2004

Afferent innervation of the quail utricle

Kathleen Ferrigan Faulkner

AFFERENT INNERVATION OF THE QUAIL UTRICLE

by

Kathleen Ferrigan Faulkner

An independent study submitted in partial
fulfillment of the requirements for the degree of:

Master of Science in Speech and Hearing

Emphasis in Audiology

Washington University
Department of Speech and Hearing

May 21, 2004

Approved by:
J. David Dickman, Ph.D., Independent Study Advisor

Introduction

The vestibular system is comprised of organs responsible for detecting linear acceleration as well as detecting positional movements of the head relative to gravity. The utricle, the end organ under investigation in this study, is specifically designed to respond to linear acceleration. Afferent nerve fiber innervations in the vestibular end organs were originally described by Retzius (1884), Ramon y Cajal (1909), Lorente de Nó (1926) and Poljak (1927). These studies noted differences in structure, terminal distribution and fiber size and were able to characterize the patterns in distinct zones of the epithelium. Lorente de Nó and Poljak identified the striola because it received the thickest fibers, most of them terminating as calyx units, while in the extrastriolar regions they observed that the afferents were thinner and had smaller terminal fields. Using specific neural tracers, the utricular afferent innervation patterns have only been studied in pigeons (Si et al, 2003) and chinchillas (Fernandez et al, 1990).

Receptor cells begin development by a process of differentiation. Following this stage, nerve fibers begin to seek out their receptor cell connections. There are two types of receptor cells, type I and type II, which have very distinct structures. There are three types of primary afferent fibers based on their morphology. Calyx units have large specialized calyceal terminals that innervate only type I receptor cells. Dimorph units have both calyceal and bouton endings that innervate both type I and II receptor cells. Bouton units have no calyceal endings and only innervate type II receptor cells. The bird afferent nerve fibers that innervate the utricle begin to form their connections at embryonic day 5 (E5) and continue through post-hatch development. The bouton afferents are the first to develop, with the dimorph afferents second. The calyx afferents begin to develop towards the end of embryogenesis but continue throughout post-hatch

development. Three regions of epithelium separate the afferent nerve fibers and allow evaluation of individual units.

We chose to study the development of bird vestibular end organ afferents because of their morphological similarity to those of mammals and amphibians and because of their rapid growth. In addition, we are interested in how altered gravity conditions affect development of the otolith system. Quail are particularly well suited for this highly specialized field of developmental biology. The aim of evaluating afferent innervation of the quail utricle is to understand the development of the nerve fibers. The methods used in this study involved the use of neural tracers, 3-D anatomical reconstruction and analysis of afferent cell types in the adult quail. This particular study is focused on the adult innervation patterns in order to achieve a full understanding of the mature nerve fiber connections. The results of this study will provide a context for continued research into the initiation and growth of the afferent innervation patterns during embryogenesis.

Methods

The methods used in our study have been described in great detail previously (Si et al, 2003). All experiments were conducted in adult quails. The methods were approved by the Central Institute of the Deaf Institutional Animal Care and Use Committees. Biotinylated dextran amine (BDA) was injected into the vestibular nuclei of the brainstem. The BDA was transported by the nerve fibers to their terminal connections in the vestibular end organs. 14 days postoperatively, the vestibular end organs and brain were harvested and placed in fixative. The utricle was trimmed and the otoconia were removed in order to visualize the BDA tracer. A solution with a chromogen (DAB and 1% nickel-cobalt in 0.1 M phosphate buffer) and an

initiator (0.3% H_2O_2) was used to deposit a dark brown precipitate on the filled nerve fibers. The tissues were embedded in plastic (Durcupan), serially sectioned at 10μm thickness and mounted on glass slides.

Afferent Reconstruction

For each section, several parameters of the epithelium were measured and identified, including the width, the location and the morphological polarity reversal line. For the reconstruction process, only darkly stained and isolated fibers were chosen for tracing. The regional location of each fiber was calculated by its location relative to the medial and lateral edge of the epithelial tissue. This calculation allowed for the reconstruction of all fibers into a composite surface map of the utricle.

Three dimensional reconstructions of the identified afferent fibers were produced using a reconstruction program (Neurolucidia, MicroBrightfield). Fibers were analyzed using a light microscope 100x oil objective fitted with a high resolution digital camera (Nikon). Several morphological parameters were measured and quantified from these reconstructions. The axon diameter was defined as the average diameter of the last 5μm of the fiber before the point it penetrated the epithelium. The length and volume of each fiber were calculated as all of the branches of the structure within the epithelium. The branch number was defined as the total number of branches. The number of boutons was calculated as the number of both terminal boutons and en passant boutons. The number of type I hair cells was calculated by visualization of the cell shape (apical neck and top) contained in calyceal terminals. A contour was drawn around the fiber tracing and the area of the contour was measured. The innervation density was determined by taking the total terminals (hair cells and terminal boutons) and dividing them by

the innervation area. The innervation angle was calculated by drawing a line though the major innervation axis of the terminal field.

Results

Neuroepithelium of the utricle

For scanning electron microscopy (SEM) visualization, the otoconia and otolith membrane were removed and the utricle was positioned flat on the mounting stud. An SEM micrograph of a quail utricle is shown in Figure 1. The perimeter of the receptor neuroepithelium was traced encompassing the surface area containing stereocilia bundles. The striola region surrounds a type II band and was characterized by a large accumulation of type I hair cells and fewer type II hair cells. The striola began posterior-medially, and ran parallel to the posterior edge, up to the posterior-lateral region, coursing parallel to the lateral edge of the epithelium. The striola continues anteriorly with a sharp turn near the anterior edge, where it continues slightly parallel to the anterior edge, in a pattern similar to that found in chicks (Matsui et al, 2003). The reversal line was traced based on the visualization of the center of the type II cell band. The middle and posterior region of the striola contained the majority of the Type I hair cells. Type I hair cells were not present beyond the striolar region in the extrastriolar zone, which exclusively contained type II hair cells.

Afferent Innervation Patterns

In the left and right utricular maculae evaluated in this study, the patterns of 50 afferent nerve fibers were evaluated. Three innervation patterns were observed, including calyx, dimorph and bouton fibers which were each characterized by their unique terminal profile.

Calyx Afferents: Calyx afferents are identifiable by their large calyceal terminals which contain type I hair cells, an example of which is shown in Figure 3. The fifteen calyx units reconstructed were contained exclusively in the striola region and their locations are shown in Figure 2. For each unit, a single, generally large unbranched parent axon entered the epithelium and ranged from 1.8 to 4.0 μm in diameter, with a mean of 2.9 (±0.7) (Table 1). The calyx units were the simplest of the afferents, for the majority of the calyx fibers ended without a branch point within the epithelium. The calyx units were also the smallest of the afferents based on length and volume. The size and structural pattern of the calyx units varied greatly with the most complex calyces containing the largest number of hair cells and encompassing the largest innervation area. The calyx afferents contained between 1 and 8 type I hair cells, with an average of 4 (±1.8) per fiber. Innervation areas for the calyx units ranged from 128.4 to 460.5 μm^2, with a mean of 302.6 (±120.1).

Two distinct patterns exist within the reconstructed calyx afferents, "flower" shaped and "rectangular" or linear shaped. The flower shaped calyces had hair cells that were all in close proximity, overlapped and came directly out of the calyceal terminal. The rectangular shaped structures had an axon that moved linearly with hair cells arising along the terminal.

Dimorph Afferents: Dimorph afferents are identifiable by their pattern which included calyceal terminals and bouton terminals, innervating both type I and type II hair cells, an example is shown in Figure 4. The fifteen dimorph units reconstructed were contained exclusively in the striola region along with calyx units, their locations are shown in figure 2. Typically, the dimorph afferents consisted of a single large axon that entered the epithelium, with a slightly

smaller average diameter as the calyx units, ranging from 1 to 3.1μm, with a mean of 2.3 (±0.6). The average number of branches was significantly greater than those of the calyx units but less than that of the bouton units. The most complex of the dimorph afferents contained 49 branch fibers (out to the 10^{th} order) with two hair cells and 27 boutons, while the simplest contained only three branches (out to the 2^{nd} order) with one hair cell and two boutons. One finding similar to that found in the pigeon dimorph afferents is a small branch extending off of the calyceal terminal body rather than off of the parent axon or another fiber. The length on average was greater than the calyx units while the volumes did not differ significantly.

The structural patterns noted could be organized into the calyceal flower or linear type classifications. The flower type consisted of centralized group or single calyx with small fibers extending into a terminal bouton away from the center of the calyx. The linear structures contained a longer axon with only few branch fibers extending from it. Typically, the calyceal terminals containing a range of 1-6 hair cells per fiber occurred either at the beginning of the or at the end of the parent axon. The calyces of the dimorph units contained a smaller number of hair cells than the calyx units, with an average of 2 (±1.3) hair cells per unit. The total number of bouton terminals varied greatly ranging from 1 to 27, with a mean of 7.9 (±6.2). The total number of terminals included the calyceal and bouton terminals with a mean of 8.2 (±5.8) terminals per fiber.

Bouton Afferents: Bouton afferents are identifiable by their en passant and bouton terminals that only terminate type II hair cells. The twenty reconstructed bouton units were contained in both the extrastriolar regions and the type II band. Their locations are shown in Figure 2. The bouton afferent fibers generally entered the epithelium with a slightly smaller average diameter than the

dimorph and calyx units, ranging from 1.1 to 2.6 μm, with a mean of 2 (±0.5). The majority of the bouton afferents crossed the epithelium without branching. Each bouton unit varied greatly in terminal pattern and complexity following entrance into the epithelium, an example of a bouton afferent is shown in Figure 5.

Bouton afferent fibers could be classified into the same two groups based on their innervation pattern, flower profiles and linear profiles. The flower types contained fibers that radiated from the parent axon in generally symmetrical patterns. The linear types were represented by a longer axon that coursed through the macula in a narrow pattern. The simplest bouton unit evaluated contained only five branches and three boutons, while the most complex contained 63 branches and 57 boutons. With an average number of 27.5 (±15.8) branches per fiber, the bouton units were much more complex than the calyx or dimorph units. The number of boutons per fiber ranged from 4 to 57 with a mean of 23.5 (±15.3) boutons per fiber. The overall length of the bouton fibers was greater than both the calyx and dimorph afferents. The innervation areas ranged from 128.4 to 460.5, with a mean of 302.6 ± 120.1.

Discussion

Afferent innervation patterns

The use of calyx, dimorph and bouton classes to describe afferent fiber types was based on previous studies of utricle afferents (Fernandez et al, 1990; Si et al, 2003). Calyx and dimorph afferents were isolated to the striolar region, while previous studies have found dimorph afferents to be located in both striolar and extrastriolar regions (Fernandez et al, 1990; Si et al, 2003). Only 15 dimorph units were chosen for reconstruction in this study, therefore it remains possible that some dimorphs may be present in extrastriolar regions. Bouton units were found both

innervating the type II band and extrastriolar regions, similar to findings in pigeons. Chinchillas, by comparison, have calyx units limited to the striolar region, bouton afferents only in extrastriolar regions and dimorph afferents found throughout the macula (Fernandez et al, 1990). Chinchillas have been described to have both simple and complex calyx units, while pigeons have not been shown to possess simple calyx fiber types; one simple calyx was observed in this study (Fernandez et al, 1990; Si et al, 2003). The average afferent diameters measured for the quail are comparable to those found in pigeons and chinchillas.

Functional considerations of afferent innervation patterns and development

Although studies to date have only evaluated the patterns of the afferent fibers in the utricle, we believe that there may be a functional correlate to when and how these fibers begin to develop. In the developing utricle, bouton afferents emerge first, beginning near embryonic day 5. Dimorph afferents appear second with calyx afferents emerging during embryogenesis and continuing during post-hatch development. Further studies are underway to examine the timing of each particular afferent during development. Preliminary results from studies in this lab have found that altering the gravitational environment of the quail embryo changes the growth rate and morphology of the vestibular afferents. There are current studies evaluating whether the location of these afferents is a determinant for their response properties.

References

Fernández C, Goldberg JM and Baird RA. The vestibular nerve of the chinchilla III. Peripheral innervation patterns in the utricular macula. *J Neurophysiol* 63:767-780, 1990.

Lorente De Nó R. Etudes sur l'anatomie et la physiologie du labyrinthe de l'oreille et du VIII[e] nerf. Deuxième partie. Quelque donnèes au sujet de l'atomie des organs sensoriels du labyrinthe. *Trab Lab Invest Biol Univ Madrid* 24:53-153, 1926.

Matsui JI, Haque A, Huss D, Messana EP, Alosi JA, Roberson DW, Cotanche DA, Dickman JD, and Warchol ME. Caspase inhibitors promote vestibular hair cell survival and function after aminoglycoside treatment *in vivo*. *J Neuroscience* 23(14):6111-6122, 2003.

Poljak S. Uber die Nervenendigungen in den vestibulären Sinnesendstellen bei den Säugetieren. *Z Anat Entwicklungsgesch* 84:131-144, 1927.

Ramon y Cajal S. *Histology of the Nervous System of Man and Vertebrates.* New York: Oxford. English translation, 1995. Original Spanish version, 1908.

Retzius G. Das gehörorgan der wirbelthiere. II. Das gehörorgan der reptilian, der vögel und der säugethiere. Stockholm: Samson and Wallin, 1884, p. 179-191.

Si X, Zakir M and Dickman JD. Afferent innervation of the utricular macula in pigeons. *J Neurophysiol* 89:1660-1677, 2003.

Table I. *Morphological parameters by afferent type*

Parameter	Calyx		Dimorph		Bouton	
	Range	Mean	Range	Mean	Range	Mean
Axon Diameter, μm	1.8 - 4.0	2.9 ± 0.7	1.0 - 3.2	2.9 ± 0.7	1.1 - 2.7	2.0 ± 0.5
Branches/fiber	0.0 - 2.0	0.4 ± 0.9	3.0 - 49.0	14.0 ± 10.9	5.0 - 63.0	27.5 ± 15.8
Terminal fiber length, μm	25.6 - 86.7	48.5 ± 20.4	40.6 - 339.3	143.0 ± 80.4	78.4 - 573.5	264.1 ± 157.3
Terminal fiber volume, μm^3	104.4 - 837.7	343.6 ± 219.7	157.6 - 607.0	337.8 ± 137.8	54.2 - 1074.7	324.5 ± 234.7
Innervation area, μm^3	128.4 - 460.5	302.6 ± 120.1	119.4 - 553.5	349.0 ± 132.1	122.9 - 1042.4	420.1 ± 276.2
Type I hair cells/fiber	1.0 - 9.0	4.2 ± 1.8	1.0 - 6.0	2.3 ± 1.3	---	---
Calyceal terminals/fiber	1.0 - 2.0	1.2 ± 0.4	1.0 - 2.0	1.1 ± 0.3	---	---
Bouton terminals	---	---	1.0 - 27.0	7.9 ± 6.2	4.0 - 57.0	23.5 ± 15.3

Mean values are ± SD.

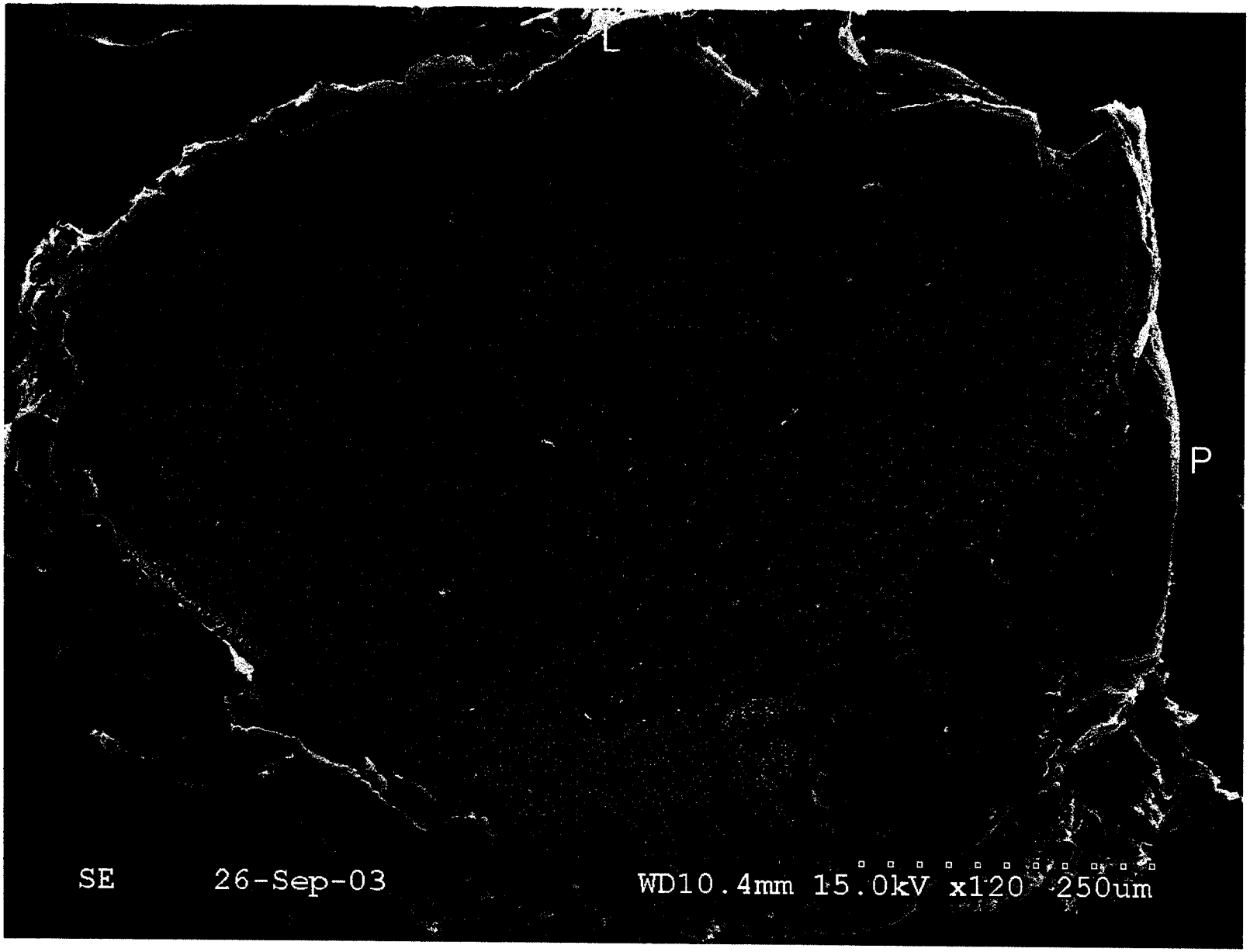

Figure 1: SEM Micrograph of Adult Quail Utricle.

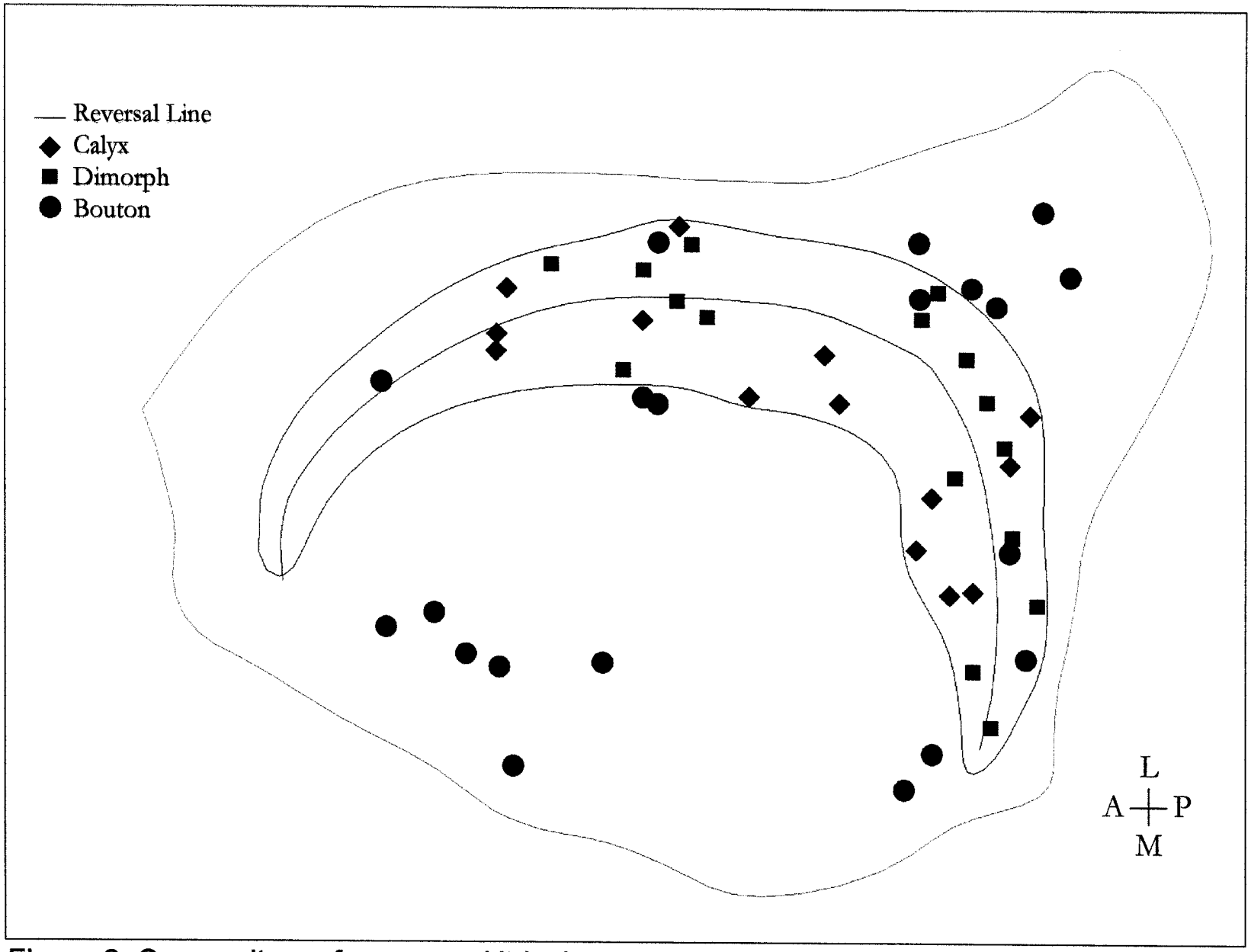

Figure 2: Composite surface map. Utricular macula is shown with reversal line and striola region indicated. Locations of 50 reconstructed afferents are indicated.

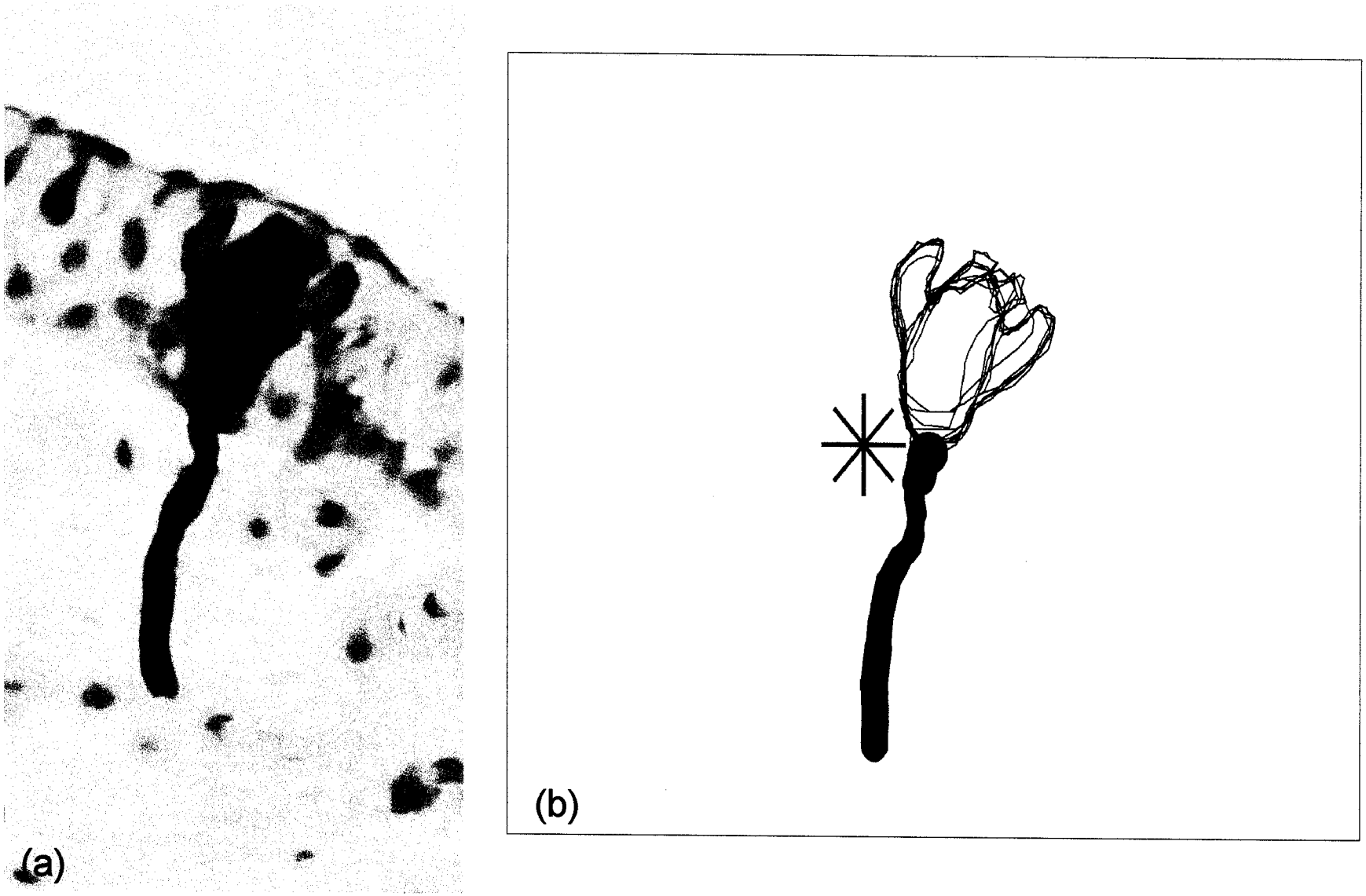

Figure 3: Calyx afferents:
(a) Photomicrograph of transverse section, (b) Anatomical reconstruction.

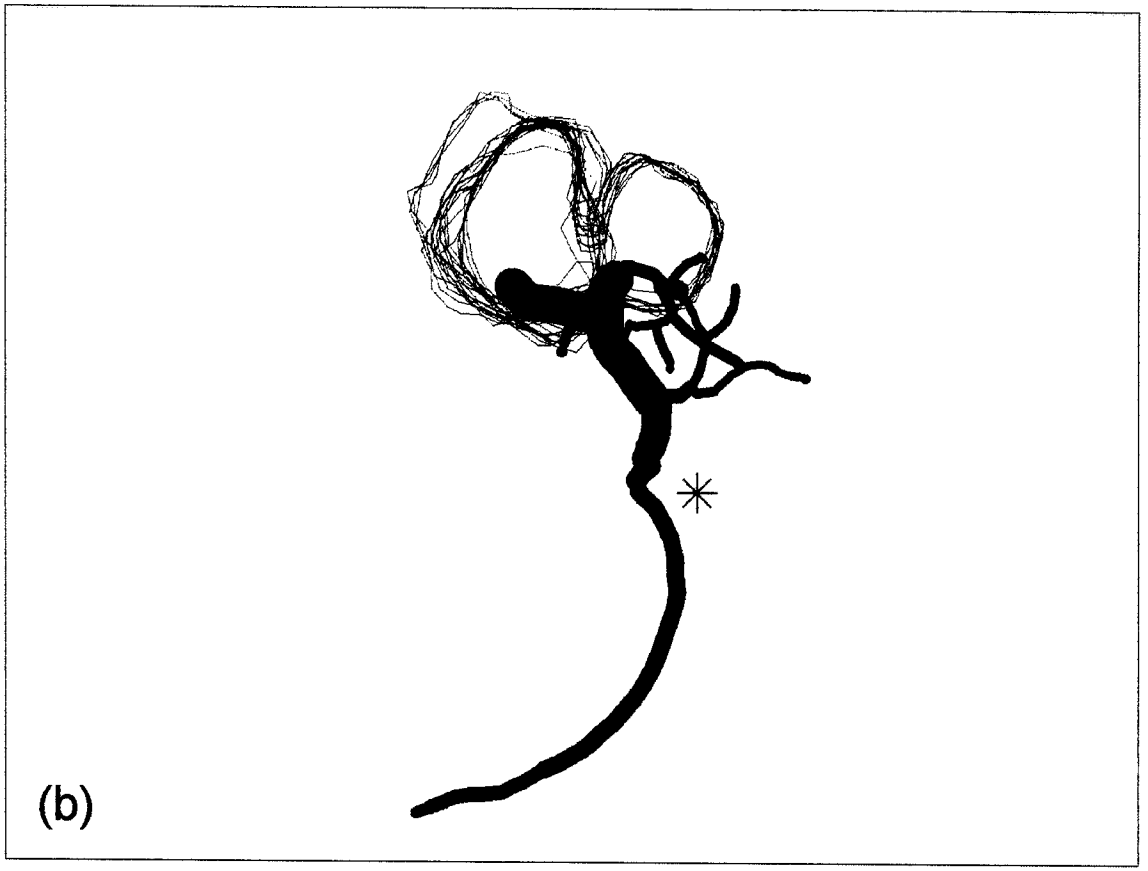

Figure 4: Dimorph afferents:
(a) Photomicrograph of transverse section, (b) Anatomical reconstruction.

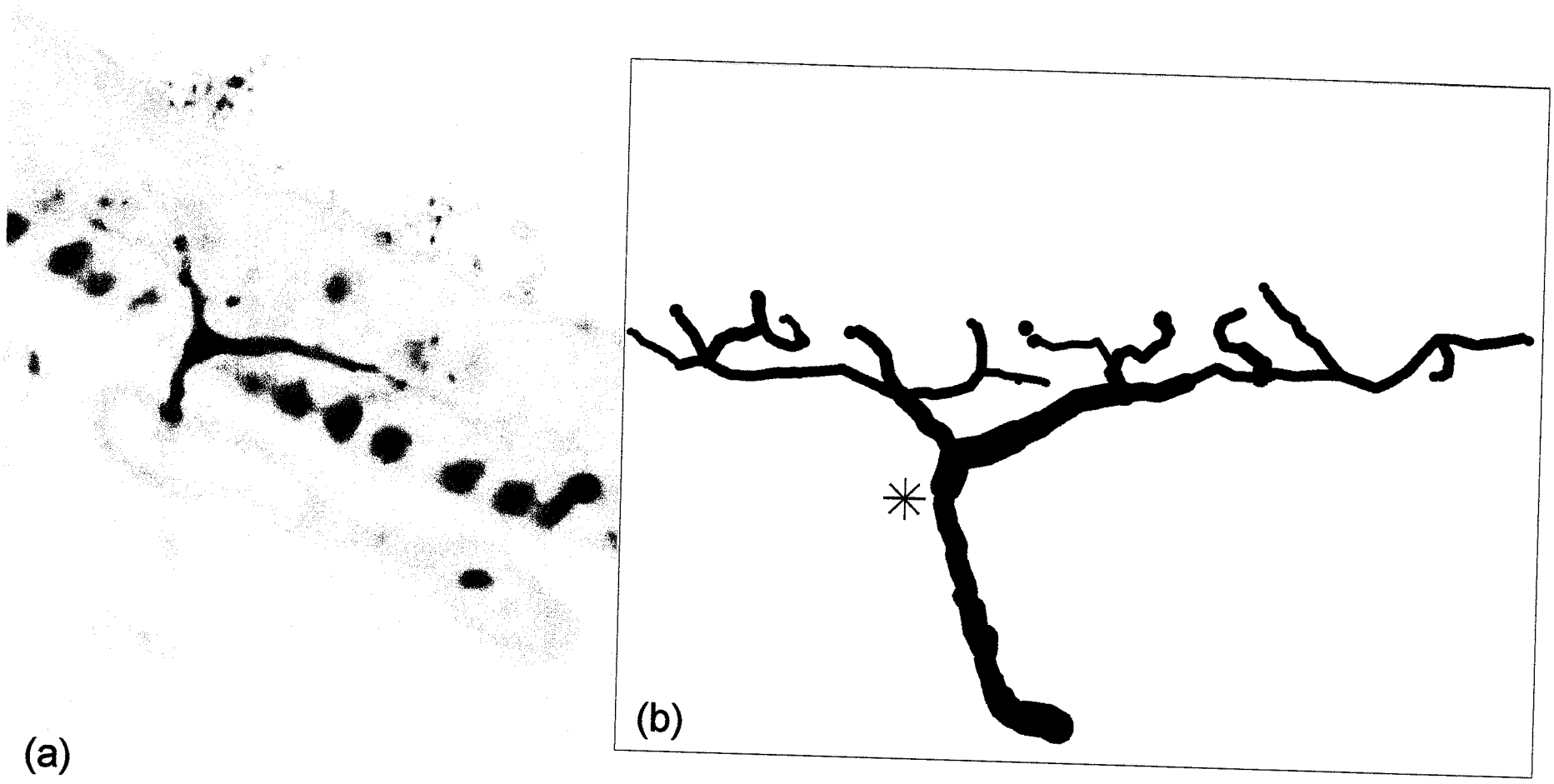

Figure 5: Bouton afferents:
(a) Photomicrograph of transverse section, (b) Anatomical reconstruction.