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 and two monkey species. 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 toward 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). All affer-

ent 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.

Keywords: reciprocal synapse, outer hair cells, efferent, type II spiral ganglion cell, outer spiral bundle, neural network

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 (Harveit 1999; Dowling 1968), olfactory bulb (Kirillova and Lin 1998; Isaacson and Strowbridge 1998), autonomic nervous system (Kawai 1996; Yagamuchi et al. 1975), carotid bulb (McDonald and Mitchell 1975; Matsumoto et al. 1980), and vestibular macula (Ross 1997;

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Dunn 1980; Lysakowski and Goldberg 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 1987; Takasaka and Shinkawa 1987) and cats (Simmons and Liberman 1988; Liberman et al. 1990) did not report the presence of reciprocal synapses. However, reciprocal synapses have been described in prenatal guinea pigs (Thorn et al. 1972; Jones and Eslami 1983), and Tanaka and Smith (1978) reported their presence in the adult chicken. In addition, reciprocal synapses were found in kittens 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, 1990; Nadol and Burgess 1990; Spoendlin and Schrott 1988). In the chimpanzee (Francis and Nadol 1993a), and also in the Japanese macaque (Sato et al. 1997, 1999), the numbers of reciprocal synapses increased from the base toward the apex and from the first toward the third row. These findings in perfused animals indicate that reciprocal synapses are not the result of postmortem 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 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 (Nadol 1981, 1983; Francis and Nadol 1993a; Sato et al. 1997). Olivocochlear fibers and processes of type IIs can be differentiated by cytoplasmic morphology (Spoendlin and Gacek 1963; Spoendlin and Schrott 1988; Liberman et al. 1990; Ginzberg and Morest 1984). 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 AND 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 ronguers, and a power drill was used to thin the otic capsule. Next, the specimens were decalcified in 0.1 M disodium ethylenediamine tetraacetic 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® (Pella, Inc., Redding, CA).

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 90 nm. 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 13,000 $\times$ using the JEOL 100CX transmission electron microscope (JEOL, Peabody, MA). The final magnification was 32,000 $\times$.

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.

FIG. 1. Examples from serial sections of a reciprocal synapse between an OHC and a VP/NR nerve terminal (NT) (1a–1c, magnification, 75,000X). 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).

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 presynaptic 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 (Fig. 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 semiquantitative categories of “low,” “medium,” and “high” neurofilament content. The differences between the categories of terminals found were analyzed using the standard *t*-test and also the two-dimensional Kolmogorov–Smirnov test (Teukolsky et al. 1992).

RESULTS

Reciprocal synapses

Reconstruction of the entire terminals proved to be essential for the identification of reciprocal synapses

FIG. 2. Prevalence of OHCs innervated by at least one terminal with a reciprocal synapse in the present study and in the chimpanzee (Francis and Nadol 1993a). Gradient from first toward third row was significant ($p < 0.001$). The data in our human specimen were not significantly different ($p > 0.5$) from that described in the middle turn of the chimpanzee (Francis and Nadol 1993a).

because their afferent and efferent components were rarely seen in the same plane of section (Fig. 1). In addition, either the efferent or the afferent components of reciprocal synapses were sometimes small and could, therefore, be missed with a semiserial 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 cisterna of the efferent component usually continued laterally as subsurface cisterna.

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 toward the third row (Fig. 2). Similarly, the average number of reciprocal synapses per OHC increased from the first toward the third row ($p < 0.001$) (Fig. 3). However, there was no statistically significant difference between the second and the third rows.

Nerve terminals innervating OHCs

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

FIG. 3. Average number of terminals with a reciprocal synapse per OHC (only OHCs with reciprocal terminals included). Gradient from first toward third row was significant ($p < 0.001$). However, there was no statistically significant difference between the second and the third row.

terminals), and were usually small (mean maximal cross-sectional area = $1.3 \mu\text{m}^2 (\pm 1.1)$).

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 terminals' data to be highly significant ($p < 0.0001$). This significant morphological dichotomy was also evident in qualitative terms (Fig. 5).

Correlation of synaptic and cytoplasmic morphology

All the terminals that formed afferent (Fig. 6a,b – VP/NR postsynaptic terminals or “classical afferents”) and reciprocal synapses (Fig. 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 (Fig. 6c). Because we do not know their neuron of origin, we will call this second group of “efferent” terminals “VP/NR presynaptic terminals.”

The prevalence of terminals of the VP/NR postsynaptic type (“classical afferents”) decreased from the first toward 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

FIG. 4. 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 syn-

napses had medium or high neurofilament content and had less than 36 vesicles. Most terminals forming only efferent synapses grouped with the larger, vesicle-rich/neurofilament-poor group (VR/NP), while others grouped with the smaller, vesicle-poor/neurofilament-rich (VP/NR) fibers. Aff = afferent, Eff = efferent, Rec = reciprocal, NF = neurofilament content.

FIG. 5. Two types of nerve fibers innervate the OHCs (magnification 36,000 $\times$). **A.** Vesicle-rich/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/

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 IIa. Both types of nerve fibers demonstrated a few dense core vesicles.

the increase of VP/NR presynaptic terminals from the first toward the third row (Fig. 7).

The decrease in the numbers of VR/NP presynaptic terminals (“classical efferents”) from the first toward 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 (Fig. 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

FIG. 6. Nerve terminals classified as VP/NR forming different types of synaptic specializations on OHCs. **A.** VP/NR presynaptic and VP/NR postsynaptic (classical afferent) side by side (magnification 34,000 $\times$). **B.** VP/NR postsynaptic (classical afferent) (magnification 74,000 $\times$). **C.** VP/NR presynaptic (magnification 64,000 $\times$). **D.** VP/NR reciprocal (magnification 69,000 $\times$). 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.

VP/NR presynaptic terminals. Furthermore, 75% of OHCs (9 cells, not shown in Fig. 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”).

FIG. 7. 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 toward 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) toward the third row was not statistically significant. The increase of the VP/NR reciprocal and presynaptic from the first toward the third row was statistically significant ($p < 0.001$). Inverse gradients of classical efferents and VP/NR reciprocal and presynaptic were evident.

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 were similar ($p > 0.5$) to those in the chimpanzee (Francis and Nadol 1993a) (Fig. 2) and higher than those 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 toward third row, of distribution of reciprocal synapses.

The presence of a subsynaptic cisterna 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 (Fig. 5). As mentioned, previous studies concluded that vesicle-rich neurofilament-poor (VR/NP) fibers are processes of the medial olivocochlear efferent system, and the nerve fibers that are vesicle-poor/neurofilament-rich (VP/NR) are likely peripheral processes of type IIs (Spoendlin and Gacek 1963; Spoendlin and Schrott 1988; Liberman et al. 1990;

Ginzberg and Morest 1984; Sato et al. 1997; Nadol 1990).

Therefore, it was expected that nerve fibers that were VR/NP formed only efferent terminals on the OHCs and that all afferent terminals were VP/NR nerve fibers. 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, Fig. 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 (present data; Nadol 1984; Francis and Nadol 1993a). Furthermore, VP/NR terminals that 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 nonclassical 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 can not 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 the early stages of ontogeny (Pujol et al.

Fig. 8. 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 be-

tween dendrites of type IIs (dendrodendritic synapses; Nadol 1983), axosomatic and axodendritic synapses formed by fibers with the morphology of olivocochlear projections (Kimura et al. 1979; Thiers et al. 2000, 2001), and the innervation of the supporting cells by the type IIs (Fechner et al. 2001).

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 post-natal week (Brown 1987; Simmons and Liberman 1988; Simmons et al. 1991; Kiang et al. 1982; Ginzberg and Morest 1984; Berglund and Ryugo 1987). Second, there is precedence for a first-order “afferent” neuron to also form “efferent” synapses on receptor cells in other sensory systems, such as the vestibular macula (Ross et al. 1997; Chimento and Ross 1996) and the carotid body (McDonald and Mitchell 1975, 1981).

This concept runs counter to the hypothesis that all “efferent” terminals are derived from the olivocochlear bundle. However, after experimental sectioning of the olivocochlear bundle, some “efferent” terminals remain. Thus, in the study of Bodian and Gucer (1980), although most efferent nerve terminals degenerated after sectioning 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 sectioning 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 sectioning of the olivocochlear projections or insufficient postsurgical survival time. Furthermore, in studies in which only the crossed olivocochlear system was sectioned (Nakai and Igarashi 1974; Iurato et al. 1978; Kimura and Wersall 1962), 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 basoapical 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 IIs.

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 toward the third row (Fig. 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

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 nonprimates (Ginzberg and Morest 1984; Hashimoto and Kimura 1987; Jones and Eslami 1983, Liberman et al. 1990). However, if some VP/NR presynaptic nerve terminals are actually processes of type IIs, 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; Kirilova and Lin 1998; Nakanishi 1995; Woolf et al. 1991; Isaacson and Strowbridge 1998).

Further studies will be necessary to test the hypothesis that the type IIs form “reciprocal innervation circuits” in mammals. Recent studies (Thiers et al. 2000, 2001; Fechner et al. 2001) demonstrate that type IIs 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 IIs may be the morphological substrate for a complex neural network in the auditory periphery (Fig. 8).

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