Afferent Innervation Patterns of the Pigeon Horizontal Crista Ampullaris

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INTRODUCTION

The vertebrate vestibular system plays a central role in the control of compensatory gaze and postural responses as well as providing cues for spatial orientation, motion perception, and navigation. As components of the vestibular system, the semicircular canals are responsible for detecting rotational head motion. Since early anatomists first began to examine the vestibular receptor system, many conserved structural components were consistently noted to be present in species ranging from amphibians to humans (Lorente de No 1926; Morita et al., 1997; Ramón y Cajal 1908; Retzius 1880, 1884; Scarpa 1789; Steifensand 1835). Most of the similarities were related to the macromechanics of canal function. They included characteristics such as the shape of the canal, position of the endolymphatic duct, presence of an enlarged ampulla housing the receptor epithelium, and critical formation of a fluid-tight cupula partition. Still, notable differences in the receptor system have also been observed. For example, a diverse variation in the relative position and orientations of the three semicircular canal pairs has been reported even between different species of birds (Hadziselimovic and Savkovic 1964). As another example, there exists a curious structure called the eminentia cruciatum that bisects the receptor epithelium, contains support cells, and appears to exist only in certain animal classes (de Burlet 1935; Dohlman 1961; Igarashi and Yoshinobu 1966; Landolt et al. 1972; Ramprashad et al. 1980; Retzius 1880; Steifensand 1835). Its function is unknown. In the center of the eminentia cruciatum lies the torus (de Burlet 1935), a small region that separates the vertical semicircular canal crista (VCC) into two distinct hemicristae (Brichta and Peterson 1994; Jørgensen 1974). In other species, such as rodents, this region is less pronounced, and it is absent altogether in primates (Desai et al. 2005; Igarashi and Yoshinobu 1966). Although all of the semicircular canal crista have similar morphology in mammalian species (Desai et al. 2005; Fernandez et al. 1988, 1995; Goldberg and Brichta 1998; Lindeman 1969; Lysakowski 1996), the horizontal semicircular canal crista (HCC) in frogs, reptiles, and birds forms a single elongated neuroepithelium that is different from that of the vertical canals (Brichta and Peterson 1994; Iigic and Brichta 1997; Ishiyama and Keels 1971; Jørgensen 1974; Landolt et al. 1975; Money et al. 1974; Myers and Lewis 1990; Schessel et al. 1991; Steifensand 1835; Suzuki et al. 1986; Weng and Correia 1990). No eminentia cruciatum is present in the pigeon HCC (Igarashi and Yoshinobu 1966; Landolt et al. 1972; Ramprashad et al. 1986). Finally, whereas there are two separate ampullary nerves supplying each VCC in birds, only a single nondividing branch innervates the pigeon HCC (Landolt et al. 1975).

With improved anatomical techniques, more recent investigations have focused on the fine morphology of the semicircular canal receptors. In reptiles, avians, and mammals, two distinct receptor cells exist (Lindeman 1969; Rusch et al. 1998; Wersäll 1956). Type I hair cells are typically amphora-shaped and are completely surrounded by a calyceal afferent terminal. Type II hair cells are more cylindrical in shape and are innervated by simple bouton-type terminals. Type I and II hair cells also differ in their regional densities in the receptor epithelium (Brichta and Peterson 1994; Desai et al. 2005; Fernandez et al. 1988, 1995; Jørgensen 1974; Lindeman 1969) and in their membrane channel properties (Correia and Lang 1990; Correia et al. 1989; Eatoek et al. 1994). In addition to two hair cell types, three different afferent innervation patterns terminate in the receptor epithelia (Brichta and Peterson 1994; Fernandez et al. 1988, 1995; Lysakowski et al. 1999; Si et al.

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2003; Zakir et al. 2003). Calyx afferents contain only calyceal terminals that innervate type I hair cells. Bouton afferents end in terminal boutons that innervate type II hair cells. Dimorph afferents have both bouton and calyceal terminals that innervate type I and type II hair cells. These three afferent innervation types also differ in their immunohistochemical reactivity (Desai et al. 2005; Desmadryl and Dechesne 1992; Leonard and Kevetter 2002; Lysakowski et al. 1999), their physiological responses to motion (Baird et al. 1988; Brichta and Goldberg 2000a,b; Hullar et al. 2005; Weng and Correia 1999), and their terminal field locations within the neuroepithelia (Brichta and Peterson 1994; Fernandez et al. 1988, 1995; Goldberg et al. 1990; Schessel et al. 1991).

Morphophysiologic studies in frogs and toadfish (which exhibit only type II hair cells) have shown that afferent discharge regularity (Honrubia et al. 1989; Myers and Lewis 1990) and physiologic diversity (Boyle and Highstein 1990; Boyle et al. 1991) were related to position of crista innervation. In turtles (Brichta and Goldberg 2000a), chinchillas (Baird et al. 1988; Fernandez et al. 1988), and squirrel monkeys (Lysakowski et al. 1995), afferent discharge and responsiveness to rotational motion were also correlated to regional location in the crista. In all of these animals, regularly firing afferents with low gains were observed to innervate the peripheral regions (bouton afferents in amniotes). All afferents innervating the central crista were irregularly firing with higher gains and more advanced phase values. Given the established nonhomogeneous distributions of receptor cell type and afferent innervation for many animal classes, we wondered whether a similar organization exists for the bird semicircular canals. Previous studies have established that a diversity of afferent response dynamics and discharge properties exists for bird canal afferents with differences in motion sensitivity between afferents innervating the vertical versus horizontal semicircular canals (Anastasio et al. 1985; Dickman and Correia 1989a,b; Landolt and Correia 1980). Thus it is surprising that to the best of our knowledge, no fine morphology investigations of semicircular canal afferents in birds have been reported. Previous investigations from our laboratory had examined the morphologic diversity in afferents innervating the saccular and utricular maculae of pigeons (Si et al. 2003; Zakir et al. 2003). Here we have continued this line of investigation by examining the receptor cell distribution and afferent innervation of the HCC. We report that an organized topography exists in the pigeon HCC, in which receptor cell type and morphologic topology are regionally distributed across the crista epithelium.

METHODS

All experiments were performed using adult male pigeons (Columba livia), ranging in age from 1 to 3 yr (400–600 g). Experimental protocols were conducted in accordance with guidelines set forth by the National Institutes of Health Guide for the Care and Use of Animals in Research as well as those approved by the Institutional Animal Care and Usage Committee. The animals were housed and cared for in the Laboratory Animal Facilities under veterinary supervision.

Neural tracer injections

Biotinylated dextran amine (BDA) was used as a retrograde neural tracer to examine HCC afferent innervation. Each animal was anesthetized with isoflurane gas (2% in O2) and placed in a stereotaxic device, such that the left horizontal semicircular canal was aligned with an earth horizontal plane (Dickman and Fang 1996). A small opening (3–4 mm) in the parietal bone was made and the underlying dura mater opened. Next, BDA (10,000 MW; Molecular Probes; 10% in saline) was passed into a small region of the vestibular nuclear complex using iontophoresis with a positive current (6–8 μA; 50% duty cycle of 7 s) for 20 min. The injection site was varied among animals purposely to provide a more complete sampling of HCC afferents due to regional variations in their central projections (Dickman and Fang 1996). After cessation of injection current, the electrode was allowed to remain in position for a few minutes and a small negative current (−0.04 μA) was applied. The electrode was then retracted, Gelfoam was placed over the brain surface, and the skin was sutured closed. After surgery, buprenorphine (0.08 mg/kg) was administered for postoperative analgesia.

Histology

After 10–14 days of post-BDA injection survival, the pigeons were anesthetized and the bony labyrinths were bilaterally exposed. Both the horizontal and posterior canals were opened, and an intralabyrinthine perfusion was performed with a 5 ml volume of 3% glutaraldehyde, 2% paraformaldehyde, and 1% Acrolein in 0.1 M phosphate buffer (PB). The animal was subsequently perfused transcardially with a 2% glutaraldehyde, 1% paraformaldehyde solution in 0.1M PB, and the whole head was placed in the aldehyde fixative for 24 h. The membranous labyrinth was then excised, and each of the canal cristae and otolith maculae were dissected free. The brain was blocked, cryoprotected in 30% sucrose solution overnight, frozen, and serially sectioned (50 μm) in the transverse plane. The cristae and brain sections were processed for BDA. First, endogenous peroxidases were blocked with a 15-min incubation in 1% H2O2 in 90% methanol at room temperature (RT). The tissue was then incubated for 16 h in a solution of 10 μg/ml avidin D-HRP (Vector A-2004) and 0.3% Triton-X100 in PB. The tissue was rinsed and reacted using the chromogen diaminobenzydine (DAB, 0.5 mg/ml) along with 0.005% each of nickel chloride and cobalt acetate (Adams 1981). Initiation of the reaction was performed with 0.05% H2O2 until a dense reaction product was visualized. The tissue was rinsed again in phosphate buffer solution (PBS). The sections were mounted on glass slides, counterstained with neutral red, and coverslipped. The reacted cristae were first photographed en bloc (Nikon) from the apical epithelial surface. Next, they were embedded into plastic (Durcupan) and serially sectioned (10 μm thickness) using a rotary microtome. The sections were mounted on glass slides and counterstained with Richardson’s stain (Richardson et al. 1960).

In several animals, the HCC (n = 3) were prepared for examination by scanning electron microscopy (SEM). The tissue was dehydrated using a series of graded acetone washes followed by two washes in 100% acetone. The tissue was then placed in 100% tetramethylsilane and allowed to desiccate at 60°C in an open container. The dried cristae were mounted on aluminum studs, gold coated, and examined using a Hitachi 2600 SEM (20 kV).

Immunohistochemistry

To examine the topographic distribution of calyceal and terminal bouton morphology across the crista receptor surface, monoclonal antibody, TuJ1, which binds to the N-terminus domain of class III β-tubulin, was utilized (Lee et al. 1990; Molea et al. 1999). TuJ1 was used so that regional differences in calyx-bearing and bouton-only type afferent topography could be distinguished across the entire crista surface. Each pigeon was anesthetized and an intralabyrinthine perfusion was performed with 5 ml 4% paraformaldehyde in 0.1 M PB. A cardiac perfusion was then delivered using with 500 ml of PBS (0.1 M sodium PB, 0.9% sodium chloride, pH 7.2), 0.1% sodium.
The location of the BDA injection site was determined in the brain stem for each animal. In three animals, some vestibular efferent somas (Dickman and Fang 1996) were filled with BDA, and these animals were eliminated from further analyses. Labeled efferents had peripheral innervation patterns that were distinctly different from afferents. Efferent terminal morphologies consisted of extremely long, multi-branched fibers that innervated large epithelial areas and contained hundreds of bouton terminals (Purcell and Perachio 1997; Si et al. 2003). Only animals without any 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 crista was examined using video microscopy on a Nikon E600 microscope with DIC infinity optics and an anatomical reconstruction program (Neurolucida, MicroBrightfield). No correction for tissue shrinkage was taken into account, 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 and location of the labeled afferent within the neuroepithelium. The section drawings were used for each animal to locate the reconstructed afferent fibers. Longitudinal sections were utilized to recreate contour wireframes, on which affrent locations were marked. Although there was no difference between using longitudinal or horizontal sections to recreate contours for the basal regions of the crista, longitudinal sections more accurately portrayed the complex folding topography of the basal-planum junction and the planum proper in vivo.

For quantification of labeled afferent morphology, only fibers that were darkly stained (to obtain complete terminal fields) and sufficiently isolated from other afferents were reconstructed. Some non-reconstructed, but identifiable afferents were also included in our topographic analyses to establish a large-scale distribution map of reconstructed, but identifiable afferents were also included in our

Afferent tracings from five horizontal canals were reconstructed. Figure 1 shows the site of a BDA injection into the vestibular nucleus; SVN, superior vestibular nucleus. Scale bar = 500 μm.

**FIG. 1.** Coronal section of the rostral portion of the left vestibular nuclear complex showing the biotinylated dextran amine (BDA) tracer injection site. Retrograde labeled vestibular fibers are observed laterally entering the brain stem. Cb, cerebellum; LVN, lateral vestibular nucleus; MVN, medial vestibular nucleus; SVN, superior vestibular nucleus. Scale bar = 500 μm.
left vestibular nuclear complex (VNC). The exact coordinates for injections were varied for each animal so that HCC afferents projecting to different regions of VNC could be examined (Dickman and Fang 1996). Consistent with our previous findings for pigeons, retrograde labeling of HCC fibers was most prevalent with lateral vestibular nucleus (LVN) injections, followed by the superior vestibular nucleus (SVN), and, to a much lesser extent, the medial vestibular nucleus (MVN). Only a small portion of the rostral inferior vestibular nucleus (IVN) was injected. Previous work from our laboratory using some of the same animals to examine macular afferent innervations showed that there was little or no bias in BDA uptake by axons of varying diameter (Si et al. 2003; Zakir et al. 2003). In fact, the distribution of axonal diameters for BDA-filled and the total population of otolith fibers was not significantly different (Si et al. 2003; Zakir et al. 2003).

**General morphology of the horizontal canal**

The HCC consists of a neuroepithelial ridge found in the ampullary enlargement of the horizontal canal. The longitudinal axis of the HCC (Fig. 2A, dashed red line) traverses the ampullary lumen (Landolt et al. 1975) and is oriented perpendicularly to the direction of endolymph flow. As shown in the scanning electron micrographs of Fig. 2, the HCC can be subdivided into two major regions, including a saddle-shaped basal epithelium (long axis of the crista) and a dorsally directed epithelial overhang (short axis of the crista) (see Landolt et al. 1972, 1975; Myers and Lewis 1990; Purcell and Perachio 1997). The basal crista begins on the postero-lateral ampullary wall as a narrow, triangular-shaped receptor epithelium (Figs. 2A and 3A, far left) that contains hair cells with extremely long stereocilia bundles. This region in the HCC is analogous to the torus of the VCC and corresponds to zone III of the crista (Flock and Orman 1983; Masetto and Correia 1997; Myers and Lewis 1990). The epithelium broadens and elevates as it traverses antero-medially to form the apex. Here, hair cells exhibit intermediate to long stereocilia bundles. Continuing along the longitudinal axis of the HCC, the epithelium narrows to form a neck or isthmus, where the basal region joins the planum. The apex and isthmus regions correspond to zone II of the crista, where hair cell density appears to decrease and stereocilia bundles are generally shorter (Fig. 2B). The epithelium then turns dorso-laterally to form the planar region, also known as zone I of the crista (Fig. 2, A and B, right), which finally extends into the planum semilunatum (Kevetter et al. 2000; Landolt 1972; Masetto and Correia 1997; Steifensand 1835). Along the entire edge of the HCC periphery, a thin row of hair cells with long stereocilia bundles is present.

Using both normal and longitudinal cross-sections, the 3D structure of the apical surface of the neuroepithelium was reconstructed for each of the five HCC. Next, a composite 3D rendering of the receptor epithelial surface was produced using longitudinal sections from a single right-sided crista. Superior, antero-lateral, and postero-medial views of the 3D renderings of the reconstructed receptor epithelium are shown in Fig. 3. The 3D views were not corrected for perspective, so that comparisons of relative size between near and far objects could be visualized.

**Afferent types and regional locations**

Two hundred and eighty-six afferents from five horizontal canal organs were identified. Figure 2, C–E, shows normal sections in the torus, isthmus (Fig. 2D, bottom), planum (Fig. 2D, top), and apex regions of the crista, respectively. BDA-labeled afferents were identified and reconstructed throughout the majority of the epithelium. As can be seen in Fig. 2, the three afferent types were not homogeneously distributed throughout the HCC. In the torus region, mostly bouton afferents were observed, while in the apex and isthmus regions, dimorph and calyx afferents were more numerous.

To better characterize the topographic distribution of afferents throughout the HCC, the 286 identified fibers (136 dimorph, 106 boutons, 46 calyx) were plotted according to epithelial location, as shown in Fig. 3. Of these, 92 (38 dimorph, 28 bouton, and 26 calyx fibers) were sufficiently labeled and isolated to perform anatomical reconstructions. Bouton units were primarily observed in the torus region (Figs. 2C and 3) and as a thick ring along the edges of the planum (see following text). Bouton units were also observed in small numbers along the extreme periphery of the entire crista (Fig. 2, A and C). Dimorph afferents were located throughout the apex (Figs. 2E and 3), isthmus (Figs. 2D and 3), and the central planum (Fig. 3). A few dimorph fibers were also located in the transition between torus and apex regions. Calyx units were observed only in the apex, isthmus, and the central planum regions (Figs. 2D and 3).

To obtain a more thorough view of the distribution of calyx-bearing versus bouton-only afferents in the HCC, we used TuJ1 (an antibody to class III β-tubulin) to elucidate the terminal structure of all fibers. First, the planum was dissociated from the basal portion of the crista for better visualization. As shown in Fig. 4, TuJ1 labeling clearly delineates the regional differences in calyceal and bouton terminal innerva-
tions throughout the crista. Calyx-bearing afferents clustered within the central planum, apex, and isthmus areas (Fig. 4, A–D). In the isthmus and apex regions, calyx-bearing fibers were located throughout the entire epithelium, except at the extreme peripheral edges. Bouton-only afferents were found in the peripheral edges of the planum (Fig. 4A) and crista base (Fig. 4E) as well as the majority of the torus region (Fig. 4, A and F). Based on differences in afferent topography, the crista could be subdivided into central and peripheral zones as indicated by the dashed white lines in Fig. 4.

Reconstruction of canal afferents

Figure 5 shows photomicrographs and anatomical reconstructions of the terminal innervation patterns for representative calyx, bouton, and dimorph afferents. With the reconstructions, two views are illustrated beneath the fiber photomicrograph. A transverse view shows the same plane as the section followed by a 90° rotated apical view of the fiber illustrating the terminal field around which an area contour was drawn. For each type of afferent, two general categorizations could be made based on terminal morphology. First, each terminal innervation pattern appeared subjectively to be arranged in either a “flower” or a linear profile, similar to pigeon macular fibers (Si et al. 2003; Zakir et al. 2003). Flower profiles (Figs. 4F and 5A) consisted of terminals (calyceal and/or boutons) arranged in a rounded or petal formation. Linear profiles (Figs. 4G and 5, B and C) consisted of the terminal field extending along a serial trajectory. Second, each afferent type could be classified as being either simple or complex, depending on the number of calyceal terminals, hair cells per calyx, and/or branch order. Although in previous studies, complex calyces contained two or more type I hair cells (Fernandez et al. 1988; 1995), for our purposes (see DISCUSSION), we classified complex calyces as contacting at least seven type I hair cells. Afferent terminal fields for bouton-bearing afferents could also be categorized with a major axis of innervation that was spatially directed along the epithelium (Brichta and Peterson 1994; Purcell and Perachio 1997). For the bouton and dimorph afferents illustrated in Fig. 5, both had innervation axes that were aligned parallel to the transverse plane of the crista (i.e., 90°).
The calyx afferent shown in Fig. 5A was located in the central zone within the planum (near the location of unit a shown in Fig. 6) and consisted of a single axon segment that ended with a single, floral profile calyceal terminal. The complex calyceal structure contained 13 type I hair cells. Figure 5B shows a bouton afferent with a complex linear profile located in the torus region (Fig. 6, h). This fiber had 34 branches, a branch order of 12, and 41 bouton terminals. After piercing the neuroepithelium, the initial axon segment divided, with the major branch running parallel to the apical surface of the neuroepithelium (orientation of 90°) for nearly 35 μm from which numerous branches emerged. The dimorph afferent shown in Fig. 5C was located in the apex (Fig. 6, k). The terminal innervation contained a single complex calyceal terminal and 25 fiber branches all arranged in a linear profile. After piercing the neuroepithelium, a calyceal terminal was formed that contained 5 type I hair cells. The fiber continued along the epithelium for 35 μm in a transverse orientation (90°), with branching segments that contained a total of 26 bouton terminals.

As shown in Fig. 6, a continuum of complexity in the innervation patterns of individual afferents was observed for all fiber types.

CALYX AFFERENTS. Figure 6, a–d, shows calyx afferents in order of increasing complexity as well as their location in the crista. On entering the neuroepithelium, all but one of the calyx afferents terminated from the parent axon with no branch points. These units ranged from the simplest having only one hair cell/calyceal terminal (Fig. 6, a) to the most complex calyx spanning nearly 40 μm in the epithelium and contacting 14 type I hair cells (Fig. 6, b). Most calyx afferents had flower shaped calyceal terminals (Fig. 6, a and b). Less common were calyx afferents with linear profiles (Fig. 6, c and d). The calyx afferent shown in Fig. 6d was unique; it was the only calyx fiber observed that branched after entering the neuroepithelium, and it contacted a total of five type I hair cells (2 in 1 terminal and 3 in the other). No correlation between calyx complexity and location within the crista was observed. Both complex and simple calyx afferents were heterogeneously distributed (see following text).

BOUTON AFFERENTS. Figure 6, e–h, depicts bouton afferent reconstructions. Bouton afferents ranged from simple flower patterns with as few as 4 bouton terminals (Fig. 6, e) to the most complex innervation pattern (Fig. 6, f) that contained 70 bouton terminals. Linear profile innervations for bouton afferents spanned from the simple (Fig. 6, g) that contained two clusters of terminal fields and a total of 33 bouton terminals to complex patterns (Fig. 6, h) that contained numerous branches and 41 bouton terminals. No bouton terminals were observed on the parent axonal fiber itself; all terminals were located on subsequent daughter branches. Orientations of the bouton afferent trees had the following occurrences: 0° (n = 2), 45° (n = 2), 90° (n = 22), and 135° (n = 2).

FIG. 4. TuJ1 labeling in the right HCC. The crista was cut at the junction between the planum (A) and the basal (B) regions to allow better visualization of the crista morphology. ---, lines separating the central zone containing calyceal-bearing afferents from the peripheral zone containing fibers with only bouton terminals. E and F: magnified views of noncalyceal bearing areas with nerve fibers and bouton terminals visible from the central periphery and torus regions, respectively. C and D: magnified views of areas within the central planum and apex, respectively, containing different calyceal terminal profiles. ■, 100 μm scale bar for A and B. □, 10 μm scale bar for each inset.

FIG. 5. Photomicrographs from cross-section (top), side view reconstructions (middle), and apical view reconstructions (bottom) of calyx (A), bouton (B), and dimorph (C) afferents. Reconstructions represent branch fibers and calyceal terminals (black), and bouton terminals (red). Orange contours depict innervation areas. All reconstructions were scaled to intrinsic size, including terminals. Measured orientation quadrants for dimorph and bouton afferents are represented by polar plots. Shaded areas correspond to orientation angles. Scale bar = 10 μm.
DIMORPH AFFERENTS. Dimorph afferents comprised the majority of the fibers observed. As shown in Fig. 6, i–l, the simplest dimorphs had flower profiles with a single calyceal terminal that contained one type I hair cell and a single branch with only one bouton terminal (Fig. 6, i). Other dimorphs had few bouton terminals but more type I hair cells, like the fiber shown in Fig. 6, j that had 13 type I hair cells but only two bouton terminals. A substantial proportion (15/38) of the dimorphs had complex innervation trees. For example, the fiber of Fig. 6, k had five type I hair cells and 26 bouton terminals. Occasionally, dimorphs with two separate calyceal terminals were observed, similar to that of Fig. 6, l, which consisted of four type I hair cells contained within two calyceal terminals and 10 bouton terminals. Similar to bouton afferents, no bouton terminals were observed directly on the parent axonal fiber. Orientations of the dimorph afferent trees had the following occurrences: 0° (n = 11), 45° (n = 4), 90° (n = 24), and 135° (n = 5).

We then wished to examine whether there was a topologic pattern according to innervation complexity and epithelial location. As shown in Fig. 7, the distribution of calyceal terminals and bouton terminals for each reconstructed afferent are plotted on a longitudinally oriented wire frame reconstruction of the HCC. Calyceal terminals are plotted in order of increasing concentration of type I hair cells per fiber for both calyx and dimorph afferents (Fig. 7A). There appeared to be no significant topological association between calyceal complexity and epithelial location. Only the regional locations of calyx-bearing versus bouton-only afferent terminal patterns were observed, as described above. A similar plot for bouton terminals per fiber for the dimorph and bouton afferents revealed a similar lack of topologic organization (Fig. 7B).

Comparisons of afferent innervation

To quantitatively compare the differences in afferent terminal morphology, statistical comparisons were made between the three different afferent types for each of the morphometric parameters quantified (Tables 1 and 2). As shown in Fig. 8, these morphometric parameters varied systematically between the calyx, dimorph, and bouton afferents. Calyx afferents had the largest axonal fiber diameters (compared with bouton afferents, but similar to dimorph fibers), the shortest fiber lengths with the smallest fiber volumes, the fewest number of branches with the lowest branch order, and the smallest innervation areas of all the fiber types. Calyx afferents also significantly exhibited the fewest number of total terminals, as compared with the dimorph and bouton afferents (Fig. 8, A).
Tables 1 and 2). No difference was noted in the number of type I hair cells innervated by a calyceal terminal between calyx and dimorph afferents. Dimorph afferents were significantly longer with more ramified branches as compared with calyx afferents, but were smaller and less complex on each of these measures than bouton afferents. Comparing boutons with dimorphs resulted in no noticeable differences in parent axon length or volume. Bouton afferents did have significantly longer fibers, with more branches and a higher branch order than any other fiber type (Fig. 8, Tables 1 and 2). Bouton afferents also had more bouton terminals per fiber than did dimorph afferents. In terms of fiber orientation, dimorph and bouton afferents were similar in the direction of the terminal fields within the receptor epithelium.

To further delineate innervation areas and number of terminals, density values were also calculated as shown in Table 1. No significant differences existed between calyceal and dimorph afferents in their type I hair cell density. However, bouton terminal density was higher for bouton afferents as compared with dimorph fibers. The density of the total number of terminals was highest for the bouton afferents, followed by the dimorph and calyx afferents (Table 2).

**DISCUSSION**

**Morphology of the pigeon horizontal canal crista**

The present results show that the horizontal semicircular canal crista in pigeons exhibits a regional organized topography in terms of cell structure and afferent innervation. The pigeon HCC is composed of a single hemicrista similar to that found in some amphibians and reptiles (Igic and Brichta 1997; Lysakowski 1996; Myers and Lewis 1990; Schessel et al. 1991). In these animals, the HCC that has a triangular-shaped torus region leading to a wider apex and a narrow isthmus. In birds, the long axis of the crista then joins a singular large planum at the antero-medial end of the epithelium. This morphology is actually quite different from the symmetrical shaped crista found in the vertical semicircular canals of pigeons (Correia et al. 1985; Landolt et al. 1972, 1975) and many other animals (Brightha and Goldberg 2000a; Brightha and Peterson 1994; Desai et al. 2005; Fernandez et al. 1988, 1995; Goldberg and Brichta 1998; Goldberg et al. 1990; Keckett et al. 2000; Lindeman 1969; Lysakowski 1996; Schessel et al. 1991). For example, the mammalian crista is saddle-shaped and consists of a continuous apical region that terminates into two opposing planum regions (Hunter-Duvar and Hinojosa 1984; Lim 1971; Lim and Lane 1969; Lindeman 1969). The regional distribution of receptor cells in the pigeon HCC was observed to be topographically organized. Type I hair cells (as evidenced by the location of calyceal terminals) were confined to a central zone that included the apex, isthmus, and central planum. Type II hair cells were observed throughout the crista but were more dense in the peripheral regions. These findings were similar to those reported for all other amniotes studied to date (Brightha and Peterson 1994; Desai et al. 2005; Fernandez et al. 1988, 1995; Jergensen 1974; Keckett et al. 1994; Lysakowski 1996; Wersäll 1956). From our SEM and neural tracing tissues, we noted that Type II hair cells often had significantly longer stereocilia as compared with type I cells. Recent quantification of stereocilia bundle structure in turtle receptor epithelia has established a correlation between bundle size, length, cell type, and regional location (Moravec and Peterson 2004; Ricci et al. 1997a,b; Xue and Peterson 2006). These studies showed that type I hair cells have many more stereocilia than type II hair cells, increasing their stiffness to deflection and purportedly their sensitivity to motion. The number of stereocilia per bundle and their length was also related to location in the epithelium. Smaller bundles with longer stereocilia were associated with peripheral hair cells, similar to our qualitative observations for hair cells in the pigeon HCC.

**HCC afferent innervations**

There are ~1,500 fibers ranging from 1 to 13 μm in diameter (mean of 2.5 μm) innervating the HCC in pigeons. Common to all amniote species studied to date, three distinct afferent innervation types were observed in the pigeon HCC, including, calyx, dimorph, and bouton fibers. These afferent types were not homogeneously distributed, but instead were found to conform to a regional topography. Calyx and dimorph afferents were restricted to the central zone of the HCC. Type II hair cells in the central crista were innervated by dimorph...
TABLE 2. Statistical comparisons for all afferents

<table>
<thead>
<tr>
<th>Comparison</th>
<th>Calyx</th>
<th>Bouton</th>
<th>Dimorph</th>
<th>Mean</th>
<th>Main Effect Value</th>
<th>Schefé Post Hoc</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fiber length</td>
<td>32.2</td>
<td>260.2</td>
<td>128.0</td>
<td>P(2,89) = 36.9</td>
<td>P &lt; 0.001</td>
<td></td>
</tr>
<tr>
<td>Fiber volume</td>
<td>330.0</td>
<td>653.6</td>
<td>613.4</td>
<td>P(2,89) = 4.5</td>
<td>P &lt; 0.05</td>
<td></td>
</tr>
<tr>
<td>Axon diameter</td>
<td>3.7</td>
<td>3.0</td>
<td>3.4</td>
<td>P(2,89) = 3.8</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Parent length</td>
<td>—</td>
<td>33.4</td>
<td>34.9</td>
<td>P(1,64) = 0.1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Parent volume</td>
<td>—</td>
<td>240.7</td>
<td>322.7</td>
<td>P(1,64) = 2.7</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Branch order</td>
<td>1.0</td>
<td>6.5</td>
<td>3.8</td>
<td>P(2,89) = 56.3</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Branches</td>
<td>1.1</td>
<td>22.0</td>
<td>9.9</td>
<td>P(2,89) = 29.5</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Innervation area</td>
<td>233.6</td>
<td>481.0</td>
<td>485.6</td>
<td>P(2,89) = 3.4</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Type I HC</td>
<td>5.6</td>
<td>—</td>
<td>5.0</td>
<td>P(1,62) = 0.5</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Terminals</td>
<td>5.6</td>
<td>23.8</td>
<td>7.5</td>
<td>P(1,64) = 31.8</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>HC I density</td>
<td>0.035</td>
<td>0.053</td>
<td>0.039</td>
<td>P(2,89) = 22.3</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Terminal density</td>
<td>0.035</td>
<td>0.058</td>
<td>0.032</td>
<td>P(2,89) = 7.7</td>
<td>NS</td>
<td></td>
</tr>
</tbody>
</table>

NS, not significant. *, one-way ANOVA only. Units of measurement are the same as shown in Table 1.
demonstrate that type II hair cells exclusively populate the torus, the peripheral planum, and the very edge of the basal periphery. The marked concentration of bouton fibers in the triangular torus region is similar to that found in the turtle and the zebra finch (Brichta and Peterson 1994; Brichta and Goldberg 2000a; Lysakowski 1996). Bouton afferent fibers were by far the most branched and the longest and had the largest axonal volumes (of the 3 afferent types). Surprisingly, HCC bouton innervation areas were similar to that of dimorphs, which was not true in pigeon maculae (Si et al. 2003; Zakir et al. 2003). Thus bouton afferents were also the most densely terminated with nearly three times the number of terminal boutons as present in dimorphs. By comparison, bouton afferents in the other amniotes studied have similar size, terminal density, and innervation areas to those of pigeons (Brichta and Goldberg 2000a; Brichta and Peterson 1994; Fernandez et al. 1988, 1995).

DIMORPH AFFERENTS. HCC dimorph afferents innervated a broad region of the crista as compared with calyx fibers. For most of the morphometric parameters measured, pigeon HCC dimorphs had intermediate values between those observed for bouton and calyx afferents. Dimorph afferents had fewer bouton terminals and fewer type I hair cells per calyceal terminal, as compared with bouton and calyx afferents, respectively.

Functional considerations of afferents and their innervation patterns

Given the regional topography of the HCC in terms of receptor cell distribution and afferent innervation, it is of interest to examine possible associations with physiological responsiveness. Morphophysiological studies in frogs and fish (where only bouton afferents exist) reported that afferent sensitivity was correlated with both number of terminal endings and location (Boyle et al. 1991; Myers and Lewis 1990). In the peripheral areas of the crista, afferents had few terminals, had lower spontaneous discharge rates, and responded to rotational motion with slow dynamics (phase values near head velocity) and low sensitivities (Boyle and Hightstein 1990; Boyle et al. 1991). The opposite was seen for afferents innervating the central crista regions, where lower irregular discharge rates, fast dynamics, and high gain slopes were observed (Boyle and Hightstein 1990; Boyle et al. 1991; Myers and Lewis 1990). Similar regional differences in afferent responsiveness have been observed in amniote species. Schessel et al. (1991) concluded that the number and type of hair cells contacted, along with afferent membrane properties, were the primary determinants of response sensitivity in lizards. In turtles, Brichta and Goldberg (1996) observed that for a given gain and phase, bouton units fired more regularly than calyx-bearing fibers. They also reported that the longitudinal location along the crista was correlated with bouton dynamic response to motion (Goldberg 2000).

Recently, many of the hair cells in the central crista region of toadfish and pigeons were shown to contain GABA as well as glutamate (Holstein et al. 2004a). Physiological experiments in toadfish have confirmed that GABA is principally involved in the observed increased gain slopes and advanced dynamic properties for afferents innervating the central crista (Holstein et al. 2004b). However, GABA does not appear to be the whole story and other components of the receptor system must play an active role in establishing the wide diversity observed in afferent discharge, sensitivity, and dynamic responsiveness. Exactly how type I hair cells and calyceal-bearing afferents differ from type II cells and bouton afferents in the function of motion detection remains an open question. The relationship between structure and function of vestibular afferents is important to understand, not only from a basic knowledge perspective but also due to renewed clinical relevance. Modern treatment regimens using ototoxic agents for vestibular hypersensitivity appear to preferentially target type I hair cells and calyceal-bearing afferents (Carey et al. 2002; Matsui et al. 2003; Minor et al. 2004). Morphophysiological studies of crista receptors and afferents to differentiate innervation type, terminal field location, and response dynamics to rotational motion deserve further attention.

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