The vestibular system is arguably the most ancient sensory system. Its evolution became an invaluable acquisition enabling vertebrates to move about in their environment. The peripheral vestibular apparatus, the semicircular canals and the otolith organs, evolved early and relatively completely. Thus, their structure and function are similar across the vertebrate phyla. Labyrinthine enteroceptors report the magnitudes and directions of angular and linear motion of the head as an animal translates and rotates through space. This information is transmitted to the central nervous system as a frequency code of impulses carried by the VIIIth cranial nerve. The code is subsequently combined with information from other sensory systems that converge on vestibular nuclear sites to compute a central estimate or vectorial representation of head and body position and motion in space called the gravitoinertial vector.

The influence of angular and linear forces is pervasive, extending throughout the central nervous system to include brain functions related to sleep, vision, audition, somatosensation, movement, digestion, cognition, autonomic function, and even learning and memory. The wide extent of this influence is evidenced by the vestibular anatomical connectivity with numerous other brainstem sites, as well as the thalamus, basal ganglia, hippocampus, cerebellum, and cerebral cortex. Physiological activity originating within the vestibular system modifies the firing of many central nervous system neurons including intrinsic cerebellar neurons, spinal and brainstem motor and interneurons, and superior collicular and cerebral cortical neurons, where, for example, the orientation of visual receptive fields can be modified by changes in head position. All of this sensory processing is largely unconscious and unrealized. Unfortunately we, as humans only become conscious of the vestibular system when it malfunctions. Thus, conditions such as vestibular neuronitis, Menieres' syndrome, Benign Positional Paroxysmal Vertigo (BPPV) etc. have a profound, incapacitating influence upon almost every aspect of our lives. Roughly 40% of hospital admissions have some component of vestibular dysfunction, and this percentage increases with age.

Responses of vestibular afferents differ in their gains to natural stimulation (spikes per second per degree per second of angular or linear velocity or acceleration) and in the angular phase of their peak responses to these same stimuli. In contrast to their auditory counterparts, vestibular afferents are generally broadly tuned. Broad tuning means that there is no characteristic or best frequency, but that each fiber generally responds with a roughly similar gain to a broad range of stimuli. However there is some frequency dependent variation in gain and phase of the afferent responses. The search for the origins of this diversity in response dynamics has been a major research goal over the previous decades.

Consideration of the labyrinthine semicircular canal structure suggests that there are five broad classes of structure that could potentially influence the formation of the response dynamics recorded from afferent fibers. They are: (1) the biomechanics of endolymph flow including endolymphatic pressure in response to head rotation, the elastic restoring forces of the cupula and stereocilia, etc.; (2) variability in the responses (magnitude of gain) of the transduction apparatus atop each sensory hair cell; (3) variation in the voltage sensitive basolateral currents in the sensory hair cells; (4) variation in the amount of transmitter released for a given receptor potential; and (5) variation of the post-synaptic response to a given quantum of transmitter released by each hair cell. These five broad categories can also be
Intracellular labeling methods have enabled investigators to identify physiological attributes of a given nerve fiber and then correlate those responses with morphological innervation patterns within the sensory epithelium and the central nervous system. Afferent labeling studies have been carried out in a variety of species and the results have led to a consensus of the role of the different patterns of afferent and efferent innervation in the shaping of afferent responses.

Hair cells are the common element in all of the acoustico lateralis organs including the vestibular semicircular canals and otolith organs, the lateral line organs, and the cochlea. The accessory structures into which these hair cell are imbedded determine the range of frequencies of stimuli to which each of these end organs respond.

Another key to understanding these diverse responses is to study the biomechanics of individual end organs. The biomechanics of canal vestibular responses can be divided into macromechanics, micromechanics, and nanomechanics. Macromechanics of the semicircular canals concerns the physical forces acting upon the gross structures of the canals such as the endolymphatic fluid, and the responses of the canal components due to their physical properties. Thus the physical laws that govern fluid flow loom large in this analysis. In contrast, in the otolithic organs it is the inertia of the otoconia versus the physical attachment of these particles to the epithelium that determine the responses. Micromechanics of the responses may be due to the attachments of the hair cell sensory cilia to the accessory structures such as the cupula or otolith membrane and otoconial mass, while nanomechanics might be the movements of molecules within the hair bundles themselves such as gating spring mechanisms or other motions that impart energy into the transduction process. In terms of the five classes of influence upon the response dynamics outlined above, biomechanics alone or in combination with the morphology and currents does not adequately account for the broad range of afferent responses. Other factors still under investigation are necessary to explain the differences in the ranges of the responses recorded in primary afferent nerves of the labyrinth versus those of the pre- and post-transduction components up to but not including the synapse. By elimination, evidence to date seems to point to a major role for the synapse in shaping response dynamics.

LABORATORY DEMONSTRATION-AFFERENT CONNECTIVITY

For the laboratory exercise, examples of low gain (LG), high gain (HG), and acceleration-sensitive (A) fiber classes will be injected with biotin-based tracers to produce a map of the peripheral neuritic distributions of the physiologically-characterized afferents within the crista. The afferents will be penetrated with sharp microelectrodes filled with tracer and labeled by passing positive current pulses of 10-20 nA (1/s; 80% duty cycle) for 2-8 min through the electrode. The dc membrane potential will be continuously monitored and current injection terminated if the membrane potential falls to $<-10$ mV or if action potentials greater than 1 mV can not be evoked by intracellular stimulation. The electrode will be withdrawn from the nerve immediately after injection.
Fig. 1. Experimental setup. The anterior canal is divided at the dorsal extreme (A) for access to the endolymphatic space. Polarization is achieved utilizing current injection ($I_e$) into the posterior section of the anterior canal via a Ag/AgCl electrode and the resulting polarization voltage measured using an endolymph-filled glass electrode within the anterior canal ampulla ($V_e$). Horizontal canal indentation (HCl) is applied to the horizontal canal lateral limb to mimic head rotation in the horizontal plane. Extracellular and/or intraxonal neural responses are measured ($V_n$) in the horizontal canal branch of the VIIIth cranial nerve (B), and discriminated spike times recorded for both electrical and mechanical stimuli. A small fenestra (H) is placed into the utricular side of the horizontal canal ampulla for the insertion of fine microelectrodes into the hair cells in the crista.

Fish are perfused and cristae containing labeled afferents are processed as described below. The cristae are processed as whole mounts for immunofluorescence visualization of the injected biocytin together with other biomarkers of interest. The biocytin is visualized using a streptavidin-AlexaFluor conjugate. Optimal injections are scanned using multiphoton laser scanning microscopy. The image stacks provide three-dimensional whole-mount visualization of the connectivity between labeled hair cells and each of the characterized afferents. The image stacks are used to determine the location of contacts on each afferent (bouton vs en passant process), and to quantify the number of hair cells that contact each afferent. In addition, each target (hair) cell will be scored for multiple synaptic contacts. The distal neuritic fields of A afferents are concentrated in the central region of the crista, whereas those of LG afferents are directed toward the periphery. Examples of injected afferents (red) in relation to GABAergic hair cells (green) are shown in Figs. 2 and 3.

Fig. 2 (above). Biocytin-injected acceleration sensitive afferent (red) in contact with GABA-immunolabeled hair cells (green) in the central crista of the toadfish. The location of the afferent dendritic field in the crista is indicated in the figure at lower left. XY: primary image plane; XZ, YZ: image rotations showing narrow belt of afferent arborizations.

Fig. 3 (left). Biocytin-injected low gain velocity-sensitive afferent (red) ramifies in the peripheral crista of the toadfish, far
away from the GABAergic hair cells (green) of the central region.

**Toadfish perfusion:** Toadfish are anesthetized by immersion in MS 222 (250 mg/L sea water, 3-aminobenzoic acid ethyl-ester methane sulphonate; Sigma) and partially immobilized by intramuscular injection of pancuronium bromide (Tradename: Pavulon, 0.2 ml of a 1 mg/ml solution), 20-30 min prior to perfusion. Secure the fish on a perfusion board in the supine position. Expose the pericardial chamber and cut the pericardium. Pass two pieces of 4.0 silk (suture) under the conus using a curved guide. Attach a small vascular clamp to the atrium, to facilitate its retrieval and cutting. Pierce the conus with an 18G catheter. Secure the plastic catheter by placing a small vascular clamp around the catheter. Withdraw the steel needle to fill the catheter with blood, attach the perfusion line (filled with PBS/heparin and attached to a variable speed peristaltic pump) and then tie the silk sutures around the catheter to prevent fixative back-flow. Do not remove the vascular clamp (the catheter may slip out).

The atrium is cut and perfusion begins by injection of approximately 50 ml of heparinized (2U/ml) room temperature (RT) phosphate-buffered saline (PBS; 0.01M, pH 7.3) for 3-5 min at 20 ml/min. Perfusion continues with injection of 100 ml of RT fixative (4% paraformaldehyde/0.2% glutaraldehyde) in phosphate buffer (PB; 0.1M, pH 7.4) for 5 min at 20 ml/min, then 400 ml of additional fixative delivered at 10-12 ml/min. Approximately 5 minutes after the beginning of the perfusion, turn the fish over (gently to avoid slippage of the catheter), and inject 5-8 ml of fixative into the cranial cavity (18 gauge needle). At the conclusion of the perfusion, harvest the labyrinths bilaterally and place them in cold PBS containing 0.02% sodium azide. Store at 4°C.

**Whole mount tissue processing for immunofluorescence:** The endorgans are further dissected under PBS to expose the sensory epithelia fully, washed with PBS, treated with 0.2% NaBH₄ in water for 45 min at RT (to reduce tissue auto-fluorescence) and then washed in PBS. The samples are then immersed in blocking buffer (5% normal goat serum; NGS) in PBS with 0.05% Triton X-100 and 0.02% NaN₃ for 5 – 18 hr at RT. After blocking, the tissue is incubated for 24 hr at RT in wells containing primary antibody(ies). After incubation in antibody, the specimens are thoroughly rinsed with PBS, reacted with secondary reagents (if necessary), rinsed, and then immobilized in Petri dishes thinly coated with 2% low-melting point Agarose (Sigma; St. Louis, MO) diluted in PBS, maintained at 37°C. Once the specimens are secured in place, cover them with cold PBS with 0.02% NaN₃.

**Labeling monoclonal antibodies with fluorescent tags.** Monoclonal antibodies can be purified from serum-free culture supernatants by thiolphilic absorption. Small aliquots of antibody (100 µg) dissolved in 0.1 M NaHCO₃ are tagged with fluorescent probes using commercial kits according to the manufacturer’s protocol (Alexa-Fluor® Monoclonal Labeling Kit, Invitrogen).

**Secondary antibodies:** AlexaFluors (Invitrogen/Molecular Probes) are more expensive, but in our experience they have the best reliability, stability, and variety. They are available in numerous stock, species- and IgG subclass- specificities, in a variety of emission colors.

**Image collection, image processing and quantitative analysis.** Whole mount specimens can be examined and image sets collected with a Zeiss Axioplan microscope with an Apotome slider, and/or a multiphoton laser scanning microscopy system. To visualize the 3-D structure of the crista, we typically collect 100-400 image planes in a single pass, resulting in data sets that are 100-400MB per channel. The 3-D reconstruction of the structure is extracted by computational re-sectioning at various angles of interest, maximum intensity projections, and opacity rendering.
The software tools currently in use include Graphic Converter to manipulate file formats; Velocity (Improvision) and Image J to generate z-axis Maximum Intensity Projections of whole stacks or selected subsets for 2D and 3D visualization and quantitation.