and ventro-posterior epithelium. All of the observed bouton afferents were located within 100 \( \mu \text{m} \) of the peripheral epithelial border, essentially forming a circumferential band around the macula (Fig. 4).

**Afferent innervation patterns**

Of the 197 identified afferents, 137 fibers satisfied our selection criteria to be anatomically reconstructed and quantified using 3D computer analyses. The locations of the reconstructed units in the saccular macula are shown in Fig. 4.
CALYX AFFERENTS. Sixty-three calyx afferents were reconstructed for quantification. As shown in Fig. 5, the size, shape, and structure of the calyx afferents varied widely. The calyx parent axon diameters were large, with a mean of 2.5 μm (Table 1). On entering the neuroepithelium, the majority (58/63) of the calyx afferents terminated from the parent axon with no branch points, as shown in Fig. 5. As such, the calyx fibers exhibited the simplest arborization patterns of the three types of afferents. Correspondingly, both the length and the volume of the calyx axons within the neuroepithelium were small (Table 1). Although there was diversity in calyx structure, two major profiles were noted. The first profile appeared rounded or "flower" shaped and contained type I hair cells that lay in close approximation (Figs. 5, A, C, and D, and 6, A–D and H–K). The second, although less numerous major calyx profile, consisted of a thin rectangular shaped structure that contained an array of hair cells arranged along a linear axis (Figs. 5B and 6, E–G). The size and structure of the calyceal terminals also varied among the afferents. The simplest calyces contained only a few type I hair cells (Figs. 5, A and B, and 6A), while the most complex calyces contained many type I hair cells (Figs. 5, C and D, and 6, H–K). The number of type I hair cells/calycal terminal ranged from 2 to 18, with a mean value of 6.5 (Table 1). No calyceal terminals or calyx afferents were observed that innervated only one type I hair cell. To examine the regional distribution of calyceal complexity, all calyx afferents were plotted according to macula location versus number of type I hair cells per calyceal terminal, as shown in Fig. 7. Although calyx afferents with the largest calyceal terminals innervated macula regions close to the reversal line, many smaller calyx afferents also innervated this region. Therefore there was no significant correlation between either number of type I hair

**FIG. 5.** Calyx afferents. Four BDA-labeled calyx units are shown. Photomicrographs (digital images, top), side (middle), and top (bottom) 3D reconstruction views are shown in each panel. Contour outlines (green) of calyceal terminal and innervation angles (arrows) are represented. A, C, and D: simple, intermediate, and large flower profile calyx afferents. B: linear profile calyx afferent. Scale bar = 10 μm.

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

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Calyx</th>
<th>Dimorph</th>
<th>Bouton</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance to reversal line</td>
<td>1.7–202</td>
<td>45–245</td>
<td>33–426</td>
</tr>
<tr>
<td>Axon diameter (μm)</td>
<td>1.5–3.7</td>
<td>1.4–3.4</td>
<td>1.2–3.2</td>
</tr>
<tr>
<td>Branch order</td>
<td>1.0–3.0</td>
<td>2.0–8.0</td>
<td>2–13</td>
</tr>
<tr>
<td>Terminal fiber length (μm)</td>
<td>1.0–9.0</td>
<td>10–379</td>
<td>78.7–638.0</td>
</tr>
<tr>
<td>Type I hair cells/terminal</td>
<td>2–18</td>
<td>6–8</td>
<td>345.8 ± 141.9</td>
</tr>
<tr>
<td>Calyceal volume (μm³)</td>
<td>52–11522</td>
<td>15–4600</td>
<td>—</td>
</tr>
<tr>
<td>Bouton terminals</td>
<td>—</td>
<td>3–44</td>
<td>10–78</td>
</tr>
<tr>
<td>Innervation area (μm²)</td>
<td>16–385</td>
<td>95–1124</td>
<td>97.1–1260.9</td>
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cells per calyceal terminal nor calyx volume and distance from the reversal line. However, most of the complex calyx afferents with calyceal terminals containing more than seven type I hair cells were located in the central striola zone near the reversal line (Figs. 6 and 7). There appeared to be no regional difference between afferents innervating the anterior or posterior regions of the macula in terms of calyceal size (Figs. 7). The calculated volume for the calyces in the calyx afferents varied widely from 52 to 11,522 µm³ (Table 1). There was an expected significant correlation between calyceal terminal size and number of type I hair cells in the calyx ($r^2 = 0.44; P < 0.05$) as shown in Fig. 8. There was also a proportional relationship between axon diameter and calyceal volume ($r^2 = 0.14, P < 0.05$). Due to the lack of axonal branching and short fiber lengths, the innervation areas for calyx afferents were small (Table 1). As shown in Fig. 9, calyx afferents varied little in their branching patterns and terminal fiber lengths, forming a near homogeneous population when viewed across the macula surface. Thus differences among units in innervation area appeared to be solely due to variation in size of calyceal terminal (Fig. 9). From the reconstruction diagrams of the surface view of the terminal field for each fiber, a measure of innervation angle was determined. A line was drawn through the long axis of the terminal field (Fig. 5), and then the angle between the field axis line and the ventral edge of the macula was obtained. By definition, an angle of 0° indicated an innervation angle directed from anterior to posterior. A 90° angle indicated an innervation angle directed toward the dorsal edge. The innervation angles for all of the calyx afferents are shown in Fig. 10. Most (87%) of the calyx afferents had innervation fields that ran parallel to the anterior-posterior axis of the macula. As a result, many of the calyx fibers had innervation angles that ran parallel to the reversal line of the striola region.

**DIMORPH AFFERENTS.** The dimorph afferents contained both calyceal and bouton terminal endings, allowing these afferents to innervate both type I and type II hair cells, as shown in Fig. 11. Dimorph afferents were broadly located throughout the macula in both the central striola and extrastriola zones (Figs. 3 and 4). In our sample, 53 dimorph afferents were identified, of which 39 were completely reconstructed for quantification (locations shown in Fig. 5). The diameter of the dimorph parent axons were also large and were not significantly different from calyx afferents (Tables 1 and 2). After entering the neuroepithelium, dimorph afferents varied greatly in the size and complexity of their arborization patterns, as shown in Fig. 12. Simple dimorphs contained a single branch fiber off the parent axon, while the most complex afferent quantified contained branch fibers to the eighth order. The simplest innervation patterns observed in dimorphs consisted of afferents with a single calyx and a single branch fiber terminating with a few bouton terminals (Figs. 11A and 12A). Often, branch fibers arose at the base, or off the side of, the calyceal terminal. These simple dimorphs had between one and several type I hair cells encased in the calyceal terminal. More complex dimorph afferents had intermediate innervation patterns that consisted of one calyceal terminal and several branch fibers with a number of en passant and terminal boutons (Figs. 11B and 12, C–E). The most complex dimorph afferents had innervation patterns that consisted of two calyces and numerous bouton terminals (Figs. 11C and 12, I–K). None of the observed saccule di-
morphs had more than two calyceal terminals. For the 39 reconstructed dimorphs, there were 69 total calyceal terminals, 15 of which contained a single hair cell. Thirty dimorphs had two calyceal terminals and nine had a single calyx. Although the number of type I hair cells in calyceal terminals for dimorph afferents varied between 1 and 6, the mean value of 3.2 was significantly less than the average 6.7 observed for calyx afferents; as were the calyceal volumes (Tables 1 and 2). In terms of en passant and terminal boutons, the dimorph afferents ranged between 3 and 44, with a mean value of 12.5 boutons per fiber (Table 1). There was no relationship between regional location in the saccular macula and number of boutons per afferent (Fig. 9). Complex and simple dimorphs appeared to be equally distributed throughout the macula (Fig. 12).

In dimorph afferents, larger branching patterns resulted in larger areas of innervation of the saccular epithelial surface, with a mean area of 413.4 \( \mu \text{m}^2 \) (Table 1). The innervation areas for dimorph afferents were significantly correlated with the fiber branch order \((r^2 = 0.27, P < 0.05)\), the axonal lengths \((r^2 = 0.60; P < 0.05)\), and number of bouton terminals \((r^2 = 0.12; P < 0.05)\), as shown in Fig. 13. Compared with calyx afferents, dimorph afferents innervated significantly larger portions of the macula surface (Fig. 9). In fact, only the simplest dimorph structures that contained a single calyceal terminal and one or a few branch fibers had innervation areas that were comparable to calyx afferents. There was no relationship between regional location relative to the reversal line and innervation area. As also apparent from the distribution plots of Fig. 9, there were no regional differences in branch order, terminal fiber length, or number of bouton terminals for dimorphs innervating the saccule.

BOUTON AFFERENTS. The bouton afferents were characterized by their innervation patterns that consisted exclusively of en passant and terminal boutons (Fig. 3) and appeared to innervate only type II hair cells, as shown in Fig. 14. A total of 58 bouton afferents were identified, all being located in the extrastriola.
(Fig. 4), and 35 bouton units were reconstructed for quantitative comparisons (locations shown in Fig. 4). The mean diameter of 2.1 μm for the bouton parent axons was significantly smaller than those of either the calyx or dimorph afferents (Tables 1 and 2). As shown in Fig. 15, the bouton afferents also varied in the size and complexity of their innervation patterns. The axons typically entered the macula without branching, although a few of the fibers had one branch beneath the neuroepithelium. The simplest bouton afferents quantified contained only two branch fibers and a few bouton terminals (Fig. 15A), while the most complex afferents had branches up to the 13th order with many terminals (Figs. 14, B and C, and 15, D–K). Similar to the calyx and dimorph afferents, bouton innervation patterns could either form a more amorphous flower profile (Figs. 14A and 15, B–D and H–K) or a more linear profile (Figs. 14B and 15, E–G). There appeared to be no correlation between fiber complexity and macular location (Fig. 15). Since the bouton afferents had the largest number of branch fibers with many long processes, the axonal lengths were significantly the largest of the three groups of fiber types (Tables 1 and 2). The regional distribution of bouton afferents plotted as a function of fiber length, number of branches, and innervation area are shown in Fig. 9 for comparison to the calyx and dimorph afferents. The innervation patterns of the bouton afferents were divergent, but several features appeared to be consistent among units as compared with other fiber types. First, the number of bouton terminals per afferent varied (Table 1) but was significantly more numerous than that observed for dimorph afferents (Table 2). In fact, the number of bouton terminals in bouton afferents was nearly four times that observed for the dimorph afferents, as observed by the regional distribution of both fiber types (Fig. 9). Second, due to the
larger number of branch fibers for the bouton afferents, the overall fiber lengths were significantly larger than either the dimorph or calyx afferents (Table 2). The innervation areas for the bouton afferents were also the largest of the three afferent types (Table 2), averaging 614.2 μm² (Table 1; Fig. 9). Similar to the dimorph afferents, the bouton afferent innervation area was also correlated with the number of fiber branches ($r^2 = 0.27; P < 0.05$), axonal length ($r^2 = 0.62; P < 0.05$), and number of bouton terminals ($r^2 = 0.11; P < 0.05$), as shown in Fig. 13. Like the dimorph afferents, the majority of the innervation angles for the bouton afferents were oriented perpendicular to the reversal line (Fig. 10).

DISCUSSION

These results clearly show that the saccular macula in pigeons is organized quite differently from the other two avian otolith organs, including the utricle and lagena. Our study sought to quantitatively examine morphological properties of afferents that innervate the saccule as part of a larger overall effort to elucidate the organized structure of the vestibular receptors in birds. Beyond a few ultrastructural observations (Chang et al. 1992, Popper and Saidel 1990) and a single recent study of afferent innervation of fish saccules (Lu and Popper 2001), to the best of our knowledge, the present study comprises the first comprehensive investigation of saccular innervation in any amniote. In fact, in terms of afferent innervation of otolith receptor organs, only the utricle has been thoroughly examined, with the major works being reported for chinchillas (Fernández et al. 1990), frogs (Baird and Lewis 1986; Baird and Schuff 1994), and pigeons (Si et al. 2003).

Morphology of the saccular macula

The results of this study coincide well with those of previous reports indicating that the general morphology of the saccular macula is similar for most avian species in terms of the shape, size, and receptor cell distribution. For example, our results are in close agreement with those of Rosenhall (1970), who described a triangular-shaped pigeon macula with a dense concentration of type I receptor cells over large portions of the neuroepithelium. Similar results were observed in cormorants (Jørgensen and Andersen 1973). In pigeons, the reversal line forms a S-curve through the central macula to the ventral-posterior region, where the line turns anteriorly and courses to the edge of the epithelium. To our knowledge, the morphological polarization maps outlined in this study were the first to observe an antiparallel opposition in the anterior dorsal edge and a strict region of posteriorly directed polarizations along the ventral edge. It is reasonable to suggest that saccular afferents in pigeons not only encode upwardly/downwardly directed accelerations, but also linear motions directed fore/aft (through excitation). How these signals are processed through convergence by central vestibular neurons remains an open question. Recently, afferents innervating the ventral saccular macula in cats were shown to provide monosynaptic excitatory input to vestibular nuclei neurons while afferents innervating the opposite side of the reversal line provided only disynaptic inhibitory input (Uchino et al. 1997b). Whether similar functional connection patterns exist in birds is presently unknown. The saccular reversal line placement appears to be quite varied among bird species. For example, in cormorants, a more centrally located reversal line (Jørgensen and Andersen 1973) has been observed. In many fish (Edwards-Walton and Fay 1995, Platt 1975; Popper and Northcutt 1983; Popper and Saidel 1990) and the few mammals examined (Lindeman 1969; Spoendlin 1964), the saccular reversal line is centrally placed.

Of interest is the comparison between the saccule and the other two vestibular otolith organs in birds, the utricle and lagena. One striking difference in the three avian otolith organs is the existence of a band of four to eight type II hair cells that straddle the central reversal line in the utricle and lagena striolar regions (Jørgensen 1970; Rosenhall 1970; Si et al. 2003). In birds, on either side of the type II band lies a region of dense concentration of type I hair cells that is fairly narrow, occupying only approximately 30–35% of the utricle and lagena epithelial surfaces. A large portion of the maculae in both organs is populated exclusively by type II hair cells. As veri-
fied in this study, the avian saccular macula has no type II band of cells along the reversal line in the striola (Jørgensen and Andersen 1973; Rosenhall 1970). In the present work, hair cell counts were not performed, but few dimorph afferents were observed in the central striola zone, suggesting that few type II hair cells are located there. In fact, it is striking that the central macula is dominated by calyx afferents. In addition, the type I receptor population of the avian saccule has extended to cover nearly 80% of the macula surface, far beyond that observed for either the lagena or utricle in birds. The highest density of type II hair cells was seen in the extrastriola, similar to the previous reports (Jørgensen and Andersen 1973; Rosenhall 1970). The morphological organization and lack of a type II band in the avian saccular macula resembles the pattern observed in mammalian otolith organs (Fernández et al. 1990). In chinchillas, the utricular macula can be proportioned into three separate

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Mean</th>
<th>Sheffe Post Hoc</th>
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<tbody>
<tr>
<td>Axon diameter</td>
<td>Calyx Dimorph</td>
<td></td>
</tr>
<tr>
<td>Branch order</td>
<td>2.5 2.5</td>
<td>NS P &lt; 0.003 P &lt; 0.03</td>
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<tr>
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<tr>
<td>Calyceal volume</td>
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<td>P &lt; 0.0001 P &lt; 0.0001 P &lt; 0.0001</td>
</tr>
<tr>
<td>Number of type I cells/terminal</td>
<td>3548.3 1356.5</td>
<td>P &lt; 0.0001 — —</td>
</tr>
<tr>
<td>Innervation area</td>
<td>200.4 413.4 614.2</td>
<td>P &lt; 0.0001 P &lt; 0.0001 P &lt; 0.0001</td>
</tr>
<tr>
<td>Number of bouton terminals</td>
<td>6.5 3.2 2 —</td>
<td>P &lt; 0.0001 — —</td>
</tr>
<tr>
<td>Innervation density</td>
<td>0.039 0.083 0.097</td>
<td>P &lt; 0.001 P &lt; 0.001 NS</td>
</tr>
</tbody>
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NS, not significant.
areas. The striolar region has a 2:1 ratio of type I/type II hair cells, while the juxtastriola and extrastriola regions have a more equivalent mixture of type I and type II hair cells (Fernández et al. 1990). Further, in mammalian otolith organs the type II band is absent and the regions adjacent to the reversal line in the central striola contain the highest concentration of type I hair cells (Fernández et al. 1990).

**Afferent innervation patterns**

In pigeon saccules, we observed three major classes of afferent innervation patterns including calyx, dimorph, and bouton fibers that were based on the descriptions provided from previous studies (Fernández et al. 1988, 1990). These vestibular afferent innervation patterns are apparently similar for all amniote vertebrates, which contain both type I and type II hair cells (Fernández et al. 1990). Further, in mammalian otolith organs the type II band is absent and the regions adjacent to the reversal line in the central striola contain the highest concentration of type I hair cells (Fernández et al. 1990).

The cytoarchitectural differences in the type I and type II hair cell distribution in the pigeon saccular macula determine much of the variation in location of different afferent innervation patterns. Since calyx afferents exclusively innervate type I hair cells, it was predicted and confirmed that these fibers would be densely located in the central striola region. Some calyx afferents were also located in the peripheral regions, most notably the dorsal epithelium. These locations included a large portion of the macula surface, which can be contrasted to the much more focused concentration of calyx afferents exclusively found near the reversal line of the smaller striola region in the pigeon utricle (Si et al. 2003). Avian saccular calyx afferents were observed to have a large range in calyceal terminal size but appear to be generally much larger than those noted for other animals. For example in mammals, complex utricle calyx afferents were observed to contain between two and four hair cells, with an average of less than three (Fernán-
dimorph and bouton afferents, saccular calyx afferents had the shortest fiber lengths, fewest branches, and smallest innervation areas. One notable feature for saccular calyx afferents was their consistent tendency to have innervation angles oriented along the anterior-posterior axis of the macula and parallel to the reversal line. These angles were similar for both the linear and flower type profiles. It is possible that the innervation angles represent a result established through guidance cues from neighboring type I hair cells during calyx afferent development. Other than exclusive type I hair cell inclusion into the calyceal structure, the cues could also serve to impede the possibility of an afferent innervating hair cells on opposite sides of the reversal line. Based on their small innervation areas, the innervation angles, and the SEM surface images of stereocilia orientations, saccular calyx afferents innervate hair cells in tight clusters all with similar morphological polarizations.

The pigeon saccular dimorph afferents were more diversely spread in the macula to essentially include all regions of the neuroepithelium. This pattern is quite similar to that observed in chinchillas (Fernández et al. 1990). In terms of structure, the dimorph afferents had the most diverse morphology of all the afferents and varied widely from quite simple innervation patterns to those of much greater complexity. Unlike the calyx afferents, many dimorphs were observed to have simple calyceal terminals containing a single type I hair cell, again more similar to the mammalian pattern (Fernández et al. 1990). Most pigeon dimorph afferents had two calyceal terminals, which is rare for calyx afferents. As a comparison, the dimorph calyceal terminals were significantly smaller and contained approximately one-half the number of hair cells compared with calyx afferents. Branching patterns varied greatly; however, in our sample, there was no apparent relationship between dimorph afferent complexity and location in the macula. Thus simple and complex dimorphs appeared to be homogeneously distributed. The angle of the innervation field for dimorph afferents was opposite to that observed for calyx afferents, with the terminal fields of most dimorphs being oriented dorso-ventrally in the macula.

In contrast, saccular bouton fibers innervated only the extrastriola. Since type II hair cells were previously noted in both the central and peripheral regions of the saccular macula (Jørgensen and Andersen 1973; Rosenhall 1970), we predicted that bouton afferents would be more widely distributed similar to the pattern observed in the pigeon utricle (Si et al. 2003). This was not the case, and all of the centrally located type II hair cells were innervated by dimorph afferents. Bouton afferents differed in complexity and could form either the linear or flower-shaped profiles. Compared with the calyx and dimorph saccular afferents, bouton fibers had the smallest axon diameters, the largest number of branch fibers, the greatest fiber lengths, and the largest innervation areas. In our sample, bouton afferents on average had nearly four times the number of terminals than did dimorph fibers. In pigeon utricles, bouton fibers with the largest innervation patterns were located near the peripheral borders, but small and large bouton afferents were also scattered throughout the utricle macula (Si et al. 2003). Similar to bird saccular dimorphs, the vast majority of bouton afferents had terminal fields that were oriented dorso-ventrally.
For all of the afferents examined, none were observed to innervate hair cells on both sides of the reversal line. For example, the calyceal terminals of calyx afferents all contained hair cells with similar kinocilia orientations. For dimorph and bouton afferents, no branching was observed to cross the reversal line. Protection from branch fibers innervating opposite sides of the reversal line may be imparted during afferent formation by the innervation angles of the terminal field. Calyx afferents dominated the central striola region and mostly had innervation angles directed parallel to the reversal line. The dimorph and bouton afferents had innervation angles directed dorso-ventrally, but many of these fibers also had innervations that ran parallel to the reversal line. In fact, bouton afferents in the posterior saccular region, were nearly all oriented parallel to the reversal line as it coursed through the macula. Given the methods employed in this study, we cannot exclude the possibility that some small branch fibers of a few dimorph or bouton afferents did innervate hair cells on both sides of the reversal line. In fact, in light of the occasional observation from SEM surface scans that a few hair cells located near the reversal line were polarized incorrectly, one could imagine that some dimorph or bouton afferents did have branches crossing the striola line and these fibers innervated the rogue hair cells. At least, similar to the observations for other otolith organs, afferents crossing the striola line appear to be a rare occurrence (Baird and Schuff 1994; Fernández et al. 1990; Si et al. 2003). The physiological significance of afferents innervating hair cells with similar morphological polarizations has been previously noted and is also reflected in the response properties of otolith afferents. For example, the vast majority of utricle and saccular afferents are cosine tuned, with one-dimensional directional and frequency response characteristics (Dickman et al. 1991; Fernández and Goldberg 1976; Si et al. 1997). If otolith afferents innervated hair cells with even moderately different morphological polarizations, spatio-temporal convergence could produce afferent responses with vastly different tuning and dynamic properties (Angelaki 1991, 1992). In contrast, the majority of vestibular nuclei neurons receiving information from only otolith afferents (as indicated by exclusive linear motion responsiveness) do show two-dimensional response characteristics (Angelaki and Dickman 2000; Angelaki et al. 1992; Bush et al. 1993).

**Adaptation of the saccular macula**

Combining the present results with the known structural and functional properties of the otolith organs in species of different animal classes suggests that the saccule has undergone remarkable selective adaptation. In a broad sense, the saccule functionally transforms to serve different roles in different animal classes, as required for sensory adaptation to the environmental niche. For example, the saccule serves as the primary auditory organ in fish (Edds-Walton and Fay 1995; Fay 1995), is sensitive to substrate-borne vibrations and head mo-
tion in amphibians (Baird and Lewis 1986; Lewis et al. 1982), and is primarily sensitive to vertical linear accelerations and head tilts in birds and mammals (Fernández and Goldberg 1976; Jones et al. 1998). On a finer scale, a number of morphological differences between the saccule and other otolith organs in birds suggest an adaptive role for terrestrial and aerial motion detection that occurred through parallel evolution for birds and mammals. As Goldberg and Brichta (1998) have pointed out, it is difficult to derive evolutionary trends in vestibular organ adaptation due to the paucity of data from different animals and due to the fact that all data comes from extant species. Still, these authors have suggested that for the semicircular canal system, a derivation from a longitudinal organization of structure and physiological properties in fish, to a combined longitudinal and concentric organization in reptiles, to a wholly concentric organization in mammals exists (Brichta and Goldberg 2000; Goldberg and Brichta 1998). Does a similar trend in otolith receptor organization exist? A number of serial steps in otolith organ structure spanning animal classes adapted to aquatic, semi-aquatic, aerial, and terrestrial habitats can be defined. First, no calyx afferents nor functionally distinct type I hair cells are present in fish or amphibian otolith organs (Baird and Lewis 1986; Baird and Schuff 1994; Chang et al. 1992; Hama 1969), but first appear in reptiles and birds (Jorgensen and Andersen 1973; Rosenhall 1970; Wersäll and Bagger-Sjöbäck 1974). However, some investigators have noted that fish have proto-typic type I hair cells (Lanford and Popper 1996, 2000). Second, in the utricle (Si et al. 2002) and lagena (personal observations) of birds, type I hair cells (Jorgensen and Andersen 1973; Rosenhall 1970), calyx, and dimorph afferents are all confined to narrow regions flanking the exclusive band of type II cells running along the reversal line in the striola. Similar to otolith organs in anamniotes, most of the avian utricle and lagena maculae are populated only by type II hair cells that are innervated by bouton afferents. In contrast, in pigeon saccular maculae, no type II hair cell band along the reversal line exists and a significantly increased distribution of type I hair cells throughout a large portion of the macula is present. In the mammalian utricle, no type II band exists, and calyx afferents are confined.
to the narrow striolar region (Fernández et al. 1990). Third, dimorph afferents were widespread throughout the pigeon sacculus macula, similar to the pattern observed in the mammalian utricle. The exception is the innermost portion of the striolar region, where few dimorph afferents were observed. In contrast, a large number of dimorph afferents populate the bird utricular striola. Fourth, in mammals bouton afferents are very few in number and are found only in the extrastriola (Fernández et al. 1990). Although apparently more numerous in pigeons, bouton afferents in the saccule were confined to a more narrow extrastriola region and were substantially decreased in both number and distribution as compared with the bird utricle.

Thus it appears that the bird saccule has adopted morphological adaptations different from the utricle or lagena that are either carried on or also selectively appear through parallel adaptations in mammalian otolith organs.

From the little information available, it would seem that the calyceal terminal is also undergoing adaptation for optimally sensing linear accelerations during terrestrial or aerial motion. For example, many more dimorph and calyx afferents populate the macula of the bird saccule and mammalian utricle compared with either bird utricle or lagena. In essence, bouton afferents decrease in number, with the type II hair cells present being mostly innervated by dimorph afferents. Second, the distinct striola region for pigeon saccules and chinchilla utricles have a high concentration of calyx afferents, few dimorph afferents, and no bouton afferents. Again, this is quite different from the pattern observed for the other avian otolith organs. Furthermore, chinchilla calyx afferents, unlike the bird, can be simple containing a single type I hair cell; a feature that apparently becomes more prominent in primates (Merchant et al. 2000; Rosenhall 1972). In pigeons, the calyceal terminals in calyx afferents contained a large number of type I hair cells. Perhaps one of the functional consequences of the development of the calyceal terminal and simpler calyx afferents is to provide a very specific innervation pattern that ensures narrowly tuned directional and dynamic response properties of the afferent (Angelaki 1991, 1992). Chinchilla striola calyx afferents were described as being quite homogeneous in their discharge characteristics and dynamics (Goldberg et al. 1990). In contrast, dimorph afferents have been shown to vary greatly in their response behavior, related more to location in the macula than to terminal morphology or number of hair cells contacted (Goldberg 1990). It would be of interest to able to compare the discharge and dynamic properties of the different afferent types in the bird utricle and saccule. These morphophysiological studies, currently in progress, should provide useful data for direct comparisons between distinctly different otolith organ structural organizations in the same species. Certainly, the otolith organs are highly structurally organized with a variety of afferent innervation patterns existing. Issues regarding the relationship between afferent structure, responsiveness, and projection pattern to the CNS remain to be clarified.

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