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MORPHOLOGY AND INNERVATION OF THE VESTIBULAR LAGENA IN PIGEONS

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Abstract—The morphological characteristics of the pigeon lagena were examined using histology, scanning electron microscopy, and biotinylated dextran amine (BDA) neural tracers. The lagena epithelium was observed to lie partially in a parasagittal plane, but was also U-shaped with orthogonal (lateral) directed tips. Hair cell planar polarities were oriented away from a central reversal line that ran nearly the length of the epithelium. Similar to the vertebrate utricle and saccule, three afferent classes were observed based upon their terminal innervation pattern, which include calyx, dimorph, and bouton fibers. Calyx and dimorph afferents innervated the striola region of the lagena, whereas bouton afferents innervated the extrastriola and a small region of the central striola known as the type II band. Calyx units had large calyceal terminal structures that innervated only type I hair cells. Dimorph afferents innervated both type I and II hair cells, with calyx and bouton terminals. Bouton afferents had the largest most complex innervation patterns and the greatest terminal areas contacting many hair cells.

Key words: vestibular, motion detection, spatial orientation, otolith.

In all terrestrial vertebrates, the utricle and saccule vestibular otolith receptors are known to function as linear accelerometers (Blanks and Precht, 1976; Fernández and Goldberg, 1976; Loe et al., 1973; Si et al., 1997). However, in many nonmammalian vertebrates and monotremes, a third otolith organ, the lagena, also exists (Retzius, 1884; Ladhams and Pickles, 1996; Rosenhall, 1970). Relatively little work has examined lagena function, since it was first described as a vestibular receptor responsive to linear motion (MacNaughtan and McNally, 1946). In frogs, lagena afferents respond to either head tilts relative to gravity, to vertical high frequency vibrations, or both (Baird and Lewis, 1986; Caston et al., 1977; Cortopassi and Lewis, 1996). In fish, all otolith organs appear to serve dual roles as both auditory and motion sensors (Fay and Edds-Walton, 1997), including the lagena (Fay and Olsho, 1979; Lu et al., 2003). In addition to motion detection, it has been suggested that the lagena also may function as a magnetic field detector due to the reported presence of ferromagnetic iron particles (Harada et al., 2001). Indeed, lagena ablation was shown to disrupt the homing ability of pigeons (Harada, 2002). More recently, c-Fos, a marker for neural activation, was used to demonstrate that lagena ablation eliminates responses to changes in magnetic field inclination in the central nervous system, suggesting that magnetoreception in homing pigeons is in large part mediated through the lagena receptor (Wu and Dickman, 2011).

What do we know about lagena morphology? In pigeons, the lagena is located at the distal end of the basilar papilla, with a hair cell population and neural innervation that are distant from the auditory receptor cells (Harada, 2002; Jørgensen, 1970; Platt et al., 2004). Morphometric examinations have shown that both type I and type II hair cells are present in the sensory epithelium of the avian lagena (Jørgensen, 1970; Rosenhall, 1970; Ricci et al., 1997). Type I hair cells and their exclusive calyx terminal nerve endings lie in the central lagena and define an area known as the striola (Werner, 1933). Coursing through the lagena striola is a distinct strip of Type II cells, known as the Type II band (Rosenhall, 1970). Both the avian utricle and lagena exhibit a type II band, whereas the saccule does not (Si et al., 2003; Warchol and Speck, 2007; Zakir et al., 2003). The morphological polarizations of avian lagenar hair cells are similar to those of the saccule, being generally directed away from a central reversal line (Rosenhall, 1970) that courses through the central lagena epithelium. More complex polarization patterns have been described in fish (Lu and Popper, 1998). Much less is known regarding lagena afferent innervation patterns. In frogs, afferent location in the epithelium was found to be correlated with differences in response sensitivity (Baird and Lewis, 1986). In fish, lagena afferent innervations were described as larger, with more complex arborizations, and more numerous terminals than those of the saccule (Edds-Walton and Popper, 2000). Detailed examination of the innervation patterns for lagena afferents in other species remains lacking.

Here, we examined the general morphology and afferent innervation patterns of the lagena in homing pigeons. The size of the receptor epithelium and morphological polarization of hair cells were measured throughout the end organ, and the afferent innervation patterns for calyx, dimorph, and bouton fiber types were reconstructed and quantified. Our findings compliment earlier works on the utricular and saccular maculae and together represent a complete body of work regarding the morphology of pigeon otolith receptor organs (Si et al., 2003; Zakir et al., 2003).

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Abbreviations: BDA, biotinylated dextran amine; PB, phosphate buffer; SEM, scanning electron microscopy.

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EXPERIMENTAL PROCEDURES

Animals

The experiments were conducted on adult homing pigeons (*Columbia livia*) that ranged in age from 1 to 3 years. All experiments were conducted using the minimum number of animals possible and all animals were anesthetized to eliminate suffering. All animal procedures were performed in compliance with the Guide for Care and Use of Laboratory Animals (National Institute of Health publication 86-23) and approved by the Institutional Animal Care and Use Committee at Washington University in St. Louis.

Lagena afferent innervation

Afferent innervation patterns were examined in five lagena receptor epithelia using retrograde tracing of biotinylated dextran amine (BDA; 10,000 MW; Molecular Probes) injected into the vestibular nuclei. Each animal was anesthetized with isoflurane (3% in O_2) and placed in a 12° nose down position in a stereotaxic device (Kopf Instruments, model 1430), which approximately aligned the horizontal semicircular canals in the stereotaxic horizontal plane (Dickman and Fang, 1996). A small flap in the parietal bone was retracted, and a glass micropipette (1.0 mm OD, 0.8 mm ID) filled with BDA (10% in saline) was lowered into the brain (0° angle) using stereotaxic coordinates for the vestibular nuclei (Dickman and Fang, 1996). BDA was injected with positive current (8 μA, 50% duty cycle, Midgard Current Source) for 20 min, then a small bucking negative current (-0.04 μA) was applied (to eliminate additional tracer release during removal) and the micropipette retracted. The injection site was purposely varied between 0.5–3.5 mm posterior to AP0 and 1–3.5 mm lateral to the midline among animals to provide a more complete sampling of lagenar afferents due to regional variations in their central projections (Si et al., 2003; Zakir and Dickman, 2006; Zakir et al., 2003). Gelfoam was then placed over the brain surface and the bone flap replaced. The skin was sutured, butorphanol (10 mg/kg im) was given for postoperative pain, and the animal was returned to its cage.

Histology

Following a postinjection survival period of 10–14 days (Si et al., 2003; Haque et al., 2006; Zakir and Dickman, 2006; Zakir et al., 2003), each pigeon was reanesthetized, the mastoid bone was opened, and an intralabyrinthine perfusion was performed using a 2% glutaraldehyde, 1% paraformaldehyde, and 1% acrolein solution (Anna Lysakowski, personal communication). The bird was subsequently perfused transcardially with sodium nitrite (1%) in saline (250 ml), followed by 750 ml of 2% glutaraldehyde and 1.25% paraformaldehyde fixative in 0.1 M phosphate buffer (PB). The head was then placed in the aldehyde fixative for 24 h at 4 °C. The next day, the membranous labyrinth was excised and the lagenar maculae were dissected free. The otoconial membrane and otoconia were removed using a fluid jet. Next, the lagenar maculae were processed for BDA using a modified diaminobenzidine (DAB) procedure (Brandt and Apkarian, 1992). Briefly, the tissue was incubated for 12 h in a solution of 0.1 M PB, 1% Triton-X100, and 0.25% avidin d-HRP (vector A-2004). The tissue was then reacted using the chromogen DAB with 1% nickel ammonium sulphate-cobalt chloride solution and 0.3% H_2O_2 until a dense reaction product was visualized. Next, the tissue was thoroughly rinsed in phosphate buffer. The tissue was next dehydrated using a series of graded alcohols, cleared with xylenes, embedded in plastic (Durcupan), and then serially sectioned (10 μm thickness) using a rotary microtome. The tissue sections were mounted on glass slides and counterstained (Richardson et al., 1960).

SEM preparation

In four additional animals, the lagenar maculae were prepared for 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 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 coated in gold. The lagenae were scanned using a Hitachi SM200 scanning electron microscope (20 kV).

Reconstruction of lagenar afferents

The lagenae and their afferent innervation patterns were examined using video microscopy (Nikon E600) and an image-based reconstruction program (Neurolucida, MicroBrightfield Inc.). 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 of the lagenae were measured, including the macular width, location of the type II band, and location of the hair cell morphological polarization reversal line.

For reconstruction of labeled afferents, only fibers that were darkly stained and sufficiently isolated from other afferents were traced in an effort to reproduce the complete innervation pattern for each unit. Afferents with partial staining (ghost fills), or those that overlapped other afferents and could not be assuredly distinguished, were not quantitatively analyzed. The regional location of each reconstructed afferent within the lagenar macula was made using relative distance to the epithelial borders and the reversal line. The three-dimensional reconstructions of identified afferents were performed using an 60× objective (dry lens, 0.95 NA). Measured morphological parameters for each of the reconstructed afferents included the following:

1. Fiber length. Summation of all branching segments.
2. Parent axon length. Length of the initial fiber segment, from epithelium penetration to the first branch point.
3. Fiber volume. Calculated by modeling each branch as a frustum (a circular cone that has been truncated) along the total fiber length using the standard equation for volume, $V=\frac{\pi}{3}(R_1^2+R_1R_2+R_2^2)$, where R1 and R2 are the radii at the two ends of the current axonal section of interest.
4. Parent axon volume. Calculated volume for the parent fiber only.
5. Axon diameter. Average diameter of the first five microns of fiber length before epithelial penetration.
6. Innervation area. After reconstruction, each tracing was rotated in 3-D space such that an observer's view was obtained orthogonal to the apical surface of the receptor epithelium. An area contour was then traced around the entire terminal field, including all calyx and bouton terminals, for each fiber such that the boundaries were that of a minimum convex polygon (Brichta and Peterson, 1994).
7. Fiber branches. Total number of branch segments in the afferent tree.
8. Branch order. Number of branching levels past the initial fiber segment, which was assigned a branch order of 1.
9. Number of bouton terminals. Number of terminal or en passant bouton endings noted.
10. Number of type I hair cells. Each constricted neck region observed in a calyx terminal was counted and used as evidence for the presence of a type I hair cell.
11. Total number of terminals. Sum of the number of bouton terminals and the number of type I hair cells observed for each fiber.