

LOCALIZATION OF NICOTINIC RECEPTORS ON HAIR CELLS AND AFFERENTS OF THE TURTLE SEMICIRCULAR CANAL

A Major Qualifying Project

Submitted to the Faculty

Of the

WORCESTER POLYTECHNIC INSTITUTE

In partial fulfillment of the requirements for the

Degree of Bachelor of Science

By

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Date: October 19, 2007

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ABSTRACT

A distinct $\alpha 9/\alpha 10$ nicotinic acetylcholine receptor (nAChR) may mediate the inhibition of type II hair cells in the turtle posterior crista. This project aims to localize these nAChRs in the hair cell layer with the use of commercial antibodies and fluorophore-conjugated nicotinic antagonists (α -bungarotoxin). An additional goal has been the optimization and development of protocols for this specific purpose. Identification and visualization of these nAChRs will provide greater insight into the complex neural pharmacology of the inner ear.

ACKNOWLEDGMENTS

I would like to convey my sincere appreciation and heartfelt gratitude to a wonderful researcher and mentor, Dr. Joseph C. Holt. This work was conducted under his constant guidance and with his untiring support, in his laboratory at the Otolaryngology Department at the University of Texas Medical Branch (UTMB) in Galveston, Texas.

I am grateful to Dr. Golda A. Leonard and Dr. Robert Leonard at UTMB who were always available for me to call on for advice in my research, and whose expertise has been a great help. I would also like to acknowledge Pat Gazzoli and Laura Teed, also at UTMB, for their kind help and assistance in several ways. In addition, I would like to thank Dr. Jill Rulfs for her role as my project advisor at WPI.

Last, but not the least, I thank my parents. Their unstinted support and constant encouragement has played a significant role throughout my education.

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I. BACKGROUND

A. Function

Organs of the vestibular nervous system, located in the inner ear, are primarily responsible for providing input regarding our balance and movement within Earth's gravitational field. Information on linear and rotational movement of the head, as well as its static position, is tracked by these organs and relayed to the central nervous system (CNS). This information can then be transmitted as instructions to muscles responsible for posture and equilibrium and also to neural structures responsible for controlling eye movements. Higher order processes such as spatial perception and navigation are also dependent on the vestibular system.

The crucial functions of this system are best realized with a simple reflection at what happens when it is stimulated in atypical ways or when it ceases to work correctly. Inappropriate motion of or damage to the vestibular organs and/or its pathways can give rise to sensations of vertigo, dizziness or a sense of imbalance, and can include feelings of discomfort or nausea. Everyday examples include the experience of spinning in circles, taking a long car ride on a bumpy road, a ride at an amusement park, the sense of imbalance during an ear infection. Sudden falls, a major cause of injury and mortality in the elderly, may also be attributed to a loss of vestibular sensitivity.

B. Overall Structure

The vestibular system is one of the more highly conserved organ systems, similar both anatomically and physiologically from fish to mammals [36]. The vestibular system starts in the ear with the membranous labyrinth, composed of a series of fluid-filled tubes and sacs, housed within channels of the temporal bone referred to as the bony labyrinth

(Figure 1A). Perilymph fills the spaces between the membranous and the bony labyrinth, while the fluid within the membranous labyrinth is known as endolymph. The organs of the vestibular system—namely the utricle, saccule and the semicircular canals—are contained within the membranous labyrinth and are the primary determinants of head movement and position [12]. It must be noted that these organs are bilaterally symmetrical, i.e., an identical set exists on each side of the head.

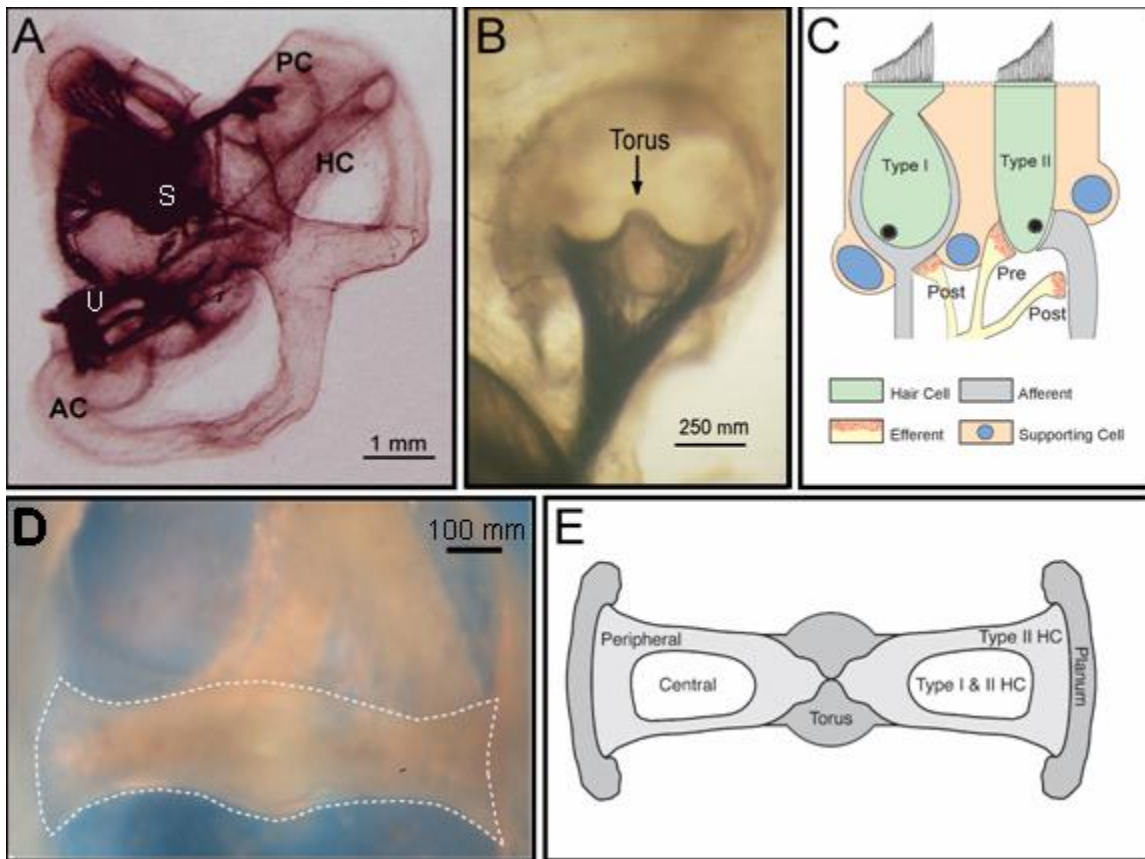


Figure 1: Introduction to the preparation. (Figures obtained from Dr. Joseph Holt)

A. Isolated turtle membranous vestibular labyrinth showing the arrangement of the vestibular end organs. The black, opaque regions are bundles of nerve fibers innervating each organ. PC, AC, HC corresponds to the posterior, anterior, and horizontal semicircular canal, respectively. **B.** Higher magnification of the posterior semicircular canal with its nerves branching to supply each side of the sensory epithelium (hemicrista). **C.** Illustration of type I and II hair cells and their innervation which along with supporting cells comprise the neuroepithelium. **D.** Photomicrograph showing a bird's-eye view of the posterior canal crista (outlined in white dashes). **E.** Illustration of the regionalization of the crista showing locations of type I and II hair cells.

The saccule and the utricle, labeled in Figure 1A, are examples of otolithic organs whose apical surfaces are covered by otoconia—small, crystalline calcium carbonate stones. Linear motion and the three-dimensional position of the head are determined as a result of the shifting of these ‘stones’ in response to head movement [12]. The strategic positions of the organs themselves are also key to the proper translation of the body’s motion. When the head is in its upright position, the saccule is oriented vertically and is capable of responding to motion in the vertical ‘y’ plane. The utricle is perpendicular to the saccule and has components in both the horizontal ‘x’ and a third ‘z’ dimension. These components are easier to understand in a real-life context—the ‘y’ plane involves the vertical motion experienced when one jumps up and down, rolling or swaying our head from left to right is an example of the horizontal motion of the ‘x’ plane and the ‘z’ plane refers to front to back movement of the head (imagine facing an open car window and pushing your head in and out, forward and backward). [17, 36]

There are three different semicircular canals, each canal named after the plane against which it is oriented. Each labyrinth has a horizontal canal and two vertical canals—the anterior and the posterior canal—whose ducts meet to form a common arm, known as the ‘common crus.’ Each canal originates and terminates within the utricular space, forming a loop structure which is marked on its end by an enlarged swelling called the ampulla. The location of the canals, as well as the characteristic swelling of the ampulla can be visualized in Figure 1A. It is the ampulla that houses the sensory neuroepithelium of the canal [12, 36].

In the semicircular canals, the epithelium is tethered to a cone-like gelatinous mass known as the cupula (or to an otolithic membrane), which spans the entire diameter

of the ampulla. When we shake our head to indicate yes or no, or turn to look behind us, the canals instantaneously move with our head. The endolymph or fluid in the canals, however, cannot move at the same speed and travels at a slower velocity. This inertial motion results in a pressure upon the gel-like cupula, oppositely displacing its mass in a mechanical movement which is then detected by the “tethered” neuroepithelium [12]. The specific steps involved in this mechanotransduction will be discussed in greater detail at a later point.

Each vestibular organ has a layer of sensory epithelium. In the utricle and the saccule, the epithelium forms a flat sheet like structure against a wall of the organ (anterior for the utricle, ventrolateral for the saccule), which is referred to as the macula. However, our studies are predominantly focused on the neuroepithelium of the semicircular canals, which is known as the ampullary crest or the crista (plural: cristae). A view of the crista as seen if looking straight down the canal is seen in Figure 1B, and a bird’s eye view (as seen from above) is provided as Figure 1D [12, 36].

The crista and the macula are mostly similar in terms of cellular makeup. Essentially, the neuroepithelium is a matrix of hair cells, supporting cells, and the synaptic connections of afferents and efferents which rests on a stroma composed of connective tissue which is penetrated by blood vessels and nerve fibers (the central area in Figure 1B and 1D) [18]. The epithelia’s placement between the perilymph (sodium-rich, potassium-deficient, typical extracellular fluid) and the endolymph (potassium-rich, sodium-deficient, intracellular fluid) acts as an interface. This separation is necessary, of course, to maintain the ionic gradient necessary for normal function of the system [12].

C. The Hair Cell

The most crucial component of the vestibular organs are the mechanically sensitive hair cells, so named for the fingerlike projections protruding from the top of each cell (Figure 1C). The apical pole of each individual hair cell is marked by a tuft of sensory hairs, called stereocilia. These stereocilia are the primary transducers of mechanical motion associated with the vestibular system, such as that caused by the movement of the cupula or otoconia. The basal half of the hair cell is marked by synaptic bodies, surrounded by vesicles and adjacent to attachments for the pre and post-synaptic membrane specializations of the hair cell and its corresponding nerve terminal. [12, 36]

An individual stereocilium has a rigid actin core surrounded by plasmalemma. Anywhere from 20-100 such stereocilia form rows on the tops of hair cells, usually in descending order with the tallest immediately adjacent to a single kinocilium [23, 38]. The kinocilium is a microtubular structure which typically serves as the point of attachment between the hair cell and the otolithic membrane or the cupula. The arrangement of the stereocilia to the kinocilium is itself a critical characteristic of the vestibular machinery. As mentioned above, the cupula is displaced in response to angular acceleration, such as shaking or nodding the head. This movement in turn deflects stereocilia and hair cells. Deflection of the stereociliary tuft (hair bundle) towards the kinocilium results in depolarization (and therefore excitation) of the hair cell, while deflection away from the kinocilium translates to a hyperpolarization (inhibition) of the hair cell. This is the fundamental principle behind how the vestibular system detects and translates head movements to a language that the central nervous system ultimately understands. [13]

A brief note must be made here about stereocilia direction in the various organs: in the semicircular canals, the hair bundles all face the same direction. Therefore, displacement of the cupula itself translates into the depolarization (in one direction) or the hyperpolarization (in the other direction) of the hair cell. In the saccule and the utricle, however, the polarity of the hair bundles changes at specific points in the macula. A line, known as the striola, may be drawn at this point to demarcate the change in polarity. Secondly, the striola transects the macula such that as one moves from one side to the other, hair cells change their orientation slightly to remain perpendicular to the striola. This endows otolithic organs with many axes of sensitivity. [18, 29, 36]

In the crista and macula of reptiles, birds and mammals, there are two types of vestibular hair cells. Type II hair cells (on the right, Figure 1C) are cylindrically shaped and are innervated by a button-shaped, or bouton, afferent ending. Both efferents and afferents make contact with the type II hair cell via bouton endings. Fish and amphibian neuroepithelia only have type II hair cells, and consequently only bouton-type afferents. Type I hair cells (on the left, Figure 1C) are flask-shaped with a rounded base and a short, constricted neck. In contrast, with bouton afferents, the afferent innervation here engulfs the entire type I hair cell and a large part of the neck, resulting in a so-called calyx ending that can be easily distinguished in the dense hair cell layer. The structural nature of the calyx afferent also prevents any efferent contact with the type I hair cell. [2]

Bouton and calyx afferents receive an electrical translation of the mechanical movement of hair cells, and relay this information from the vestibular end-organs to the brain. Efferents, on the other hand, carry directions received from the brain to target organs. Efferents innervating the vestibular organs originate near the facial nuclei in the

brain stem, where they travel in nerve VIII and branch profusely to each vestibular organ. They can be distinguished from afferents due to their highly vesiculated appearance at the EM level. Efferents may end as boutons on calyx afferents, on afferents innervating type II hair cells or on type II hair cells themselves (Figure 1C). [12]

Finally, supporting cells constitute a large part of the layer, wrapping around hair cells and filling the spaces in-between. They are crowded with secretory-like granules and organelles, and their basal portion is nucleated. Supporting cells are also important in the synthesis and maintenance of the macula and cupula, in the removal of the neurotransmitter from synaptic spaces and in the regulation of the ionic environment of the perilymph and endolymph. [18]

D. Mechanotransduction by Vestibular Hair Cells

Mechanotransduction in the vestibular system may be defined simply as a conversion of mechanical stimulus into electro-chemical activity. The slightest movement of our head must be conveyed firstly to the vestibular system (which happens mechanically) and then it must be transmitted to the brain chemically and then electrically. When hair bundles are deflected by movement of the cupula, mechanoelectric transducer (MET) channels either open or close, depending on the direction of movement. Recall from the discussion of the epithelia that when stereocilia are deflected towards the kinocilium, excitation (or depolarization) of the hair cell takes place, and when deflected away from the kinocilium, inhibition (or hyperpolarization) of the hair cell occurs. When MET channels open as a result of deflection towards the kinocilium, a depolarizing transducer current is created as endolymphatic potassium ions move into the hair cell. Voltage-sensitive calcium channels in the basolateral

plasmalemma of the hair cell open, leading to an influx of calcium ions and a subsequent release of the afferent neurotransmitter, glutamate, from vesicles around the synaptic ribbons onto the afferent synapse. Glutamate is packaged in vesicles that fuse with the plasmalemma when cytosolic calcium is high, consequently releasing their contents. The released glutamate then diffuses across the synapse, binds to the post-synaptic receptor, and then depolarizes the afferent terminal. This depolarization is then translated into action potentials, which are transmitted to the brain to be read as messages about the position and movement of the head. [5, 10, 12]

If opening the MET channels leads to depolarizing and neurotransmitter release, then it follows that the closing of MET channels leads to hyperpolarization and inhibition of release. Instead of calcium channels opening, they close, resulting in a decrease in neurotransmitter release. Less post-synaptic receptor activation results in less depolarization of the afferent terminals, and therefore a decrease in action potentials. [5, 10, 12]

E. The Turtle Crista

The chosen animal model in this lab is the red-eared turtle (*Trachemys scripta elegans*) which appropriately suits our research needs. Anatomically and physiologically similar to the the mammalian vestibular system, the turtle inner ear is populated by both type I and type II hair cells and a crista that is relatively easy to extract and manipulate. All discussion from this point onwards, therefore, is focused specifically on the turtle crista and its characteristics. The panels A-E are all representative of the turtle crista (A, B and D were actually extracted from a red-eared turtle). Figure 1E characterizes the turtle crista into the regions as described below.

Each vertical crista of the turtle is composed of two triangular shaped ‘hemicristae,’ The hemicrista is widest at the ‘planum’ and tapers off towards the center at the ‘torus,’ a non-sensory region (labeled in Figure 1E and identifiable in 1D). The horizontal crista resembles half of the vertical crista, consisting of a single hemicrista. The crista has been demarcated into two zones—the central and the surrounding peripheral zone—based on the cellular makeup of the regions. Type I hair cells are only present in the central zone, which is also populated by type II hair cells. Type II hair cells are also present in the peripheral zones, i.e., towards the torus and out at the ends of the planum. [28, 34]

F. Vestibular Afferents

The central zone of the crista, rich in both type I and type II hair cells, is innervated by either calyx, bouton or dimorphic afferents, while the type II-only peripheral zone is rich in bouton fibers. Calyx afferents, as mentioned earlier, engulf the entire type I hair cell, surrounding most of the body and neck and leaving mostly the sensory hair bundle exposed on the apical end. Boutons are bud or button-shaped afferent endings on type II hair cells. Dimorphic afferents have branches that extend out as individual calyces on type I hair cells and as bouton endings on type II hair cells. (The calyx and bouton afferents are the grey structures innervating the hair cells in Figure 1C.) [2, 8, 9]

The bouton afferents can be further divided into three classes—boutons near the planum (BP), near the torus (BT) and in the mid-central areas of the hemicrista (BM). This is not simply a regional classification; electrophysiological characterization has established varied response patterns to efferent and mechanical stimulation for each

group of afferents. Stimulation of the efferent component for the BT afferents has been seen to produce a pronounced, long lasting inhibition. BM afferents display a shorter inhibitory period, with a quick excitatory rebound. Calyx or CD afferents, in complete contrast, exhibit a prolonged excitatory response to efferent stimulation. This varied pattern of responses confirms that an impressive and complex machinery exists within each hair cell and afferent; it also indicates that crucial differences in receptors and ion channels can have a significant effect on efferent actions and ultimately neuronal expression. (See Figure 2 for a visual characterization of this phenomenon.) [1, 9, 11, 12, 17]

G. Vestibular Efferents

As described previously, vestibular efferents may directly contact calyx afferents, or end on afferents innervating type II hair cells and/or on type II hair cells themselves (yellow structures in Figure 1C). The neuronal terminology used to describe the efferent-afferent and efferent-hair cell connection has been modified to better characterize these various connections of the vestibular system. The efferent component is termed as post-synaptic when it connects to any afferent, be it calyx, bouton or dimorphic; however, an efferent on the type II hair cell itself is referred to as a pre-synaptic component. It is thought that a single efferent can provide both pre and post-synaptic terminations in the hair cell layer. [8, 9, 12, 17]

H. Efferent Neurotransmitters of the Vestibular System

The predominant neurotransmitter of the efferent vestibular system is acetylcholine (ACh). It is not, however, the only efferent neurotransmitter, although it is the one that has been the most researched. Adenosine triphosphate (ATP), gamma

aminobutyric acid (GABA) and ACh have all been identified as neurotransmitters in this vestibular efferent system. They may work through interactions with both ionotropic receptors and G-protein coupled (i.e., metabotropic) receptors. Ionotropic receptors are ligand-gated ion channels that are activated very quickly and are likely to underlie rapid actions, while metabotropic receptors initiate signal transduction pathways and are relatively slower in their neural modulation. Both are discussed in greater detail at a later point.

GABA, as mentioned previously, has been implicated as both an efferent and an afferent neurotransmitter. Preliminary studies have suggested that ATP may be co-localized with ACh and perform in a similar fashion. In addition, calcitonin gene related peptide (CGRP), enkephalin and substance P are some of several neuropeptides that have also been proposed as participants in the system. There is strong evidence that CGRP, already widely distributed in the central and peripheral system, is present in efferent axons synapsing on afferents (i.e., the post-synaptic component) and is involved in the slow excitation of hair cells. [12]

I. Acetylcholine (ACh)

ACh is perhaps best characterized for its role in stimulating skeletal muscle fibers and its opposite role in inhibiting the cardiac muscle via the vagus nerve (i.e., slowing the heart rate). Throughout the body, it is largely involved in the “involuntary” effects of the nervous system. In the peripheral autonomic nervous system, ACh is the neurotransmitter of all preganglionic autonomic fibers, all postganglionic parasympathetic fibers and some postganglionic sympathetic fibers. These fibers are all labeled as ‘cholinergic.’ Chemically, this choline ester exhibits behavior similar to both muscarine (an agonist of

muscarinic receptors, a metabotropic-type receptor) and nicotine (agonist of nicotinic or ionotropic receptors). The two behaviors are important characteristics of ACh, resulting in the formation of two distinct families of ACh receptors with a large variability in pharmacology. [14]

In the peripheral vestibular system, efferent neurons are characterized as cholinergic, based on the presence of the choline acetyltransferase enzyme (ChAT), which is responsible for the synthesis of ACh. Acetylcholinesterase (AChE) is necessary for the degradation of ACh in synaptic clefts and is also prolific among vestibular efferents. Cholinergic (i.e., nicotinic and muscarinic) receptors regulate the action of the ACh on their target structures. The two types of ACh receptors, briefly mentioned previously, are the ionotropic nicotinic acetylcholine receptors (nAChRs) and the metabotropic muscarinic acetylcholine receptors (mAChRs). Physiological experiments with tubocurarine, a nAChR blocker, and atropine, a mAChR blocker, have demonstrated the existence of both receptors in vestibular organs, since the proposed effects of ACh released by efferent stimulation are significantly compromised with the application of either drug. [14]

J. Muscarinic Acetylcholine Receptors (mAChRs)

mAChRs belong to the metabotropic or G-protein coupled receptor group. As their name suggests, these receptors use G-proteins as a signal transducer. The pathway is initiated by the binding of a ligand, molecules of ACh, to a specific region on the seven transmembrane protein receptor. The shape or conformation of the receptor changes in response to the binding of the ligand. A change in conformation activates the components of the coupled G-protein—usually consisting of an α , β subunit and a γ subunit. All three

subunits activate other downstream proteins and ion channels within the membrane of the cell. Eventually (within tens of milliseconds), this signaling cascade leads to neural modulation, usually resulting in some inhibition or excitation. The complexity of these signal transduction pathways means that the mAChRs operate relatively slowly (in comparison to nicotinic receptors). mAChRs on the hair cells and afferents are likely to be responsible for some of ACh's slow excitatory actions. [14]

K. Nicotinic Acetylcholine Receptors (nAChRs)

nAChRs are ionotropic receptors that form ligand-gated ion channels in the plasma membrane. These ion channels are permeable to sodium, potassium and calcium ions. Activation by acetylcholine, therefore, rapidly increases the membrane permeability to these cations. Typically within a couple of milliseconds (significantly faster than mAChRs in the neural world), the influx of cations can lead to depolarizing currents which may further activate other voltage gated ion channels thereby propagating the effects. Depending on what nAChR and/or subsequent ion channel gets activated, the final result may be rapid inhibitory and/or excitatory responses in hair cells and afferents, occurring almost instantaneously after ACh binding. [14, 31]

Structurally, nAChRs are formed by the pentameric arrangement of five distinct subunits surrounding an internal channel. nAChRs are pentameric, ligand-gated ion channels whose pharmacology and physiology can be related to the subunits that compose them. To date, 10 alpha subunits (α), 4 beta (β) subunits, and 1 each gamma (γ), delta (δ), and epsilon (ϵ) subunit have been described. Functional nAChRs may be comprised of single α , multiple α , single α and single β subunits, etc. Homomeric receptors are composed of only a single type of α -subunit, while heteromeric receptors

are a combination of both α and β units (for example, $\alpha 4\beta 2$) or 2 α subunits (e.g., $\alpha 9/\alpha 10$). [3, 22, 31, 33]

Three pharmacological/physiological categories of nAChRs exist: 1) nAChRs of the skeletal muscle and those that are present in fish electric organs, 2) neuronal nAChRs that do not bind the snake toxin α -bungarotoxin (or α -BTX, discussed later) and 3) neuronal nAChRs that do bind α -BTX [12]. Receptors that contain the $\alpha 7$ or $\alpha 9$ subunits can be homomeric and have been pharmacologically characterized as binding to α -BTX. The $\alpha 9$ nAChR is especially significant in that it is the only nAChR classified whose presence has been absolutely confirmed in the vestibular hair cell organs, in both type I and type II hair cells [4, 32, 39, 21, 30]. Its pharmacology and functional role in the vestibular system is therefore a subject of great importance and interests to those studying the efferent system.

II. INTRODUCTION

A. The $\alpha 9$ Nicotinic Acetylcholine Receptor ($\alpha 9$ nAChR)

As mentioned above, the $\alpha 9$ nAChR has been observed to be present in vestibular hair cells. Luebke et al described its location in the rat: antibodies to the $\alpha 9$ nAChR labeled afferents of either the calyx or the dimorphic type in the central zone with some dimorphic afferents in the peripheral zone [30, 32]. In their experiments, the $\alpha 9$ nAChR protein was not found in bouton-only afferents in any area of the rat crista. The regionalization of this receptor protein in the turtle has not yet been well-defined or confirmed, and is part of the work included in this study.

Pharmacologically, $\alpha 9$ containing nAChRs in hair cells display several physiological and pharmacological characteristics that cannot be ascribed to the $\alpha 9$ nAChR alone. It is thus the general belief that in the vestibular system, the recently described $\alpha 10$ subunit couples with the $\alpha 9$ nAChR in the ear, to result in its characteristic pharmacological behavior [6, 39]. While this $\alpha 9/\alpha 10$ nAChR class has been described only recently, it has been suggested that its evolutionary appearance may have preceded that of any of the other nAChRs [21, 39, 40]. The $\alpha 9/\alpha 10$ nAChRs are functionally unique in their behavior and pharmacological properties. $\alpha 9$, $\alpha 9/\alpha 10$ and the $\alpha 7$ nAChR are all noted for their significant permeability to calcium, corroborating their importance since this calcium is crucial to their physiological actions [3, 14, 22, 30, 32]. The $\alpha 9/\alpha 10$ nAChR response to ACh is antagonized competitively by nicotine; this nAChR is also blocked by atropine (actually a muscarinic blocker), d-tubocurarine, curare and strychnine [45]. The $\alpha 7$ and $\alpha 9/\alpha 10$ receptors: they are reversibly blocked by the snake venom toxin α -bungarotoxin (α -BTX), and are the only nAChRs that display this

susceptibility [22, 40, 46]. Therefore, α -BTX has proven to be a useful tool, since $\alpha 9/\alpha 10$ nAChRs rich-regions.

Functionally, $\alpha 9/\alpha 10$ nAChRs have been implicated as mediators of hair cell inhibition in vestibular organs [16, 17]. Efferent activation of these receptors on type II hair cells therefore leads to an influx of calcium ions [17]. As the local levels of calcium increase due to the calcium ion flowing into the cell, small-conductance, calcium-dependent potassium channels (SK channels) are activated, resulting in a significant efflux of potassium ions from the cell [25]. This phenomenon rapidly results in membrane hyperpolarization and inhibition of neurotransmitter release in the type II hair cells in the BM and BT afferent crista zones—decreased neurotransmitter release translates into lesser action potentials, as illustrated in Figure 2A. A different, currently-unidentified nAChR (hypothesized to be the $\alpha 4$ nAChR) has been suggested to play a role in post-synaptic afferent excitation by efferents [1, 17].

Since the inhibitory function of the $\alpha 9/\alpha 10$ nAChR requires efferent stimulation, the type II hair cell in the BM and BT zones, which makes direct contact with an efferent, expresses this receptor. The type I hair cell is encapsulated by its afferent and therefore is not likely to be innervated directly by efferents [13, 43]. However, it is intriguing to note that several studies have found the $\alpha 9$ nAChR to be still present in these calyx hair cells. At this point there is no explanation for this observation since the $\alpha 9$ receptor protein likely fulfills no apparent function here; however, it is hypothesized its presence is possibly a remnant of evolution or the organism's development [36]. At one point during inner ear development, calyces are not fully formed and efferents can obviously connect directly to the type I hair cell: in this case, the $\alpha 9$ nAChR might be crucial in such a case

to facilitate hair cell inhibition before the afferent arrives. However, SK channels, which are needed for inhibition, have not been positively identified in type I hair cells indicating the need for further investigation [36, 44].

B. Immunohistochemistry

We are interested in localizing efferent nAChRs in the vestibular organs. One way of localizing cellular proteins in tissue is to use immunohistochemistry (IHC). While the basic principles of IHC are fairly straightforward, the actual process requires a significant amount of optimization and trial-and-error. IHC protocols must be adapted for various tissue types, antigens of interest, antibodies and detection systems used (fluorescence vs peroxidase, live tissue vs fixed tissue, etc.). The antibody titer will determine the intensity of the signal and must be adjusted to obtain the optimal image.

The primary need for any histological process is the preservation of the cells and the tissues in as reproducible a state as possible. A fixative is usually used immediately following extraction of tissue from the live organ of interest to essentially “freeze” or “fix” the tissue in that state, usually by cross-linking molecules in the cellular membranes. However, fixation can potentially change protein structure and conformation, which will duly have an affect on the antigenicity of the protein. [19, 37]

In a recent study by Moser et al which evaluated a wide variety of antibodies from different manufacturers directed against key nAChRs (including several used in this project). Through immunohistochemical experiments, the authors demonstrated that the same immunoreactivity was observed in brain tissue from both wild-type and knock-out mice for each nAChR-specific antibody [37]. This suggests that with conventional IHC

techniques—which involve the use of traditional fixatives—several popular commercial antibodies are not suitable and do not provide accurate or reliable results.

To preserve staining quality and antigenicity, it is possible to use unfixed tissue with a cryostat cutting technique. This is a more sensitive technique, since the tissue must be treated gently and carefully without proper fixation, but often yields clearer, more trustworthy results since the protein denaturing affects of fixation are not present. A third alternate way, and one used in this study, is to carry out the ligand-labeling or antibody staining steps of the protocol prior to fixation. This protocol provides the benefit of preserving the antigen structure and sensitivity during treatment with a specific antibody or label, and also prevents tissue disintegration or damage since fixation follows incubation. A conventional primary and secondary antibody series is not necessarily possible with this technique, however, since those require long periods of incubation during which cell lysis in the dying tissue would build up excess artifact. This too can be overcome by treating with a molecule that recognizes the antigen of interest directly. Examples of such molecules are receptor ligands and ion channel antagonists, and include the two used in this study, α -bungarotoxin (α -BTX) and apamin.

C. α -Bungarotoxin

Exactly this type of substance has been used in this study to overcome the difficulties of fixative based IHC and at the same time achieve clear, reproducible results. Our focus, discussed previously, is on the $\alpha 9$ nAChR in the hair cell layer and $\alpha 4$ nAChR on afferents. The $\alpha 9$ nAChR has been especially difficult to isolate and label in the hair cell structure, and the turtle model adds to the complexity of the problem since antibodies against the turtle are difficult to find. Our experience with IHC of the hair cells so far has

led us to strongly suspect that the paraformaldehyde-based fixative used in our studies is compromising the antigenicity of the $\alpha 9$ nAChR. With experimentation of different fixatives and varied antibody types, we have struggled to optimize the IHC protocols to obtain some type of lucid and reproducible staining. To this end, we have found that α -bungarotoxin (α -BTX) has initially proven to be an extremely useful tool.

The α -bungarotoxin toxin is a potent blocker of ACh. This 8kDa protein is present in the venom of the banded krait, *Bungarus multicinctus*. It is a 74 amino acid polypeptide, cross-linked by five disulfide bonds. Its amino acid sequence is homologous to other toxins obtained from cobra and sea snakes. [41]

α -BTX binds to the nAChR, acting as a competitive antagonist of ACh. In vestibular hair cells, it has been seen to reversibly bind to $\alpha 9$ nAChR in several physiological studies. In fact, the $\alpha 9$ inhibitory response in the turtle posterior crista was successfully blocked by the application of α -BTX to the exposed vestibular epithelia [17]. Studies on rat end organs have also confirmed α -BTX binding on type II vestibular hair cells and in some type I chalice, which is consistent with the $\alpha 9$ nAChR localization described by Luebke et al [30].

D. Apamin

Apamin is a known neurotoxin, purified from bee venom as a 10 amino acid peptide with two disulphide bridges [26]. It plays an important role in neural pharmacology as a blocker of calcium sensitive potassium channels, and specifically the small-conductance potassium channels (SK channels) that are present on type II hair cells (discussed earlier) [25]. In physiological experiments conducted in the turtle crista and presented by Holt et al in 2006, apamin successfully converted the normal inhibitory

response to excitation, indicating that this toxin was binding to SK channels and actually unmasking $\alpha 9$ nAChR mediated excitation [17]. Based on its pharmacological qualities, one can anticipate that apamin could be a potentially useful tool in labeling the SK-channel rich type II hair cells in the central and peripheral zones of the turtle posterior crista. IHC experiments conducted in this study explore the potential of this compound for this purpose.

III. SPECIFIC PURPOSE

Previous studies in this laboratory have shown that afferents in the vestibular periphery of the turtle crista produce varied responses to efferent stimulation. Stimulating the efferent component for the bouton afferent near the torus (the BT units), results in a pronounced, long lasting inhibition. Bouton afferents in the central zone, or BM units, are also inhibited but for a shorter period with a quick excitatory rebound. Calyx or CD afferents, on the other hand, exhibit a prolonged excitatory response to efferent stimulation. [1, 11, 17, 34, 36]

The schematic shown in Figure 2 illustrates this phenomenon, separating the efferent response into a bouton and a calyx category. The spikes represent action potentials during the presentation of a single efferent shock train stimulation (at=0) for the boutons and the calyx afferents in the hair cell layer. 20 shocks at 200/s at t=0 were administered to the efferent fibers. It is obvious that the bouton response is inhibited, indicating hyperpolarization, since the spikes completely disappear at this stimulation. In the blue graph (calyx response), we observe the opposite to be true, with an immediate increase in the number of spikes or action potentials, indicating depolarization and excitation of the afferent since efferents do not synapse on the type I hair cell.

Preliminary studies designed to investigate the receptor behind each physiological response used three known pharmacological blockers were used: strychnine, ICS and α -BTX. The graphs in red represent data from bouton afferents, blue represent the calyx response. Response after application of the blocker is represented by the black line. It is observed that the bouton inhibitory response is blocked by all three substances. Meanwhile, the calyx excitatory response remains almost unaffected. [1, 17]

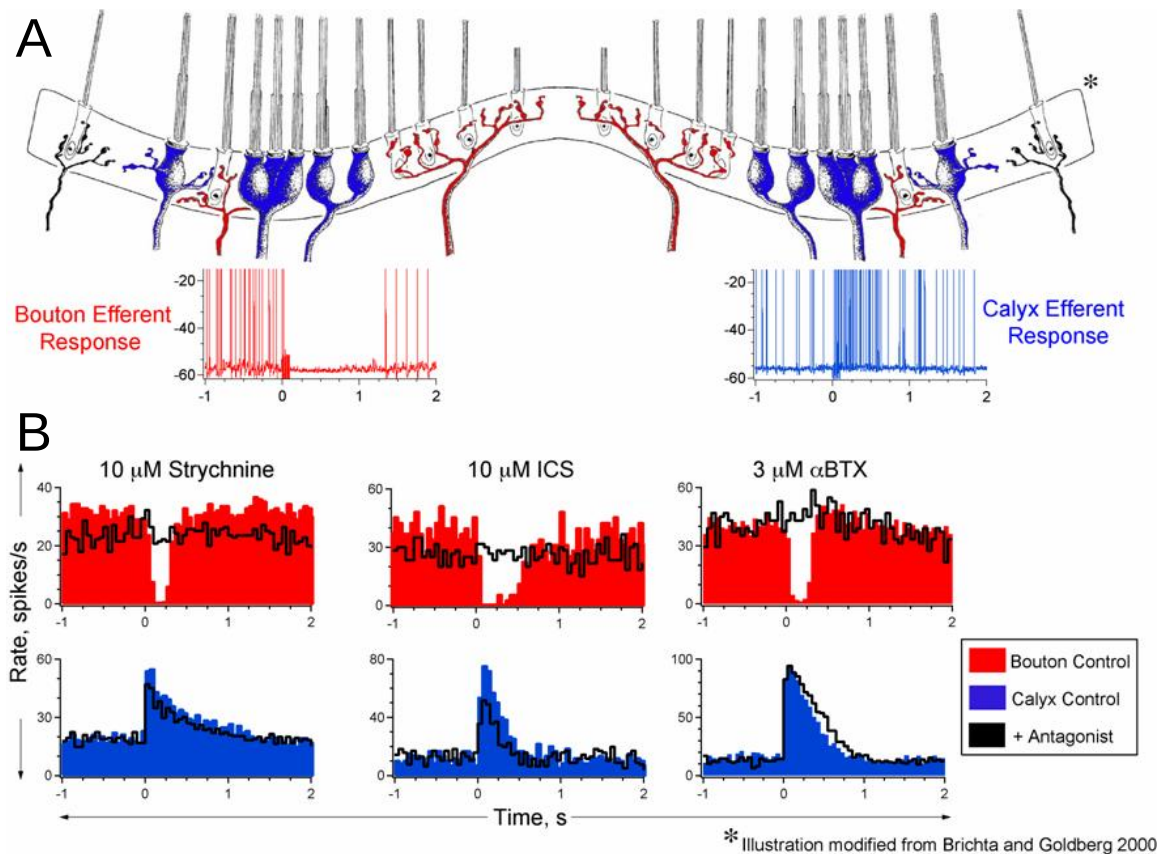


Figure 2: Summary of efferent responses in the turtle posterior crista and their pharmacology. [1]
A. Schematic of a longitudinal section through the center of the turtle posterior crista illustrating the relative location of hair cells and their respective afferents. Bouton afferents (**red**) can be seen innervating type II hair cells near the torus (BT) and more medially (BM). Bouton afferents near the planum (BP) are shown in black. Calyx and Dimorphic afferents (CD) innervating type I hair cells in the central zone are shown in **blue**. A typical efferent response for BT/BM afferents is also shown in red on the left and that for the CD is shown in blue on the right. **B.** In the top row, efferent responses in BT/BM afferents are shown before (red bars) and after (black line) the application of various cholinergic antagonists. In the bottom row, efferent responses in CD afferents are shown before (blue bars) and after (black line) the application of the same antagonists.

We can make two conclusions from this physiological data. Firstly, the component responsible for the response in the bouton afferent and the type II hair cell is pharmacologically different from the component on the calyx afferent (it is likely a nAChR composed of a different nAChR subunits). Secondly, the inhibitory component in

the bouton afferent shares the pharmacologic and therefore is blocked by strychnine, ICS and α -BTX. The $\alpha 9/\alpha 10$ nAChR fits this description.

The specific purpose of this study is to confirm, using various immunohistochemical methods, the identity of this receptor and to localize its presence in the hair cell layer of the turtle crista, and to compare with the nAChR on calyx afferents.

IV. MATERIALS & METHODS

A. **Reagents, Primary and Secondary Antisera and Toxins**

1. Reagents: All chemicals and reagents used in this study were purchased from the Sigma Aldrich Chemical Company (St. Louis, MO).

2. Primary antibodies: The primary antibodies used in this study are listed below, grouped by manufacturer. The specific dilution of each, as used in our experiments, is also given in parenthesis.

2-1. Obtained from Chemicon International (Temecula, CA).

1. Rabbit IgG anti-rat, goat IgG anti-guinea pig against calretinin or CR (1:1000)
2. Goat IgG anti-human against choline acetyltransferase or ChAT (1:200)
3. Rabbit IgG anti-bovine directed against synapsin I (1:1000)
4. Rabbit IgG anti-rat directed against the vesicular acetylcholine transporter or VACHT (1:200)
5. Rabbit IgG anti-guinea pig directed against the $\alpha 4$ nAChR (1:200)

2-2. Obtained from the laboratory of Dr. Miles Epstein (University of Wisconsin-Madison Medical School) [24]

Rabbit IgG anti-chicken directed against ChAT (1:1000)

2-3. Obtained from the laboratory of Dr. Anna Lysakowski (University of Chicago) [30].

Rabbit IgG anti-turtle directed against the $\alpha 9$ nAChR (1:200)

2-4. Obtained from the Sigma-Aldrich Chemical Company (St Louis, MO).

Rabbit IgG anti-bovine directed against neurofilament-200 or NF-200 (1:200)

3. Secondary antibodies: All secondary antibodies used were fluorophore-conjugated with either Alexa Fluor® 488 or 594 (both were used in different experiments). These were obtained from Molecular Probes (Eugene, OR).

1. Donkey IgG anti-rabbit (1:500)
2. Chicken IgG anti-goat (1:500)
3. Goat IgG anti-rabbit (1:500)
4. Rabbit IgG anti-goat (1:500)

4. 4',6-diamidino-2-phenylindole (DAPI): DAPI was included in our mounting medium (Vector Laboratories, Burlingame, CA) which was used as needed to coverslip slides.

5. Toxins:

1. α -Bungarotoxin or α -BTX: Fluorophore-conjugated with Alexa Fluor® 488 and 594 and obtained from Molecular Probes. The results provided were obtained with a concentration between 10-20uM.
2. Apamin: Fluorophore-conjugated with Alexa Fluor® 488 and custom synthesized by Molecular Probes. The results provided were obtained with a 1uM concentration of the substance.
3. α -Bungarotoxin: Unlabeled α -BTX for control experiments was obtained from the Sigma Aldrich Chemical Company and from Biotium, Inc (Hayward, CA). A 1mM concentration of the toxin was used.
4. Methyllaconitine or MLA: This drug was obtained for control experiments from Tocris, Inc (Ellisville, MD), and used at a concentration of 570uM.

B. Tissue Preparation and Labeling

1. α -Bungarotoxin Labeling:

Red-eared turtles (*Trachemys scripta elegans*) of both sexes and ranging in length from 4 to 6 inches were purchased from Charles D. Sullivan Company Inc. (Nashville, TN). They were housed according to the University of Texas Medical Branch (UTMB) animal care guidelines. The experimental protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of UTMB.

Turtles were deeply anaesthetized with Nembutal (40 mg/kg, I.M.). Following decapitation, the head was divided into two halves and the vestibular labyrinth was exposed on each side. Each side was treated with 25 μ L of 10-20 μ M α -bungarotoxin conjugated to Alexa Fluor® 488 or 594. Each half was constantly oxygenated and incubated in the dark for 30 minutes, at which time, a second dose of α -BTX 488/594 was applied and the half-head was further incubated for an additional 30 minutes. The halves were then transferred to 4% paraformaldehyde (PF) in 0.1M phosphate buffer (PB, pH 7.4) for 1-2 hours, following which the vestibular organs were dissected out and placed in 30% sucrose overnight at 4°C. From this point onward, IHC processing was carried out in the tissue catcher, seen in Figure 3, a device designed in this laboratory which is used to maximize convenience and efficiency with tissue processing. Post-cryoprotection with sucrose, the organs were embedded in fresh sucrose-gelatin. 40- μ m frozen sections of the semicircular canal cristae were cut and collected in a tissue catcher. Subsequently, the gelatin was dissolved by warming the tissue catcher. The tissue sections were then transferred to slides using an eyelash.

All slides were cover-slipped with Vectashield containing DAPI, which labels DNA and is an excellent marker for cell nuclei. A standard Nikon fluorescence

microscope and a Nikon LSM-S10 confocal microscope were used to visualize and capture images of the stained tissue.



Figure 3: Photograph of the Tissue Catcher

This device consists of a small glass tube with a small-meshed screen at one end which allows for removal of fluid while retaining either whole organs or tissue sections. The removal of various bathing media is controlled by a one-way valve in series with the glass tube. This system works well under gravity-flow conditions but is also fitted with a port to apply gently suction.

2. Controls:

For control experiments, the labyrinth was pre-treated for 15 minutes with unlabeled α -BTX (1mM), followed by an hour incubation in a mixture of 1mM unlabeled α -BTX and 10uM α -BTX 488. Alternatively, MLA, an antagonist of the $\alpha 9$ nAChR, was used in the same manner. The subsequent steps were carried out as described above.

3. Double Labeling Experiments:

In double-labeling experiments, α BTX-treated organs were further processed following fixation with 4% PF. The organs were first permeabilized with 4% Triton-0.1M PB for 1 hour, followed by an incubation in blocking serum (0.5% fish gelatin, 1% BSA and 0.5% Triton-X in 0.1M PB) to reduce non-specific background, for another hour. Organs were then incubated with an antibody against the calcium-binding protein, calretinin (CR) at RT for 16-24 hours. Double labeling with an antibody to the neurofilament protein (NF-200) was also conducted in place of CR in some sets of tissue. The following day, organs were transferred into the corresponding Alexa Fluor® 488 or 594 secondary antibody. Following the secondary incubation, organs were rinsed and cryoprotected in 30% sucrose before being embedded and cut as described above. In some experiments, the embedding and sectioning preceded the double-labeling procedure.

Immunohistochemical double-labeling of nicotinic receptor subunits with antibodies against CR or NF-200 was performed in both whole organs and sections after fixation with 4%PF.

4. Tissue Markers:

Several anatomical markers were used to demarcate and regionalize distinct cellular components of the crista epithelium. DAPI, a DNA marker which fluoresces blue under UV light, was used to label cell nuclei. CR, as previously mentioned, is a calcium-binding protein and was used in our studies to label afferents and demarcate the crista into the torus and planum zones based on the population of calyx and bouton afferents [27]. NF-200 was used to label all neural fibers, both afferents and efferents, in the crista.

The use of these markers was helpful in creating a clearer image of the sensory epithelium and the neural network, allowing better visualization of the staining of interest.

Three other antibodies used to demarcate efferent fibers and terminals were synapsin I, ChAT and VACht. Synapsin, a synaptic protein located in nerve terminals, serves as a label for efferent terminals (it is believed that it is absent in hair cell and afferent terminals) [7, 15, 42]. Choline acetyltransferase (ChAT) is responsible for the synthesis of ACh and the vesicular acetylcholine transporter (VACht) is a protein necessary for the transport of acetylcholine into synaptic vesicles. Both the enzyme and the transport protein are present in the efferent terminals, making antibodies directed against the two ideal for marking efferents in the hair cell layer [21, 24]. Organs were permeabilized with triton, blocked and then incubated with a primary antibody against either synapsin, ChAT or VACht for 16-24 hours. Usually these sets were also double-labeled with calretinin. Subsequent steps were carried out as described for the double-labeling experiments.

V. RESULTS

A. Afferent and Efferent Cell Markers

The use of cell markers was very beneficial to this study, allowing clearer visualization of the protein of interest against a better defined and demarcated background. Markers for afferent and efferent neurons clarified the cellular elements of the sensory epithelium and allowed for a greater understanding of nAChR-specific staining patterns by providing a ‘reference point’ for better evaluation.

Figure 4 presents a longitudinal section of a posterior semicircular canal crista, where antibodies to calretinin (CR, in green) label calyx afferents in the central zone, some bouton afferents near the torus (BT), and type II hair cells near the planum (BP). While nuclei of both hair cells and supporting cells are stained by DAPI, the intensity of the labeling in supporting cells beneath the hair cells is noticeably greater. This is a result of the dense layer of supporting cells around and between hair cells which forms a ‘packing’ with the nuclei of individual cells located much closer to each other. As a result, a thick row of tightly packed supporting cell nuclei appears more dense with DAPI staining than the hair cell layer they support. At higher magnifications (lower three panels), calretinin clearly labels calyx afferents, bouton afferents near the torus and type II hair cells near the planum.

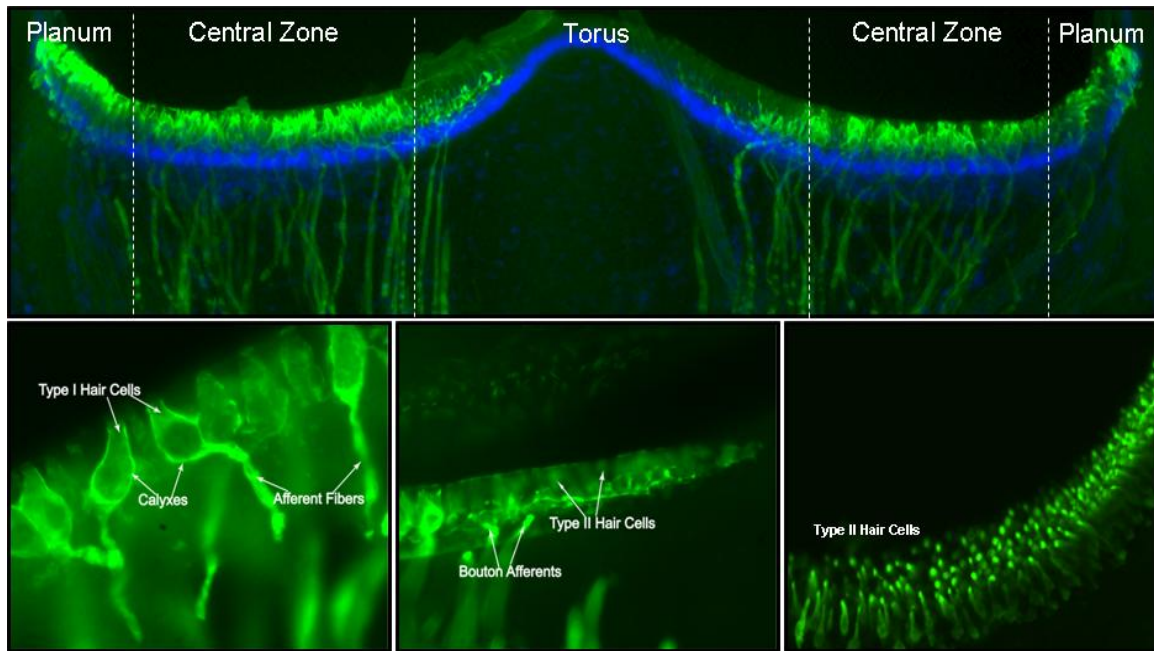


Figure 4: Calretinin (CR) labeling in the turtle posterior crista

As seen in this longitudinal section of a posterior semicircular canal crista (top panel), CR (green) is labeled in calyx afferents in the central zone, in bouton afferents near the torus, and in type II hair cells near the planum (upper panel). The nuclei of supporting cells are highlighted by the intense blue DAPI staining. At higher magnifications (lower three panels), calretinin clearly labels calyx afferents (shown at 40X) in the central zone. The outlines of type I hair cells are easily visualized. Bouton afferents near the torus (shown at 20X) and type II hair cells near the planum (15X) are also labeled. The boutons in the torus appear as small puncta along the base of type II hair cells.

Staining with polyclonal antibodies against NF-200 results in a ‘meshwork’ labeling present throughout the crista, with a significantly stronger signal in the hair cell layer (Figure 5). It is likely that both afferents and efferent neurons branching out into the hair cell layers are labeled for this protein.

Double labeling with antibodies against both calretinin and NF-200 results in some co-labeling in the central zone, while the peripheral zone is largely labeled for neurofilament only. Calretinin, it can be inferred from this staining pattern, is only present in a subset of afferent neurons in the hair cell layer. The entire region, however, is rich with neurofilament labeling.

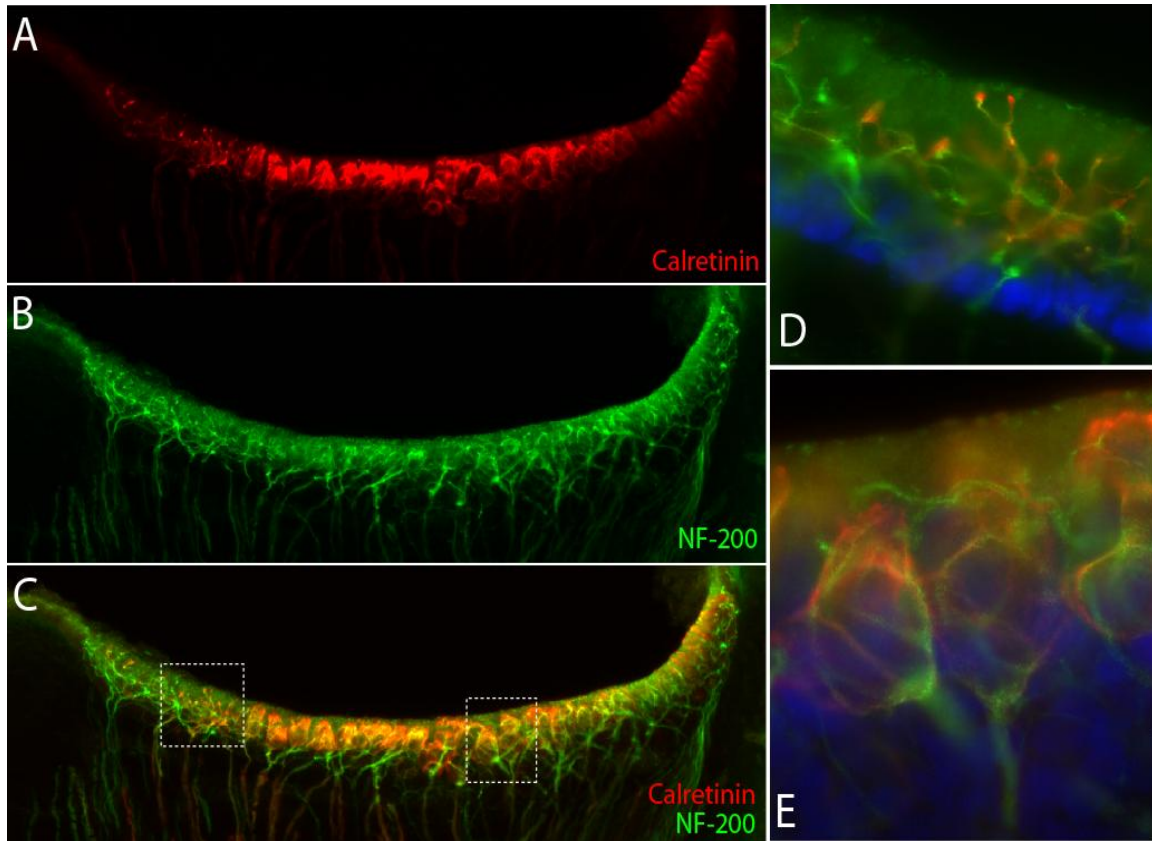


Figure 5: Neurofilament-200 (NF-200) labeling in the turtle posterior crista

Panels 5A-5C show longitudinal sections of posterior semicircular canal hemicristae with calretinin and NF-200. A. Again, CR labels calyx afferents in the central zone, some BT afferents, and type II hair cells near the planum. B. NF-200 labels all fibers including boutons, calyx, and presumably efferent neurons. C. Composite of CR and NF-200 labeling revealing differences in staining patterns. D,E At higher magnifications (40X), CR and NF-200 differentially label bouton afferents whereas it appears that all CD afferents are labeled with both.

Labeling with efferent markers was equally successful in characterizing their distribution in the hair cell layer. The enzyme ChAT is responsible for the synthesis of ACh and is expected to be localized to efferent neurons. Figure 6A is an image of a hemicrista stained with antibodies to this enzyme, which labels as bright green punctae visible from the torus to the planum. Synapsin is a key synaptic protein present in efferent nerve terminals and is believed to be absent or have no association with hair cell synaptic ribbons, making the protein mostly specific to efferents [7, 15, 42]. Antibodies to

synapsin resulted in dense punctate labeling that ran along the base of the hair cells. Since this antigen is a synaptic protein located in nerve terminals, it is logical to infer that the punctae are representative of efferent terminals branching out to hair cells. Any staining below the blue DAPI layer probably marks efferent to afferent connections. It must be noted that staining for both ChAT and synapsin, while present intermittently along the entire layer, was strongest in intensity near the torus. A polyclonal antibody directed against VACHT (the protein involved in the transport of acetylcholine and present in the ACh-containing vesicles) resulted in a thick band of staining along the entire length of the hair cell layer. The labeling resembles dense co-localization of punctae-like structures seen with synapsin.

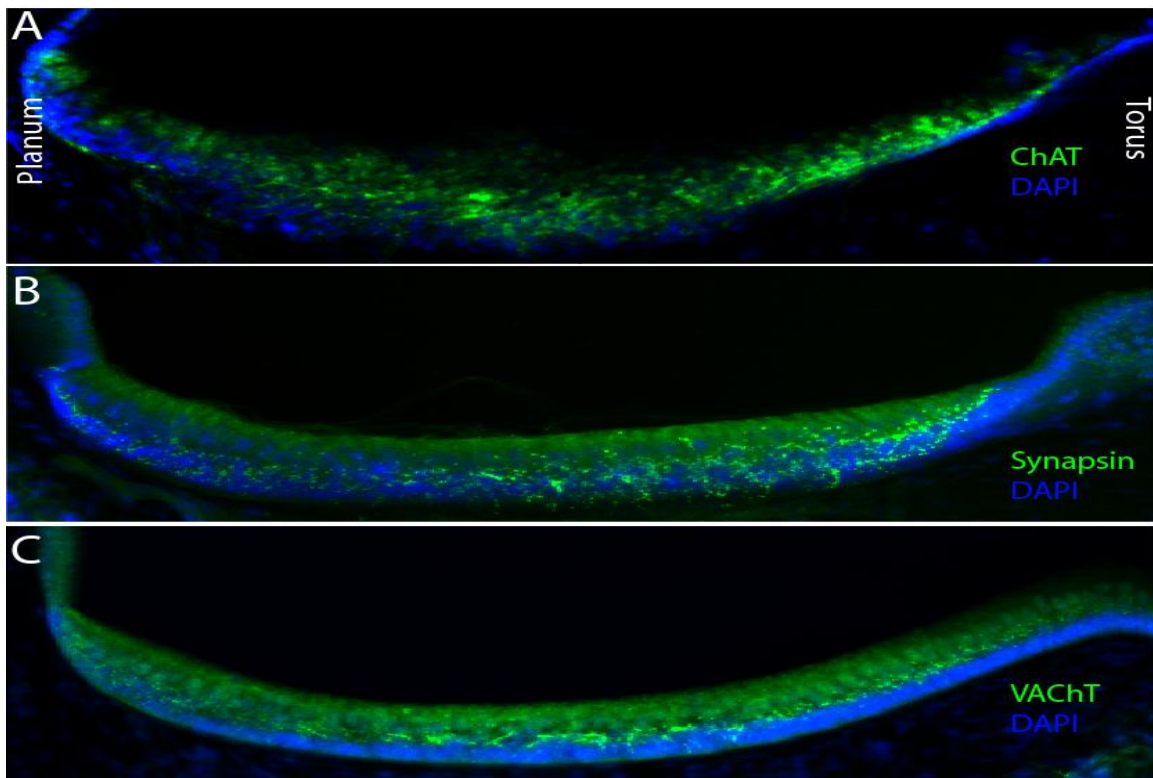


Figure 6: Efferent markers in the turtle posterior canal hemicrista
A-C. Efferent terminals can be identified using antibodies to choline acetyltransferase (ChAT), Synapsin, and vesicular acetylcholine transporter (VACHT).

It is important to note that the labeling patterns of ChAT and VACHT are not necessarily identical to the staining seen for synapsin. Synapsin is likely present in all efferent nerve terminals, while ChAT and VACHT are likely specific to cholinergic nerve terminals only. It has not been shown yet that all vestibular efferents in the crista are cholinergic, and so the varying stain patterns may provide support for the presence of other vestibular neurotransmitters in this neural network.

B. $\alpha 9$ and $\alpha 4$ nAChR Labeling

We were largely unsuccessful in our attempts to localize the $\alpha 9$ and the $\alpha 4$ nAChR in fixed tissue with commercial antibodies against the chick and guinea pig receptor. Despite trying various different manufacturers, concentrations and incubation methods, labeling seen for either protein was either persistently weak and very sparse, or difficult to visualize against a very high non-specific background (Figure 7A, B and C). At this point we believe that, as suggested by Moser et al, this low quality and unreliable staining might be a result of using these commercial antibodies with a conventional fixation IHC protocol [37]. In addition, we strongly suspect that the fixation solution (4% paraformaldehyde) and procedure we use in our IHC processing affects the antigenicity of the nicotinic receptors in the vestibular periphery. This theory is supported with our attempts to reproduce the characteristic α -BTX 488/594 staining in tissue that had been fixed prior to incubation with the toxin, as per the standard protocol we use for a normal antibody reaction. Figure 7D presents an image of a crista treated in this manner and it is clear to see that staining was essentially non-existent, indicating that it is a step in the fixation process that changes the antigen through cross-linking or denaturation, preventing its interaction with α -BTX. After our failed attempts to stain fixed tissue with

this conjugated α -BTX, we attempted to try the compound on live, unfixed tissue, with a slightly modified protocol.

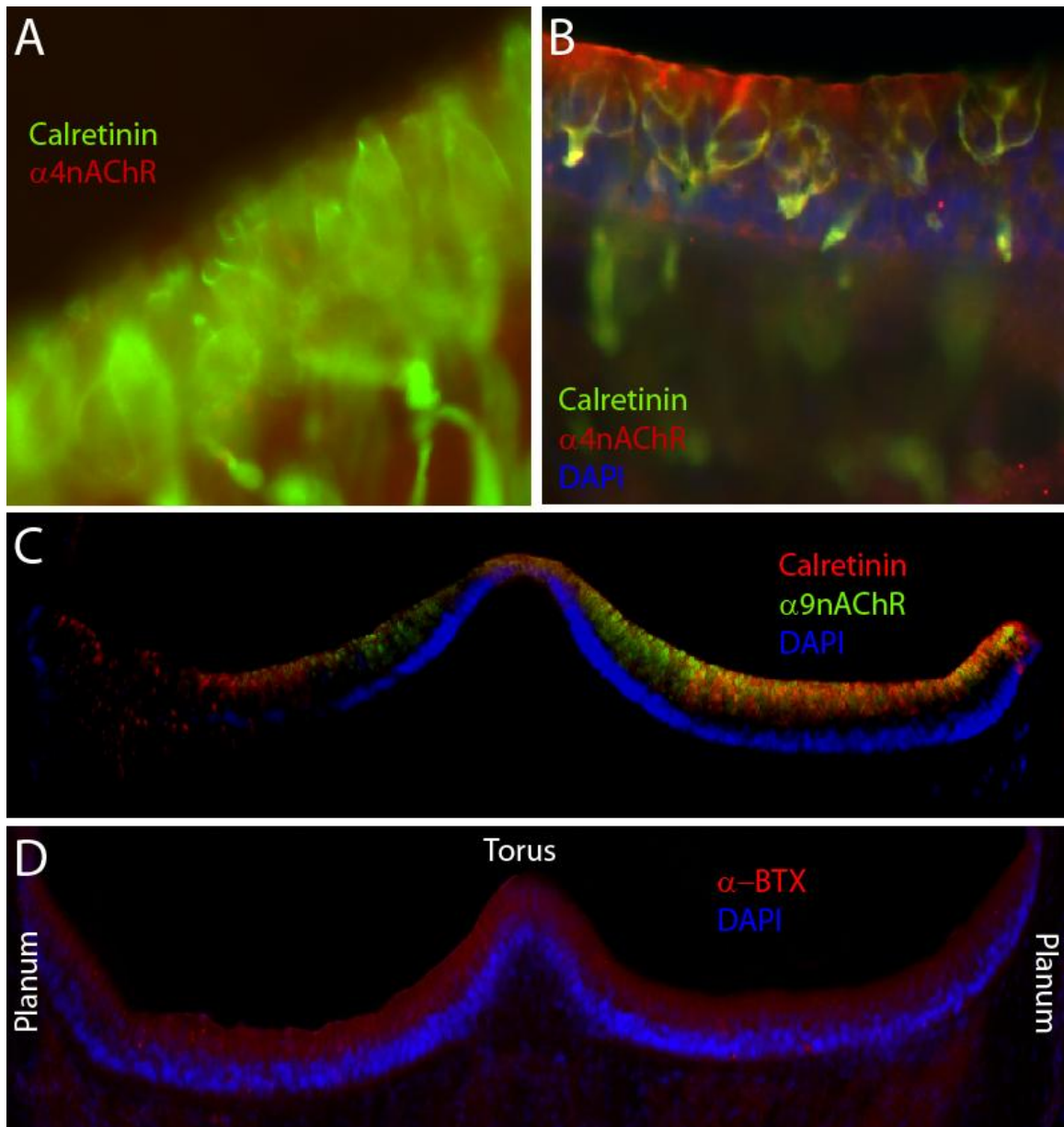


Figure 7: Immunohistochemistry with antibodies against $\alpha 9nAChR$ and $\alpha 9nAChR$ subunits and AlexaFluor-594 α -BTX in fixed tissue from turtle posterior canal crista.

C. α -Bungarotoxin Labeling

Alexa Fluor® 488 and 594 conjugated α -BTX applied to the live, unfixed posterior cristae of the turtle resulted in a dense punctate labeling pattern in the hair cell layer (Figure 8A). It appears that type II hair cells are strongly bound by the toxin, as the staining observed was largely concentrated near the torus, in the peripheral zone which is populated only by type II hair cells only. In addition, double labeling with calretinin, which allows for a clearer visualization of calyx and bouton afferents, demonstrates that there is very little co-labeling visible in the type I hair cell populated central zone. There is some indication of some faint labeling in calyx afferents, while the densest staining appears close to the torus.

α -BTX-treated tissue was also processed with an antibody against NF-200. NF labels all efferents and afferents but no hair cells. Figure 9 shows that there is no co-labeling observed with the two proteins, further strengthening the case that the α -BTX staining is localized to hair cells. This double labeling experiment also serves to better characterize the NF-200 staining, since it is possible to see two kinds of fibers in the magnified images. Fibers that appear to end around and near the α -BTX punctae are possibly efferents innervating type II hair cells and boutons, other fibers appear to terminate in the hair cell space without necessarily making any connections with the punctae—these are likely to be afferents.

As is obvious in all the α -BTX figures, the central stroma of the crista is also stained a vivid green. This is non-specific background, a result of the direct application of the fluorophore-conjugated molecules to the tissue, which is trapped in the stroma and difficult to wash off once the tissue is fixed after treatment (at least without also affecting the desired signal).

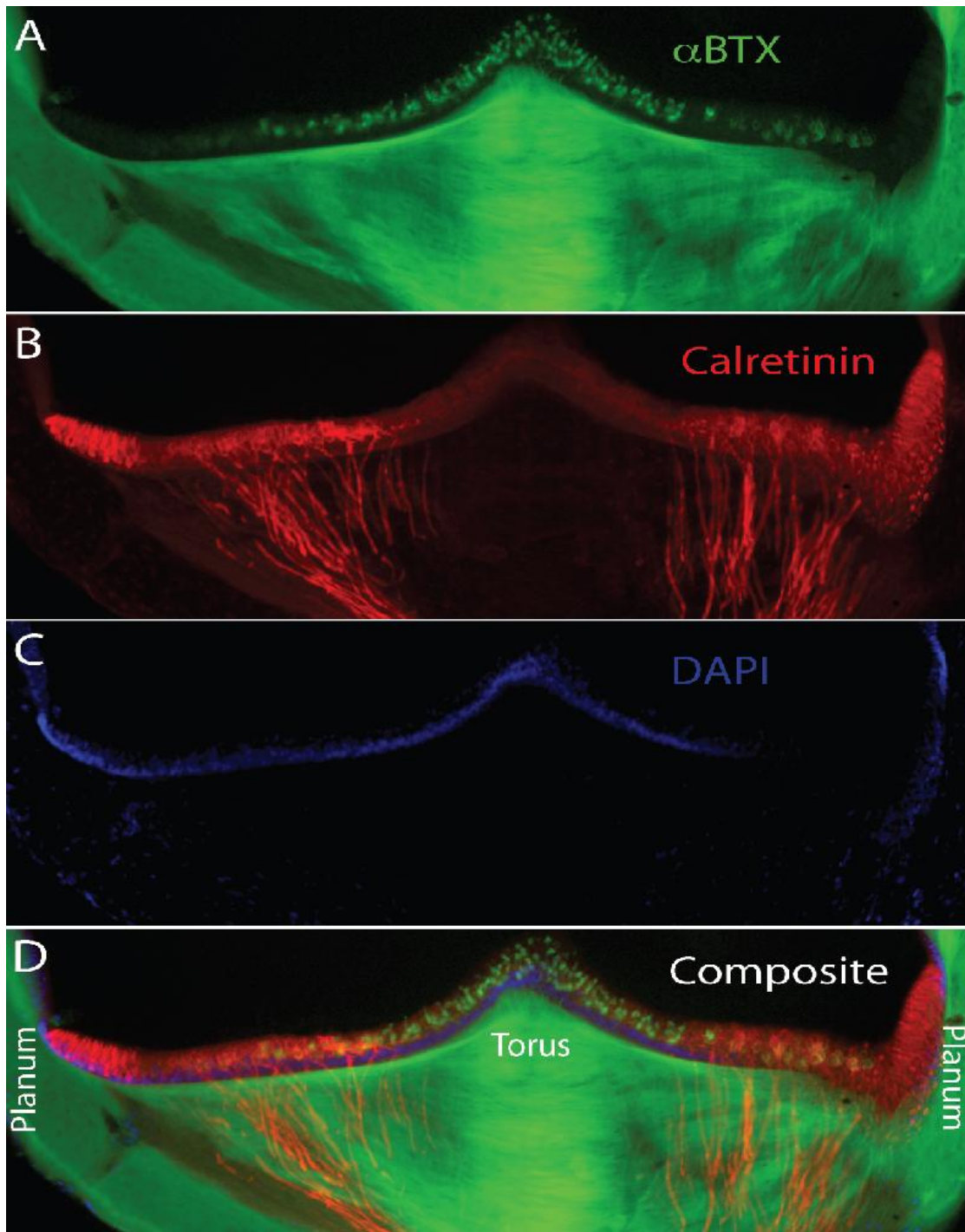


Figure 8: Immunohistochemistry with Alexa Fluor 488 α -BTX in live, unfixed tissue from the turtle posterior canal crista.

Panel A shows a longitudinal section of the crista labeled with the α -BTX 488 alone. Panel B is an image of the same section, labeled with an antibody against calretinin: calyx and BT afferents have been labeled, along with type II cells near the planum. In panel C, DAPI stains the nuclei of the hair cells and the supporting cells, and panel D is simply a composite of A, B and C, demonstrating the localization of the α -BTX staining to the BT zone.

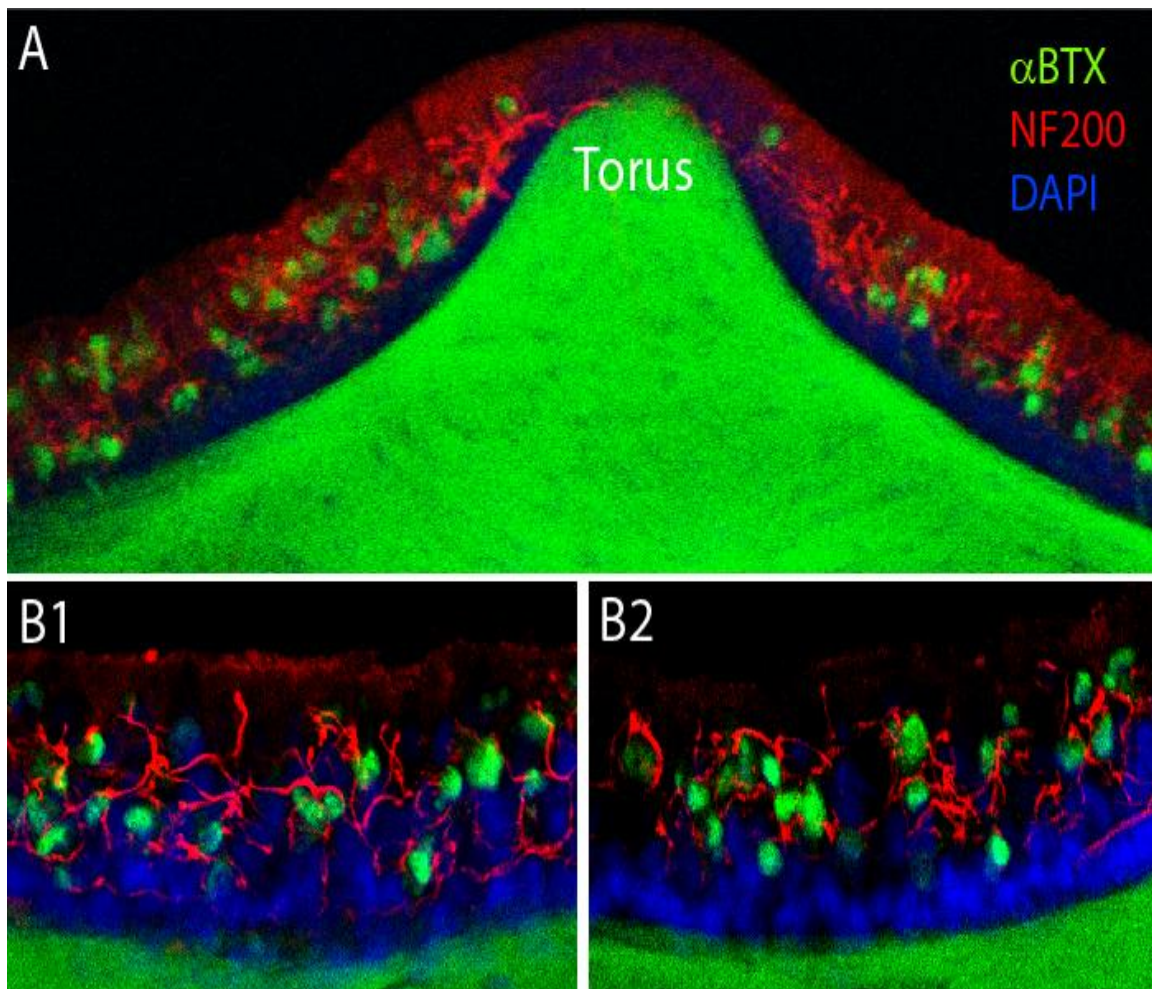


Figure 9: Immunohistochemistry with Alexa Fluor 488 α -BTX in live, unfixed tissue from the turtle posterior canal crista.

Panel A shows a section of the crista obtained with confocal microscopy. The characteristic punctate α -BTX staining is seen in **green** against the background of the neurofilament-200 labeling in **red** (10X). Panel B1 and B2 are confocal images taken at a higher magnification (20X). Green punctae are in the hair cell layer, above the DAPI stained supporting cell layer, and fibers stained with neurofilament end at or close to the punctae at several points.

To investigate the reliability of the α -BTX staining observed, i.e., to confirm that the punctae seen are indeed indicative of the location of the $\alpha 9$ nAChR, several controls were carried out. Our first technique was to use antagonists of the $\alpha 9$ nAChR: if the α -BTX fluorophore is binding to this receptor, then pre-incubating the tissue with a significantly large amount of unlabeled antagonist should block any fluorescent labeling. Methyllycaconitine (MLA) is a plant alkaloid that has been confirmed to bind to α -

bungarotoxin sites in the brain and is a known antagonist of $\alpha 7$ and the $\alpha 9/\alpha 10$ nAChR [47]. Incubation of the posterior cristae in a mixture of α -BTX 488/594 and MLA, with the MLA concentration being several-fold greater than the α -BTX, was successful in eliminating any labeling in our initial control experiments (Figure 10).

Repeating the pre-incubation control experiments with unlabeled α -BTX has, so far, not provided absolutely definitive results. There is some sparse, weak labeling that remains in these sections, despite the high concentration (100 fold greater) of unlabeled α -BTX being used. The labeling observed is definitely not as robust or prolific as that usually seen with α -BTX 488/594. Experiments to optimize the assay are currently being performed.

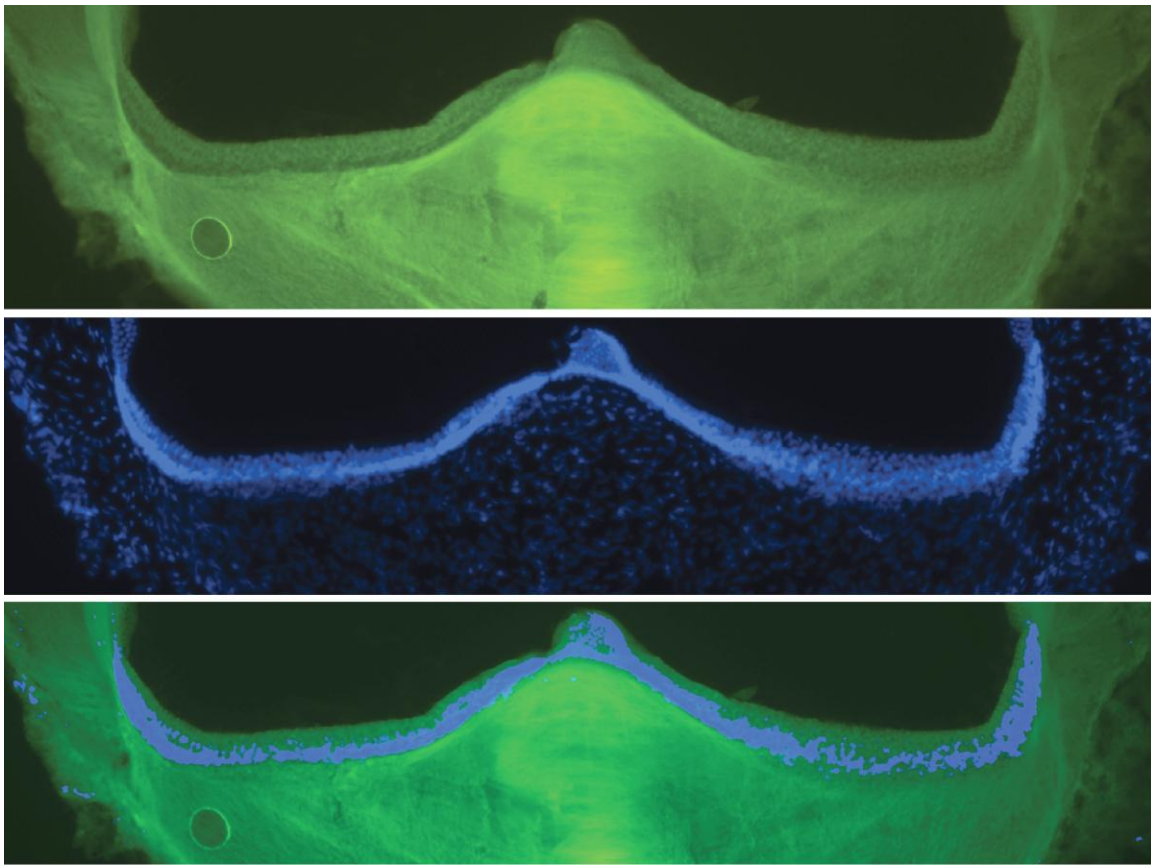


Figure 10: Blocking α -BTX labeling with preincubation with methyllycaconitine.

D. Apamin Labeling for SK Channels

Apamin is known to be a pharmacological blocker of small-conductance potassium channels (SK channels) present on the type II hair cell [20, 26]. The Alexa Fluor® 488 conjugated apamin was initially tested in a physiological experiment where the efferent response of the BT/BM afferents was recorded before and after the direct application of apamin. As expected, the normal inhibitory response was converted to excitation, indicating that this toxin was binding to SK channels in the hair cell layer and unmasking $\alpha 9$ nAChR-mediated excitation. Tissue from this same experiment was fixed, cut and visualized. As seen in Figure 11, apamin labeled proteins present in the peripheral zones near the torus, specific to type II hair cells. The characteristic cylindrical structures of these hair cells are visible in the intense layer of staining present on both sides of the non-sensory torus, and it appears that this labeled protein is relatively abundant in these cells. This initial IHC processing combined with the pharmacological data obtained from the same tissue appears to indicate that the binding sites of the apamin 488 are indeed SK channels on the type II hair cell.

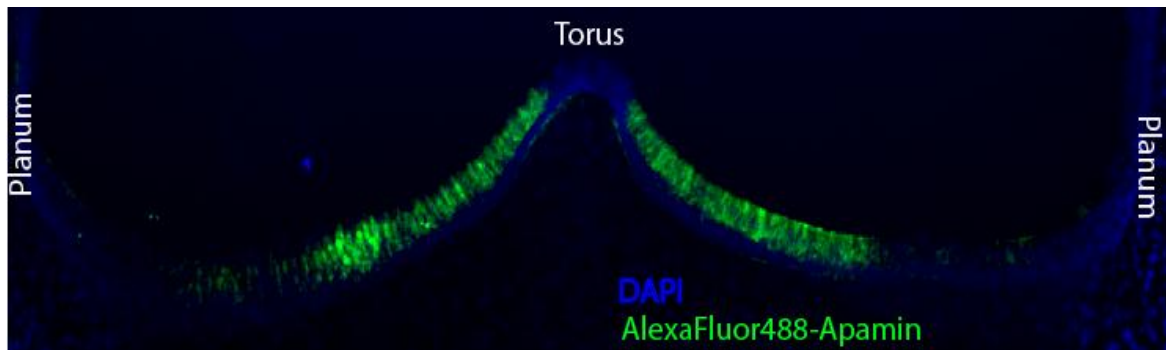


Figure 11: Labeling observed with application of Alexa Fluor 488 apamin to live, unfixed tissue from the turtle posterior crista.

VI. DISCUSSION

A. Characterizing the Crista

CR, NF-200, ChAT, VACHT, synapsin and DAPI all proved to be excellent markers for the turtle crista, highlighting key cellular elements in the hair cell layer and providing definition to the structural design. The immunoreactivity of each of these markers in the turtle vestibular periphery has not been previously presented in terms of a scientific publication, making the data presented here novel and a beginning for greater exploration.

The staining for CR observed remained consistent in all our experiments and resembles that seen in other organisms [27, 36]. Type I hair cells in the central zone are very well outlined by CR-stained calyx afferents, bouton fibers are clearly visible (often punctae-like) near the torus (BT), and type II hair cells near the planum (BP) are brightly labeled with the use of a polyclonal anti-CR antibody. Afferents in the stroma are also labeled as they travel up into the hair cell space. Calretinin is essentially our most successful and reliable marker, allowing simple, efficient and clear demarcation of the crista into its hair cell zones, and afferent fiber types.

Staining with antibodies against NF-200 has been similarly successful, though with a somewhat higher non-specific background than seen with the CR staining. It appears that antibodies to the neurofilament protein do not discriminate between efferent and afferent fibers in the turtle crista. We infer this based on the observation that not all fibers that are stained in the hair cell space seem identical in connectivity, as seen in Figure 9. Double labeling with an efferent marker, such as antibodies against ChAT or efferent synapse proteins, would provide a clearer answer regarding the NF-200 reactivity.

In any case, NF-200 has proved to be very useful in confirming that the α -BTX punctae is most likely localized to the type II hair cell themselves and definitely not to the afferent and efferent neural fibers in the hair cell space.

Antibodies to ChAT, VACht and synapsin have all been effective in providing information about the efferent network in the neuroepithelia of the turtle crista. Efferent terminals have been highlighted with staining for all three proteins; however, the labeling patterns are not necessarily identical, which points to other aspects of exploration. Not all efferent fibers in the crista are believed to be cholinergic—the differences in staining between ChAT, VACht and synapsin may provide evidence for this conjecture. Since synapsin is present in all efferent terminals (contacting both boutons and hair cells) and ChAT and VACht exists only in cholinergic efferent terminals, staining for synapsin results in denser, more widespread labeling than the other two proteins. Further exploration of their immunoreactivity might be one way of learning more about efferent pharmacology in the vestibular periphery.

DAPI, a classic marker for cell nuclei, was used in all our IHC processing and proved to be excellent for regionalizing the hair cell layer. The supporting cell layer is stained intensely (as a result of the thickly packed nuclei) and there is a clear divide between these supporting cells and where the less-intensely labeled hair cell layer begins.

B. α -Bungarotoxin Binding in Vestibular Hair Cells

Our IHC experiments with the Alexa Fluor® 488 and 594 conjugated α -BTX result in a very characteristic staining pattern in the hair cell layer of the turtle posterior crista, running along the type II hair cell populated peripheral zone, adjacent to the torus. This staining is intense with α -BTX concentrations as low as 3 μ M, but is only present in

fresh, unfixed tissue. The toxin must be applied to freshly extracted tissue and incubated in Ringer's solution, followed by fixation. If fixed tissue is incubated in a 3, 10 or 20uM α -BTX solution, no obvious labeling is seen, suggesting that the fixation steps may have a deleterious effect on the protein the α -BTX is interacting with.

Pharmacologically, as discussed previously, α -BTX appears to block the inhibitory response the $\alpha 9$ nAChR is responsible for, as observed in turtle electrophysiological preparations. α -BTX, therefore, clearly has an antagonistic relationship with the $\alpha 9$ nAChR and must bind to the receptor to be able to block its hair cell action. But based on this information, we might infer that the punctae observed in α -BTX treated tissue are indeed labeling the $\alpha 9$ nAChR. This staining does not appear to be non-specific, as it has been repeatedly reproduced and is always seen in the same regions with the same characteristics. Double labeling with other neural markers (namely CR, NF-200 and DAPI) appears to confirm the localization of these punctae to vestibular hair cells and not to efferents or afferents in the neural space. However, only preliminary controls have been run at this point to absolutely authenticate the identity of these labeled proteins, and so more testing is required to confirm the same.

We have carried out control studies with MLA, another cholinergic antagonist of the $\alpha 9$ nAChR which has successfully blocked the inhibitory $\alpha 9$ response in pharmacological studies. When tissue was treated with an excess of MLA and incubated with α -BTX 488, no labeling was visible in the entire hair cell layer. This control was repeated with an improved surgical technique that ensured proper accessibility of the crista for the solutions. This second time, surprisingly, we observed sparse but obvious labeling in the peripheral zones in most of the tissue sections. The second type of control

was conducted with the same protocol, using unlabeled α -BTX at a 100-fold greater concentration than the α -BTX 488. While it was weak and sparse in each control set, labeling was never completely blocked out as it was expected to, despite the literal flood of unlabeled α -BTX that preceded the labeled protein.

We cannot ignore or reject the staining pattern—it is reproducibly, consistently, and lucidly present in the type II hair cell region—and α -BTX has been characterized to have a nAChR binding site in other organisms [21, 38] apart from its pharmacological ability to block the α 9 response in the turtle. On the other hand, at this preliminary stage, we do not have the control data to support a conclusion regarding the localization of the α 9 receptor protein with this fluorophore toxin. Our current hypothesis is that the labeling observed is real (i.e., there is an authentic protein interaction taking place between the hair cell and the receptor protein), and we are considering multiple possibilities to gain greater insight into what might be taking place at the molecular level.

Firstly, we evaluated the possibility that the unlabeled α -BTX might not be effective, since the quality of the purified toxin can vary from manufacturer to manufacturer. We tested its potency in a physiological prep and observed that the inhibitory response was clearly blocked when a 3 μ M solution of α -BTX was applied to the canals. The unlabeled α -BTX being used was therefore in proper working condition pharmacologically. On the other hand, the quality of the α -BTX fluorescent toxin must also be considered. The process of synthesis (it was obtained from Molecular Probes) might have affected the toxin and its chemical properties: perhaps it is now binding to a different protein than it should be? Our next step is therefore to test the fluorophore-

conjugated α -BTX pharmacologically and ensure that it is working to block the inhibitory response, with about the same dose as the unlabeled toxin has been seen to do.

Another related theory to consider is that the conjugation of a fluorophore to the purified α -BTX molecule, an artificial synthesis process, may have enhanced the potency of the toxin and actually increased its affinity many times. This would boost its ability to bind to the target protein, negatively affecting the competitive antagonism by unlabeled α -BTX. One way to test this theory is to try both the fluorophore-conjugated and unlabeled α -BTX in a physiological prep separately (using the same concentration for each) and recording the speed at which the inhibitory response is blocked. To be considered an appropriate antagonist for the other, each compound must display its effect in relatively the same amount of time when used at the same concentration.

A third possibility is also related to the artificial attachment of the fluorophore to α -BTX. Since we do not have detailed knowledge at this point of how the chemical structure is manipulated to obtain a conjugated toxin, it is worthwhile to consider if this created α -BTX is now involved in a different, abnormal, molecular mechanism. Instead of binding at its target site on the hair cell, perhaps the 488/594 fluorophore is being internalized by the hair cell itself and pulling in the α -BTX along with it, resulting in the labeling pattern we observe. This internalization method need not be receptor-mediated, and therefore application of the unlabeled α -BTX (which is specific to the receptor) does not block the process. To investigate this further, it might be helpful to study the fluorophore and investigate its qualities, perhaps obtaining it by itself and trying it with the same IHC protocol. A physiological experiment, once again, could provide

information as to whether the compound is indeed interacting with the receptor or participating elsewhere.

It might also be true that the concentration of α -BTX 488/594 being used in our IHC experiments may be too high. In pharmacological studies conducted in this lab, a 1 μ M solution of α -BTX is sufficient and potent enough to block the response of the α 9 nAChR. However, having failed to observe the same vivid labeling at a 1 μ M concentration, we use a concentration that is usually in the range of 3 μ M to 20 μ M (10 μ M was considered to be optimal after various trial and error experiments) in our IHC experiments. Naturally, the two methods—of running through a solution directly on the live, exposed organ in a physiological experiment versus incubating the organ in the solution in a histochemical staining experiment—are very different and may influence the dosage required. But, we believe that it is likely that the 3-20 μ M concentration of fluorophore toxin we use in the IHC experiment is in such excess that it is saturating the binding sites on the hair cell layer, so that even a large amount of unlabeled α -BTX may be unable to out-compete effectively. If the maximum response is reached with an excess dose of the fluorophore toxin, the system is saturated and it is likely that even a 100-times greater amount of the unlabeled toxin is not enough to appreciably change the labeling. This can only be investigated by continuing to optimize the IHC protocol and experimenting with surgical and incubation techniques that will allow us to improve our staining with even lower concentrations of the labeled α -BTX.

C. Apamin Binding in Vestibular Hair Cells

The neurotoxin, apamin, has been characterized as a blocker of the small-conductance potassium channels (SK channels) present on the type II hair cell [20, 26]. The SK

channels are key players in the inhibitory response that the $\alpha 9$ nAChR is responsible for, since it is the efflux of K^+ ions from these channels that leads to membrane hyperpolarization and inhibition of neurotransmitter release [25]. Based on this role, it follows that SK channels are likely to be located structurally close to the $\alpha 9$ nAChR protein itself. Initial experiments with custom synthesized apamin conjugated to the Alexa Fluor® 488 fluorophore have provided encouraging results. This conjugated toxin was initially tested pharmacologically where, as expected, the normal inhibitory response was blocked with the use of apamin, confirming that this toxin had successfully bound to SK channels in the hair cell layer. Labeling was largely present in the peripheral zone near the torus which is solely populated by type II hair cells. The pattern was not punctate at this low magnification, but appeared to be dense and widespread in this region of the hair cell space. The cylindrical structures of the hair cells are actually visible in the intense layer of staining present on both sides of the non-sensory torus.

The appropriate pharmacological response described above combined with this visual data on the physical location of this apamin-bound protein in the hair cell layer (and more importantly in type II hair cells), strongly corroborates the identity of these proteins as SK channels. In addition, the fact that this apamin labeling does not resemble that seen with the conjugated bungarotoxin compound lends support to the authenticity of the bungarotoxin staining pattern, since it cannot be attributed to the Alexa Fluor 488 fluorophore. If the presence of the fluorophore was compromising the IHC labeling, then staining with apamin 488 would resemble staining with α -BTX 488, which we demonstrate to not be the case.

Fluorophore-conjugated apamin, with further experimentation to reduce non-specific background and obtaining a clearer signal, may be very useful in characterizing the type II hair cell in terms of its physical structure and constituents. In addition, combined staining with α -BTX 594 could prove to be an intriguing way of exploring both the apamin and the inconclusive α -BTX labeling.

D. Future Outlook

Nicotinic acetylcholine receptors form a crucial part of the complex, intricate machinery underlying the mechanisms driving excitation and inhibition in the vestibular hair cells and afferents. To truly gain insight into how mechanical head movements can be affected by efferent signals, the characterization of these nAChRs is important and necessary. Localizing these nAChRs in the hair cell layer is essential to this process: a large part of understanding *how* a receptor works is knowing *where* it exists and what role that location plays in the larger picture. Additionally, our knowledge of the vestibular hair cells and the communication between afferents, efferents and the two types of hair cells remains inadequate, with many unidentified components and unexplained characteristics.

With this study, our first step has been to structurally define and illuminate the turtle crista and demonstrate its value as an animal model for immunohistochemical studies of the vestibular nervous system. The vestibular organs of the turtle are not that different from the mammalian system. The essential preservation of this system evolutionarily—combined with factors such as the surgical ease in removing the labyrinth, the presence of both types of hair cells and the success of various markers in

regionalizing its hair cell layer—all support the use of the turtle as an excellent and convenient model for our nAChR studies.

Potentially, the techniques presented in this study involving the use of a fluorophore-conjugated nicotinic antagonist could hold great value since it could provide reliable, lucid results without the many negative aspects of commercial antibodies (described by Moser et al and Huang et al among others) [19, 37]). Fixation is not necessary, the question of serum compatibility or reactivity between species need not be considered, concerns regarding the quality of the antibody (and the various additional experiments to test the manufacturer's claims) as well as the many variables that accompany primary and secondary antibody use do not come into play at all with this technique. With appropriate optimization of the protocol, we are optimistic that this method could be used with greater ease, efficiency and reliability, and prove to be an excellent benefit to our own studies as well as others.

Identifying the $\alpha 9$ nAChR using a fluorophore-conjugated α -BTX antagonist was the primary aim of this study. At this preliminary stage, our data remains inconclusive, but we have made large strides in our knowledge of the receptor proteins and the current immunohistochemical methods available to isolate them. We are in the midst of designing various other physiological and anatomical experiments that could provide a better explanation for our results as well as direct us to more definitive conclusions regarding the $\alpha 9$ nAChR and its presence on the type II hair cell. As discussed in the previous section, many possibilities exist and each must be explored to allow us to paint a clearer picture of what is likely taking place at the molecular level. To this end, the use of

other neural markers (hair cell specific and additional efferent and afferent markers) and other nicotinic and ion channel antagonists should also prove useful.

VII. REFERENCES

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