3.22 Afferent Synaptic Mechanisms

M A Rutherford and W M Roberts, University of Oregon, Eugene, OR, USA
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Glossary

**active zone (AZ)** The area where synaptic vesicles fuse with the presynaptic membrane. The AZ is juxtaposed to the postsynaptic terminal across the synaptic cleft.

**afferent** The flow of signals from the periphery toward the central nervous system (CNS).

**auditory nerve fiber (ANF)** The axon of an auditory afferent neuron that projects to the brainstem in the vestibulocochlear (eighth cranial) nerve. In mammals, ANFs have cell bodies in the spiral ganglion.

**characteristic frequency (CF)** The frequency of maximum sensitivity to sound.

**cytomatrix at the active zone (CAZ)** The cytoskeletal meshwork comprised of multidomain proteins that aggregate through specific protein–protein and protein–lipid interactions. The CAZ creates a scaffold upon which the presynaptic machinery is assembled.

**hair cells** The sensory receptors of the auditory system and related sense organs of the ear and lateral line of vertebrate animals.
inner hair cell (IHC) One of the highly innervated hair cells in the mammalian ear that are primarily responsible for the perception of sound. They are located in a single row near the modiolar (inner) edge of the organ of Corti.

root mean square (RMS) A type of average computed by first squaring a set of values, then computing the mean of the squared values, then taking the square root of the mean. The RMS average deviation of $N$ samples from their mean is $\sqrt{\frac{1}{N} - 1}$ times the standard error of the mean.

sound pressure level (SPL) The RMS pressure change during a sound, expressed on the logarithmic decibel (dB) scale, measured in the vicinity of the listener during a specified time period.

spiral ganglion The cell bodies of the afferent neurons that innervate the organ of Corti; housed in the bony spiral lamina of the mammalian cochlea.

spontaneous rate (SR) Frequency of spikes in an auditory nerve fiber in the absence of an applied sound stimulus.

synaptic body or ribbon (SB) The most obvious presynaptic structure at hair cell afferent synapses, and the subject of many carefully designed experiments to reveal its function(s). There is general agreement that the SB serves an important purpose, which is not presently known. It harbors synaptic vesicles attached to it via fine filaments that are thought to either facilitate or impede the movement of vesicles along its surface.

synaptic vesicle (SV) Spherical lipid bilayer that contains neurotransmitter.

synaptic vesicle cycle The recurrent processes by which SVs are continuously created, used and recreated. Exocytosis of the neurotransmitter is triggered by calcium and involves fusion of the SV membrane with the plasmalemma and/or the formation of a transient aqueous connection between the SV and the extracellular space. After exocytosis, SVs are reformed, refilled, and reenter the exocytic pathway.

threshold The SPL needed to produce a criterion response. Defined in this way, threshold is a measure of sensitivity to sound: low threshold means high sensitivity. Sensitivity is usually expressed in units of response/stimulus, but in auditory physiology threshold is traditionally defined in units of stimulus only, omitting explicit reference to the response criterion used in a particular experiment.

3.22.1 Introduction

We will begin with an overview of the hair cell afferent synapse and related structures, with a focus on mammalian inner hair cells (IHCs) and spiral ganglion neurons. Our review will then turn to electrophysiological studies and discussions of the specializations that may endow these first auditory synapses with their ability to maintain fast and reliable, information-rich signaling to the brain. Readers unfamiliar with the basics of cellular neuroscience will better appreciate the unique properties of auditory afferent synapses after reviewing the properties of conventional chemical synapses described in the many excellent neuroscience textbooks (e.g., Delcomyn, F., 1998; Kandel, E. R. et al., 2000; Hille, B., 2001; Nicholls, J. G. et al., 2001). Additional information specific to hair cell afferent synapses can be found in recent literature reviews (Nouvian, R. et al., 2006; Moser, T. et al., 2006a; 2006b).

3.22.2 Afferent Neurons of Acousticolateralis Sensory Systems

The primary afferent neurons in the auditory and vestibular organs of all vertebrate species, and in related mechanosensory and electrosensory organs of the lateral line system of aquatic vertebrates are bipolar neurons that have cell bodies located in or near the ear. They extend one process peripherally to the sensory epithelium, the other centrally to the brainstem. The peripheral processes have often been called dendrites, but most are myelinated and can properly be called axons. This distinction is likely to be important for the assembly and maintenance of afferent synapses because axons and dendrites use different protein-targeting signals (Burack, M. A. et al., 2000). The afferent neurons in auditory and vestibular organs have cell bodies located close to the corresponding sensory epithelium, and therefore have short peripheral axons.
Lateral line afferent neurons have long peripheral axons that extend from cell bodies in the lateral line ganglia near the ear to sensory neuromasts on the head and trunk (Dambly-Chaudiere, C. et al., 2003).

### 3.22.2.1 Spiral Ganglion Neurons

In mammals, the cell bodies of auditory afferent neurons reside in the spiral ganglion, which parallels the inner (modiolus) edge of the organ of Corti (Figure 1(a)). Type I spiral ganglion neurons are responsive to acoustic stimulation and constitute 90–95% of cochlear nerve afferents (Spoendlin, H., 1969; Liberman, M. C., 1982). Intracellular labeling with horseradish peroxidase and subsequent histological processing revealed that they contact only the IHCs (Robertson, D., 1984). Both the peripheral and the central axons of a type I afferent neuron are myelinated and have a cluster of voltage-gated Na\(^+\) channels (Na\(^+_v\), 1.6) at each node of Ranvier. Na\(^+\) channels are also found in the unmyelinated axon segment between the postsynaptic terminal and the most distal peripheral node, suggesting that spikes may be initiated at or near the site of synaptic input (Hossain, W. A. et al., 2005), as occurs in vertebrate skeletal muscle (Kandel, E. R. et al., 2000).

Minimizing the distance from the synapse to the spike initiation site is expected to minimize attenuation and low-pass filtering of excitatory postsynaptic potentials (EPSPs). The cell bodies of type I spiral ganglion neurons are also wrapped in a loose myelin-like sheath that may speed conduction, reduce temporal jitter, or decrease the probability of conduction failure across the cell body during high-frequency firing (Hossain, W. A. et al., 2005).

In mammals, the peripheral axons of type I afferents (also known as radial fibers) remain unbranched and contact only a single IHC. Unlike most neurons, which receive synaptic inputs from hundreds or thousands of separate sites of synaptic contact (active zones; AZs), each type I afferent neuron typically receives sensory input from a single hair cell AZ (Spoendlin, H., 1969; Liberman, M. C., 1980; Spoendlin, H., 1985; Hashimoto, S. et al., 1990). In other vertebrates, most auditory afferent neurons make multiple postsynaptic contacts on one or more hair cells (Lewis, E. R. et al., 1982).

Each mammalian IHC provides the sole input to 5–30 type I afferent axons, which vary in number by species and hair cell position on the cochlear tonotopic axis (Kiang, N. Y. S. et al., 1982; Liberman, M. C., 1982; Fuchs, P. A. et al., 2003). The mammalian cochlea thus represents a case of synaptic divergence in which each IHC controls many private communication channels to the brain. The function of this unusual arrangement is only partially understood, but it is easy to speculate that the dynamic membrane potential of the hair cell contains more information than can be transmitted across one synapse and encoded in a single spike train.

Information is degraded by events that alter a signal in an unpredictable way (noise). The synaptic noise introduced by the stochastic nature of exocytosis can be reduced by having synaptic currents from many AZs converge on the spike initiation zone of one afferent axon, as occurs at the calyx synapses of vestibular hair cells (Goldberg, J. M., 1996); but the mammalian auditory system uses a different strategy in which the maximum possible separation of information channels to the brain is maintained by having each synaptic AZ control action potential generation in a separate afferent axon. If separate information channels later converge onto a single higher-order neuron in the brainstem (e.g., a globular bushy cell in the cochlear nucleus that detects coincident input), this arrangement can reduce the effects of variability in the spike conduction time from the ear to the central nervous system (CNS) and other noise sources that are downstream of the afferent synapse.

A small population of afferent axons in the mammalian cochlea (type II; Figure 1(a)) are unmyelinated (Liberman, M. C. et al., 1990). Like the type I afferents, they are labeled by antibodies against Na\(^+\), 1.6 and may initiate action potentials near their distal tips (Hossain, W. A. et al., 2005). These outer hair cell (OHC) afferents are thought to monitor the state of the organ of Corti, but not to contribute directly to the perception of sound, the role ascribed to the IHCs. Very little is known about the physiology of type II afferents, and they will not be considered further in this chapter.

Although the peripheral neural network in the ear is less elaborate than that of the retina and contains far fewer cell types, electron micrographs of the organ of Corti do show several additional types of synapses that could provide important functional interactions, including local feedback (Raphael, Y. and Altschuler, R. A., 2003; Sobkowicz, H. M. et al., 2003). These include connections between IHCs and OHCs via afferent and efferent fibers that contact both hair cell types (Sobkowicz, H. M. et al., 2004).

Nothing is known about the function of these synapses in auditory signal processing. Efferent synapses are typically seen on the postsynaptic
terminals of mammalian type I afferents, where they may modulate synaptic transmission from the hair cell to the afferent axon. Efferent axons also make inhibitory cholinergic synapses directly onto hair cells in most vertebrate auditory and vestibular organs, including mammalian IHCs and OHCs (see Chapter Efferent System).

3.22.2.2 Auditory Nerve Fibers

The central axons of spiral ganglion neurons project to the brainstem in the eighth cranial nerve, where they are called auditory nerve fibers (ANFs). Nearly all recordings of action potentials in mammalian ANFs are presumed to be from axons of type I spiral ganglion neurons because they are larger and much more numerous than the axons of type II neurons (Liberman, M. C., 1982).

*In vivo* recordings from single ANFs show that nearly all fire action potentials spontaneously (i.e., in a quiet laboratory environment with no experimentally imposed stimulus). The baseline or spontaneous firing rate (SR) in some ANFs exceeds 100 spikes s$^{-1}$, while others fire fewer than 5 spikes s$^{-1}$, with no apparent correlation between a fiber's characteristic frequency (CF) and SR (Kiang, N. Y. S. *et al.*, 1965; Tsuji, J. and Liberman, M. C., 1997). CF varies with position along the tonotopic axis whereas SR has been correlated with both axon diameter and subcellular synaptic structure in the guinea-pig and cat (Liberman, M. C., 1980; Merchán-Pérez, A. and Liberman, M. C., 1996; Tsuji, J. and Liberman, M. C., 1997). In these studies of afferent structure/function,
intracellular recording and labeling of individual ANFs followed by serial-section electron microscopy (EM) showed that ANFs of high SR belong to spiral ganglion neurons that have large diameter peripheral axons and contact IHCs predominantly on the pillar face (Figure 1(b)); however, this segregation of axonal diameters around the hair cell circumference was not observed in one study of the gerbil (Slepecky, N. B. et al., 2000). Low- and intermediate-SR fibers of the cat and guinea-pig contact the modiolar face and have synapses with larger presynaptic dense bodies (called synaptic bodies, SBs, or ribbons) and more vesicles than synapses with high-SR fibers. Because the 5–30 afferent synapses on each IHC display this range of ultrastructural characteristics and modiolar/pilar position, and because ANFs of the same CF differ in SR, it is inferred that each IHC synapses onto both high-SR fibers and intermediate-/low-SR fibers.

To quantify the strength of a sound, most experimenters report the sound pressure level (SPL), expressed on the logarithmic decibel scale (dB). Each 20 dB increase in SPL corresponds to a tenfold multiplication of root mean square (RMS) sound pressure. The zero point (0 dB) is often defined as 20 μPa, which is approximately the amplitude of a barely perceptible sound at frequencies where human hearing is most sensitive (2–5 kHz). Compared to the standard atmospheric pressure of ~100 kPa, 20 μPa amounts to a change of only about 1 part in 5 billion.

An ANF’s sensitivity to sound (Figure 2) is typically measured as the SPL needed to increase its firing rate by a criterion number of spikes per second above the SR, or alternatively, to produce a criterion increase in ANF discharge synchrony with the sound stimulus (Rose, J. E. et al., 1967; 1971; Johnson, D. H., 1980). This measure, which is often called the fiber’s threshold, depends on the response criterion, stimulus duration, and other factors that are specific to the experimental protocol (Heil, P. and Neubauer, H., 2003). The term threshold is typically used in biology to mean a stimulus level below which there is no response (e.g., the threshold for generating an action potential), and therefore implies the existence of a strong nonlinearity in the stimulus–response relation (a cut-off below which there is no response). The use of the term threshold in auditory physiology is quite different. It carries no implication of a threshold nonlinearity, only that the response has fallen below some criterion level. In fact, the initial stages of auditory processing are constructed to avoid threshold nonlinearities.

A threshold nonlinearity in the peripheral auditory system would be detrimental to subsequent neural processing because it would eliminate information that could otherwise be recovered by temporal averaging (integration) or filtering, which can in principle pull an arbitrarily small periodic signal out of aperiodic background noise. This ability is crucial for systems designed to detect small noisy signals. For example, a sinusoidal signal that is superimposed on additive white noise can be passed through a narrow-band linear filter that leaves the signal unchanged while reducing the noise in proportion to the bandwidth of the filter. Narrowing the filter’s bandwidth can thus increase the signal-to-noise ratio as much needed to detect an arbitrarily small periodic signal, albeit at the expense of temporal resolution (Bracewell, R. N., 1999). The auditory system’s use of temporal integration to improve signal-to-noise ratio is seen experimentally as an inverse relation between threshold and sound duration (Jeffress, L. A., 1968; Heil, P. and Neubauer, H., 2001; 2003). The observation that sounds too soft to be audible can nevertheless influence the audibility of subsequent sounds (Plack, C. J. et al., 2006) supports the idea that subthreshold sounds still produce some response.

The peripheral auditory system eliminates any threshold cut-off in the afferent response to faint sounds by eliminating all slack from the system, ensuring that arbitrarily weak sounds evoke a change in ANF firing rate that is proportional to the sound amplitude, at least for low-frequency tones. This begins with mechanosensory transduction, which uses feedback to maintain partial activation of the transduction channels even in complete silence. The resulting resting transduction current places the hair cell’s resting membrane potential in a range where the presynaptic calcium channels are also partially activated (Koschak, A. et al., 2001; Xu, W. and Lipscombe, D., 2001; Johnson, S. L. et al., 2005). This steady-state activation of the calcium current (i.e., the random, thermally driven, independent gating of individual calcium channels that occurs at constant membrane potential) may be sufficient to catalyze baseline transmitter release (Brandt, A. et al., 2005) that drives the so-called spontaneous spiking in ANFs (Liberman, M. C. and Kiang, N. Y. S., 1978; Sewell, W. F., 1984; Glowatzki, E. and Fuchs, P. A., 2002; Keen, E. C. and Hudspeth, A. J., 2006). Thus, each stage of processing is maintained in a partially active state even in complete silence, thereby avoiding a threshold nonlinearity and the resulting loss of...
information about small signals. Because several steps in auditory reception involve discrete all-or-none events (e.g., the opening of individual ion channels, the fusion of individual synaptic vesicles (SVs) with the plasmalemma, and the generation of individual action potentials in ANFs), the presence of thermally driven noise in the system is crucial to maintaining high sensitivity to small sounds (Indresano, A. A. et al., 2004). This phenomenon has been studied in many physical systems under the odd name of stochastic resonance.

At frequencies above a few kilohertz, where sound perception relies on nonlinear transduction at the foot of the stimulus–response curve to produce the

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**Figure 2** Tuning curves and phase locking in avian (emu) auditory nerve fibers (ANFs). (a) The sound pressure level (SPL) required to evoke a criterion response, in this case, a 20 Hz increase in ANF firing rate above the spontaneous rate (SR), plotted as a function of the frequency of the tonal stimulus. This type of measurement is often called the fiber's threshold, although it does not mean that the fiber fails to respond to less intense sounds, only that the response falls below the experimenter's criterion. Data from two ANFs are shown. Each panel shows one fiber's tuning curve as a continuous line and phase histograms (insets) from the same fiber, each spanning a time frame of two cycles on the x-axis and displaying the probability of a spike in particular time bins within the cycle. The stimulus for the insets was at the frequency-level combinations indicated by the associated filled, inverted triangles. The phase relationship of spike occurrence to the period of the tonal stimulus changes as a function of frequency, but at the characteristic frequency (the low point on the curve) the phase relationship does not vary with changes in sound intensity (dB SPL). (b) Tuning curves for four ANFs of different characteristic frequency. (c) When vector strength (a measure of the degree of phase locking, on a scale of 0 to 1, derived from phase histograms like those shown in (a)) is plotted as a function of stimulus frequency around characteristic frequency for 73 different afferent fibers, it is apparent that cochlear ANFs in the emu exhibit a sharp decline in phase-locked firing at frequencies above 1 kHz. Reprinted with permission from Manley, G. A., Köppl, C., and Yates, G. K., J. Acoust. Soc. Am., Vol. 101, pages 1560-1573, 1997. Copyright 1997, American Institute of Physics.
DC component of the receptor potential (see Section 3.22.6), a nonlinear response to small sounds may be unavoidable.

Fibers that are the most sensitive to sound also have the highest SR. The equivalent statement in terms of threshold is that SR varies inversely with threshold. In cats, ANFs have been divided into three classes based on SR: low-SR fibers (<0.5 Hz) have the highest threshold (20–60 dB higher than high-SR fibers), high-SR fibers (≥18 Hz) have the lowest thresholds, and medium-SR fibers have an intermediate sensitivity (Merchan-Perez, A. and Liberman, M. C., 1996). Because ANFs with the same CF can have high-, intermediate-, or low-SR, it seems likely that a single IHC forms synapses with representatives of all three functional groups.

Unlike many types of neurons that fire either with a fairly constant interspike interval or in bursts, the exponential distribution of interspike intervals for baseline activity in mammalian ANFs suggests that their activity in the absence of sound is driven by independent random events (i.e., it is a Poisson process), except during a neural refractory period following each spike (Johnson, D. H. and Kiang, N. Y. S., 1976) rather than by an oscillation in the pre-or postsynaptic membrane potential or by any of the other well-known mechanisms that underlie bursting or rhythmic firing in neurons. The independent events may be the brief openings of individual presynaptic calcium channels, in which case the afferent synapses onto ANFs with higher SRs could have more calcium channels or a higher probability of transmitter release in response to the opening of a single calcium channel. Another possibility to explain the correlation between an ANF’s sensitivity and its SR is that the voltage dependence of the calcium channels at high-SR synapses is shifted such that the channel’s resting open probability is increased, which would also move the resting potential to a steeper place on the channel’s sigmoidal activation curve. In all of these cases, high SR would be associated with greater sensitivity to sound. Since a single hair cell can make synapses onto several ANFs that have widely varying SRs, these differences must be specific to individual synapses.

If the Poisson events that trigger exocytosis are the openings of individual calcium channels, then a simple calculation shows that the probability of exocytosis associated with each channel opening is likely to be very small. Calcium channels in hair cells have mean open times of <1 ms and a steady-state open probability of ~10% at the resting potential, implying a mean closed time of <10 ms. If calcium channels in IHCs have properties similar to these, then each channel is expected to generate >100 openings per second at the resting potential, and the ensemble of ~100 calcium channels at a typical AZ are expected to generate >10^7 openings per second. Given that the highest SRs in ANFs are ~100 s−1, fewer than 1% of calcium channel openings are predicted to trigger exocytosis.

### 3.22.2.3 Innervation Patterns and the Function of Auditory Organs

The pattern of afferent innervation onto hair cells contributes greatly to the response properties observed in afferent fiber recordings. To illustrate this, we will consider two very different auditory organs of the inner ear: the mammalian cochlea and the frog sacculus.

As mentioned above, sensory information from a single IHC in the mammalian cochlea diverges onto multiple ANFs, which can be regarded as a set of parallel channels that relay information from a single IHC to the brain. Because each IHC has a slightly different CF, and a slightly different phase relationship to the sound, restricting each ANF’s input to a single IHC preserves these two important features of the cochlear tonotopy. Having multiple outputs from each IHC with different sensitivities (range fractionation) may be important for maintaining a high signal-to-noise ratio for faint sounds in some ANFs while avoiding saturation by loud sounds in other ANFs. Even if some of the ANFs have identical response properties, maintaining redundant information channels can be important for increasing the signal-to-noise ratio. Two important sources of noise are uncertainty in when (or whether) a spike will be generated in a given ANF and variation in the conduction time of ANF spikes to the brain. This noise can, in principle, be reduced by combining information transmitted over separate channels that have independent noise sources. Each ANF postsynaptic to a given IHC shares a common input signal (the hair cell’s membrane potential), but the subsequent steps of synaptic transmission, spike generation and conduction to the brain are carried out independently in each AFN, and therefore are subject to independent noise. The innervation pattern of the mammalian cochlea thus appears to be optimized to provide the spectral and temporal information needed for complex computations involved in speech recognition and generating a map of auditory space.
The frog sacculus has a very different structure and pattern of innervation (reviewed in Smotherman, M. S. and Narins, P. M., 2000). It is (in addition to its function as a vestibular organ) exquisitely sensitive to both airborne sound and substrateborne vibration (Koyama, H. et al., 1982). The frog saccular hair cells reside in a roughly circular patch of sensory epithelium beneath the otolithic membrane, and all are attached to it through their kinocilia. There is no evidence of tonotopic organization. Instead, the hair cells fall into only a few anatomical classes, suggesting that many may be functionally equivalent to each other (Edmonds, B. et al., 2000). Unlike the mammalian cochlea, each saccular afferent neuron branches to contact many hair cells (Lewis, E. R. et al., 1982). Summation of synaptic inputs from multiple hair cells is expected to increase the sensitivity and, perhaps more important, the signal-to-noise ratio of ANFs from the frog sacculus, which is expected to contribute to the organ’s extreme sensitivity to sound and vibration.

3.22.3 Hair Cells

Hair cells are part of the tight epithelium that separates endolymph from perilymph. Each hair cell is sealed to its neighbors by tight junctions that form an extracellular diffusion barrier across the epithelium and divides the cell membrane into an apical domain that is primarily involved in mechanosensory transduction and a basolateral domain where the synapses reside (Figure 1(b)). Hair cells are both pre- and postsynaptic, and have other neuronal properties, including voltage- and calcium-gated ion channels that, in concert with the transduction current and the membrane's electrical capacitance, determine how the membrane potential changes with time. The hair cell's compact shape has important consequences for both the electrical signal and the ensuing calcium signal that controls transmitter release at afferent synapses.

3.22.3.1 Hair Cell Shape in Relation to Synaptic Signaling

The hair cell body has a diameter-to-length ratio that is too large to allow significant spatial variation in the membrane potential, except in OHCs of the mammalian cochlea, where extensive internal membranes lining the plasmalemma might impede intracellular current flow (Nakagawa, T. et al., 2006). Other types of hair cells, including IHCs of the mammalian cochlea, are expected to be nearly isopotential even to the tips of the stereocilia (Roberts, W. M. et al., 1990). This means that the electrical signal for synaptic transmission is the same at each of the afferent synaptic sites, and implies that the different properties of the afferent synapses on a single hair cell must arise downstream of the electrical signal. Similarly, the membrane capacitance is expected to attenuate high-frequency voltage oscillations to the same degree at all AZs in a given hair cell, leaving only a steady depolarization in response to high-frequency sounds (Section 3.22.6).

The hair cell’s large radius of membrane curvature compared to the diameter of a single synaptic AZ (several micrometers vs. hundreds of nanometers) simplifies the understanding and modeling of afferent synaptic transmission because for some purposes the membrane can be regarded as a planar boundary separating two infinite spaces (Neher, E. and Augustine, G. J., 1992; Roberts, W. M., 1994). Clearly, the hair cell’s fraction of volume is less than infinite and cannot serve as an infinite sink for calcium entering at synapses, but as long as calcium extrusion can keep pace with calcium entry, the details of how and where calcium is pumped back into the extracellular space are expected to be less important than in the confined space of a typical presynaptic bouton.

3.22.3.2 The Synaptic Body and Synaptic Vesicle Pools

Afferent synapses on all hair cells and related electroreceptors are distinguished by a prominent presynaptic structure, the synaptic body (SB). One or more SBs are found at each site of synaptic contact, in close association with the presynaptic plasma membrane (Figures 3 and 4). Numerous functions have been proposed for the SB (Sjostrand, F. S., 1958; Bunt, A. H., 1971; Gray, E. G. and Pease, H. L., 1971; Osborne, M. P. and Thornhill, R. A., 1972; Wagner, H.-J., 1978; King, T. S. and Dougherty, W. J., 1982; Schmitz, F. et al., 2000; Lenzi, D. and von Gersdorff, H., 2001; Parsons, T. D. and Sterling, P., 2003), but none proved.

The hair cell contains a large population of clear-core vesicles that are presumed to contain the neurotransmitter, glutamate. These SVs have been partitioned into several conceptual pools, defined either structurally with EM or functionally.
Experiments on a wide variety of chemical synapses in many animals have shown that SVs can generally be assigned to one of three groupings: the readily releasable pool (RRP), the recycling pool, and the reserve pool (reviewed in Rizzoli, S. O. and Betz, W. J., 2005). Parallel definitions of these pools at hair cell afferent synapses are useful to facilitate comparisons with more conventional chemical synapses, although this is hampered somewhat by their different anatomical and functional characteristics.

At both types, maximal activation of the presynaptic calcium current exhausts a small SV pool, defined as the RRP, within a few milliseconds. At conventional synapses, the RRP comprises 1–2% of the total vesicle population, but considering the hair cell cytoplasm as an unrestricted volume for SV movement containing hundreds of thousands of SVs (Lenzi, D. et al., 1999; Holt, M. et al., 2004), the RRP in hair cells comprises a miniscule fraction of the total (Moser, T. and Beutner, D., 2000; Schnee M. E. et al., 2005; Rutherford, M. A. and Roberts, W. M., 2006).
At conventional synapses, the RRP is generally thought to correspond to the docked vesicles, those seen in EM to be in contact with the plasmalemma at the AZ. The corresponding pool of morphologically docked vesicles at ribbon synapses consists mostly of vesicles that occupy the space between the SB and the plasmalemma at each AZ (Figures 3 and 4). Differences in the precise criteria used for anatomical classification of vesicles can cause estimates of the size of the docked vesicle pool to differ by a factor of two, even in careful quantitative studies.

There is ambiguity about how to classify vesicles that are tethered to the SB but do not contact the plasma membrane. Direct evidence that these vesicles even participate in transmitter release under normal conditions is scant (Siegel, J. H. and Brownell, W. E., 1986), but assuming that they do, are they part of the RRP, the recycling pool, or some other pool? One reason to think that even though they reside far from the plasmalemma they might still be in the RRP is that they may be able to fuse with each other, forming a chain that connects to the cell surface (reviewed in Parsons, T. D. and Sterling, P., 2003; Edmonds, B. W. et al., 2004). Figure 4(c) shows what may be a chain of fused vesicles. If the morphological criterion that has been used to determine whether a vesicle is docked to the plasmalemma is applied to vesicles on the SB, then many of these vesicles are morphologically docked to each other, and thus could conceivably be ready to fuse together, although the calcium signal is expected to be highly attenuated more than a few vesicle diameters from the calcium channels on the cell surface (Roberts, W. M., 1994). Alternatively, the type of structures shown in Figure 4(c), which were observed after extended depolarization in high potassium (Lenzi, D. et al., 2002), could reflect membrane retrieval.

The available physiological evidence indicates that the RRP in the frog sacculus and mouse cochlea is much smaller than the total pool of SB-associated vesicles, and is close to the number of vesicles docked at the plasmalemma (Khimich, D. et al., 2005; Rutherford, M. A. and Roberts, W. M., 2006), which supports the more conventional hypothesis that only vesicles docked at the plasmalemma are part of the RRP, and that other SB-associated vesicles are part of the recycling vesicle pool that replenishes the RRP. Physiological correlates of these anatomical definitions are discussed in more detail in the following sections of this chapter.

Tens of thousands of clear-core vesicles are found in the hair cell cytoplasm within several hundred
nanometers of the cell surface, and hundreds of thousands more reside in the vast cell interior. They occur at a low average density in frog saccular hair cells (0.4% of dense packed = ~100 vesicles per \( \mu m^3 \)), but are much more concentrated within 700 nm of each synapse (5–10% of dense packed = ~1200–2400 vesicles per \( \mu m^3 \); Lenzi, D. et al., 1999; 2002). The contribution of these vesicles to synaptic transmission under normal physiological conditions is unclear. At conventional synapses, there is a physiologically distinct (but sometimes not anatomically distinct; Rizzoli, S. O. and Betz, W. J., 2005) recycling pool of SVs that are reused preferentially by local endocytosis (Pyle, J. L. et al., 2000; Stevens, C. F. and Williams, J. H., 2000), whereas vesicles in the reserve pool enter the exocytic pathway only under strong stimulus conditions that also cause bulk endocytosis and vesicle reconstitution through an endosomal compartment. It remains unknown whether hair cell afferent synapses have distinct recycling and reserve SV pools.

The AZ at most brain synapses contains only a few docked vesicles, and a single presynaptic action potential seldom evokes exocytosis of neurotransmitter from more than one of them, with frequent failures (Dobrunz, L. E. and Stevens, C. F., 1997; for a review see Sudhof, T. C., 2000). In contrast, the docked vesicle pool is considerably larger at AZs in hair cells of the turtle papilla (mean of 30; Schnee, M. E. et al., 2005), mouse cochlea (16–30, IHC; Khimich, D. et al., 2005), and the frog sacculus (mean of 43; Lenzi, D. et al., 2002). Furthermore, both pre- and postsynaptic assays of SV exocytosis (see Sections 3.22.5–3.22.7) have indicated that more than one SV can fuse in response to a single, brief presynaptic stimulus (reviewed in Fuchs, P. A., 2005). The stimulus to open voltage-gated calcium channels (VGCCs) in hair cells is not an all-or-none action potential that lasts on the order of 1 ms, rather, they respond to depolarizations of varying duration and intensity with graded release of neurotransmitter. Thus, while some generalizations can be made across all synapse types, there may be important differences between SV pools at conventional synapses and hair cell afferent synapses.

### 3.22.3.3 Calcium Buffering in Hair Cells

Exogenous calcium buffer can be introduced into a hair cell through a micropipette recording electrode, and has been shown to influence synaptic transmission. Interestingly, a high concentration of calcium buffer with fast binding kinetics is required to interfere with exocytosis from the RRP, whereas refilling of the RRP can be inhibited by low concentrations of slower buffer (Moser, T. and Beutner, D., 2000). It is concluded that although both processes are regulated by calcium, refilling of the RRP involves signaling over a longer distance, and is therefore more susceptible to disruption by calcium buffers. To understand the interaction between calcium ions and calcium buffers that can diffuse in cytoplasm one must think about the rates of binding and diffusion, not just equilibrium binding of calcium to the buffer. A brief introduction to the essential concepts needed to understand the nonequilibrium interaction between a mobile calcium buffer and the calcium ions that enter through a calcium channel and diffuse into a large cytoplasmic volume is given below.

High concentrations of calcium-binding proteins can limit the spread of calcium signals within a cell (Roberts, W. M., 1994). This may be particularly important in hair cells, because they use calcium signals for several distinct cellular processes, including feedback inhibition of the transduction current, which involves calcium entry and action at the stereociliary tips (see Chapter Hair Cell Transduction and Adaptation: Physiology and Molecular Mechanisms), and efferent synaptic transmission see Chapter Efferent System at postsynaptic sites, which intermingle with afferent presynaptic sites on the hair cell’s basolateral surface. In addition to localized transmembrane calcium signals, hair cells also exhibit localized calcium-induced calcium release from intracellular stores near sites of efferent contact (Lioudyno, M. et al., 2004).

The equilibrium interaction between calcium and a molecule that has a single calcium-binding site is characterized by a single number, the dissociation constant, \( K_D \) (units: molar, M), or its reciprocal, \( K_A \) (M\(^{-1}\)):

\[
K_D = \frac{[Ca][B]}{[CaB]}
\]

where [Ca] and [B] are the concentrations (M) of free Ca and free buffer, and [CaB] is the concentration of calcium bound to the buffer. Free means that they are able to interact with each other (i.e., not already bound to something else, hidden inside an organelle, etc.); whether or not they are free to diffuse in the cytoplasm is a separate issue that is also important. Time does not appear in the units of these variables. It is assumed that enough time has elapsed for equilibrium to be attained.

The equilibrium equation does not apply near an open calcium channel because equilibrium is never
reached while the calcium current persists (which requires an extrusion mechanism to keep the cell from filling up with calcium). The existence of a calcium current is prima facie evidence of disequilibrium, at least locally around the mouth of the channel. We will not specifically consider the calcium pump here, except to note that the maximum flux of calcium ions through a single pump \( \approx 10^4 \text{s}^{-1} \) is roughly one thousandth the flux through an open calcium channel \( \approx 10^7 \text{s}^{-1} \), so that the local calcium gradient around a single pump is miniscule compared to the gradient near the mouth of a single calcium channel. Therefore, it makes sense to consider the calcium extrusion process to be distributed over a large region of cell surface, and ignore its effect on the localized region of extremely high free calcium near an open calcium channel.

Two parameters, \( k_{\text{on}} \) \( \text{(M}^{-1}\text{s}^{-1} \) and \( k_{\text{off}} \) \( \text{s}^{-1} \), are needed to describe the kinetics of calcium binding to a single-site buffer. \( k_{\text{on}}[B] \) and \( k_{\text{off}} \), respectively, the rate constants for calcium binding to and unbinding from the buffer. The three parameters, \( k_{\text{on}}, k_{\text{off}}, \) and \( K_D \) are related by \( K_D = k_{\text{off}}/k_{\text{on}} \), so any two specify the third.

A calcium ion is initially free as it enters the cell through a calcium channel. The mean time required to encounter and bind to a buffer molecule is \( 1/(k_{\text{on}}[B]) \), which for the buffering conditions encountered in many hair cells \([B] \gg K_D\) is close to the equilibration time constant \( \tau \) in response to a step change in free calcium:

\[
\tau = \frac{1}{k_{\text{off}}([B] + K_D)}
\]

During this time, a free calcium ion diffuses a characteristic distance of

\[
\lambda = \sqrt{\tau D_{Ca}}
\]

where \( D_{Ca} \) is the diffusion coefficient for free calcium in cytoplasm (Neher, E. and Augustine, J. G. 1992; Roberts, W. M., 1993). The parameters \( \tau \) and \( \lambda \) are, respectively, the time and space constants for calcium binding to the buffer in the vicinity of an open calcium channel.

If the distance between the presynaptic calcium channels and the calcium sensors that trigger exocytosis is substantially less than \( \lambda \) then the buffer has little effect. If the distance is substantially greater than \( \lambda \) then the buffer has time to come into equilibrium with global free calcium and binding kinetics are not important. If the distance is similar to \( \lambda \) then the binding kinetics are important and the buffer’s effects can be complex. The diffusion coefficient for the buffer in cytoplasm is also important because slow diffusion reduces the rate at which calcium-free buffer is replenished near the open channel, which reduces the local \([B]\), and in turn locally increases both \( \tau \) and \( \lambda \). At hair cell afferent synapses, exocytosis of RRP vesicles falls into the first category, where cytoplasmic buffers are irrelevant, but some other important calcium-dependent steps in the SV cycle appear to fall into the complex category, where buffer concentration, binding kinetics, and diffusion coefficient all come into play.

To assign approximate values to \( \tau \) and \( \lambda \) one needs to know the concentration and \( k_{\text{on}} \) for the predominant diffusationally mobile calcium buffer(s) in the cell. Many types of hair cells are intensely labeled by fluorescent antibodies against one or more of the diffusible calcium-binding proteins calbindin-D28k, calretinin, parvalbumin-\( \alpha \), and parvalbumin-\( \beta \) (also known as oncomodulin or PV-3). For example, some hair cells in the frog sacculus contain calretinin at a concentration above 1 mM, which provides more than 5 mM calcium-binding sites (Edmonds, B. et al., 2000). Other frog saccular hair cells contain predominantly parvalbumin \( \beta \), at the extraordinary concentration of 4 mM (Heller, S. et al., 2002), which amounts to 8 mM calcium-binding sites. OHCs in the mammalian cochlea contain different calcium-binding proteins than are found in the IHCs, and appear to express them at a tenfold higher concentration (calbindin-D28k and parvalbumin-\( \beta \) equivalent to 5 mM calcium-binding sites; Hackney, C. M. et al., 2005). These extreme concentrations of calcium buffer are certain to have important consequences for many calcium signals in the cell. It is also worth remembering that some calcium-binding proteins may serve both as buffers and as calcium sensors that influence exocytosis or participate in other steps in the SV cycle.

For typical values of \( K_D = 1 \mu\text{M} \) and \( k_{\text{on}} = 10^7 \text{M}^{-1}\text{s}^{-1} \), the buffer space constant and time constant in a cell that has a moderate calcium buffer concentration \([B] = 100 \mu\text{M} \) are \( \lambda = 450 \text{ nm} \) and \( \tau = 1 \text{ ms} \). Increasing the buffer concentration 100-fold to 10 mM decreases \( \lambda \) to 44 nm and \( \tau \) to 10 \mu\text{s} \). From this calculation, one can see that an extremely high concentration of a calcium buffer can reduce the buffering space constant from approximately the diameter of the entire AZ (Figure 3(c)) to approximately the diameter of a single SV, thereby minimizing the overlap of calcium...
domains around each open calcium channel, particularly at barely perceptible sound intensities where only a few calcium channels are open at each afferent synapse. The close association between calcium channels and AZs (Lewis, R. S. and Hudspeth, A. J., 1983; Art, J. J. and Fettiplace, R., 1987; Roberts, W. M. et al., 1990; Issa, N. P. and Hudspeth, A. J., 1994; Martinez-Dunst, C. et al., 1997; Rodriguez-Contreras, A. and Yamoah, E. N., 2001; Zenisek, D. et al., 2003; Brandt, A. et al., 2005) means that steep spatial calcium gradients and the nonequilibrium calcium binding to buffer are important at afferent synapses.

At distances on the order of \( \lambda \) from an open channel, the buffer serves as a calcium sink, and can greatly reduce the free calcium concentration (Roberts, W. M., 1994). At larger distances, where free calcium falls below the buffer’s \( K_D \), the buffer becomes a calcium source as it dumps its bound calcium, although the large volume available to receive this calcium means that the calcium rise associated with unbinding from the buffer is orders of magnitude smaller than the calcium concentration near the source. Because the buffer molecules are large compared to a calcium ion, bound calcium diffuses more slowly than free calcium. The net result is that the buffer can greatly increase total calcium near an open calcium channel, and can slightly increase free calcium at certain distances from the channel, but its main effect is to greatly reduce the extremely high concentration of free calcium that accumulates near the channel (i.e., at distances on the order of \( \lambda \)).

More detailed discussions of these and other properties of mobile calcium buffers are in the literature (e.g., Stern, M. D., 1992; Roberts, W. M., 1993; 1994; Dargan, S. L. and Parker, I., 2003). One interesting result is that the average distance that a calcium ion travels while remaining bound to the buffer is >1 \( \mu \)m, which is large compared to the AZ diameter. Thus, the mobile buffer gives a calcium ion only one brief chance to exert its effect. Either it binds to its target, such as the calcium sensor that triggers exocytosis, or it is captured by the mobile buffer and is carried away. If the buffer has reasonably fast binding kinetics (\( k_{\text{on}} = 10^7 \text{M}^{-1} \text{s}^{-1} \)) and provides 5–10 mM of mobile binding sites for Ca (Edmonds, B. et al., 2000; Heller, S. et al., 2002), then the average time available for a calcium ion to encounter and bind to its target (i.e., the binding time constant, \( \tau \)) is only 10–20 \( \mu \)s.

### 3.22.4 Ribbon Synapses – A Small but Diverse Class

The SBs of hair cells are homologous to the presynaptic ribbons found in rods, cones, and bipolar cells in the retina, and pinealocytes in the pineal body. Like hair cells, these cells usually do not make action potentials. Because of their anatomical, physiological, and molecular similarities, we include the afferent synapses of hair cells in the class of ribbon synapses, but one should also keep in mind the differences, including, for example, the slow-time scale of the visual system compared to the auditory system.

Ribbon synapses are understood to be specialized for sustained transmitter release. For example, vertebrate photoreceptors release neurotransmitter continuously in the dark (Rea, R. et al., 2004) and the hair cell afferent synapse is active in the absence of an applied stimulus (Siegel, J. H., 1992). However, the hair cell is also capable of releasing transmitter phasically (e.g., EPSPs that are correlated with the periodicity of the stimulus for afferent fibers at frequencies up to a kilohertz; Figures 1(c) and 2(c), see also Section 3.22.6). The details of synaptic physiology that vary among ribbon synapses from different tissues and cell types are likely to be essential for their function (for a review, see Sterling, P. and Matthews, G., 2005). Nevertheless, the first synapse in the pathways for vision, hearing, balance, and lateral line senses in all vertebrate animals is a ribbon synapse.

### 3.22.4.1 The Synaptic Body (Ribbon) and Its Vesicles

The term ribbon originates from the flat, almost 2D morphology of the presynaptic structures observed in transmission EMs of retinal photoreceptors and bipolar cells. Serial reconstructions of gluteraldehyde-fixed hair cells from many preparations, including mammalian cochlea, have shown that the SB in hair cells has a more spherical or ellipsoid shape. IHCs rely exclusively on ribbon synapses for afferent transmission. The role of SBs in OHCs is more uncertain as they (the SBs) are far fewer and often seen only during development (Smith, C. A. and Sjostrand, F. S., 1961; Siegel J. H. and Brownell W. E., 1981; Sobkowicz, H. M. et al., 1986).

Although SBs are heavily stained by osmium, which typically labels lipid membranes, they are thought to be composed primarily of protein...
(Krstic, R., 1976). The osmiophilic nature of the SB is well-known because OsO₄ is a common EM fixative and is electron-dense. Perhaps more relevant to function, the ribbon has been reported to bind both calcium and phosphate (Schmitz, F. and Drenckhahn, D., 1991), although there is no evidence that it binds or releases these ions in the process of synaptic transmission.

SBs at AZs are always found in association with ∼40 nm-diameter clear-cored vesicles, which are attached to it by thin filaments (Usukura, J. and Yamada, E., 1987) and often cover its surface nearly completely (Figures 3 and 4; Lenzi, D. et al., 2002). SBs can sometimes be found in the cell interior, either singly or in clusters, where they are also covered in vesicles (Miller, M. R. and Beck, J., 1987). The molecular identity of the filaments is not known, except that they are thought not to contain synapsins I or II, which form filaments linking SVs to cytoskeleton at other synapses (Hirokawa, N. et al., 1989), but are absent from ribbon synapses (von Kriegstein, K. et al., 1999). Based on antibody labeling, the filaments could contain the KIF3A subunit of the kinesin 2 motor complex (Muresan, V. et al., 1999), which forms 50 nm-long rods in vitro (Yamazaki, H. et al., 1995), or possibly one or more of the many other KIF3 isoforms that may cross react with the antibody.

SBs and ribbons can be regarded as elaborations of the cytomatrix at the active zone (CAZ) seen at all synapses (Zhai, R. G. and Bellen, H. J., 2004), but in EM the SB appears as a separate structure, connected to the CAZ by filamentous links (Usukura, J. and Yamada, E., 1987; Lenzi, D. et al., 2002). The tight association between the SVs and the SB, and between the SB and the presynaptic plasma membrane is evident in cells that were broken open prior to fixation (Figure 3(b)). If the binding were weak or disrupted by exposure to the high-calcium concentration of extracellular saline, these structures would not have remained behind while the rest of the cytoplasm was washed away.

### 3.22.4.2 Molecular Specializations of Ribbon Synapses

Understanding of the molecular composition of hair cell afferent synapses has begun (Figure 5), but is far from complete (Wagner, H.-J., 1997). A protein, Ribeye

![Figure 5 Molecular specializations of the hair cell ribbon synapse.](image)

**Figure 5** Molecular specializations of the hair cell ribbon synapse. (a) Localization of proteins associated with the synaptic ribbon and plasma membrane density of the cytomatrix at the active zone (CAZ), labeled in (b). Voltage-gated calcium channels (VGCCs) are clustered underneath the ribbon and juxtaposed to postsynaptic α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptors. In some hair cells, presynaptic calcium-sensitive voltage-gated (BK) potassium channels intermingle with the VGCCs. Glutamate transporters in supporting cells clear neurotransmitter from the cleft. Bassoon anchors the ribbon to the Cytomatrix at the active zone (CAZ) plasma membrane density via its interaction with the RIBEYE. Bassoon, RIBEYE/CtBP2, CtBP1, and Piccolo have been observed at hair cell afferent synapses. The existence of the other proteins at the hair cell’s ribbon synapse is by inference from observations at retinal ribbon synapses (tom Dieck, S. et al., 2005). Reproduced from The Journal of Cell Biology, 2005, 168, 825–836 by Copyright permission of The Rockefeller University Press; and Reprinted from Curr. Opin. Neurobiol. Vol. 13, Fuchs, P. A., Glowatzki, E., and Moser, T., The afferent synapse and cochlear hair cells, 452–458, Copyright 2003, with permission from Elsevier.
(Schmitz, F. et al., 2000), has been identified that appears to be a component of all synaptic ribbons, and is expressed nowhere else. This finding justifies exclusion of T-bar synapses of arthropods (e.g., Drosophila) from the class of ribbon synapses because the Drosophila genome contains no DNA sequences that code for a homolog of the unique exon 1 of the Ribeye protein (Bradford, Y. and Roberts, W. M., unpublished observations). Ribeye is one of two known protein products of the CtBP2 gene. The other product, CtBP1, is a transcription regulator (reviewed in Chinnadurai, G., 2002). A recent report suggests that the CtBP1 protein, which is the product of a closely related gene and also a known transcription regulator, is a presynaptic component of both hair cell (Moser, T., personal communication) and retinal (tom Dieck, S. et al., 2005) ribbon synapses, as well as conventional synapses, raising the possibility that the ancestral CtBP protein had a presynaptic function in addition to being a transcriptional corepressor, and conceivably could link synaptic activity to gene expression. The CAZ-associated protein Bassoon was recently shown to anchor the ribbon at the photoreceptor synapse via a physical interaction with Ribeye (tom Dieck, S. et al., 2005). Bassoon and the related protein Piccolo, are also present at hair cell afferent synapses (Khimich, D. et al., 2005). Bassoon appears to interact with both Ribeye and CtBP1, which are both localized to ribbons even in the absence of functional Bassoon protein.

Drosophila has a single CtBP gene that produces a transcription regulator essential for embryonic development (Nibu, Y. et al., 1998; Poortinga, G. et al., 1998), but it is not known whether CtBP is present at synapses in Drosophila. Similarly, the Ciona genome (a urochordate) has a single CtBP gene that lacks the coding sequence for Ribeye (Bradford, Y. and Roberts, W. M., unpublished observations). The simplest evolutionary scenario consistent with the data is that a gene duplication event early in the vertebrate lineage, but after the divergence of urochordates from vertebrates, gave rise to paralogous genes, CtBP1 and CtBP2. The CtBP2 gene then acquired an alternate first exon, which became involved in aggregation of CAZ components (Schmitz, F. et al., 2000) into large structures (ribbons), with important, but largely unknown consequences for afferent synaptic transmission in the eye and ear. Ribeye expression became limited to the small class of ribbon synapses, while CtBP1 continues to be expressed at other synapses. An additional gene duplication gave rise to two CtBP2/ribeye genes in teleost fishes (Wan, L. et al., 2005). Besides Ribeye, only one other protein, the B16 antigen, has been proposed to be a unique component of all ribbon synapses. This protein is known only as an antigenic epitope (amino sequence: DTYQHPPKDS; Balkema, G. W., 1991; Nguyen, T. H. and Balkema, G. W., 1999). A search of the human genome and expressed sequence tag (EST) databases shows that only one gene, aconitase, contains a nucleotide sequence that codes for this exact amino acid sequence. Aconitase is a transcription regulator and a regulator of iron metabolism, but reportedly is not present in synaptic ribbons (Nguyen, T. H. and Balkema, G. W., 1999). Human CtBP2 and Ribeye contain a sequence (TYxxPP) near the C-terminus that is part of the B16 antigen and thus might bind the B16 antibody, but synaptic ribbons in fish reportedly also bind the B16 antibody (Balkema, G. W., 1991) even though this sequence is entirely absent from their CtBP2 and Ribeye proteins. Thus, the component of the ribbon that binds antibodies raised against the DTYQHPPKDS peptide remains unknown.

There are other proteins that may be unique to ribbon synapses but are not expressed at all ribbon synapses. The $\alpha_{1D}$ calcium channel is found only in hair cells and is essential for normal hair cell $Ca^{2+}$ current, neurotransmitter release, and for hearing (reviewed in Moser, T. et al., 2006a). Most synapses express N, P/Q, or R-type calcium channels, but hair cells express L-type calcium channels (Cav 1.3) formed by this unique splice variant. This channel opens at unusually hyperpolarized voltages (Koschak, A. et al., 2001; Brandt, A. et al., 2003; Rodriguez-Contreras, A. and Yamoah, E. N., 2003; Schnee, M. E. and Ricci, A. J., 2003), which may cause the transmitter release responsible for the baseline action potential rate (i.e., SR) inafferent neurons. Retinal rods express a different L-type calcium channel at ribbon synapses, Cav 1.4 ($\alpha_{1V}$; Morgans, C. W., 2001).

Another protein that may be unique to ribbon synapses is complexin IV (Pabst, S. et al., 2000; Reim, K. et al., 2006). Based on similarity of complexin I and II, and the ability to rescue synaptic transmission in complexin I/II knockout mice, both complexin III and complexin IV are potential calcium-dependent regulators of synaptic transmission. Complexins I and II are known to bind to the soluble NSF (N-ethylmaleimide-sensitive factor) attachment receptor (SNARE) complex that forms the core of the vesicle fusion machinery at many synapses (McMahon, H. T. et al., 1995; Reim, K. et al., 2001; Chen, X. et al., 2002; for a review, see Sudhof, T. C., 2004). Complexins III and IV have both been
described at ribbon synapses in the retina, but only complexin IV was found to be unique to retinal ribbon synapses. It was not reported whether complexins III or IV are present in the ear.

Otoferlin is another protein that may be involved in synaptic transmission by hair cells. This protein was discovered by genetic mapping of familial deafness (Yasunaga, S. et al., 1999), and is essential for hearing. The otoferlin gene is large and complex, having 48 exons and many splice variants (Yasunaga, S. et al., 2000). It is similar to the FER-1 protein of Caenorhabditis elegans, which has been implicated in vesicle membrane fusion (Achanzar, W. E. and Ward, S., 1997). Otoferlin is a candidate to substitute for synaptotagmin as the calcium sensor for transmitter release (Yasunaga, S. et al., 2000).

Several other presynaptic proteins, including synapsins and synaptotagmins, are notable by their absence from ribbon synapses (Safieddine S. and Wenthold R. J., 1999; von Kriegstein, K. et al., 1999). Syntaxin 1, a component of the core SNARE complex is also missing, replaced by syntaxin 3 (Morgans, C. W. et al., 1996).

### 3.22.4.3 Anatomy of the Synaptic Body: Heterogeneity of Structure

SBs come in a bewildering variety of shapes and sizes. Most synapses have one SB at the synaptic complex, others have two or three (Liberman, M. C. et al., 1990). In addition to the typically spherical or ellipsoidal shapes of SBs in hair cells from mice, turtles, frogs, and chicks (Sneary, M. G., 1988; Martinez-Dunst, C. et al., 1997; Lenzi, D. et al., 1999; Khimich, D. et al., 2005), SBs of different geometries and densities have been observed in EM (Giannessi, F. and Ruffoli, R., 1996). In many types of hair cells, the SB appears as a uniformly dense blob, with no internal structure but it also can have a laminar appearance, as in some cochlear IHCs. In old world monkeys the SBs of IHCs nearly always appear ring shaped (Bodian, D., 1980). It is unclear how much of this variability is an artifact of fixation and staining. For example, in the frog retina, synaptic ribbons of a lamellar nature were observed with conventional transmission electron microscope (TEM) thin sections, but were not so apparent when freeze-etching and freeze-substitution methods were employed for tissue preparation (Usukura, J. and Yamada, E., 1987).

SBs vary in size and number among organs, within and between species, as well as among cells within a particular organ. Furthermore, SBs vary in size and shape within individual cells. The dimensions of the SB are typically submicron, ranging from ~200 nm for the shortest principle axis of the ellipsoid SBs in mouse IHCs (Khimich, D. et al., 2005) to ~400 nm in hair cells of frog and goldfish sacculus (Gleisner, L. et al., 1973; Hama, K. and Saito, K., 1977; Jacobs, R. A. and Hudspeth, A. J., 1990; Lenzi, D. et al., 1999). Estimates of the number of ribbon-containing synapses per cell ranged from 10 in mouse cochlear IHCs (Khimich, D. et al., 2005) to 85 in the high-frequency hair cells of the turtle papilla (Sneary, M. G., 1988).

Several studies have provided clues regarding the function of SBs in hair cells by demonstrating that the size and number of synaptic ribbons, as well as the numbers of associated calcium channels and SVs, is correlated with the position of the hair cell on the tonotopic axis of the organ. In the turtle papilla, high-frequency cells have three times more SBs, higher vesicle densities near SBs, more calcium channels, and larger calcium currents than do lower-frequency cells (Sneary, M. G., 1988; Art, J. J. et al., 1995; Schnee, M. E. et al., 2005). Upon depolarization, the high-frequency cells released more vesicles and with faster kinetics. A model of SV trafficking that incorporated all of these data predicted that these differences result in transmission being optimized to the CF of the hair cell (Schnee, M. E. et al., 2005). In the papilla of the chick, the diameter of the SB ranged from 120 to 210 nm with increasing CF from apical to more basal positions along the tonotopic axis (Martinez-Dunst, C. et al., 1997). These two studies used different experimental approaches to reach a similar conclusion; that the morphology of the SB has influence over the numbers of SVs that are maintained at well-defined positions with respect to the source of calcium influx. SVs close to the center of the AZ may be more responsive to calcium influx if the channels are clustered there (Furukawa, T. and Matsuura, S., 1978; Furukawa, T. et al., 1982; reviewed for the case of CNS synapses in Schneggenburger, R. and Neher, E., 2005).

A study in the mammalian cochlea has reported presynaptic morphological correlates of ANF SR and threshold (Merchan-Perez, A. and Liberman, M. C., 1996). Analysis of ultrastructure at the EM level showed that the size and complexity of SBs tended to increase as SR decreased, accompanied by an increase in the total number of SVs clustered at the AZ. These data suggest that the size of the SB has something to do with the regulation of vesicle release that determines the baseline afferent discharge rate.
and sensitivity to faint sounds, but the relationship is opposite what is expected based on the prevailing view that the ribbon collects vesicles and conveys them to the release sites. Instead, these data seem to suggest that a larger SB may somehow inhibit SV fusion for weak sounds. Clearly, more work needs to be done to understand the relationship between SB morphology and postsynaptic properties.

### 3.22.5 The Synaptic Vesicle Cycle in Hair Cells

The basic features of the SV cycle are similar at conventional chemical synapses and ribbon synapses. Both baseline and evoked EPSCs at ribbon synapses require presynaptic calcium influx through channels (Robertson, D. and Paki, B., 2002; Keen, E. C. and Hudspeth, A. J., 2006) and are completely and reversibly blocked by antagonists of the α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA)-type glutamate receptor (Glowatzki, E. and Fuchs, P. A., 2002; Keen, E. C. and Hudspeth, A. J., 2006; Holt, J. C. et al., 2006). The calcium-controlled release of glutamate into the synaptic cleft is quantal, involving exocytosis from SVs that fuse with the plasmalemma and are later retrieved for reuse.

#### 3.22.5.1 Endocytosis

Various routes for vesicle recycling at chemical synapses have been proposed, including two controversial types of fast, local reuse in which the neurotransmitter leaves the SV through a fusion pore without full collapse of the SV membrane into the plasmalemma. In one of these hypothetical fast recycling routes, named kiss-and-stay, the vesicle remains docked to the AZ where it is presumably refilled with glutamate after discharging its contents. In the other, named kiss-and-run, the vesicle undocks and enters a local recycling pool. Slower recycling pathways involve full fusion of the SV with the plasmalemma, followed by clathrin-dependent endocytosis of vesicles or bulk membrane retrieval and reprocessing of SVs either directly or via an endosomal route (reviewed in Sudhof, T. C., 2004). The extent to which each of these processes contributes to the SV cycle under natural physiological conditions remains unclear.

Recent work in the cochlea showed that IHCs may be able to draw upon the vast supply of preformed vesicles present throughout the cytoplasm, and thus may not have to rely on endocytic routes for replenishment of releasable SVs for even the longest stimuli used in many experiments (Griesinger, C. B. et al., 2005). For example, a hair cell that contains 1 million vesicles could, in principle, sustain the exocytosis of 10 vesicles per millisecond for 100 s, although it is hard to imagine how the hair cell could accommodate the resultant threefold increase in surface area. Clearly, in the long run endocytosis must still keep pace with exocytosis.

#### 3.22.5.2 Vesicle Mobility

The movement of vesicular membrane through the cytoplasm is an essential step in the SV cycle, except for the case of kiss-and-stay. At conventional synapses, the vesicle-associated phosphoproteins, called synapsins, play essential roles in regulating the movement and clustering of SVs (Rosahl, T. et al., 1995). Studies on hippocampal synapses in synapsin knockout mice have shown that synapsins contribute to the SV cycle by maintaining the reserve pool and the RRP at glutamatergic and GABAergic terminals, respectively (Gitler, D. et al., 2004). The synapsins tether SVs to actin filaments in a phosphorylation-dependent manner (for a review, see Hilfiker, S. et al., 1999).

Synapsins are absent at ribbon synapses, supporting the idea that vesicle clustering and mobilization are different in these cells (von Kriegstein, K. et al., 1999). Recent work in retinal bipolar cells utilized EM, confocal microscopy, and total internal reflection microscopy to demonstrate that cytoplasmic vesicle movement is rapid, random, and not altered by calcium influx or cytoskeletal interactions (Holt, M. et al., 2004, reviewed in Sterling, P. and Matthews, G., 2005). The authors concluded that SVs 2 are highly mobile in bipolar cells ($D = 1.5 \times 10^{-2} \mu m s^{-1}$), and that random vesicle movement (i.e., diffusion) is sufficient to account for the translocation of vesicles from the large cytoplasmic pool to release sites.

If baseline spike rates in ANFs are due to fusion of SVs, then the diffusion coefficient reported for SVs in bipolar terminals is far too low to support continuous resupply of release sites at mammalian hair cell synapses that sustain SRs of $>100$ Hz unless some additional mechanism exists to concentrate vesicles in the vicinity of the synapse. Even assuming that diffusion is used only to transport vesicles to the SB, whereupon they are instantly transported to release sites by some other means, and ignoring the
plasmalemma and other barriers that restrict diffusional access to the SB, the maximum steady-state rate at which vesicles can reach the SB by diffusion is

\[ F = 4\pi DRC \]

where \( C \) is the global concentration of vesicles in the volume. Substituting \( D = 1.5 \times 10^{-2} \mu m^2 s^{-1} \), \( F = 100 s^{-1} \), and \( R = 100 \text{nm} \), and solving for \( C \) yields \( C = 5000 \text{vesicles} \mu m^{-4} \), which is more than 50 times the density reported in the nonsynaptic regions of hair cells. This calculation shows that the average concentration of SVs throughout the cytoplasm is too low to continuously supply the SB with vesicles by diffusion unless some additional process operates to maintain the locally high concentration of SVs within a few hundred nanometers of the SB (Lenzi, D. et al., 1999; 2002). Experiments like these, combined with the observed presence of synaptic ribbons in cell types that support continuous exocytosis have led to the view that the structural and molecular specializations of ribbon synapses assist high rates of sustained vesicle supply to AZs, but crucial questions remain unanswered. For example, data for the refilling of the RRP by vesicles on the SB are indirect; it is still unknown whether or not vesicles actually move on the ribbon.

### 3.22.5.3 Docking and Priming

In addition to translocation to the AZ, other things must occur before a vesicle is ready for exocytosis. Two important but relatively poorly understood steps are called docking and priming, each of which likely embodies a series of associated events. Docking refers to the placement of a SV in contact with the inner leaflet of the plasma membrane whereas priming refers to the molecular reactions, some ATP-dependent, required to make a vesicle immediately ready for fusion upon calcium influx. It is unclear whether a vesicle must first be docked in order to be primed.

For clues to the relationship between fast exocytosis and docking/priming at ribbon synapses, we look to experimental evidence from retinal bipolar cells. Here the ribbon is plate-like and the anatomical picture of rows of vesicles tethered to both sides of the ribbon led to the model in which the vesicles move along the ribbon to docking sites. One hypothesis is that during a 100 ms strong depolarization all vesicles on the ribbon are depleted via exocytosis. Assuming that this exocytosis requires movement of vesicles along the ribbon to the plasmalemma, several lines of evidence argue against the involvement of ATP-dependent, kinesin-mediated translocation of vesicles to the plasmalemma (Heidelberger, R. et al., 2002). This observation led to the alternative hypothesis that SVs on the ribbon can fuse with each other (reviewed in Parsons, T. D. and Sterling, P., 2003), creating chains that extend to the plasmalemma. This mechanism can account for the large EPSCs recorded postsynaptically at hair cell synapses (see Section 3.22.6.3), and implies that vesicles on the ribbon can be primed and fuse with each other. However the protein Munc13-1, thought to be necessary for priming (Rosenmund, C. et al., 2003), was found to cluster with calcium channels at the plasma membrane and not on the ribbon (tom Dieck, S. et al., 2005), implying that the final priming steps must take place near the membrane.

### 3.22.5.4 Transmitter Release and Clearance

Glutamate released into the synaptic cleft binds to postsynaptic AMPA receptors (Ruel, J. et al., 2000; Glowatzki, E. and Fuchs, P. A., 2002; Keen, E. C. and Hudspeth, A. J., 2006), causing a depolarization of the postsynaptic membrane and, if the depolarization is sufficient, one or more action potentials. N-Methyl-D-aspartate (NMDA) receptor antagonist had no effect on ANF activity, suggesting that they are functionally absent. The pattern of spikes depends on the temporal pattern of neurotransmitter release, the kinetics of AMPA receptor activation, stochastic spike generation, and neuronal refractoriness. Most in vivo studies have focused on the firing properties of ANFs in response to pure tones of varying intensities.

If quantal release of neurotransmitter is to accurately reflect the temporal pattern of the sound stimulus, then the chemical signal in the synaptic cleft must rapidly be terminated, which involves uptake of glutamate from the synaptic cleft by excitatory amino acid transporters (e.g., GLAST) in surrounding supporting cells and afferents (Furness, D. N. and Lehre, K. P., 1997; Furness, D. N. and Lawton, D. M., 2003). Voltage-clamp recordings from inner phalangeal cells in the cochlea of normal rats and GLAST knockout mice showed the presence and absence, respectively, of currents produced by these transporters (Hakuba, N. et al., 2000). Normal glutamate reuptake from the synaptic cleft is also thought to be important for the prevention of noise-induced damage to afferent terminals, which can be mimicked by GLAST blockers (Hakuba, N. et al., 2000; Rebillard, G. et al., 2003) and prevented by AMPA receptor blockers.
3.22.5.5 Relating Anatomical and Functional Vesicle Pools

The hair cell's compact shape has proved fortuitous for experimental measurements of synaptic exocytosis by observing the increase in electrical capacitance associated with the increased cell surface that occurs when SVs fuse with the plasmalemma (Figures 6(a)–6(d); Lindau, M. and Neher, E., 1988; reviewed in Nouvian, R. et al., 2006). Because the surface area of a single SV can be estimated, capacitance changes can be translated into numbers of SVs exocytosed, although given the uncertainties in SV diameter and specific membrane capacitance, such calculations are probably only accurate to within a factor of two. These functionally derived SV counts can be compared to numbers obtained with anatomical studies at the EM level in order to test hypotheses about the relationships between the structure and the function of SV pools. The reliability of this technique in accurately monitoring the release of neurotransmitter has been verified by combining capacitance

![Figure 6](image-url)

**Figure 6** Exocytosis from inner hair cells, recovery from synaptic vesicle pool depletion, and adaptation/disadaptation of auditory nerve fiber responses. (a) Plasma membrane capacitance changes (upper) caused by depolarization-induced synaptic vesicle exocytosis that depends on calcium influx (lower). Two 15 ms depolarizations to the peak of the IV curve for calcium (−15 mV) were separated by 100 ms. Although the calcium currents evoked by the paired pulses were the same, the exocytic response to the first pulse was greater than the response to the second pulse (secretory depression). (b, c) The amplitude of stimulus-evoked capacitance changes is plotted as a function of the duration of the depolarization for different intracellular calcium-buffering conditions. (c) The first 50 ms of (b) at higher temporal resolution. With the cell’s endogenous buffer present, the readily releasable pool (RRP) is depleted within the first 10 ms, after which exocytosis continues at a slower rate that is sustained for at least 1 s. The slow calcium buffer (EGTA) of 5 mM abolishes the sustained release while leaving exocytosis of the RRP intact. The fast calcium buffer (BAPTA) of 5 mM inhibits both fast exocytosis of the RRP and the slower component of exocytosis. (d) Strong depolarizations sufficient to deplete the RRP were followed by a second identical stimulus with varying interstimulus intervals as indicated on the x-axis. Recovery of the RRP as a function of time after pool depletion has both rapid and slow components. (e) Auditory nerve fiber spike rate *in vivo* as a function of time after sound onset. The sound is interrupted by 10 ms (upper) or 160 ms (lower). During a sound presentation, the spike rate is initially very high followed by two phases of fast reduction in firing rate called rapid and short-term adaptation, with time courses of about 10 and 100 ms, respectively. The 10 ms interruption (upper) is not enough for recovery from rapid adaptation of the spike rate whereas the 160 ms interruption (lower) is sufficient for nearly complete recovery of the initial spike rate. Note the depression of baseline rate and its recovery after sound offset. Panels (a)–(d) are for the mouse: Reproduced with permission from, Copyright (2000) National Academy of Sciences, U. S. A., Moser, T. and Beutner, D., Kinetics of exocytosis and endocytosis at the cochlear inner hair cell afferent synapsis of the mouse. Proc. Natl. Acad. Sci. U S A 97, 883–888. Panel (e) is from the chick: JARO, Vol. 5, 2004, Evidence that rapid vesicle replenishment of the synaptic ribbon mediates recovery from short-term adaptation at the hair cell afferent synapse, Spassova, M. A., Avissar, M., Furman, A. C., Crumling M. A., Saunders, J. C., and Parsons, T. D., Copyright Springer, with kind permission of Springer Science and Business Media.
measurements with simultaneous detection of glutamate release in bipolar cells of the vertebrate retina (von Gersdorff, H. et al., 1998).

To estimate the size of the RRP in hair cells, synaptic transmission is first inhibited by hyperpolarization (to fill vesicle pools), then a depolarizing voltage stimulus that maximally stimulates exocytosis is delivered, and the ensuing capacitance increase is observed (Figures 6(b) and 6(c); reviewed in Fuchs, P. A. et al., 2003). Pairs of brief strong depolarizations that evoke maximal calcium currents typically cause paired-pulse depression (Figures 6(a) and 6(d)), implying that the first stimulus partially or totally depletes the RRP, which is refilled more slowly. The size of the RRP can also be measured by applying strong continuous depolarizations of various durations, which evoke continuous calcium currents from the L-type calcium channels at ribbon synapses. Exocytosis of the RRP is seen as an initial rapid increase in capacitance that lasts only a few milliseconds before giving way to a sustained secretory component (SSC) that lasts for seconds (Figure 6(b); Moser, T. and Beutner, D., 2000; Spassova, M. A. et al., 2004; Rutherford, M. A. and Roberts, W. M., 2006). Generally, the size of the RRP measured by change in capacitance is in good agreement with the size of the docked SV pool, as measured by EM, and is smaller than the pool of vesicles on the SB. The presence of a SB and the size of the RRP are both important for normal ANF responses (Khimich, D. et al., 2005; see also Section 3.2.2.7.2).

The density of vesicles on the SB and in the cytoplasm surrounding the AZ is sensitive to the physiological state of the hair cell before and during fixation (Lenzi, D. et al., 2002). In frog saccular hair cells, prolonged depolarization by high extracellular potassium reduces the density of vesicles on the SB from 79% to 42% of 3D dense-packed, with the greatest reduction occurring in the number of docked SVs in the narrow space between the SB and the plasmalemma. Fixation under resting conditions yielded intermediate values (Lenzi, D. et al., 2002). Whole-cell measurements of SV exocytosis support the notion that all of the docked, SB-associated vesicles can fuse with the plasmalemma within 10 ms (Rutherford, M. A. and Roberts, W. M., 2006). Vesicles in the cytoplasm that are not attached to the SB are also massively depleted by prolonged depolarization (from 10% to 2% of 3D dense-packed in reconstructed volumes within 800 nm perpendicular to the plasmalemma; Figure 4).

Exocytosis of the SSC is slow compared to the RRP (but still a fast resupply rate of hundreds of vesicles per second per AZ), and may indicate the rate at which the RRP is replenished from a recycling pool in the presence of a continuing calcium influx. Dialyzing the cell with the calcium buffer EGTA to inhibit the spread of free calcium away from the AZ (Figures 6(b) and 6(c)) blocked the SSC but not the initial exocytic burst from the RRP (Spassova, M. A. et al., 2004; Moser, T. and Beutner, D., 2000), which suggests that refilling of the RRP is stimulated by long-distance calcium signaling that can be blocked by a slow calcium buffer. Exocytosis of the RRP was inhibited only with the fast calcium buffer BAPTA, as is expected for the case of tight spatial coupling between the release-ready vesicles and the calcium channels (i.e., the RRP docked at the AZ). At present, it is unknown whether the SSC involves vesicles on the SB, other cytoplasmic vesicles that move to the AZ, or possibly even vesicles that are not located at AZs. Experiments that combine capacitance measurements with other techniques to monitor the flow of membrane through the SV cycle will be required to answer such questions.

3.22.6 Physiology of the Hair Cell Afferent Synapse

Precise encoding at the first synapse in the auditory system is absolutely critical for the complete range of behavioral interactions with the acoustic world. To produce a neural representation of the acoustic environment in the CNS, the mammalian cochlea first decomposes complex stimuli into separate frequency components, each of which has an amplitude and a phase. The simplest statement that can be made about sensory encoding in the mammalian cochlea is that the amplitude of the sound at a given frequency is encoded by the spike rate in the ANFs of corresponding CF, while phase and other temporal patterns (e.g., amplitude modulations) are specified by the timing of ANF spikes. Decades of work have produced a wealth of detail about sensory encoding in the auditory system, but much remains to be discovered. The following will barely scratch the surface of what is known. For additional information, the mechanisms underlying the temporal precision of sound coding at the IHC ribbon synapse have been recently reviewed (Moser, T. et al., 2006b).
3.22.6.1 Encoding of Phase

For low-frequency sinusoidal (tonal) stimuli, action potentials in ANFs occur within a narrow time window relative to each cycle of the sinusoid (Anderson, D. J. et al., 1970). The discharge of the ANF is said to be phase-locked to the stimulus (Rose, J. E. et al., 1967). Phase locking has been observed experimentally in the auditory pathways of mammals, birds, reptiles, amphibians, and fish, and is thought to be an important mechanism for the temporal encoding of low-frequency sound. For example, phase differences between the two ears provide important binaural cues for localizing sounds horizontally (Knudsen, E. I. and Konishi, M., 1978; see Chapter Encoding of Interaural Timing for Binaural Hearing).

Phase locking declines as frequency increases until action potentials in the auditory nerve become nearly random with respect to the stimulus period. The frequency where this transition occurs in the cochlear nerve is generally between 1 and 4 kHz, but it varies among species and even within an individual cochlea. In part, the decline of phase locking with increasing frequency can be attributed to the low-pass filtering of the AC component of the receptor potential by the hair cell membrane (Figure 7). When intracellular receptor potentials were recorded in vivo from a guinea-pig cochlear IHC, the receptor potential was quasinonuniform with the periodicity of the stimulus for low-frequency tones (Palmer, A. R. and Russell, I. J., 1986; Cheatham, M. A. and Dallos, P., 1993). The AC (periodic) component of the receptor potential began to decline at 600 Hz and was not detectable above 3.5 kHz, leaving only a DC (steady) depolarization (Figures 7(a) and 7(b)). The diminution of the presynaptic AC component was mirrored by a decline in postsynaptic phase locking (Figure 7(c)).

Recordings of spike trains in the saccular branch of the eighth nerve in frogs have shown that vibrations at frequencies ≤50 Hz evoke a single, phase-locked spike for each cycle of the stimulus (Koyama, H. et al., 1982; Christensen-Dalsgaard, J. and Narins P. M., 1993). When expressed as spikes per second, the response thus decreases in proportion to frequency below 50 Hz. Because for low frequencies the depolarizing phase of the hair cell receptor potential may be

![Figure 7](https://example.com/figure7.png)

**Figure 7** The relationship between receptor potentials in inner hair cells (IHCs) and phase locking in auditory nerve fibers. (a) Intracellular recordings of receptor potentials in one IHC of the guinea-pig cochlea in response to loud (80 dB sound pressure level) tones at the frequency (Hz) indicated by the right side of each trace. The receptor potential has an oscillatory (AC) component and a steady-state (DC) component. The AC component dominates at low frequencies but is almost absent above 3000 Hz. (b) The ratio of AC to DC component across frequency for the receptor potentials from nine different IHCs. (c) The synchronization index (a measure of phase locking to the tonal stimulus) plotted as a function of tone frequency. (Reprinted from Palmer, A. R. and Russell, I. J. 1986. Phase-locking in the cochlear nerve of the guinea-pig and its relation to the receptor potential of inner hair-cells. Hear Res. 24, 1–15, with permission from Elsevier.)
relatively long (e.g., 5 ms for 100 Hz) compared to the duration required for fusion of a single vesicle, the synapse could generate a single spike per cycle if synaptic exocytosis occurred primarily within a narrow time window in response to the onset of each depolarizing phase of a periodic stimulus, and was subsequently suppressed until the next depolarization. This possibility was tested using capacitance measurements as the assay of SV exocytosis (Rutherford, M. A. and Roberts, W. M., 2006). A 30 ms depolarization to −55 mV was interrupted with a return to −80 mV, thus creating a pair of 10 ms depolarizations separated by 10 ms. If the synapse responds primarily to stimulus onset, then the response to the interrupted depolarization is expected be twice the response to a maintained 30 ms depolarization, even though the interposed hyperpolarization reduces the total calcium influx. The total capacitance change in response to the interrupted stimulus was nearly twice as large as the response to the sustained depolarization. The result may reflect the mechanism by which hair cell limits the ANF discharge to a single phase-locked spike per stimulus cycle at low frequencies.

3.22.6.2 Encoding of Frequency

In general, all ANFs phase lock to low frequencies, regardless of their CF. Therefore, the timing of ANF spikes can provide information about the frequency of a low-frequency sound independent of the fiber’s CF (Figure 2). For frequencies above 3–4 kHz, the alternative to temporal coding is place-rate coding (Javel, E. and Mott, J. B., 1988), which does not rely upon the temporal (fine timing) pattern of spikes to communicate frequency. In the case of the mammalian cochlea, which performs exquisite mechanical tuning, frequency can be determined from peaks in action potential rates in ANFs that innervate specific CF regions (labeled lines) in the organ of Corti. Therefore, an increase in the mean firing rate of an ANF that originates from a particular place in the organ of Corti is a mechanism for specifying frequency to the brain. Auditory nuclei in the brain stem may use different decoding algorithms for different frequency ranges in order to interpret firing patterns of the auditory nerve.

3.22.6.3 Baseline and Evoked Synaptic Activity

Whole-cell recordings from postsynaptic afferent terminals demonstrated EPSCs (Figure 8) resulting from baseline and evoked SV exocytosis from rat IHCs (Glowatzki, E. and Fuchs, P. A., 2002) and from hair cells of the frog amphibian papilla (Keen, E. C. and Hudspeth, A. J., 2006). In both cases, EPSCs were fast (1–2 ms for the rat, 2–3 ms for the frog) with very rapid onset and exponential decay. Sensitivity to AMPA receptor antagonists provided a functional confirmation of previous in vivo and EM studies (Matsubara, A. et al., 1996), showing that the glutamatergic receptor subunits GluR2/3 and 4 are present.

The amplitude distribution of EPSCs recorded under baseline conditions (i.e., extracellular saline with normal concentration of potassium) showed ~20-fold variation in the rat and ~10-fold variation in the frog with mean sizes of 190 and 100 pA, respectively (Figure 8). This is remarkable considering that spontaneous glutamatergic EPSCs recorded elsewhere in the CNS are distributed normally with much smaller variation around a quantal size of 20–40 pA.

Presynaptic depolarization via paired whole-cell recordings in the frog, and by perfusion with high-potassium saline in the rat, greatly increased the EPSC rate (Figure 8(a)) while the amplitude distribution remained relatively unchanged (Figures 8(d) and 8(e)). In both preparations, individual small events (miniature EPSCs) were observed, but the amplitude distributions were skewed to larger events for both baseline and evoked EPSCs. For example, in the rat the peak in the amplitude distribution changed from about 18 pA in 5.8 mM potassium (baseline, Figure 8(d), left) to about 36 pA in 40 mM potassium (evoked, Figure 8(e), left), as the mean rate changed from about 1.5 to 27 events per second. Interestingly, the mean EPSC size was slightly less for evoked EPSCs, perhaps due to SV depletion with the sharp increase in EPSC frequency. Combined with the positive shift in the peak of the amplitude distribution, this results in a tightening of the EPSC amplitude distribution (e.g., smaller interquartile range), although the total range remains unchanged (cf. Figures 8(d) and 8(e), left). In both frog and rat hair cells, the larger EPSCs were sometimes multi-phasic, but the durations of the larger monophasic events were not appreciably longer than the smaller monophasic events (Figure 8(b), frog; Figures 8(c)–8(e), rat), which suggests that if the large EPSCs are due to the fusion of several SVs, then these fusion events are highly synchronized (reviewed in Parsons, T. D. and Sterling, P., 2003; Singer, J. H. et al., 2004). Two possible mechanisms might explain such multi-quantal events. One possibility is that the opening of
a single presynaptic calcium channel, or a few channels that happen to be close together, triggers the synchronized fusion of several nearby vesicles. Another is that several vesicles undergo nonsynchronous fusion with each other, but their contents are not released until one of them fuses with the plasmalemma.

When spikes are present in intracellular recordings from ANFs, the peak EPSP amplitude is obliterated by the action potential that it triggers, but the slope of the initial rise can be used instead to measure the EPSP size. In vivo recordings from afferent units in the guinea-pig cochlea demonstrated a range of EPSP slopes (Siegell, J. H., 1992). Similar to the EPSCs recorded in vitro and discussed above, the distribution of EPSP slopes was positively skewed, suggesting the existence of synchronous multiquantal release events. Interestingly, nearly all EPSPs that did not occur during neural refractory periods triggered action potentials.

Figure 8 Excitatory postsynaptic currents (EPSCs) in afferent fibers indicate the size and frequency of glutamate release from hair cells. (a) EPSCs recorded in afferent fibers of the frog amphibian papilla while the presynaptic hair cell was depolarized to the voltages indicated at the right of each trace. The predominant effect of depolarization was an increase in EPSC frequency, with little change in EPSC amplitudes. (b) The rise and decay times for baseline EPSCs (frog) are tightly distributed despite a wide range of amplitudes. (c) EPSCs evoked from rat cochlear afferent fibers in response to inner hair cell depolarization by 40 mM extracellular potassium, aligned in time, exhibit a large range in the amplitude of monophasic events. (d, e) Left: EPSC amplitude distributions from rat afferent fibers show the number of events observed in each amplitude bin along the x-axis. Baseline EPSC amplitudes from four afferents are shown in (d), evoked EPSC amplitudes from one afferent in (e). The distributions are positively skewed, suggestive of multivesicular release events from individual hair cell active zones. The range of baseline EPSC amplitudes observed in a normal concentration of extracellular potassium (5.8 mM) spans the entire range of EPSC amplitudes evoked by depolarization in high potassium (see text). Mean amplitudes were 190 pA (baseline) and 133 pA (evoked). Right: The relationship between EPSC amplitude and 10–90% rise time is very similar for baseline (d) and evoked (e) events. (a, b) Reproduced from Keen, E. C. and Hudspeth, A. J. 2006. Transfer characteristics of the hair cell’s afferent synapse. Proc. Natl. Acad. Sci. U. S. A. 103, 5537–5542; Copyright (2006) National Academy of Sciences, U. S. A. (c–e) Glowatzki, E. and Fuchs, P. A., 2002. Transmitter release at the hair cell ribbon synapse. Nat. Neurosci. 5, 147–154; Copyright (2002) Nature Publishing Group.
What then is the consequence of multivesicular release to synaptic function? One possibility is that large EPSCs trigger action potentials with less temporal jitter (Anderson, D. J. et al., 1970; reviewed in Fuchs, P. A., 2005), but this leaves open the question of whether there is some value in having a large variance in EPSP amplitude rather than all being equally large. An intriguing possibility has to do with the role of efferent innervation on the input resistance of afferent terminals. The terminal bouton of a type I afferent is typically postsynaptic to both an IHC and an efferent axon (see Chapter Efferent System). Spikes in the efferent axon release transmitter that opens postsynaptic channels, thereby reducing the input resistance of the afferent terminal. The effect of efferent activity may therefore be to reduce the size of the EPSP produced by a given EPSC. Since efferent activity increases in loud environments, the effect may be to render small EPSCs from the hair cell unable to trigger ANF spikes in loud environments. In this case, the wide distribution of EPSC amplitudes associated with multiquantal events may be important for efferent control of ANF firing rate. Similarly, efferent innervation directly onto hair cells is proposed to evoke inhibitory conductances in hair cells during strong stimuli. The expected effect would be a decrease in hair cell depolarization and calcium influx. Since efferent modulation of synaptic activity is expected to be largely absent from in vitro preparations, the effects of such inhibition on the EPSC amplitude and frequency in response to varying presynaptic stimulus levels are unknown.

If large EPSCs are due to the fusion of vesicles with each other on the SB before one of them fuses with the plasmalemma, this could be a mechanism to integrate sound amplitude over many cycles while preserving phase locking of glutamate release to the sound waveform. For this to work, the fusion of SVs with each other on the SB would have to be triggered by the relatively small changes in free calcium that are predicted to occur at distances of tens to hundreds of nanometers from the plasmalemma, whereas the fusion of the resulting multivesicular structure with the cell surface membrane would be phase locked to the large, rapid calcium changes that occur close to the presynaptic calcium channels. In favor of this hypothesis, large irregularly shaped structures that appear to be attached to the SB are seen much more frequently at stimulated synapses than inhibited or resting synapses (Lenzi, D. et al., 1999; 2002, Figure 4). Opposed to this hypothesis, the mean EPSC amplitude did not increase following stimulation (Glowatzki, E. and Fuchs, P. A., 2002; Keen, E. C. and Hudspeth, A. J., 2006).

### 3.22.6.4 Tuning Mechanisms in Hair Cells

Auditory organs of fish, amphibians, reptiles, and birds all show frequency selectivity resulting from the properties of individual hair cells. These mechanisms are different from the frequency tuning seen in the mammalian cochlea, which is the result of the gradient in stiffness of the basilar membrane (acellular) and active mechanical feedback from hair bundle motion and OHC motility (cellular). Our focus will be on tuning in the other four classes of vertebrate animals.

#### 3.22.6.4.1 Frequency-selective augmentation of membrane potential oscillation

In the turtle auditory papilla, there is no apparent frequency tuning extrinsic to the hair cell (i.e., transduction currents are equal over a broad frequency range). Instead, each cell is intrinsically tuned to a different frequency band by a process that has been called electrical resonance. The resonant frequency is determined primarily by the number and kinetics of the cell’s calcium-activated potassium channels, voltage-activated potassium and calcium channels, and electrical capacitance (Ricci, A. J. et al., 2000; and reviewed in Fettiplace, R. and Fuchs, P. A., 1999). Each hair cell preferentially amplifies the receptor potential in a frequency band, with peak amplification near the resonant frequency. The tonotopic axis in this organ is generated by the systematic variation of the hair cells’ preferred frequencies along the length of the sensory epithelium. Low-frequency cells have low channel density and slower potassium channel kinetics than high-frequency cells. These variations in channel kinetics are caused by differential expression of potassium channel genes and differential splicing of the mRNAs (Jones, E. M. C. et al., 1999). The result is a continuum of resonant frequencies along the papilla. Similar electrical tuning has been studied in the frog sacculus (Armstrong, C. E. and Roberts, W. M., 1998; 2001) and chick cochlea (Correia, M. J., 1992), but there is no evidence of electrical tuning in the mammalian cochlea.

#### 3.22.6.4.2 Synaptic frequency selectivity

Experiments employing capacitance measurements in hair cells typically use very strong depolarizations...
that evoke maximal calcium currents in order to measure vesicle pool sizes by depletion. This approach has been very fruitful for understanding vesicle pools, but the stimuli used for these experiments are much larger than in vivo receptor potentials, drawing into question the physiological relevance of the evoked exocytosis. A recent study of frog saccular hair cells (Rutherford, M. A. and Roberts, W. M., 2006) used smaller, sinusoidal fluctuations in membrane potential as the voltage-clamp stimulus to mimic the size and frequency of in vivo receptor potentials and looked for frequency selectivity downstream of the known mechanical and electrical tuning mechanisms in these cells. Changes in membrane capacitance were measured in response to small sinusoidal voltage changes (±5 mV centered at −60 mV) delivered at 5, 50, and 200 Hz to test the hypothesis that exocytosis would be largest at 50 Hz, the frequency where the sacculus is most sensitive to airborne and groundborne vibrations. Consistent with this hypothesis, exocytosis was twice as large at 50 Hz than at 5 or 200 Hz, even though the total calcium influx was the same at the three frequencies. This preference for 50 Hz disappeared when stronger sinusoidal stimuli (±10 mV centered at −55 mV) were applied, which is reminiscent of other tuning mechanisms that are saturated by large stimuli (e.g., de Boer, E. and Nuttall, A. L., 2000). It is important to note that the ±5 mV sinusoidal stimulus, while small compared to the stimuli used in most voltage-clamp studies, is well above the amplitude of oscillations that occur at sensory threshold (Dallos, P., 1985). The exocytosis in response to the small stimulus at 50 Hz corresponded to the net fusion of 1.7 SV per AZ per stimulus cycle. This amounts to twice the number of vesicles in the docked pool fusing during the 1 s stimulus, and suggests that new vesicles can dock and be primed for release during the stimulus. This synaptic tuning, operating in series with mechanical and electrical tuning, is expected to contribute to the overall tuning of the sacculus, which has been measured by action potential firing rates and sensitivity in afferent axons of the eighth cranial nerve (Koyama, H. et al., 1982; Lewis, E. R. and Narins, P. M., 1985; Yu, X. et al., 1991; Christensen-Dalsgaard, J. and Narins, P. M., 1993). Similar synaptic tuning could contribute to frequency selectivity at other ribbon synapses, particularly in auditory and vestibular organs where synaptic transmission results in phased-locked action potentials in the postsynaptic neuron.

### 3.22.7 Auditory Nerve Fiber Firing Patterns Mirror Presynaptic Vesicle Pool Dynamics

Until recently, physiological studies of cochlear output have focused on in vivo recordings of spike trains in ANFs in response to sound stimuli (e.g., pure tones of varying SPL). Advances in patch-clamp techniques in vitro, such as capacitance measurements as a presynaptic assay of SV exocytosis, have allowed more direct comparisons between presynaptic activity and postsynaptic spike trains.

#### 3.22.7.1 Adaptation of the Auditory Nerve Response

Adaptation of ANF responses is thought to be necessary for the peripheral processing of speech (Delgutte, B., 1980). Two types of fast adaptation have been described to explain the reduction in ANF discharge rate following the onset of a sustained acoustic stimulation (Figure 6(e)). These have been called rapid adaptation and short-term adaptation, with durations of approximately 10 and 20–90 ms, respectively. Although the precise time courses can vary by species, SR of the fiber, and sound intensity; both processes are thought to arise from mechanisms downstream of IHC receptor potential in vivo, which is not reduced in amplitude during a sustained stimulus (Russell, I. J. and Sellick, P. M., 1978; Holton, T. and Weiss, T. F., 1983). Fast adaptation is thought to involve presynaptic depression (as described below), but not via inactivation of the presynaptic calcium current which shows no significant decline over this time course under voltage clamp (Parsons, T. D. et al., 1994; Moser, T. and Beutner, D., 2000; Schnee, M. E. and Ricci, A. J., 2003; Eisen, M. D. et al., 2004).

Fast adaptation of ANF firing and its subsequent recovery seem to be predominantly due to depletion and refilling of the RRP. Recovery from adaptation takes hundreds of milliseconds (Smith, R. L., 1977; Harris, D. M. and Dallos, P., 1979; Chimento, T. C. and Schreiner, C. E., 1991), and this time course is thought to reflect refilling of the RRP. In the chick, using a combination of in vivo single unit recordings of ANF activity and in vitro patch-clamp recordings of SV fusion, it was found that sound-evoked ANF activity adapted and recovered with similar time courses as RRP depletion and recovery (Figure 6; Spassova, M. A. et al., 2004). After RRP exhaustion,
its recovery was 95% complete after a 200 ms rest period, similar to the time course of recovery from adaptation. In comparison, analogous recovery in retinal bipolar neurons takes about 100 times longer (20 s; von Gersdorff, H. and Matthews, G., 1997). In the mouse IHC, RRP refilling is biphasic, possibly reflecting the refilling of two functionally distinct types of AZs (Moser, T. and Beutner, D., 2000). Differences among AZs in a single IHC could provide the basis for the variable properties of ANFs (Merchan-Perez, A. and Liberman, M. C., 1996). Indeed high- and low-SR fibers exhibit different times needed for recovery from prior stimuli (Relkin, E. M. and Doucet, J. R., 1991) that are a close match to the biphasic refilling of the RRP (Figure 6(d)).

3.22.7.2 The Role of the Synaptic Body in Auditory Nerve Fiber Spike Timing

Computational attempts at predicting psychophysical performance on auditory tasks based on the activity of ANFs have generally concluded that the probabilistic nature of action potentials (the variability of spike timing during repeated presentations of the same stimulus) limits the precision of acoustic information sent to the brain (Mountain, D. C. and Hubbard, A. E., 1996). Upon repeated presentation of a pure tone, an ANF will fire a first spike with variable latency. The variance of first spike latency in mice with a targeted deletion of the gene for the cytomatrix AZ protein Bassoon was increased by a factor of 12 compared to controls (Buran, B. N. et al., 2006). Bassoon is thought to anchor SBs at AZs by interacting with Ribeye (Khimich, D. et al., 2005; tom Dieck, S. et al., 2005); ultrastructural inspection demonstrated a 90% reduction in the number of SBs anchored at AZs. This work suggests that the presence of SBs at release sites helps to reduce the temporal jitter of ANF discharge, and hence improve phase locking.

In the absence of an applied stimulus, the temporal firing pattern of ANFs in vivo is often represented as a poison process, but on a short timescale it is more accurately described as a renewal process that is a function of neurotransmitter concentration in the cleft (presynaptic effect) and neural refractoriness (postsynaptic effect), the combination of which causes the firing probability to be depressed for more than 20 ms following a spike (Gaumond, R. P. et al., 1983). Furukawa and co-workers recorded synaptic potentials from afferent units in the saccular branch of the eighth nerve of the goldfish and observed a 20 ms delay in firing upon a decrement in the intensity of tone. They attributed this delay to the time required for SVs to refill the depleted low-threshold release sites between the SB and the calcium channels at the plasmalemma. Furthermore, quantal analysis of the size of EPSPs showed a negative correlation between the amplitude of two consecutive events (i.e., large EPSPs tend to be followed by smaller ones and vice versa). These data support the idea that adaptive rundown of EPSPs is the result of SV depletion (Furukawa, T. and Matsuura, S., 1978; Furukawa, T. et al., 1978).

Additional evidence for the involvement of the SB in maintenance of the functional RRP comes from the Bassoon mutant mice, in which the first phase of exocytosis and the calcium current are both reduced (Khimich, D. et al., 2005). The large reduction of SB-associated docked SVs at AZs in the mutant provides additional support for the contribution of these docked vesicles to the functional RRP measured in capacitance experiments. The reduced calcium current supports previous evidence that calcium channel expression is coregulated with and restricted spatially to SV release sites adjacent to SBs (Martinez-Dunst, C. et al., 1997; Zenisek, D. et al., 2003). Intriguingly, the sustained component of the capacitance rise was relatively intact in the mutant mice compared to the 50% reduction in ANF discharge rate after short-term adaptation to tonal stimuli (Buran, B. N. et al., 2006). One interpretation is that the sustained component in the mutant mice is relatively asynchronous or not properly localized to postsynaptic fibers. In contrast, the sustained exocytosis in wild-type mice is likely to reflect synaptic release as indicated by the massive sustained release measure by EPSCs in high potassium and higher levels of sustained ANF discharge rate in vivo. The sound-evoked onset firing rate (before adaptation) and steady-state firing rate (after fast adaptation) were decreased and threshold was increased, in accordance with the reduction of the functional RRP measured presynaptically. In spite of this, the shapes of rate-level functions were normal in the mutants. Indeed a recent modeling study showed that the effect of increasing the maximum number of transmitter quanta held in the immediate store (analogous to the concept of increasing the size of the RRP) is to scale the release rate linearly across the entire dynamic range (Sumner, C. J. et al., 2002).

Work with the Bassoon mutant mouse has shown that the localization of synaptic ribbons to hair cell
AZs is required for transmitter release to be precisely synchronized to the onset of sound at each AZ, which is required for synchronous auditory signaling carried to the brain stem (Khimich, D. et al., 2005; Buran, B. N. et al., 2006). If simultaneously activated fibers provide input to a single neuron in the cochlear nucleus, then converging synaptic inputs arriving closely in time could be the physiological basis for a coincidence detector to differentiate between baseline activity and stimulus-evoked (synchronous) hair cell afferent synaptic activity. It seems probable that the precise configurations of SBs and their associated cytomatrices at AZs are important for establishing both the sensitivity and the phase relationship of ANF spikes to the sound.

### 3.22.8 Synapse to Psychophysics

One of the goals of auditory research is to provide an explanation of the behavior of the auditory system based upon the functions of its cellular components and the circuits they make. For example, numerous experiments have examined the ability of the auditory system to detect weak sounds. These experiments have been carried at the level of ANFs, cortical neurons, and behavior, in a variety of mammals, including humans. Recently it has been proposed that the inverse relationship between the duration and the SPL needed to attain a threshold criterion for detection seen at all levels of auditory processing can be explained by the properties of hair cell afferent synapses. One interpretation of these results is that the statistics of individual events, which may correspond to the excocytosis of individual SVs at the afferent synapse, underlies the trade-off between strength and duration for barely detectable sounds (Heil, P. and Neubauer, H., 2003) that is measured in psychophysical experiments of sound perception.

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