The Outer Spiral Network and its Innervation by the Olivocochlear System

by

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M.D.
Universidade Federal de Pernambuco, 1998
M.Sc.
Harvard –MIT Division of Health Sciences & Technology, 2006

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Abstract

Outer hair cells (OHCs) are key elements of the mammalian hearing system, which amplify sound-evoked signals transmitted into the inner ear. OHCs are innervated by peripheral projections of olivocochlear (OC) and type-II spiral ganglion neurons.

Type-II neurons innervate up to 100 OHCs, but their function is unknown. It has been suggested that type-II neurons interconnect neighboring OHCs through reciprocal synapses, which are bi-directional (afferent and efferent) synaptic interactions (Nadol, 1981). Since reciprocal synapses on the OHCs have been shown to be prevalent only in aged primates, they were thought to be a pathological finding. In addition to their interactions with OHCs, type-II neurons are also innervated by OC neurons. Synapses between OC and type-II neurons (OC/type-II synapses) have been described (Smith and Rasmussen, 1963), but these interactions have not been characterized in detail.

Serial and semi-serial section transmission electron microscopy were used to study the synaptic interactions of type-II neurons with OHCs and OC neurons in a young human and in adult cats.

A high prevalence of nerve terminals with reciprocal synapses was observed in the young human and in adult cats. These reciprocal terminals were processes of type-IIs, and not of OC neurons. Reciprocal type-II terminals were found in all frequency regions studied in cats, but were most prevalent below 4,000Hz. All the type-II fibers traced to more than one OHC in an adult cat had reciprocal interactions with OHCs. Type-II fibers/terminals were heavily innervated by OC neurons, which preferentially targeted terminals with reciprocal synapses that were predominantly afferent in an adult cat. The innervation patterns of type-IIs and OC neurons in the cat were similar to that found in comparable frequency regions of primates.

Type-II neurons have reciprocal synaptic interactions with OHCs and form an “outer spiral network”, which may functionally integrate the OHCs. The OC system may modulate this network through OC/type-II synapses. The outer spiral network and its innervation by the OC system seem to be relevant to OHC function, and further research is needed to determine their role in hearing.

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CHAPTER 1 - Background

The role of type-II spiral ganglion neurons (type-IIs) in the auditory system is unknown and there are fundamental aspects of their interactions with other cells inside the cochlea that remain unresolved. In this PhD thesis, we will focus on the synaptic interactions of these type-II neurons in the peripheral auditory system. We will employ serial section electron microscopy to quantitatively describe how these neurons are innervated, and how they innervate other cells. Some of the questions to be addressed are related to the possible impact of maturation and age-related degeneration in the type-II's synaptic interactions, while others are related to possible differences between primates and non-primate mammals. We hope that the systematic characterization of the synaptic interactions of the type-II neurons to be presented in this thesis will shed light into their role in mammalian auditory function.

In this thesis, we will be studying the synaptic interactions of the type-IIs in the human and in the cat, and there are reasons for studying both. We studied human specimens because it is important to have a detailed understanding of these interactions in humans. At the same time, the identification of the commonalities in the synaptic interactions of type-IIs of humans and non-primates is pivotal in the search for the functional role of the type-IIs in human hearing. In case the synaptic interactions cat’s type-II neurons happen to be similar to the ones found in humans, the information generated from physiological experimentation related to the type-IIs synaptic interactions in this species may be extrapolated to humans.

All of the key cellular elements present in the human cochlea are common to all mammalian species, including the two types of mechano-electrical transducer cells (inner & outer hair cells), two types of sensory neurons (type-I & type-II spiral ganglion neurons), and two types of neurons that send descending projections to the cochlea (medial & lateral olivocochlear neurons). The inner hair cells (IHCs) and outer hair cells (OHCs) are the cochlear mechano-transducers. All these cochlear elements work as an elaborate system that enables the transduction of the mechanical vibrations contained in the sound stimulus into electrical impulses, which are transmitted to the brain for interpretation.

The functioning of the peripheral auditory system is quite complex. In brief, the sound-evoked mechanical vibrations are transmitted into the organ of Corti and the OHCs amplify these vibrations (Dallos, 1992). The amplified mechanical energy is transduced into receptor
potentials by the IHCs, which are then transmitted to type-I spiral ganglion neurons (type-Is) through chemical synapses. Action potentials are generated in the type-Is innervating these IHCs and coded electrical impulses are transmitted to the central nervous system (Nobili et al., 1998). The descending lateral OC neurons synapse on the peripheral processes of type-I neurons and are thought to modulate their input to the central nervous system, while the medial olivocochlear (OC) neurons synapse on the OHCs and modulate their function as cochlear amplifiers (Guinan, 2006). The type-II neurons innervate the OHCs (Kiang et al., 1982; Leake-Jones and Snyder, 1982; Liberman, 1982a), but their functional role is unknown. It has been hypothesized that they bring information to the central nervous system about the operational status of the OHCs (Kim, 1986).

Type-II neurons have synaptic interactions in the cochlear nucleus (Berglund et al., 1996), spiral ganglion (Kimura et al., 1979) and within of organ of Corti (Bodian, 1978; Dunn, 1975; Fechner et al., 2001; Nadol, 1990b). In this study, we will focus on the synapses on their cell bodies (located in the spiral ganglion) and on the synapses in the OHCs area of the organ of Corti. The first step in the study of these synaptic interactions is the morphological identification of the cell types within the spiral ganglion and in the OHCs area. In the study of the synapses on the cell bodies within spiral ganglion, it is important to distinguish the type-II neurons from the type-I neurons, while in the OHC area it is important to distinguish the type-II neurons from the OC neurons. As a starting point, we will describe the cytology of the type-II neurons. Subsequently, we will describe what is known about the synaptic interactions of type-II neurons, while describing the gaps in the current knowledge that will be addressed in this thesis.

**Cytology of the Type-II Spiral Ganglion Neurons**

**In the Spiral Ganglion**

Studies employing electron microscopy have provided ultrastructural characterization of the type-II neurons at the level of the spiral ganglion. In a morphometric study in human specimens (Nadol and Burgess, 1990), it was found that the parameters that more accurately divided the spiral ganglion cells into two groups were the areas of the cell body and the ratio of diameters of central and peripheral processes. The type-II neurons were characterized as the ones with cell area ≤300 μm², cell diameter ≤21 μm, cell circumference ≤68 μm, nuclear area
≤90 μm, nuclear diameter ≤10 μm, and nuclear circumference ≤32 μm. The mean diameter of central processes of type-Is were found to be larger than the ones of type-II neurons, but the mean diameters of the peripheral processes of both types of neurons was similar. The average ratio of the diameter of the central process to that of the diameter of the peripheral process was 2.94 for the type-Is and 1.13 for the type-II neurons.

The type-Is are usually located in the central portions of the spiral ganglion, while the type-II are usually in the periphery in loose clusters close to portions of the OC intraganglionic spiral bundle (Kimura et al., 1979). Studies in humans have found that both types of spiral ganglion cells are almost exclusively bipolar (Nadol et al., 1990), with about 90 (Spoendlin and Schrott, 1988) to 94% (Ota and Kimura, 1980) being type-Is, and 6 to 10% being type-IIs. However, since the type-II cells are found in clusters, the sampling techniques used in their quantification may underestimate their numbers (Chiong et al., 1993).

The type-IIs have unmyelinated cell bodies and central/peripheral projections in all mammals, while the type-Is are myelinated throughout in most species, except humans (Adamo and Daigneault, 1973; Romand and Romand, 1987; Rosenbluth, 1962; Ruggero et al., 1982; Thomsen, 1966). In humans, 94% of the cell bodies of type-Is are unmyelinated, while their central/peripheral projections are myelinated. The myelination of the cell bodies of type-Is in humans increases with age, with absence of myelinated neurons in the spiral ganglion of newborns (Arnold, 1982; Chiong et al., 1993) and 5.8% of myelination in a study of patients between 65 and 92 years of age (Ota and Kimura, 1980).

The type-I and type-II neurons have distinct cytoplasmic features in human adults. The cytoplasm of type-II neurons contains many neurofilaments arranged in bundles, and commonly presents aggregates of rough endoplasmatic reticulum close to the nucleus. Their nucleus has variable shape, commonly presenting fine chromatin substance and a loosely arranged nucleolus. In contrast, the type-Is contain a spherical nucleus with loose chromatin and a pronounced round nucleolus. The cytoplasm of the large neurons is rich with mitochondria and presents prominent rough endoplasmatic reticulum with ribosomes scattered throughout the cytoplasm. While the central and peripheral processes of type-IIs are unmyelinated, the central processes of type-Is became myelinated at a distance of about 4 to 38 μm from the cell body, while the peripheral processes became myelinated at a distance of 5 to 26 μm from the cell body (Arnold, 1987;
In the Organ of Corti

The study of the type-II projections from the cell bodies into the organ of Corti has been performed with neuronal tracing and immunohistochemical techniques. Several previous studies have used horseradish peroxidase (HRP) tracing to study morphology of individual type-II neurons. HRP is a 40 KDa macromolecule that is actively uptaken and transported retrogradely by neurons (Adams, 1977; 1992). This cytochemical technique has been used in the tracing of the central and peripheral processes of individual type-II neurons (Berglund and Ryugo, 1987; Brown, 1987a; Fechner et al., 2001; Liberman, 1982a; Simmons and Liberman, 1988a). The type-II fibers in the cochlear nerve are thin (mean diameter ~ 0.5μm) and have uniform diameter. The peripheral processes of type-II neurons enter the organ of Corti and then cross directly through the lower portion of the inner spiral bundle, without branching or contacting the IHCs. Some type-II fibers enter the inner spiral bundle briefly, to spiral past one pillar cell before turning outward to the tunnel of Corti. These nerve fibers cross the tunnel of Corti at a level close to the nuclei of the pillar cells and then spiral basally among Deiter’s cells, with the exception of some apical nerve fibers that can spiral both basally and apically (Brown, 1987a; Wright and Preston, 1976). During this spiral course, the fibers gradually rise in level relative to the OHCs and eventually give off branches ending in small terminals on the OHCs. The course of type-II fibers within the outer spiral bundle can be divided into two regions: an initial “spiral region” in which no endings are given off to OHCs and a “terminal region”, where branches with endings are produced. It is interesting to note that the type-IlIs’ peripheral processes increase in diameter during the basalward spiral until the terminal region and then become gradually thinner as terminals are formed within the terminal region.

In the cases where the HRP injection site encompasses the intraganglionic spiral bundle, OC fibers can be labeled with this neuronal tracer (Brown, 1987b). It has been demonstrated that OC fibers travel through the intraganglionic spiral bundle and spiral lamina. Once within the organ of Corti, they usually project radially to the OHCs, with some of them spiraling for a relatively short distance within the inner tunnel spiral bundle. These nerve fibers cross the tunnel of Corti in variable heights from the basilar membrane, but most of them (> 85%) have an
elevated position and can be distinguished from the type-II fibers that travel close to the tunnel’s floor. At the level of the OHCs, OC fibers are distinct from type-IIs because they do not spiral as much as the type-IIs and form larger endings that, especially for fibers in the apical turn of humans and guinea pig, end mainly on OHCs of the first row (Brown, 1987b; Nadol, 1990b).

The morphology of individual type-II neurons have also been studied with the use of antibodies targeted at intermediate filaments, such as neurofilaments (NFs) and peripherin, which are present in their cytoskeleton. The term “intermediate” comes from the fact that the polymers formed by these proteins have diameters ranging from 7-11 nm, which are intermediate between the larger tubulin polymers (22-25 nm) and the smaller actin microfilaments (5-7 nm). Microtubules and intermediate filaments are the main cytoskeletal elements of nerve cells, generating the framework of neuron shape (Peng et al., 1986). Because the intermediate filaments are the most stable and the least soluble cellular constituents (Anniko et al., 1987), it is presumed that one of their primary functions is mechanical support. These proteins are interconnected to microtubules and microfilaments of actin; hence, they may also have a role in effecting transport of organelles within the cell. NFs share with other cytoskeletal proteins the function of integrating space and regulating the size and configuration of neurons. While many small neurons are maintained with limited, if any, NF proteins, large neurons, especially those with long cellular processes, are richly endowed with NFs (Schlaepfer, 1987). In mammals, NFs are heteropolymers composed of three subunit proteins with apparent molecular weights of 200kDa (NF 200 or H NF); 160 kDa (NF 160 or M NF); and 68 kDa (NF 68 or L NF). The 200 kDa NF protein antibodies seem to be the most specific to the cell bodies of type-IIs, but they are not specific to the dendrites of type-IIs at the level of the outer spiral bundle, as they label the OC fibers as well (Anniko and Arnold, 1990; Berglund and Ryugo, 1986; 1991; Dau and Wenthold, 1989; Despres et al., 1994; Hsu et al., 1997).

Conversely, antibodies against peripherin are quite specific to the type-IIs and do not seem to label OCB fibers. In an immunohistochemical study of the adult rat cochlea and cochlear nucleus (Hafidi, 1998), peripherin was found in the cell bodies, peripheral and central processes of neurons with type-II morphology. More specifically, peripherin-positive neurons had the following features: 1) had large eccentric nucleus; 2) were often found in groups of 2 or 3 cells; 3) were often located near the intraganglionic spiral bundle fibers; 4) represented about 8% of the whole ganglion population; and 5) had smaller cell bodies than non-immunoreactive
cells. In surface preparations of the organ of Corti, peripherin-like positive fibers within the spiral ganglion were observed only to arise from immunostained perikarya. The intraganglionic spiral bundle fibers, as well as radial fibers from the main spiral ganglion neuronal population were peripherin-negative. Within the organ of Corti, peripherin-positive fibers crossed near the IHCs without innervating these cells, or sometimes coursed along 3 to 5 IHCs before turning radially to the tunnel of Corti (the height within the tunnel of Corti is not mentioned, but the pictures seem to show that the stained fibers are in contact with the tunnel's floor). Tunnel crossing fibers formed three networks of fiber bundles that traveled underneath the neural poles of the OHCs. Peripherin-positive fibers were not observed to end at the IHC level and this argues against staining of the fibers of the lateral olivocochlear bundle. Furthermore, the central projections of the labeled fibers were present within both ventral and dorsal divisions of the cochlear nucleus, with especially intense staining within the granule cell lamina, which is the main area of projection of central processes of type-II neurons (Berglund et al., 1996; Brown et al., 1988).

The morphological descriptions of the type-II and OC neurons at the level of the organ of Corti using light microscopy (Berglund and Ryugo, 1987; Brown, 1987a; b; Ginzberg and Morest, 1983; Kiang et al., 1982; Liberman and Simmons, 1985; Perkins and Morest, 1975; Simmons and Liberman, 1988a) have been correlated with the ones of ultrastructural studies. Electron microscopic studies have suggested that the two populations of the nerve fibers described with light microscopy have distinct ultrastructural features (Dannhof and Bruns, 1993; Dunn, 1975; Ginzberg and Morest, 1984; Liberman et al., 1990; Simmons, 1986; Simmons and Liberman, 1988b; Spoendlin and Gacek, 1963). The population described as large and vesicle-rich in ultrastructural studies has a spatial distribution in the cochlea that corresponds to the one used for the OC neurons in light microscopy studies. On the other hand, nerve fibers of the other population are small and poorly vesiculated at an ultrastructural level and correspond to the type-II fibers of the light microscopy studies.

The peripheral distribution patterns of nerve fibers in humans are similar to the ones found the in experimental animals. But since planned degeneration studies are not possible in humans, the classification of these fibers can only be done by analogy with the ones found in animals. It is assumed that the inner radial fibers are the terminal portions of the type-I neurons and that the basilar fibers within the tunnel of Corti and the outer spiral fibers are peripheral
projections of type-II neurons. The upper tunnel radial fibers are probably the medial OC neurons, while the inner spiral fibers are probably lateral OC fibers.

The type-II fibers at the bottom of the tunnel of Corti in humans tend to be hidden in invaginations of the supporting cells, and there is on average one fiber between two outer pillar cells. Below the OHCs, they form the outer spiral bundles, which in the human organ of Corti are large bundles of several hundred fibers between the Deiter’s cells, sometimes visible as dark masses in light microscopy. These outer spiral bundles are composed of type-II fibers, which contain many neurotubules and have calibers ranging from 0.1 to 1 μm. The impressive number of outer spiral fibers (a multiple of the outer spiral fibers found in the animals) originating from the few basilar fibers is most probably the consequence of long spiral extension (>1mm) of these fibers (Nadol, 1983; Spoendlin and Schrott, 1988).

***Synaptic Interactions of Type-II Spiral Ganglion Neurons***

**In the Spiral Ganglion**

The only report of synapses on cell bodies of type-II neurons in non-primates was made in the rat (Ivanov et al., 1992). Those synapses were found in neonate white rats, but not in older animals studied. Ultrastructural investigations of other animals, including mice (Romand and Romand, 1987), chinchilla (Ruggero et al., 1982), guinea pig (Thomsen, 1966), rat (Rosenbluth, 1962), or cat (Adamo and Daigneault, 1973) did not report evidence of synapses on the cell bodies of type-II ganglion cells, but these studies were not explicitly trying to demonstrate or quantify them.

The first description of synapses on cell bodies of type-IIs was made in humans (Kimura et al., 1979). It was observed that some varicose nerve fibers penetrated the junctions of the ensheating satellite cells and myelin lamellae and partly invaginated into the cell bodies to form synapses. The varicose nerve fiber contained numerous mitochondria, synaptic vesicles, including some dense cored vesicles. The typical asymmetrical thickenings of the synaptic membranes with accumulation of vesicles on the presynaptic side of the nerve fiber were clearly demonstrated. Synapses on type-II’s cell bodies were not described as a common occurrence in this study (3 out of 17 neurons – 17.6%) and they were quantified in only one specimen (from a
37-year-old subject). Synapses on the type-II’s cell bodies were also shown in newborns, but there was no quantification of this phenomenon (Arnold, 1982).

Synapses were subsequently observed on the peripheral, central processes and cell bodies of type-II neurons in adult macaque monkeys (Kimura et al., 1987). The synapses occurred most frequently on their peripheral processes within the spiral ganglion. In one case, the fiber forming a synapse on the peripheral process of a type-II neuron was traced to another type-II neuron. This type of synapse would then be similar to the type-II/type-II synapses (also known as dendrodendritic) synapses that occur within the outer spiral bundles (Bodian, 1978; Nadol, 1990a; Nadol and Burgess, 1990). In a macaque monkey submitted to an experimental midline section of the crossed OC fibers at the floor of the fourth ventricle, atrophic changes of some synapses on cell bodies of type-II neurons were observed (Kimura, 1986). In a human specimen (Kimura et al., 1979), and also in the monkey (Kimura, 1986), nerve fibers synapsing on the cell bodies of type-II neurons were traced back to the intraganglionic spiral bundle, which contain the OC fibers (Brown, 1987b; Liberman and Brown, 1986). This second variety of synapse on the type-II cell bodies are probably related to the OC/type-II (also know as axo-dendritic) synapses that occur in the tunnel of Corti and outer spiral bundle (Dunn, 1975; Liberman et al., 1990).

In the chapter 2 of this thesis, we will provide a quantitative assessment of the synapses on the cell bodies of type-II neurons in human subjects of different ages, while describing the ultrastructural features of these synapses. In this study, we will address the possible effects of maturation and degeneration of the peripheral auditory system on the synaptic interactions of type-II neurons and will characterize the neurons that synapse on the type-IIs.

In the Organ of Corti

There are several types of synapses present in the OHC region of the organ of Corti, including: 1) synapses between OC neurons and OHCs (at the neural pole and supranuclear); 2) between OC and type-II neurons; 3) among type-II neurons; 4) between type-II neurons and OHCs; and 5) between type-II neurons and supporting cells (Fechner et al., 2001; Liberman et al., 1990; Nadol, 1988). These synaptic interactions have been studied with the use of neuronal tracing techniques, immunohistochemistry and electron microscopy.

The use of the neuronal tracing techniques like HRP labeling relies on the identification of varicosities or nerve contacts with light microscopy, or on the identification of HRP filled
terminals with electron microscopy. The number of OHC terminals generated by individual OC fibers has been quantified in the guinea pig cochlea (Brown, 1987b), and the number of OHC contacts by type-II fibers has been counted in the cat (Simmons, 1986). In a study in which the HRP injections were made into the root of the cochlear nerves in cats (Leake-Jones and Snyder, 1982), they were able to label small unmyelinated neurons and confirmed that these were type-II neurons by remounting the tissue and by analyzing it with the transmission electron microscope (TEM). In the same study, numerous small, HRP-filled presumed type-II fibers were also observed passing near the bases of inner pillars and crossing the tunnel of Corti close to the basilar membrane, and projecting into the outer spiral bundles. These labeled fibers contacted OHCs and classical afferent synapses were demonstrated.

Several studies have employed immunohistochemistry for known neurotransmitters / modulators or synaptic markers in the study of synaptic interactions in the OHC area. Acetylcholinesterase (ChAT) – immunostaining has been found in nearly all the OC terminals synapsing with the OHCs (Altschuler et al., 1985b). A study of the cynomolgous monkey (Hozawa and Kimura, 1990) reported ChAT activity on OC fibers forming synaptic contacts on the cell processes and cell bodies of type-IIs. OC fibers have positive immunostaining for several other neurotransmitters / modulators, including: CGRP (Kitajiri et al., 1985; Sliwinska-Kowalska et al., 1989; Vetter et al., 1991), enkephalin (Altschuler et al., 1984b; Eybalin and Altschuler, 1990), and dynorphin B (Altschuler et al., 1985a). These substances are probably co-localized in some of these terminals (Puel, 1995).

GABA-like immunostaining is also present in OC fibers contacting OHCs (Eybalin et al., 1988; Fex and Altschuler, 1984; Fex et al., 1986). These GABAergic fibers form several kinds of synaptic contacts: (1) OC efferent synapses with the OHCs; (2) OC/type-II synapses; and (3) occasional supranuclear synapses on OHCs. This GABA-related immunostaining is found also in the upper turns of the cochlea, were ChAT unstained medial OC terminals are observed (Eybalin and Altschuler, 1990). In a study on isolated OHCs (Plinkert et al., 1989), GABA-like receptor epitopes were found at the lower pole of the cells. This immunostaining was more pronounced in OHCs of the apical turns.

Synaptophysin is a 38 kD calcium-binding protein which is integral to the small synaptic vesicles of neurons, and immunostaining against synaptophysin has been shown to label OC terminals (Liberman et al., 1990). In a study on six human specimens (Nadol et al., 1993), the
inner spiral bundle, tunnel spiral bundle, upper tunnel crossing and the base of OHCs were stained at a light microscopic level. The staining at the base of OHCs was observed to decrease from base to apex and from the first to the third row, similar to the ChAT-positive large OC terminals. Synaptophysin immunostaining was used in another study for the determination of the number of OC/type-II contact at the level of the tunnel of Corti and outer spiral bundle across different frequency regions in the cat (Liberman et al., 1990), and as many as 130 synapses/mm were identified in the outer spiral bundle.

Several ultrastructural studies have reported the presence of OC/type-II synapses at the level of the outer spiral bundle (Bodian, 1978; Ginzberg and Morest, 1984; Iurato et al., 1978; Nadol, 1983; Nadol and Burgess, 1990; Sato et al., 1997; Smith and Rasmussen, 1963; Spoendlin and Schrott, 1988), but these synaptic interactions were not fully characterized and quantified. Similarly, type-II/type-II synapses have been found in primates (Bodian, 1978; Nadol, 1983; 1988; Nadol and Burgess, 1990; Sato et al., 1997), but they were not quantified.

In chapter 3 of this thesis we will provide detailed characterization and quantification of the OC/type-II synapses and of type/type-II synapses in a human infant. We will also provide a detailed description of OC/type-II synapses in the outer spiral bundle of an adult cat, which will be presented in chapter 5. This study in the cat includes tracing of individual OC and type-II fibers and determination of the location of OC/type-II synapses on the type-II neurons (synapse on the fiber vs. terminal).

The synapses of OC terminals onto OHCs have been described in several ultrastructural studies (Bodian and Gucer, 1980; Dunn, 1975; Ginzberg and Morest, 1984; Iurato et al., 1978; Kimura and Wersall, 1962; Liberman et al., 1990; Nadol, 1983; Nadol and Burgess, 1990; Simmons and Liberman, 1988b; Spoendlin and Gacek, 1963), and they are characteristically large and vesicle filled. In an electron microscopy study of the chimpanzee, the terminals with efferent synapses were divided into two groups based on their size (Francis and Nadol, 1993b). The prevalence of large efferent terminals decreased from base to apex and from the first to third row of each turn, whereas the incidence of small efferent terminals increased from base to apex and from the first to third row in each turn. The distribution of the large OC terminals matches the distribution of the classically defined cholinergic efferent system (Churchill and Schuknecht, 1959; Schuknecht et al., 1959). The nature of the sub-population of relatively small efferent terminals with efferent synapses on OHCs is not well defined and in this thesis we are going to
address the possibility that some of terminals with such characteristics in humans and cats originate from type-II neurons.

Neurons with type-II morphology have been found to form reciprocal synapses in primates, and these synapses are characterized by the presence of synaptic membrane specializations of opposite polarity between two cells. More specifically, reciprocal synapses have been found on OHCs of humans subjects (Nadol, 1981; 1983; 1990b; Spoendlin and Schrott, 1988), in a chimpanzee (Francis and Nadol, 1993a), and in a Japanese macaque (Sato et al., 1999; Sato et al., 1997). Reciprocal synapses were present on 56% of OHCs in humans (Nadol 1984) and on 74% of OHCs in the chimpanzee (Francis and Nadol 1993a). Nevertheless, the somewhat advanced age of the studied subjects (human and chimpanzee, not the Japanese macaque) raised the possibility that the high prevalence of reciprocal synapses could be an age-related degenerative finding.

In chapter 4 of the thesis we will present a quantitative serial section analysis of the nerve terminals contacting the OHCs in a human infant (8-month old). The study includes a morphometric analysis to assist in the classification of terminals as OC or type-IIs. We will determine whether terminals with type-II morphology commonly form reciprocal synapses on the OHCs of young humans.

Reciprocal synapses were not reported as common in previous studies of non-primate mammals (Ginzberg and Morest, 1984; Hashimoto and Kimura, 1988; Jones and Eslami, 1983; Kimura and Wersall, 1962; Liberman et al., 1990; Simmons and Liberman, 1988b; Spoendlin and Gacek, 1963). They were found, however, in newborn cats submitted to transection of the crossed OC bundle (OCB) (Pujol and Carlier, 1982). In another study in which the OCB was transectioned in the chinchilla, terminals identified as type-IIs were apposed to sub-synaptic cisternae, which are efferent synaptic specializations (Iurato et al., 1978). The authors in both studies suggested that the appearance of efferent synaptic specializations apposed to type-II terminals was a pathological reaction to the OCB transection. Other studies in normal cats, showed sub-synaptic cisternae apposed to type-II terminals, but they were not identified as efferent synaptic specializations (Dunn and Morest, 1975; Simmons, 1986).

We have raised the hypothesis that reciprocal synapses are also prevalent in the cat. In chapter 5, we will describe the innervation of a normal adult cat with serial section electron microscopy. The study of this animal will include detailed morphometric analysis of the
terminals and their synaptic interactions with the OHCs. The terminals will be traced to their parent fibers, and the interactions formed by individual type-II fibers on neighboring OHCs will also be described. The analysis will also include a cat submitted to neonatal transection of the OCB and other two adult cats, which will be analyzed with semi-serial sections through samples from seven different frequency regions.
CHAPTER 2 – Prevalence and Ultrastructural Morphology of Axosomatic Synapses on Spiral Ganglion Cells in Humans of Different Ages


Abstract

Axosomatic synapses were found on human spiral ganglion cells (HSGCs). Ultrastructural characterization and calculation of the prevalence of these synapses were performed by electron microscopic serial sections of both type-I and type-II HSGCs. Specimens from subjects of ages 1 day, 14 days, 21 years and 51 years were studied. Synapses on type-I HSGCs were extremely rare. In contrast, axosomatic synapses were present on approximately 50% of type-II HSGCs of a young adult. This prevalence seemed to vary by age. Thus, no synapses were found in a 1-day old neonate, few in a 14-day old, and on approximately 15% of the type-II SGCs from a 51-year old specimen. The origin of the pre-synaptic fibers is unknown. The fact that some neurons seemed to originate from the intraganglionic spiral bundle suggest that at least some of these fibers may be of olivocochlear origin. Based on ultrastructural characteristics there may be more than one type of nerve fiber synapsing on type-II HSGCs. Although the physiological function of these synapses is unknown, hypothetically they may allow pre-synaptic neural modulation at the level of the spiral ganglion.
Introduction

Axosomatic synapses have been described in the spiral ganglion of some species. However, there is little data concerning their prevalence and origin of pre-synaptic fibers.

Kimura et al. (1979) published the first description of axosomatic synapses on type-II spiral ganglion cells (SGCs) in the human. The synapses were quantified in only one specimen from a 37-year old female, and a prevalence of 17.6% (3 out of 17 neurons) was found. Arnold (1982) also described synapses on type-II SGC in human newborns, but without obvious pre-synaptic vesicles. No prevalence data were provided. More recently Rask-Andersen et al. (1997) confirmed the presence of synapses on type-II cells in the human spiral ganglion. In addition, they described “contacts” between nerve fibers and type-I SGCs, but without morphologic evidence of synaptic vesicles. The presence of synapses on type-II spiral ganglion cells has also been described in the macaque monkey (Kimura et al., 1987). The only report of axosomatic synapses on type-II SGCs in non-primates was presented by Ivanov et al. (1992), in a neonate (10-day old) white rat. In a serial section ultrastructural analysis of SGCs in the mouse (Romand and Romand, 1987) synaptic contacts between nerve fibers and ganglion cells of any type were found. Similarly, other studies reported no synapses on the cell bodies of type-II ganglion cells in the chinchilla (Ruggero et al., 1982), cat (Adamo and Daigneault, 1973), rat (Rosenbluth, 1962) or guinea pig (Thomsen, 1966). However, serial section techniques were not employed.

The origin of the fibers establishing axosomatic synapses on the type-II spiral ganglion cells in primates is uncertain. Putative origins of these fibers include processes of type-I or type-II spiral ganglion cells, autonomic fibers, and neurons of the olivocochlear bundle (OCB). Published data suggest that, at least part of these fibers originate from the OCB. Thus, experimental midline section of the crossed olivocochlear efferent fibers at the floor of the fourth ventricle in the macaque monkey resulted in degenerative changes of some nerve terminals synapsing on type-II SGCs (Kimura, 1986). In the same species (Kimura et al., 1987) and also in the human (Kimura et al., 1979) some of these fibers were traced back to the intraganglionic spiral bundle, which is thought to be part of the olivocochlear efferent system. In addition, in the macaque monkey, one fiber that synapsed on the peripheral process of a type-II neuron was traced back to a dendritic process of another type-II SGC (Kimura et al., 1987).
Despite numerous similarities between the normal anatomy of the human inner ear and that of many mammalian species, there are also many differences between them. They include the presence of synapses on both types of SGCs (Kimura et al., 1979; Nadol, 1988), percentage of spiral ganglion cells that are myelinated (Ota and Kimura, 1980) and the presence of reciprocal synapses between afferent fibers and outer hair cells in the human (Nadol, 1988; 1990b). The reciprocal synapses have also been found to be common in the Japanese macaque (Sato et al., 1997) and chimpanzee (Francis and Nadol, 1993a). These differences warrant further study of the anatomy of the human inner ear.

The objectives of this study are: (1) to quantify the prevalence of synapses within the human spiral ganglion in human subjects of different ages; and (2) to characterize the ultrastructure of these synapses.

**Material & Methods**

**Clinical Histories**

Case 1: A 1-day old neonate, born at 35 weeks of gestation, died 7 hours after birth of hypoxemia secondary to hyaline membrane disease. The weight at birth was 3.2 Kg and the height was 34 cm. There were no associated congenital anomalies. The interval between death and fixation of the inner ears was 3 hours.

Case 2: A 14-day old neonate died as a consequence of congenital heart disease. No further clinical data were available. The interval between death and fixation was 1.5 hours.

Case 3: A 21-year old man died of metastatic osteogenic sarcoma. Medical treatment included several courses of chemotherapy including methotrexate, adriamycin, and cisplatin, which were completed 1 year prior to death. There were no complaints of hearing loss or vertigo. There was a history of military and civilian noise exposure. An audiogram done 10 months before death demonstrated normal hearing acuity below 4 kHz and bilateral high-frequency sensorineural loss. Speech discrimination was normal bilaterally. Light microscopy showed loss of OHCs in the basal turn and a morphologically normal spiral ganglion. Comparison of the histology of this specimen with published cases of cisplatin ototoxicity (Hinojosa et al., 1995; Schuknecht, 1993) suggested that the observed otopathology was unlikely
to be secondary to cisplatin ototoxicity and was more consistent with noise trauma. The interval between death and fixation was 3.5 hours.

Case 4: A 51-year old airplane pilot died of cardiac arrest after several hours of unsuccessful attempts at resuscitation. His hearing was reported to be within normal limits by his wife, but no audiograms were available. The interval between death and fixation time was 1.75 hours.

Histological Methods

Fixation for all cases was achieved with 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. Details of tissue preparation have been reported elsewhere (Nadol, 1977). After thinning the otic capsule with a power drill, the specimens were decalcified in 0.1 M disodium ethylenediamine tetra-acetic acid (EDTA) with 2% glutaraldehyde in 0.1 M phosphate buffer and postfixed in 2% osmium tetroxide in 0.1 M phosphate buffer. The tissue was stained with aqueous uranyl acetate, dehydrated in graded alcohols, exchanged with propylene oxide, and embedded in Epon®. Serial sections were cut in a radial direction in an effort to section both axons and dendrites in a longitudinal plane.

Serial sections of approximately 80 nm were performed on representative samples of the basal, middle and apical turns of the spiral ganglion. Every 10th section was stained with 5% uranyl acetate and Sato's triple lead. Electronmicrographs (JEOL 100CX) were taken at a magnification of 3,450X. Montages of the entire section were created for analysis.

Analytical Methods

The following numbers of serial sections were analyzed: 430 from the middle turn and 300 from the apical turn of case 1; 180 from the middle turn and 180 from the apical turn of case 2; 500 from the basal turn and 380 from the middle turn of case 3; and 420 from the basal turn and 600 from the middle turn of case 4.

The differentiation between type-I and type-II HSGCs was based on both morphometric and cytologic criteria. Plotting maximal cross-sectional area (μm²) by numbers of cells in a bin width of 20 μ² (Figures 1-4) confirmed a bimodal distribution of spiral ganglion cells (Nadol et al., 1990). Maximal cross-sectional areas were used to identify type-II SGCs. A cut-off point of ≤ 300μ² was used in specimens from cases 2 and 3 and ≤ 200μ² in specimens from cases 3 and
4. In addition the following cytologic characteristics were used to verify assignment to type-I and type-II categories. Type-I HSGCs (Figure 5) were characterized by a spherical nucleus with loose chromatin and a rounded nucleolus. The cytoplasm of the type-I cells was rich in mitochondria, rough endoplasmatic reticulum and ribosomes but contained few intracellular neurofilaments. In contrast, the type-II HSGCs (Figure 6) were characterized by a nucleus of variable shape with fine chromatin substance and an irregular, loosely arranged nucleolus. The cytoplasm of the type-II cells was rich in neurofilaments, which were usually organized into bundles. In addition, these type-II cells characteristically had fewer mitochondria and ribosomes than the type-I HSGCs (Kimura et al., 1979; Ota and Kimura, 1980).

Cells were numbered and followed through serial sections. The individual maximal cellular area, perimeter, diameter and nuclear area were measured by computerized planimetry using a MOP-3 digital analyzer system (Carl Zeiss, Inc.). Only cells that were completely serially sectioned were included in the calculation of prevalence of synapses. In addition, the measurements from cells that were not completely serially sectioned but had sufficient sections to assure identification of the maximal cross-sectional area were included in the database of cell morphometry.

A synapse was defined by the presence of an asymmetrical pre and post-synaptic membrane thickening of apposed neural cell membranes and an accumulation of cytoplasmic vesicles abutting the pre-synaptic membrane.

Statistical analysis of the differences in the prevalence of synapses in the various turns and ages was done using Fischer's exact test and Mantel-Haenszel Chi-Square. Linear regression analysis was used for comparison of the means of HSGC's maximal cross-sectional areas in the different subjects.
Figure 2.1– Sizes of Human Spiral Ganglion Cells – 1-day Old Neonate

Histogram of maximal cross-sectional area of type-I and type-II HSGC’s in the 1-day old neonatal specimen (case 1). Unlike older specimens (Figs. 2-4) a bimodal distribution was not obvious. Thus, in addition to cell size, cytologic parameters were also used to differentiate type-I from II HSGCs.

Figure 2.2 – Sizes of Human Spiral Ganglion Cells – 14-day Old Neonate

Histogram of maximal cross-sectional areas of type-I and type-II HSGC’s in the 14-day old neonatal specimen (case 2). There was a clear bimodal distribution of cell size.
Figure 2.3 – Sizes of Human Spiral Ganglion Cells – 21-year Old Adult

Histogram of maximal cross-sectional areas of type-I and type-II HSGCs in the 21-year old adult specimen (case 3). There was a clear bimodal distribution of cell size.

Figure 2.4 – Sizes of Human Spiral Ganglion Cells – 51-year Old Adult

Histogram of maximal cross-sectional areas of type-I and type-II HSGCs of the 51-year old adult specimen (case 4). A bimodal distribution was present but with a less clear boundary compared to cases 2 and 3.
Figure 2.5 - Electronmicrograph of a Type-I Human Spiral Ganglion Neuron

Typical type-I HSGC (case 4). The cell had a round shape and a spherical nucleus with loose chromatin and a prominent nucleolus. There were many mitochondria and ribosomes. Scale bar = 0.5 μm.

Figure 2.6 - Electronmicrograph of a Type-II Human Spiral Ganglion Neuron

Typical type-II HSGCs (case 4). The nucleus was usually round, with a diffuse nucleolus and denser chromatin than type-I HSGCs. There was an abundance of neurofilaments and relative paucity of mitochondria. Scale bar = 0.5 μm.
Results

Cell Morphometry and Morphology

In cases 2-4, there was a clear delineation between type-I and type-II HSGCs (Figures 2-4; Table 1). Because the type-I HSGCs of 1-day old neonate (case 1) were significantly smaller than those of the other specimens the differentiation of type-I from type-II HSGCs based on morphologic criteria was more difficult in this specimen (Figure 1). Furthermore, the type-IIs located in the apical turn of case 1 had cytoplasmatic characteristics similar to those of the 21-year old (Figure 7), while the type-IIs of the middle turn looked consistently immature (Figure 8). These cells of the middle turn tended to have a round shape, lower neurofilament content and were most often isolated from other cells and nerve fibers. However, the difference between the cell sizes of the type-IIs of the middle and apical turns in this specimen was not statistically significant. The morphometric data on the 14-day old specimen (case 2) showed that by this age the HSGC had attained adult cell size.

<table>
<thead>
<tr>
<th>Case 1 - 1-day old</th>
<th>Type I HSGCs</th>
<th>Type II HSGCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Middle turn</td>
<td>240 ± 34 (n=107)</td>
<td>134 ± 29 (n=14)</td>
</tr>
<tr>
<td>Apical turn</td>
<td>283 ± 43 (n=75)</td>
<td>144 ± 31 (n=36)</td>
</tr>
<tr>
<td>Total</td>
<td>258 ± 44 (n=182)a</td>
<td>141 ± 32 (n=50)</td>
</tr>
<tr>
<td>Case 2 - 14-day old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle turn</td>
<td>589 ± 86 (n=52)</td>
<td>229 ± 38 (n=16)</td>
</tr>
<tr>
<td>Apical turn</td>
<td>613 ± 115 (n=76)</td>
<td>189 ± 51 (n=26)</td>
</tr>
<tr>
<td>Total</td>
<td>603 ± 106 (n=128)</td>
<td>204 ± 51 (n=42)</td>
</tr>
<tr>
<td>Case 3 - 21-year old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal turn</td>
<td>766 ± 167 (n=130)</td>
<td>176 ± 34 (n=18)</td>
</tr>
<tr>
<td>Middle turn</td>
<td>502 ± 102 (n=325)</td>
<td>163 ± 32 (n=58)</td>
</tr>
<tr>
<td>Total</td>
<td>577 ± 173 (n=455)</td>
<td>166 ± 34 (n=76)</td>
</tr>
<tr>
<td>Case 4 - 51-year old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal turn</td>
<td>323 ± 68 (n=171)</td>
<td>104 ± 28 (n=35)</td>
</tr>
<tr>
<td>Middle turn</td>
<td>414 ± 82 (n=97)</td>
<td>116 ± 21 (n=22)</td>
</tr>
<tr>
<td>Total</td>
<td>356 ± 86 (n=268)a</td>
<td>109 ± 26 (n=57)</td>
</tr>
</tbody>
</table>

Table 2.1- Morphometric Analysis of Spiral Ganglion Neurons of Different Ages

Means and standard deviation of maximum cross-sectional areas (μm²) for the type-I and type-II HSGC populations. Total=average from cell measurements of both turns in the same specimen. n=number of cells measured. p<0.01 - comparing the averages from cell measurements in both turns in the same specimen with the average found in case 3.
Figure 2.7- Type-II Spiral Ganglion Neuron of Apical Turn of the 1-day Old Subject

The neurofilament content was similar to the mature type-II HSGCs (compare with Figs. 6, 11, 12, 17 and 18). Scale bar = 1 μm.

Figure 2.8- Type-II Spiral Ganglion Neuron of Middle Turn of the 1-day Old Subject

There was a paucity of neurofilaments. Scale bar = 1 μm.
The data on the 51-year old subject (case 4) showed a uniform decrease in the cellular dimensions compared to both cases 2 (14-day old) and case 3 (21-year old). The differentiation between type-I and type-II HSGCs was performed without difficulty in this specimen when the morphometric data were employed in combination with the cytological criteria. Several abnormal nerve fibers (Figure 9) and two examples of pseudomonopolar cells (Figure 10) were observed in case 4, in contrast to the other specimens studied.

Type-II HSGCs were found in clusters in all specimens. Although apposition of cell bodies (Figure 11) and cell processes (Figure 12) of type-II cells were observed, no membrane specialization was seen between these cells.

Figure 2.9 - Abnormal (giant) Nerve Fiber in the 51-year Old Subject

There was a high neurofilament content, central accumulation of vesicles (arrowheads) and a diameter at least five times larger than adjacent nerve fibers. Scale bar = 0.5 μm.
Figure 2.10 - Pseudomonopolar Type-I Spiral Ganglion Neurons in the 51-year Old Subject
The axonal (A) and dendritic (D) processes originated from the same neuronal hillock (arrow).
Scale bar = 0.5 μm.

Figure 2.11 - Apposition of Two Type-II Spiral Ganglion Neurons in the 21-year Old Subject
These cells were usually organized in clusters, with frequent apposition of cell membranes. There were also axosomatic synapses with 'dense' (d) and 'clear' (c) nerve fibers. Scale bar = 1 μm.
Synapses on the Cell Bodies of Spiral Ganglion Neurons

The prevalence of axosomatic synapses are presented in table 2. Axosomatic synapses on type-I HSGCs were rare and were only observed in the basal turn of the 21-year old specimen (case 3) (Figure 13). In one case, the same fiber also synapsed on a type-II HSGCs.

<table>
<thead>
<tr>
<th>Cells with axosomatic synapses (%)</th>
<th>Type I HSGCs</th>
<th>Type II HSGCs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Case 1 – 1-day old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle turn</td>
<td>0 (n = 107)</td>
<td>0 (n = 14)</td>
</tr>
<tr>
<td>Apical turn</td>
<td>0 (n = 75)</td>
<td>0 (n = 36)</td>
</tr>
<tr>
<td>Total</td>
<td>0 (n = 182)</td>
<td>0 (n = 50)</td>
</tr>
<tr>
<td>Case 2 – 14-day old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Middle turn</td>
<td>0 (n = 52)</td>
<td>0 (n = 16)</td>
</tr>
<tr>
<td>Apical turn</td>
<td>0 (n = 76)</td>
<td>4 (n = 26)</td>
</tr>
<tr>
<td>Total</td>
<td>0 (n = 128)</td>
<td>2 (n = 42)</td>
</tr>
<tr>
<td>Case 3 – 21-year old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal turn</td>
<td>1.8 (n = 53)</td>
<td>46.6 (n = 15)</td>
</tr>
<tr>
<td>Middle turn</td>
<td>0 (n = 153)</td>
<td>52.1 (n = 46)</td>
</tr>
<tr>
<td>Total</td>
<td>0.5 (n = 206)</td>
<td>50.8 (n = 61)</td>
</tr>
<tr>
<td>Case 4 – 51-year old</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Basal turn</td>
<td>0 (n = 133)</td>
<td>13.3 (n = 30)</td>
</tr>
<tr>
<td>Middle turn</td>
<td>0 (n = 85)</td>
<td>15 (n = 20)</td>
</tr>
<tr>
<td>Total</td>
<td>0 (n = 218)</td>
<td>14 (n = 50)</td>
</tr>
</tbody>
</table>

Table 2.2 - Prevalence of Axosomatic Synapses on Human Spiral Ganglion Neurons

N = total number of cells studied. \( ^{a}p<0.01 \) - comparing with the prevalence found in case 3.
\( ^{b}p>0.05 \) - comparing with the prevalence found in case 3.
Axosomatic synapses on type-II HSGCs had maximal prevalence (≈ 50% of type-II cells) in the 21-year old specimen (case 3). They were not found in the 1-day old neonate (case 1) and were rare (≈ 2% of type-II cells) in the 14-day-old specimen (case 2). In the 51-year old specimen there was a statistically significant (p<0.01) decrease (≈ 15% of type-II cells) in the prevalence of these synapses on type-II HSGCs, when compared to case 3. There was no statistically significant difference in the prevalence of synapses between the turns studied within the same specimen.

Two different types of synaptic contacts on type-II HSGCs were observed. The most common type of pre-synaptic nerve fiber (“clear nerve fiber”) contained sparse neurofilaments (Figure 14). The second type of presynaptic nerve fiber (“dense nerve fiber”) had a much higher neurofilament content, and their vesicles were more loosely arranged in the nerve terminals (Figure 15). In both the clear and dense types there were membrane specializations adjacent to the vesicular accumulation (Figure 14-16). The membrane specializations consisted of pre-synaptic linear condensations paralleling the synaptic cleft and attached to mitochondria. They formed a double pre-synaptic thickening, one at the cleft and one in an intracytoplasmic location. There were fine perpendicular bands linking the pre-synaptic condensations at the cleft with those in an intracytoplasmic location. Mitochondria were concentrated in the area of the intracytoplasmic condensations, as compared to other areas of the nerve terminal. The morphology of these membrane specializations was consistent with the “mitochondria-associated
adherence complex”, a term coined by Spirou et al. (1998) and also described by Gray (1963), Tolbert and Morest (1982), and Cant and Morest (1979).

In addition to synapses on cell bodies, synapses between cell processes were also found within the spiral ganglion. In several cases the post-synaptic cell could be identified as a dendritic process of a type-II HSGC (Figure 16). Although in most cases the origin of both fibers could not be identified, they were frequently found adjacent to the intraganglionic spiral bundle. In general, type-II HSGCs were frequently surrounded or adjacent to fibers of the intraganglionic spiral bundle (Figure 17).

There were no differences in the morphological characteristics of the synapses found in the 21-year old (case 3) compared to those found in the 14-day old neonate (case 2) and 51-year old subject (case 4). Moreover, synapses found on the few myelinated type-II HSGCs (Figure 18) were not morphologically different than the ones found on unmyelinated type-II HSGCs. Pre-synaptic bodies or sub-synaptic cisternae were not observed in synapses within the spiral ganglion. The prevalence of dense core vesicles was low and similar between the two described types of synaptic terminals.

Figure 2.14 - “Clear” Axosomatic Synapse on a Type-II Spiral Ganglion Neuron

The vesicles (v) were localized at a small area of the nerve terminal of the 21-year old subject (case 3). Mitochondria (M) seemed to accumulate parallel to the synaptic membrane. Membrane specializations were present at the synaptic cleft (small arrows) and parallel to it in an intracytoplasmic location (arrowheads). These membrane specializations formed presynaptic “double thickenings” near the mitochondria. Scale bar = 0.1 μm.
Figure 2.15 - "Dense" Axosomatic Synapse on a Type-II Spiral Ganglion Neuron

The neurofilament content of this nerve terminal of the 21-year old subject (case 3) was higher and the vesicle accumulation was more diffuse than that seen in Fig. 14. "Double thickenings" were similar to those seen in "clear" synapses (arrowhead) and they were physically linked to mitochondria (M). Scale bar = 0.1 µm.

Figure 2.16 - Mitochondria-Associated Adherens Complex in a Synapse of a Type-II Neuron

Synapse between two cell processes in case 3 (21-year old specimen). In this case, the postsynaptic cell was identified as a peripheral process of a type-II HSGC. The synaptic morphology was identical to that of "clear" axosomatic synapses, including the vesicle accumulation (V); "double thickenings" (arrowheads); and accumulation of mitochondria (M). The intracytoplasmic thickenings have been described elsewhere as "mitochondria-associated adherence complex". Scale bar = 0.1 µm.
Figure 2.17 - Type-II Neuron with Several “Clear” and “Dense” Axosomatic Synapses

The type-II neuron of the 21-year old subject was surrounded by fibers of the intraganglionic olivocochlear spiral bundle (IOSB). c = clear synapse. d = dense synapse. Scale bar = 0.5 µm.

Figure 2.18 - "Clear" Axosomatic Synapse on a Myelinated Type-II Neuron

Myelinated type-II neurons were rare in the 21-year old specimen, but they also had “clear” axosomatic synapses. When followed in serial sections, this synapse contained double thickenings. Scale bar = 0.5 µm.
Discussion

Axosomatic Synapses on Spiral Ganglion Neurons

The prevalence of axosomatic synapses on type-II HSGCs found in case 3 was larger than the 17.6% reported in a 37 year old female (Kimura et al., 1979). However, in that study semi-serial sections were analyzed and a relatively small number of neurons (17 compared to 61 in our study of case 3) were studied.

The existence of axosomatic synapses on type-II SGCs has been well described in humans (Kimura et al., 1979) and in the macaque monkey (Kimura et al., 1987). Their presence in non-primates has not been completely ruled out by previous studies. The finding of synapses on type-IIs only during the early neonatal period in the rat (Ivanov et al., 1992) cannot be explained based on the data obtained in humans in our study, in which no synapses were found in the 1-day old neonate (case 1).

Synapses have been described on the peripheral and central processes and cell bodies of spiral ganglion cells (SGCs) in non-primates and humans. Some olivocochlear fibers originating in the lateral superior olivary complex (SOC) synapse on the peripheral processes of type-I SGCs at a plane just below the IHC within the inner spiral bundle. Other olivocochlear fibers, originating in the medial SOC, synapse directly on OHCs and also on peripheral processes of type-II HSGCs within the outer spiral bundle (Ginzberg and Morest, 1984; Liberman et al., 1990). Moreover, the central processes of type-II SGCs have been shown to receive synapses at the level of the cochlear nucleus in the mouse (Berglund et al., 1996).

The morphological characteristics of the synapses on HSGCs found in our study were similar to those described by Kimura et al. in the human (1979) and macaque monkey (1987). The presence of two distinct morphological types of pre-synaptic fibers ("clear" and "dense") suggests that they may originate from two different cell types or neural networks.

The tracing of pre-synaptic fibers to the intraganglionic spiral bundle and the observed relative paucity of neurofilaments in this type of pre-synaptic fiber are consistent with an origin of "clear nerve fibers" from the OCB.

Although we have no direct proof, the type-II HSGCs may be the cell of origin of the pre-synaptic "dense nerve fibers". This type of nerve fibers demonstrated a neurofilament content that was similar to type-II HSGC's cell processes. Synapses between type-II HSGCs have
already been observed (Kimura et al., 1979). Moreover, the presence of dendro-dendritic synapses between type-II nerve fibers at the level of the outer spiral bundle in primates (Bodian, 1978; Nadol, 1988; 1990b) suggests that neuronal connections between type-II SGCs exist and therefore may also occur within the spiral ganglion. The formation of clusters of type-IIs cells and frequent cell membrane apposition would support this theory.

Nerve terminals of the autonomic (sympathetic) nervous system are characterized by the presence of dense core vesicles. This type of vesicle was uncommon in both “clear” and “dense” pre-synaptic nerve fibers. Therefore, the hypothesis of innervation of type-II HSGCs by the autonomic system is not supported by our study.

The morphology of the synaptic contacts on type-II HSGCs suggests a high level of neural activity. Gray (1963) published the first description of electron dense plaques parallel to the pre-synaptic membrane and physically attached to mitochondria. The same type of structure has been described in neurons with high synaptic rates within the cochlear nucleus (Cant and Morest, 1979; Tolbert and Morest, 1982). It has been hypothesized that these intracytoplasmic condensations are important for membrane stabilization at synapses with high rates of vesicle membrane turnover. These intracytoplasmic condensations have been named “mitochondria-associated adherence complex” by Spirou (1998) in large terminals of the lateral nucleus of the trapezoid body in the cat.

The physiologic role of synapses on type-II cells is speculative. However, their presence and morphological characteristics raise the possibility that the olivocochlear efferent bundle may modify neural activity of the type-II SGCs in addition to their known direct modulation of electromotility of the OHCs (Kim, 1986).

Maturation of the Inner Ear

Chiong et al. (1993) described full differentiation of type-I and type-II HSGCs at term. However, the youngest specimen in our study suggested immaturity of some SGCs. In addition, other studies also described some degree of incomplete maturation in neonates (Arnold, 1982).

The suggestion in our study of a gradient of maturation of the type-II in the neonate toward the apex is consistent with larger cell sizes and higher counts of type-II HSGCs in the apex described by Chiong et al. (1993). Postnatal maturation of type-II SGC has been observed in the kitten and rat (Romand and Romand, 1987; Romand, 1983; Romand and Romand, 1984).
A study on rats demonstrated that the morphological changes and differentiation of both type-Is and type-IIIs together with their synaptogenesis are associated with changes in cochlear microphonic and auditory nerve potentials (Ivanov et al., 1992; Uziel et al., 1981).

Our morphometric data is consistent with the findings of Chiong et al. (1993), which suggested that the HSGCs reach adult size during the neonatal period. However, the formation of synapses on the type-II SGCs may occur latter. The precise age at which this system is mature and the functional implications of this event for the developing child are unknown.

A study of 42 pre-term human neonates revealed the presence of evoked and spontaneous otoacoustic emissions (OAE) from at least 33 weeks of gestation, suggesting that the OHCs are functional. Nevertheless, there was no suppression of the OAE by contra-lateral sound, suggesting immaturity of the medial olivocochlear system (Morlet et al., 1993). Abdala et al. (1999) reported that OAE was equally likely to be suppressed or enhanced by noise presented contra-laterally on pre-term babies. The development of the innervation of SGCs might be correlated with the maturation of the olivocochlear efferent bundle and further research is needed to assess this possibility.

**Degeneration of the Inner Ear**

In a study on patterns of degeneration of the human spiral ganglion (Zimmermann et al., 1995) it was demonstrated that degeneration of the spiral ganglion may result in significant changes in the diameters of the cell bodies. These findings were present also in an ultrastructural analysis of a profoundly deaf patient (Nadol, 1990a), which also manifested a higher incidence of pseudomonopolar type-I HSGCs (6%) compared to the normative control (0%).

The lack of a recent audiogram and detailed otologic history makes critical analysis of the data obtained from the 51-year-old specimen (case 4) largely speculative. However, the uniform decrease in the cellular dimensions of the type-I SGCs, the slight decrease in cellular density, and the presence of pseudomonopolar and abnormal nerve fibers suggests that the SGCs of this specimen were in process of degeneration. The decreased prevalence of axosomatic synapses on the type-IIIs in this subject may possibly be an additional degenerative finding when compared to the 21-year old studied (case 3).

Castor et al. (1994) published a study on the influence of aging on the function of the medial olivocochlear (MOC) system in humans. The results suggested that there is an age-
related decrease in the suppression of OAE by contra-lateral sound. It was hypothesized that the
decrease in the efficiency of the MOC system might in part explain the increasing difficulty of
signal extraction in noise with aging. Further study of the degenerative changes of the OCB and
other putative inputs to the type-II HSGCs is warranted.
CHAPTER 3 - Axodendritic and Dendrodendritic Synapses Within Outer Spiral Bundles in a Human

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Abstract

Axodendritic and dendrodendritic synapses have been described at the level of the outer spiral bundle (Nadol, 1983; Bodian, 1978). The objectives of this study were to quantify these synaptic interactions and to describe their ultrastructural morphology in a young human subject.

The temporal bone of an 8-month old infant was processed for transmission electron microscopy and semi-serial section reconstructions of the 3 outer spiral bundles were performed. The nerve fibers forming the outer spiral bundles were found to segregate into two morphological groups: (1.) vesicle-rich and neurofilament-poor (VR/NP); (2.) vesicle-poor and neurofilament-rich (VP/NR). Synapses between VR/NP and VP/NR nerve fibers (NFs) and synapses between two VP/NR nerve fibers were quantified.

Presumed axodendritic synapses (i.e., between VR/NP and VP/NR NFs) were numerous and their numbers decreased from the first towards the third row. Presumed dendrodendritic synapses (i.e., between two VP/NR NFs) were also frequent but their numbers did not vary significantly among different rows.

The presence of axodendritic synapses may provide the morphological basis for modulation of the function of the type-II spiral ganglion cells by the olivocochlear efferent system. Similarly, numerous presumed dendrodendritic synapses may provide a morphological substrate for interaction between dendrites of type-II spiral ganglion cells.
Introduction

Synaptic interactions between fibers contained in the outer spiral bundles (OSB) have been reported in several mammals. They are of two types, namely, axodendritic and dendrodendritic synapses. Axodendritic synapses have been the most commonly described (Ginzberg and Morest, 1984; Iurato et al., 1978; Jones and Eslami, 1983; Nadol, 1983; Nadol and Burgess, 1990; Sato et al., 1997; Smith and Rasmussen, 1963; Spoendlin and Schrott, 1988). In one study (Liberman et al., 1990), as many as 130 synapses per radial millimeter were found in the OSB. In contrast, dendrodendritic synapses have been described only in humans (Nadol, 1983) and in old-world monkeys (Bodian, 1978).

The terminologies used in the literature for the synaptic interactions within the OSBs are based on the concept that there are only two types of nerve fibers (NFs) present at this level. These are the axons originated in the superior olivocochlear complex (olivocochlear efferent system) and dendrites of type-II spiral ganglion cells (type-II). These NFs can be differentiated by morphological criteria (Dannhof and Bruns, 1993; Francis and Nadol, 1993a; b; Ginzberg and Morest, 1984; Jones and Eslami, 1983; Liberman et al., 1990; Liberman et al., 2000; Nadol, 1983; Nadol and Burgess, 1990; Sato et al., 1997; Spoendlin and Schrott, 1988; Spoendlin and Gacek, 1963). Thus, efferent fibers of the olivocochlear bundle are generally described as vesicle-rich and neurofilament-poor (VR/NP), whereas dendrites of type-II are described as vesicle-poor and neurofilament-rich (VP/NR). Therefore, synaptic interactions between VR/NP and VP/NR NFs have been described as axodendritic and synapses between two VP/NR NFs have been called dendrodendritic.

Two types of synaptic interactions involving the same neurons that form the OSB (olivocochlear bundle fibers and type-II) have also been reported at the level of the human spiral ganglion (axosomatic synapses) (Kimura et al., 1987; Kimura et al., 1979; Nadol, 1988; Rask-Andersen et al., 2000; Thiers et al., 2000). The first type is similar to axodendritic synapses found in the OSB, since the presynaptic NFs had morphological characteristics of olivocochlear bundle fibers and synapsed on cell bodies of type-II. The synapses of the second type were similar to dendrodendritic synapses, as the presynaptic NFs had morphological characteristics of peripheral processes of type-II and these NFs synapsed on cell bodies of other
type-IIIs. In one human specimen these axosomatic synapses (both types) were found on up to 50% of the type-IIIs (Thiers et al., 2000).

The objective of this study was to quantify synapses in the three rows of the OSB and to characterize their ultrastructural morphology in a human infant.

**Material & Methods**

**Case History**

An 8-month old male infant died of pulmonary edema. No further clinical data were available and the interval between death and fixation was 1 hour.

This specimen was chosen because of its excellent preservation and normal light microscopic examination of the organ of Corti and spiral ganglion.

**Histological Methods**

Both cochleas were fixed *in situ* by perilymphatic perfusion of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. Both temporal bones were removed with a bone plug cutter. After removing excess bone with rongeurs, the otic capsule was thinned with a power drill. The middle turn of the right cochlea was selected for the present study, while the left cochlea and the basal and apical portions of the right cochlea were used for other studies. The specimens were immersed in the same fixative solution for two weeks and were subsequently placed in 0.1 M disodium ethylenediamine tetra-acetic acid (EDTA) with 1% glutaraldehyde in 0.1 M phosphate buffer for decalcification. This EDTA solution was changed weekly in order to shorten the time required for decalcification. Subsequently, the cochlea was bisected, and the tissue was postfixed in reduced osmium tetroxide (reduction achieved with addition of 10mg/ml of potassium ferrocyanide) in 0.1 M phosphate buffer for 1 hour. The tissue was then stained *en bloc* with 5% aqueous uranyl acetate for 1 hour. Dehydration was achieved by placing the tissue in 2 five-minute changes of distilled water, 2 ten-minute changes of 50%, 70%, and 80% ethanol, 2 fifteen-minute changes of 95% ethanol, 6 twenty-minute change of 100% ethanol, and then in two 2 fifteen-minute changes of 100% propylene oxide. The tissue was finally embedded in 50/50 propylene oxide / Poly/Bed® for 1 hour, left uncapped overnight in 20/80 propylene oxide / Poly/Bed®, and exchanged with 100% Poly/Bed®.
The specimen from the middle turn of the right cochlea was oriented in the radial plane in order to obtain sections perpendicular to the tunnel of Corti. A total of 1000 serial sections were cut at a thickness of approximately 90nm. The sample size corresponded to a linear distance including 11 radial rows of outer hair cells (OHCs) (or approx. 0.09mm). The sections were subsequently placed on formvar-coated grids (10 sections per grid) and were stained with 5% uranyl acetate (double staining) and Sato’s triple lead citrate. Virtually all 1000 sections (less than 1% of sections were unusable) were analyzed under a magnification of 13,000X using the JEOL 100CX transmission electron microscope and every third section was photographed at a magnification of 5,000X and further magnified photographically to a final magnification of 13,000X. Upon development of the electronmicrographs the pictures taken from the same grid were organized in sequence and were superimposed for analysis. Reconstruction of the outer spiral bundles was performed by analysis of these semiserial electronmicrographs. The synaptic interactions found within the OSB were counted, and the NFs that formed them had their morphological characteristics studied.

**Results**

**Morphology of Nerve Fibers of the Outer Spiral Bundle**

There was a clear segregation between two groups of NFs contained in the OSB: vesicle-rich and neurofilament-poor NFs (VR/NP – Figure 1), and vesicle-poor and neurofilament rich NFs (VP/NR – Figure 2). The morphology of the VR/NP NFs did not change significantly within the OSB. However, the VP/NR NFs significantly changed in size and vesicle content along their spiral course. These VP/NR NFs had a larger cross-sectional diameter and contained more vesicles near their synapses with either other NFs or with OHCs, and were usually of smaller diameter without identifiable vesicles when distant from these synapses.
Figure 3.1 - Electronmicrograph of a Vesicle-Rich and Neurofilament-Poor (VR/NP) Neuron

The VR/NP neuron forms classical efferent synapse at the base of an outer hair cell (OHC). The cytoplasm of these neurons was usually filled with vesicles, with little or no neurofilaments. These were radial NFs at the level of the outer spiral bundle, which were usually large and whose diameter did not change significantly when away from synapses. SC – supporting cell. Scale bar = 1μm.

Figure 3.2 - Electronmicrograph of a Vesicle-Poor and Neurofilament-Rich (VP/NR) Neuron

The VP/NR neuron forms classical afferent synapse at the base of an outer hair cell (OHC). These neurons had few vesicles and their cytoplasm was usually filled with neurofilaments. These were spiral NFs at the level of the outer spiral bundle, which were usually smaller than the VR/NP NFs when close to the synapses. Their diameter decreased even more when the segments of these NFs were away from their synaptic interactions with other NFs and OHCs. Scale bar = 1μm.
When a NF could be traced from a synapse within the OSB up to the neural pole of an OHC, it was found that VR/NP NFs formed only classical efferent synapses with the OHCs, whereas the VP/NR NFs formed synapses with the OHCs of the afferent and reciprocal types.

**Synapses Between VR/NP and VP/NR NFs**

Synapses between VR/NP and VP/NR NFs had a substantial concentration of presynaptic vesicles and had small electrondense bodies attached to the presynaptic membrane (Figure 3). The vesicles were usually round and of uniform size, and the few dense core vesicles present were usually distant from the synaptic specialization. In a number of cases, there were membrane specializations in close proximity to the synapse. These were characterized by the presence of presynaptic electrondense condensations that were parallel to the cell membrane, close to the vesicle accumulation, and physically associated with mitochondria (Figure 4). These structures were not quantified in the present study, but were especially common in the NFs located in a plane just below the neural poles of the outer hair cells (OHCs) and within the tunnel of Corti.

![Figure 3.3 - Electronmicrograph of a Presumed Axodendritic Synapse](image)

A VR/NP NF (putative axon originating in the superior olivocochlear complex) synapsed on a VP/NR NF (putative dendrite of a type-II). An accumulation of clear and small vesicles at the synapse was evident. Dense core vesicles were not common and were usually found at a distance from the synapse. Small electrondense triangular shaped bodies (arrows) were frequently seen attached to the presynaptic membrane when followed in serial sections. This same VR/NP NF formed a classical efferent synapse and the VP/NR NF formed an afferent synapse on an OHC (not in this plane of section). SC- supporting cell. Scale bar = 1μm.
Synapses of VR/NPs on VP/NR NFs were abundant and their number decreased steeply from the first towards the third row (Figure 5). There was also a decrease in the absolute number of VR/NP NFs from first towards third row. The calculated density of this type of synapse was 2,233 synapses/mm (201/0.09mm), as measured in the radial direction. They were also found at the level of the tunnel of Corti at an intermediate level between the lower and upper tunnel NFs, but the synapses at this site were not quantified.
Synapses of VR/NP NFs on VP/NR NF’s (presumed axodendritic synapses) were counted in the 3 rows of outer spiral bundles. Axodendritic synapses were numerous and decreased substantially from the first towards the third row. The number of VR/NP NFs also decreased from the first towards the third row (not shown).

**Synapses Between VP/NR NFs**

VP/NR to VP/NR synapses were smaller than the ones formed by VR/NP NFs but the classical synaptic membrane condensation and vesicle accumulation were clearly discernible (Figure 6). Frequently, the dark cytoplasm of the VP/NR NFs made the identification of these synaptic specializations difficult. No unequivocal presynaptic electron-dense condensations parallel to the cell membrane and associated with mitochondria were found at synapses between VP/NR NFs. Some of these same VP/NR NFs also formed reciprocal synapses with OHCs (Figure 6).

Synapses of VP/NRs on other VP/NR NFs were also abundant and their numbers did not vary significantly in different rows (Figure 7). Similarly, the absolute number of VP/NR NFs did not seem to change significantly among outer spiral bundle rows. The calculated density of these VP/NR to VP/NR synapses was 1,366 synapses/mm (123/0.09mm). Synapses of VR/NPs on other VR/NP NFs were not observed.
Figure 3.6 - Electronmicrographs of a Presumed Dendrodendritic Synapse

The VP/NR1 NF formed a synapse on another VP/NR NF (VP/NR2) (6a and 6b). Synaptic membrane thickening and an accumulation of vesicles (arrow) were evident. The same NF (VP/NR1) also formed a reciprocal synapse on an OHC approximately 1.3μm away from the supposedly dendrodendritic synapse (6c – 6e). Serial sections of the neural pole of the OHC demonstrated both the afferent (AF - 6c) and the efferent (EF – 6d and 6e) components of the reciprocal synapse. Scale bar = 1μm.

Figure 3.7 - Number of Presumed Dendrodendritic Synapses by Row in a Human Infant

Synapses between VP/NR NF’s (presumed dendrodendritic synapses) were counted in the 3 rows of outer spiral bundles. The number of dendrodendritic synapses did not change significantly among the 3 rows. The absolute number of VP/NR NFs also did not seem to change in the different rows (not shown).
Discussion

The data presented here are consistent with the previous descriptions of the two types of NFs contained in the OSB (Dannhof and Bruns, 1993; Francis and Nadol, 1993a; Jones and Eslami, 1983; Liberman et al., 1990; Liberman et al., 2000; Nadol, 1983; Sato et al., 1997; Spoendlin and Gacek, 1963). Thus, the morphological characteristics of the VR/NP NFs found in our study are similar to the descriptions of the axons from the superior olivary complex, and the VP/NR NFs found in this specimen are morphologically analogous to dendrites of type -IIs.

The finding that the VR/NP NFs formed classical efferent synapses on OHCs (Figure 1) and the VP/NR NFs formed afferent (Figure 2) and reciprocal (Figure 6) synapses on the OHCs also give support to the assumption that VR/NP NFs are processes of olivocochlear bundle fibers and the VP/NR NFs are dendrites of type-IIs. Therefore, there is good evidence that synapses of VR/NP NFs on VP/NR NFs are axodendritic and the synapses between two VP/NR NFs are dendrodendritic.

The estimated density of presumed axodendritic contacts, found in the present study (2,233 synapses/mm), was several times higher than the number reported in cats using light microscopy (160 synapses/mm). Interspecies differences and/or better detection of these synapses using transmission electron microscopy may explain this difference.

The finding that the incidence of presumed axodendritic synapses decreased from the first towards the third row may be explained by an associated steep decrease in the absolute number of VR/NP NFs. The high number of presumed axodendritic synapses suggests that type-IIIs are profusely innervated by fibers of the olivocochlear bundle and that the olivocochlear efferent system performs its peripheral function not only via its axosomatic synapses on the OHC but also via synapses on type-II NFs. The total number of type-IIIs receiving axodendritic synapses probably ranges between 50 and 100% based on the finding that up to 50% of type-IIIs are innervated at the level of the cell body (Thiers et al., 2000) and the synapses on their dendrites are also numerous, as shown in the present study.

The presynaptic electrondense plaques associated with mitochondria were found in many presumed axodendritic synapses (Figure 4). These membrane specializations have been described in axosomatic synapses on type-IIIs (Kimura et al., 1987; Rask-Andersen et al., 2000;
Thiers et al., 2000) and have also been observed in neural circuits with especially high activity outside the cochlea (Gray, 1963; Rowland et al., 2000; Spirou et al., 1998).

The calculated density of presumed dendrodendritic synapses was also high, and may be an underestimate because of their small size and the electron-dense cytoplasm of the VP/NR NFs. There was neither a substantial change in the incidence of presumed dendrodendritic synapses nor a decrease in the absolute number of VP/NR NFs in the different rows.

The role of dendrodendritic synapses is speculative because the function of the type-IIIs is unknown. However, synapses between first order afferent neurons have been described in the vestibular macula (Ross, 1997). In the retina and in the olfactory bulb, dendrodendritic synapses are responsible for lateral inhibition and enhancement of contrast detection (Anton et al., 1993; Kirillova and Lin, 1998; Nakanishi, 1995). A similar mechanism may therefore exist among OHCs of the auditory receptor via dendrodendritic synapses of type-IIIs.

The finding of numerous putative axodendritic and dendrodendritic synapses on NFs that have morphological characteristics of dendrites of type-IIIs suggests a morphological basis for complex neural interactions (Figure 8) (Fechner et al., 2001). Further studies will be necessary for a deeper understanding of the physiologic function of this putative neural network.
Presumptive olivocochlear projections (VR/NP NFs) synapse on cell bodies (axosomatic synapses) and on the dendrites (axodendritic synapses) of presumptive Type-IIs (VP/NR NFs). Type-IIs interact with each other through dendrodendritic synapses at the level of the OSB and possibly at the level of the spiral ganglion as well (not represented). These Type-IIs also innervate supporting cells (Nadol and Burgess, 1994; Fechner et al., 2001) and form afferent and reciprocal synapses on OHCs.

Figure 3.8 – Diagram of Synaptic Interactions of the Neurons that Innervate the OHCs

Presumptive olivocochlear projections (VR/NP NFs) synapse on cell bodies (axosomatic synapses) and on the dendrites (axodendritic synapses) of presumptive Type-IIs (VP/NR NFs). Type-IIs interact with each other through dendrodendritic synapses at the level of the OSB and possibly at the level of the spiral ganglion as well (not represented). These Type-IIs also innervate supporting cells (Nadol and Burgess, 1994; Fechner et al., 2001) and form afferent and reciprocal synapses on OHCs.
CHAPTER 4 - Reciprocal Innervation of Outer Hair Cells in a Human Infant

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Abstract

Reciprocal synapses are characterized by the presence of both afferent and efferent types of synaptic specializations between two cells. They have been described at the neural poles of outer hair cells (OHCs) in humans with advanced age (Nadol 1984) and two monkey species (Francis and Nadol 1993a; Sato et al. 1997). Our objective was to study the innervation of the OHCs and determine if reciprocal synapses were present in a young (8-month old infant) human subject. We studied the synaptic and cytoplasmic morphology of 162 nerve terminals innervating 29 OHCs using serial section transmission electron microscopy.

Seventy-six percent of all OHCs were innervated by terminals with reciprocal synapses. This prevalence increased from the first towards the third row (p<0.001) and 100% of OHCs in the third row demonstrated at least one reciprocal synapse. The prevalence of terminals with reciprocal synapses was higher in the human infant than in older human subjects and was very similar to what has been reported for the chimpanzee. Reciprocal synapses occur in sufficient numbers to be physiologically significant in primates.

The nerve terminals were found to segregate into two groups on the basis of their cytoplasmic morphological characteristics: 1) vesicle-rich/neurofilament-poor (VR/NP), and 2) vesicle-poor/neurofilament-rich (VP/NR) terminals. All afferent and reciprocal terminals were of the VP/NR variety. The majority of the efferent terminals originated from VR/NP nerve fibers (classical olivocochlear morphology), but 23.5% of the efferent terminals were VP/NR.

The hypothesis that peripheral processes of type-II spiral ganglion cells form classical afferent, reciprocal and a number of purely presynaptic terminals on OHCs is discussed. The presence of different types of synaptic specializations on OHCs formed by nerve fibers of the same type (VP/NR) suggests the existence of reciprocal neuronal circuits between OHCs sharing the dendritic arborization of a type-II spiral ganglion cell.
Introduction

A reciprocal synapse is characterized by the presence of synaptic membrane specializations of opposite polarity between two cells. This type of synaptic relationship has been described in both the central and peripheral nervous systems. Reciprocal synapses are common in the brain (Famiglietti, 1970; Guan et al., 1995; Marcos et al., 1996), retina (Dowling, 1968; Hartveit, 1999), olfactory bulb (Isaacson and Strowbridge, 1998; Kirillova and Lin, 1998), autonomic nervous system (Kawai, 1996; Yamauchi et al., 1975), carotid bulb (Matsumoto et al., 1980; McDonald and Mitchell, 1975) and vestibular macula (Dunn, 1980; Lysakowski and Goldberg, 1997; Ross, 1997). In the organ of Corti, reciprocal synapses are characterized by the presence of both afferent and efferent types of synaptic membrane specializations between a single nerve fiber and a hair cell (Nadol, 1981).

Several studies describing the ultrastructure of the neural poles of the outer hair cells (OHCs) and inner hair cells (IHCs) in adult guinea pigs (Hashimoto and Kimura, 1988; Takasaka and Shinkawa, 1987) and cats (Liberman et al., 1990; Simmons and Liberman, 1988b) did not report the presence of reciprocal synapses. However, reciprocal synapses have been described in prenatal guinea pigs (Jones and Eslami, 1983; Thorn et al., 1972), and Tanaka and Smith (1978) reported their presence in the adult chicken. In addition, reciprocal synapses were found in kitten after section of the olivocochlear spiral bundle (Pujol and Carlier, 1982), and were described innervating IHCs in a tissue culture preparation of the organ of Corti in the mouse (Sobkowicz et al., 1993).

In the organ of Corti of primates, reciprocal synapses have been described only at the base of OHCs (Nadol, 1981; 1983; Nadol and Burgess, 1990; Spoendlin and Schrott, 1988). In the chimpanzee (Francis and Nadol, 1993a), and also in the Japanese macaque (Sato et al., 1999; Sato et al., 1997), the numbers of reciprocal synapses increased from the base towards the apex and from the first towards the third row. These findings in perfused animals indicate that reciprocal synapses are not the result of post-mortem changes or fixation artifacts.

Reciprocal synapses were present on 56% of OHCs in humans (Nadol, 1984) and on 74% of OHCs in the chimpanzee (Francis and Nadol, 1993a). Nevertheless, the somewhat advanced age of the studied subjects (human and chimpanzee) raised the possibility that the high prevalence of reciprocal synapses could be an age-related degenerative finding. The first
The objective of this study was to test the hypothesis that reciprocal synapses are also present in the innervation of OHCs of young humans.

The role of the reciprocal synapses on OHCs is unknown. Elucidation of the origin of the nerve fibers forming reciprocal synapses may provide clues. There are two plausible candidates for the origin of nerve terminals with reciprocal synapses, namely, fibers of the olivocochlear bundle and peripheral processes of type-II spiral ganglion cells (type-IIs). Currently, there is evidence that reciprocal synapses originate from type-IIs (Francis and Nadol, 1993a; Nadol, 1981; 1983; Sato et al., 1997). Olivocochlear fibers and processes of type-IIs can be differentiated by cytoplasmic morphology (Ginzberg and Morest, 1984; Liberman et al., 1990; Spoendlin and Schrott, 1988; Spoendlin and Gacek, 1963). Thus, olivocochlear efferent fibers are classically described as vesicle-rich/neurofilament-poor (VR/NP), whereas dendrites of type-IIs are described as vesicle-poor/neurofilament-rich (VP/NR). Based on these classical descriptions, we examined the morphology of all fibers synapsing on OHCs. Thus, our second objective was to determine, in a young human specimen, if we could classify the nerve fibers synapsing on OHCs as VP/NR (possibly processes of type-IIs) or VR/NP (possibly olivocochlear fibers) and correlate cytoplasmic morphology with synaptic morphology.

Material & Methods

Case History

An 8-month old infant died of pulmonary edema. No further clinical data were available. The interval between death and fixation was 1 hour. This specimen was chosen because of its excellent preservation and normal light microscopic examination of the organ of Corti and spiral ganglion. The protocol for removal and study of human temporal bones was approved by the Human Studies Committee of the Massachusetts Eye & Ear Infirmary.

Histological Methods

Fixation was achieved by perilymphatic perfusion of 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer. Excess temporal bone was then removed with rouggers, and a power drill was used to thin the otic capsule. Next, the specimens were decalcified in 0.1 M disodium ethylenediamine tetra-acetic acid (EDTA) with 1% glutaraldehyde.
in 0.1 M phosphate buffer. Subsequently, the cochlea was bisected, postfixed in reduced osmium tetroxide in 0.1 M phosphate buffer, stained en bloc with aqueous uranyl acetate, dehydrated in graded ethanol, exchanged with propylene oxide, and embedded in Poly/Bed®.

A sample of the middle turn was oriented in the radial plane in order to obtain sections perpendicular to the tunnel of Corti. A total of 1000 serial sections were cut with a thickness of approximately 90nm. The sections were subsequently stained with 5% uranyl acetate (double staining) and Sato’s triple lead citrate. Almost every section was photographed (less than 1% of sections were unusable) at a magnification of 13,000X using the JEOL 100CX transmission electron microscope. The final magnification was 32,000X.

The neural poles of a total of 29 OHC (8 in the first row; 9 in the second row; 12 in the third row) and 162 nerve terminals (56 in the first row; 51 in the second row; 55 in the third row) were reconstructed in their entirety by serial section electron microscopy.

**Analytical Methods**

After complete analysis of the serial section reconstruction of the entire area of apposition of the terminals and OHCs, the type of synaptic specialization was specified (afferent, efferent or reciprocal) and quantified in each row of OHC. The Mantel-Haenszel Chi-Square and the Fisher’s exact tests were used for the statistical evaluation of this quantification.

An afferent synapse was defined by the presence of vesicles abutting a membrane thickening (with or without pre-synaptic bodies) in the OHC; i.e., the terminal appeared to be postsynaptic to the OHC. In contrast, an efferent synapse was defined by the presence of vesicles within the terminal abutting the interface with an OHC, with an apposed subsynaptic cistern within the OHC. A reciprocal synapse was defined by the presence of morphologic evidence for an afferent and an efferent synapse between the same terminal and OHC (Figure 1).

The average number of reciprocal synapses per OHC was quantified and evaluated by analysis of variance of the logarithmic count. The prevalence of OHCs innervated by at least one terminal with a reciprocal synapse was also quantified and the results were compared with published numbers from the middle turn of the chimpanzee (Francis and Nadol, 1993a) using logistic regression tests.

The maximal cross sectional areas of the terminals were measured and the number of synaptic vesicles contained in the same plane of section was counted. Finally, the terminals were
classified into semi-quantitative categories of “low”; “medium”; and “high” neurofilament content. The differences between the categories of terminals found were analyzed using standard t-test and also the two-dimensional Kolmogorov-Smirnov test (Teukolsky et al., 1992).

Figure 4.1 - Reciprocal Synapse Between an OHC and a VP/NR Nerve Terminal

The afferent component of the reciprocal synapse (AF) was characterized by the presence of clear vesicles (v) abutting the OHC cellular membrane and presynaptic bodies (arrows) in the OHC. The efferent component (EF) demonstrated clear (v) and a few dense core vesicles (DCV) in the nerve terminal (VP/NR NT) and a subsynaptic cistern (SSC) in the OHC. Even though dense core vesicles were frequently present, usually there were only a few of them. The SSC often extended laterally as a subsurface cistern (asterisk).
Results

**Synaptic Interactions of the OHCs**

Reconstruction of the entire terminals proved to be essential for the identification of reciprocal synapses because their afferent and efferent components were rarely seen in the same plane of section (Figure 1). In addition, either the efferent or afferent components of reciprocal synapses were sometimes small and could, therefore, be missed with a semi-serial section technique.

The afferent and efferent components of the reciprocal synapses were indistinguishable from the afferent and efferent synapses found separately. In the reciprocal synapses, presynaptic bodies of the afferent component were common, and the subsynaptic cistern of the efferent component usually continued laterally as subsurface cistern.

Reciprocal synapses were observed in the 3 rows of OHCs. The total number of OHCs (weighed average) forming reciprocal synapses with at least one terminal was 76.3%. This prevalence increased significantly \((p<0.001)\) from the first towards the third row (Figure 2). Similarly, the average number of reciprocal synapses per OHC increased from the first towards the third row \((p<0.001)\) (Figure 3). However, there was no statistically significant difference between the second and the third rows.

![Figure 4.2 - OHCs Innervated by More Than one Terminal with a Reciprocal Synapse](image)

*Figure 4.2 - OHCs Innervated by More Than one Terminal with a Reciprocal Synapse*

Bar graph shows the prevalence of OHCs innervated by at least one terminal with a reciprocal synapse in the present study and in the Chimpanzee (Francis and Nadol 1993). Gradient from first towards third row was significant \((p<0.001)\). The data in our human specimen was not significantly different \((p>0.5)\) from that described in the middle turn of the chimpanzee (Francis and Nadol, 1993a).
Figure 4.3 – Average Number of Terminals with Reciprocal Synapse per OHC

Bar graph shows the average number of terminals with a reciprocal synapse per OHC (only OHCs with reciprocal terminals included). Gradient from first towards third row was significant (p<0.001). However, there was no statistically significant difference between the second and the third row.

Nerve Terminals Innervating the OHCs

The data on size, vesicle counts and neurofilament content was plotted (Figure 4). A clear segregation between two groups of terminals was present. Terminals of the first group were rich in vesicles (over 69 vesicles; mean 228.2 (+/- 118.1), had low neurofilament content (low neurofilament terminals), and were usually large (mean maximal cross sectional area – 5.2 \( \mu \text{m}^2 \) (+/- 2.1). In contrast, the terminals of the second group had few vesicles (less than 36 vesicles; mean 12.9 (+/- 7.8), had medium or high neurofilament content (medium and high neurofilament terminals) and were usually small (mean maximal cross sectional area: 1.3 \( \mu \text{m}^2 \) (+/- 1.1).

Using the standard t-test, we found the difference between the means of the two groups of terminals for both cell size and the number of vesicles to be significantly different (p<0.001 in both cases). A two-dimensional Kolmogorov-Smirnov test also showed the difference between the two-dimensional distributions of the two groups of terminal’s data to be highly significant (p<0.0001). This significant morphological dichotomy was also evident in qualitative terms (Figure 5).
Figure 4.4 - Morphometric Analysis of Terminals with Different Synaptic Specializations

Morphometric data and types of synaptic specialization of 162 nerve terminals analyzed at their maximal cross sectional diameter. There was a clear segregation of two groups of terminals by number of vesicles, size and neurofilament content (p<0.0001). The synaptic morphology was determined by serial section reconstruction. All the terminals forming afferent and reciprocal synapses were had medium or high neurofilament content and had less than 36 vesicles. Most terminals forming only efferent synapses grouped with the larger, vesicle rich and neurofilament poor group (VR/NP), while others grouped with the smaller, vesicle poor and neurofilament rich (VP/NR) fibers. Aff – afferent. Eff – efferent. Rec – reciprocal. NF – Neurofilament content.

Figure 4.5 - Two Morphological Distinct Types of Terminals Contact the OHCs

(A) Vesicle-rich and neurofilament-poor (VR/NP) nerve fiber. These nerve fibers had large dimensions, large number of clear vesicles distributed throughout the terminals and little cytoplasmic neurofilament. This description has been classically used for peripheral projections of the olivocochlear bundle. (B) Vesicle-poor and neurofilament-rich (VP/NR) nerve fiber. These nerve fibers were usually small, had few vesicles and were filled with neurofilaments. This is the classical description used for peripheral projections of type-IIs. Both types of nerve fibers demonstrated a few dense core vesicles. SC – Supporting Cell.
Correlation of Synaptic and Cytoplasmic Morphology

All the terminals that formed afferent (Figures 6a and 6b - VP/NR postsynaptic terminals or “classical afferents”) and reciprocal synapses (Figure 6d - VP/NR reciprocal terminals) belonged to the group of fibers with less than 36 vesicles, more neurofilaments and smaller size. The majority of the terminals that were found to form only efferent synapses were of the group with more than 69 vesicles, fewer neurofilaments and larger size (VR/NP presynaptic terminal or “classical efferents”). However, 23.5% of the terminals that formed only “efferent” synapses were of the VP/NR nerve fiber group (Figure 6c). Because we do not know their neuron of origin, we will call this second group of “efferent” terminals of “VP/NR presynaptic terminals”.

Figure 4.6 - VP/NR Terminals Forming Different Types of Synaptic Specializations on OHCs

(A) VP/NR presynaptic and VP/NR postsynaptic (classical afferent) side by side, (B) VP/NR postsynaptic (classical afferent); (C) VP/NR presynaptic; (D) VP/NR reciprocal. The variability in the size of the terminals was small and they were indistinguishable by number of vesicles and neurofilament content. Note that the different nerve fibers could not be differentiated by their cytoplasmic morphology, implying that they have a common type of neuron of origin. SC – Supporting Cell.
The prevalence of terminals of the VP/NR postsynaptic type ("classical afferents") decreased from the first towards the third row, but this finding was not statistically significant. On the other hand, the increasing gradient of the number of VP/NR reciprocal terminals was significant \((p<0.001)\), as was the increase of VP/NR presynaptic terminals from the first towards the third row (Fig. 7).

Figure 4.7 – Number of Terminals of Different Types Contacting OHCs of the Three Rows

Types of nerve terminals on different rows of OHCs and correlation between cytoplasmic content and synaptic morphology. There was a statistically significant \((p<0.001)\) decrease in the numbers of VR/NP presynaptic (classic efferents) from the first towards the third row, so that, in the third row, there were no terminals of this type. The decrease of the VP/NR postsynaptic (classic afferents) towards the third row was not statistically significant. The increase of the VP/NR reciprocal and presynaptic from the first towards the third row was statistically significant \((p<0.001)\). Inverse gradients of classical efferents and VP/NR reciprocal and presynaptic were evident.

The decrease in the numbers of VR/NP presynaptic terminals ("classical efferents") from the first towards the third row was also statistically significant \((p<0.001)\). It should be noted that there was no "classical efferent" terminals innervating the OHCs of the third row (Figure 7). In other words, the only "efferent" synapses on the OHCs of the third row were part of a VP/NR reciprocal synapse or were VP/NR presynaptic terminals. Furthermore, seventy five percent of OHCs (9 cells - not shown in Figure 7) in the third row did not demonstrate any VP/NR presynaptic terminals. Consequently, the only form of "efferent input" to these cells was through the "efferent" components of the VP/NR reciprocal terminals.
The analysis of all the terminals as a group (irrespective of cell row), resulted in the finding that 48% of the terminals were VP/NR postsynaptic ("classical afferents"), 31% were VP/NR reciprocal, 5% were VP/NR presynaptic and 15% were VR/NP presynaptic ("classical efferents").

Numerous synaptic interactions between VR/NP and VP/NR nerve fibers and between two VP/NR nerve fibers have been found at the level of the outer spiral bundle and tunnel of Corti in the same specimen. They will be described in a separate publication.

Discussion

Reciprocal Synapses

The prevalence and characteristic distribution of the reciprocal synapses by row described in our study was similar \( p>0.5 \) to that in the chimpanzee (Francis and Nadol, 1993a) (Figure 2) and higher than the one found in older human specimens (Nadol, 1984). The finding of reciprocal synapses in a specimen at this young age strongly suggests that the presence of reciprocal synapses in humans is not an age-related degenerative finding. Furthermore, the present findings support the concept of an ascending gradient, from first towards third row, of distribution of reciprocal synapses. The presence of a subsynaptic cistern and presynaptic vesicles in the VP/NR terminals suggests an “efferent” physiologic function.

Innervation of OHCs

The scatter plot in Figure 4 is consistent with the current view that there are only two types of neurons that form the outer spiral bundle and innervate the OHCs (Figure 5). As mentioned, previous studies concluded that vesicle rich and neurofilament poor (VR/NP) fibers are processes of the medial olivocochlear efferent system, and the nerve fibers that are vesicle poor and neurofilament rich (VP/NR) are likely peripheral processes of type-IIIs (Ginzberg and Morest, 1984; Liberman et al., 1990; Nadol and Burgess, 1990; Sato et al., 1997; Spoendlin and Schrott, 1988; Spoendlin and Gacek, 1963).

Therefore, the fact that nerve fibers that were VR/NP formed only efferent terminals on the OHCs and that all afferent terminals were VP/NR nerve fibers was expected. However, nerve fibers that were indistinguishable from the ones that formed these classical afferent
terminals were also presynaptic to the OHCs (VP/NR reciprocal and VP/NR presynaptic terminals - Figure 6). This suggests that some “efferent” synapses may originate from processes of type-IIs.

VP/NR nerve fibers that are presynaptic to OHCs have been previously described. Thus, terminals with reciprocal synapses described in primates were VP/NR (Francis and Nadol, 1993a; Nadol, 1984). Furthermore, VP/NR terminals, which are purely presynaptic and predominate in the third row, have been found in the cat (Liberman et al., 1990). These VP/NR presynaptic terminals may correspond to “efferent” terminals described in mammalian OHCs, which are small, predominate in the apex (Francis and Nadol, 1993b), and have non-classical immunohistochemical properties (Eybalin and Altschuler, 1990).

Two theoretical possibilities exist to explain the finding that nerve fibers with the same cytoplasmic morphology (VP/NR terminals) can be purely postsynaptic (“afferent”), reciprocal or purely presynaptic (“efferent”) to OHCs. The first possibility is that they originate from three different subpopulations of neurons with similar morphology. The second possibility is that the same neuron can have three distinct types of synaptic relationships with different OHCs. Even though we cannot exclude the first possibility, the second one is theoretically more likely for the following reasons. Even though type-I neurons might transiently project to the OHC region during early stages of ontogeny (Pujol et al., 1998), there is no evidence for more than two types of neurons (i.e. type-IIs and olivocochlear fibers) innervating OHCs in mammals after the second postnatal week (Berglund and Ryugo, 1987; Brown, 1987a; Ginzberg and Morest, 1984; Kiang et al., 1982; Simmons and Liberman, 1988b; Simmons et al., 1991). Secondly, there is precedence for a first order “afferent” neuron to also form “efferent” synapses on receptor cells in other sensory systems, such as in the vestibular macula (Chimento and Ross, 1996; Ross, 1997) and in the carotid body (McDonald and Mitchell, 1975; McDonald and Mitchell, 1981).

This concept runs counter to the notion that all “efferent” terminals are derived from the olivocochlear bundle. However, after experimental section of the olivocochlear bundle some “efferent” terminals remain. Thus, in the study of Bodian and Gucer (1980), although most efferent nerve terminals degenerated after section of the olivocochlear bundles, total absence of efferent nerve terminals was not confirmed by serial section reconstruction of the neural poles of the OHCs. In addition, residual “efferent” nerve terminals were found after section of the olivocochlear bundle in other studies (Kimura and Wersall, 1962; Smith and Rasmussen, 1963;
Spoendlin and Gacek, 1963). The presence of these residual “efferent” terminals was attributed to incomplete section of the olivocochlear projections or insufficient postsurgical survival time. Furthermore, in studies in which only the crossed olivocochlear system was sectioned (Iurato et al., 1978; Kimura and Wersall, 1962; Nakai and Igarashi, 1974), it was observed that the “efferent” terminals of the apical half of the cochlea were relatively unaffected. This was interpreted as a result of a baso-apical gradient of innervation by crossed olivocochlear projections. However, an alternate hypothesis exists, namely that some “efferent” (presynaptic) terminals, in the apical half of the cochlea, may not originate from olivocochlear projections, but rather from collateral projections of type-IIIs.

It is interesting to note that the increasing gradient of VP/NR nerve fibers with “efferent” membrane specializations (VP/NRs reciprocal and presynaptic) was complementary to the decrease of VR/NP presynaptic terminals (“classical efferents”) from the first towards the third row (Figure 7). This decreasing gradient of the classical cholinergic olivocochlear efferents (VR/NP presynaptic) has been well described in the literature (Ishii and Balogh, 1968; Nadol et al., 1993).

Reciprocal Innervation of the OHCs

We propose the use of the term “reciprocal innervation” to describe this neuron/receptor cell interaction via reciprocal synapses. Classical “afferent” and “efferent” innervation implies hair cell to neuron or neuron to hair cell transmission. However, this unidirectionality may not apply to some neurons innervating the OHCs, especially those forming reciprocal synapses.

Reciprocal synapses have not been reported to be as common on OHCs of non-primates (Ginzberg and Morest, 1984; Hashimoto and Kimura, 1988; Jones and Eslami, 1983; Liberman et al., 1990). However, if some VP/NR presynaptic nerve terminals are actually processes of type-IIIs, one neuron may form an afferent synapse with one OHC and an “efferent” synapse with another cell, creating a “reciprocal innervation circuit”. A similar type of neural organization (combination of reciprocal, afferent and “efferent” terminals formed by the same “interneuron” on different cells – reciprocal interaction) is very common at the level of second order neurons in the olfactory bulb and retina. These interneurons have been suspected of forming “reciprocal inhibition circuits” (Anton et al., 1993; Chen et al., 2000; Isaacson and Strowbridge, 1998; Kirillova and Lin, 1998; Nakanishi, 1995; Woolf et al., 1991).
Further studies will be necessary to test the hypothesis that the type-IIIs form “reciprocal innervation circuits” in mammals. Recent studies (Fechner et al., 2001; Thiers et al., 2000; 2002a) demonstrate that type-IIIs are probably profusely innervated by fibers of the olivocochlear efferent system, form dendrodendritic synapses, and also innervate supporting cells in mammals. These findings lend credence to the hypothesis that type-IIIs may be the morphological substrate for a complex neural network in the auditory periphery (Figure 8).

Figure 4.8 - Hypothetical Neural Network in the Outer Spiral Bundle

A hypothetical reciprocal innervation circuit (neural network) at the level of the outer spiral bundle. In this case a peripheral process of a type-II (which can innervate up to 100 OHCs – Simmons and Liberman 1988; Sato et al. 1997) forms different types of synaptic specializations (afferent, reciprocal and efferent) on different OHCs. The other components of the network may be synapses between dendrites of type-IIIs (dendrodendritic synapses - Nadol 1983); axosomatic and axodendritic synapses formed by fibers with the morphology of olivocochlear projections (Kimura 1979; Thiers et al. 2000, 2001); and the innervation of the supporting cells by the type-IIIs (Fechner et al. 2001).
CHAPTER 5 – The Outer Spiral Network and its Innervation by the Olivocochlear System in Cats

Abstract

Outer hair cells (OHCs) are key elements of the mammalian hearing system, which amplify sound-evoked signals transmitted into the inner ear. OHCs are innervated by peripheral projections of olivocochlear (OC) and type-II spiral ganglion neurons.

Type-II neurons innervate up to 100 OHCs, but their function is unknown. It has been suggested that type-II neurons interconnect neighboring OHCs through reciprocal synapses, which are bi-directional (afferent and efferent) synaptic interactions (Nadol, 1981). Since reciprocal synapses on the OHCs have been shown to be prevalent only in primates, they were not thought to be an universal mammalian feature. In addition to their interactions with OHCs, type-II neurons are also innervated by OC neurons. Synapses between OC and type-II neurons (OC/type-II synapses) have been described (Smith and Rasmussen, 1963), but the interactions between individual fibers/terminals have not been characterized in detail.

Synaptic interactions of type-II neurons with OHCs and OC neurons in humans and cats were studied with serial and semi-serial section transmission electron microscopy. Analysis included three normal cats and an animal submitted to neonatal OC bundle transection.

A high prevalence of nerve terminals with reciprocal synapses was observed in the cat and these terminals were processes of type-IIs, and not of OC neurons. Reciprocal type-II terminals were found in all frequency regions studied, but were most prevalent below 4,000Hz. All the type-II fibers traced to more than one OHC had reciprocal interactions with OHCs. Type-II fibers/terminals were heavily innervated by OC neurons, which preferentially targeted terminals with reciprocal synapses that were predominantly afferent. The innervation patterns of type-IIs and OC neurons in the cat were similar to that found in comparable frequency regions of primates.

Type-II neurons have reciprocal synaptic interactions with OHCs and form an “outer spiral network”, which may functionally integrate the OHCs. The OC system may modulate this network through OC/type-II synapses. The outer spiral network and its innervation by the OC system seem to be relevant to OHC function, and further research is needed to determine their role in hearing.
Introduction

The inner hair cells (IHCs) and outer hair cells (OHCs) are the mechano-transducers of the mammalian auditory sensory organ – the cochlea. They transform sound stimuli into coded electrical impulses, which are sent to the central nervous system. The inner hair cells provide most of the sound-evoked information that is transmitted into the central nervous system, but this information is pre-processed inside the cochlea by the OHCs (Nobili et al., 1998). These OHCs act as motor elements to amplify mechanical energy contained in the sound stimulus, and are key to the remarkable sensitivity of the mammalian auditory system (Dallos, 1992).

The OHCs in adult mammals are innervated by medial olivocochlear (OC) neurons and by type-II spiral ganglion neurons (type-IIs) (Guinan, 2006; Raphael and Altschuler, 2003; Spoendlin, 1985), which have been found to have distinct morphological features in both light and electron microscopy studies. Light microscopy studies employing neuronal tracing techniques (Brown, 1987b; Fechner et al., 2001; Ginzberg and Morest, 1983; Guinan et al., 1983; Liberman and Brown, 1986; Warr and Boche, 2003; Warr and Guinan, 1979; Wilson et al., 1991) have shown that OC neurons send descending projections to the cochlea through the olivocochlear bundle (OCB), which cross the tunnel of Corti and project radially to the OHCs. Ultrastructural studies have reported that nerve fibers with this spatial distribution are large and filled with vesicles (Liberman et al., 1990; Smith and Rasmussen, 1963). The type-II neurons give rise to peripherin-positive nerve fibers that cross the tunnel of Corti at its floor and spiral underneath the OHC rows to give rise to terminals contacting up to 100 OHCs, preferentially of the same row (Berglund and Ryugo, 1987; Brown, 1987a; Hafidi, 1998; Kiang et al., 1982; Simmons, 1986). These type-II fibers have been found to be relatively thin, neurotubule/filament-filled and poorly vesiculated at an ultrastructural level (Dannhof and Bruns, 1993; Dunn, 1975; Ginzberg and Morest, 1984; Liberman et al., 1990; Simmons, 1986; Simmons and Liberman, 1988b; Spoendlin and Gacek, 1963). It has also been suggested that neurons of the lateral OC system (Guinan et al., 1983; Vetter et al., 1991) and immature type-I spiral ganglion neurons (Perkins and Morest, 1975; Simmons et al., 1991) innervate the OHCs, but the morphological characteristics of these neuronal pathways are still not well determined.

OC nerve fibers give rise to large and vesicle-filled OC terminals, which form efferent synapses with the OHCs (Spoendlin and Gacek, 1963), while type-II fibers give rise to small,
neurotubule/filament-filled and poorly vesiculated type-II terminals that form afferent synapses on OHCs (Liberman et al., 1990). In efferent synapses on OHCs, the synaptic vesicles are within the nerve terminal and this terminal is apposed to a sub-synaptic cistern located at the inner surface of the OHC membrane. The location of synaptic vesicles in relation to the sub-synaptic cisternae of efferent OHC synapses indicates that the transmission of information at that point is from nerve terminal towards OHC. In afferent synapses on OHCs, there is a narrowing of the cleft with pre and post-synaptic membrane condensation in the region where the vesicles inside the OHCs aggregate. The location of the synaptic vesicles in relation to the membrane specialization suggests that the transmission of information at that point is from OHC towards nerve terminal.

Studies in primates suggested that a substantial number of nerve terminals interact with the OHCs through reciprocal synapses (Francis and Nadol, 1993b; Nadol, 1981; Sato et al., 1997; Spoendlin and Schrott, 1988; Thiers et al., 2002b), but this type of synaptic interaction was not previously reported as common in non-primate mammals (Ginzberg and Morest, 1984; Hashimoto and Kimura, 1988; Jones and Eslami, 1983; Liberman et al., 1990; Simmons and Liberman, 1988b). In a reciprocal synaptic interaction an afferent synapse is paired with an efferent synapse at the same cell membrane interface between a nerve terminal and an OHC, and the polarity of the transmission of information between the nerve terminal and OHC is seemingly bi-directional (OHC to terminal & terminal to OHC).

Based on the morphological features of the nerve terminals with reciprocal synapses in primates, it has been suggested that they are derived from type-II neurons, not from OC neurons (Francis and Nadol, 1993a; Nadol, 1984; Thiers et al., 2002b). The presence of reciprocal synaptic interactions between type-II fibers and OHCs would be especially relevant because of the innervation patterns characteristic of individual type-II fibers. As mentioned, a given type-II fiber can give rise to terminals contacting up to 100 OHCs (Sato et al., 1999; Simmons, 1986). If the type-II terminals from the same parent type-II fiber have bi-directional (reciprocal) interactions with the innervated OHCs, this fiber could hypothetically enable bi-directional intercommunication among OHCs (Nadol, 1981; Simmons, 1986; Spoendlin, 1982; Thiers et al., 2002b). The presence of reciprocal interactions between type-IIs and OHCs as a prevalent mammalian feature – and not limited to primates - could enable the study of these interactions in more depth, as invasive physiological experimentation in primates is more difficult.
In the investigation to be presented, we determined the prevalence of type-II terminals with reciprocal synapses in the cat, described their characteristics, compared their features with the ones found in primates, and assessed the distribution of reciprocal type-II terminals across OHC rows and in different frequency regions. The analysis to be presented allowed us to determine whether the type-II fibers form a neural network within the outer spiral bundle, as suggested to be the case in humans (Thiers et al., 2002b), and provided preliminary insights into the ways in which neighboring OHCs might communicate with each other via reciprocal interactions.

The other feature of the OHC innervation that was quantitatively addressed in this study is the seemingly extensive innervation of the type-II neurons by the OC fibers. These synapses will be referred to as OC/type-II synapses and have been found in previous studies to target different parts of the type-II neurons, especially along their course within the outer spiral bundle (Dunn, 1975; Liberman et al., 1990; Thiers et al., 2002a). The relative distribution of the OC/type-II synapses targeting the fiber or the terminal segments of the type-II neurons was not addressed quantitatively in these previous studies and we were particularly interested in how these synapses were positioned in relation to type-II fibers/terminals forming reciprocal synaptic interactions with the OHCs.

We chose the cat as the non-primate animal model to be studied because this species has been used extensively in hearing research and has a cochlea that is specialized to hear both low and high frequency sounds. Having a substantial portion of the cat cochlea coding sounds in the frequency range that is comparable to the one preferentially coded in primates (relatively low frequencies) was important because we wanted to directly compare the OHC innervation of cats and primates, while minimizing the effects frequency-dependent anatomical variations. Four adult cats were studied, and one of the cats was previously submitted to neonatal complete surgical transection of the OCB (which contains the peripheral projections of the medial and lateral OC neurons to the cochlea). It is likely that virtually all terminals studied in this experimental specimen originate from type-II neurons, and the analysis of its OHC innervation has assisted in the determination of the source of terminals innervating the OHCs.
Material & Methods

Animals Studied
The adult cats selected for our morphological study underwent single-fiber recordings, which showed normal cochlear function in both ears and at all regions (Table 1).

<table>
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<th>Analytical Method</th>
<th>Specimen</th>
<th>Frequency Region</th>
<th># OHCs</th>
<th># Terminals</th>
<th>Row(s)</th>
<th>Longitudinal</th>
<th>Parallel to Cuticular plate</th>
</tr>
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<tr>
<td>Serial Section</td>
<td>Cat # 1</td>
<td>700 Hz</td>
<td>18</td>
<td>421</td>
<td>1,2,3</td>
<td>X</td>
<td></td>
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<tr>
<td></td>
<td>Cat # 2</td>
<td>1,000 Hz</td>
<td>5</td>
<td>58</td>
<td>2</td>
<td>X</td>
<td></td>
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<tr>
<td></td>
<td>(Cut OCB)</td>
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<td></td>
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<tr>
<td>Semi-Serial Section</td>
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<td>16</td>
<td>239</td>
<td>1,2,3</td>
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<td>1,2,3</td>
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<td>186</td>
<td>1,2,3</td>
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<td>227</td>
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Table 5.1 - Description of Database in the Study of Cats
The four different cats were studied with different sampling methodologies and planes of section. Serial section involved analysis of every section through the neural poles of the studies OHCs, while in the semi-serial section analysis every fifth section was studied. The frequency region determination was based on the cochlear frequency map of the cat (Liberman, 1982), and the number of terminals refers to elements contacting the OHCs studied in each frequency region.

Histologic Preparation
All animals were perfused intravascularly with a solution containing 2.5% glutaraldehyde and 1.5% paraformaldehyde in 0.065 M phosphate buffer at pH 7.3. Immediately after perfusion, the cochleae were dissected and both the round and oval windows opened. Additional fixative was then perfused into the cochlear scalae and the ears were left in fixative for several days. At the end of the post-fixation, the ears were perfused with 1% osmium tetroxide for one hour and then rinsed, dehydrated and embedded in epoxy resins. Once polymerization was complete, the plastic and bone were drilled away, and the organ of Corti was cut into about 16 pieces. These pieces were re-embedded, thinned, and mounted in microscopic slides for light microscopy evaluation as surface preparations of the organ of Corti. The total length of each cochleae was measured by using computerized planimetry of drawing tube sketches, and the frequency
correlates of each piece was determined from the relative position along the cochlear spiral based on the cochlear frequency map for the cat (Liberman, 1982b).

Before the analysis under the Transmission Electron Microscope (TEM), the pieces of the organ of Corti corresponding to the frequency regions of interest were removed from the slides used for light microscopy and were further trimmed with razor blades to eliminate bone remnants. Serial ultrathin (~90nm) sections were cut in two different planes: parallel to the outer spiral bundle and longitudinal axis of OHCs (cats # 1 and # 4) and parallel to the cuticular plate (cats #s 2 and 3) (Figure 1). The ultrathin sections were harvested in ribbons on Formvar coated slot grids, stained with uranyl acetate and lead citrate, and examined either on a Philips CM 10 TEM (cats #s 1 and 4) or on a Jeol 100CX TEM (cats # 2 and 3).

Analytical Strategy

In the analysis of cat # 1, we employed serial section electron microscopy, which involved cutting, photographing, printing and analyzing every section through the neural poles of randomly selected six neighboring OHCs (in each row) and their underlying outer spiral bundle. No sections were missing in the serial section reconstruction of cat # 1, which allowed us to perform complete reconstructions of membrane specializations between terminals and OHCs, while enabling the tracing of type-II and OC terminals to their parent fibers within the reconstructed outer spiral bundles of the three OHC rows. The analysis of cats # 2, 3, and 4 employed semi-serial section electron microscopy (photographing and printing every 5th section), which was used to determine how universal the findings of the serial section analysis of cat # 1 were. Semi-serial section electron microscopy with analysis of every five sections did not allow full reconstruction and tracing of outer spiral bundle elements, but enabled a quantitative assessment of the relative prevalence of reciprocal type-II terminals in different cochlear regions.
Schematic representation of the organ of Corti showing the planes of section employed in the study, and the course of nerve fibers that innervate the OHCs, which are the peripheral projections of olivocochlear (OC) neurons and type-II spiral ganglion neurons. The longitudinal plane of section is perpendicular to the bottom of the OHCs and parallel to the respective OHCs' rows. The plane parallel to the cuticular plate is tangential to the bottom of the OHCs. OC fibers (in green) are radial, that is, they cross the tunnel of Corti and directly project to the bottom of the OHCs. The peripheral projections of type-II spiral ganglion neurons (in yellow) cross the tunnel of Corti close to its floor, and spiral basally to ultimately give rise to terminals contacting up to 100 OHCs.

The plane of section parallel to the outer spiral bundle and perpendicular to the neural poles of the OHCs was chosen to enable the tracing of type-II fibers along their spiral course underneath the reconstructed OHCs. The plane of section perpendicular to the neural poles helped in the study of the membrane appositions (synapses) between terminals and OHCs in the bottom (and basal/apical sides) of the neural poles. Visualization of synaptic structures becomes difficult when the plane of section is parallel to the apposed membranes, and a considerable number of terminals (53%), especially the ones on the modiolar or lateral sides of the neural poles, were still not in a plane of section that allowed full characterization of their synaptic morphology. The plane of section parallel to the cuticular plate (used for cats #s 2 and 3) is not optimal to visualize the terminals at the bottom of the OHCs, but can show the ones that are in the modiolar, lateral, apical, and basal sides of the neural poles. The proportion of modiolar and lateral type-II terminals that were reciprocal were quantified in order to determine if the exclusion of these terminals in cat # 1 was causing a selection bias.
The frequency region chosen for the serial sections analysis of cat # 1 (700 Hz) is in the area with highest number of OHC contacts per type-II fiber (apical half of the cat cochlea) (Simmons, 1986), and roughly corresponds to the upper middle turn region previously studied in a human subject by our group (Thiers et al., 2002a; b). The overall magnification (29,000X) was also comparable to that used in that human study (32,000X) and we also studied the three rows of OHCs. We wanted to analyze a region comparable to the one studied in humans because we wanted to make quantitative comparisons between the two datasets, while minimizing frequency-dependent changes in cochlear innervation that are known to occur in primates and in the cat (Francis and Nadol, 1993a; b; Liberman et al., 1990). The most important difference in the methodology used in the human and the analysis of cat # 1 was cutting the neural poles of the human OHCs in the radial plane, instead of the longitudinal plane used for cats #s 1 and 4.

Cats # 2 and # 3 were studied with semi-serial section in a lower magnification (12,000X) to maximize the visualization of neural poles with fewer photographs. The objective of the study of the cat submitted to neonatal surgical section of the OCB (cat # 2) was to assist in the morphometric classification of fibers/terminals innervating the OHCs. We also determined whether the previously described terminals with efferent (reciprocal) synapses that remain after surgical section of the OCB have morphology of type-II neurons. Only type-II terminals were expected to be present in the 1,000 Hz frequency region in this experimental specimen, and the relative prevalence of type-II terminals with reciprocal synapses was compared with a similar frequency region/row of cat # 3.

The semi-serial section analysis of cat # 3 was designed to address frequency specific changes in the innervation of the OHCs. Seven frequency regions were studied in cat # 3 (350Hz; 750Hz; 1,300Hz; 1,600Hz; 3,200Hz; 6,400Hz, and 12,000Hz), with the specific goal of determining if and how the relative prevalence of type-II terminals with reciprocal synapses are correlated with frequency region. Cat # 4 was studied with semi serial section and in a magnification (13,500X) that allowed direct comparison with the data obtained from cats # 2 and # 3. The only difference in the analytical methodology of cat # 4 was that it was studied in the longitudinal row, similarly to cat # 1. Sectioning in the longitudinal plane facilitated determination if reciprocal innervation circuits could be documented in more than one animal.
Neonatal Section of the Olivocochlear Bundle (OCB)

Cat # 2 was submitted to surgical OCB section at the age of two days. The animal was anesthetized with isoflurane (0.5-2% mixed with O₂) and pentobarbital (20-40 mg/kg). After a longitudinal skin excision was made, the muscle layers overlying the skull were separated between the nuchal ridge and foramen magnun. A hole was then made in the skull, the dura was incised, the cerebellum gently elevated to expose the floor of the forth ventricle. The OCB was sectioned with a microknife, using surface landmarks, such as the midline furrow and the facial genua as guides. Off-midline cuts of ~4mm in depth were made along the sulcus limitans with the knife angled ~45° from vertical in the dorsomedial to ventrolateral direction. After OCB section the cerebellum was repositioned, the dural flaps were reapposed, the skull defect was closed with gelfoam®, and finally muscle layers and skin were sutured to close the wound. The animal was closely monitored until full recovery and was returned to its mother as soon as possible. Analgesics were administered as needed. Eleven months after surgery, physiological recordings were made and the animal was prepared for histologic evaluation. Data from this animal has been previously published (Liberman et al., 2000).

The completeness of the OCB section was ascertained by light microscopy evaluation of plastic-embedded surface preparations. An observer blinded to the identification of experimental groups measured the diameters of the combined thickness of fascicles of OC fibers as they crossed the tunnel of Corti at a distance of 10μm from the inner pillar feet. These measurements were made with computerized planimetry of drawing tube sketches at an overall magnification of 2,200X, using high high-power Nomarski optics without special staining. A quantitative estimate of the OHC innervation was calculated through the division of the added fascicular diameter in each dissected piece of the organ of Corti by the length of that piece. It has been shown in a previous study (Liberman and Gao, 1995) that average fascicular diameter is well correlated with the volume of OC terminals remaining on OHCs in guinea pigs submitted to partial OCB section. Counts of fascicles of OC fibers and OC terminals on OHCs were reported elsewhere (specimen 93042L), and OC fibers were found to be virtually absent, while there was no decrease in numbers of type-II fibers/terminals in comparison with controls (Liberman et al., 2000).
Morphological Criteria

We defined OC fibers as the fibers that are neurotubule-poor, vesicle-filled, and do not project longitudinally for more than two OHC widths. The OC terminals are the ones with more than 70 vesicles at their plane of maximal cross-sectional area. The type-II fibers are neurotubule-rich, vesicle-poor fibers which spiral for more than two OHC widths, while the type-II terminals are the terminals with less than 40 vesicles at their plane of maximal cross-sectional area.

The synaptic interactions between terminals and OHCs were characterized by en face reconstructions of their membrane interfaces. The following morphological criteria were employed in the characterization of synaptic specializations: 1) Membrane interface was defined as the zone of apposition between a nerve terminal and an OHC in which the distance between the external surface of the two apposing cell membranes is equal or smaller than 35nm; 2) Synaptic cleft was defined as the space between the nerve terminal and the OHC in the zone of contact; 3) Synaptic vesicle was defined by a roughly spherical cytoplasmic structure with diameter between 35 – 100nm located within 140 nm of the internal surface of the apposing membranes; 4) Afferent synaptic vesicle was defined as a vesicle located inside the OHC within 140nm of the afferent synaptic specialization; 5) Afferent synaptic specialization was defined as a continuous (observed in at least two adjacent serial sections) pre and post-synaptic membrane condensation in the area of apposition with corresponding narrowing of the synaptic cleft; 6) Efferent synaptic vesicle was defined as a vesicle located inside the nerve terminal within 140nm of the inner membrane of the nerve terminal apposed to the efferent synaptic specialization; 7) Efferent synaptic specialization (subsynaptic cistern) was defined as a continuous (observed in at least two adjacent serial sections) flat saccular structure that is directly apposed to the inner surface of the OHCs of the area of apposition. The distance of the subsynaptic cistern from the membrane is under 15nm and its height from OHC membrane is under 70nm.

The synaptic interactions between OC fiber/terminals and type-II fiber/terminals were characterized by a pre and post-synaptic membrane condensation with clustering of vesicles in the OC side.
Results

Distinguishing Type-IIs from OC Terminals

The nerve fibers/terminals present in the outer spiral bundle in the normal adult cat # 1 (Figure 2) were systematically studied with serial section electron microscopy and two morphologically distinct types of fibers were identified. Fibers of the first group were called (outer) radial fibers and had morphological features of OC fibers. These radial fibers were neurotubule-poor, vesicle-filled, did not project laterally (spiral) for more than two OHC widths, and were progressively less common towards the third row of OHCs. Fibers of the second group were called outer spiral fibers and had morphological characteristics of type-II fibers. They were neurotubule-rich, vesicle-poor, spiraled for more than two OHC widths and were abundant in all three OHC rows. Some vesicle-poor and neurotubule-rich fibers (type-II morphology) left the field of view before their longitudinal span could be determined and were excluded from the outer radial vs. spiral classification.

All the nerve fibers classified as outer radial or spiral were given identification codes and were traced to their respective terminals contacting the reconstructed OHCs, which were also given identification codes. The morphometric analysis of the terminals contacting the OHCs took into account the number of vesicles in the plane of maximal cross-sectional area, size of membrane interface with the OHC, and neurofilament content. This systematic analysis showed that the terminals traced to outer radial fibers and the ones traced to outer spiral fibers had distinct features, just like their parent fibers (Figure 3). Terminals traced to outer radial fibers had at least 70 vesicles at their plane of maximal cross-sectional area and were relatively large. These terminals also had morphological characteristics of previously described OC terminals. On the other hand, terminals traced to outer spiral fibers had less than 40 vesicles at their plane of maximal cross-sectional area and were relatively small. These terminals had morphological characteristics of previously described type-II terminals.
Figure 5.2 – Innervation of the OHCs in the Normal Adult Cat

Nerve fibers present in the first row of cat #1 could be characterized by their neurotubule content (high vs. low), vesicle content (high vs. low), and the degree to which they spiral longitudinally underneath the OHCs' rows (radial vs. spiral). The longitudinal span of the studied fibers was measured by OHC width. Neurotubule-poor and vesicle-rich ones with a longitudinal span of less than two OHCs (purple) were called radial (OC) fibers (green), while the neurotubule-rich and vesicle-poor fibers that had a longitudinal span of more than two OHCs were called spiral (type-II) fibers (yellow). The area within the rectangle is depicted in higher magnification in the upper panel of figure 4. The uncolored cellular processes are supporting cells. Orientation: left – toward basal turn; top – toward reticular lamina. Scale bar = 1μm.
Figure 5.3 - Olivocochlear & Type-II Fibers/Terminals in the Cat

Electronmicrographs showing the fibers and terminals of the outer spiral bundle (OCs & type-IIs) of the first row of cat # 1, with examples of synapses from OCs onto a type-II fiber (upper panel) and onto a type-II terminal (lower panel). The arrows in the upper panel represent the polarity of the synaptic specialization from the OC fibers to the type-II fibers. The same qualitative differences in vesicle and neurotubule/filament content between the OC and type-II fibers and terminals can be appreciated both at the level of the outer spiral bundle (upper panel) and at the level of the neural contacts with the OHC (lower panel). The type-II terminals did not have high neurotubule content like their parent fibers (unless they are en passant terminals), but they still presented high neurofilament content, which is also characteristic of type-II spiral ganglion cells. The OC terminal of the lower panel formed a large efferent synaptic specialization with the OHC, while the type-II terminals form reciprocal specializations, which are combinations of afferent (downward arrows) and efferent (upward arrows) synapses. The subsynaptic cistern apposed to the OC terminal was indistinguishable in morphological terms to the ones apposed to the type-II terminals. Both electronmicrographs had the same magnification and the upper panel corresponds to the area within rectangle of figure 3. The uncolored cellular processes are supporting cells. Orientation: left – toward basal turn; top – toward reticular lamina. Scale bar = 1µm.
In order to determine if these fibers/terminals with morphological characteristics of type-II terminals could have originated from nerve fibers contained in the OCB (cochlear projections of medial and lateral OC neurons), we have also performed a comparative analysis of terminal morphology (with vesicle counts) of the terminals innervating the OHCs of the cat in which the OCB had been transected (cat # 2). All the fibers/terminals observed in this animal had type-II morphology (Figure 4). The fibers were indistinguishable from the spiral fibers described in cat # 1 (type-II morphology), and the terminals had less than 40 vesicles at their plane of maximal cross-sectional area (average = 9.3 (+/- 4.3)). These terminals were also characteristically small, but their membrane interface area were not fully reconstructed and measured with serial section electron microscopy like the ones of cat # 1.

Based on the presented morphometric analysis (Figure 5) we will heretofore refer to the outer radial, neurotubule-poor, vesicle-filled fibers as OC fibers, and the terminals with more than 70 vesicles at their plane of maximal cross-sectional area as OC terminals. Likewise, the outer spiral, neurotubule-rich, vesicle-poor fibers were labeled as type-II fibers, and the terminals with less than 40 vesicles at their plane of maximal cross-sectional area as type-II terminals. Terminals with less than 40 vesicles traced to neurotubule-rich, vesicle-poor fibers (type-II morphology) that clearly projected in the lateral direction (spiral) but were not fully reconstructed because they exited the field of view were also classified as type-II terminals. These terminals were also labeled as type-IIs because they belonged in the same morphometric group as the ones traced to spiral (type-II) fibers and to the ones present in the cat with cut OCB.
The upper panel shows a representative sample of the innervation of second row OHCs of an adult cat (cat # 2 - 1,000 Hz frequency region) submitted to neonatal transection of the olivocochlear bundle (OCB), while the lower panel shows a sample of the corresponding region/row from a normal adult cat (cat # 3 - 1,300 Hz frequency region). The qualitative and quantitative (figure 10) comparison between the normal adult cats (cat # 3 and 4) with an animal with cut OCB was designed to determine whether the terminals labeled as type-II and forming reciprocal synapses did not originate from fibers of the OCB, which contain the descending projections of the medial and lateral superior olivary complex. In the experimental animal (cut OCB), only type-II fibers and terminals (colored in yellow) were observed, while there were numerous OC terminals (colored in green) in the same corresponding region/row in the normal cat. The morphological characteristics of the type-II terminals and fibers in both animals were indistinguishable. The OHCs are colored in purple and the uncolored cellular processes are supporting cells. Orientation: left – toward apical turn; top – toward lateral wall. Scale bar = 1μm.
Morphometric Analysis of Terminals Contacting the OHCs

Scatter plot depicting the size of the area of apposition and number of vesicles (log scale) of 258 terminals contacting 18 OHCs of the 700 Hz region of cat #1. The terminals traced to radial and vesicle-filled fibers (labeled as OC fibers -- empty squares) had at least 70 vesicles at their plane of maximal cross-sectional area and were relatively large. These terminals had morphological characteristics of peripheral projections of olivocochlear neurons and were referred to as OC terminals (filled green circles). The terminals traced to spiral and neurotubule-filled fibers (labeled as type-II fibers -- crosses) had less than 40 vesicles at their plane of maximal cross-sectional area and were relatively small. These terminals had morphological characteristics of peripheral projections of type-II spiral ganglion neurons and were referred to as type-II terminals (filled yellow circles). The morphometric segregation between the two groups of terminals sharing the same morphological features (OC vs. type-IIIs) is clear-cut. The OC terminals were found to form only efferent synapses on the OHCs, while the type-II terminals had reciprocal synaptic interactions with the OHCs. Type-II terminals refer to the yellow colored elements on figures 2, 3, 4, 6 and 15, while OC terminals are colored in green.

Figure 5.5 - Morphometric Analysis of Terminals Contacting the OHCs
Synaptic Specializations of Individual Type-II Terminals

The synaptic interactions of the terminals contacting the 18 OHCs of cat # 1 were systematically characterized and OC terminals formed only efferent synapses on the OHCs, while the type-IIs had reciprocal synaptic interactions with the OHCs (Figure 6). The efferent synaptic specializations of both OC and type-II terminals were characterized by the presence of a sub-synaptic cistern directly apposed to the inner surface of the OHCs at the membrane interface. In the afferent synaptic specializations of type-II terminals, we found pre and post-synaptic membrane condensations at the area of apposition with corresponding narrowing of the synaptic cleft. In the reciprocal synaptic interactions we found both afferent and efferent synaptic specializations at the same membrane interface between a type-II terminal and an OHC.

The method of \textit{en face} reconstruction was used to quantitatively characterize the synaptic interactions between OC and type-II terminals with the OHCs of cat # 1. This analytical method rendered 2-Dimensional representations of the membrane interface between individual terminals and OHCs, which allowed determination of the total area of the membrane interface, interpretable synaptic area, combined size of afferent specializations, afferent proportion of interpretable synaptic area, combined size of efferent specializations, and efferent proportion of interpretable synaptic area for both type-II and OC terminals. The type-II terminals with only two or less informative sections through their area of apposition (53% of the terminals) were excluded from the database because the analysis of such terminals did not render enough information for an accurate characterization of their form of synaptic interactions with the OHCs.

Vesicles were counted at the plane of maximal cross-sectional area, and the number of afferent and efferent vesicles of the reconstructed terminals. The efferent vesicles were located opposite to the efferent synaptic specialization (sub-synaptic cisternae) and within 140nm of the inner membrane of the nerve terminal. OC terminals had on average 190 vesicles/µm$^2$ of efferent synapse, while type-II terminals had 19 vesicles/µm$^2$ of efferent synapse. Afferent synaptic vesicles were within a distance of 140nm from the afferent synaptic specialization inside the OHC and type-II terminals had on average 62 vesicles per µm$^2$ of afferent synapse.
Serial sections through a type-II terminal (yellow) contacting an OHC (purple) are shown, together with the corresponding en face reconstruction. This 2-Dimensional representation of the membrane interface and specializations was created from synaptic specializations observed in each studied serial section. The anatomical information from each serial section was registered in a corresponding line of a tracing paper, which had horizontal evenly spaced lines corresponding to the average distance between serial sections (90nm). The linear graphic representations of each serial section were connected with outlines drawn around the contiguous specializations of the same type (afferent - blue or efferent - red) present in neighboring sections. These reconstructed synaptic specializations characteristically had the shape of plaques on the membrane interface, which were commonly fragmented in two-three pieces (up to six) without readily identifiable stereotyped patterns. The area measurements of the total membrane interface and synaptic specializations were extracted from digitized versions of the outlines with NIH's ImageJ® software. This 2-Dimensional en face reconstruction methodology was used for both type-II and OC terminals. The uncolored cellular processes are supporting cells. Orientation: left – toward basal turn; top – toward reticular lamina. Scale bar = 1μm.

Most (86%) of the reconstructed type-II terminals contacting the OHC studied in cat # 1 had reciprocal synapses, which had varying degrees of afferent versus efferent (directional) synaptic predominance (Figure 7). Some terminals presented only one form of synaptic specialization (100% afferent or 100% efferent) and the morphometric analysis of these terminals showed that they tend to be amongst the smallest of all type-II terminals. These terminals were usually small collateral branches of type-II fibers that were also the source of larger reciprocal terminals contacting the same OHC or their neighbors. The type-II terminals with the largest areas of membrane interface, on the other hand, were invariably reciprocal. The morphological characteristics of these relatively large type-II terminals (and their parent fibers) were indistinguishable from other type-II terminals, even though there was overlap in their area of membrane interface with the smallest OC terminals. Type-II terminals frequently presented invaginations into the neural poles of their innervated OHCs, which also served to increase the size of their membrane interface, and many of these type-IIs with large membrane interfaces with the OHCs were en passant terminals.
Figure 5.7 - Synaptic Directional Predominance & Size or Type-II Terminals

Scatter plot of individual type-II terminals with synaptic directional predominance in the Y axis and size in the X axis. The terminals with only one form or synaptic specializations (100% afferent or 100% efferent) were among the smallest of the type-II terminals, while the largest ones were invariably reciprocal with varying directional predominance. More specifically, these 100% afferent or 100% efferent type-II terminals were usually small collateral limbs of type-II fibers, which invariably gave rise to other larger reciprocal terminals contacting the same OHC or neighboring ones (reciprocal circuits). In other words, no reconstructed type-II fiber that was traced to terminals on more than one OHC was found to give rise to only to terminals that were all 100% afferent or 100% efferent. The four depicted 100% efferent type-IIs were readily distinguishable from the OC terminals, based on their size (not overlapping with OCs terminals), average number of cross sectional vesicles (5 vesicles), high neurofilament content, low average number of efferent vesicles per efferent synapse area (13 vesicles/um²), and the morphological characteristics of their parent (invariably spiral type-II) fibers. On the other side of the size spectrum, the largest type-II terminals had membrane interface areas that were comparable to ones of the smallest OC terminals, but these type-II terminals were invariably reciprocal and had robust afferent synaptic specializations, which has never been described for OC contacts or other types of descending projections from the central nervous system that directly innervates mammalian peripheral sensory organs. These type-II terminals with relatively large contact areas were not larger than other type-IIs per se and did not originate from larger type-II fibers with distinct morphology. They achieved a large contact area by running tangentially to the bottom of the OHCs (en passant terminals) and also by forming membrane invaginations into their neural poles.
Reciprocal Type-II Terminals as a Function of OHC Row

The average directional synaptic predominance of individual type-II terminals contacting individual OHCs, or the OHCs of a given row, varied substantially (Figure 8). The analysis of the shifts in average synaptic predominance of terminals contacting individual OHCs suggests that the type-II fibers innervating these OHCs, as a group, vary their form of interaction from OHC to OHC, by having more afferent interactions with a given OHC and more efferent interactions with a neighboring one. Moreover, type-II terminals innervating the OHCs of the third row tended to have less afferent directional predominance that the ones of the second or first row.

We next addressed the row-specific changes in the innervation of the OHCs (Figure 9). We took the perspective of individual OHCs (of each row), and estimated how much of the synaptic specializations found in their neural poles was: 1) efferent from OC terminals; 2) efferent from type-II terminals; or 3) afferent from type-II terminals. The average estimated combined size of OC efferent synapses declined towards the third row, where four out of the six OHCs were not contacted by any OC terminal. The efferent type-II specializations increased slightly towards the third row, where it dominates as a source of efferent synapses to the OHCs. The sizes of the type-II afferent specializations were also similar (with upward trend) across rows and were consistently larger than the type-II efferent specializations across the three rows.

Reciprocal Type-II Terminals as a Function of Frequency Region

We have employed semi-serial (every 5th) section electron microscopy to address the relative prevalence of reciprocal type-II terminals in different animals (cats # 2, 3, and 4) and frequency regions [350 Hz (cat # 3), 750 Hz (cat # 3), 1,000 Hz (cat # 2), 1,300 Hz (cat # 3), 1,600 Hz (cat # 4), 3,200 Hz (cat # 3), 6,300 Hz (cat # 3), and 12,000 Hz (cat # 3)]. The terminals contacting the OHCs in all these specimens were classified according to the same morphological criteria used for cat # 1, but en face reconstruction and membrane interface area measurements were not performed as serial sectioning is needed for that. Furthermore, absolute prevalence of type-II terminals with reciprocal synapses can only be determined with serial section analysis as the afferent or efferent components of the reciprocal synapses can be easily missed when random sections are studied for each terminal. Therefore, the comparisons of the
prevalence of reciprocal type-II terminals across frequencies between cats # 2, 3, and 4 are in relative terms, not absolute.

The relative prevalence of reciprocal type-II terminals in the cat with cut OCB (cat # 2) was similar to the relative prevalence of comparable frequency regions of cats # 3 and 4 (Figure 10). As described in a previous publication in which the same specimen was analyzed (Liberman et al., 2000), the OCB section apparently eliminated all the OC terminals in this experimental animal, but did not seem to affect the relative prevalence of type-II terminals. As the type-II terminals with reciprocal synapses did not disappear or presented discernible changes after neonatal section of the olivocochlear bundle, they most likely originated from peripheral projections of type-II spiral ganglion neurons and not from descending projections of superior olivocochlear complex contained in the OCB. The normal cats (#s 1, 3, and 4) had numerous OC terminals in similar frequency regions, and the morphological characteristics of the type-II terminals and fibers in all studied animals were indistinguishable from each other.

The quantitative comparisons across different animals and frequency regions showed that the relative prevalence of reciprocal type-II terminals did not vary substantially in the frequency regions and in the animals studied below 4,000 Hz, but there was a drop in their relative prevalence in cochlear regions coding for higher frequencies (6,300 Hz and 12,000 Hz). The use of the same plane of section (parallel to the cuticular plate) across frequency regions of cat # 3 allowed direct comparisons within the same animal, but had some issues because of the changes in the cat’s cochlear anatomy as we move from the relatively low frequency region of the cat cochlea (below 4,000 Hz ~ 43% distance from the base) to the high frequency region (Liberman, 1982b; Simmons, 1986). In the higher frequencies, the angle of the OHCs with respect to our plane of section (cuticular plate) straightens and the even smaller type-II terminals tend to be positioned closer to the center the neural pole. When the contacts are in this position, a plane of section parallel to the bottom of the neural poles oftentimes do not yield images perpendicular to the apposing cell membranes, which are necessary for proper visualization of synaptic structures.
Figure 5.8 - Synaptic Directional Predominance of Type-II Terminals of Individual OHCs

Scatter plot of the relative predominance of afferent versus efferent synaptic specialization of individual type-II terminals contacting neighboring OHCs of cat #1, with the weighed average for each OHC. Synaptic specializations of type-II terminals were exclusively afferent or efferent, therefore the proportions of the synaptic area occupied by each type of specializations were complementary to each other in a way that a terminal with 25% of efferent specialization necessarily has 75% of afferent specialization. The scatter plot shows how type-II terminals with different directional predominance were distributed across neighboring OHCs of the same row (six OHC in each row). The average (weighted by total interface area) directional predominance of the type-II terminals innervating the same OHC varied among neighboring OHCs and among different rows. Some OHCs (#s 1.6; 2.2; and 3.3) had considerably less afferent (and correspondingly more efferent) directional predominance than their immediate neighbors. OHCs of the third row tended, as a group, to have less afferent directional predominance that the ones of the first and second row.
Figure 5.9 - Combined Sizes of Type-II & OC Synaptic Specializations of Individual OHCs

Line graph depicts row-specific averages of the combined sizes of synaptic specializations within individual OHCs. The estimate of the row-specific combined sizes of the OC (efferent) and type-II (afferent and efferent) synaptic specializations per OHC resulted from the multiplication of the number of terminals (OC or type-IIs) in each row times the average sizes of the different types of synaptic specializations per terminal. The average sizes of the different types of synaptic specializations (OC efferent; type-II efferent; or type-II afferent) per terminal was computed through the multiplication of the row-specific average terminal sizes times the average proportion of their synaptic area that is occupied by afferent or efferent synaptic specialization. The line graph clearly shows the decline in the average combined size of OC efferent synapses toward the third row and illustrates the finding that a substantial number (four out of six) of the OHCs of the third row were not contacted by any OC terminal. As for the sizes of the type-II efferent specializations, they showed some increase towards the third row, where it basically dominates as a source of efferent synapses. The sizes of the type-II afferent specializations were essentially the same across rows and were consistently larger than the type-II efferent specializations. The relative drop of the sizes of type-II synapses in the second row was due to the smaller average sizes of the sample of type-II that were studied in this row. Some of the larger type-II terminals in this second row were not in a plane of section suitable for en face membrane interface reconstruction and were excluded from the database.
Figure 5.10 – Prevalence of Reciprocal Type-II Terminals Across Frequencies

Line graph shows the proportion of type-II terminals in cats #2, 3, and 4 with semi-serial section documentation of reciprocal synapses. Cat #2 (1,000 Hz region) was an adult cat submitted to neonatal section of the olivocochlear spiral bundle. Cat #3 was studied in several frequency regions (350, 750, 1,300, 3,200, 6,300, and 12,000 Hz), while cat #4 was studied in the 1,600 Hz region with a longitudinal plane of section. The data for cat #3 shows a decline in the proportion of terminals identified as reciprocal in the two highest frequency regions (6,300 and 12,000 Hz) that were analyzed, while the probability of finding reciprocal synapses did not vary substantially in the lower frequency regions.
We have also addressed the correlation of the number of informative sections (clear visualization of at least two thirds of the apposing membranes) through individual terminals of all animals/frequencies (excluding cat # 1) with the changes in prevalence of reciprocal type-II terminals (Figure 11). It was found that this probability increases to 85% when we get three or more informative sections through the same terminal, which is close to the 86% rate of the serial section analysis of cat # 1, in which only terminals with three or more sections were analyzed. The average numbers of informative sections for individual specimens were matched to their relative prevalence of reciprocal type-II terminals, and the average for the higher frequency samples may explain some of the differences across frequency regions. The lower numbers of informative sections through individual type-II terminals in the higher frequencies is only partially to blame because the relative proportions of reciprocal type-II terminals were still below the average found for the terminals analyzed in aggregate (line in Figure 11).

Even though the drop of reciprocal type-II terminals in the basal half of the cat cochlea might be an artifact, there is also a clear decrease in the absolute number of type-II terminals in the higher frequencies (Figure 12). Type-II terminals of the cochlear basal half also tend to be concentrated in the lateral side of the OHCs’ neural poles, while the reciprocal type-II terminals of the apical 60% of the cochlea were distributed evenly in the neural pole. Type-II terminals with reciprocal synapses were as likely to be found in the lateral, modiolar, basal, or apical portions of the OHCs’ neural poles.
Reciprocal Synapses of Type-II Terminals by Number of Informative Sections

Informative sections are the samples through individual type-II terminals that allow clear visualization of at least two thirds of the apposing membranes OHC and terminal and accurate identification of afferent/efferent membrane specializations. The depicted line graph (in black) refers to an aggregate representation of all type-II terminals studied with semi-serial section analysis (cats # 2, 3, and 4), showing the proportion of type-II terminals individually analyzed with one, two, or >= three informative sections, for which a reciprocal synapse could be documented. The superimposed scatter plot represents the analysis of specific specimens and frequency regions. On the X-axis, we have the average number of informative sections through type-II terminals of each specimen, while on the Y-axis we have the proportion of these type-II terminals that have documented reciprocal synapses. The comparison of the specimen-specific data with the aggregate information presented in the line graph suggests that the type-II terminals of the 6.3 KHz and 12 KHz frequency regions of cat # 3 have a smaller proportion of documented reciprocal synapses than would be expected solely from their corresponding average number of informative sections. The smaller average number of informative sections through individual type-II terminals of these high frequency regions (6.3 KHz and 12 KHz) could also explain, to some extent, the differences in reciprocal synapse documentation across regions shown in figure 10.
Figure 5.12 - Number of Type-II Terminals per OHC in Different Frequency Regions

Line graphs show the total number of type-II terminals per OHC as quantified by semi-serial section analysis in the different frequency regions of cat # 3. The counts of the same population of type-II terminals that were localized in the lateral and in the modiolar side of the OHC neural pole are also shown. There was no clear-cut spatial segregation of the type-II terminals in the low frequency regions until ~4KHz as the terminals were evenly distributed around the neural poles of the OHCs and the type-II fibers spiraled mostly tangential to the semi-spherical bottom of the OHCs. The modiolar type-II terminals in these low frequency regions were indistinguishable in morphological terms from the lateral ones and were as likely to have reciprocal synapses. The overall picture in the high frequency regions (6.3 KHz and 12 KHz) was quite distinct from the one described for the 3.2 KHz region and below. In these frequency regions above 4 KHz, there were less type-II terminals in absolute terms and a smaller proportion of them were documented as reciprocal (Figure 14). Furthermore, these type-II terminals were clustered on the lateral side of the neural pole. Also characteristically, these type-II terminals came from perpendicular projections of type-II fibers that spiraled at a distance of about 10 micrometers below the neural poles of the OHC they were innervating, instead of being tangential to them like their low frequency counterparts.
Reciprocal Circuits Formed by Type-II Fibers

None of the 30 type-II fibers that could be traced to terminals contacting more than one neighboring OHC had terminals that were exclusively 100% afferent or 100% efferent. In other words, all the described type-II fibers formed reciprocal circuits. In these reciprocal circuits, the terminals of a given type-II segment contacting neighboring OHCs had synaptic interactions of varying synaptic directional predominance with the targeted OHCs. Reciprocal circuits were found to be a pervasive feature of the OHC innervation in all three rows of cat # 1, and we were able to document this phenomenon without difficulty once we started to trace type-II fibers to their (mostly reciprocal) terminals contacting neighboring OHCs.

The reciprocal circuits found in cat #1 were depicted in two ways. The first one was a 3-Dimensional surface reconstruction of a type-II segment forming a reciprocal circuit in the third row of cat # 1, which was built with superimposition of 15 digital electronmicrographs of serial sections using the Amira® software (Figure 13). The objective of the 3-D reconstruction was to display the anatomical features of a type-II segment forming a reciprocal circuit with neighboring OHCs, while enabling measurements of elements of the circuit. In the example shown the fiber gave rise to two terminals and both of them have stalks, but the other traced type-II fibers innervated the OHCs through variable combinations of terminals with stalks and en passant contacts.

The second representation of reciprocal circuits was through a diagram showing only the type-II segments that contained reconstructed terminals contacting more than one OHC, which also included the location of the OC/type-II synapses they received (Figure 14). The analysis of type-II fiber segments suggested that the interaction of these neurons with the OHCs is essentially reciprocal, even though they had mostly afferent interactions with some OHCs and mostly efferent interactions with others. As described in the light microscopy literature (Simmons, 1986), individual type-II fibers were seen to give rise to more than one terminal per OHC, and commonly skipped OHCs. Despite the substantial variability in the way individual type-II fibers innervate the OHCs, it was observed that the great majority (89%) of the fibers running within the outer spiral bundle close (within 5-10μm) to the neural pole of the six OHCs of the first row formed at least one contact with these OHCs. In other words, type-II fibers running very close to the bottom of a group of OHCs were usually actively interacting with them.
The upper panel is a 3-D surface reconstruction of fifteen superimposed serial sections of the third row outer spiral bundle of the 700 Hz region of cat # 1. The 3-D model shows a segment of a type-II fiber, its terminals (colored in yellow), and the neural poles of neighboring OHCs (colored in purple), while the electronmicrograph of the lower panel shows the ultrastructural features of the same cellular elements. The depicted type-II terminals are characteristically vesicle-poor and neurofilament-rich and their shared parent type-II fiber is characteristically vesicle-poor and neurotubule-rich. The depicted type-II terminals are reciprocal, that is, they both have afferent and efferent synaptic specializations with the neighboring OHCs they innervate. It follows that the type-II fiber shown forms a reciprocal circuit with its innervated OHCs, which is characterized by the presence of synaptic specializations of opposing directions between one shared type-II fiber and the different OHCs it innervates. The 3D model also allows the visualization of the geometry of the stalks connecting the type-II terminals to the shared type-II fiber that are not aligned to the plane of section. Based on this reconstruction, one can determine the distance between the two type-II terminals (distance between the two stalks + the length of the stalks), which is of 7.5 μm. The bidirectional nature and relative short distance between the reciprocal synaptic contacts may enable intercommunication between the OHCs innervated by the same type-II fiber, which probably contact many other OHCs (up to 100 in this frequency region - Simmons, 1986). The uncolored cellular processes are supporting cells. Orientation: left – toward basal turn; top – toward reticular lamina. Scale bar = 1 μm.
Figure 5.14 - Reciprocal Circuits and their OC/type-II Synapses

Diagram showing only the type-II fibers that were traced to reconstructed terminals contacting more than one OHC, also showing the OC/type-II synapses they received. All type-II fibers that met this criterion were found to form reciprocal circuits, which means that they had synaptic specializations of opposing directions between themselves and the different OHCs they innervate. Conversely, none of the type-II fibers that were traced to more than one OHC was seen to form exclusively 100% afferent or 100% efferent terminals on neighboring OHCs. This diagram documents the pervasive nature of the reciprocal circuits formed by type-II fibers, while also showing how type-II fibers are innervated by OC fibers. OC/type-II synapses on the reciprocal circuits were commonly positioned close to type-II terminals with afferent directional synaptic predominance, but this relationship was not clear-cut. It should be noted, however, that the diagram actually represents a limited view of the type-II/OHC interactions. This is because our analyzed specimen is just a small segment of the innervation field of the depicted type-II fibers, as they are known to contact up to 100 neighboring OHCs in this frequency region. Furthermore, not all the type-II fibers/terminals contacting OHCs are represented in the diagram. OHCs of the first row were contacted, on average, by 14.6 type-II fibers and corresponding 21.6 type-II terminals. OC fibers profusely innervated the OHCs and the reciprocal circuits formed by type-II fibers. Not all the OC/type-II synapses present underneath the 18 reconstructed OHCs are shown here as they were also found on uncharacterized terminals and on fibers that were not traced to more than one OHC.
OC Synapses onto Type-II Fibers & Terminals

The location of OC/type-II synapses was also addressed quantitatively in the cat # 1. OC/type-II synapses were quite common and 216 examples were documented in the outer spiral bundles underneath the 18 studied OHCs (6 in each row). These OC/type-II synapses targeted the type-II neurons both on their fiber segments and also on their terminals (Figure 15). The OC/type-II synapses were most common in the first row, where about half of them targeted type-II fibers, while the other half targeted terminals (or their corresponding stalks). The smaller number of OC/type-II synapses towards the third row of OHCs was directly correlated with the progressively smaller number of OC fibers present at the level of the outer spiral bundle in the second and third rows.

The interactions among all the neural elements of the outer spiral bundle of the first row of cat # 1 were also quantified. We have traced 17 segments of OC fibers to their respective 32 terminals, and 52 segments of type-II fibers to their respective 105 terminals. Individual OC segments (one OC fiber + X number of OC terminals) were seen to form up to 23 (average = 9.6) OC/type-II synapses and innervate up to 16 (average = 7.8) distinct type-II segments (one type-II fiber + X number of type-II terminals). Individual type-II segments were found to receive as many as 11 (average = 3.3) OC/type-II synapses originating from as many as 6 (average = 2.7) OC segments.

Type-II terminals have been seen to receive as many as 3 (average = 0.3) OC/type-II synapses from distinct OC segments, and these synapses seemed to preferentially target type-II terminals with more afferent directional synaptic predominance (Figure 16).
Figure 5.15 - OC Synapses on Type-II Terminals & Fibers

Bar graph represents the differential count of OC/type-II synapses targeted at terminals and fibers within the distance corresponding to the width of one OHC (approximately 10 micrometers) in cat #1. This differential quantification is represented for each OHC row and it is clear that synapses on fibers are common only in the first row. In the second and third row, OC/type-II synapses are considerably less common and occur primarily on type-II terminals, not fibers. The decreased prevalence of OC/type-II synapses towards the third row coincides with an absolute decline on the number of OC fibers in these regions.
Correlation Between Synaptic Directional Predominance of Individual Type-II Terminals and Number of OC/type-II Synapses

Figure 5.16 – Synaptic Directional Predominance & Number of OC/type-II Synapses

Scatter plot shows the correlation between synaptic directional predominance of type-II terminals and their number of OC/type-II synapses in the first row of cat #1. In the Y-axis, the synaptic directional predominance (proportion of synaptic area occupied by afferent specialization) of individual type-II terminals is depicted. In the X-axis we have the number of OC-type-II synapses per terminal. The presented scatter plot shows that OC-type-II synapses were not randomly distributed. They were quite rare in type-II terminals that are mostly (>75%) efferent, and become increasingly prevalent in the type-II terminals with more afferent predominance ($r = 0.56$). The graph shows data from the row with more OC/type-II synapses (first row) for the sake of clarity, but analysis of all three rows shows similar correlation between afferent predominance and number of OC/type-II synapses. The type-II terminals not targeted by OC/type-II synapses were as likely to be mostly afferent or efferent, while the ones with one of more OC/type-II synapses were more likely to be mostly afferent.
Discussion

Reciprocal Synapses on OHCs is not Limited to Primates

The terminal morphology and synaptic interactions formed by type-II and OC terminals in cats do not seem to be different than the ones of previously studied primate species, namely: human (Nadol, 1981; 1990b; Spoendlin and Schrott, 1988; Thiers et al., 2002b), chimpanzee (Francis and Nadol, 1993a; b), and Japanese macaque (Sato et al., 1999; Sato et al., 1997). The ultrastructural morphometric analysis performed in a human specimen (Thiers et al., 2002b) also showed two populations of terminals that could be distinguished based on number of vesicles at the plane of maximal cross sectional area (< than 40 & > than 70 vesicles), in which vesicle-poor and neurofilament-rich (type-II morphology) terminals had reciprocal interactions with the OHCs, while the vesicle-rich and neurofilament-poor (OC morphology) terminals formed only efferent synapses.

An ultrastructural study of the chimpanzee (Francis and Nadol, 1993a) showed that the cytoplasmic characteristics of the terminals that were 100% afferent and the ones with reciprocal synapses were similar to each other, even though the reciprocal ones tended to be slightly larger. A companion paper describing the terminals with efferent synapses in the same chimpanzee specimen (Francis and Nadol, 1993b) described a subpopulation of small terminals that had efferent specializations. Their size was comparable to the 100% afferent terminals described in the same specimen and also tended to be smaller than most of the reciprocal terminals. We have found a comparable pattern in the cat (Figure 7), in which the 100% afferent and the type-II variant of the 100% efferent terminals tended to be smaller than the reciprocal ones.

The other feature of the OHC innervation that was found to be similar in the cat (present study), human (Thiers et al., 2002b), chimpanzee (Francis and Nadol, 1993a) was the increase towards the apical turn and/or third row in the relative contribution of reciprocal terminals as sources of efferent synapses on OHCs. In cat # 1 of the present study, 94% of the efferent synaptic area of the third row of OHCs was from type-II terminals. In the chimpanzee, it was also found that over 90% of the total area of synaptic contact of an OHC of the apical turn was from reciprocal terminals, while in the 12 OHC of the third row in a human specimen 100% of the efferent synapses were from type-II terminals. This increase in relative contribution of efferent synapses from type-II terminals has to do more with the decrease in the prevalence of
OC terminals towards the apical turn/third row than with an absolute increase in OHC reciprocal innervation by type-IIIs. The estimated combined size of OC efferent area per OHC presented for cat #1 (Figure 9) decreases markedly towards the third row, and is comparable to the one previously reported in the cat for a similar frequency region (Liberman et al., 1990). The absolute decrease in number of OC terminals towards apical turn/third row, and the existence of OHCs in the third row of OHCs devoid of OC terminals is well documented in the literature (Dunn, 1975; Ginzberg and Morest, 1984; Kimura and Wersall, 1962; Liberman, 1990; Simmons, 1986).

The most noticeable difference between the reciprocal synapses described in the cat and the primates was the rarity of the pre-synaptic bodies on their afferent component. The rarity of pre-synaptic bodies in the type-II terminals of cats has been reported in several previous ultrastructural studies (Berglund, 1990; Dunn and Morest, 1975; Ginzberg and Morest, 1983; Liberman et al., 1990; Spoendlin and Gacek, 1963) and the reason for this inter-species difference remains unclear. The rarity of pre-synaptic bodies in cats needs to be taken into consideration in the evaluation of previous ultrastructural investigations. The pre-synaptic body serves as a convenient marker of afferent synapses (and therefore of type-II terminals) and their virtual absence in cats probably made the identification of type-II reciprocal terminals more difficult, especially in the previous studies in which a representative number of individual terminals were not reconstructed with serial sections.

Previous ultrastructural studies in cats did not report that reciprocal synapses were a common feature in the OHC innervation, and two scenarios might explain why they were not recognized. Some relatively small terminals with efferent specializations were classified as atypical efferent terminals based on their synaptic specializations in the cat (Liberman et al., 1990). These terminals were vesicle-poor and were more common in the third row of OHCs, so it is possible that they were type-II terminals. On the other hand, terminals classified by previous authors as type-IIIs based on their morphological characteristics or tracing to outer spiral fibers had sub-synaptic cisternae apposed to them (Dunn and Morest, 1975; Dunn, 1975; Ginzberg and Morest, 1984; Simmons, 1986), but these membrane specializations were not interpreted as indicators of efferent synaptic neurotransmission. Analysis of the electronmicrographs presented in the cited studies in cats suggests that differences in the classification schemes of terminals and synaptic specializations are more likely than inter-animal (or histological processing) differences
between the four cats presented here and the ones studied in these previous ultrastructural investigations.

Synaptic specializations suggestive of reciprocal synapses have been reported (but not quantified) on OHCs of guinea pigs (Furness et al., 2002; Jones and Eslami, 1983; Thorn et al., 1972), and in a cat submitted to neonatal transection of the OCB. In the study in which reciprocal synapses were found after OCB transection (Pujol and Carlier, 1982), the finding was interpreted as pathological, but a quantitative comparison with a normal animal of similar age was not presented. In another study (Iurato et al., 1978), the OCB of the chinchilla was transected and terminals classified by the authors as afferent (type-IIIs) were shown to have efferent specializations (sub-synaptic cisternae) with the OHCs. These putative efferent specializations were thought to result from the OCB transection, but evaluation of normal controls was not presented to support this hypothesis. In general, the interpretation of the results of the other OCB transection studies (Kimura and Wersall, 1962; Morrison et al., 1975; Nakai and Igarashi, 1974; Smith and Rasmussen, 1963; Spoendlin and Gacek, 1963) in relation to the presence/absence of reciprocal type-II terminals is difficult because we do not have a way to systematically analyze the morphological characteristics of the terminals with efferent synaptic specializations that were reported to remain after OCB section. In the current study, we had the opportunity to analyze the synaptic specializations of terminals that remained after OCB transection in a specimen (cat # 2) that was described in a published investigation (Liberman et al., 2000). Our data analysis suggests that terminals with efferent (reciprocal) specializations that remain after OCB transection have type-II morphology.

The presence of reciprocal synapses between afferent neurons and receptor cells of other sensory systems indirectly suggests that type-IIIs are also be capable of engaging in reciprocal synaptic interactions. Of the hair cell-based sensory transducers, reciprocal synapses have been found on IHCs in the mouse (Sobkowicz et al., 2003); and on hair cells of the vestibular macula in the rat (Ross, 1997), crista ampullaris in the chinchilla (Lysakowski and Goldberg, 1997); crista ampullaris in the bullfrog (Dunn, 1980); and paratympanic organ in the chicken (Giannessi, 1989). Afferent neurons were also seen to form reciprocal synapses on receptor cells of the carotid body (Matsumoto et al., 1980); muscle spindles (Luo et al., 1995); taste buds (Delay and Roper, 1988); and intrapulmonany neuroepithelial bodies (Taha and King, 1986). The pre-synaptic vesicle clustering found in these synapses, and in other reciprocal synapses in
sensory organs like the retina and olfactory bulb, does not have as many vesicles as the OC efferent synapses on OHCs (Dacheux et al., 2003; Dowling, 1968; Gabriel et al., 2000; Landis et al., 1974; McGuire et al., 1984; Rebiere and Dainat, 1981). The relatively non-impressive vesicle clustering does not mean that they are not engaged in neurotransmission. In the case of the terminals formed by sensory neurons in the vestibular macula, they do present the molecular machinery (N-type voltage-gated Ca++ channels, SNARE proteins, syntaxin, synaptogamin, synapsin I, synaptophysin, rab 3A, and SNAP 25) for synaptic vesicle docking and Ca++ dependent neurotransmitter exocytosis, which is primarily located close to their hair cell contact (Dememes et al., 2000). Synaptophysin has been found on efferent vesicles of reciprocal synapses of human OHCs (Nadol et al., 1993) and it can be speculated that other related proteins involved in synaptic transmission are present inside the reciprocal type-II terminals.

The nature of the neurotransmitter of the efferent portion of the reciprocal type-II terminals is unknown, and it is not impossible that they use the same neurotransmitters than the OC neurons. In the universe of neurotransmitters/modulators that have been shown in immunohistochemical studies to be present in the OHC region (including: acetylcholine, GABA, CGRP, dynorphins and enkephalins), all of them seem be co-localized in the large, vesicle-filled OC terminals (Altschuler et al., 1984a; Altschuler et al., 1985a; Altschuler et al., 1984b; Eybalin et al., 1988; Fex and Altschuler, 1984; Maison et al., 2003; Maison et al., 2006; Schrott-Fischer et al., 2002; Sliwinska-Kowalska et al., 1989; Vetter et al., 1991). It is difficult to draw inferences about the nature of the efferent neurotransmitter used by type-lls from these cited immunohistochemical studies because they were not explicitly designed to detect the probably relatively small immunoreactivity (IR) of the efferent vesicles inside type-II terminals, which have one tenth of the number of vesicles than the OC ones.

Analysis of the OHC innervation in relatively low frequency regions, where the OC terminals are expected to be less prevalent, and/or in animals submitted to OCB section may be more productive. For that matter, previous studies in guinea pigs have shown absence of choline-acetyltransferase-like (ChAT) IR after OCB section, thus suggesting that acetylcholine is used primarily by the OC system (Altschuler et al., 1985b). On the other hand, GAD-like IR is present in the nerve fibers innervating OHCs of preferentially the third row and apical turns in guinea pigs (Fex and Altschuler, 1984), where ChAT IR is reportedly scanty (Altschuler et al., 1985b) and large OC terminals are not prevalent (Brown, 1987b; Hashimoto and Kimura, 1988;
Kimura and Wersall, 1962). This finding was considered by previous authors as suggestive of the existence of a separate source of efferent OHC terminals - the lateral OC neurons.

In light of the results presented in our study, one might consider that efferent vesicles of type-II neurons may account for some of the aforementioned GAD-like IR in the third row and in the low frequency regions. Most terminals in the third OHC row of cat # 1, and the terminals with efferent specializations in the 1,000 Hz frequency region of cat # 2 (submitted to surgical elimination of the lateral and medial OC innervation) had type-II morphology, while outer spiral fibers (type-II morphology) have been shown to have GABA-like IR in humans (Schrott-Fischer et al., 2002) and guinea pig (Fex et al., 1986). The corresponding OHCs of the apical turns in guinea pigs (Plinkert et al., 1993) and mice (Maison et al., 2006) have increased expression of GABAa receptors, and this GABA-ergic innervation have been proposed to affect the stiffness of their lateral wall through bicuculline (GABAa receptor antagonist)-sensitive GABA-mediated Ca++ signaling (Batta et al., 2004).

The efferent specializations of the reciprocal type-II terminals (sub-synaptic cisternae) were found occasionally to be continuous with ones apposed to OC terminals and also to sub-surface cisternae of OHCs in the described cat # 1 and in a human specimen (Thiers et al., 2002b). These sub-synaptic/surface cisternae are thought to be Ca++ reservoirs involved in Ca++-induced-Ca++-release (Benedeczky et al., 1994; Lioudyno et al., 2004; Sridhar et al., 1997; Szonyi et al., 2001), and it is possible that the type-II fibers affect intracellular Ca++ levels in OHCs through activation of the efferent components of their reciprocal synapses. There are numerous Ca++ mediated intracellular processes, which can take effect in different time scales. At this point in time, one cannot exclude the possibility that the type-II are involved in relatively fast Ca++ mediated changes in membrane potential (Fuchs and Murrow, 1992), nor in slower Ca++-dependent changes in electromotility or protein phosphorylation (Frolenkov et al., 2000). It is also possible that this hypothetical local role of the type-IIIs through reciprocal synapses involves long term trophic support to the innervated OHCs, since, to our knowledge, an in vivo animal model with normal OHC function and absent type-II neurons has not been generated.

The neurotransmitter in the afferent type-II synapse is another key element of the reciprocal synapse on OHCs that needs clarification. The evidence supporting the role of glutamate as the OHC afferent neurotransmitter is not conclusive, but include: glutamate IR in OHCs (Altschuler et al., 1989); L-[3H]glutamine uptake by OHCs in a in vivo radioautographic
study (Ryan and Schwartz, 1984); presence of glutamate/aspartate transporter (GLAST) close to the type-II’s afferent synapses (Furness et al., 2002); presence of AMPA (GluR4) receptors around the OHC base (Kuriyama et al., 1994); expression of receptors for AMPA (GluR2-4), kainate (GluR5-6, KA1-2), and NMDA (NR1, NR2a-d) in the cell bodies of small (type-IIs?) spiral ganglion neurons (Niedzielski and Wenthold, 1995); and finally presence of glutaminase activity in the OHC region in a microchemical assay, which was not related to the OC fibers (Wiet et al., 1990). The evidence against an important role for glutamate include: absence of glutaminase IR in the OHC area after OCB section (Fex et al., 1985), and the preferential damage of type-I versus type-II afferent terminals after kainic acid intracochlear injection (excitotoxin which targets glutaminergic neurons) in the adult animals (Pujol et al., 1985); and lack immunogold labeling of AMPA receptors (GluR1-4) (Matsubara et al., 1996). Despite the current controversy in the nature of the neurotransmitter in the afferent type-II synapse, pre-synaptic Ca ++ channels (Cav1.3) have been shown in association with afferent synaptic proteins (CtBP2/RIBEYE) in the neural poles of mature OHCs (Knirsch et al., 2007). This molecular machinery probably supports Ca ++-mediated neurotransmitter exocytosis and subsequent signaling to the type-II terminals.

The molecular underpinnings of the reciprocal type-II synapses are unknown, but it is possible that they employ molecular mechanisms analogous to the ones used by their well-studied counterparts in the retina and olfactory bulb, which are quite similar to each other (Nakanishi, 1995). Notably, both the bipolar/amacrine (retina) and the mitral/granule (olfactory bulb) reciprocal synapses work through functionally integrated pairs of excitatory (glutamate) and inhibitory (mostly GABA) synapses (Dacheux et al., 2003; Gabriel et al., 2000; Isaacscon and Strowbridge, 1998; Marc and Liu, 2000; Marc et al., 1990). It has been shown in studies of the rat’s olfactory bulb that the first neuron of the loop (mitral cell) release glutamate that activates both AMPA and NMDA receptors in the granule cells (the second neuron of the feedback loop) (Schoppa et al., 1998). The Ca ++ that enters the cell through the glutamate (NMDA) receptors on granule cells directly triggers GABA release from these neurons (Chen et al., 2000), but the GABA release in lateral connections of these granule cells can also be elicited through the activation of N- and P/Q-type voltage gated Ca ++ channels (Isaacscon and Strowbridge, 1998). The GABAergic inhibitory neurotransmission back at the mitral cell is mediated the activation of GABAa receptors, which results in self-inhibition of the mitral cell through hyperpolarization.
The whole cycle of self-inhibition mediated by the mitral/granule cells' reciprocal synapses has been estimated to take $2.4 \pm 0.4$ msecs (Mori and Takagi, 1978).

**Type-II Fibers Form an “Outer Spiral Network” with the Innervated OHCs**

The most compelling evidence that type-II fibers give rise to reciprocal terminals is the tracing of numerous individual spiraling neurotubule-filled and vesicle-poor (type-II) fibers to combinations of reciprocal, 100% afferent, and small 100% efferent terminals contacting neighboring OHCs. We could not find examples of type-II fibers traced only to 100% afferent terminals, or alternatively, to small 100% efferent terminals, which could be indicators of the existence of subpopulation of fibers with type-II morphology that were only afferent or only efferent in relation to their innervated OHCs. In contrast, we have evidence that individual type-II fibers are essentially reciprocal in relation to their innervated OHCs, with the interaction between an individual type-II terminal and an individual OHC possibly being shaped by the directional (afferent vs. efferent) predominance of their synaptic interaction(s).

There is also evidence that the type-II fibers in the frequency regions below ~4,000 Hz (~apical half) have more reciprocal synaptic interactions with their innervated OHCs than the fibers of the basal half. Cat #1 was studied at a frequency region that corresponds to 82% distance from the basal end of the cat cochlea. Previous HRP reconstructions of type-II fibers that quantified the number of OHC contacts per fiber across the cat's cochlear spiral (Simmons, 1986) showed that there is a transition point around the 40-50% distance from the base (corresponding to ~3,000 to 5,000Hz), in which the number of OHC terminals per type-II fiber and their spiral length decreases as we move towards the base. In the apical half, the type-II fibers are more numerous, have greater spiral length, and they tend also to be thicker - 0.8μm on average as opposed to 0.2μm in the basal half. These apical fibers were also found to be relatively thicker when crossing the tunnel of Corti, and tended to travel mostly in a straight line (shortest distance between two points) between the OHC neural poles they contacted. In contrast, the basal type-II fibers usually travel at about 10μm from the neural poles and innervate the OHCs through relatively thin stalks/terminals. Our data from cat #3 support the notion of a transition point in the frequency region around 4,000Hz, with type-II fibers basal to this region traveling away from the OHC’s neural poles and forming fewer contacts with the OHCs.
The functional properties of the type-II neurons are still not well characterized (Brown, 1994; Robertson et al., 1999), but recent studies have disclosed relevant information. An in vitro study of type-II neurons in mice (Reid et al., 2004) showed that they had low action potential thresholds, with the apical ones having slower accommodation than the basal ones. It was suggested that the slower accommodation of these type-II neurons could help them in the integration of multiple synaptic inputs and enhance the temporal resolution of their response. In another in vitro study, using rat cochlear slices (Jagger and Housley, 2003), type-II neurons were shown to have inactivating outward (A-type) currents with (voltage-dependent) fast kinetics of inactivation and recovery from inactivation. Similarly, these biophysical characteristics were thought to help them in the temporal integration of multiple inputs into an intensity-graded firing rate. Another relevant contribution was a study that combined immunohistochemistry in the mouse cochlea with computational modeling to investigate of the generation/conduction of action potentials by type-II neurons (Hossain et al., 2005). They showed a high concentration of Na_1.6 (Na++ channel involved in the generation and propagation of action potentials) in the type-II terminals and in the terminal regions of the type-II fibers. Their model suggested that the Na++ channels of the type-II neurons were strategically located to enable generation and propagation of action potentials in the outer spiral bundle, through the cell body and probably into the central nervous system.

We have shown in the present study that nerve fibers with type-II morphology give rise to terminals with reciprocal interactions with neighboring OHCs. The role of these reciprocal synapses may be limited to individual OHCs, but they could also mediate bi-directional signal transmission among OHCs innervated by the same type-II fiber. Evidence for OHC intercommunication mediated by type-IIs is indirect and is based on the fact that anatomically similar reciprocal circuits in the retina and olfactory bulb are engaged in intercommunication and functional integration of sensory elements working in parallel (Isaacson and Strowbridge, 1998; Nakanishi, 1995). The possibility that the type-IIs mediate OHC intercommunication through reciprocal synapses has been alluded to by several investigators (Berglund, 1990; Brownell, 1982; Fechner et al., 2001; Francis and Nadol, 1993a; Giannessi, 1989; Glueckert et al., 2005a; Guinan, 2006; Gummer, 1991; Nadol, 1983; Sato et al., 1999; Simmons, 1986; Spoendlin, 1982; Thiers et al., 2002b), based on the discovery of reciprocal synapses on human OHCs (Nadol, 1981), and on other indicators that the type-IIs have a “peripheral emphasis”. This peripheral
emphasis (Spoendlin, 1972; 1975; 1979) was inferred from the finding that the cell bodies of type-II neurons do not degenerate like the type-I spiral ganglion cells after transection of the auditory nerve, and that the thickest portions of the type-II projections are within the outer spiral bundle.

Even though the reciprocal circuits formed by type-II neurons seem to be well suited for the mediation of OHC intercommunication, several events would need to take place for such intercommunication to happen and none of them have been demonstrated in physiological experiments. Taking the example of the reciprocal circuit portrayed in Figure 13, the sequence of events may include: 1) afferent input from one OHC generates a shift in membrane potential in the type-II terminal; 2) an electrical impulse is transmitted through the fiber and reaches the terminal on the neighboring OHC; 3) a change in membrane voltage in the terminal triggers efferent synaptic transmission on the reciprocal terminal contacting the second OHC; and 4) the efferent input elicits functional changes in this second OHC. The likelihood that these events would take place cannot be ascertained, but its conceptualization brings about several fundamental questions that will be tentatively discussed here. Definitive answers needs to come from further research.

The first question is whether the type-IIs are limited to orthodromic (towards the cell body) signal transmission. The capacity of type-IIs to conduct action potentials (orthodromic or antidromic) has not been fully characterized yet (Robertson et al., 1999), but in a previous study in the pig (Brown, 1994) antidromic conduction of electrical impulses through putative type-II fibers was observed. The demonstrated electrical conduction was from the level of the cochlear nucleus to the spiral ganglion, and did not include the outer spiral fibers.

The second question is whether the impulse transmitted (antidromically or orthodromically) by the type-II is able to modulate functional properties of remotely located terminals/OHCs. The ability of an afferent fiber to affect remotely located receptor cells through reciprocal synaptic specializations was suggested in a study of the cat’s carotid body (McDonald and Mitchell, 1981). Electrically evoked action potentials antidromically conducted through the afferent nerve reached the receptor (glomus) cells and changed their chemoreceptor activity, supposedly by activating efferent (reciprocal) synaptic specializations present at their recep-
The third question is how far (in numbers of OHCs) would a type-II be able to conduct an electrical impulse? The range and speed of conduction of an electrical impulse through type-II fibers cannot be firmly determined until we learn more about their biophysical properties and their ability to conduct action potentials. Neurons with high resistivity than can conduct receptor potentials for more than 10mm exist, so electrotonic conduction by the type-IIs cannot be discarded (Hudspeth et al., 1977). However, the above-mentioned discovery of a high concentration of Na,1.6 in the type-II terminals and fibers are suggestive that type-II can conduct action potentials (Hossain et al., 2005). One can tentatively calculate the conduction speed of putative type-II fibers from the data obtained from fibers were antidromically stimulated at the level of the root of the cochlear nerve from (Brown, 1993). In this experiment, the impulses took 6.1msecs to travel 2.32mm on putative type-II fibers, and the resulting conduction velocity was 0.38m/s, which fits exactly with the velocity of 0.38m/s estimated for an unmyelinated fiber with 0.5μm of diameter1 (Hoffmeister et al., 1991; Wyatt et al., 2005). The diameter of 0.5μm is average for the type-II fibers in the cochlear nerve (Brown, 1987a; Brown et al., 1988; Brown and Ledwith, 1990), but at the level of the outer spiral bundle the type-II fibers are 50 to 100% thicker (Hossain et al., 2005; Simmons, 1986), which should result in a proportionally higher conduction velocity. But even at the speed of 0.38m/s, the longest of the 78 outer spiral fibers (~1,000μm or 125 OHCs) reconstructed in a study of the cat cochlea (Simmons, 1986) could conduct a impulse from beginning to end of the spiral length of its OHC-terminal region in ~2.6msecs. Adding a hypothetical reciprocal synaptic delay of ~2.4msecs (Mori and Takagi, 1978), the intercommunication between OHCs 1mm apart could take as little as 5msecs (2.6 + 2.4). Using an example from our own dataset, the intercommunication between the two OHCs depicted in Figure 13 (terminals are distanced by 7.5μm) would take 2.42msecs (2.4 + 0.02). A hypothetical OHC intercommunication initiated in one of the these two cells would be virtually reduced to the delay of reciprocal synaptic interaction (afferent in one cell/efferent on another), and an action potential generated remotely would activate the reciprocal terminals of the OHCs almost simultaneously.

1 The formula used to calculate conduction velocity was obtained from direct observations of mammalian unmyelinated nerve fibers and employs the equation: \( \Theta = 0.24s \), where \( \Theta \) is conduction velocity and \( s \) is the circumference of the unmyelinated nerve fiber [Hoffmeister B, Janig W, Lisney SJ. 1991. A proposed relationship between circumference and conduction velocity of unmyelinated axons from normal and regenerated cat hindlimb cutaneous nerves. Neuroscience 42(2):603-611].
The forth question is how would a type-II fiber be able to integrate sensory input of the operational status of the OHCs (Kim, 1986), send such information to the cochlear nucleus (Brown et al., 1988), while also mediating local feedback loops with the OHCs? Several computational modeling studies of the afferent neurons of the vestibular macula have tried to address this issue (Chimento and Ross, 1996), since these neurons also seem to intercommunicate neighboring hair cells while projecting to the vestibular nuclei (Ross, 1997). These studies have suggested that these two roles (local modulation & sensory integration) are not mutually exclusive. Their models predict that all processes with efferent specializations emanating from a single fiber receive synchronous depolarization sufficient to provide an unified output to all innervated hair cells simultaneously. The character of the feedback to the vestibular hair cells is unknown, as the nature of the efferent neurotransmitters has not been determined yet, but they are thought to be inhibitory. A relevant prediction from the models is that neural responses (which get transmitted centrally) are shaped by the feedback cycles from the modulated vestibular hair cells, in a way the responses to stimuli that carry certain features, like directional input, get reinforced (Ross et al., 2000).

Assuming that type-II fibers mediate OHC intercommunication, their highly complex reciprocal circuits may work in conjunction as a network. As mentioned, the individual type-II fibers have extensive and overlapping innervation fields (Berglund, 1990; Simmons and Liberman, 1988a). They constitute the outer spiral bundle, which amount to a collection of more than 100 fibers coursing longitudinally below the rows of OHCs (Glueckert et al., 2005b; Spoendlin and Schrott, 1988). Despite the bundle denomination, collections of type-II fibers do not appear to operate as a bundle when close to neural poles of the OHCs. The type-IIs form elaborate reciprocal interactions with the OHCs, synapse on each other through type-II/type-II synapses (Bodian, 1978; Nadol, 1983; Nadol and Burgess, 1990; Thiers et al., 2000; 2002a), and innervate the supporting cells (Burgess et al., 1997; Fechner et al., 2001; Nadol and Burgess, 1994), thus constituting a highly complex neural network (Thiers et al., 2002b). We will hereafter refer to this outer spiral bundle neural network, as outer spiral network for brevity.

The presence of type-II/type-II contacts (also known as dendro-dendritic synapses) and the innervation of supporting cells by type-II fibers also seem to be important features of this outer spiral network. The type-II/type-II contacts have found to be common in primates (Bodian, 1978; Nadol, 1983; Nadol and Burgess, 1990; Thiers et al., 2000; 2002a), and may allow
communication among type-II fibers that innervate different groups of OHCs. The innervation of the supporting (Hensen’s and Deiter’s) cells by type-II fibers has been found to be prominent in the apical turns of the guinea pig (Fechner et al., 1998), and it was suggested that the type-II fibers formed a local axon reflex in which information from the OHCs gets transmitted to the supporting cells. Such local reflex could be involved in the modulation of structural changes that occur in supporting cells in response to acoustic trauma (Wang et al., 2002). These local axon reflexes have been described in the nociceptive capsaicin-sensitive fibers of the somatosensory system, and have been characterized as “efferent” effects of sensory neurons, which involve the release of vaso-active substances by the nerve endings in response to noxious stimuli (Maggi et al., 1987; McDonald et al., 1996). The cochlear ganglion cells also seem to be capsaicin-sensitive (Zheng et al., 2003), and the type-II neurons share features (unmyelinated; peripherin-positive) with the small dorsal root ganglion neurons that carry nociceptive information (Hafidi, 1998; Parysek and Goldman, 1988). By analogy, it can be hypothesized that the outer spiral network is involved the modulation of the functional status of OHCs and supporting cells in response to intense acoustic trauma, and an axon reflex may mediate temporary reduction of cochlear amplification and prevention of permanent damage to cochlear elements.

The outer spiral network has also been implicated in the modulation of some sound-evoked OHC responses, as measured by changes in otoacoustic emissions, in normal conditions (Guinan, 2006). More specifically, it was hypothesized that some OHCs responses to ipsilateral tones are mediated by activation of efferent components of reciprocal circuits. Changes in stimulus-frequency otoacoustic emissions (SFOAEs) produced by ipsilateral tones in one of these studies had a decay time constant of 194msecs, which was considered too slow for an OC fast effect (~100msecs) and too fast for an OC slow effect (>10 secs) (Guinan et al., 2003; Sridhar et al., 1995). Other studies attributed changes SFOAEs with time constant of 516 msecs (Goodman and Keefe, 2006) and changes in $2f_1 - f_2$ DPOAEs with time constant of 1sec (Liberman et al., 1996) to intrinsic cochlear effects, which may involve the outer spiral network. Furthermore, neural mediated, non-OC, and possibly GABAergic changes in $f_2 - f_1$ DPOAEs (initial increment, followed by slow decrement) that takes effect over minutes have been described (Kirk and Johnstone, 1993; Kujawa et al., 1995). A local neural feedback loop was implicated in this effect (Lowe and Robertson, 1995), and the outer spiral network is a plausible candidate.
Modulation of the Outer Spiral Network by OC Neurons

OC/type-II synapses have been found in various species (human, chimpanzee, old world monkey, Japanese macaque, cat, guinea pig, chinchilla, rat, and mouse), and throughout the peripheral portion of the type-II neurons. More specifically, OC/type-II synapses have been described at the level of their cell body (Arnold, 1982; Ivanov et al., 1992; Kimura et al., 1987; Kimura et al., 1979; Nadol, 1988; Rask-Andersen et al., 2000; Rask-Andersen et al., 1997; Thiers et al., 2000), in the tunnel of Corti (Dunn, 1975; Liberman et al., 1990), and within the outer spiral bundle (Altschuler et al., 1984b; Dunn, 1975; Eybalin et al., 1988; Ginzberg and Morest, 1984; Iurato et al., 1978; Jones and Eslami, 1983; Liberman et al., 1990; Nadol, 1983; 1990b; Nadol and Burgess, 1990; Sato et al., 1997; Shnerson et al., 1981; Smith and Rasmussen, 1963; Spoendlin and Schrott, 1988; Thiers et al., 2002a). The fact that they are found in so many species, coupled with the high prevalence described here and in other studies (Dunn, 1975; Liberman et al., 1990; Thiers et al., 2000; 2002a), are indicators that the OC/type-II interactions are a prominent feature of the OHC innervation.

The numbers of OC/type-II contacts per OHC width reported in cat # 1 are comparable with the ones found in a human specimen (Thiers et al., 2002a) (Figure 17), specially considering that every third section was analyzed in the human study, as opposed to every section in cat # 1. The decrease in the numbers of OC/type-II synapses towards the third row in the cat and in the human is explained by the decreased number of OC fibers available to synapse on the type-IIs, especially in third row of OHCs. This finding could be seen as an indicator that some type-II neurons in the low frequency regions of the cochlea are not influenced by the OC system through OC/type-II synapses. However, in a study of the cat employing light microscopy (Liberman et al., 1990), it was found that the number of putative OC/type-II synapses in the tunnel of Corti in the frequency regions between 200 and 2,000 Hz is high (~ 80/mm) and comparable to the one found in the outer spiral bundle in the same animal. Moreover, OC/type-II synapses may also occur close to the extremity of the terminal region of outer spiral fibers, since they spiral towards the cochlear base and could reach frequency regions with progressively more OC innervation. As mentioned, type-II neurons of the low frequency region may also be innervated at the level of the cell body, even though this phenomenon has been shown primarily in primates (Arnold, 1982; Kimura et al., 1979; Rask-Andersen et al., 2000; Rask-Andersen et al., 1997; Thiers et al., 2000).
The OC/type-II synapses commonly have morphological hallmarks of highly active neurotransmission. In humans, these OC/type-II synapses commonly have an accessory synaptic specialization called Mitochondria-Associated Adherens Complex (MAC) (Thiers et al., 2000; 2002a), and many cases of the synapses found in cat # 1 had this specialization as well (data not shown). These synapses have been found in highly active synapses of the auditory system (Cant and Morest, 1979; Gray, 1963; Spirou et al., 1998; Tolbert and Morest, 1982), and they are thought to enable fast neurotransmission and vesicle turnover. As for the neurochemicals used in these OC/type-II synapses, immunohistochemical studies have shown immunoreactivity for several neurotransmitters/peptides, and most of them are inhibitory at the level of the organ of Corti. The neurotransmitters found in pre-synaptic (OC) side of OC/type-II synapses are probably co-localized and include GABA (Eybalin et al., 1988; Usami et al., 1988), Acetylcholine (Eybalin and Pujol, 1987), CGRP (Sliwinska-Kowalska et al., 1989), and enkephalin (Altschuler et al., 1984b; Scholtz et al., 1998). On the post-synaptic side, type-II neurons have been shown to have GABAb (Maison et al., 2007a), nicotinic cholinergic (Bao et al., 2005; Morley et al., 1998), and opioid (μ, κ, and δ) receptors (Jongkamonwiwat et al., 2006).

The OC/type-II synapses in the first row of cat # 1 were equally likely to be on nerve fibers or on terminals. Since we did not have reconstructions of the entire type-II fibers, and many terminals were en passant, we cannot assume that a synapse on a given terminal is more distal (in relation to the type-II cell body) than another synapse on the fiber. As for the 17 pre-synaptic OC fibers, they probably originate from a considerably smaller number of parent olivocochlear neurons (5-10?), as these the OC neurons commonly branch at a level proximal to the outer spiral bundle - outside the area reconstructed in this study (Brown, 1987b; Warr and Boche, 2003). It is possible, then, that many of the terminals originated from a given parent type-II fiber are being contacted by the same parent OC neuron, in a way that the firing of this OC neuron causes a virtually simultaneous change in all innervated type-II terminals (and fiber segments).
Column graphs representing the number of OC/type-II synapses per OHC width (approximately 10 micrometers) in the three rows of OHCs of cat # 1 and of a young human subject (Thiers, et al., 2002). The observed gradient can be explained simply by the fact that there are progressively less OC fibers/terminals towards the third row of OHCs. The frequency region chosen for the serial section study in the cat (700 Hz) was chosen to enable direct comparison of its innervation patterns with the human specimen described in Thiers et al, 2002. The numbers in the cat are comparable with the human's and the difference in the count of OC/type-II synapses in the first row can be at least partially attributed to the fact that every third section (instead of bona fide serial section) were analyzed in the human study.
The OC neurons seem to be well positioned to modulate the firing of type-II neurons. Support to this notion comes from the finding that OC neurons target preferentially reciprocal type-II terminals with afferent directional synaptic predominance, and tend to synapse on fiber segments close to these mostly afferent reciprocal terminals. This finding is relevant in a context in which the location of an excitatory synapse in the dendritic arborization of a neuron affects the afferent input’s ability to trigger an action potential (Bui et al., 2007; Maltenfort et al., 2004). An isolated input at a distal point of the dendritic arborization may not be enough for the action potential generation, but the temporal coincidence of many inputs substantially increases the chances of action potential generation. Conversely, if the afferent inputs are all inhibited at the same time, the chances of temporal summation of inputs and action potential generation can be substantially reduced (Williams and Stuart, 2003). Assuming that OHC’s afferent synapses are excitatory and that neighboring OHCs get stimulated by cochlear motion almost simultaneously, the afferent input to a given type-II neuron innervating these OHCs could be temporally related and generate an action potential. In this scenario, the activation of OC neurons synapsing on the fiber/terminals formed by this type-II neuron could potentially diminish the strength of temporal related inputs to the type-II fibers and therefore decrease the probability of action potential generation. In other words, the OC neurons could be counteracting temporal summation of afferent inputs possibly performed by the type-II fibers. In relation to the ability to prevent generation/conduction of action potentials into the central nervous system, a similar effect may be achieved through more proximally located synapses at the tunnel of Corti or cell body, so apical type-IIs may be modulated as well. By modulating the probability of generation of action potentials in type-II neurons, the OC system may be affecting both the local (intra-cochlear) and sensory (central) processes mediated by the type-IIs.

In case the activity of the outer spiral network involves action potentials, a decrease in the probability of generation of action potentials by type-II neurons through OC activation may secondarily affect the local function of such network. In a recent study (Maison et al., 2007b), it was shown that after interruption of long (~70 secs) continued stimulation of the OC system, there was a temporary slow enhancement of response amplitude as measured by DPOAEs, which was not mediated by cholinergic synapses on OHCs. It is conceivable that this effect was mediated by temporary changes in the activity of the outer spiral network caused by continued activation of the OC/type-II synapses.
The OC system may also modulate the sensory input conveyed by the type-IIIs into the central nervous system, and disruption of the OC/type-II synapses may affect the strength of this medial OC reflex. It has been shown in a recent study (Maison et al., 2007a) that disruption of the GABA\textsubscript{b} receptor they found on type-II neurons causes elevated hearing thresholds (with changes in DPOAEs) and increased resistance to permanent acoustic injury. The authors hypothesized that decreased activity of the OC/type-II synapses mediated by the GABA\textsubscript{b} receptor were increasing the sensory input conveyed type-II neurons into the brainstem, resulting in abnormal reinforcement of the medial OC reflex. The proposed consequence was that over stimulated OC neurons were suppressing the cochlear amplifier, causing threshold elevation and protection from acoustic injury. The suggestion from anatomical studies that type-II and OC signals are integrated at the level of the cochlear nucleus by neurons that share input from the two systems (Benson and Brown, 1996; 2004) are supportive of the notion that the sensory information about operational status of the OHCs conveyed by the type-II is used indirectly to modulate the activity of the OC system (Berglund, 1990; Brown, 1994; Kim, 1986).

Further studies will be necessary to better characterize the interactions of the outer spiral network with the OHCs and also with the supporting cells. The innervation of the outer spiral network by the OC system also needs more detailed anatomical and functional characterization. The presented findings are suggestive that the function of the outer spiral network and of the OC system are interrelated, so that their relationship needs to be taken into consideration in future discussions about neural modulation of OHCs.
CHAPTER 6 – Summary of Findings & Future Directions

In this summary, we will briefly review the key findings of the thesis, while also addressing how future research may help to elucidate the functional properties of this newly disclosed outer spiral network and of its innervation by the OC system.

1. Terminals with reciprocal synapses seem to derive from type-IIs, and not from OC neurons

The morphometric analysis of the terminals contacting the OHCs in the young human specimen (Figure 4 – Chapter 4) and in cats #s 1 and 2 (cut OCB) (Figure 5 – Chapter 5) suggested the terminals forming reciprocal synapses with the OHCs do not originate from the OC system. It follows that the neurons with OC morphology are probably not the only source of efferent synapses on the OHCs, and the type-IIs may have a local role in the modulation of the OHCs through the efferent components of their reciprocal synapses.

The functional properties of the efferent neurotransmission between type-IIs and OHCs are unknown. The first step towards elucidation of these properties can be the determination of the neurotransmitter(s) used by the type-IIs on their OHCs contacts. Double immunostaining with the type-II specific anti-peripherin antibody (Hafidi, 1998; Maison et al., 2007a; Reid et al., 2004) and known neurotransmitters may provide clues. One can also focus the study on regions of the cochlea where OC terminals are not prevalent, like in the third row of OHCs in the 700Hz region in the cat (Figure 9 – Chapter 5) or in the apical turn of the guinea pig. The study of adult animals submitted to long-term transection of the OCB may also help, as the number of reciprocal type-II terminals in such experimental condition does not seem to be substantially different than the one of normal animals in comparable frequency region (Figure 10 - Chapter 5). It will also be important to determine whether the type-II terminals with the OHCs have the molecular machinery to enable synaptic vesicle docking and Ca$$^{++}$$ dependent neurotransmitter exocytosis, like the one present in the supposedly reciprocal hair cell contacts of vestibular afferent neurons (Dememes et al., 2000). Again, an immunohistochemical study employing double immunolabeling with peripherin and N-type voltage gated Ca$$^{++}$$ channels or SNARE proteins may provide answers.
2. Presence of reciprocal type-II terminals is not a degenerative finding associated with aging and is not exclusive to primates

Reciprocal type-II terminals were found to be at least as common in the 8-month old human specimen (Figure 2 – Chapter 4) as in the older human and chimpanzee specimens that were evaluated previously (Francis and Nadol, 1993a; Nadol, 1984). The study of cat # 1 involved detailed analysis of the membrane interface between terminals and OHCs (Figure 6 and 7 – Chapter 5), and reciprocal synaptic specializations were also found to be common. It is likely that reciprocal synapses between type-IIs and OHCs are also present in other mammals, but further research is needed to determine if that is the case.

*En face* reconstruction of membrane interfaces based on serial section electron microscopy in a plane of section perpendicular to the studied membranes is currently the gold standard for the study of anatomical features of reciprocal synapses. However, such time-consuming approach may not be necessary if the objective is simply to determine if the type-IIs also have efferent (reciprocal) specializations with the OHCs in a given animal. A more focused approach employing labeling of type-IIs, transection of the OCB, or even a semi-serial section analysis like the one performed for cats # 2, 3, and 4 presented in the chapter 5 of this thesis may provide the answer to such question.

3. Reciprocal type-II terminals are present across frequency regions, but seem to be more prevalent in the frequency regions below 4,000Hz

Type-II terminals with reciprocal synapses are distributed throughout the cat cochlea, but have a higher prevalence in the low frequency half (Figure 10 and 11 – Chapter 5). The finding that reciprocal type-IIs are in all studied regions indicates that they may be relevant to the coding of both low and high frequency sounds. However, it is possible that they are more important in the processing of low frequencies.

Unfortunately, we are still not able to selectively affect the function of type-II neurons in order to test such hypothesis. The determination of the molecular machinery used in their reciprocal synapses or transmission of electrical impulses through type-II fibers may enable the use to selective antagonists to determine their role in (low and/or high frequency) hearing. Based on such information, one may also be able to create viable knockout animals that present type-II neuron-specific dysfunction. A substantial amount of new information on the neurobiology and
electrophysiology of type-IIs will be needed before we can move on to studies that will provide definitive answers on their functional properties.

4. Directional predominance of reciprocal synapses vary along a continuum

There was substantial variability in synaptic directional predominance of the reciprocal interactions of individual type-II terminals with the OHCs, with some being mostly afferent and others mostly efferent (Figure 8 – Chapter 5). This finding suggests that the interactions between individual type-II neurons with individual OHCs are quite complex, and the other factor that adds to such complexity is that a given type-II fiber sometimes gives rise to more than one terminal contacting the same OHC (Figure 8 – Chapter 5). It is not known to what extent directional synaptic predominance of reciprocal terminals shapes the interaction of type-II neurons with individual OHCs. It is also unclear if individual OHCs have substantially more terminals with afferent (or efferent) predominance than their neighbors.

Immunohistochemical labeling of the afferent and/or efferent components of the reciprocal synapses could enable a more precise description of the variation in synaptic directional predominance of individual type-II terminals. One could also determine how neighboring OHCs compare to each other in terms of their form of interaction with the ensemble of type-II neurons that innervate them. Immunohistochemical labeling of the OC/type-II synapses can also assist in this effort, as these synapses tend to be present preferentially on reciprocal terminals with afferent predominance. It would be interesting to see how the synaptic directional predominance of type-II terminals change as function of cochlear region, stage of development, studied species, and presence of pathological conditions.

5. Synaptic interactions of type-IIs with OHCs seem to be essentially reciprocal

All of the 30 type-II fibers that could be traced to terminals contacting more than one OHC formed reciprocal circuits with the innervated OHCs in the 700 Hz frequency region of cat # 1. In other words, we could not find type-II fibers that were traced only to 100% afferent or 100% efferent terminals contacting neighboring OHCs. This finding runs counter to the notion that subpopulations of spiraling fibers with type-II morphology are exclusively afferent or efferent in the relation to the OHCs.
To our knowledge, systematic tracing of type-II fibers to reconstructed terminals on neighboring OHCs had not been performed before in any species, and further research is needed to verify if this finding can be replicated in other frequency regions and animals. It will also be important to gather data on a higher number of reciprocal circuits, as their composition seem to be quite variable (Figure 16 – Chapter 5) and it is difficult to identify patterns from a relatively small number of data points. Reconstruction of the entire “terminal region” of individual type-II fibers will also be important. One may be able to determine whether there are systematic changes in the synaptic directional predominance of the type-II terminals as one move towards the distal end of the type-II fibers.

6. Type-II neurons form a local neural network that seem to interconnect OHCs

Type-II neurons form reciprocal circuits with the OHCs and also innervate the supporting cells. These neurons forming reciprocal circuits have overlapping innervation fields and share common cellular targets (OHCs and supporting cells) (Figure 16 – Chapter 5). The ensemble of type-IIs is thought to constitute a neural network based on the finding that they have bi-directional interactions with the innervated OHCs. This neural network at the level of the outer spiral bundle was named outer spiral network for brevity. We hypothesize that the outer spiral network may have a role in functional integration of the OHCs, and also supporting cells.

It will be important to test the hypothesis whether the outer spiral network allows bi-directional neural signal transmission among OHCs sharing the same type-II fibers. One possible approach to test such hypothesis would the use of in vitro patch-clamping of neighboring OHCs in cochlear explants. The creation of such preparation will probably be a challenging undertaking, but it may allow substantial expansion of our knowledge on the neural-based longitudinal intercommunication among OHCs, possibly mediated by the outer spiral network. In case such longitudinal communication is demonstrated, it may be possible to use pharmacological agents that affect specific mediators of synaptic transmission and electrical signal conduction to study the mechanisms used by the type-IIs in their interaction with OHCs and supporting cells.

7. Type-II neurons interact with each other through type-II/type-II synapses
In humans, type-II/type-II synapses seem to be common at the level of the outer spiral bundle and spiral ganglion (Figure 15 – Chapter 2; Figure 6 – Chapter 3) (Nadol, 1990b). These synapses have been described also in the old world monkey (Bodian, 1978), macaca fuscata (Kimura et al., 1987) and in the cynomologus monkey (Thiers, unpublished). These synapses were found in the cat # 1 (data not shown), but the plane of section parallel to the type-II fibers does not seem to be optimal for the identification of these synapses. In the analysis of the outer spiral bundle of human specimen presented in Chapter 3, a plane of section perpendicular to the type-II fibers was employed, which probably helped in the identification of these type-II/type-II synapses.

The type-II/type-II synapses were found to be unidirectional (not reciprocal) and it is not known if they are inhibitory or excitatory, or which neurotransmitters are involved. It will be important to determine if these synapses are also present in other mammals and if their prevalence systemically change depending on the cochlear frequency region studied. The function of these synapses is also unknown, but they may enable communication among type-II fibers innervating different groups of OHCs.

8. OC/type-II synapses are ubiquitous and have ultrastructural features of highly active synapses

In the outer spiral bundle, OC/type-II synapses are common in the regions where OC terminals are prevalent (Figure 17 – Chapter 5), and they occur at the level of type-II nerve terminals and fibers (Figure 13 – Chapter 5). OC/type-II synapses also occur in the tunnel of Corti (Dunn, 1975; Liberman et al., 1990) and spiral ganglion (Kimura et al., 1987; Kimura et al., 1979). In the spiral ganglion, the prevalence of OC/type-II synapses on the cell bodies of type-IIIs in humans varied in individuals of different ages (Table 2 – Chapter 2), and it was quite high in a young adult (50% of type-IIIs). These OC/type-II synapses were found to have morphological features that are similar to the ones of other highly active synapses (Spirou et al., 1998), specially as it relates to the presence of the “mitochondria-associated adherens complex” (MAC) (Figure 16 – Chapter 2; Figure 4 – Chapter 3). Evidence for the presence of several types of neurotransmitters in pre-synaptic (OC) side of OC/type-II synapses have also been reported, including GABA (Eybalin et al., 1988; Usami et al., 1988), acetylcholine (Eybalin and
Pujol, 1987), CGRP (Sliwinska-Kowalska et al., 1989), and enkephalin (Altschuler et al., 1984b; Scholtz et al., 1998).

It is possible that immunolabeling targeted at molecular elements of the poorly understood MAC specialization or to some of the neurotransmitters found in the OC/type-II synapses will allow the identification of these synapses without the need for serial section electron microscopy. The development of a marker for OC/type-IIIs synapses that can be used in immunohistochemical studies would be an important step in the study of these synaptic interactions. This marker may enable the quantification of these synapses in different cochlear regions, stages of development, disease states, and species. Data presented in chapter 2 was suggestive of a correlation of prevalence of OC/type-II synapses on the cell bodies with maturation and age-related degeneration of the type-II and/or of the OC system. There were not enough data points to systematically investigate the importance of maturation and degeneration, but a quantitative approach using immunohistochemistry at a light microscopy level may allow the testing of such hypothesis.

9. OC neurons preferentially target type-II terminals that are predominantly afferent

The OC/type-II synapses were not randomly distributed at the level of the outer spiral bundle of cat # 1 (Figure 14 – Chapter 5). The type-II terminals without OC/type-II synapses were as likely to be predominantly afferent or efferent, but the ones targeted by one or more OC/type-II synapses tended to have afferent synaptic directional predominance. There was also a tendency for the OC/type-II synapses on the type-II fibers to be closer to the terminals with afferent predominance (Figure 16 – Chapter 5), but this relationship was not clear-cut. The reason behind the preferential placement of the OC/type-II synapses on the predominantly afferent type-II terminals is unknown, but the OC system seems to be well positioned to modulate the afferent input from the OHCs into the type-II neurons.

Little is known about the biophysical properties of type-II neurons in mature animals. As a consequence, our ability to build realistic compartmental neuronal models to study the effect of the location of afferent inputs from OHCs and OC/type-II synapses in the relation to the cell bodies of type-IIIs is very limited. The determination of the types of Na⁺, K⁺ and Ca⁺⁺ channels present on the type-IIIs will be quite important, as will be the characterization of the synaptic
inputs from the OHCs and OC neurons, and their output back to the OHCs. Electrophysiological studies that address the conduction velocity of type-II neurons at the level of the outer spiral network in different frequency regions will also be helpful.

10. OCB transection does not seem to affect anatomical integrity of outer spiral network

Even though the OC/type-II innervation has been found to be quite prominent in the cat (Figure 17 – Chapter 5) (Dunn, 1975; Liberman et al., 1990), the presence of the OC system does not seem to be essential for the maintenance of the synaptic interactions between type-II and OHCs (Figures 4 and 10 – Chapter 5) (Liberman et al., 2000). This finding indicates that the outer spiral network may be able to operate in the absence of the OC system, even though the normalcy of its function in such condition cannot be ascertained.

The study of animals with transected OCB may allow the study of the possible role of the outer spiral network in modulation of OHCs function without an important confounding factor, which would be the presence of the OC system innervating the same OHCs. The study of the efferent components of the reciprocal synapses with immunohistochemical techniques may also be easier in this experimental condition. The likely small immunoreactivity of type-II efferent specializations would not be obscured by the one of the OC terminals, which have ten times more vesicles than the type-IIIs (Chapter 5).
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