Is the Utricular Striola Specialized to Encode High Frequency Stimuli?

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This thesis entitled

Is the Utricular Striola Specialized to Encode High Frequency Stimuli?

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ABSTRACT

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The vestibular system comprises morphologically and physiologically distinct hair cells and afferent neurons. These hair cells and afferent neurons respond heterogeneously to stimuli as a function of their position on the vestibular sensory epithelium. To date, little is known about the cellular sources of this signal heterogeneity within the hair cells and afferent neurons of the vestibular system. This thesis explores regional differences in pre- and post-synaptic proteins that may play a role in producing signal heterogeneity within the utricle of the turtle, *Trachemys scripta.*

Immunohistochemistry, confocal microscopy, and three-dimensional reconstructions of the vestibular system were used to quantify sizes, frequencies, and colocalization between synaptic ribbons and the GluR4 subunit of the AMPA receptor.

Notable results of my research include increased sizes of synaptic ribbons, increased frequencies of synaptic ribbons and GluR4 subunits, and increased colocalization between synaptic ribbons and GluR4 subunits within striolar Zone 2 of the turtle utricle. These results support previous work characterizing striolar regions of the vestibular system as being preferentially suited to encode high frequency stimuli.

Approved: __________________________________________________________

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INTRODUCTION

The Vestibular System

The vestibular system plays a pivotal role in providing the sense of balance in animals. Often overlooked in importance, the vestibular system’s normal functioning is critical for everyday life. Malfunction of this system can lead to unstable balance and posture and blurred vision. Approximately 5% of all visits to the family physician include complaints of dizziness (Post and Dickerson, 2010). With such a large number of clinical visits being related to dizziness, it is clear from a medical standpoint that a more fundamental understanding of the vestibular system is needed.

Primitive vestibular systems in vertebrates comprise at least one semicircular canal and one otoconial organ, whereas in jawed vertebrates there are typically three semicircular canals and at least two otoconial organs, the utricle and saccule. The semicircular canal(s) sense accelerations of the head during rotational movements, while the otoconial organ(s) measure linear head accelerations: linear translations of the head and linear accelerations due to gravity during head tilt. The way in which one of these otoconial organs, the utricle, sends its information to the central nervous system is the focus of this thesis.

Hair Cell-to-Afferent Junction

The sensory surface of the utricle, the macula, is a neuroepithelium comprising many sensory hair cells. These hair cells function as mechano-sensitive receptors. The
hair cells have hair bundles, or grouped stereocilia with a single kinocilium, that extend from the tops of the hair cell bodies (Figure 1). Bending of the hair bundles is induced indirectly by the movement of crystals that rest above the bundles. The crystals, known as otoconia, rest on top of a gelatinous membrane. This membrane lies above the hair cells and is coupled to the hair bundles. The gel-bundle coupling is due to the tall kinocilium, and for some hair bundles, the longest stereocilia of the hair bundles being directly embedded within the gelatinous membrane (Xue and Peterson, 2006). When the head experiences linear accelerations or tilts, the otoconia and gelatinous membrane shift in response. Because the gelatinous membrane and hair bundles are coupled, the effects of gravity or linear accelerations result in the movement or bending of the hair bundles.

With the bending of the hair bundles there is a concurrent shift in the receptor potential of the hair cell. This change in the receptor potential is caused by the opening of mechano-electrical transduction (MET) channels near the tips of the hair bundles. The endolymph fluid bathing the apical surface of the hair cells is unusually high in potassium ion concentration. This high extracellular potassium concentration results in an influx of potassium when the MET channels open. The resulting depolarization of the hair cell causes an opening of voltage gated Ca^{2+} channels in the basolateral portion of the hair cell. The opening of the Ca^{2+} channels results in an influx of Ca^{2+} into the hair cell, which induces the release of neurotransmitters into the synaptic cleft. The synaptic cleft is the space between the membrane of the hair cell (presynaptic side) and the membrane of the afferent neuron that innervates the hair cell (postsynaptic side). The neurotransmitter glutamate is contained within vesicles in the hair cell bodies, and these vesicles cluster
around a presynaptic structure called a synaptic ribbon (Figure 1). The synaptic ribbon is an organelle that tethers vesicles at the active zone of the synapse in visual, auditory, and vestibular systems (Logiudice and Matthews, 2009). Vesicles at the active zone of the synapse can quickly be released, which allows for a sustained response to sensory stimuli, especially high frequency stimuli (Logiudice and Matthews, 2009). On the postsynaptic side of the synapse the signal is received by the afferent neuron and is carried by the afferent to the central nervous system. Thus, the hair cell-to-afferent signal is key in the transmission of information about head movements to the central nervous system.

**Figure 1.** Hair cell-to-afferent junction.

Hair bundles experience a force caused by head movements. This force opens channels that cause the hair cell receptor potential to change. On the presynaptic side, neurotransmitters are held within the vesicles tethered to the synaptic ribbon. These vesicles are represented by the grey circles around the dark black circle, the ribbon, at the synapse in the image. The receptor potential change causes the vesicles to release neurotransmitters onto the postsynaptic density of the afferent neuron. The signal produced in the afferent neuron is then transmitted to the central nervous system (CNS). (Peterson, unpublished.)
On the presynaptic side of the synapse of amniotes are two types of hair cells.

Type I hair cells are enclosed by a cup-like afferent nerve ending called a calyx (Figure 1). Type II hair cells, on the other hand, are contacted by afferent nerve endings called boutons. These two hair cell types are contacted by three types of afferents (Figure 2). Pure calyx afferents innervate only type I hair cells, pure bouton afferents innervate only Type II hair cells, and dimorphic afferent units innervate both type I and type II hair cells from the same parent axon. Reptiles, birds, and mammals have calyx, bouton, and dimorphic afferents. Fish and amphibians, in contrast, have only type II hair cells and thus only bouton afferents. (Lysakowski, 1996)

Figure 2. Afferent morphology of the vestibular system

Calyx, bouton, and dimorphic afferent neurons. Signals are received from each hair cell and are sent to the central nervous system (CNS). (Peterson, unpublished.)
The organization of hair cells along the sensory surface of the utricle (macula) divides it into two major regions: the striola and the extrastriola (Figure 3). Relative to the extrastriola, the striola is characterized by larger hair cells, specialized hair bundles (Xue and Peterson, 2006), a higher concentration of type I hair cells, and the presence of pure calyx afferents (Lysakowski and Goldberg, 2004). The distinctive organization of the hair cells and afferents in the striola and the extrastriola raises the question of whether each region sends distinctive signals to the central nervous system. One line of evidence for this is that afferents innervating the striola and extrastriola differ in their physiological responses to head movements.

**Figure 3.** The turtle utricle

The striola is the crescent-shaped black band; the extrastriola refers to the two adjacent regions, lateral and medial extrastriolae. Scale: 100 µm (Peterson, unpublished.)
Afferent Heterogeneity

Common physiological features measured in an afferent neuron’s signal are the resting discharge rate, discharge regularity, response dynamics, including gain and phase, and whether the afferent exhibits phasic or tonic responses to stimuli. The *resting discharge rate* is defined as the number of spikes, or action potentials, per second in the absence of any stimulation. Essentially, the resting discharge means that all fibers have a tonic output. What purpose does a resting discharge of the afferents serve? It has been postulated by Lowenstein and Sand (1936) that a resting discharge allows for two different features within the afferent. A resting discharge can produce bidirectional responses to vestibular stimuli (i.e., the system can respond by either increasing or decreasing its response), and it may provide the input necessary to produce normal postural tone (Lowenstein and Sand, 1936). Interestingly, the resting discharge rate of afferents differs according to the species, the afferent’s discharge regularity, and the organ analyzed. For example, for regularly discharging fibers, in turtles the resting discharge rate is approximately 10-30 spikes/second (Brichta and Goldberg, 2000); it is between 50-120 spikes/second in fish (Boyle and Highstein, 1990) and between 60 to 120 spikes/second in mammals (Lysakowski *et al.*, 1995). These rates are all considerably lower in irregularly discharging fibers.

*Discharge regularity* is a measure of how regularly or irregularly spaced action potentials occur within an afferent signal. Regularly discharging afferents have a standard interval in the spacing of their action potentials, whereas irregular afferents do not (Figure 4). Irregularly discharging afferents have been shown to have thick to medium
parent axons, have phasic-tonic response properties, and are highly sensitive to rotational or linear forces. Conversely, regularly discharging afferents typically have medium to small parent axons, have tonic response properties, and show a low sensitivity to rotational and linear forces (Lysakowski and Goldberg, 2004). Intracellular injections of physiologically characterized afferents indicate that calyx afferents are irregularly discharging, whereas bouton afferents are typically regularly discharging. Dimorphic fibers exhibit a range of regularities depending on epithelial locus. Because calyx afferents are restricted to the striola (Goldberg, 1991; Si et al., 2003), their distinct physiological properties suggest that the striola plays a distinctive role in utricular signaling.

**Figure 4.** Regularly vs. irregularly discharging afferent neurons

The top image shows a regularly discharging afferent, in which the interval between the spikes is constant. The bottom image shows an irregularly discharging afferent, in which the interval between the spikes is variable. (Lysakowski and Goldberg, 2004)
Additional functional distinctions between afferents in the striola versus the extrastriola are their *response dynamics*. The response dynamics of vestibular afferents are typically described in terms of phase and gain. The phase of an afferent refers to the timing of the afferent’s peak output with respect to the peak input of a sinusoidal stimulus. In Figure 5A, the stimulus mimics sinusoidal head movement, and stimulus amplitude is an indirect measure of head velocity. In this example, a posterior semicircular canal afferent that discharges regularly shows a characteristic phase shift that slightly leads the maximal stimulus amplitude with low frequency stimuli (Figure 5A). These same units show phase lags when compared to stimulus amplitude at high stimulus frequencies. Irregularly discharging units show slightly different phase responses, in which both low and high frequencies cause phase leads between 20 to 40 degrees. Figure 5B shows phase and gain responses of three types of turtle posterior canal afferents. Bouton planum (BP) units are bouton afferents near the wall of the posterior canal. Calyx-dimorphic (CD) units are both calyx and dimorphic units grouped together. BP (blue) and CD (green) units are typical regularly and irregularly discharging units, respectively. In turtles, unlike in mammals, there is also a specialized type of unit, bouton torus (BT; red) units, which is an irregularly discharging bouton unit. Figure 5B, the lower graph shows phase responses of the three afferent types vs. stimulus frequency. All three afferent types show large phase leads to low frequency stimuli. With increasing frequency stimuli, the phase of BT and BP units converge at a phase near 0 (no lead or lag), while the CD units remain slightly phase advanced. Gain, on the other hand, is defined as the amount of output relative to the input, or in this case the number of spikes
per second in the afferents per micron of canal indentation (Upper graph in Figure 5B).

Irregularly discharging afferents (red, green) show a fivefold frequency dependent
increase in gain, whereas regularly discharging units (blue) show a nearly constant gain
of 1 above approximately 0.2 Hz. Overall, irregularly discharging units have large phase
leads and high gain, and regularly discharging units have little phase shifting and low
gain.
Figure 5. Physiological responses of posterior canal afferents (Rowe, unpublished)
A. Sinusoidal stimulus (top), compared to the number of afferent spikes (bottom) fired in response to this stimulus. The black bar indicates the peak afferent spiking compared to the timing of the peak stimulus input. This afferent neuron shows a slight phase lead.

B. Bode plot comparing changes in gain and phase of 3 afferent types versus frequency. BT (red) and CD (green) units are irregularly discharging units, whereas BP (blue) units are regularly discharging units. The upper graph shows gain versus frequency. BP units show a gain of 1 after approximately 0.2 Hz, while CD units and BT units show increasing gain across all frequencies. The lower graph shows phase versus frequency. Greater phase shifts are indicated by responses further from 0 on the Y axis. BT and CD units show larger phase shifts through high and low frequencies, while BP units show much less phase shifting at higher frequencies. Error bars in both graphs are limited to a single direction in order to keep the graphs from becoming cluttered.

Finally, I will look at phasic versus tonic responses of vestibular afferents. Phasic afferents, not to be confused with phase shifts of afferents, are afferents that show adaptation to a stimulus. These units are rare in the vestibular system. Tonic afferents, conversely, show a more constant output to a stimulus (Fernandez and Goldberg, 1976). A combination of phasic and tonic properties, or afferents that adapt quickly, but not completely, are seen in afferents of mammalian and non-mammalian vertebrates (Baird and Lewis, 1986). Curiously, phasic-tonic units tend to be irregularly discharging afferents located in the striola, whereas tonic afferents tend to be regularly discharging units located in the extrastriola. Figure 6 summarizes different aspects of afferent heterogeneity.
Irregularly versus regularly discharging afferents and how they differ with respect to epithelial locus, parent fiber size, phasic and tonic properties, and their responses to angular and linear forces. Other differences are given, but are not discussed within this proposal. (Lysakowski and Goldberg, 2004)

Spatial Organization of Afferents

It is clear that many physiological properties are factors in the heterogeneous signals arriving at the CNS from vestibular afferents. Interestingly, while there are many distinct afferent signals from the vestibular system, there is also spatial organization of the afferents. The afferents are spatially organized morphologically and physiologically.
For example, in Figure 5 three distinct types of afferents were recorded. These afferents not only show different physiological properties, but also differ in their location along the posterior semicircular canal. The bouton-planum units (BP), which are characterized by low gains and little phase advancement, are found predominately at the margins of the sensory epithelium of the semicircular canal. On the other hand, the bouton-torus units (BT) are characterized by high gains and moderate phase leads at low frequencies and are found predominately within the central region of the posterior semicircular canal. As with the semicircular canals, the otoconial organs show similar spatial organization of their afferent populations. In mammals, the striolar region comprises calyx and dimorphic afferents and their corresponding type I and type II hair cells. The innervating afferents of striolar hair cells tend to be irregularly discharging units with high gains and phase advances (Eatock and Lysakowski, 2006). The extrastriola is characterized by dimorphic and bouton afferents and their corresponding hair cells. The afferents within the extrastriola tend to be more regularly discharging with low gains and little phase advancement (Eatock and Lysakowski, 2006). These data indicate that while afferents do send heterogeneous signals to the CNS, afferents that send similar signals to the CNS tend to be spatially organized into striolar and extrastriolar subdivisions of the sensory surface. One important question regarding the vestibular periphery is the role of the striola versus the role of the extrastriola. As striolar afferents tend to be irregularly discharging with high gains and phase advances they appear to be suited to respond to high frequency stimuli (i.e. rapid movements).
The Striola and High Frequency Stimuli

As characterized above, afferents in the striola tend to have high gains and tend to be phase advanced. Additionally, afferents in the striola have been characterized as phasic-tonic, or fast adapting. A phasic-tonic, or fast adapting afferent, would be required to respond to a high frequency of stimulation. In Figure 7 the dashed black line represents a slow head movement (slow stimulus). The solid red lines represent multiple afferents with different abilities to adapt to a stimulus. The peak response of afferent 1 occurs at approximately 300 units of time before the peak of the slow stimulus. On the other hand, the peak response of afferent 4 occurs at approximately 50 units of time before the peak of the slow stimulus. Compared to afferent 4, afferent 1 adapts sooner to the same slow stimulus and is the most phasic (fastest adapting) afferent. Conversely, compared to afferent 1, afferent 4 adapts later to the same slow stimulus and is the least phasic (slowest adapting) afferent. With reference to a slow stimulus, both afferents 1 and 4 are considered “phase advanced”. If we now consider a fast head movement (fast stimulus - dotted purple line) applied to the same afferents, we can see how a fast adapting afferent (number 1) could faithfully encode each fast stimulus while a slow adapting afferent (number 4) could not faithfully encode each fast stimulus. Afferent number 1 could reach its peak response and adapt prior to the peak of a fast stimulus. This fast adaptation would allow afferent number 1 to sequentially encode the second peak of a fast stimulus. With reference to the fast stimulus stimulus, afferent 1 would be considered “phase advanced”. Afferent number 4 would have its peak response and adapt after the peak of the fast stimulus. This slow adaptation would not allow afferent number
4 to sequentially encode the second peak of the fast stimulus. With reference to a fast stimulus, afferent 4 would be considered “phase lagged”. As striolar afferents have been characterized as phasic-tonic and phase advanced (Lysakowski and Goldberg, 2004), the idea follows that the striola may preferentially encode high frequency stimuli, and there is a need to describe potential cellular sources of these distinct striolar physiological characteristics. Both pre-synaptic and post-synaptic mechanisms could result in the unique physiological characteristics of striolar afferents.
Figure 7. Stimulus and adaptation responses in afferents.

The Y-axis corresponds to the response of the afferents. The dashed black line corresponds to a stimulus slow stimulus over time. The dashed purple line represents a fast stimulus over time. The red lines correspond to afferents 1-4’s responses over time.

Adaptation is generally thought of as a decrease in gain that is proportional to stimulus amplitude and occurs with a time constant. So for a stimulus that has a sigmoidal profile, i.e., rises from zero to some value over some period of time, if the adaptation time constant is long relative to the stimulus time constant, then the response will reach a peak at the same time as the stimulus does and then decline even if the stimulus doesn’t, due to the decrease in gain. However, if the adaptation time constant is shorter than the stimulus time constant, then the response peak will occur before the stimulus peak and also be smaller due to the fact that the gain has declined even before the stimulus reached its peak. As the adaptation time constant becomes increasingly shorter, the response peaks occur progressively earlier and are progressively smaller, as shown in the figure. If the stimulus was a sin wave, the earlier responses would be manifested as a phase advance.
Cellular Sources of Afferent Heterogeneity

It is possible that both pre-synaptic and post-synaptic mechanisms could modulate the afferent signal arriving at the central nervous system. Pre-synaptically, there exists evidence of hair cell tuning. (Xue and Peterson, 2006; Nam et. al., 2005; Martinez-Dunst et. al., 1997; Art, Fettiplace and Wu, 1993; Eatock, 2000). This tuning results in hair cells being most sensitive to certain types of stimuli or head movements. For example, Xue and Peterson (2006) showed variations in hair bundle structure across the turtle utricular macula. Striolar bundles tended to have shorter kinocilia and smaller KS ratios (Kinocilia to tallest Stereocilia ratio), whereas extrastriolar bundles tended to have taller kinocilia and larger KS ratios. Nam et al. (2005) modeled differences in turtle utricular hair bundle responses to fluid forces using finite element analysis. Components of the hair bundle model included kinocilia height, stereocilia height, and cellular linkages between stereocilia and the kinocilium. Interestingly, bundles that were modeled based on turtle striolar hair bundle structure showed distinct differences in response sensitivity (gain) compared with bundles modeled based on turtle extrastriolar hair bundle structure (Nam et. al., 2005). This lends support to the idea that the striola may encode high frequency stimuli (the hair bundle must be sensitive enough to respond to very rapid stimulation). The differences that exist across turtle utricular hair bundles likely play a role in producing the dynamic responses from hair cells during stimulation. These dynamic responses may result in heterogeneous signals within the corresponding innervating afferent neuron. The results from Xue and Peterson (2006) and Nam et al. (2005) provide
support with regard to pre-synaptic hair cell tuning as a source of afferent heterogeneity in turtle utricle.

In addition to the above results, the synaptic ribbon has been hypothesized to play a role in afferent signal heterogeneity (Liberman et al., 2011; Martinez-Dunst et al., 1997; Schnee et al., 2005). Specifically, differences in synaptic ribbon size and shape may play potential roles in the signals produced within the afferent neuron innervating hair cells.

Post-synaptically, mechanisms have been postulated that affect the afferent signal routed to the central nervous system. Liberman et al. (2011) show how post-synaptic densities in mouse cochlea differ in their glutamate receptor subunit distribution across and within cochlear hair cells. Liberman et al. (2011) hypothesize that post-synaptic densities with large amounts of GluR2/3 subunits may favor rapid synaptic transmission. Thus, differences in post-synaptic receptor composition may affect signals sent to the CNS.

As described in the next two sections, I will explore both pre-synaptic and post-synaptic mechanisms that could result in afferent heterogeneity. Specifically I will explore the pre-synaptic ribbons in hair cells and postsynaptic glutamate receptors in afferents.

The Synaptic Ribbon

The synaptic ribbon is a proteinaceous structure that lies within the active zone of a synapse. Functionally, the synaptic ribbon tethers large numbers of vesicles at the
active zone of a synapse. These vesicles in turn release their contents into the synaptic cleft. The exact mechanism through which the vesicles attached to the synaptic ribbon release their contents into the synaptic cleft is unknown (Matthews and Fuchs, 2010).

In general, synaptic ribbons are found in sensory systems that are tonically stimulated. These sensory systems include the visual system, auditory system, and the vestibular system. The tonic input of stimuli to these systems must be matched by a tonic response by the innervating afferent neuron. One important step in matching the tonic stimulus with a tonic response is the timing and release of the proper amount of vesicles at the synapse. The faithful encoding of a tonic signal from the receptor to the afferent neuron is assisted by the synaptic ribbon’s ability to release the contents of multiple vesicles into the synapse cleft (Matthews and Fuchs, 2010). The synaptic ribbon allows a hair cell to release vesicles quickly enough to represent both tonic and high frequency stimulations with high temporal resolution. Interestingly, the size and shape of synaptic ribbons differs across and within the different systems mentioned above.

To date, research on synaptic ribbons from visual and auditory systems has provided many insights into the role of the synaptic ribbon (Matthews and Fuchs, 2010). Within the auditory system, synaptic ribbons differ in their size based on their relative position along the cochlea. Anatomically, the cochlea is arranged in a tonotopic fashion, with hair cells near the base responding optimally to high frequency sounds and hair cells near the apex responding optimally to low frequency sounds. As the optimal frequencies of the hair cells increase, the size of the synaptic ribbons within these hair cells also increases. Within the chick and turtle basilar papilla (auditory organ; corresponds to
cochlea in mammals), the size of the synaptic ribbon was shown to increase in hair cells as the hair cell’s optimal frequency increased. Thus, hair cells optimally responding to the highest frequencies generally had the largest sized synaptic ribbons at their synapses (Martinez-Dunst et al., 1997; Schnee et al., 2005).

The preceding research on synaptic ribbons suggests synaptic ribbon size as a possible source of afferent heterogeneity. How might this relate to the striola and the encoding of a high frequency stimulus? The encoding of a high frequency signal would require a synaptic ribbon that could maintain the temporal fidelity of the stimulus. In accordance with the Martinez-Dunst et al. (1997) and Schnee et al. (2005) results, as the size of the synaptic ribbon increases, it is possible that the synaptic ribbon could hold more vesicles at the active zone of the synapse. This readily releasable pool of vesicles could allow for a greater vesicle release rate, and thus be crucial for responding at synapses that encode high frequency stimuli. Simply put, a greater number of vesicles at the synapse could support the elevated release rate needed in response to a high frequency stimulus. This increased rate of vesicle release in some hair cell populations could enable some afferents to encode high frequency stimuli.

Hypothesis 1

Differences in the sizes of synaptic ribbons across the cochlea and basilar papillae of mice and turtles raises an interesting question of whether these size differences are also present in the vestibular end organs. Martinez-Dunst et al. (1997) and Schnee et al. (2005) both show the size of synaptic ribbons increasing as the optimal response frequencies of
auditory hair cells increases. As with auditory afferents and their specific regions of innervations along the cochlea, utricular afferent signals show heterogeneity according to their position in either the striola or extrastriola. One possible source of this afferent heterogeneity could be differences in ribbon size between the utricular striola and extrastriola. **I, therefore, hypothesize that synaptic ribbon size will differ between the utricular striola and the utricular extrastriola. Specifically, if the striola is preferentially suited to respond to high frequency stimuli, I hypothesize that synaptic ribbon size will be greater in the utricular striola than in the utricular extrastriola.**

**Glutamate Receptors**

One potential source of afferent heterogeneity is the receptor proteins on the postsynaptic (afferent) side of the synapse. These postsynaptic receptors are the proteins that bind the neurotransmitter released from the hair cell. The predominant receptor at the hair cell-to-afferent junction is the glutamate receptor (Niedzielski and Wenthold, 1995). Glutamate receptors are classified into two groups, the ionotropic receptors, which function as ion channels, and the metabotropic receptors, which induce activity in second messenger systems (Hassel and Dingledine, 2006). Both of these groups have multiple subgroups. Only the ionotropic group will be explored in the proposed research.

Ionotropic receptors are functionally defined by their ion channels properties. When a neurotransmitter binds to an ionotropic channel, it opens a pore through which ions can cross from extracellular to intracellular spaces, or *vice versa*. Glutamate
receptors allow for ionic flow when the neurotransmitter glutamate binds to the receptors.

Three different subtypes of glutamate receptors exist: AMPA; NMDA; and kainite receptors (Figure 8) (Davies and Watkins, 1981; Davies and Watkins, 1985; Krogsgaard-Larsen et al., 1980). These receptors differ in their structure, their distribution within the nervous system, and their affinity for glutamate (Kennedy, 2000; Hassel and Dingledine, 2006). Within the utricle the AMPA and NMDA receptors are predominant. I will examine the AMPA receptors as a potential source of afferent heterogeneity.

**Figure 8.** Ionotropic glutamate receptors

Ionotropic glutamate receptors are organized into three groups: NMDA, AMPA, and kainate receptors. These receptor groups are further split into different subunits which bind together to form the fully functional receptors. AMPA receptors are composed of GluR1 through GluR4 receptor subunits. (Tocris Biosciences, 2011). All four subunits of AMPA receptors are present within the red eared slider turtle (Fowler et al., 1999).

**Glutamate Receptor Subunits**

AMPA glutamate receptors, AMPAR’s, are composed of four functionally distinct protein subunits. These subunits are referred to as GluR1 through GluR4 (Hollmann et al., 1989; Boulter et al., 1990; Sakimura et al., 1990; Keinanen et al., 1990). The quaternary three dimensional structure of glutamate receptors pairs two
homomeric dimers together to form a fully functional, heterotetrameric receptor (Greger et al., 2007) (Figure 9). Homomeric refers to the similarity of the two protein subunits that bind together to form a single dimer. Two dimers then pair together to form a receptor with four total protein subunits, called a tetramer. In vivo, the receptor is preferentially composed of dimers with different receptor subunits (Mansour et al., 2001). For example, a fully functional glutamate receptor could consist of a dimer of GluR3 subunits, and then another dimer of GluR2 subunits, but rarely two dimers of GluR1 subunits. Each glutamate receptor subunit confers its different properties on the functional receptor (Dingledine et al., 1999). Two particularly interesting differences between subunits are their desensitization rate and their rate of recovery from desensitization (Grosskreutz et al., 2003).
A tetrameric glutamate receptor, with four receptor subunits. The receptor is composed of two homomeric dimers and typically forms a heteromeric tetramer. The yellow highlighting indicates the plasma membrane. Above the yellow highlighting is the extracellular space, while below the yellow highlighting is the intracellular space. Thus, NTD corresponds to the N-terminal domain of the AMPA receptor, while C-terminus corresponds to the C-terminal domain of the AMPA receptor. M2 corresponds to the AMPA receptor reentrant loop. The S1/S2 regions define the ligand binding domains of the protein. Sodium, potassium, and calcium ions are permeable through the AMPA receptor, though the presence of the GluR2 subunit in an AMPA receptor eliminates calcium permeability. (Isaac et al., 2007).

**Desensitization**

Desensitization is generally defined as a state of receptor inactivation when a ligand is still bound to the active site of the receptor (Sun et al., 2002). Essentially, desensitization determines the decay time of the synaptic signal carried by the afferent, while the recovery from desensitization determines the upper bound of frequency transmission that can occur at the hair cell-to-afferent junction (Grosskreutz et al., 2003).
Interestingly, glutamate receptor subunits differ in their desensitization and recovery from desensitization rates. The GluR4 subunit’s desensitization and recovery from desensitization rates are below 10 milliseconds (ms). While other glutamate receptors subunits also have fast desensitization rates, their rates of recovery from desensitization all exceed 30 ms, with the GluR1 subunit recovering the slowest from desensitization at a rate of 178 ms (Grosskreutz et al., 2003). With a fast desensitization and recovery from desensitization, the GluR4 subunit is suited for high frequency synaptic transmission.

**Hypothesis II**

Differences in the glutamate receptor subunits’ rates of desensitization and rates of recovery from desensitization raises the interesting question of whether the heterogeneous afferent signals that are known to exist in the utricle could be due in some part to different glutamate receptor subunit compositions within afferents. The striola appears to be well suited to respond to high frequency stimulation. Irregularly discharging fibers show large phase leads, which is an indication that the units are fast adapting. Phase leads are seen in fast adapting units because the units have their peak output, and then adapt to the signal, before the peak stimulus intensity. Fast adapting units can respond better to high frequency stimuli than slow adapting units because they can fire more often. One potential cause of the adaptation in afferents is desensitization of the glutamate receptors. The rate of desensitization could therefore determine whether the afferent units can be fast adapting. The rate of recovery from desensitization, as stated
previously, shapes the ability to respond to high frequency stimulation. The GluR4 subunit confers both rapid desensitization and rapid recovery from that desensitization to a fully functional glutamate receptor. *I, therefore, hypothesize that the GluR4 subunit will be expressed more in the utricular striola.*
MATERIALS AND METHODS

Dissection

*Trachemys scripta*, the red-eared slider turtle, was used for all experiments. The turtles were euthanized and decapitated according to IUCAC guidelines. The lower jaw was removed from the head, and the skull then bisected. The half heads were placed in phosphate buffered saline (PBS) and fine dissected to remove the vestibular labyrinth from the head. The vestibular labyrinth was then transferred to a smaller dish and placed in freshly prepared 4% paraformaldehyde (EM grade formaldehyde), and allowed to fix, undisturbed, for 10 minutes. The labyrinth was then transferred to a dish containing PBS with 2.5% Triton X-100 (PBST). The anterior and horizontal ampullae and the utricle were fine dissected from the rest of the vestibular labyrinth. The utricle was then placed in a new container with 3 ml of PBST and microwaved for 20 minutes. After microwaving, the utricle was embedded at 45°C, in a 4% agarose gel.

Turtle Zones and Sectioning

The utricle in the agarose gel is positioned to cut lateral to medial sections of the tissue. The utricle is then cut into 35 µm-thick slices on a Leica Vibratome. This orientation allows for a cross sectional view that contains both striola and extrastriola, called the transect. Within turtle utricle, our lab has further subdivided the striola and extrastriola into zones by their bundle morphology and the presence of calretinin. Zone 1 corresponds to the lateral extrastriola (LES); Zone 2 is a striolar region that does not
contain calyx units; Zone 3 is a striolar region that contains calyces; and Zone 4 corresponds to the medial extrastriola (MES) (**Figure 10**).

**Figure 10.** The turtle utricle with zones

The schematic below shows the striola and the extrastriola and their corresponding zones in turtle utricle. The dark black arrows laterally and medially correspond to the cut of the transect of the utricle. The light grey arrows show general trends of hair bundle polarities at each area. The turtle utricle is approximately 1 mm x 1 mm (Peterson, unpublished).

**Extrastriola – Zone 1 & Zone 4**
Zone 1 corresponds to the lateral extrastriola (LES).
Zone 4 corresponds to the medial extrastriola (MES).

**Striola – Zone 2 & Zone 3**
Zone 2 is indicated by the darkest shade of grey and contains the line of polarity reversal (dashed black line within zone 2).
Zone 3 is indicated by the moderate shade of grey and is just medial to zone 2. Only zone 3 contains calyx type afferents in turtle.
Immunohistochemistry

*GluR4 / CtBP2 / Phalloidin*

The sections were blocked in PBS with 10% normal donkey serum for 1 hour at room temperature (RT). The sections were then washed with PBST three times for 15 minutes at RT. After washing, sections were incubated in PBS with primary antibodies for approximately 20 hours at 4°C. Primary antibodies were rabbit anti-GluR4 (Millipore, Cat#06-308, Lot#1745296, 1:400 dilutions) and mouse anti-CtBP2 (BD Biosciences, Cat#612044, Lot#76115, 1:200 dilutions). Importantly, the anti-CtBP2 antibody is a marker for the synaptic ribbon (Schmitz, 2009). The sections were then washed with PBST eight times for 15 minutes at RT. After washing, sections were incubated in PBS with secondary antibodies for approximately 20 hours at 4°C. The secondary antibodies were donkey anti-rabbit 649 (JacksonImmuno, Cat#711-495-152, Lot#90029, 1:400 dilutions) and donkey anti-mouse 488 (JacksonImmuno, Cat#715-485-150, Lot#89132, 1:400 dilutions). The sections were washed 4 times at RT with PBST, and then incubated for 1 hour at RT with Phalloidin 546 (Invitrogen, Cat#A22283, 1:100 dilutions). After incubation with Phalloidin 546, the sections were washed an additional 4 times at RT with PBST. The sections were then mounted with number 1.5 coverslips using Slowfade Gold (Invitrogen) as the mountant. The preparations were imaged on a Zeiss 510 laser scanning confocal microscope using a C-Apochromat 63x water, 1.2 NA, coverslip correction objective.
The sections were blocked in PBS with 10% normal donkey serum for 1 hour at room temperature (RT). The sections were then washed with PBST three times for 15 minutes at RT. After washing, sections were incubated in PBS with primary antibodies for approximately 20 hours at 4°C. Primary antibodies were rabbit anti-Calretinin (Millipore, Cat#AB5054, Lot#JC1666019, 1:400 dilutions) and mouse anti-CtBP2 (BD Biosciences, Cat#612044, Lot#76115, 1:200 dilutions). The sections were then washed with PBST eight times for 15 minutes at RT. After washing, sections were incubated in PBS with secondary antibodies for approximately 20 hours at 4°C. The secondary antibodies were donkey anti-rabbit 649 (JacksonImmuno, Cat#711-495-152, Lot#90029, 1:400 dilutions) and donkey anti-mouse 488 (JacksonImmuno, Cat#715-485-150, Lot#89132, 1:400 dilutions). The sections were washed 4 times at RT with PBST, and then incubated for 1 hour at RT with Phalloidin 546 (Invitrogen, Cat#A22283, 1:100 dilutions). After incubation with Phalloidin 546, the sections were washed an additional 4 times at RT with PBST. The sections were then mounted with number 1.5 coverslips using Slowfade Gold (Invitrogen) as the mountant. The preparations were imaged on a Zeiss 510 laser scanning confocal microscope using a C-Apochromat 63x water, 1.2 NA, coverslip correction objective.

Controls

Five separate controls were performed for each of the antibodies utilized in the proposed research. Initially, each antibody was subjected to a 1) dilution series to
evaluate the proper concentration of antibodies needed for the research. At a high enough dilution, the signal should disappear if it is due to the primary antibody. The antibodies were then evaluated by 2) western blot analysis and 3) pre-adsorption analysis. Western blot analysis was utilized to ensure that the antibodies are staining the correct sized protein. Pre-adsorption is the saturation of the primary antibodies with their respective immunizing peptides prior to their incubation in tissues. Incubation with the pre-adsorbed antibodies should abolish any tissue staining. Rabbit-anti GluR4 antibody with the corresponding antigenic peptide sequence was used for the pre-adsorption control (Tocris Biosciences, Cat#2111, HTGTAIRQSSGLAVIASDLP). Approximately 5 µl of anti-GluR4 antibody was pre-adsorbed with 25 µl of the Tocris peptide. Both western blot and pre-adsorption controls allow for greater certainty about the staining specificity of the primary antibodies. The fourth control 4) Incubation with an inappropriate secondary antibody was utilized to ensure the specificity of the secondary antibodies. The tissue was incubated in the primary antibodies, but then incubated in goat anti-mouse secondary antibodies instead of the proper goat anti-rabbit antibodies. Staining should be abolished if the incorrect secondary antibodies are specific to their target primary antibodies. The fifth control 5) was an omission of the primary antibody. If the staining pattern is produced by the primary antibody, the staining pattern should be eliminated by omission of the primary antibody.
Western Blot

Turtle Brain

Turtle hind brain was dissected in PBS and then transferred to 2x Laemmeli sample buffer (0.5M Tris-HCl, pH 6.8, 10% SDS, 20% glycerol, 5% 2-mercaptoethanol, bromophenol blue) for homogenization. Approximately 10 swipes were taken with a Teflon pestle in a polished glass homogenization tube. The homogenized tissue was centrifuged at 13,000 g for 10 minutes. The supernatant was collected and diluted 10x with 2x Laemmeli sample buffer. This diluted sample was boiled in water for approximately 10 minutes. The samples were loaded into a Novex 8-16% Tris-Glycine Gel (Invitrogen, Cat# EC60452BOX) and run at 100V, 100mA for approximately 120 minutes. Precision Plus Protein WesternC standards (Bio-Rad, Cat#161-0376) were used, which required a second, separate secondary antibody to visualize. The samples in the gel were transferred to a PVDF membrane at 25V, 100mA for approximately 120 minutes. After transferring, the membrane was blocked for 3 hours at RT with 5.0% bovine serum albumin (BSA) and 5.0% normal goat serum in 50 ml of PBS with 2.5% Tween-20. The membrane was briefly washed twice at RT with 50 ml of PBST (2 x 2 min.). Rabbit anti-GluR4 (Millipore, Cat#06-308, Lot#1745296, 1:800 dilutions) was incubated at 4°C for approximately 16 hrs in PBS. The membrane was then washed 5 times (1x20min., 4x10min.) in 50 ml of PBS with 2.5% Tween-20 at RT. Immun-Star Goat Anti-Rabbit HRP Conjugate (Bio-Rad, Cat# 170-5046, 1:10000 / 1:20000 / 1:40000 dilutions) and Precision Protein StrepTactin-HRP Conjugate (Bio-Rad, Cat#161-0381, 1:10000 dilutions) secondary antibodies were both added for 1 hour at RT. The membrane was
then washed 5 times (1x20min., 4x10min.) in 50 ml of PBS with 2.5% Tween-20 at RT. Immun-Star WesternC chemiluminescent kit (Bio-Rad, Cat#170-5070, 2ml) was used to activated conjugated HRP substrates on the secondary antibodies. The Bio-Rad Chemidoc XRS system and Chemidoc XRS quantity one software were used to visualize and analyze all bands.

_Turtle Vestibular Periphery_

A second methodology was utilized for western blots within turtle vestibular tissue. As the GluR4 protein appears to be in low concentration within turtle vestibular periphery, a membrane fractionation protocol was used in hopes of concentrating the GluR4 protein by concentrating the membrane portion of tissue used in the western blot. Turtle vestibular periphery was dissected in Ringer’s solution and then flash frozen in dry ice and stored at -80°C. The tissue was homogenized with a Teflon pestle in a polished glass homogenization tube in 200 µl Hepes homogenization buffer (0.32M sucrose, 10mM Hepes, pH 7.4, 2mM EDTA) containing 1:20 dilutions of protease inhibitors (Sigma, Cat # P8340-1ML). The putative membrane fraction was obtained by a series of centrifugations. The tissue was centrifuged at 4°C at approximately 2500 rpm (591g) for 20 minutes. The supernatant (S1) was collected and stored on ice. The pellet was resuspended in 1.7 ml of Hepes homogenization buffer. The resuspended pellet was then centrifuged again at 2500 rpm (591g) for 20 minutes at 4°C. The supernatant (S2) was collected and pooled with supernatant S1. The pooled supernatants were brought up to 4 ml with the Hepes homogenization buffer. The pooled supernatants were then
ultracentrifuged at 60,000 rpm (200,000g) for 45 minutes at 4°C. The pellet from the ultracentrifugation was resuspended in 100 µl of Hepes buffer (50mM Hepes, pH 7.4, 2mM EDTA, 1:20 dilution of protease inhibitors). The resuspension was passed 5 times through a 26G needle and then solubilized in 1% Triton X-100 for 1 hour at 4°C. The tissue solution was then mixed in a 1:1 ratio with 2x Laemmeli sample buffer (0.5M Tris-HCl, pH 6.8, 10% SDS, 20% glycerol, 5% 2-mercaptoethanol, bromophenol blue). The tissue was boiled for 20 minutes and centrifuged with a bench top centrifuge for 15 minutes. The remaining SDS-PAGE procedure was identical to that used above in turtle brain tissue.

Confocal Microscopy

The preparations were imaged on a Zeiss 510 laser scanning confocal microscope using a C-Apochromat 63x water, 1.2 NA, coverslip corrected objective. I collected overlapping stacks of the entire central transect at 63x magnification and used them to construct a montage of the transect (Figure 11). The images were collected as stacks of 15 optical sections. The sections were 2x oversampled, making each confocal stack approximately 4 µm in the z-dimension. All laser intensities were maintained at 30% of maximum intensity. Ribbon size analyses were taken from two turtles from which three separate transects were montaged, whereas GluR4 size analyses and colocalization frequencies were taken from five turtles from which five separate transects were montaged. See Appendix A for additional details on confocal microscopy and deconvolution.
The turtle utricle has been subdivided into four anatomically distinct zones. Zone 1 and Zone 4 are the lateral and medial extrastriola, respectively. Zone 2 and Zone 3 are striola. Zone 2 is a region defined by a lack of calretinin, whereas Zone 3 is defined by the presence of calyces.

This section was stained with anti-calretinin, anti-CtBP2, phalloidin, and a nuclear stain known as sytox orange. The anti-calretinin antibody (red) stains hair cells in zones 1, 3, and 4, and calyx afferents in zone 3. The anti-CtBP2 is indicated by the green puncta (white arrows point to CtBP2 puncta), while the phalloidin and sytox are indicated by the yellow hair bundles and yellow-green basal support cell nuclei, respectively. The white bar indicates approximately 10 µm. The inset shows the entire montage of the utricle. The inset shows that the medial extrastriola, Zone 4, exceeds the other zones in size (Miller et al., 2007).
Amira Methods

The confocal LSM’s were loaded into Amira 5.3.3 (Mercury systems). Each confocal stack’s voxel size was recorded in the Amira software. The confocal stacks were deconvolved using the appropriate blind deconvolution module (see Appendix A). Each confocal stack was montaged with its adjacent confocal stack along the transect of the utricle (Figure 11). This montage was merged together within the Amira software. The montage contained puncta that corresponded to GluR4 and CtBP2 staining. An example of this montage may be seen in a following section on GluR4 and CtBP2 colocalization (pg. 72). A threshold intensity of each puncta was selected and Amira was used to segment both the GluR4 and CtBP2 puncta into separate connected “regions”. More specifically, each segmented punctum was analyzed by Amira to see if there was another punctum above or below, i.e. if Amira detected any segmented regions that were connected to each other in any direction, it added them together to form a larger segmented area called a region. Each region corresponded to a single GluR4 or CtBP2 punctum. Each of these regions was identified and was placed in a spreadsheet generated by Amira. Additional information regarding each region’s volume and center of mass in X, Y, and Z coordinates was contained in the spreadsheet. The volume of each segmented region, or punctum, was used to calculate the radius of a sphere with the same volume. Therefore, we have assumed each punctum to be spherical and to have a certain radius based on its segmented volume. See Appendix B for additional details on reconstruction and quantification using the Amira software.
Statistical Analysis

Size distribution Analysis

The radius of each punctum was mapped against its corresponding X coordinate to give a size distribution of puncta across the transect. This analysis was completed for CtBP2 puncta (ribbons) and GluR4 puncta. Descriptive and inferential statistics were performed in S-Plus, or Statistica.

Statistical Tests for Size Distribution

Box plots were generated for CtBP2 and GluR4 puncta size as a function of zone. (Figure 10). A Kruskal Wallis one-way analysis of variance was performed with post-hoc comparisons to identify significant differences between zones.

Matlab Analysis

Colocalization Analysis

The term colocalization is used to mean a CtBP2 punctum and a GluR4 punctum within one radius of each other. Why are colocalizing puncta used? The use of an electron microscope is necessary to identify synapses, but by using colocalized GluR4 and CtBP2 puncta, we can increase the probability that we are observing pre- & post-synaptic elements of a synapse. More specifically, two puncta were considered colocalized if the center points of each puncta were separated by a distance equal to or less than the sum of the radii of the pair. This criterion assures that colocalized puncta are touching. In MATLAB, an array of colocalized points was generated with X, Y, and Z
locations. This array of colocalized points was normalized for distance across the transect. Five cases were also averaged together to create one unified case.

**Monte Carlo Analysis, Defining Statistical Significance for Colocalization**

Statistical significance for each data set was determined using a Monte Carlo procedure to determine the likelihood of co-localization among randomly distributed CtBP2 ribbons and GluR4 puncta at equivalent densities. Each utricle was divided into 10 µm bins along its X axis. The bins with the three highest densities of puncta were selected and an equivalent volume was calculated in units of µm as $3 \times 10 \times Y \times Z$, where $Y$ is the distance from the minimum to the maximum y value in the selected bins, and $Z$ is the distance from the minimum to the maximum z value in the selected bins. An equivalent puncta density was then calculated by summing the number of puncta for three selected bins and dividing by the equivalent volume. An equivalent ribbon density was calculated in a similar manner. The sizes of puncta and ribbons were the average sizes of the puncta and ribbons, respectively, within the three selected bins. Using these values, 10000 surrogate data sets were created by randomly positioning the puncta and ribbons within the equivalent volume and finding the number of co-localized pairs for each surrogate using the same procedure as for the original data. The mean and standard deviation of the resulting distribution of co-localization percentages was then calculated. The percent co-localization within each bin of the original data was considered statistically significant if it exceeded the mean plus 3 standard deviations, corresponding to a significance level <0.01, i.e., the probability of the observed co-localization within
each bin occurring by chance was < 1%. This significance is indicated in the colocalization plots as a dashed horizontal red line. Colocalization frequencies falling below this dashed red line were considered statistically insignificant, while colocalization frequencies falling above the dashed red line were considered statistically significant.
RESULTS

Immunohistochemical Controls

The dilution series antibody control for the rabbit-anti-GluR4 antibody showed optimal staining at a dilution of 1:400 and an absence of anti-GluR4 antibody staining at a dilution of 1:6400. Incubation of tissue with an inappropriate secondary (anti-mouse Alexafluor instead of anti-rabbit Alexafluor) showed an absence of anti-GluR4 antibody staining. During the inappropriate secondary antibody test, a positive control was simultaneously produced. The positive control was tissue processed identically to the inappropriate secondary antibody control, with the exception of using appropriate secondary antibodies. This positive control showed proper anti-GluR4 antibody staining of the tissue.

The pre-adsorption control for the anti-GluR4 antibody showed complete saturation of the primary antibody by the immunizing peptide. Thus, anti-GluR4 antibody staining was abolished with the pre-adsorption control (Figure 12). A positive control for the pre-adsorption of the anti-GluR4 antibody showed puncta-like staining (Figure 13). This positive control was processed identically to the pre-adsorption control with the exception that the primary antibody was not pre-adsorbed with the immunizing peptide. These controls strengthen the argument that the staining pattern created in the turtle vestibular tissue is due to the anti-GluR4 antibody.
Figure 12. Pre-adsorption control for rabbit-anti-GluR4 antibody.

The tissue was stained with pre-adsorbed rabbit anti-GluR4 antibody and phalloidin. The phalloidin is indicated by the yellow staining in the image. The image was taken from the striola of turtle utricle. There is an absence of GluR4 puncta staining (red) within this tissue, likely indicating saturation of the anti-GluR4 antibody with the immunizing peptide. The white arrow indicates an estimation of the line of polarity reversal.

Phalloidin is used to stain hair bundles, but due to the low fixation necessary to produce GluR4 staining, the hair bundles are poorly fixed and are easily mechanically deformed or sheared from the epithelial surface of the utricle during dissection. During dissection of this specific sample, mechanical trauma resulted in the bundles being sheared from the utricular surface.
Figure 13. Positive control for pre-adsorption control for rabbit-anti-GluR4 antibody.

The tissue was stained with rabbit anti-GluR4 antibody and phalloidin. The image was taken from the striola of turtle utricle. There is red punctate staining (anti-GluR4 antibody) within this tissue, likely indicating that the anti-GluR4 antibody stained the GluR4 protein. The white arrow indicates the line of polarity reversal.

Western Blot Control for Turtle Hindbrain

The western blot on turtle hindbrain showed bands for the rabbit anti-GluR4 antibody at approximately 110 kDa and 40 kDa (Figure 14). The secondary antibody was serially diluted from approximately 1:10000 dilutions to approximately 1:40000 dilutions. As the secondary antibody dilution increased, it appears that the band at approximately 40 kDa is eliminated (Figure 14), suggesting that the band at 40 kDa was due to overly concentrated secondary antibodies on the blot. The blot at 1:40000 dilutions showed a single band at approximately 110 kDa, which is the expected weight for the GluR4 protein. Research within Dr. Joyce Keifer’s laboratory has also produced a similar
110 kDa band within turtle hindbrain for the same rabbit anti-GluR4 antibody (Wei and Keifer, 2009).

**Figure 14.** Western blot analysis of turtle hindbrain lysate

25 µm of turtle hindbrain was loaded into three lanes of the gel. WesternC protein standards were loaded into adjacent lanes of the turtle tissue. The WesternC protein standards ranged from 250 kDa to 10 kDa. The PVDF membrane with the transferred turtle hindbrain proteins was cut into three sections. These three sections were incubated in rabbit 1:1000 dilutions of anti-GluR4 primary antibody. The membranes were then incubated in serially diluted samples of goat anti-rabbit HRP conjugate secondary antibody and streptavidin HRP conjugate secondary antibody.

The right two lanes (R) and middle two lanes (M) show a set of standard proteins and a lane of turtle hindbrain lysate that were incubated primary antibody and in 1:10000 dilutions and 1:20000 dilutions of the secondary antibody, respectively. The turtle brain lane shows one band at approximately 110 kDa (black arrow) and a second at approximately 40 kDa (orange arrow).

The left two lanes (L) show a set of standard proteins and a lane of turtle hindbrain lysate that were incubated primary antibody and in 1:40000 dilutions of the secondary antibody. The turtle brain lane shows one band at approximately 110 kDa (black arrow), which is the proper weight for the turtle GluR4 protein.
Western Blot Control for Turtle Vestibular Periphery

For the western blot with turtle vestibular tissue, a second method using tissue centrifugation and the creation of a membrane fraction through centrifugation was used. The western blot showed several faint bands ranging from approximately 10 kDa to 75 kDa (Figure 15, image M). After visualizing the membrane for GluR4 antibody activity, the membrane was subjected to coomassie blue staining. The coomassie blue staining shows the most abundant proteins that are present on a membrane and was used as to check for the presence of proteins at the expected weight of the GluR4 protein (110 kDa). The coomassie staining results showed similar bands of protein on the membrane as were seen in the western blot (Figure 15, image R). The similarity between the coomassie blue staining and the western blot staining supports the idea that the bands seen on the western blot were due to over concentrated primary or secondary antibodies indiscriminately binding to all proteins on the blot. As a control to show that the primary antibody was not causing the banding pattern on the western blot, a blot incubated with secondary antibodies only was used on the same batch of turtle vestibular periphery lysate. This secondary antibody only control showed very similar bands as the original western blot that was stained with primary antibody, but was somewhat inconclusive due to band intensity differences between the two western blots (Figure 15, image L).

Overall, while the results did not show the expected GluR4 band at 110 kDa, they additionally did not show an intense band at any other weight. If an additional band of significant intensity had appeared, this would support that idea that the GluR4 antibody was staining the vestibular periphery tissue non-specifically. While inconclusive, it
appears that the banding pattern generated in the western blot is likely due to using over concentrated secondary antibodies in the procedure.

![Image of western blot analysis of turtle vestibular periphery lysate]

**Figure 15.** Western blot analysis of turtle vestibular periphery lysate

Turtle lysate and standard band descriptions are similar to Figure 13. The PVDF membrane in image M was incubated in 1:600 dilutions of rabbit anti-GluR4 primary antibody. The PVDF membranes in images L and M were both incubated in 1:10000 dilutions of goat anti-rabbit HRP conjugate secondary antibody and streptavidin HRP conjugate secondary antibody.

The western blot image with rabbit anti-GluR4 primary antibody (M) showed several faint bands ranging between approximately 15 kDa and 75 kDa. A band corresponding to the expected weight of the GluR4 protein (110 kDa) was not seen on the blot.

The coomassie blue image (R) showed several bands in the tissue from turtle vestibular periphery. The coomassie blue bands appeared very similar to the bands within the western blot that was incubated with primary antibody and secondary antibodies (anti-GluR4).

An additional western blot control was performed on the same batch of vestibular...
periphery tissue by incubating with 1:10000 dilutions of goat anti-rabbit HRP conjugate secondary antibody and streptavidin HRP conjugate secondary antibody only (L). The secondary antibody only control (L) showed similar banding patterns as the membrane incubated with the rabbit-anti-GluR4 primary antibody (M). Black arrows indicate similar bands between all 3 blots. The similarity between the bands in images L and M lends credibility to the notion that the bands in image M were likely due to an over concentrated use of secondary antibodies, though these results cannot be considered definitive. As not all bands in image L and image M match, there is a possibility that the bands seen in blot M were also due to a non-specific primary antibody activity.

Synaptic Ribbon Size

Three CtBP2 / Calretinin / Phalloidin cases were imaged and analyzed. Raw data from case 59 are shown in Figure 16. Case 59 (Figure 16) appears to show variations in the diameter of synaptic ribbons between the different zones within the image. Zone 2 and zone 3 CtBP2 puncta appear to have larger radius synaptic ribbons, while zone 1 puncta appear to have relatively smaller radius synaptic ribbons. Two additional cases were completed, using the same methodology, and produced similar images as case 59. In order to test synaptic ribbon size differences between zones, statistical analysis of synaptic ribbon radius was performed on one case from tissue sample 59 and on two cases from tissue sample 60.
**Figure 16.** Turtle utricular striola and lateral extrastriola stained with anti-CtBP2 and anti-calretinin

This striolar section of turtle utricle was stained with anti-CtBP2 (green), anti-calretinin (red) and phalloidin (yellow). The anti-CtBP2 stains synaptic ribbons in the pre-synaptic density of the synapse. The anti-calretinin antibody labels cell bodies in zones 1, 3, and 4 and afferents within zones 2 and 3. Thus, the anti-calretinin antibody could be used as a marker for zone boundaries within the utricular transect. Phalloidin stains hair bundles. The vertical white arrow indicates the line of polarity reversal. The CtBP2 puncta (green) appear to be larger within zones 2 and 3 versus zone 1. Zone 4 CtBP2 puncta (not pictured) appear to show similar CtBP2 puncta size as zone 1 puncta.
Box plots were generated for the three cases (Figure 17). In the box plots, zones 1 through 4 within the turtle utricle were compared based on CtBP2 puncta radius. Descriptive statistics for all three cases are found in Table 1.

In the box plots, non-overlapping confidence intervals suggest statistically significant differences. In order to test statistical significance between the zones, a non-parametric Kruskal-Wallis ANOVA was performed. An alpha value of 0.05 was selected for the Kruskal-Wallis analysis. Table 2 shows p-values for differences between CtBP2 radii between the zones for cases 59, 60, and 60 transect 2.

The box plots and the non-parametric ANOVA indicate that:

a) In all 3 cases, ribbons in zone 2 were larger than ribbons in other zones,
b) The difference between zone 2 and zone 1 was statistically significant in 1 case.
c) The difference between zone 2 and zone 3 was statistically significant in 2 of 3 cases.
d) The difference between zone 2 and zone 4 was statistically significant in all cases.

As previously stated, synaptic ribbon size appears to be related to an ability to encode high frequency stimuli (Martinez-Dunst et al, 1997.; Schnee et al., 2005). These results indicate that zone 2 synaptic ribbons may be better suited to encode high frequency stimuli than zone 4 ribbons and, perhaps, ribbons in zone 3. Interestingly, zone 3 synaptic ribbons, which are also considered part of the striola, were not larger than zone 4 synaptic ribbons.
<table>
<thead>
<tr>
<th>Case</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
<th>Zone 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Case 59</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median value (µm)</td>
<td>0.012</td>
<td>0.044</td>
<td>0.022</td>
<td>0.017</td>
</tr>
<tr>
<td>Confidence Interval (minimum – maximum) (µm)</td>
<td>0.009 – 0.014</td>
<td>0.026 – 0.062</td>
<td>0.013 – 0.031</td>
<td>0.014 – 0.020</td>
</tr>
<tr>
<td>Range (minimum – maximum) (µm)</td>
<td>0.006 – 0.143</td>
<td>0.006 – 0.450</td>
<td>0.006 – 0.694</td>
<td>0.006 – 0.187</td>
</tr>
<tr>
<td><strong>Case 60</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median value (µm)</td>
<td>0.020</td>
<td>0.027</td>
<td>0.015</td>
<td>0.017</td>
</tr>
<tr>
<td>Confidence Interval (minimum – maximum) (µm)</td>
<td>0.017 – 0.024</td>
<td>0.021 – 0.033</td>
<td>0.012 – 0.018</td>
<td>0.014 – 0.020</td>
</tr>
<tr>
<td>Range (minimum – maximum) (µm)</td>
<td>0.006 – 0.150</td>
<td>0.006 – 0.140</td>
<td>0.006 – 0.290</td>
<td>0.006 – 0.200</td>
</tr>
<tr>
<td><strong>Case 60 transect 2</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median value (µm)</td>
<td>0.034</td>
<td>0.052</td>
<td>0.027</td>
<td>0.025</td>
</tr>
<tr>
<td>Confidence Interval (minimum – maximum) (µm)</td>
<td>0.028 – 0.041</td>
<td>0.042 – 0.063</td>
<td>0.022 – 0.032</td>
<td>0.021 – 0.028</td>
</tr>
<tr>
<td>Range (minimum – maximum) (µm)</td>
<td>0.006 – 1.220</td>
<td>0.006 – 0.465</td>
<td>0.006 – 0.645</td>
<td>0.006 – 0.325</td>
</tr>
</tbody>
</table>
Case 59

Radius (μm)

Case 60

Case 60
transect 2

Zone
**Figure 17.** Box plots of synaptic ribbon radius across zones

Box plots were generated for all three cases. For each zone, the box plots show CtBP2 puncta radii median values (red circle), the 95% confidence interval of the median (dark blue hourglass), the interquartile range (light blue box) and whiskers that show the largest CtBP2 radii that falls below 1.96 times the interquartile range. The N below each box represents the total puncta sample size for that zone, whereas the number following the sample size is the percentage of outliers versus total puncta sample size for that zone. The whiskers were used to designate the radius of CtBP2 puncta that were to be considered outliers. If the CtBP2 punctum fell above the whisker value, the punctum was considered an outlier (black circles). Non-overlapping confidence intervals suggest statistically significant differences. We tested these differences (**Table 2**). These conventions also apply to box plots in **Figure 18**.

<table>
<thead>
<tr>
<th></th>
<th>Zone 2</th>
<th>Zone 3</th>
<th>Zone 4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Case 59</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone 1</td>
<td>0.000**</td>
<td>0.000</td>
<td>0.021</td>
</tr>
<tr>
<td>Zone 2</td>
<td></td>
<td>0.402</td>
<td>0.000</td>
</tr>
<tr>
<td>Zone 3</td>
<td></td>
<td></td>
<td>0.253</td>
</tr>
<tr>
<td><strong>Case 60</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone 1</td>
<td>1.000</td>
<td>0.031</td>
<td>0.307</td>
</tr>
<tr>
<td>Zone 2</td>
<td></td>
<td>0.001</td>
<td>0.012</td>
</tr>
<tr>
<td>Zone 3</td>
<td></td>
<td></td>
<td>1.000</td>
</tr>
<tr>
<td><strong>Case 60 transect 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone 1</td>
<td>0.176</td>
<td>1.000</td>
<td>0.021</td>
</tr>
<tr>
<td>Zone 2</td>
<td></td>
<td>0.047</td>
<td>0.000</td>
</tr>
<tr>
<td>Zone 3</td>
<td></td>
<td></td>
<td>0.655</td>
</tr>
</tbody>
</table>
GluR4 Subunit Size

Five cases were stained with CtBP2 / GluR4 / Phalloidin and were imaged and analyzed for GluR4 puncta size as a function of their position along the utricular transect. Images from these cases can be found within the GluR4 / CtBP2 colocalization section of this thesis (pg. 72). In order to investigate GluR4 puncta size differences between zones, statistical analyses were performed that were comparable to the methods used for determining synaptic ribbon size distributions. Box plots were generated for all five cases stained with anti-CtBP2, anti-GluR4, and phalloidin (Figure 18). Zones 1 through 4 within the turtle utricle were compared based on GluR4 puncta radii. Descriptive statistics for all three cases are found in Table 3.

In 4 of the 5 cases, zone 2 GluR4 puncta confidence intervals are non-overlapping with both lateral and medial extrastriolar GluR4 puncta confidence intervals. The exceptional case (50) shows zone 2 GluR4 puncta confidence intervals to be overlapping with lateral extrastriolar GluR4 puncta confidence intervals, but not with medial extrastriolar GluR4 puncta confidence intervals. The zone 3 GluR4 puncta median radii values are always larger than extrastriolar GluR4 puncta median radii values, but the box plots for zone 3 GluR4 puncta show variable overlapping of confidence intervals with all other zones across the cases. This suggests that zone 3 puncta are not different from puncta in the extrastriola.

In order to test statistical significance between the zones, a non-parametric Kruskal-Wallis ANOVA was performed. An alpha value of 0.05 was selected for the
Kruskal-Wallis analysis. **Table 4** shows p-values for differences between GluR4 radii between the zones for all five cases.

Overall, zone 2 GluR4 puncta are generally statistically different in size from lateral and medial extrastriolar GluR4 puncta. Zone 3 GluR4 puncta are intermediate in size.
Figure 18. Box plots of GluR4 puncta radius across zones

Refer to Figure 17 for a general description of the box plots.

The box plots generated for all 5 cases show a general trend of striolar GluR4 puncta having the largest radii. Of the striolar GluR4 puncta, zone 2 shows the largest median puncta radii value in 4 of the 5 cases.

In cases 43, 44, 55, and 57, zone 2 GluR4 puncta all have the largest median radii. In
these cases, zone 3 GluR4 puncta confidence intervals appear to overlap with zone 2 GluR4 puncta confidence intervals. Thus, zone 2 and zone 3 GluR4 puncta may not statistically differ in their size. While zone 2 GluR4 puncta confidence intervals appear to not overlap with extrastriolar GluR4 puncta confidence intervals, zone 3 confidence intervals are substantially overlapped with extrastriolar GluR4 puncta confidence intervals. Thus, zone 2 GluR4 puncta appear to statistically differ in size from both lateral and medial extrastriolar GluR4 puncta, while zone 3 GluR4 puncta do not.

In case 50, zone 2 and zone 3 confidence intervals are also overlapping. Additionally, case 50 shows striolar GluR4 puncta as having overlapping confidence intervals with zone 1 GluR4 puncta, but not zone 4 GluR4 puncta. Thus, in case 50, it appears that striolar GluR4 puncta do not statistically differ in size from lateral extrastriolar (zone 1) GluR4 puncta, but do statistically differ in size from medial extrastriolar (zone 4) GluR4 puncta. We tested all zonal differences statistically (Table 4).

Overall, while the striola appears to have the largest radii GluR4 puncta, differences between striolar GluR4 puncta sizes and extrastriolar GluR4 puncta sizes are inconsistent across box plots. Interestingly, in cases 50 and 55 there are stark increases in overall GluR4 puncta size versus the other three cases.
Table 3. Box Plot Descriptive Statistics of GluR4 Radii in Turtle Utricle

<table>
<thead>
<tr>
<th>Case 43</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
<th>Zone 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median value (µm)</td>
<td>0.022</td>
<td>0.033</td>
<td>0.025</td>
<td>0.022</td>
</tr>
<tr>
<td>Confidence Interval (minimum – maximum) (µm)</td>
<td>0.020 – 0.023</td>
<td>0.030 – 0.035</td>
<td>0.021 – 0.029</td>
<td>0.020 – 0.024</td>
</tr>
<tr>
<td>Range (minimum – maximum) (µm)</td>
<td>0.010 – 0.030</td>
<td>0.010 – 0.080</td>
<td>0.008 – 0.086</td>
<td>0.007 – 0.110</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Case 44</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
<th>Zone 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median value (µm)</td>
<td>0.033</td>
<td>0.050</td>
<td>0.041</td>
<td>0.031</td>
</tr>
<tr>
<td>Confidence Interval (minimum – maximum) (µm)</td>
<td>0.030 – 0.036</td>
<td>0.042 – 0.059</td>
<td>0.036 – 0.045</td>
<td>0.029 – 0.033</td>
</tr>
<tr>
<td>Range (minimum – maximum) (µm)</td>
<td>0.007 – 0.157</td>
<td>0.007 – 0.283</td>
<td>0.011 – 0.422</td>
<td>0.007 – 0.205</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Case 50</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
<th>Zone 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median value (µm)</td>
<td>0.072</td>
<td>0.073</td>
<td>0.078</td>
<td>0.044</td>
</tr>
<tr>
<td>Confidence Interval (minimum – maximum) (µm)</td>
<td>0.063 – 0.082</td>
<td>0.062 – 0.084</td>
<td>0.068 – 0.089</td>
<td>0.040 – 0.048</td>
</tr>
<tr>
<td>Range (minimum – maximum) (µm)</td>
<td>0.007 – 0.371</td>
<td>0.008 – 0.270</td>
<td>0.012 – 0.494</td>
<td>0.007 – 0.366</td>
</tr>
</tbody>
</table>
Table 3: continued

<table>
<thead>
<tr>
<th>Case 55</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
<th>Zone 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median value (µm)</td>
<td>0.049</td>
<td>0.064</td>
<td>0.063</td>
<td>0.045</td>
</tr>
<tr>
<td>Confidence Interval (minimum – maximum) (µm)</td>
<td>0.041 – 0.056</td>
<td>0.053 – 0.075</td>
<td>0.044 – 0.083</td>
<td>0.041 – 0.050</td>
</tr>
<tr>
<td>Range (minimum – maximum) (µm)</td>
<td>0.007 – 0.273</td>
<td>0.012 – 0.693</td>
<td>0.011 – 0.301</td>
<td>0.007 – 0.394</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Case 57</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
<th>Zone 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median value (µm)</td>
<td>0.027</td>
<td>0.039</td>
<td>0.028</td>
<td>0.025</td>
</tr>
<tr>
<td>Confidence Interval (minimum – maximum) (µm)</td>
<td>0.024 – 0.030</td>
<td>0.031 – 0.046</td>
<td>0.024 – 0.031</td>
<td>0.023 – 0.027</td>
</tr>
<tr>
<td>Range (minimum – maximum) (µm)</td>
<td>0.007 – 0.248</td>
<td>0.007 – 0.210</td>
<td>0.007 – 0.180</td>
<td>0.007 – 0.210</td>
</tr>
<tr>
<td>Case</td>
<td>Zone 1</td>
<td>Zone 2</td>
<td>Zone 3</td>
<td>Zone 4</td>
</tr>
<tr>
<td>------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
<td>--------</td>
</tr>
<tr>
<td>Case 43</td>
<td><strong>0.000</strong></td>
<td>0.581</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Zone 2</td>
<td>0.052</td>
<td><strong>0.000</strong></td>
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<td></td>
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<tr>
<td>Zone 3</td>
<td></td>
<td>0.855</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 44</td>
<td><strong>0.000</strong></td>
<td><strong>0.002</strong></td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Zone 2</td>
<td>0.820</td>
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<td>Zone 3</td>
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</tr>
<tr>
<td>Case 50</td>
<td>1.000</td>
<td>1.000</td>
<td><strong>0.000</strong></td>
<td></td>
</tr>
<tr>
<td>Zone 2</td>
<td>1.000</td>
<td><strong>0.000</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone 3</td>
<td></td>
<td><strong>0.000</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 55</td>
<td><strong>0.016</strong></td>
<td>0.430</td>
<td>1.000</td>
<td></td>
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<tr>
<td>Zone 2</td>
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<td><strong>0.000</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Zone 3</td>
<td></td>
<td><strong>0.040</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Case 57</td>
<td><strong>0.013</strong></td>
<td>1.000</td>
<td>1.000</td>
<td></td>
</tr>
<tr>
<td>Zone 2</td>
<td></td>
<td><strong>0.013</strong></td>
<td><strong>0.000</strong></td>
<td></td>
</tr>
<tr>
<td>Zone 3</td>
<td></td>
<td></td>
<td>1.000</td>
<td></td>
</tr>
</tbody>
</table>

** Highlighted values indicate significant differences between zones

GluR4 / CtBP2 Colocalization

Five GluR4 / CtBP2 / Phalloidin cases were imaged and analyzed. Raw data from cases 43 and 57 are shown below in Figures 19-22. Rabbit anti-GluR4 antibody staining
corresponds to the red puncta in the images, while the mouse anti-CtBP2 antibody staining corresponds to the green puncta in the images. Phalloidin staining is indicated by the yellow staining within the hair bundles and along the surface of the utricular epithelium.

The Striola and Lateral Extrastriola

Figure 19 shows raw data from the striolar and lateral extrastriolar regions of case 43 of the GluR4 / CtBP2 / Phalloidin immunohistochemical cases. The GluR4 puncta (red) appear to be most dense within the striolar region of the utricle. The CtBP2 puncta (green) also appear to be most dense within the striolar region of the utricle. Surrounding the polarity reversal line (white arrow) there appears to be many colocalizations (=overlap) between the red GluR4 puncta and the green CtBP2 puncta, resulting in a yellow coloration. Moving medially towards zone 4, the GluR4 and CtBP2 colocalization appears to occur less frequently. Case 57 shows similar colocalization between the GluR4 and CtBP2 puncta (Figure 20).
Figure 19 – Raw data from the striolar region of case 43.

This section was stained with anti-GluR4, anti-CtBP2, and phalloidin. The anti-GluR4 antibody is indicated by the red puncta and stains glutamate receptor subunit 4. The anti-CtBP2 is indicated by the green puncta, while the phalloidin is indicated by the yellow hair bundles. The white arrow indicates the line of polarity reversal. LES indicates the lateral extrastriola and MES indicates the medial extrastriola. Centered around the line of polarity reversal and extending approximately 25 microns laterally and 75 microns medially is the utricular striola. Zone 2 extends 25 microns laterally and 25 microns medially from the polarity reversal line. Zone 3 begins at the medial border of Zone 2 and extends another 50 microns medially. Thus, Zone 3 is approximately 50 microns in length along the transect.

Case 43 raw data appears to show considerable colocalization (=overlap) between CtBP2 puncta and GluR4 puncta within the striola, especially centered around the line of polarity reversal. This colocalization is most obviously noted by the appearance of yellow dots created by red puncta and green puncta overlapping.
Figure 20 – Raw data from striolar region of case 57

This section was stained with anti-GluR4, anti-CtBP2, and phalloidin. Case 57 appears to show relatively high GluR4 colocalization frequencies with CtBP2 puncta in zone 2 and moderate levels of colocalization with CtBP2 puncta in zone 3 of the striola.
The Medial Extrastriola

Figure 21 shows raw data from the medial extrastriolar region of case 43 of the GluR4 / CtBP2 / Phalloidin immunohistochemical cases. The GluR4 puncta (red) appear to be sparse within this extrastriolar region of utricle. Two examples are indicated by white arrows. Conversely, the CtBP2 puncta (green) appear to be moderately dense within the extrastriolar region of the utricle. There appears to be little to no colocalization (=overlap) between the red GluR4 puncta and the green CtBP2 puncta. Case 57 shows somewhat similar colocalization patterns between the GluR4 and CtBP2 puncta in the medial extrastriola (Figure 22). In the medial extrastriolar region of case 57 there appears to be slightly more colocalization between the GluR4 puncta and the CtBP2 puncta compared to case 43 (white arrows). In both case 43 and case 57, the medial extrastriolar colocalization frequency between GluR4 puncta and CtBP2 puncta appears to be much less than the striolar GluR4 puncta and CtBP2 puncta colocalization frequency.

In order to determine whether there were statistical differences of the colocalization frequency between the GluR4 puncta and the CtBP2 puncta as a function of their distance across the utricle, statistical analysis of the colocalization frequency was performed on five cases.
Figure 21 – Raw data from the far extrastriolar region of case 43

This section was stained with anti-GluR4, anti-CtBP2, and phalloidin. The anti-GluR4 antibody is indicated by the very faint red puncta (white arrows). The anti-CtBP2 is indicated by the green puncta, while the phalloidin is indicated by the yellow utricular epithelium. The medial extrastriolar staining shows GluR4 puncta and CtBP2 puncta that very rarely overlap. The frequency of overlap or colocalization between the GluR4 and CtBP2 puncta appears to be much lower in the utricular extrastriola versus the utricular striola.
Figure 22 – Raw data from far extrastriolar region of case 57

This section was stained with anti-GluR4, anti-CtBP2, and phalloidin. The medial extrastriolar staining shows very faint GluR4 puncta and CtBP2 puncta that occasionally colocalize (white arrows). The frequency of overlap or colocalization between the GluR4 and CtBP2 puncta appears to be much lower in the utricular extrastriola versus the utricular striola, though case 57 appears to show slightly more colocalization in the extrastriola than case 43 shows in the extrastriola.
Statistical Analysis of Colocalization Results

A colocalization analysis was performed within Matlab on 5 cases that were stained with anti-GluR4 antibodies, anti-CtBP2 antibodies and phalloidin. The Matlab analysis analyzed the density distributions of GluR4 puncta and CtBP2 puncta (synaptic ribbons) as a function of distance across the transect of the utricle. Additionally, the Matlab analysis showed the colocalization frequency between GluR4 puncta and CtBP2 puncta as a function of distance across the transect of the utricle.

Case 44 Results

Figure 23 shows the Matlab analysis for case 44. Figure 24 shows the Monte Carlo analysis for case 44, which was used to define a statistical significant level of colocalization frequency between GluR4 puncta and CtBP2 puncta. Case 44 shows increased densities of GluR4 puncta and CtBP2 puncta and the highest levels of colocalization between GluR4 puncta and CtBP2 puncta centered around the line of polarity reversal. Thus, in case 44, synaptic GluR4 subunits appear to be most prevalent within or near the striola. While adjacent extrastriolar regions surrounding the striola appear to have increased GluR4 and CtBP2 densities and moderately high colocalization frequencies, these results seem to taper off and reach insignificant levels especially within zone 4.
Figure 23. Colocalization analysis and spatial distribution for case 44

The x-axis on each graph from the Matlab analysis corresponds to the normalized distance across the entire transect of the utricle. A value of 1 corresponds to the far end of the medial extrastriola and a value approximating -0.3 corresponds to the far end of the lateral extrastriola. The zero coordinate on the x-axis corresponds to the line of polarity reversal of the utricle. The line of polarity reversal is also indicated by a solid vertical red bar within the second graph showing colocalized GluR4 and CtBP2 puncta, and in the middle graph showing colocalization frequencies between GluR4 and CtBP2 puncta. The horizontal dashed red line within the colocalization frequency graph indicates the percentage of CtBP2 puncta (synaptic ribbons) colocalized to GluR4 puncta by chance.

The top graph is a spatial mapping of every GluR4 puncta (green dots) and every CtBP2 puncta (red dots) versus distance across the utricular transect. Density distributions of the GluR4 and CtBP2 puncta are shown in the bottom two graphs of the figure. GluR4 puncta are designated by green bars, whereas the CtBP2 puncta are designated by the red bars. For the bottom two graphs, the y-axis corresponds to the number of CtBP2 or GluR4 puncta found within each bar (bin) of the graph. GluR4 puncta and CtBP2 puncta show density distributions that range from 0 to 30 puncta per bin. Zone boundaries are approximated under the bottom three graphs. Within the striolar region of the transect, corresponding to approximately -0.05 to +0.10 on the x-axis, the analysis shows the largest densities of GluR4 and CtBP2 puncta per bin. Within the striolar region, both GluR4 and CtBP2 puncta range in density from approximately 10 puncta to 30 puncta per bin. Extrastriolar regions to the utricle show a density distribution of GluR4 and CtBP2 puncta that tends to be less dense in number of puncta per bin than compared to the striola. Far medial extrastriolar GluR4 and CtBP2 puncta only occasionally exceed 10 puncta per bin.

In the middle graph, red bars show the percent of GluR4 puncta colocalized with a ribbon. Green bars show the percent of ribbons that colocalize with a GluR4 punctum. The dashed red line corresponds to case 44’s level of colocalization significance for synaptic ribbons. A Monte Carlo analysis was performed to calculate the level of colocalization significance. The level of colocalization considered significant was found to approximate 18% colocalization between GluR4 and CtBP2 puncta in case 44 (Figure 23). The second graph from the top designates a colocalized GluR4 puncta with a CtBP2 puncta as an open blue circle. The heaviest colocalization between puncta centers directly around the line of polarity reversal, or within the striolar region of the utricle. The striolar colocalization between puncta tapers both laterally and medially on the x-axis until approximately +/- 15%. Extrastriolar regions of the transect show sparse colocalization between puncta that tends to be statistically insignificant.
Figure 24. Monte Carlo analysis for case 44

The dashed red line in Figure 23 corresponds to case 44’s level of colocalization significance. In order to calculate this level of colocalization significance, 10,000 random surrogates were created and analyzed for their amount of colocalization within a defined space (see Materials and Methods). The probability distribution of these random surrogate tests are shown above. The x-axis corresponds to the percent of colocalized puncta, whereas the y-axis corresponds to the total percentage of the 10,000 random surrogate trials for which there was a certain level of colocalization between GluR4 and CtBP2 puncta.

For case 44, a mean percent of colocalization between puncta was calculated from the random surrogate analysis and was approximately 7.46% with a standard deviation of 3.44%. Statistically significant colocalization was selected to be approximately 3 standard deviations from the mean of 7.46%. Thus, the level of colocalization considered significant was found to approximate 18% colocalization between GluR4 and CtBP2 puncta. This level of colocalization corresponds to the dashed red line in Figure 23.
Case 50 Results

Figure 25 shows the Matlab analysis for case 50. Figure 26 shows the Monte Carlo analysis for case 50. Cases 44 and 50 differed most between all five cases.

Interestingly, case 50 shows increased densities of GluR4 puncta and CtBP2 puncta in what appears to be zone 3, but the highest levels of colocalization between GluR4 puncta and CtBP2 puncta are still centered around the line of polarity reversal within zone 2. While GluR4 puncta and CtBP2 puncta densities were high in zone 3, the percent of GluR4 puncta colocalized with ribbons was markedly decreased throughout most of zone 3 (red bars below dashed line). While case 44 showed a tapering effect of puncta densities and colocalization frequency toward the medial extrastriola (zone 4), case 50 showed a different result. In case 50, medial extrastriolar regions adjacent to the striola appeared to have increased GluR4 and CtBP2 densities and moderately high colocalization frequencies.
Figure 25. Colocalization analysis and spatial distribution for case 50

In the bottom two graphs, GluR4 puncta show density distributions that range from approximately 0 to 25 puncta per bin., whereas CtBP2 puncta show density distributions that range from 0 to 20 puncta per bin. Within the striolar region, both GluR4 and CtBP2 puncta range in density from approximately 10 puncta to 25 puncta per bin. Within extrastriolar regions of the utricle density distribution of GluR4 and CtBP2 puncta tend to be less dense in number of puncta per bin than compared to the striola. Far medial extrastriolar GluR4 and CtBP2 puncta only occasionally exceed 10 puncta per bin.

Colocalization analysis between GluR4 puncta and CtBP2 puncta is shown on the middle graph. The level of colocalization between synaptic ribbons and GluR4 puncta considered significant was found to approximate 34% colocalization (Figure 26). Colocalization between both puncta is relatively heavy (versus the MES) surrounding approximately -14% and +5% from the line of polarity reversal. The heaviest colocalization between puncta centers directly around the line of polarity reversal, or within zone 2 of the utricle. Interestingly, zone 3 colocalization frequencies are decreased relative to zone 2 colocalization frequencies, though both zones are considered part of the striola. The most lateral and most medial extrastriolar regions of the transect show sparser colocalization between puncta that tends to be statistically insignificant. Interestingly, case 50 differs from case 44 as colocalization between puncta significantly peaks again around – 15% and +18% from the polarity reversal line, which is significantly beyond the striolar region of the utricular transect.
For case 50, a mean percent of colocalization between puncta was calculated from the random surrogate analysis and was approximately 15.05% with a standard deviation of ±6.27%. Statistically significant colocalization was selected to be approximately 3 standard deviations from the mean of 15.05%. Thus, the level of colocalization considered significant was found to approximate 34% colocalization between GluR4 and CtBP2 puncta. This level of colocalization corresponds to the dashed red line in Figure 23.

**Figure 26. Monte Carlo analysis for case 50**

Averaged Cases

All 5 cases analyzed for puncta density distribution and puncta colocalization frequency showed results similar to cases 44 and 50. For this reason, all 5 cases were pooled to create an averaged graph showing the average density distribution of the GluR4 and CtBP2 puncta and the average colocalization frequency between the GluR4 and the
CtBP2 puncta (Figure 27). Table 5 gives puncta density distributions and puncta colocalization frequencies across all four zones for the averaged results.

In the averaged results, the GluR4 subunits in the far extrastriolae only occasionally exceed 10 puncta per bin and only occasionally colocalize above significant levels. Interestingly, as with case 50, the averaged results show a significant colocalization frequency between GluR4 and CtBP2 within extrastriolar regions closest to the striolar (near lateral and medial extrastriola).

Within the striola, the GluR4 subunit was most dense and most highly colocalized to CtBP2 puncta, especially within zone 2, but, the region corresponding to zone 3 shows a marked decrease in colocalization frequency between GluR4 and CtBP2 puncta.
Figure 27. Average colocalization and spatial distribution of 5 cases

Averaged results show striolar regions have the highest density distribution ranging from approximately 15 to 20 GluR4 puncta per bin (bottom graph) and approximately 10 to 20 CtBP2 puncta per bin (middle graph). As with colocalization frequency in case 44 and case 50, both GluR4 puncta and CtBP2 puncta density distributions in the averaged case show a pronounced tapering effect on puncta density moving both laterally and medially along the x-axis. In the top graph, colocalization frequencies also show similar trends as case 44 and case 50. Striolar regions, especially zone 2, show very high colocalization frequencies between GluR4 and CtBP2 puncta. As with case 50, the averaged results show a marked increase in colocalization frequency in the near medial extrastriolar region of the utricular transect (approximately +10% to +20% from the line of polarity reversal). This near medial extrastriolar region of the utricular transect has been termed...
the juxtastriola. Colocalization frequencies in the far medial extrastriola and the lateral extrastriola show sparse colocalization, only occasionally reaching the statistically significant 25% puncta colocalization mark.

Overall, the highest GluR4 and CtBP2 puncta densities and GluR4 and CtBP2 puncta colocalization frequencies appear to center around the line of polarity reversal found within the striolar region of the utricular transect.
**Table 5.** GluR4 and CtBP2 Puncta Density Distributions and Colocalization Frequency from Averaged Results

**Ranges from approximately +10% to +20% on the x-axis**

***Ranges from approximately +20% to +100% on the x-axis***

<table>
<thead>
<tr>
<th>GluR4 Density Range</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
<th>Juxtastriola Zone 4**</th>
<th>Far Zone 4***</th>
</tr>
</thead>
<tbody>
<tr>
<td>(number of puncta / bin)</td>
<td>1 - 15</td>
<td>15 - 20</td>
<td>16 - 18</td>
<td>6 - 16</td>
<td>2 - 12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>CtBP2 Density Range</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
<th>Juxtastriola Zone 4**</th>
<th>Far Zone 4***</th>
</tr>
</thead>
<tbody>
<tr>
<td>(number of puncta / bin)</td>
<td>3 - 14</td>
<td>14 - 20</td>
<td>7 - 16</td>
<td>9 - 15</td>
<td>3 - 9</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Range GluR4 Colocalization %</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
<th>Juxtastriola Zone 4**</th>
<th>Far Zone 4***</th>
</tr>
</thead>
<tbody>
<tr>
<td>(green bars)</td>
<td>10% - 45%</td>
<td>50% - 70%</td>
<td>30% - 50%</td>
<td>18% - 40%</td>
<td>0% - 35%</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Range CtBP2 Colocalization %</th>
<th>Zone 1</th>
<th>Zone 2</th>
<th>Zone 3</th>
<th>Juxtastriola Zone 4**</th>
<th>Far Zone 4***</th>
</tr>
</thead>
<tbody>
<tr>
<td>(red bars)</td>
<td>7% - 52%</td>
<td>45% - 60%</td>
<td>17% - 40%</td>
<td>18% - 50%</td>
<td>0% - 36%</td>
</tr>
</tbody>
</table>
DISCUSSION

Methodological Considerations

There are several methodological considerations to take into account regarding the results of this research. To demonstrate that CtBP2 puncta and GluR4 puncta were properly characterized within the utricle, several criteria needed to be met. These criteria included showing antibody specificity, establishing appropriate methodology for tissue preparation, and accurately collecting and analyzing data. Each of these criteria will be discussed.

Western Blot in Vestibular Periphery

Two distinct Western blot controls were used to show the specificity of the rabbit anti-GluR4 antibody within turtle tissue. The first western blot control used turtle brain lysate and produced a properly weighted band for the GluR4 subunit (~110 kDa). This western blot result using turtle brain lysate was consistent with results from Dr. Joyce Keifer’s laboratory using the same rabbit anti-GluR4 antibody (Wei and Keifer, 2009).

Though the anti-GluR4 antibody showed positive western blot results in turtle brain lysate, these western blot results in turtle brain do not wholly equate to specificity of the anti-GluR4 antibody within the vestibular periphery. Therefore, an additional western blot test was performed with the anti-GluR4 antibody in the vestibular periphery.

Initial western blot techniques on the vestibular periphery were identical to the techniques used for the turtle brain lysate experiments and proved to be ineffective. A number of possible reasons for these results exist: 1) By using the entire portion of
homogenized vestibular periphery tissue, the GluR4 protein was not concentrated enough to show a band on a western blot; 2) As the GluR4 protein appeared to only strongly stain the striolar region in immunohistochemical sections, and the striola is a relatively small part of the utricle, it is possible that the GluR4 protein is of very low abundance within the vestibular periphery and is not concentrated enough to show a band on a western blot. One potential way to solve these issues was to concentrate the GluR4 subunit. Therefore, we attempted to extract only the membrane portion of the vestibular periphery tissue using ultracentrifugation, and thus to concentrate the membrane associated proteins (i.e. AMPA receptors).

The anti-GluR4 antibody results from the western blot on the membrane fraction of vestibular periphery tissue showed several non-specific bands that were very faint. As the vestibular periphery western blot results did not show a specific band at the proper weight for the GluR4 protein, it is possible that the bands produced were the result of non-specific staining of the anti-GluR4 antibody. While it is possible that the primary antibody was producing these non-specific bands, results from staining the vestibular periphery tissue with the secondary antibodies only showed that at least some of the inappropriate bands were likely the product of non-specific binding of the secondary antibodies. Thus, due to non-specific banding and the lack of a proper weighted band, we cannot definitively say that the anti-GluR4 antibody is specifically binding to the GluR4 protein within turtle vestibular periphery tissue. However, the anti-GluR4 antibody appears to specifically bind to the GluR4 protein in turtle brain tissue, and immunohistochemical results using the anti-GluR4 antibody in vestibular periphery tissue
show specific staining of a post-synaptic component, suggesting that it may be the GluR4 subunit. The absence of an appropriately weighted band in peripheral tissue in both Western blots and Coomassie blue staining suggests that we may not have been able to produce a sufficient concentration of GluR4 protein with the methods we used.

**Fixation Effect on Tissue**

One challenging aspect of working with the GluR4 protein is that it is part of a tetrameric AMPA receptor, which is additionally situated within the post-synaptic density. The post-synaptic density is called a density because it comprises scaffolding proteins, ionotropic proteins, metabotropic proteins, enzymes and other physiological elements necessary for a synapse to function. These many elements of the post-synaptic density produce a physical barrier that can hinder the penetration of antibodies (Fritschy, 2008).

The problem of antibody penetration into the post-synaptic density is exacerbated when tissue has been fixed with formaldehyde. Though formaldehyde significantly reduces enzymatic activity and provides rigidity and longevity of tissue by cross linking proteins, it can also greatly reduce antibody penetration at certain cellular sights by producing a physical barrier. Cellular areas with many closely associated proteins (e.g. the synapse) become tightly locked together due to strong cross linking of the proteins by formaldehyde (Fritschy, 2008). This process is one way in which an epitope can be masked, or blocked, from being stained by an antibody specifically produced to bind to it. For this reason, all tissue used for research regarding the GluR4 protein was required to
be minimally fixed. Though minimal fixation was necessary to produce GluR4 staining, it comes with its own issues.

One potential issue that arises from poor fixation of tissue is non-specific staining of the tissue by the primary or secondary antibodies. Mechanical trauma of tissue has been shown to increase the likelihood of non-specific IgG binding to histological sections (Fritschy, 2008). As the tissue is lightly fixed, some mechanical trauma of the tissue is assumed to have happened during dissection, and thus some of the GluR4 staining may be attributed to non-specific staining.

A second potential issue is that of variable fixation of tissue. Formaldehyde must also penetrate tissues and requires a certain amount of time to fully fix a tissue preparation. If formaldehyde is not given enough time to penetrate deeper sections of the tissue, there will be variations in the fixation level throughout the sample. These variations could produce differences in staining intensity (Fritschy, 2008). If this were the case, the ability to accurately measure the volume of puncta across a three dimensional space would be jeopardized.

In hopes of counteracting the above fixation issues, turtle utricles were always carefully dissected in PBS first to reduce mechanical trauma. Once the utricle was removed from the surrounding vestibular tissue, this much smaller piece of tissue was placed in formaldehyde. Though the fixation was limited to 10 minutes, the smaller tissue sample hopefully allowed for a more uniform fixation of the utricular tissue.
GluR4 Puncta and Amira Thresholding

One confounding issue that arose during this research was the question of "what is a punctum?" Within the raw data and deconvolved data there were several punctum-looking objects that were below the utricular epithelium, at the level of the cuticular plate in the utricular epithelium, and above the utricular epithelium. The location of these puncta does not correspond well with published data about the location of glutamate receptors or synaptic ribbons (Bonsacquet et al., 2006). For our analysis, any punctum that fell outside of the utricular epithelium was disregarded because all hair cell-to-afferent synapses are within the epithelium. GluR4 and CtBP2 puncta that fell outside of the utricular epithelium showed little to no colocalization (i.e. any colocalization was well below chance levels).

A second confounding issue was that of the segmentation process and finding a proper thresholding value to properly select GluR4 puncta. An optimal thresholding value was determined by eye to obtain each channel’s “true” puncta. Thresholding by eye is conventionally used as a thresholding method (Dondzillo et al., 2010). One issue that arose from this segmentation process was that of two individual puncta in the raw data being merged and counted as a single puncta within Amira. For example, if two GluR4 puncta were in close spatial proximity to each other, the segmentation process could not distinguish the puncta as two discrete objects. While this merging of two puncta during segmentation did not occur often, it did occasionally result in artefactual puncta with exceptionally large volumes and radii. These large puncta are probably the outliers in the box plots.
Matlab Analysis

The Matlab analysis assumes two puncta to be colocalized when the two puncta are physically overlapping, i.e. when the center points of the two puncta were separated by a distance equal to or less than the sum of their radii. This criterion for puncta colocalization has been previously successfully utilized (Dondzillo et al., 2010). As the distance across the synapse is approximately 20 nanometers, and the punctate fluorescence produced was measured in microns, it appears that the fluorescence produced from a pre-synaptic punctum and fluorescence produced from a post-synaptic punctum should always overlap. For this reason, the criterion for colocalization was set to be very conservative, only assuming colocalization between two puncta if they overlapped. Interestingly, when looking at the raw data from the anti- GluR4 and anti-CtBP2 immunohistochemical cases, it appears that some CtBP2 puncta and GluR4 puncta are very close, but these puncta never physically overlap. This situation especially appears to be the case within the medial extrastriolar region of the utricular transect. It is possible that if the colocalization criterion were relaxed, there may be increased colocalization frequencies within the medial extrastriola.

Alternative Explanations

In addition to the interpretive challenges raised by the methodological considerations, two other possible interpretations of the results of this research will be discussed.
Is there really more GluR4 in the striola?

One alternative explanation for the differences in puncta frequency lies in the ability to detect the signal generated from the anti-GluR4 antibody. Thus, as the anti-GluR4 antibody becomes more concentrated at the post-synaptic density, the signal intensity will increase, the size of the puncta will increase, and the ability to detect the signal generated from the anti-GluR4 antibody will also increase. Within the striola, there are increased numbers of GluR4 puncta and increased sizes of the GluR4 puncta versus the extrastriola. This raises an interesting question. Is the GluR4 protein more concentrated within the striolar region of the utricle, or could the post-synaptic densities within the striolar region just be larger than the post-synaptic densities within the extrastriolar regions? A larger post-synaptic density is going to have more total receptors than a smaller post-synaptic density, and thus is going to show more intensely stained puncta and larger diameter puncta than a smaller post-synaptic density. This would allow for easier detection of these puncta and could potentially bias puncta frequencies within certain zones of the utricular transect. Additionally, while a larger post-synaptic density may have more receptors than a smaller post-synaptic density, it isn’t clear whether the ratio of AMPA receptor subunit types (e.g. GluR4 subunit, GluR3 subunit, GluR2 subunit) is going to be different between the large and small post-synaptic densities. If the ratio of the GluR4 subunit to the other AMPA receptor subunits remained the same in post-synaptic densities of striolar regions and extrastriolar regions of the utricle, there would be little support for the idea that the striola is specialized to encode high frequency stimuli.
The raw data from the anti-GluR4 and anti-CtBP2 immunohistochemical cases shows distinct intensity differences and size differences between GluR4 puncta in the striola and GluR4 puncta in the extrastriola. These differences could be due to there actually being more GluR4 subunit within the striolar regions, or could be due to the post-synaptic densities being larger within the striolar regions.

While there is no research regarding the size distributions of post-synaptic densities within the vestibular system, there is some data within mouse cochlea regarding size distributions of post-synaptic densities. Liberman et al. (2011) have shown through ultrastructural observations that afferents with low spontaneous discharge rates have approximately equaled size post-synaptic densities as afferents with high spontaneous discharge rates. Interestingly, afferents within the vestibular system also differ in their spontaneous discharge rates as a function of their position within the sensory epithelium (Lysakowski and Goldberg, 2004). So, the Liberman results suggest that these different afferents may have similarly sized post-synaptic densities. Whether post-synaptic densities are equivalent in size across zones in the turtle utricle is an important issue regarding the accuracy of the GluR4 protein frequency results and size results. One approach to answering this question may lie in utilizing antibodies to the remaining AMPA receptor subunits 1, 2, and 3. These additional AMPA receptor subunit antibodies could be co-incubated with the anti-GluR4 antibody, and the resulting puncta sizes of each AMPA subunit could be analyzed. The comparison of the relative sizes of each AMPA subunit puncta would give an estimate of the expression of different AMPA receptor subunits at each synapse.
As a side note, this issue does not arise with the CtBP2 antibody, as the CtBP2 protein is reported to be the major structural component of the synaptic ribbon (Schmitz, 2009). Thus, the fluorescence generated from the anti-CtBP2 antibody can be used as a marker for relative sizes of synaptic ribbons within a single tissue preparation.

Simply More Hair Cells in Striola?

One alternative explanation for the CtBP2 puncta and GluR4 puncta being more densely concentrated within the striola than the extrastriola is that there are simply more hair cells within the striolar region. If there were more hair cells within the striolar region of the utricle, this dense concentration of hair cells would appear to skew the number of puncta as a function of distance across the utricle. In mammals, hair cell density is thought to be lower in the striola (Lysakowski and Goldberg, 2004). In turtles, we do not have the quantitative data on spatial variation in hair cell density necessary to eliminate this alternative explanation.

CtBP2 Size / GluR4 Size and GluR4 Expression

The Striola and Encoding High Frequency Stimuli

Within the striola, zone 2 showed the largest radii CtBP2 puncta and GluR4 puncta, the highest densities of CtBP2 puncta and GluR4 puncta, and the highest levels of colocalization between CtBP2 and GluR4 puncta. These observations support the idea that zone 2 may be preferentially suited to encode high frequency stimuli.
With reference to CtBP2 size, several lines of research point to the size of the synaptic ribbon increasing as the optimal frequency increases within auditory hair cells (Martinez-Dunst et al., 1997; Schnee et al., 2005). The larger the synaptic ribbon, the more suited it may be to release the large amounts of neurotransmitters needed for faithfully encoding a high frequency stimulus. On the post-synaptic side of the synapse, these released neurotransmitters must bind to receptors that faithfully encode this same stimulus to the afferent nerve. As GluR4 puncta are the largest and most frequent within zone 2, it appears that zone 2 may have a large concentration of the GluR4 subunit within its post-synaptic densities. Within this post-synaptic density, the AMPA GluR4 subunit has an exceptionally fast desensitization and fast recovery from desensitization rates compared to other AMPA receptor subunits. These fast desensitization and recovery from desensitization rates would allow the GluR4 subunit to rapidly adapt to a stimulus and then quickly reset to respond to a second stimulus. This rapid adaption appears to be necessary to respond to high frequency stimuli. The results regarding synaptic ribbon size, GluR4 size, and GluR4 distribution support the idea that zone 2 may encode high frequency stimuli. Interestingly, hair bundle structures in zone 2 have been shown to be stiffer than in other zones (Spoon et al., 2005; Moravec et al., 2005), which should also help specialize zone 2 hair cells for high frequency signaling.

Zone 3 and GluR4 Puncta Distribution

Within the turtle utricle, zone 3 is a very physiologically and morphologically distinct part. Zone 3 of the turtle utricle is the only area of the utricle where calyx-type
afferents innervate hair cells. These calyx afferents envelop the entire hair cell, and for this reason calyx afferents differ from the characteristic bouton, or button-like, afferents found in all other zones of the utricle. As the calyx is cup-like and tightly envelopes the hair cell, one could speculate that the entire area between a calyx and its corresponding hair cell has the potential to be synaptically active. In fact, there is some evidence to support the notion that the shape of the calyx may play a significant synaptic role at the hair cell-to-afferent synapse. Research from Lim et al. has shown how the close apposition of the calyx to the hair cell may allow for $K^+$ to accumulate within the intercellular space (Holt et al., 2007; Lim et al., 2011). This accumulation of $K^+$ would drive the resting membrane potential of the calyx towards a more depolarized state, and thus closer to the afferents threshold for firing. With this in mind, it seems possible that the neurotransmitters released from a single synaptic ribbon could diffuse within the intercellular space between the calyx and hair cell and provide input to multiple, spatially separated post-synaptic receptors within the calyx afferent. If this were the case, many post-synaptic densities (or GluR4 puncta) may be present in zone 3 without corresponding ribbons. This possibility is supported by the raw data and by the averaged Matlab results as there appears to be multiple GluR4 puncta without a corresponding CtBP2 punctum within zone 3 of the turtle utricle (Figure 26).

In both the raw data and the Matlab analysis, GluR4 puncta densities are very high within zone 3. Conversely, in both the raw data and Matlab analysis, CtBP2 puncta (ribbon) densities are decreased in zone 3 versus zone 2 and versus parts of zone 1 and zone 4. When looking at the percent of CtBP2 puncta colocalized with a GluR4 punctum
(green bars), one can see that zone 3 colocalization frequencies in Figure 26 are decreased versus adjacent zones, but all fall above the line of colocalization significance. Conversely, when looking at the percent of GluR4 puncta colocalized with a CtBP2 punctum (red bars), one can see that zone 3 colocalization frequencies in Figure 26 fall mostly at or below the line of colocalization significance. Thus, a high percentage of CtBP2 puncta (ribbons) are associated with GluR4 puncta, whereas a lower percentage of GluR4 puncta are associated with ribbons. Many of these non-colocalized GluR4 puncta appear to line the calyx (Figure 13, far left). It seems a plausible explanation that the non-colocalized GluR4 puncta in zone 3 may actually be receiving input from a synaptic ribbon elsewhere in the calyx afferent. Thus, the results regarding zone 3’s ability to encode a high frequency stimulus may be artefactually low due to the unique morphology and physiology of the calyx afferent in this zone.

**Juxtastriola and CtBP2 and GluR4 Colocalization**

Interestingly, in the Matlab analysis the juxtastriolar region of zone 4 appears to show increased levels of colocalization between both GluR4 puncta and the CtBP2 puncta. While this region is considered extrastriolar, there is some evidence within our laboratory that this juxtastriolar region differs from the rest of zone 4. Afferent terminals in this region are more similar to those in zone 2 than to terminals in the rest of zone 4, suggesting that the juxtastriola may actually be part of the striola (Huwe et al., 2011). It is possible that the afferents within the juxtastriolar region of zone 4 are physiologically distinct from other utricular afferents, but further research is needed to
completely characterize this region of the turtle utricle. Until this research is completed, it is difficult to speculate about the function of the increased colocalization frequencies between GluR4 puncta and CtBP2 puncta in this extrastriolar region.

Conclusion

In conclusion, these results support the idea that zone 2 of the turtle utricle may function to encode high frequency stimuli. CtBP2 puncta are largest within zone 2 of the turtle utricle and may allow for increased rates of neurotransmitter release into the synapse. GluR4 puncta are most dense within zone 2 of the turtle utricle and may allow for the necessary rapid adaptation within the afferent that is encoding a high frequency stimulus. Zone 3 in the turtle utricle showed inconsistent results regarding synaptic ribbon size and GluR4 puncta density. For these reasons, it cannot be said that the entire striola is suited to encode high frequency stimuli.
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Confocal Microscopy

Confocal microscopy has improved upon widefield fluorescence microscopy through more effective elimination of out-of-focus light. This is accomplished by: a) sampling the image one point at a time; and b) using a pinhole to block light from above and below the point. The pinhole is located in a conjugate plane of the focal plane of the objective (hence the term confocal microscope), which allows it to pass in-focus light from a point in the specimen, while blocking out-of-focus light from above and below this point. The elimination of out-of-focus light results in an image of a relatively thin “optical slice” within the specimen. A disadvantage of this system is a reduction of image brightness, since some of the light from the point being imaged is also lost. For this reason, low intensity fluorescent objects may be difficult to visualize with confocal microscopy (Pawley, 2006). This is one reason that the numerical aperture of the objective is especially important for confocal microscopy.

Numerical Aperture

The major determinant of an objective’s resolving power is its numerical aperture. The magnitude of the numerical aperture of an objective is a measure of the angle of the solid cone of light that enters the objective and thus to the amount of light from the focal plane that will be focused on the pinhole. The magnitude of the numerical aperture is also
related inversely to the size of the point spread function of the objective, and thus to its resolving power.

The Point Spread Function

In ideal confocal microscopy, excitation light is focused to a single point in the specimen, and emitted fluorescence from that point is then focused to a point in the confocal plane, where the pinhole is located. However, this ideal can never be realized, due to diffraction of light as it passes through the aperture of the objective. In general, for any optical system, the image of a point source of light is not a point, but a 2D diffraction pattern. The exact shape of the pattern is related to the shape of the aperture of the optical system; for a circular aperture, it is a central spot surrounded by a radial pattern of maxima and minima like that shown in Figure 28B. The mathematical description of this pattern is the Bessel function. The central circle of the point spread function contains approximately 84% of all the photons from the light source and is known as the Airy disk. Surrounding the Airy disk are successive rings that contain progressively less and less photons. Hence, an intensity profile of a point spread function will have a large central peak with progressively smaller peaks radiating outwards (Figure 28A). As the numerical aperture of the objective increases, diffraction decreases which has the effect of making the central peak of the point spread function (the Airy disk) narrower. This narrowing of the numerical aperture is increases the resolution of the objective.
**Figure 28. The Point Spread Function**

**A.** The point spread function converted to an intensity profile. The units in Y are intensity (number of photons), where the unit in X is distance. The central peak, known as the Airy disk, is the largest due to it containing 84% of all photons from the point source.

**B.** Light passing through the pinhole produces the circular grating pattern known as the point spread function. The point spread function has alternating light (yellow) and dark (grey) rings, with the central sphere, or Airy disk, containing 84% of all photons from the point source.

**FWHM and the Rayleigh Criterion**

More formally, the resolving power of an objective can be specified as the full width at half maximum (FWHM) of the Airy disk profile, i.e., in order to resolve two points in an image, they must be separated by at least the FWHM. (Figure 29). This definition of resolution is somewhat arbitrary. An alternative, known as the Rayleigh criterion, states that two points in an image are resolvable if the peaks of the Airy discs are separated by a distance at least as great as the distance from the peak to the first minimum of the PSF.
Figure 29. Full Width at Half Maximum
Single point resolution is directly related to the intensity profile of the Airy disk. The central peak of the Airy disk (the orange line) defines the maximum resolvable size as its full width when the peak is at half its maximum intensity.

Resolution and the Point Spread Function in 3D

When attempting to visualize any object, the light from the object is convolved with the point spread function of the objective to produce the image. More specifically, an image is the result of convolving every point in the object with the point spread function of the objective. If the point spread function of an objective is too large, the image is blurred, and small details of the object will not be resolved in the image. In confocal microscopy, the point spread function is actually three-dimensional, i.e., the excitation light is focused to a small volume of tissue. Interestingly, this volume of tissue is not uniform in all three dimensions, with the Z-dimension being 2-3 times greater than that of either the X or Y dimensions. The larger point spread function in the Z-dimension produces what is known as axial smearing.

One method used to try and accurately reconstruct the true size of an object blurred by an objectives point spread function is called deconvolution.
Deconvolution

Simply stated, deconvolution is a method used to enhance image quality. Functionally, deconvolution is a mathematical algorithm that attempts to recover the true size of an object by reversing the effects of the point spread function of the objective. There are two versions of this procedure: Blind deconvolution and Non-blind deconvolution. Both require information about the 3D dimensions of the PSF.

Non-blind deconvolution utilizes PSF dimensions obtained by directly measuring the true PSF of the objective. One way to obtain these measurements involves creating an image of a microsphere of known size, and with dimensions much smaller than the PSF. It is possible to extract the dimensions of the PSF from such an image, since the dimensions of the image are dominated by the dimensions of the PSF if the microsphere is sufficiently small. Non-blind deconvolution requires much care as the preparation of the microspheres must as closely as possible mimic the preparation of the tissue used for the images one wishes to deconvolve. This is important because the point spread function of the objective is dependent on the path that light takes between the tissue and the objective. Therefore, matching the refractive indices of the mountant and the immersion medium, and matching the thickness of the coverslip are all critical to measuring a point spread function as it exists in your tissue.

Blind deconvolution utilizes PSF dimensions that are estimated from the magnification of the objective, numerical aperture of the objective, the refractive index of
the immersion medium, and the absorption and emission wavelengths of the
fluorophores. This method has the major advantage that it is simple and quick to perform.
Blind deconvolution may also provide an advantage in giving a generally correct point
spread function for the tissue, where in real tissue the true point spread function varies
across and through the tissue (Pawley, 2006).

Oversampling

A digital image is said to be oversampled if the pixel spacing in the image is less
than the resolution criterion set by the dimensions of the PSF. For objects near the
resolution limit, oversampling ensures that image is indeed the convolution of the object
with the PSF. The increase in the digital resolution of the image from oversampling does
not actually change the optical resolution, but can result in a more accurate digital
representation of the object and facilitate further image enhancement and processing.
Thus, an oversampled image is desirable either when the known dimensions of the PSF
are used to accurately estimate the dimensions of the object, or when the known
dimensions of the object are used to accurately estimate the PSF. An undersampled
image, where the pixel spacing is greater than the resolution limit, will produce image
artifacts that compromise any further image analysis. On the other hand, while generally
advantageous, oversampling results in larger image files and has the effect of increasing
the amount of photobleaching that occurs in objects. This increase in photobleaching is
generally due to the extended scan times necessary for scanning the increased number of
pixels (in either X, Y or Z). Therefore, it is important to keep in mind that the image
clarity obtained by oversampling will be constrained by photobleaching and the confocal user’s time.
APPENDIX B – AMIRA METHODS

Loading LSM’s and Organization

Multichannel Zeiss confocal lsm stacks collected were loaded into Amira 5.3.3 (Mercury systems). Each confocal stacks voxel size was incorporated into the Amira software. Channel 1, which corresponded to GluR4 staining, was filtered through a difference of Gaussian technique. The deconvolution module was subsequently added to each channel of the lsms and the appropriate blind deconvolution information was added to perform the deconvolution (Figure 30). After deconvolution of each channel, a new multichannelfield was created for each stack and the appropriate deconvolved channels were added to their new respective multichannelfield (Figure 31).
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Figure 30. Amira deconvolution module
Blind deconvolution was utilized for a confocal microscope. Blind deconvolution utilizes PSF dimensions that are estimated from the magnification of the objective, numerical aperture of the objective, the refractive index of the immersion medium, and the absorption and emission wavelengths of the fluorophores.
A multichannel field was created for each confocal image stack from the Create drop down box near the top of the Amira screen (Figure A). In Figure B, two of the three multichannel fields have had the appropriate channels linked to the multichannel field module (black arrows). The third multichannel field was selected and right clicked. Channel 1 was selected from the drop down box and then the corresponding deconvolved channel 1 (orange arrow) from the third confocal image stack was selected. The third multichannel field was selected and right clicked again. Channel 2 was selected from the drop down box and then the corresponding channel 2 from the third confocal image stack was selected (blue arrow). This process was repeated for channel 3.
Creating a Montage

An orthoslice module was added to each multichannelfield to allow for visualization of the channels. Each multichannelfield was then montaged with the transform editor in the properties box of the multichannelfields. The montaging was completed by viewing each orthoslice and aligning the orthoslices together in the 3D work area. Overlapping areas of each multichannelfield were cropped within Amira. The cropped segment was always the lsm’s segment which had been scanned twice during the initial collection on the Zeiss confocal microscope.

Merging

The confocal stacks were now merged together by linking each deconvolved lsm channel, from each multichannel field, to the merge module within Amira (Figure 32). The merge module allows you to merge channels together by adding (porting) each channel to the module. Once all proper deconvolved channel one files were ported to the merge module, the module was applied. The output of the merge module is a new deconvolved channel one, in which all of the original deconvolved channel ones ported to the module have been merged together according to their alignment within the transform editor. This process was repeated for channels two and three.
Figure 32. Merging channels in Amira

Each channel from the first multichannel field was selected and a merge module was added to the file. A standard algorithm was chosen from the properties box of the merge module to perform the merging.

The merging process links each appropriate channel from one multichannel field to all other appropriate channels selected from other multichannel fields.
Thresholding and Segmentation

In Amira, a localthreshold module was attached to each merged channel and was used to segment the signal from the background in the images (Figure 33). The signals in channels one and three were puncta-like. Therefore, the localthreshold module allowed us to select and segmented out punctum based on their signal intensity. Within the localthreshold module, the hysteresis algorithm was chosen to perform the thresholding of puncta. The hysteresis algorithm works by selecting a seed threshold value (a higher value) and then selecting a low threshold (a lower value). The seed threshold will select any point in the image with the selected intensity. The low threshold will then grow outward to its selected intensity from the points chosen by the seed threshold (hence the name “seed” threshold). The criterion for selecting the seed and low thresholds was observer based. The observer selected threshold values based on optimally representing the puncta within the medial extrastriolar region of the raw LSM files. In Table 6 below are the selected thresholding values across cases. A new object called a Labelfield is created once the localthreshold module has been applied. The new Labelfields contain the segmented puncta from each channel.
**Figure 33.** A local threshold module was attached to deconvolved files

The file produced from the merge module was right clicked and a LocalThreshold module was added to the merged file. Within the properties box of the LocalThreshold module, the hysteresis function algorithm was chosen to perform the segmentation of the puncta.
<table>
<thead>
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<th>Case Name</th>
<th>Seed Threshold</th>
<th>Low Threshold</th>
<th>Seed Threshold</th>
<th>Low Threshold</th>
</tr>
</thead>
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<td>Channel 1 GluR4</td>
<td>Channel 3 CtBP2</td>
<td>Channel 3 CtBP2</td>
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<td>400</td>
<td>300</td>
</tr>
</tbody>
</table>

Quantification of Segmented Puncta

In Amira, connectedcomponents modules were added to the new Labelfields created from the localthreshold modules. This module calculates the number of connected regions in a three dimensional space. More specifically, each segmented puncta was analyzed by this module to see if there was another corresponding puncta above or below. If the module detected any segmented regions that were connected to each other in any plane, it added them together to form a larger segmented area called a region (Figure 34). Within the connectedcomponents module, there are options to set a minimum voxel number for selecting regions. As some artifacts tend to occur in the segmentation process, a minimum voxel number of 20 was selected for finding three dimensional regions corresponding to a three dimensional puncta. The output of this module, which contains information about regions volumes and their position in a merged coordinate plane, was used for further analysis of the data in MATLAB.
Figure 34. Segmentation into connected “regions” with Amira

The above image is a single plane from a deconvolved confocal image stack that was stained with rabbit-anti-calretinin and mouse-anti-CtBP2. Channel 3, which corresponded to CtBP2 puncta, was segmented into regions using the localthreshold module. The green spots in the above image, indicated by orange arrows, are segmented CtBP2 “regions”. The red circle near the middle of the image corresponds to the line of polarity reversal. The blue circles adjacent to the red circle indicate borders between different zones along the utricle.

Processing of Data

The spreadsheet that was generated by Amira contained information about the volume of each CtBP2 and GluR4 puncta, contained information about the center of mass of each CtBP2 and GluR4 puncta in X, Y, and Z coordinates, and contained an X
coordinate corresponding to the line of polarity reversal (Figure 35). The volume of each segmented region, or puncta, was used to calculate the radius of a sphere with the same volume. Therefore, we have assumed each puncta to be spherical and to have a certain radius based on its segmented volume.

Figure 35. The spreadsheet containing puncta information generated by Amira

The spreadsheet contained information regarding each puncta’s volume, contained information about the center of mass of each puncta in X, Y, and Z coordinates, and contained an X coordinate corresponding to the line of polarity reversal.