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Frequency Selectivity is Conferred by Membrane Resonance in a Sensory System of Non-mammalian Vertebrate, *Rana Castebiana*

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Non-mammalian Vertebrate, *Rana Castebiana*

A Thesis Presented

by

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ABSTRACT

FREQUENCY SELECTIVITY IS CONFERRED BY MEMBRANE RESONANCE IN A SENSORY SYSTEM OF NON-MAMMALIAN VERTEBRATE, RANA CASTEBIANA

MAY 2019

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In the amphibian auditory system, a subset of hair cells is known to be frequency tuned via electrical resonance. This tuning is thought to contribute to frequency selectivity of the information leaving the auditory periphery via the auditory afferent fibers. At the same time, while most, if not all, afferent fibers are shown to be frequency tuned, electrical resonance has only been experimentally demonstrated in a subset of amphibian auditory hair cells. In this thesis, we validate and use a novel Zap current method to probe the electrical resonance of the bullfrog amphibian papilla hair cells. We uncover the existence of two previously unknown types of electrically resonant auditory hair cells. We then show the existence of resonant hair cells across the length of amphibian papilla, with the range of frequency tuning that is nearly indistinguishable from that previously reported in the of auditory fibers. Therefore, this work further validates amphibian hair cell frequency resonance as the possible mechanism underlying frequency selectivity of the subsequent stages in auditory signal transduction.

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CHAPTER I

INTRODUCTION

The inner ear of the modern vertebrates has also universally evolved a process of decomposition of the frequency components of an incoming sound stimulus into its constituent frequencies and selective amplification of the signal at those frequencies (Webster et al., 1992). This ability is fundamental for the sense of hearing, but its mechanisms are not fully known for vertebrate species despite extensive research and the important evolutionary perspective such knowledge would provide (Manley, 2000). In mammalian vertebrates, this decomposition and amplification is achieved mechanically through the action of the basilar and tectorial membranes (Ulfendahl, 1997; von Békésy, 1960). In non-mammalian vertebrates, on the other hand, the tectorial membrane only exhibits uniform mechanical filtering (Lewis and Leverenz, 1983; Purgue and Narins, 2000), while the frequency decomposition is thought to be performed by electrically tuned hair cells (Crawford and Fettiplace, 1978; Fettiplace and Fuchs, 1999).

Organization of frequency decomposition in a hair cell containing sensory system

What are the significant steps that may contribute to the signal selectivity and amplification? The arrival of a sound wave and the deflection of the hair bundle opens cation-permeable mechanotransduction (MET) channels at the tip of the hair cell's stereocilia. The resulting inward current elicits graded depolarization of the hair cell membrane voltage. These changes propagate to the basolateral membrane of the cell

where synaptic contact is formed with afferent fibers (Hudspeth, 1985; Fettiplace and Kim, 2014). Small, graded changes in membrane potential control calcium influx through the voltage-gated calcium channels (VGCCs) that are located at each synaptic contact. This calcium influx triggers vesicular release machinery clustered together with the synaptic ribbons (presynaptic electron dense bodies specialized for the organization of vesicular release), resulting in the release of glutamate neurotransmitter (Rutherford and Roberts, 2006).

To address the question of frequency selectivity in a hair cell containing sensory system, it is useful to think not only of the frequency selectivity properties of a hair cell basolateral membrane, but also of the possible roles of the properties of synapse itself (Rutherford and Roberts, 2005; Schnee, 2005) and/or afferent fiber membrane properties such as channel composition (Oak and Yi, 2014) in defining frequency tuning previously observed at the level of the auditory fiber action potentials (Frishkopf and Goldstein, 1963; Feng et al., 1975; Ronken, 1991).

Frequency tuning of basolateral Ca^{2+} influx

Tight spatial control of the Ca^{2+} influx around the ribbon-associated vesicle release machinery is shown to be involved in the precise timing control of resulting vesicle release and release synchronicity (Frank et al., 2010; Graydon et al., 2011). Hair cell exocytosis is triggered by the calcium influx through a population of Ca^{2+} channels clustered close to a presynaptic electron dense body (synaptic ribbon) at a hair cell-

afferent fiber synaptic membrane (Frank et al., 2010). This calcium inflow is executed within tight spatiotemporal constraints that are facilitated by the ability of these channels to quickly increase Ca^{2+} concentration in the small spatial domains between the synaptic ribbon and the cell membrane (Graydon et al., 2011).

Replenishment of synaptic vesicles that is necessary for the sustained release is also dependent on this Ca^{2+} influx, a relationship that displays tonotopic differences in mammalian inner hair cells (Johnson et al., 2008; Quiñones et al., 2012). Existing data points to the tonotopic relationship between several ionic currents including calcium current, and resonant frequencies of hair cells (Smotherman and Narins, 1999; Schnee and Ricci, 2003).

Frequency tuning of presynaptic vesicle release

The resulting vesicle release activity, which includes both exocytosis and compensatory endocytosis, can be measured using capacitance changes that detect net change in cell surface area that results from both of these processes (Rutherford and Roberts, 2005; Patel et al., 2012). Although vesicle release in hair cells is shown to possess tonotopic differences (Schnee et al., 2005) and frequency selectivity (Rutherford and Roberts, 2006, Schnee, 2011, Patel et al., 2012), existing studies did not attempt to isolate the vesicle release tuning itself from the possible tuning of the preceding step, Ca^{2+} entry into the cell. At the same time, disordered function of ribbon synapse (temperature-sensitive auditory neuropathy) is known to be associated with the lack of

proper adaptation being demonstrated at the level of auditory brainstem responses (Wynne et al., 2013).

Neurotransmitter vesicle release occurs in amphibian hair cells at the rates up to several hundred vesicles per second for several seconds (Parsons et al., 1994), allowing for small graded changes in response to facilitate the encoding of incoming stimulus frequency. This is thought to be facilitated by action of the presynaptic specializations such as synaptic ribbons. These specializations spatially organize vesicles according to a voltage-dependent gradient that ensures efficient replenishment of vesicles at a synapse (Lenzi et al., 2002; Schnee, 2005). Ultimately, it is thought that cellular machinery of presynaptic dense bodies underlies the precision (von Gersdorff, 2001) and the tuning of vesicle release to certain frequencies (Schnee, 2005). At the same time, reports of tonotopic differences in calcium buffering (Quinones, 2012) and drastic effects of calcium concentration on endocytosis kinetics (Beutner et al., 2001) point at the importance of calcium entry for the balance of exocytosis and endocytosis. Therefore, it appears that the machinery of neurotransmitter vesicle release is poised to be an important mechanism for frequency selectivity.

Frequency tuning of postsynaptic events and the afferent fiber

Through the events of calcium influx and vesicle release, information contained in the receptor potentials of hair cells is ultimately transmitted across the hair cell-afferent synapse by the released chemical neurotransmitter glutamate (Glowatzki and Fuchs,

2002; Rutherford and Roberts, 2006). Neurotransmitter action at the postsynaptic membrane evokes EPSC events in the afferent fiber. These events can be divided into quantal (simple) and multiquantal. The latter events are shown to be more effective at generating the action potentials in lower vertebrate afferent fibers than single EPSCs (Schnee, 2013). Large events are thought to arise from the synchronous release of multiple vesicles at a single ribbon synapse (Li et al., 2009). Other studies in non-amphibian lower vertebrates also suggest that complex events can be generated through summation across multiple synaptic contacts that a single afferent fiber forms with a hair cell (Schnee, 2013). This synchronicity of the multiple vesicle release again points at the importance of the presynaptic vesicle release and replenishment machinery for the maintenance of the frequency following and as a potential point for adaptation, both in terms of the delay before the action potential is generated, and the ability to generate the spike in response to stimulation.

At the same time, it is known that afferent fibers possess mechanisms that enhance temporal precision (Oak and Yi., 2014) and filter redundant information (Avissar et al., 2007). Based on mathematical models of mammalian IHC synapse, spontaneous neurotransmitter release at the hair cell-afferent fiber synapse is thought to possess a best frequency at which information transmission by the auditory nerve fiber is favored (Kumsa, 2012). Afferent fibers are also known to be tonotopically organized and displaying frequency tuning with distinctive v-shaped peaks (Frishkopf and Goldstein, 1963; Feng et al., 1975; Ronken, 1991).

Overall, frequency tuning of membrane voltage oscillations, Ca^{2+} current and vesicle release can act as series of sequential filters, tuning ESPC generation to an incoming stimulus frequency (Patel et al., 2012). Still, the question stands how the afferent fiber in lower vertebrate auditory system is able to maintain the same sharpness of tuning as any individual presynaptic hair cell, while making synaptic contacts with multiple individually tuned hair cells (Lewis et al., 1982; Ronken 1991; Smotherman and Narins, 2000, Frolov and Li, 2017). In an afferent fiber, firing efficiency is thought to depend on EPSC frequency in a nonlinear manner. Relationship between this nonlinearity and the refractory period (time when new action potential cannot be fired, even in presence of threshold EPSC) defines the afferent fiber response to trains of stimuli in the auditory fiber model (Goldwyn, 2012).

Inner ear of non-mammalian vertebrates as model system for frequency selectivity research

Partial electrical tuning and tonotopy has been previously demonstrated in a variety of species and hearing organs (Fettiplace and Fuchs, 1999; Fuchs et al., 1988; Hudspeth and Lewis, 1988; Tan et al., 2013, Smotherman and Narins, 1999), but to date, it remains unclear whether this tonotopy and tuning are necessary and sufficient to explain the frequency decomposition in the non-mammalian inner ear. Partially, this lack of clarity can be explained by the limitations of the step current injection method previously used in auditory research to produce and analyze dampening oscillations of the sensory receptor membrane voltage, leading to the recent development of new current

injection methods less reliant on the assumptions of modeling the sensory receptor as simple electrical circuit (Frolov and Li, 2017).

Frequency selectivity is one of the fundamental questions in sensory systems research, and investigation of both adaptation and frequency selectivity at every step of the sensory system circuit is of paramount importance for the creation of a well-linked picture of how adaptation affects signal recognition and behavior (Whitmire and Stanley 2016). Still to this date, the exact location of the frequency selectivity mechanism(s) and their relative contribution of the hair cells, as well as the higher levels of sensory processing, is poorly understood in non-mammalian vertebrates (Fritz et al., 2007, Wen et al., 2009, Shamma and Fritz, 2014). Therefore, the questions of where the mechanisms of frequency selectivity are located in the periphery of the hair-cell containing sensory system, and the relative contribution of the hair cell to these phenomena, still present an important problem for the current research in auditory signal encoding, adaptation and recovery from adaptation.

Amphibian inner ear presents a unique opportunity to study frequency tuning due to its exceptional anatomy that includes three separate end organs, each responding to a particular range of frequencies. It includes frequency filtering elements arranged both in series and in parallel. Serial filtering is represented by sequentially linked tympanic membrane and single columella ossicle (both impose mechanical filtering with a broad range peak of 0.4 – 2 kHz on the sound they transmit, due to their physical properties), as

well as tectorial membrane of the end organs (Lewis and Leverenz, 1983; Purgue and Narins, 2000; Smotherman and Narins, 2000) (Fig.1).

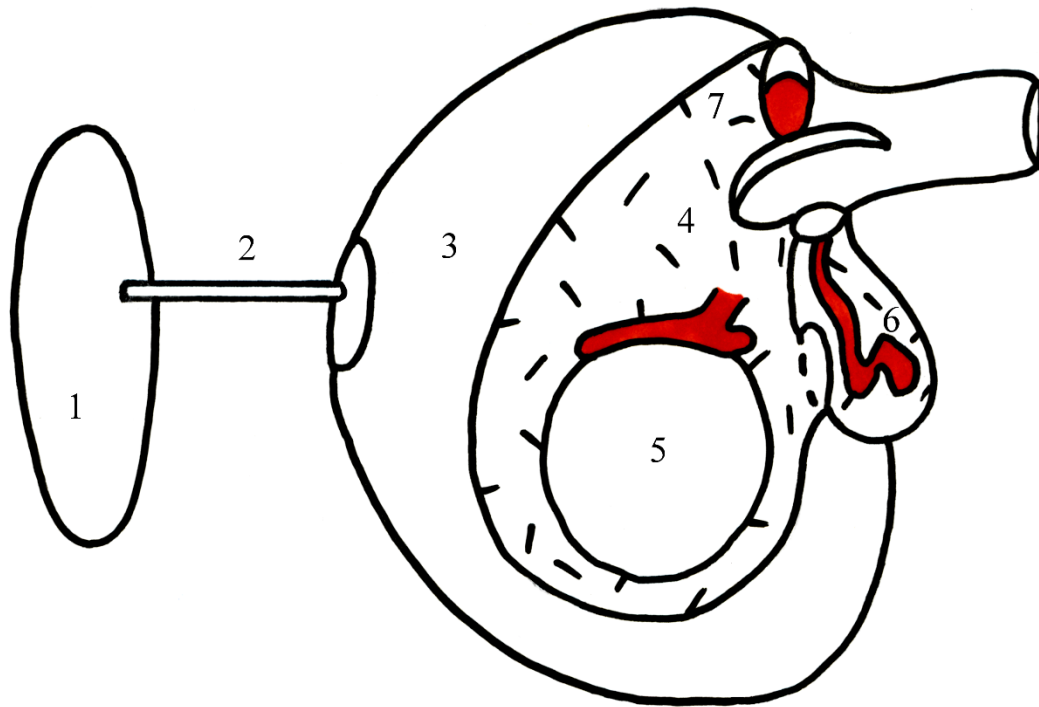


FIGURE 1. The structure of the amphibian outer and inner ear. (1) Tympanic membrane, (2) Columella ossicle, (3) Round window, (4, in red) Sacculus, (5) Saccular otoconial mass, (6, in red) Amphibian papilla, (7, in red) Basilar Papilla. Image created by Tiffany Fox, A.S, B.S., MHFA and used with permission.

Frequency selectivity stages in the amphibian inner ear

The amphibian inner ear end organs lack basilar membrane that is characteristic of the higher vertebrates, but possess tectorial membrane that is vibrated by the sound arriving through the contact membranes. Thus far, there is no indication available of the mechanism by which the amphibian papilla tectorial membrane may be able to provide

tonotopic parallel filtering (frequency deconvolution) through its mechanical properties. It can nevertheless exhibit uniform mechanical filtering based on stiffness and weight (Lewis and Leverenz, 1983; Purgue and Narins, 2000).

Therefore, any parallel filtering (deconvolution) in the amphibian inner ear must be represented by a combination of rough deconvolution into low and high frequencies by perilymphatic and endolymphatic canals and much more precise frequency deconvolution achieved in hair cells. Parallel arrangement of filtering elements provides frequency deconvolution of the sound wave frequency contents, in the sense of resolving the arriving sound into its constituent frequency components. Perilymphatic and endolymphatic canals and their contact membranes direct particular ranges of sound frequencies to the separate end organs within the inner ear, creating two separate sound processing streams with band-pass peak frequencies around 500 Hz for amphibian papilla and 1100 Hz for basilar papilla (Purgue and Narins, 2000).

Frequency deconvolution is believed to happen mainly in auditory hair cells, which form an array of frequency filters arranged tonotopically in parallel (Lewis et al., 1982a; Smotherman and Narins, 1999). Accordingly, lower vertebrate (including amphibian) hair cells are thought to be able to selectively process and signal the arrival of a specific frequency at the hair bundle. For this, they have to possess the ability to selectively amplify signals at certain characteristic frequencies while suppressing signals at other frequencies. This amplification is made possible by the electrical tuning, that depends on the membrane voltage resonance of hair cells (Lewis et al., 1982; Crawford

and Fettiplace, 1987; Pitchford and Ashmore, 1987; Hudspeth and Lewis, 1988; Smotherman and Narins, 1999, Manley, 2000). This resonance is revealed through the dampening oscillations resulting from step current stimulation (Crawford and Fettiplace, 1981; Hudspeth and Lewis, 1988; Manley, 2000). This view is well supported for the rostral part of the bullfrog auditory end organ, amphibian papilla, known to respond to low-frequencies (Pitchford and Ashmore, 1987; Smotherman and Narins, 1999). The cells from the caudal, high-frequency regions did not initially seem to possess electrical resonance (Smotherman and Narins, 1999).

According to the existing literature, for lower vertebrates, the location of the electrical resonance is believed to be auditory hair cells membrane, most possibly owing to its ion channels that allow for the voltage response resonance process to occur. This in turn, allows individual hair cells to selectively amplify and signal the arriving signal possessing specific frequencies, while suppressing signals at other frequencies in a process called electrical tuning (Lewis et al., 1982; Crawford and Fettiplace, 1987; Pitchford and Ashmore, 1987; Hudspeth and Lewis, 1988; Smotherman and Narins, 1999, Manley, 2000). This is different from the well-known mechanical tuning that underlies the frequency selectivity in the mammalian inner ear.

Modeling and existing research of the amphibian hair cell resonance

Neuronal membranes were long thought to be well-described by the equivalent RLC circuit (where capacitive element (C) is in parallel, or “shunted”, by an inductive

(L) and a rectifying resistive element (R) connected in series) (Cole, 1941). The ability of the ion-permeable membranes to display a feature called “natural frequency”, expected from that equivalent circuit, was first described in the squid axon and took a form of dampening small-signal oscillations in response to the depolarizing current injection (Mauro, 1970). Oscillatory response of such equivalent RLC circuit (dampened harmonic oscillator, Hudspeth, Lewis 1988) was taken as the basis for the development of the mathematical model with which to study the response of the actual hair cells. Dampened oscillations in response to current step are predicted by such model and were observed experimentally in the turtle hair cells. These oscillations were fit to a decaying amplitude sine wave to obtain resonant frequency (f_R) and time constant of decay (T). In turn, those are used to calculate tuning quality factor (Q_e). From f_R and Q_e , hair cell tuning curve is constructed to represent the input-output relationship of either the hair cell, or its equivalent circuit (Crawford, Fettiplace 1981).

Existing reports that directly explore the existence of electrical tuning in the amphibian papilla region of lower vertebrate inner ear rely on the calculations derived from the RLC circuit model, but no definitive proof of this model's applicability to all hair cells has been provided in literature. Also, dampening oscillations resulting from the step current stimulation seem to be another requirement to reveal the existence of the frequency filtering (Pitchford and Ashmore, 1987; Hudspeth and Lewis, 1988, Smotherman and Narins, 1999).

This oscillation has proven to be absent in certain populations of amphibian hair cells, such as in the caudal region of the amphibian papilla, and therefore the authors concluded that either this region's hair cells do not possess electrical resonance, or these oscillations were abolished by the method employed. It is of note that one possible reason for this abolition is mentioned to be the dissociation procedure that was used to isolate hair cells, more precisely, the use of papain (Smotherman, Narins 1999). It is also known that in cases where the oscillations are not abolished by enzymatic dissociation with papain, their frequency can be exaggerated by the treatment, which produces altered conventional (f_R) and (Q_e) measures of hair cell electrical resonance (Armstrong, Roberts 1998).

In the literature, caudal region of amphibian papilla is believed to process high-frequency sound. This conclusion is made on the basis of a body of works exploring the tuning of the single units in the eights nerve of the bullfrog. In short, sound stimulation of the intact ear was performed in the anesthetized animal and electrophysiology recordings were made from the single units in the eights nerve of the bullfrog. This revealed frequency selectivity in the auditory units, including those that project to the caudal region of the amphibian papilla, as evidenced by the intracellular dye-injection into the axon (Frishkopf, Goldstein 1963, Lewis et al., 1982, Ronken 1991).

Therefore, the question arises as to how frequency selectivity in the auditory fibers contacting these non-oscillating cells can be supported, without the benefit of electrical tuning in the hair cell itself. While a certain level of mechanical filtering is present in

amphibian inner ear, such as that conferred by tympanic membrane, single columella ossicle, and the tectorial membrane of end organs (Lewis and Leverenz, 1983; Purgue and Narins, 2000; Smotherman and Narins, 2000), these sequentially arranged filtering elements can be thought as simply limiting the range of naturally-occurring frequencies that can reach the inner ear to those frequencies relevant for the animal behavior in its environment. Perilymphatic and endolymphatic canals and their contact membranes do present an example of the parallel filtering (deconvolution), but at the level that appears to be too rough for such sound frequency discrimination tasks as, for example, conspecific vocalization.

Parallel frequency filtering in the amphibian papilla

These anatomical features can indeed direct different sound frequencies to the separate endorgans within the amphibian inner ear, but only effectively separating the sound processing into 1-3 kHz wide band-pass streams with peak frequencies around 500 Hz for amphibian papilla and above 1100 Hz for basilar papilla (Purgue, Narins 2000). Amphibian papilla tectorial membrane was proposed to provide some degree of frequency resolution due to the gradient of its thickness and mass, and it has been proposed a traveling wave akin to that of the mammalian cochlea can exist in the amphibian papilla (Lewis, Leverenz 1983, Purgue, Narins 2000). Still, no definitive data exists to date that would prove that amphibian papilla tectorial membrane can provide frequency deconvolution through a process characteristic for the mammalian inner ear,

where basilar membrane confers mammalian hair cells their frequency selectivity on the basis of their position along its mass and stiffness gradient.

Parallel arrangement and possible frequency filtering achieved in the hair cells seems to be mirrored by the organization of the auditory nerve units. It was shown that in the auditory fibers innervating amphibian papilla (AP) increase in their characteristic frequency from the rostral to caudal part of the amphibian papilla (Lewis, 1982). According to other reports, there exist at least two populations of units with characteristic best excitatory sound frequencies of 250 Hz and 600 Hz (Feng, 1975). These two groups of fibers can be classified by measuring the bandwidth of the tuning curve 10 dB above characteristic frequency threshold (W10dB). This measure was also found to increase concomitantly with characteristic frequency of the unit (Ronken, 1991). Increasing characteristic frequency of the AP fibers also coincided with the increasing the time delay between the stimulus onset and the recorded neural response. It was proposed that this delay may be attributed to the filter function of the frequency-tuned hair cell (Hillery, Narins 1984).

Therefore, current literature points at an unexplored possibility that frequency filtering function is indeed carried out by both rostral and caudal amphibian hair cells before information transduction to the auditory fibers, and the lack of experimental data on the electrical tuning of these cells stems from the inability of the conventional methods to resolve the resonant properties of all hair cells.

Novel method for the hair cell frequency selectivity research

In my Masters research, I have used an alternative approach, using Zap current as the straightforward experimental measure of the cellular oscillatory properties in amphibian hair cells. The Zap signal is a sine wave of a fixed peak-to-peak amplitude that changes in frequency over time. Because of that property, hair cell tuning can be conveniently determined by converting the voltage response of the cell to the frequency domain using Fast Fourier Transformation (Hutcheon, Yarom 2000). This eliminates the need for the equivalent circuit assumption that was made in the previous literature and the use of curve fitting, therefore eliminating the need for the dampening oscillation to exist to perform electrical tuning analysis. We have used the Zap current to directly determine the peak frequency and tuning curve of the hair cells. This was accomplished in the minimally perturbed amphibian papilla that has not undergone any mechanical or enzymatic dissociation to avoid confounding the resulting frequencies with papain or mechanical damage to the hair cells.

I used whole-cell patch-clamp in acute intact (not mechanically stretched, neither enzymatically dissociated) preparation of bullfrog (*Rana castebiana*) amphibian papilla. We stimulated hair cells using both conventional step current and a novel Zap current, where the current input is delivered in a sine wave manner, with the frequency increasing over time at a constant rate (Puil et al., 1986; Hutcheon and Yarom, 2000). Briefly, I utilized whole-cell patch-clamp in amphibian papilla hair cells to apply a sine wave of

uniform peak to peak amplitude and increasing frequency (Hutcheon and Yarom, 2000, Yan et al., 2012; Yang et al., 2014).

This technique was used to probe the tonotopic frequency selectivity by systematically acquiring patch-clamp recordings of the cells along the hair-cell sensory epithelium of amphibian papilla, seeking to map the electrical response to the injection of the Zap current. This was an improvement on the previously established method of probing the frequency tuning of hair cells by injecting a step current (Crawford and Fettiplace, 1978; Fettiplace and Fuchs, 1999), as such method depended on the detection of a dampened oscillation strong enough to measure its quality factor (Q_e) and on modeling the cell as a simple electronic circuit with a capacitor in parallel with series combination of a resistor and an inductor (Crawford and Fettiplace, 1981). By dispensing with those assumptions, we were able to directly probe the frequency selectivity of any hair cell in an unbiased manner.

Therefore, Zap stimuli allowed us to sweep multiple frequencies over the course of the stimulation and record membrane voltage oscillatory responses. We studied both the rostral low-frequency caudal high-frequency parts of the amphibian papilla (Smotherman and Narins, 1999). Tuning curves were obtained, representing the relationship between the amplitude of hair cell response and stimulation frequencies, with the peak of the curve thought to indicate the frequency to which a hair cell is tuned (best frequency). The shape of the curve is determined by the ability of hair cells to selectively amplify (respond with larger voltage oscillations to) a particular range of frequencies

around the best frequency, with narrower range of amplified frequencies producing a sharper tuning curve (Crawford and Fettiplace, 1987). This sharpness is defined by the W10dB value, the measurement of the tuning curve width 10 dB below the peak (Fig. 2) (Ronken, 1991). Eventually, the hair cells frequency tuning is thought to confer frequency selectivity of the information leaving the auditory periphery via the auditory afferent fibers.

CHAPTER II

PROBING ELECTRICAL TUNING OF H

The results of the study briefly described above were published in the journal *Synapse* (Frolov and Li, 2017) and are reproduced below verbatim from the publication, with explicit permission of the Master's Thesis Committee and according to the journal *Synapse* and Wiley Online Library guidelines for re-use of the Final Published Versions of the papers or parts thereof for any publication authored or edited by the Contributor (excluding journal articles). With respect to these guidelines, the text of the article is reproduced below with minimal changes to the text and figures.

Introduction

The inner ears of modern vertebrate species came from multiple line-ages of evolution that all underwent dramatic and parallel improvements (Manley, 2000). Regardless of the differences in anatomy, they all decompose acoustic signals into series of frequency components first and then process them in parallel thereafter (Webster et al., 1992). This process of decomposition, which is often referred to as frequency tuning, is achieved mechanically in the mammalian cochlea through the basilar and tectorial membranes (Ulfendahl, 1997; von Békésy, 1960). For non-mammalian vertebrate species such as reptiles, amphibians and birds, however, frequency tuning has been attributed to electrical tuning of hair cells (Crawford and Fettiplace, 1978; Fettiplace and Fuchs, 1999).

Electrical tuning of auditory hair cells was first discovered in the turtle basilar papilla where hair cells responded to step current injection with dampened voltage oscillation at approximately their characteristic frequencies (Crawford and Fettiplace, 1978). Because a step function can be viewed as a sum of simultaneous sinusoidal functions at different frequencies (i.e., Fourier Series), this result argues that these hair cells are electrically tuned to respond best to one frequency while suppressing others. Aiming to quantify this intrinsic property of hair cells, the same authors demonstrated in a follow-up study that these hair cells behave like an electrical circuit where a capacitor is connected in parallel with a series combination of a resistor and an inductor (Crawford and Fettiplace, 1981). Based on this model, a tuning curve can be calculated from the quality factor (Q_e), which can be obtained by fitting the dampened voltage oscillation with a sinusoidal function whose amplitude decays exponentially (Crawford and Fettiplace, 1981). Since then, the step current method has become a standard in characterizing electrical tuning of hair cells, and it has revealed electrical tuning in hair cells from a variety of hearing organs in different species (Fettiplace and Fuchs, 1999; Fuchs et al., 1988; Hudspeth and Lewis, 1988; Tan et al., 2013), including the frog amphibian papilla (Smotherman and Narins, 1999). However, this method relies on modeling hair cells with a simple electronic circuit, which may not be accurate enough to capture the interplay of membrane capacitance with all different types of voltage-gated ion channels in hair cells. More importantly, this method cannot be applied to hair cells that do not oscillate, because the quality factor (Q_e) cannot be determined. For example, in the frog amphibian papilla, while low frequency hair cells in the rostral end displayed dampened voltage oscillation with step current injection, high frequency hair cells in the

caudal end did not (Smotherman and Narins, 1999). Although non-oscillating hair cells probably have less tuning capability, the lack of oscillation is by no means any indication of no electrical tuning. In fact, any neuron (or receptor cell) can at least act as a low-pass filter (Hutcheon and Yarom, 2000), so that non-oscillating hair cells are likely to have some electrical tuning. In the present study, we developed a Zap current method that allows us to unbiasedly assess electrical tuning of all hair cells. A Zap current is a sinusoidal current where the peak-to-peak amplitude is kept constant while the frequency is increased linearly with time. By applying this new method to hair cells in the intact amphibian papilla of bullfrogs, we revealed an underappreciated heterogeneity of electrical tuning in this hearing organ.

Materials and Methods: Electrophysiology

All animals are handled according to an animal protocol reviewed and approved by the University IACUC. Adult bullfrogs (*Rana Catesbeiana*) were purchased from Rana Ranch (Twin Falls, Idaho, USA) and maintained in a centralized animal care facility. The bullfrogs were sacrificed by quick double pithing followed by decapitation. The otic capsules from both sides were carefully dissected out and placed in an artificial perilymph solution containing the following (in mM): 95 NaCl, 2 KCl, 2 CaCl₂, 1 MgCl₂, 25 NaHCO₃, 3 Glucose, 1 creatine and 1 sodium pyruvate, pH adjusted to 7.30 with NaOH and continuously bubbled with 95% O₂ and 5% CO₂. The whole hearing epithelium from the amphibian papilla was carefully isolated and transferred to a recording chamber continuously perfused (2–3 ml min⁻¹) with the same artificial perilymph solution throughout the recording. The tissue was visualized through an

upright microscope (BX51WIF, Olympus, Tokyo, Japan) with a 603 water-immersion objective, and hair cells were identified based on their unique morphology and hair bundles. While tissue splitting is necessary for postsynaptic afferent fiber recordings in this hearing organ (Keen and Hudspeth, 2006; Li et al., 2009), we managed to record from hair cells in the intact epithelium without splitting. With a positive pressure of 3 PSI maintained in the patch pipettes, we found it is possible to penetrate the tissue surface and form GΩ seals with a reasonable success rate. Patch pipettes were pulled from a vertical pipette puller (PC-10, Narishige, Tokyo, Japan), and they have a typical resistance of 10 MΩ when filled with an internal solution containing (in mM) 80 K-gluconate, 30 KCl, 10 HEPES, 2 EGTA, 3 Mg-ATP and 0.5 Na-GTP (pH 7.30, osmolarity 230 mOsm). Patch-clamp recordings were made at room temperature (22°C) through a Heka amplifier (EPC10/2, Heka, Lambrecht/Pfalz, Germany). The voltage signals were low-pass filtered at 2 kHz and sampled at 100 kHz. A liquid junction potential of 10 mV was corrected offline.

Materials and Methods: Data Analysis

All data analyses were performed in Igor Pro 6.0 (WaveMetrics, OR) with custom-written functions and routines. Zap currents were generated according to the following function:

$$I_{\text{zap}}(t) = I_{\text{step}} + I_{\text{zap}} \cdot \sin(2 \cdot \pi \cdot V_f \cdot t^2)$$

Where A_{zap} is the amplitude of the sinusoid, V_f is the speed of frequency sweeping. This current was superimposed on a step current (A_{step}) that starts 0.5 s earlier.

An example of such current templates is shown in Figure 1B where A_{Zap} , V_f , and A_{step} are 20 pA, 300 Hz s²¹, and 250 pA, respectively. Between stimuli, hair cells were kept under the current-clamp mode with enough negative current injected to maintain a membrane potential of 290 mV. Upon stimulation, A_{step} was adjusted in real time to reach a desired membrane potential, i.e., 255 mV unless stated otherwise. From the voltage response to I_{Zap} , a tuning curve can be calculated through Fast Fourier Transform (FFT). From such a tuning curve, f_e was determined as the frequency with the maximum voltage response (V_{max}), $f_{3dB, left}$ and $f_{3dB, right}$ were determined as the frequency where $0.708 \cdot V_{max}$ (given that $20 \cdot \log_{10}(0.708) \approx -3$ dB) is crossed on the left and right of f_e , respectively, W_{3dB} is defined as $f_{3dB, right} - f_{3dB, left}$, and Q_{3dB} is defined as f_e/W_{3dB} .

In response to the step current (A_{step}), some hair cells showed classic dampened voltage oscillation, which was fitted to a sinusoidal function with the amplitude decaying exponentially (Catacuzzeno et al., 2003; Li et al., 2014):

$$V(t) = A \cdot \sin(2\pi \cdot f_e \cdot t + \phi) \cdot e^{-t/\tau} + V_{SS}$$

Where A is the peak amplitude of voltage oscillation, f_e is the resonance frequency, ϕ is the phase, τ is the time constant of single exponential decay, and V_{SS} is the steady-state membrane potential. With f_e and τ yielded from the curve fitting, the quality factor (Q_e) can be calculated with the following formula (Crawford and Fettiplace, 1981):

$$Q_e = \sqrt{(\pi \cdot f_e \cdot \tau)^2 + 0.25}$$

For a given Q_e and f_e , a tuning curve can be calculated through the following equation (Crawford and Fettiplace, 1981):

$$A(f) = A_0 \cdot \sqrt{\frac{Q_e^2 + f_e^2 / f^2}{(f/f_e - f_e/f)^2 \cdot Q_e^2 + 1}}$$

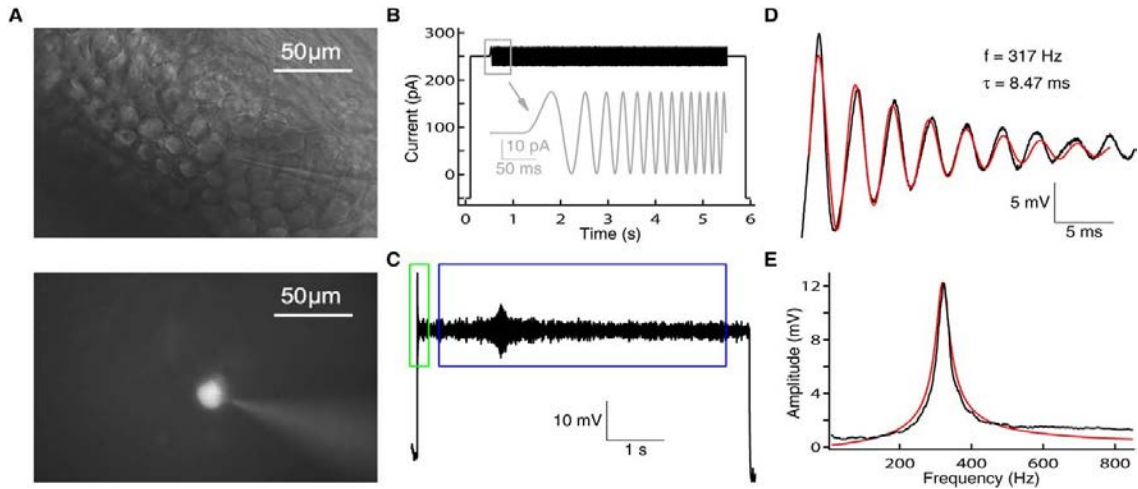


FIGURE 2. Probing electrical tuning of hair cells with Zap current injection. (A), image of an intact bullfrog amphibian papilla (upper panel) while patch-clamp recording was being established on one hair cell, as indicated by diffusion of a fluorescence dye (Alexa 594) into the cell through the patch pipette (lower panel). (B), typical template for Zap current injection. It consists of a step current of 250 pA and a Zap current of 40 pA (peak-to-peak) with the frequency sweeping from 0 to 1.5 kHz in 5 s. The inset shows the beginning part of the Zap current (in gray box) in a different temporal scale (in gray). (C), voltage response of a hair cell to the current template in B. (D), part of the trace in C (in green box), showing the voltage response to the initial step current. This is a classic example of dampened voltage oscillation, and the trace was fitted very well to a sinusoidal function with the amplitude decaying exponentially (in red), which yielded a resonance frequency (f_e) and a time constant (τ). See “Materials and Methods” for details. (E), two tuning curves for the same hair cell in C. The black curve was obtained from the voltage response to the Zap current (in blue box in C) through Fast Fourier Transform, and the red curve was calculated from f_e and τ in D (see Materials and Methods for details). Note that the two curves are in close agreement to each other.

Results

The traditional step current approach to assess electrical tuning of hair cells has been successfully applied to hair cells in many different hearing organs (Fettiplace and Fuchs, 1999), but it requires hair cells to have dampened voltage oscillation in response to step current injection (Crawford and Fettiplace, 1981). Therefore, the tuning capability of non-oscillating hair cells, as discovered in the caudal end of the frog amphibian papilla (Smotherman and Narins, 1999), remains to be quantitatively determined. In the present study, we decided to adopt the Zap current approach, which has been proven to be a powerful tool to reveal the intrinsic frequency preferences of neurons in the brain (Hutcheon and Yarom, 2000). We chose the bullfrog amphibian papilla to carry out our study because it has both oscillating and non-oscillating hair cells, allowing us to validate and expand the new method, respectively. Furthermore, unlike the previous studies where mechanical or enzymatic treatment was applied to expose hair cells (Armstrong and Roberts, 1998; Smotherman and Narins, 1999), we made whole-cell patch-clamp recordings directly in the intact epithelium where the microenvironment surrounding hair cells is maximally preserved (Figure 1A).

We first focused on the oscillating hair cells in the rostral end of the bullfrog amphibian papilla. We designed a current template to consist of a step current and a Zap current superposed with a delay of 0.5 s, and applied it to hair cells under current-clamp (Figure 1B,C).

Consistent with the previous study (Smotherman and Narins, 1999), the voltage response to the step current shows typical dampened voltage oscillation (Figure 1D). We fitted the response to a decaying sinusoidal function, which yielded a resonance frequency (f_e) and a decaying time constant (τ). On the basis of these two parameters, we calculated the quality factor (Q_e) and the tuning curve (Figure 1E, red) with the formula outlined in Crawford and Fettiplace (1981; see Materials and Methods for details). Meanwhile, based on the voltage response to the Zap current, a Fast Fourier Transform (FFT) directly yielded a second tuning curve for the same hair cell, which is in close agreement with the first one from the step current method (Figure 1E).

Electrical tuning of hair cells relies on the interplay of voltage-gated ion channels and membrane capacitance (Hudspeth and Lewis, 1988). Therefore, the tuning capability, quantified as Q_e , has been shown to depend on the steady-state membrane potential level (Smotherman and Narins, 1999). To further validate the new Zap current method, we wanted to find out if it can capture this well-established feature of electrical tuning. We varied the amplitude of the step current to bring the steady-state membrane potential to different levels and calculated the tuning curve at each membrane potential level (Figure 2A,B). Based on these tuning curves, Q_{3dB} and f_e were determined and plotted against Q_e and f_e obtained from the step current method, respectively (Figure 2C,D). The estimates of f_e from the two methods are in close agreement with a linear correlation coefficient (r) of 0.999 (Figure 2C).

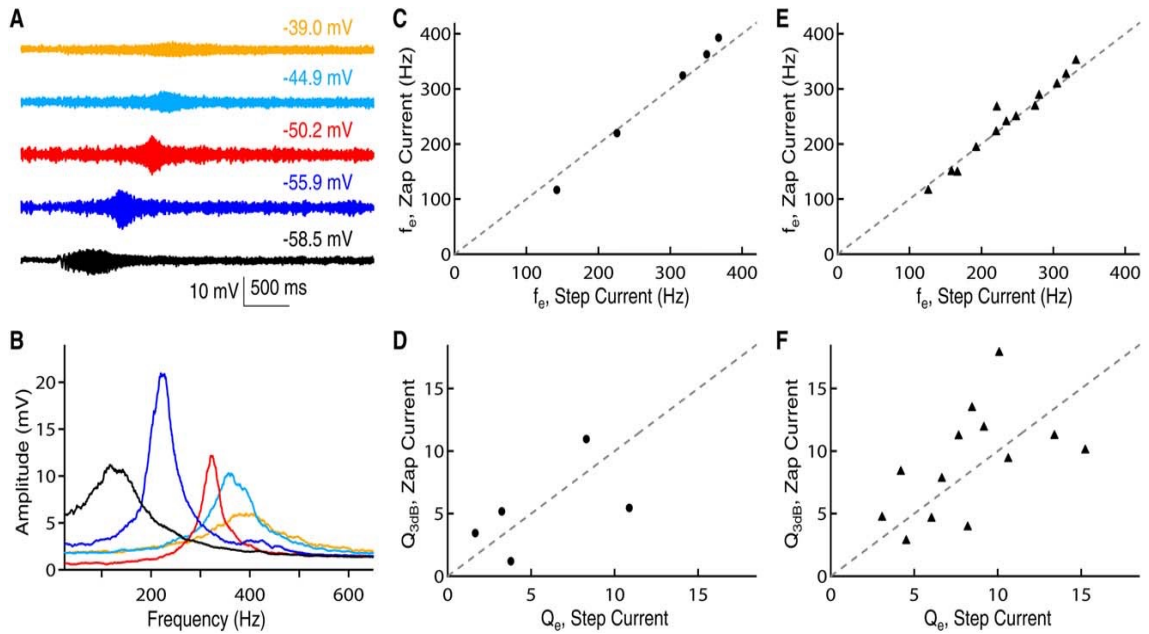


FIGURE 3. The Zap current method yields similar estimates of electrical tuning as the step current method. (A), voltage responses to Zap current injection at different steady-state membrane potentials. The Zap current was kept the same while the step current was varied to reach different steady-state membrane potentials. All 5 traces were obtained from the same hair cell. (B), tuning curves from voltage responses in A. C and D), the resonance frequency (f_e , C) and the sharpness of the tuning curve in B (Q_{3dB} , D) are plotted against the values obtained from the classic step current method. The dashed line depicts a line with the unity slope through the origin. E and F), based on the tuning curves obtained from 13 hair cells, f_e (E) and Q_{3dB} (F) are plotted against the values obtained from the step current method. For each cell, the data values were determined from voltage responses at a single steady-state membrane potential close to 255 mV

Q_{3dB} from the Zap current method and Q_e from the step current method results are also positively correlated ($r = 0.560$). Furthermore, across a total of 13 hair cells, the estimates of f_e from the two methods are also in close agreement ($r = 0.980$, Figure 2E), and Q_{3dB} and Q_e are positively correlated ($r = 0.538$).

We then moved on to the non-oscillating hair cells in the caudal end of the bullfrog amphibian papilla. Consistent with the previous study, these hair cells did not show voltage oscillation in response to step current injection (Figure 3A). With the new

Zap current method, we were able to determine their tuning curves (Figure 3B), showing a broad tuning up to 800 Hz. Surprisingly, we discovered a new type of hair cells in the rostral end. Like the typical oscillating hair cells, these hair cells oscillated in response to step current injection, and the amplitude of oscillation also decreased significantly following the onset of the step current (Figure 3C). Unlike the typical oscillating hair cells, however, the oscillation in these hair cells continued as long as the step current is on and the amplitude of oscillation became stable after the initial decrease (Figure 3 inset). Because of the continuous oscillation, the decay time constant of oscillation cannot be obtained through curve fitting (see Equation 2), and therefore the tuning curve cannot be calculated through the step current method. With the new Zap current method, we were able to determine the tuning curve, and it revealed extremely sharp tuning in these hair cells (Figure 3D). More interestingly, it also revealed a second peak at approximately twice the frequency for the first peak, a phenomenon reminiscent of harmonics. Lastly, we calculated W_{3dB} from all hair cells and plotted against their f_c (Figure 4), showing a greater heterogeneity of electrical tuning in the amphibian papilla. For comparison, we also included data from seven hair cells in the sacculus, which are homogeneously tuned to a narrow frequency range of 10–40 Hz (Figure 4, in red). The diversity of electrical tuning in the amphibian papilla closely resembles the frequency tuning measured at afferent fibers in vivo (Ronken, 1991), suggesting the electrical tuning within hair cells may be sufficient to account for the overall frequency tuning in this hearing organ.

Discussion

In the present study, we developed and validated a new Zap current method to measure electrical tuning of hair cells in non-mammalian hearing organs (Crawford and

Fettiplace, 1978; Fettiplace and Fuchs, 1999). Compared to the traditional step current method (Crawford and Fettiplace, 1981), the new method offers three significant advantages. First, it can be applied unbiasedly to all hair cells, including both oscillating and non-oscillating hair cells. Second, it yields a tuning curve directly from experimental data, requiring no assumptions regarding the underlying cellular mechanisms. Third, the Zap current method is able to reveal additional peaks in the tuning curve, a sign for high-order tuning (see Figure 3D). In contrast, the step current method assumes the tuning curve has only one peak (see Equation 4 in the Materials and Methods). The Zap current approach has been applied to different neurons in the central nervous system (Hutcheon and Yarom, 2000), and it remains a simple and powerful tool to reveal the intrinsic frequency preferences for sub-threshold synaptic inputs (Hu et al., 2002; Yang et al., 2015). In the hearing field, a similar stimulus called the chirp sound has been used in acoustic stimulation to compensate the frequency-dependent delay of neural responses caused by the mechanical traveling wave in the Organ of Corti in the cochlea (Elberling and Don, 2008).

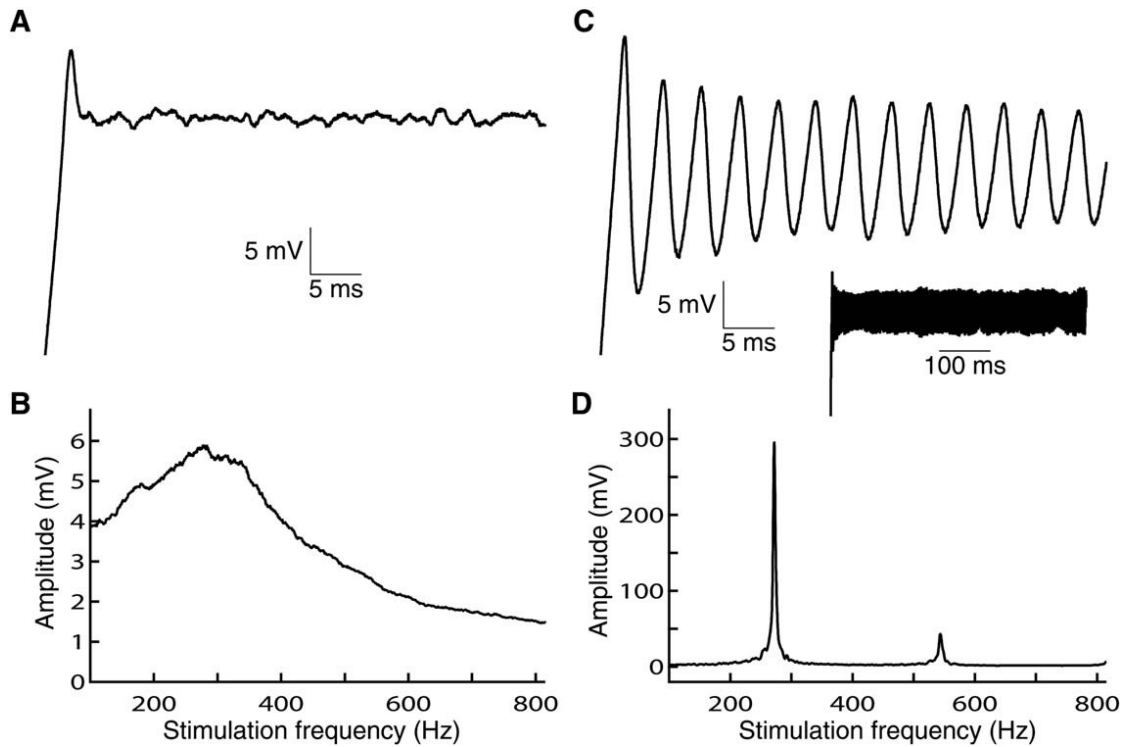


FIGURE 4. Heterogeneity of electrical tuning in hair cells of the intact amphibian papilla. (A), voltage response to step current injection, recorded from a hair cell on the caudal end of the amphibian papilla. Due to the lack of voltage oscillation, the tuning capability of this hair cell cannot be determined with the step current method. (B), tuning curve of the hair cell in A, obtained through the Zap current method. (C), voltage response to step current injection, recorded from a hair cell on the rostral end. After the initial decrease, the oscillation continued and the amplitude became stable, as shown in the inset. The continuous oscillation makes it impossible to estimate its tuning capability based on the step current method. (D), tuning curve of the hair cell in C, obtained through the Zap current method. This hair cell shows extremely sharp frequency tuning with a primary and secondary peak at 272 and 544 Hz, respectively retinal bipolar cells (Baden et al., 2011).

We therefore wanted to adopt this approach to study electrical tuning of hair cells. We designed a current protocol with both step and Zap current injections and applied it to hair cells with dampened voltage oscillations in the bullfrog amphibian papilla. We found that the new Zap current method yields similar estimates of electrical tuning as the traditional step current method. Furthermore, when hair cells are depolarized to different

steady-state membrane potential levels, the Zap current method follows the step current method and captures the voltage-dependence of electrical tuning (Hudspeth and Lewis, 1988; Smotherman and Narins, 1999).

Having validated the new Zap current method, we applied it to hair cells in both ends of the intact bullfrog amphibian papilla. First, we found that while oscillating hair cells in the rostral end are sharply tuned, non-oscillating ones in the caudal end are broadly tuned. With the recordings obtained from the intact hearing epithelium, we ruled out the possibility that the lack of oscillation in the caudal hair cells is caused by enzymatic or mechanical treatment in cell preparation (Armstrong and Roberts, 1998; Smotherman and Narins, 1999). Our finding that these hair cells are broadly tuned suggests that electrical tuning may not be necessary for phase-locking as *in vivo* recordings from afferent fibers receiving input from these hair cells showed prominent phase-locking (Dunia and Narins, 1989; Hillery and Narins, 1984). Second, we discovered a third type of hair cells, located in the rostral end, which oscillate continuously in response to step current injection. These hair cells are extremely sharply tuned, and their tuning curves show multiple peaks that are reminiscent of harmonics, suggesting high-order tuning. This continuous oscillation in response to step current injection was also observed in the retinal bipolar cell terminals (Burrone and Lagnado, 1997; Hull et al., 2006), suggesting this may be a general property shared by cells using L-type Ca^{21} channels for triggering exocytosis. Lastly, when we pooled the data from all hair cells and plotted $W_{3\text{dB}}$ against f_e , we found a whole spectrum of electrical tuning, which seems to match the frequency tuning of afferent fibers in the same species (Ronken, 1991).

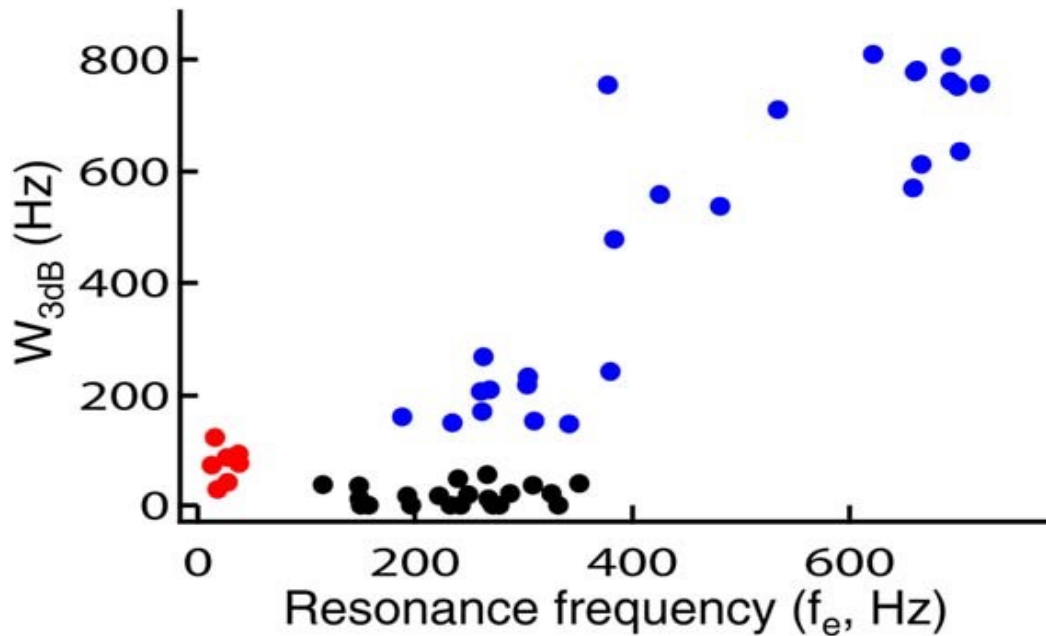


FIGURE 5. Electrical tuning capability of hair cells in the bullfrog amphibian papilla. For all cells, tuning curves were obtained through the Zap current method, and the sharpness of electrical tuning, quantified as W_{3dB} , is plotted against the resonance frequency (f_e). The black and blue dots represent data from the rostral and caudal end of the papilla, respectively, while the data from the sacculus are drawn in red for comparison.

The newly developed Zap current method revealed a greater heterogeneity of electrical tuning in non-mammalian hair cells, which seems to further suggest electrical tuning alone is sufficient to account for all frequency tuning in non-mammalian hearing organs. However, as auditory signals go through stages of processing, frequency tuning convolves and accumulates, so that systematic and independent examination of both electrical and mechanical tuning is required. Contrary to what the literature may suggest, these two major tuning mechanisms are by no means mutually exclusive. In the frog amphibian papilla, for example, there is indirect evidence for traveling waves, a characteristic of mechanical tuning (Hillery and Narins, 1984). In the guinea pig cochlea, the overall frequency tuning, measured either by the receptor potential of hair cells or by

the spiking of afferent fibers, matches the tuning of the basilar membrane motion, which suggests no significant contribution from electrical tuning (Sellick et al., 1982). However, the experimental data in this study and many similar studies were collected at a location of 16–19 kHz, at which electrical tuning is unlikely to be feasible. In addition to mechanical and electrical tuning, exocytosis from hair cells is believed to be frequency-selective (Patel et al., 2012; Rutherford and Roberts, 2006), making ribbon synapses a third source for frequency tuning in the inner ear. To untangle contributions of different tuning mechanisms, it is necessary to examine how each tuning mechanism aligns or misaligns with one another in frequency domain. The newly developed Zap current method, which can be applied unbiasedly to all hair cells including non-oscillating mammalian hair cells, provides a promising new tool to quantitatively examine electrical tuning and permits in-depth inquiries in the governing principles of frequency tuning in all vertebrate species.

CHAPTER III

DISCUSSION

This thesis work aims to advance the understanding of frequency selectivity within the first step of the auditory signal transduction: hair cell receptor potential, and opens further lines of inquiry into hair cell-afferent fiber synaptic signaling at the level of Ca^{2+} influx, EPSC generation, and eventual generation of the afferent fiber action potentials. All of these stages of auditory signal transduction occurs at the auditory periphery and therefore can play a major role in shaping the frequency selectivity of the auditory response before the signal reaches more central stages of the auditory pathway.

Our data revealed three types of hair cells in the amphibian papilla. The first type of hair cells, located in rostral (low-frequency) part of the amphibian papilla, demonstrated typical dampened oscillations in response to the step current injections. These oscillations decayed to zero as shown previously (Pitchford and Ashmore, 1987; Smotherman and Narins, 1999). In response to Zap current injection, these cells displayed pronounced tuning. Second type of hair cells, located also in the rostral (low-frequency) part, demonstrated oscillation in response to step current injection that persisted as long as the depolarizing current step remained on.

Novel cell types discovered in amphibian papilla display differences in frequency resonance

These cells also possessed the sharpest frequency tuning (lowest W10dB). The third type of hair cells was located in the caudal (high-frequency) end of the amphibian papilla. We demonstrated that while no oscillations were observed in these hair cells in response to step current injection, Zap current stimulation revealed higher voltage response over a particular range of frequencies. The tuning curve of these cells was much less sharp compared the responses of the first and second group of hair cells. Next, I plotted the W10dB against the resonant frequencies for all amphibian papilla hair cells. Our finding was that W10dB values match closely the range of previously reported W10dB values of the auditory fibers (Ronken, 1991; Frolov and Li, 2017). Given that every afferent fiber contacts several hair cells (Lewis et al., 1982; Smotherman and Narins, 2000), my data leads me to expect a somewhat broader tuning curve from afferent fiber than from any of the individual hair cells innervated by that particular fiber, even if that those hair cells are located close together along the tonotopic axis. This is reinforced by observation that the more sharply tuned low-frequency afferents seem to form connections to more hair cells (>6), compared to less sharply tuned high-frequency afferents (1-4 hair cells) (Lewis et al., 1982; Ronken, 1991). Below, I attach the more detailed description of these results published in the journal Synapse (Frolov and Li, 2017).

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Relationship between hair cell frequency selectivity and frequency tuning of basolateral Ca²⁺ influx

Taken together, my data and the preceding literature suggest the existence of an additional response-shaping mechanism to account for the observation above. Such mechanisms may constitute a frequency tuning of the hair cell-afferent fiber synapse, downstream from the receptor potential (Schnee, 2005; Patel et al. 2012). Another possibility would be a separate frequency tuning of the afferent fiber itself (Verschooten, 2012). Ultimately, both of these stages and their interaction may influence the frequency tuning of the auditory periphery outputs, which take form of action potentials (APs) in afferent fibers (Megela, 1984; Kumsa, 2012; Schnee, 2013).

Therefore, as a possible future direction to my Master's thesis research, it would have been useful to look at the Ca^{2+} entry and respective excitatory postsynaptic current (EPSC) that reflects the glutamate neurotransmission. In order to stimulate Ca^{2+} current across a hair cell membrane, a Zap voltage stimulus could be employed similar to Zap current stimulus. This stimulus would consist of a sine wave voltage command, with its frequency increasing over the time of stimulation at a constant rate. The oscillatory voltage changes will elicit Ca^{2+} current that can be recorded and its amplitude and frequency analyzed to determine if calcium currents in hair cells is tuned to a particular frequency range of voltage changes. Zap voltage-stimulated calcium current is expected to include Ca^{2+} influx in the synaptic region and subsequent neurotransmitter releases in a frequency-dependent manner (Rutherford and Roberts 2006; Patel et al., 2012). Simultaneously, paired whole-cell patch-clamp technique can be used to obtain simultaneous recordings of EPSCs in the afferent fiber (Li et al., 2009; Graydon et al., 2011; Schnee et al. 2013).

One possibility is that Ca^{2+} current and vesicle exocytosis, as reflected by afferent fiber EPSC generation, directly follows the frequencies of the hair cell membrane voltage change. Another possibility is the existence of a particular frequency range of a hair cell membrane's voltage changes that will elicit higher Ca^{2+} current amplitude. This will point at the existence of band-pass filtering in the Ca^{2+} influx step of a hair cell response to the stimulus.

This would allow to establish a relationship between the frequency of the membrane voltage oscillations driven through the hair cell, calcium current across the hair cell membrane, and resulting neurotransmitter release as reflected in the afferent fiber EPSC generation. Additionally, it would be of interest to study the frequency tuning of neurotransmitter vesicle exocytosis and endocytosis in hair cells. Using paired patch-clamp recording, it would be possible to simultaneously record capacitance changes of the hair cell membrane and EPSCs in the afferent fiber. Capacitance recordings will be used to measure net membrane area change during stimulation. Membrane area change is a measure combining neurotransmitter vesicle release (exocytosis) and recycling (endocytosis) processes occurring at the presynaptic membrane. While previous studies used different frequency sine waves to stimulate hair cells, we propose to use different frequency stimulation trains of short, square voltage pulses. The rise-time of the sine waveform can possibly affect the resulting Ca^{2+} current, thus confounding the dependence between Ca^{2+} influx and vesicle release. Trains of square pulses will engage the maximum number of calcium channels with each stimulation pulse. At the afferent

fiber, EPSC amplitude and frequency over the course of the hair cell stimulation will be measured. Average amplitudes and frequencies of the EPSCs will be analyzed at each frequency of the square pulse stimulation for potential frequency tuning of neurotransmitter release. Net sum of EPSC amplitudes at each frequency will also be used as a measure of exocytosis (Li et al., 2009), as opposed to membrane capacitance measurement that reflects both exocytosis and endocytosis (Rutherford and Roberts, 2006; Patel et al., 2013). Comparing these two measures would allow to calculate the rate of endocytosis and assess the balance of exocytosis and endocytosis at each frequency. We expect the existence of best frequency when two processes are balanced in a way that favors the synaptic transmission (Schnee et al., 2005).

Relationship between hair cell frequency selectivity and frequency tuning of postsynaptic events and the afferent fiber.

Complex EPSC and the resulting action potential fidelity appear to be solely dependent on the rate of neurotransmitter release at the multiple presynaptic sites contacted by a single afferent fiber. The relationship between frequency tuning of the Ca^{2+} influx, vesicle release and EPSC generation would be important to pursue in the future studies of how frequency tuning at these sequential steps translates into the information transmission- and physiologically-relevant changes of the AP generation in the afferent fiber.

It is known that afferent fibers possess mechanisms that enhance temporal precision (Oak and Yi., 2014) and filter redundant information (Avissar et al., 2007). Based on mathematical models of mammalian IHC synapse, spontaneous neurotransmitter release at the hair cell-afferent fiber synapse is thought to possess a best frequency at which information transmission by the auditory nerve fiber is favored (Kumsa, 2012).

Afferent fibers are also known to be tonotopically organized and displaying frequency tuning with distinctive v-shaped peaks (Frishkopf and Goldstein, 1963; Feng et al., 1975; Ronken, 1991). The sharpness of the afferent fiber tuning curves (defined by W10dB value) is in the same range as the sharpness of tuning curves we have found for the hair cells contacted by these fibers (Ronken, 1991, Frolov and Li, 2017). Frequency tuning of membrane voltage oscillations, Ca^{2+} current and vesicle release can act as series of sequential filters, tuning ESPC generation to the incoming stimulus frequency (Patel et al., 2012).

Still, the question stands how the afferent fiber in lower vertebrate auditory system is able to maintain the same sharpness of tuning as any individual presynaptic hair cell, while making synaptic contacts with multiple individually tuned hair cells (Lewis et al., 1982; Ronken 1991; Smotherman and Narins, 2000, Frolov and Li, 2017). In the afferent fiber, firing efficiency is thought to depend on EPSC frequency in a nonlinear manner. The relationship between this nonlinearity and the refractory period (time when

new action potential cannot be fired, even in presence of threshold EPSC) defines the afferent fiber response to trains of stimuli in the auditory fiber model (Goldwyn, 2012).

A particular frequency of membrane voltage oscillations may exist that will elicit EPSCs of higher amplitude of frequency compared to all other frequencies. This can be predicted from the observations of tonotopic variation and frequency selectivity of hair cell exocytosis (Schnee 2005; Rutherford and Roberts, 2006; Patel et al., 2013) and the proposed importance of EPSC temporal summation for afferent fiber action potential generation (Li et al., 2009; Schnee, 2013). Increased amplitude or frequency of the EPSCs will reflect higher vesicle release. This frequency may or may not be the same frequency that elicits additional Ca^{2+} influx, and the comparison of these two frequencies will provide additional insight into the relationship between the Ca^{2+} influx and vesicle release.

Ultimately, the physiological output of the auditory periphery are action potentials generated in the afferent fiber. From the mathematical modeling, we can expect that AP generation dependence on the EPSC frequency will have a band-pass filtering quality (Goldwyn, 2012). Straightforwardly, we can then predict the number of action potentials generated is expected to rise supralinearly, reach a peak, and then decrease when the interval between individual AP's will become comparable to the fiber's refractory periods.

Whether the decrease will be gradual or abrupt is not clear. If the band-pass tuning curve is found, AP generation best frequency and tuning peak W10dB value ranges can be directly compared to those obtained *in vivo* using auditory stimulus, as well as to the range of best frequency and tuning peak widths of the amphibian papilla hair cells. This will provide a unique possibility to align the frequency filtering properties (frequency tuning curves) of multiple sequential signal transduction steps, from the hair cells membrane potential change to the AP generation in the fibers.

Alternatively, it may be found that fiber AP generation frequency is linearly or supralinearly following the EPSC frequency, without any filtering within the range of frequencies characteristic for the amphibian papilla end organ. This would indicate an alternative finding that the frequency selectivity of the auditory afferent fiber is fully shaped by the preceding frequency filtering steps.

Understanding the mechanisms of amphibian auditory frequency selectivity will allow further scientific inquiries in the growing area of research focused on the cellular frequency resonance and selectivity of the neuronal frequency input and output. We also expect that the current and following studies will promote the exploration of the novel engineering approaches in the cochlear implant construction, by providing the experimental basis for mathematical description and modeling of sequential frequency selectivity and frequency tuning sharpening mechanisms that exist in the amphibian hair cell-afferent fiber synapse.

Additionally, it will further advance the use of amphibian auditory system as a model for the exploration of the signal transduction in the sensory systems, using auditory system as our main model. While hair cell electrical resonance and frequency tuning appears to be a major phenomenon in the lower vertebrate hearing systems, to the best of our knowledge no current experimental data exists that directly explores the relationship between hair cell electrical resonance and the signal transduction in the first synapse of the auditory pathway. It is my hope that the current Thesis will advance this understanding.

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