Distribution of the Glutamate/Aspartate Transporter GLAST in Relation to the Afferent Synapses of Outer Hair Cells in the Guinea Pig Cochlea

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ABSTRACT

The glutamate/aspartate transporter GLAST is known to occur in the plasma membrane of supporting cells and their glialike processes around the synaptic region of inner hair cells of the mammalian cochlea. Its function there is presumably to take up glutamate following the release of this putative amino acid neurotransmitter from the inner hair cells. In this study, we have investigated whether GLAST is also associated with the outer hair cells using postembedding immunogold labeling. This is interesting because it is uncertain whether the outer hair cells have a functional synapse at which glutamate may be released. However, earlier ultrastructural studies of the afferent synapses in outer hair cells in several mammalian species have shown features normally associated with synaptic activity. These observations are confirmed and extended here in guinea pig where these afferent synapses have presynaptic bodies, putative synaptic vesicles, and coated pits associated with them. Immunoreactivity for GLAST was found along the plasma membranes of Deiters' cells, especially around the synaptic region of the hair cell, on processes wrapped around approaching nerve fibers. Semiquantitative analysis of the distribution of immunogold labeling of Deiters' cells confirmed that it was densest in the region adjacent to the synapses. There was also more labeling in apical than in basal regions of the cochlea in three of the four animals examined, suggesting an association

with the number of afferent synapses, which are more numerous in apical regions. Interestingly, labeling also occurred in other regions of the cell membrane away from the afferent terminals. This suggests that glutamate uptake is also required away from the immediate vicinity of synapses, perhaps as a consequence of glutamate dispersal resulting from the mechanical displacement of the cochlear partition during stimulation. Nonetheless, the particular association of GLAST with the synaptic region of the outer hair cell implies that the latter have active afferent synapses at which glutamate is released.

Keywords: glutamate transport, cochlea outerhair cells, afferent synapses, Deiters' cells

INTRODUCTION

The high affinity Na⁺-dependent glutamate/aspartate transporter GLAST, first cloned by Storck et al. (1992) from rat brain, has been shown previously to be present in the mammalian inner ear (Furness et al. 1993; Li et al. 1994; Furness and Lehre 1997; Takumi et al. 1997). In the central nervous system, GLAST is thought to be involved in the uptake of glutamate into glial cells following its release at glutamatergic synapses, along with other glutamate transporters that are present either in glia or in neurons (see reviews by Danbolt 1994; Saier 1999). This uptake is necessary because glutamate is excitotoxic when present in relatively high extracellular concentrations, causing neuronal damage and death probably as a consequence of overactivation of glutamate receptors (see review by Meldrum 1993). Transporter-mediated activity has also been implicated in the subtle control of

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synaptic activity since blockage of glutamate uptake alters the time course of synaptic currents (Kinney et al. 1997; Turecek and Trussell 2000). While the other major glial glutamate transporter, GLT-1, may predominate in some brain regions, GLAST protein and GLAST mRNA levels are particularly high in the hippocampus and cerebellar molecular layer (Torp et al. 1994; Lehre et al. 1995), regions containing substantial glutamatergic innervation.

In the inner ear, GLAST has been shown to occur in the supporting cells surrounding the synaptic region of cochlear inner hair cells (IHCs) (Furness and Lehre 1997) and vestibular hair cells (Takumi et al. 1997). It is well known that the IHCs have highly active, probably glutamatergic, synapses with cochlear nerve fibers that provide the main route for neural output from the cochlea to the brain stem cochlear nuclei (see review by Eybalin 1993). A single row of IHCs is present along the spiral sensory epithelium (organ of Corti) and these cells form synapses with the type I neurons of the spiral ganglion, whose central projections represent 90%–95% of the afferent fibers of the cochlear nerve (Spoendlin 1972, 1985). Therefore, the function of GLAST in these locations is presumably to take up glutamate following its release from the synapses of these hair cells.

The other sensory cell type in the cochlea is the outer hair cell (OHC). There are at least three times as many of these than there are IHCs. They form three rows that are innervated by the remaining 5%–10% of afferent cochlear nerve fibers. This innervation is composed of the peripheral processes of type II spiral ganglion cells whose central processes are weakly myelinated or unmyelinated (Spoendlin 1969) but which also end in the cochlear nuclei (Berglund and Brown 1994; Berglund et al. 1996; Hurd et al. 1999). The density of afferent innervation to the OHCs increases toward the apex of the cochlea in many mammalian species (cat: Liberman et al. 1990; see review by Eybalin 1993). There is also evidence in guinea pig that there are more afferent endings in the third or outermost row of OHCs than the other rows (Hashimoto and Kimura 1988). Information on the physiological properties of these afferent fibers has proved difficult to obtain (Robertson et al. 1999). Therefore, the extent to which the OHC–afferent synapse is functionally active and what, if anything, these cells signal to the brain stem are still in question. In fact, the OHCs are thought to contribute to cochlear function mainly by means of a biomechanical, as opposed to neural, response that produces cycle-by-cycle amplification of the motion of the cochlear partition (Dallos 1997). In this respect, they have a relatively extensive efferent innervation compared with the IHCs which utilizes the neurotransmitters acetylcholine (ACh) and possibly GABA (Eybalin 1993).

While questions remain about the function of their synapses, immunolabeling for both glutamate (Altschuler et al. 1989; Usami et al. 1992) and aspartate (Usami and Ottersen 1996) is greater in the OHCs than the surrounding supporting cells, as is also the case in IHCs. This suggests the possibility that the OHCs are glutamatergic or aspartatergic, although the presence of elevated levels of these amino acids is not sufficient by itself to indicate a neurotransmitter role. There is also evidence that all spiral ganglion neurons, including those innervating OHCs, express a range of glutamate receptors (Niedzielski and Wenthold 1995). However, there are conflicting reports as to whether the α -amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) type GluR4 receptor subunit found at IHC synapses is also present at those of the OHCs (Kuriyama et al. 1994; Matsubara et al. 1996).

The synaptic ultrastructure of OHCs in some species also suggests the possibility that some of these cells have active synapses. For example, synaptic bars and vesicles reminiscent of those found at the IHC synapse have been reported in apical regions of the cat cochlea (Liberman et al. 1990). In humans (Nadol 1990) and other primates (Sato et al. 1999), there is evidence of reciprocal synapses on OHCs that have characteristics of both afferent and efferent synapses associated with the same nerve terminal. In guinea pigs, counts have shown that 26% of OHC afferent endings in the base of the cochlea and that 16% in the apical region have synaptic bars (Hashimoto and Kimura 1988), although the morphology of these is quite variable (Swetlitschkin and Vollrath 1988).

By analogy with IHCs and vestibular hair cells, if OHCs do have significant synaptic activity and use glutamate as a neurotransmitter, there would probably also be GLAST in the supporting cells associated with them. The basal region of each OHC is enclosed in the cup-shaped cell body of a Deiters' cell that surrounds the afferent and efferent endings. The main aim here has been to investigate whether GLAST is present in these cells and, if so, whether its distribution is consistent with glutamate uptake around the afferent terminals. In addition, further observations of the comparative ultrastructural features of the synaptic region of guinea pig OHCs have been made that support the suggestion that these cells may have active afferent synapses.

MATERIALS AND METHODS

Animals and antibodies

Six pigmented guinea pigs were used in this study. Animals were maintained and used in accordance with the "Principles of Laboratory Care" (NIH publication No. 85-23, revised 1983) and the Animals

(Scientific Procedures) Act, 1986. The polyclonal antibody used was antibody A1 which was raised against a synthetic peptide, based on the sequence for GLAST (Storck et al. 1992) starting at the first amino acid. It was a gift from Dr. N.C. Danbolt, University of Oslo. This antibody was identical to one of those used in the previous study on IHCs in which it was shown to recognize a protein in the cochlea of similar molecular mass to that of GLAST isolated from rat brain (Furness and Lehre 1997).

Preparation for conventional transmission electron microscopy of OHC synapses

Two guinea pigs were given an overdose of sodium pentobarbitone (1 ml/kg, Pentoject, Animalcare, York, UK) decapitated, and the bullae removed. The cochleae were opened by making a small hole in the apex with a needle and removing the round window, fixed by perfusion with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer, and then immersed in this fixative for 2 h. After washing in buffer, they were postfixed in 0.1% osmium tetroxide in the same buffer for 1 h, and dissected into segments that were dehydrated and embedded in Spurr resin according to previously published methods (Furness and Hackney 1985). Radial ultrathin (100 nm) sections were obtained from both apical and basal regions and were stained in 2% ethanolic uranyl acetate (20 min) and 2% lead citrate (5–7 min) prior to examination in a JEOL 100 CX transmission electron microscope operated at 100 kV.

Electron microscope immunocytochemistry for GLAST

Postembedding immunogold labeling was carried out on ultrathin sections of tissue prepared using freeze-substitution embedding in low temperature resin (Chaudhry et al. 1995; Furness and Lehre 1997). The cochleae of four young adult guinea pigs, aged between 2 and 3 months, were obtained as before, opened, and fixed by perfusion with 2.5% glutaraldehyde and 1% paraformaldehyde in 0.1 M sodium phosphate buffer (NaPi). They were then dissected into small segments which were cryoprotected by sequential immersion in 10%, 20%, and 30% glycerol in NaPi for 30 min each and then in 30% glycerol overnight at 4°C. For freezing, they were dipped in glycerol, placed on pin-stubs or small screws covered with a piece of double-sided sticky tape, and rapidly plunge-frozen in liquid propane cooled in liquid nitrogen using a Reichert KF80 plunge freezer. Segments were transferred in liquid nitrogen to the chamber of a Reichert AFS cryosubstitution unit precooled to –90°C, immersed in anhydrous methanol

containing 0.5% uranyl acetate, and gradually brought up to –45°C at the rate of 4°C/h. After washing with anhydrous methanol, they were infiltrated with increasing concentrations of Lowicryl HM20 resin in methanol (1:1 followed by 2:1, 2 h each), then pure resin twice (2 h, overnight). The resin was finally polymerized with UV light at –45°C for 24 h and subsequently brought up to room temperature over 35 h.

For immunolabeling, ultrathin sections of organ of Corti from apical and basal cochlear regions were cut with a diamond knife in the radial plane and collected on nickel grids coated with quick-drying adhesive (Coat-quick “G,” Agar, Stansted, UK). The grids were placed inside a moist chamber in drops of the following solutions in sequence: fresh 1% sodium borohydride and 0.05 M glycine in 0.05 M tris-buffered saline (gTBS) (10 min), gTBS (10 min X3), 1% human albumin (HA) in gTBS (10 min), primary antibody diluted to 1–20 µg/mL in HA-gTBS (overnight at 4°C), HA-gTBS (10 min X3), goat antirabbit IgG conjugated to 30 nm gold particles (British Bio-Cell, Cardiff, UK) diluted at 1:20 in HA-gTBS (1 h), HA-gTBS (10 min X3), and 0.1 M NaPi (10 min X2). Sections were finally stained in 2% aqueous uranyl acetate and observed using a JEOL 100 CX transmission electron microscope operated at 100 kV.

Semiquantitative analysis

A preliminary analysis of the distribution of labeling was carried out in the OHC region following postembedding labeling to determine whether there was any particular association of GLAST with the synaptic pole of the hair cell. A photographic montage of the OHCs and their Deiters' cells was taken from sections in which complete OHCs were visible and from both apical and basal cochlear locations for each animal used in this study. For each hair cell to be analyzed, a line was drawn along the longitudinal axis which was then subdivided by a series of lines drawn across it, parallel to and starting from the apical membrane, which were spaced apart equally to form bins. The size of the spacing and the number of bins thus obtained depended on the original magnification and the length of the hair cell, but within each animal were identical for each row and for both apical and basal locations. The length of Deiters' cell membrane in each bin was measured using an odometer and the number of gold particles counted to obtain a labeling density.

In the light of the preliminary data, which was limited in scope because only cells in which the full length was visible in the section were considered, a more detailed analysis was performed in a restricted region around the synaptic pole. The reduction in

area analyzed allowed many more Deiters' cell/hair cell pairs to be obtained, as sections containing only the lower part of a hair cell could be included. For this, a series of nine bins of fixed size ($10\ \mu\text{m} \times 4.3\ \mu\text{m}$) were drawn, centered on the orthogonal to the long axis of the hair cell (Fig. 1). The central bin (bin 5) contained the base of the hair cell and the four bins on either side extended upward along the hair cell and downward along Deiters' cell. Deiters' cell membrane lengths and number of particles lying on the membranes were then determined in these bins, as above, for all Deiters' cell/hair cell pairs visible in all sections. This much larger sample permitted statistical analysis of the distribution of labeling and the amount of Deiters' cell membrane to be performed.

RESULTS

Synaptic ultrastructure

It is well known that both afferent and efferent fibers make contact with the basal end of the OHC and that afferent innervation is denser near the apical region of the cochlea. The examination of apical and basal OHCs performed in this study confirmed that several nerve terminals contacted the hair cell base, although these were not always unambiguously identifiable in a single section as afferent or efferent by means of their ultrastructural characteristics. Afferent terminals, however, appeared to be more common on apical hair cells and efferents on basal ones, as expected from previous studies (Fig. 2a,b).

Presumed efferent terminals had large numbers of vesicles in them (Fig. 2c,d). In many sections, especially from the apical regions, there appeared to be no subsynaptic cisternae in the hair cell adjacent to these efferent endings, although serial sectioning was not performed, so this feature may have been present out of the plane of sections that were examined. Afferent terminals did not have these large numbers of vesicles, and, in some cases, at the point of contact one or more dense bars could be observed in the presynaptic cytoplasm adjacent to the plasma membrane of the hair cell (Fig. 2).

A comparison of afferent terminals between apical and basal cochlear locations showed that the former (Fig. 2a,c,d) often had more than one synaptic bar visible in the same section plane, whereas the latter tended to have only one (Fig. 2b). The synaptic bars were rod-shaped and varied in position so that some appeared to be nearly perpendicular to the membrane, while others seemed to lie parallel to it (Fig. 2d). Both variants were noted in association with a single synapse in apical hair cells. Vesicles of varying size were present around the synaptic bars, some of which appeared to be connected to them. In general,

FIG. 1. Schematic diagram showing the arrangement of bins used for analysis of gold particle distributions in Deiters' cells (DC) around the synaptic pole of the outer hair cell (OHC). A line was drawn along the long axis of the cell and 9 bins of equal size were drawn along this line, the central one (bin 5) containing the hair cell base.

these were larger than the vesicles in the presumed efferent terminals visible in the same sections (Fig. 2d). There were also coated vesicles in the surrounding cytoplasm and coated pits in the membrane of the hair cell around the synaptic region. Dense material lining the pre- and postsynaptic membranes was apparent (Fig. 2d,e).

Within the afferent terminals identified by the presence of synaptic bars in the hair cell cytoplasm there were small numbers of both clear and dense-core vesicles. Interestingly, clusters of vesicles of a similar diameter to those present in the efferent endings could sometimes be seen (Fig. 2d). There was, however, no clear evidence of a subsynaptic cisterna on the inside of the plasma membrane of the hair cell adjacent to these clusters, a characteristic of reciprocal synapses in primates (Nadol 1990), although again serial sections were not taken and this structure, if present, may have been missed.

Immunocytochemistry

Immunogold labeling for GLAST was seen in the OHC region of both apical and basal locations in the cochlea. The labeling was seen to occur mainly along the basolateral membranes of Deiters' cells (Fig. 3). Where the Deiters' cell body associated with an OHC

FIG. 2a. Transmission electron micrograph (TEM) of the synaptic region of an apical outer hair cell (OHC) following conventional preparation showing presumed afferent (A) endings onto the cell. In this example, two of these have distinctive presynaptic dense bodies. Scale bar = 1 μ m. **b** TEM of a basal OHC from the same animal showing fewer afferent (A) and two efferent (E) endings onto the cell. Note that one afferent has a single presynaptic dense body. Scale bar = 1 μ m. **c** Example of an apical OHC base from a second animal. Two afferents (A) with presynaptic bodies associated with them and an efferent ending (E) are visible. Details of the afferents are shown in **d** and **e**. Scale bar = 1 μ m. **d** This synapse has three presynaptic bodies,

one of which lies nearly perpendicular to the cell membrane (arrow) while the others lie parallel to it (arrowheads). Note the numerous vesicles of variable size around the presynaptic bodies that are larger than those of the adjacent efferent (E) terminal. However, a cluster of small vesicles (v) comparable in size to the efferent vesicles is also present within the afferent terminal. Scale bar = 0.2 μ m. **e** In this example, a single presynaptic body is visible in this section plane. Note the distinct presynaptic vesicles, one of which may be attached to the dense body (arrow). Note also the coated pits (cp) in the OHC membrane and coated vesicles in the presynaptic cytoplasm in close proximity to the synapse. Scale bar = 0.2 μ m.

from one row came close to another OHC from the adjacent row, it was also labeled along the membrane opposite that cell (Fig. 3, inset). Deiters' cells had little labeling on their apical surfaces where they joined the reticular lamina that constitutes the upper surface of the organ of Corti. Scattered particles were occasionally noted on the OHC membrane but these cells did not appear to be systematically labeled.

In the region beneath the base of the OHC, the Deiters' cell membranes were extensively convoluted, producing glialike processes interdigitating between nerve fibers and wrapping around them (Fig. 4). To quantify the extent of these membranes, an analysis of the length of the Deiters' cell membrane was made in bins drawn along the long axis of the hair cell, starting from its apex. This confirmed that the greatest amount of Deiters' cell membrane was found around the base of the hair cell and in the region just beneath it (Fig. 5). For cells from apical regions, these convoluted membranes extended along a greater proportion of the hair cell length. These

projections from Deiters' cells frequently had gold particles associated with them (Fig. 4).

To determine how the distribution of immunogold labeling related to the different regions of Deiters' cells, a preliminary analysis was performed along the entire length of each Deiters' cell/hair cell pair, in which the total number of gold particles was counted in each of the bins described above. The number of particles was found to increase in the bins located at or just below the region surrounding the hair cell base (Fig. 5). This confirmed a qualitative impression that labeling was at its greatest in the region of the Deiters' cell cup. However, particles were not confined to this region. Thus, weaker GLAST immunoreactivity was also found at some distance from the synaptic region, toward the reticular lamina in the Deiters' cell, especially the longer ones from the apical region of the cochlea.

The increase in the number of particles around and below the hair cell base could have resulted from two different factors: first, it might have simply

FIG. 3. Transmission electron micrograph of guinea pig organ of Corti following postembedding immunogold labeling with anti-GLAST. Gold particles (arrowheads) are confined almost completely to the plasma membranes of Deiters' cells (DC). Note the OHC base (OHC) being contacted by distinct efferent (E) nerve terminals. Scale bar = 0.5 μ m. Detail of a Deiter's cell showing labeling (arrowheads) on both sides of the cell where these face hair cells of two different rows. Scale bar = 0.5 μ m.

reflected the increased surface area of Deiters' cell membrane in these regions, as described above, and second, it could have resulted from an increase in the density of GLAST in the membrane. To test the latter, the number of particles per 100- μ m length of Deiters' cell membrane was determined in each bin and plotted as before. This increased in the region around the hair cell base (Fig. 5), although the difference in this value between the different regions of the Deiters' cell was not as pronounced as the difference in number of particles (Fig. 5). This suggests that it is the increase in the amount of membrane present rather than an increase in the density of the GLAST in the membrane that is the more important factor in determining the amount of labeling present.

To investigate these patterns of labeling more thoroughly and provide sufficient data for statistical treatment, a more extensive analysis was performed, concentrating on the region of the Deiters' cell encompassing the synaptic region of the hair cell. Nine bins of fixed size were drawn along the long axis of the hair cell, centered so that bin 5 always contained the hair cell base. This enabled a larger number of samples of Deiters' cell/hair cell pairs to be collected

(a total of 50 apical and 67 basal pairs) than in the preliminary analysis, as it was no longer necessary to confine the analysis to sections containing the entire length of the hair cells, which were obtained less frequently. This revealed some variability between animals, but a number of consistent features were apparent. Cells from the apex of the cochlea showed most clearly a concentration of labeling associated with the base of the hair cell. Thus, in all animals, the number of particles per bin was greatest in bins containing, or just below, the base of the hair cell, usually in bin 6 (Fig. 6a). In cells from the basal region, an increase in labeling was also apparent nearer to the synaptic pole of the hair cell, although it was spread out over a greater number of bins. As noted above these bins also contained the most membrane.

The extent to which these increases in the number of particles were due to increased membrane length or to increased density of GLAST in the membrane was also investigated further. The correlation between mean number of particles per bin and mean length of membrane per bin (Fig. 6b) was tested. In three of the four animals (3445, 3446, 3428), these were significantly correlated (Table 1). This was true for both apical and basal regions of the cochlea. Interestingly, while the relationship between membrane length and number of particles in 3445 and 3446 appeared similar in both apical and basal regions, in 3428 there were more particles for the same amount of membrane in the basal region than in the apical region. Sections from the fourth animal were comparatively weakly labeled and showed no significant correlation.

The correlation between mean number of particles per bin and mean number of particles per 100 μ m per bin (Fig. 6c) was also tested. This correlation was usually weaker than the correlation between membrane length and total number of particles; in fact, it was significant in the apical region in only one animal and in the basal region in three of the four used for immunocytochemical labeling (Table 1).

In order to assess the overall significance of the trends seen in some of the individual animals, the data from all four animals were pooled and plotted together (Fig. 7). In cells from the apical region of the cochlea, the mean Deiters' cell membrane length was clearly greatest in bins 5–7, and in cells from the basal region, it was greatest in bins 4–9 (Fig. 7a). In order to pool the data on labeling across animals, the number of particles in each bin was adjusted by dividing it by the total number of particles counted in each animal, thus accounting for overall labeling variations between animals and between the different labeling runs. The mean adjusted number of particles per bin across all animals was again found to be greatest in bin 6 in apical cells, while in basal cells it

TABLE 1			
Correlation coecients			
Correlation category	Apical	Basal	Combined
Individual animal data			
Number of particles/bin vs. membrane length (3445)	0.82	0.83	0.82
Number of particles/bin vs. membrane length (3446)	0.96	0.82	0.92
Number of particles/bin vs. membrane length (3428)	0.95	0.94	0.68
Number of particles/bin vs. membrane length (3498)	−0.01	−0.15	−0.11
Number of particles vs. particles per 100 μm (3445)	0.3	0.94	0.66
Number of particles vs. particles per 100 μm (3446)	−0.07	0.86	0.5
Number of particles vs. particles per 100 μm (3428)	0.94	0.2	0.53
Number of particles vs. particles per 100 μm (3498)	0.17	0.77	0.59
Pooled data from all animals			
Number of particles/bin vs. membrane length	0.83	0.88	0.86
Number of particles vs. particles per 100 μm	0.4	0.53	0.52

^aSummary of the different correlation coefficients (*r*) obtained by comparing (1) the number of particles per bin and membrane length and (2) the number of particles per bin with the number of particles per unit length of membrane, for the individual experimental animals and for pooled adjusted data. These are derived from the scatterplots shown in Figs. 6 and 7. Significant *r* values (*p* > 0.05) are **boldface**. Note that in three of the four animals and in the pooled data there is a strong significant correlation between length of membrane and number of particles per bin. In contrast, the correlation between the total number of particles and the number of particles per unit length of membrane per bin is weaker and not always significant.

FIG. 4. Transmission electron micrograph of a Deiters' cell (DC) showing extensive processes (P) in the synaptic region, some of which interdigitate with and wrap around the nerve fibers (N) approaching the base of the outer hair cell (OHC). These processes also come close to the afferent (A) and efferent (E) terminals. Scale bar = 0.5 μm. Inset: These Deiters' cell processes (P) are also labeled (arrowheads). Scale bar = 0.5 μm.

was more widely spread through bins 5–9 (Fig. 7b). Using these adjusted values, the mean labeling density per unit length of membrane was also plotted as a function of bin number. In contrast to the adjusted number of particles per bin, this showed little evidence of an increase specifically in the bins around the base of the cell (Fig. 7c). The correlation between the measurements of labeling and membrane length

was examined as before using the pooled, adjusted values (Fig. 7d,e). This showed that there was a significant correlation between mean membrane length and mean adjusted number of particles per bin in both the apical and basal regions when considered separately and when the data from these regions were combined (Table 1). However, as in some of the individual animal data, there was a weaker correlation

3446

FIG. 5. Graphs based on the preliminary analysis of complete cells showing the actual number of gold particles per bin, density of gold particles (number per 100 μm) along Deiters' cell membrane, and membrane length in two Deiters' cell/hair cell pairs from apical and basal locations. Values were obtained from bins of equal width located along the long axis of the associated hair cell, starting from a line drawn parallel to the hair cell apex. The bin containing the hair cell base is indicated with an arrow. Because hair cells and Deiters' cells are greater in length at the apex than at the base and the bin size is fixed, the

between the mean number of particles per bin and the mean adjusted labeling per unit membrane. This was just significant for the combined data from both regions but not significant when apical or basal regions were analyzed separately (Table 1).

To determine whether there was any correspondence between the expected innervation density and the amount of GLAST in different cochlear regions, the mean adjusted number of particles (from all bins) was plotted for each hair cell row in apical and basal regions (Fig. 8a). In three out of the four animals used for immunocytochemistry (3445, 3446, 3498), labeling toward the apex of the cochlea was greater than labeling in basal regions. In order to test whether these differences were significant, the number of gold particles found in bins 5–7 for each Deiters' cell (i.e., concentrating on the more heavily labeled bins) was compared between apex and base for each animal using a Mann–Whitney U test. Labeling was significantly higher ($p < 0.01$) in the apical regions of two of these three animals (3445 and 3446). In animal 3428 there was no significant difference between the two regions (Mann–Whitney U test, $p = 0.37$). Interestingly, as noted above, this animal showed a shift in the relationship between membrane length and number of particles per bin in the basal region compared with the apical region.

Comparison of the different Deiters' cell rows showed considerable variation between the different

number of bins representing the basal Deiters' cell/hair cell (DC/OHC) pair is smaller than that representing the apical one. Both hair cells are from row 2. Note that the maximum number of particles coincides with the synaptic region adjacent to and just below the hair cell base. In addition, the number of particles per unit length also increases in this region, but, in apical Deiters' cells, this increase is less pronounced as the density of labeling remains high toward the top of the cell. Deiters' cell membrane lengths per bin also increase in the synaptic region and below, where the membrane becomes highly convoluted.

animals. However, when the data were pooled using adjusted values (as described above), the second row in the apical turn was clearly more heavily labeled than the others in this region (Fig. 8b). A Kruskal–Wallis analysis of the pooled data showed a significant difference ($p < 0.05$) between rows only in the apical region of the cochlea, which can be ascribed to the difference between the second row and the other two rows.

DISCUSSION

Functional implications of GLAST around the OHC base

This study has shown that GLAST is present in the membranes of Deiters' cells, the supporting cells associated with OHCs. It is concentrated around the base of the hair cell and is found in extensive membrane processes below it. These processes wrap around the nerve fibers and enlarge the surface area of the cell in a manner reminiscent of glial cell processes in the brain. This is similar to the situation in the IHC region (Furness and Lehre 1997) where labeling is found in supporting cells and their processes, with the greatest concentration around the hair cell base. In addition, as in the IHCs, GLAST does not appear to be present in the membrane of the OHCs themselves. It thus seems likely that GLAST in the cochlea plays a role in the uptake of glutamate or possibly aspartate into sup-

FIG. 6. Data obtained from the more extensive analysis of the region around the hair cell base, from all sections of all four of the animals used for immunolabeling in this study. Note that the bins here are smaller than those in Figure 5 and cover an equivalent area of each of the Deiters' cells analyzed in all sections in this study. **a** Histograms showing the mean number of particles per bin for apical (white bars) and basal (black bars) Deiters' cells. In apical regions, the greatest number of particles occurs closer to the base of the hair cell, especially in bin 6. In basal regions, there is an increase in gold particles toward the lower bins, but they are less concentrated than in the apical Deiters' cells. **b** Scatterplots and trend lines showing the relationship between the mean number of particles per bin and the

mean membrane length per bin in apical (white diamonds, solid trend line) and basal (black squares, dashed trend line) regions. Note that in three of the four animals (3445, 3446, 3428), these values are linearly related, and in two of the three, the apical and basal locations appear to have a similar quantitative relationship. However, in 3428, the line for the basal region is displaced upward compared with the apical region because more particles are present relative to the amount of membrane. **c** Scatterplots comparing mean number of particles per bin and number of particles per 100 μm of membrane. There is some evidence of a relationship between these two parameters, particularly in basal regions of 3445 and 3446 and in apical regions of 3428 (see Table 1 for correlation coefficients).

porting cells, a role similar to that which it plays in the glial cells in the brain (Chaudhry et al. 1995; Lehre et al. 1995). This uptake may protect cochlear neurons from glutamate-induced damage (see below) and might also be important in the shaping of the synaptic responses of the auditory nerve fibers. It is unlikely that the presence of this transporter is related to any of the other known cochlear neurotransmitters since the glutamate transporters are distinct from those used to take up other neuroactive substances or their breakdown products (Kanner 1994).

In the central nervous system, an increase in labeling density compared with other regions of the glial cell membrane suggests that GLAST is apparently concentrated in the perisynaptic area of glutamatergic synapses (Chaudhry et al. 1995). Interestingly, this increase in density does not appear to be the only factor that accounts for the increase in labeling found around the synaptic region of the hair cells. In addition, there is an increase in the area of Deiters' cell membrane in this region. The relative

contribution of these two factors is variable but overall it is the amount of membrane which has the greater influence on the amount of GLAST present.

In two of the four animals used in this study, there was significantly more labeling in apical than in basal cochlear locations, and the same trend was present, but not significant, in a third animal. This may reflect the fact that there are greater numbers of afferent synapses on OHCs in apical locations (Hashimoto and Kimura 1988; Liberman et al. 1990), suggesting that more GLAST is required there because more glutamate is released. However, it may be that only a proportion of the afferent synapses is active. For example, if the presence of synaptic bars is an indicator of activity, then it might be more appropriate to relate the density of GLAST labeling to the number of bars rather than to the number of afferent terminals. In fact, in the present study, more synaptic bars appeared to be associated with apical terminals than with basal terminals, which is consistent with the differences in GLAST in

FIG. 7. Histograms and scatterplots based on the data shown in Figure 6 but pooled across all four of the animals used for immunolabeling. In all graphs, data from the cochlear apex are represented in white bars or diamonds and data from the cochlear base are shown in black. To pool the data relating to numbers of gold particles, values have been adjusted by dividing the number of particles in each bin by the total number of particles in all bins for that animal. **a** Histogram showing how the length of Deiters' cell membrane varies with position (bin number) along the hair cell/Deiters' cell pair. Note that in apical regions, Deiters' cell membranes are most extensive in bins 5–7, coinciding with the synaptic pole of the hair cell. **b** Histogram showing how the amount of labeling (mean adjusted number of particles per bin) varies along the length of the hair cell/Deiters' cell pair. Note that in apical cochlear regions, the area of Deiters' cell just below the hair cell base (bin 6) contains the highest labeling, while in basal regions the heaviest labeling is more widespread around the synaptic pole (bins

5–8). **c** Histogram showing how the density of labeling (mean adjusted number of particles per 100 μm of membrane) varies in different regions of Deiters' cell. The lack of a clear peak indicates that there is no substantial increase in the density of GLAST in the region near to the synaptic pole of the hair cell. **d** Scatterplot showing the relationship between the mean adjusted number of particles per bin (as in **b**) and mean membrane length per bin (as in **a**) in both apical (solid trend line) and basal (dashed trend line) locations. The concentration of points along a single line appears to indicate that the number of particles is closely related to the amount of membrane in a region. **e** Scatterplot showing the relationship between mean adjusted number of particles per bin (as in **b**) and labeling per 100 μm of membrane per bin (as in **d**). This shows a comparatively weak correspondence between the amount of GLAST per unit area of membrane and total labeling. The correlation coefficients for **d** and **e** and their significance are shown in Table 1.

apical compared with basal locations. This finding differs, however, from that of Hashimoto and Kimura (1988) who reported little consistency between the number of bars and the number of afferent endings. Indeed, in their study, a similar number of synaptic bars was found in hair cells of the third row of basal regions as in all rows from apical regions, despite the up to fourfold difference in the number of presumed afferent endings. Unfortunately, directly determining whether there is a relationship between the number of bars and the density of GLAST labeling was not possible in the present study. This was because the fragility of the sections prepared by freeze substitution and low temperature embedding, coupled with the harshness of their treatment during immunolabeling, meant insufficient labeled serial sections could be collected.

Based on sensitivity to kainate (Pujol et al. 1985) and synaptic morphology, it has been suggested that the afferent innervation of the OHCs in the apical region may retain remnants of the immature innervation (Eybalin 1993). This may also be reflected in different functional properties of the afferents compared with the basal cochlear locations that could, in turn, alter the requirement for GLAST. Thus, it may be that the apical concentrations of GLAST change with maturity, reflecting alterations in synaptic function at different stages of auditory development.

A further finding was that the greatest labeling tended to occur in the second row of Deiters' cells. This may simply reflect the position of these cells in the center between the outermost and innermost rows. Certainly there was labeling where the mem-

FIG. 8a. Histograms based on the data obtained from the region around the hair cell base showing the mean adjusted number of particles per bin for each row in both apical (white bars) and basal (black bars) cochlear regions of each animal. Note that in three animals (3445, 3446, 3428) labeling is greater overall in apical regions while in 3498 it is greater in basal regions. **b** Histogram of the pooled data showing that there is a greater amount of labeling associated with row 2 overall in the apical region of the cochlea.

branes of Deiters' cells lay next to the bases of OHCs from adjacent rows.

Although the concentration of GLAST around the OHC base implies that their afferent synapses are functional, it has proved difficult to record physiological responses to sound from the type II cells of the spiral ganglion (Roberston et al. 1999). Nevertheless, ultrastructural studies in cats and primates show evidence of synaptic structures similar to those seen in IHCs (Spoendlin 1969, 1972; Liberman et al. 1990; Nadol 1990; Sato et al. 1999). The ultrastructural observations made here, including the presence of vesicles and coated pits in the adjacent membrane, indicate that this is true also for guinea pigs. In the present material, however, some differences were observed in synaptic structure between guinea pigs and the other species. In cats, the afferent terminals themselves lack vesicles and some, but not all, have presynaptic dense bodies with associated vesicles in the hair cell. In the current study of guinea pig hair cells, clearly identifiable afferent synapses had one or more dense presynaptic bars surrounded by vesicles.

In primates, there are reciprocal synapses that have the characteristics of both afferent and efferent synapses (Nadol 1990). In the present study of the guinea

pig, clusters of vesicles were also found within some nerve terminals that were associated with presynaptic bars in the hair cell, which might be indicative of reciprocal synapses. In apical hair cells, subsynaptic cisternae were not observed either adjacent to the presumed efferents or to these potentially reciprocal synapses, although it is possible that they were missed because serial sections were not taken. The former is consistent with the findings of Hashimoto and Kimura (1988) who noted that only half of the second-row OHC efferents had subsynaptic cisternae. It is possible that the variable presence of subsynaptic cisternae results from changes that occur during fixation or as a consequence of physiological activity. Certainly dynamic changes have been suggested to account for variations in the appearance of the laminated cisternae in the lateral cortex of these cells (Forge et al. 1993), and the precise structure of the latter is altered if OHCs are forced to contract prior to fixation (Evans 1990).

More importantly, however, the terminals with presynaptic bars and vesicles also showed evidence of an endocytotic process occurring in the form of coated pits and coated vesicles in the synaptic region. These features suggest that these could be active afferent synapses. Together with the particular association of GLAST with the OHC base, this implies that these cells release a neurotransmitter, presumably glutamate, and require an uptake process.

It is not clear, however, what postsynaptic receptor for glutamate is present on the afferent terminals. Although punctate immunofluorescence labeling was found for AMPA-type receptors around the OHC base (Kuriyama et al. 1994), a lack of immunogold labeling was found in another study (Matsubara et al. 1996). This suggests that glutamate receptors of this type, if they are present at all, are at low density. It is possible, however, that other glutamate receptors are present. Immunolabeling for one subunit of an N-methyl-D-aspartate (NMDA) receptor, NR2A, is found transiently in the outer spiral fibers that innervate the OHC. This subunit is expressed in all cochlear turns in the rat up to postnatal day 10 but declines and is absent from all but the apicalmost turn by postnatal day 18 (Knipper et al. 1997). Evidence from *in situ* hybridization and immunocytochemical studies suggests that as well as the AMPA receptor GluR2-4, all spiral ganglion cell bodies express NMDA (NR1, NR2a-d) and kainate (GluR5 and 6, KA1 and 2) receptor types (Niedzielski and Wenthold 1995). A fourth glutamate receptor type, $\delta 1$, has been detected in all spiral ganglion neurons and the IHCs themselves (Safieddine and Wenthold 1997). It has yet to be shown whether any of these receptors are localized to the synaptic region of the OHCs, although it is conceivable that they are since they are present in the neurons. If not,

another possibility is that an unusual receptor variant is present, as is the case for the acetylcholine receptor of the efferent synapse on OHCs (Glowatzki et al. 1995). Thus, although GLAST is concentrated around the OHC bases and the afferent synapses have ultrastructural characteristics suggesting that they are active, other components of functional synapses have yet to be demonstrated conclusively.

Functional implications of GLAST in other cochlear locations

The current study also showed that GLAST is present in Deiters' cells away from the synaptic region, albeit in smaller quantities. One possible reason for this is that it may be associated with the chemical synapses which have been described as occurring on Deiters' cells themselves, especially in apical cochlear locations; the neurotransmitter at these synapses is unknown (Burgess et al. 1997). None of these synapses were noted, however, in the material examined in the present study and so no direct correlation between them and GLAST could be made. In addition, however, Hensen's cells, which are similarly innervated, did not appear to label strongly for GLAST.

The presence of GLAST away from the hair cell bases and the fact that it also occurs on membranes at a distance of at least one cell removed from the IHC synapse (Furness and Lehre 1997) may suggest a need for glutamate uptake beyond the immediate vicinity of the synapses. One reason for this could be the fact that the organ of Corti undergoes mechanical displacement during sound stimulation and the OHCs actively contribute to this motion (Dallos 1997). This may give rise to the dispersal of glutamate throughout the perilymph, the fluid that bathes the hair cell basolateral and synaptic regions.

Role of GLAST in prevention of excitotoxicity

It has been argued that the presence of GLAST may help to protect neuronal elements in the cochlea from glutamate neurotoxicity (Furness and Lehre 1997). Under ischemic conditions, where the transporters fail to function properly (Danbolt 2000), baseline glutamate levels in gerbil perilymph rise over 100-fold from approximately 0.35 to 40 μM (Hakuba et al. 1997). This indicates that there is a substantial source of the amino acid in the cochlea that is normally kept under tight control. Moreover, in mice lacking GLAST, baseline perilymphatic glutamate levels are elevated compared with controls, and noise overstimulation results in a considerable and sustained increase in glutamate concentration that does not occur in normal mice expressing GLAST (Hakuba et al. 2000). In fact, noise overstimulation (Robertson 1983), aminoglycoside

antibiotics (Hackney et al. 1992), and hypoxia (Pujol et al. 1991) all cause afferent nerve terminals at the IHC base to swell, producing damage which resembles excitotoxic changes in the brain. Noise overstimulation also produces terminal swelling to a greater extent in mice lacking GLAST than in normal mice, and this damage is less reversible (Hakuba et al. 2000). In this context, it has been demonstrated *in vitro* that neurons are very sensitive to glutamate in the absence of glial uptake. In cultures of cerebral cortical tissue from embryonic rats, 50% of neurons die within 24 h when exposed to 4 μM of glutamate for 30 min in the absence of glial glutamate uptake. The same neuronal mortality requires 205 μM of glutamate in the presence of astrocytes in the culture (Rosenberg et al. 1992). Given that glutamate levels in perilymph may reach 40 μM following ischemia as noted above (Hakuba et al. 1997), a requirement for glutamate uptake by the supporting cells seems eminently necessary to protect potentially glutamate-sensitive cochlear nerve cells from damage.

Despite this, there is little reported evidence of excitotoxic damage to the OHC afferent synapses, particularly in adult animals, compared with those of the IHC following ototoxic or excessive noise trauma or ischemia. Possible explanations for this lower sensitivity are that OHCs do not release much glutamate and/or that these afferent synapses are normally inactive or require much higher levels of stimulation to become active. It has also been shown that application of AMPA causes swelling of the afferent nerve terminals ending on IHCs but does not affect the OHC afferents. However, NMDA antagonists appear to reduce aminoglycoside-induced hair cell loss, perhaps by interfering with an excitotoxic process (Basile et al. 1996), but whether this involves the OHC afferents is not known and requires further investigation.

In conclusion, the distribution of GLAST around the OHCs within the cochlea is consistent with two roles: (1) its involvement in a localized synaptic glutamate uptake mechanism associated with the OHC afferents, perhaps contributing to shaping the as yet uncharacterized activity at these synapses, and (2) participation in a more widespread cochlear uptake requirement arising from mechanical dispersal of glutamate throughout the perilymphatic compartment that protects the neuronal elements from excitotoxicity.

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