Brief Communications

The Glutamate–Aspartate Transporter GLAST Mediates Glutamate Uptake at Inner Hair Cell Afferent Synapses in the Mammalian Cochlea

Elisabeth Glowatzki,[1] Ning Cheng,[2] Hakim Hiel,[1] Eunyoung Yi,[1] Kohichi Tanaka,[4] Graham C. R. Ellis-Davies,[5] Jeffrey D. Rothstein,[3] and Dwight E. Bergles[1,2]

Departments of [1]Otolaryngology–Head and Neck Surgery, [2]Neuroscience, and [3]Neurology, Johns Hopkins University, Baltimore, Maryland 21205, [4]Laboratory of Molecular Neuroscience, School of Biomedical Science and Medical Research Institute, Tokyo Medical and Dental University, Tokyo 113-8510, Japan, and [5]Department of Pharmacology and Physiology, Drexel University College of Medicine, Philadelphia, Pennsylvania 19102

Ribbon synapses formed between inner hair cells (IHCs) and afferent dendrites in the mammalian cochlea can sustain high rates of release, placing strong demands on glutamate clearance mechanisms. To investigate the role of transporters in glutamate removal at these synapses, we made whole-cell recordings from IHCs, afferent dendrites, and glial cells adjacent to IHCs [inner phalangeal cells (IPCs)] in whole-mount preparations of rat organ of Corti. Focal application of the transporter substrate D-aspartate elicited inward currents in IPCs, which were larger in the presence of anions that permeate the transporter-associated anion channel and blocked by the transporter antagonist D,L-threo-β-benzyloxyaspartate. These currents were produced by glutamate–aspartate transporters (GLAST) (excitatory amino acid transporter 1) because they were weakly inhibited by dihydrokainate, an antagonist of glutamate transporter-1 (excitatory amino acid transporter 2) and were absent from IPCs in *GLAST*$^{-/-}$ cochleas. Furthermore, D-aspartate-induced currents in outside-out patches from IPCs exhibited larger steady-state currents than responses elicited by L-glutamate, a prominent feature of GLAST, and examination of cochlea from GLAST–Discosoma red (DsRed) promoter reporter mice revealed that DsRed expression was restricted to IPCs and other supporting cells surrounding IHCs. Saturation of transporters by photolysis of caged D-aspartate failed to elicit transporter currents in IHCs, as did local application of D-aspartate to afferent terminals, indicating that neither presynaptic nor postsynaptic membranes are major sites for glutamate removal. These data indicate that GLAST in supporting cells is responsible for transmitter uptake at IHC afferent synapses.

Key words: **glutamate transporter; GLAST; EAAT1; AMPA receptor; organ of Corti; BAC transgenic; hair cell; ribbon synapse**

Introduction

Auditory signals are transmitted to the brain by converting graded depolarization of inner hair cells (IHCs) to trains of action potentials in auditory nerve fibers. This initial transduction occurs at ribbon synapses between IHCs and afferent dendrites of auditory nerves, in which release of glutamate triggers activation of AMPA receptors (Fuchs et al., 2003). At excitatory synapses in the CNS, glutamate is cleared by high-affinity transporters present in the membranes of neurons and glia (Danbolt, 2001). By limiting the accumulation of extracellular glutamate, transporters prevent tonic activation of receptors and excitotoxicity. Transporters also act on a rapid timescale to shape the activation of synaptic and extrasynaptic receptors because they compete with receptors for binding of glutamate as it exits the synaptic cleft (Bergles et al., 1999). Both immunocytochemical and reverse transcription-PCR analyses indicate that glutamate transporters are expressed in the mammalian organ of Corti (Li et al., 1994; Furness and Lehre, 1997; Rebillard et al., 2003); however, the mechanisms of glutamate clearance in the cochlea are not well understood because there have been no functional studies of glutamate transporters in this region.

In the CNS, glutamate transporters reach their highest density in astrocyte membranes (Lehre and Danbolt, 1998). By extending processes to excitatory synapses, astrocytes create a barrier to diffusion and bring transporters near sites of release. Although most neurons express excitatory amino acid carrier 1 (EAAC1), recent studies indicate that glutamate transporter-1 (GLT-1) and glutamate aspartate transporter (GLAST), two glutamate transporters expressed by astrocytes, are responsible for the majority of glutamate uptake near excitatory synapses (Palmer et al., 2003; Brasnjo and Otis, 2004; Huang et al., 2004a,b). In the mammalian cochlea, astrocyte-like cells, including inner phalangeal cells (IPCs) and border cells located near IHC afferent synapses, exhibit immunoreactivity to GLAST (Furness and Lehre, 1997;

Received April 10, 2006; revised June 14, 2006; accepted June 15, 2006.

This work was supported by National Institute of Neurological Disorders and Stroke Grant NS44261 (D.E.B.) and National Institute on Deafness and Other Communication Disorders Grants DC06476 (E.G.), DC00276 (Paul A. Fuchs), and P30D005211. We thank Paul Fuchs for initial support and Naoko Nishiyama and Melissa Regan for assistance with mouse breeding and genotyping.

Correspondence should be addressed to Dwight E. Bergles, Department of Neuroscience, Johns Hopkins University Medical School, 725 North Wolfe Street, Wood Basic Science Building 813, Baltimore, MD 21205. E-mail: dbergles@jhmi.edu.

DOI:10.1523/JNEUROSCI.1545-06.2006

 0270-6474/06/267659-06$15.00/0

Hakuba et al., 2000; Furness and Lawton, 2003), and GLAST-deficient mice exhibited an impaired recovery from acoustic overstimulation (Hakuba et al., 2000), suggesting that this transporter plays an important role in preventing glutamate accumulation in the cochlea. Nevertheless, the high quantal content of EPSCs at IHC afferent synapses (Glowatzki and Fuchs, 2002) and the high rate of release sustained by IHCs may pose unique challenges for transporter-mediated uptake.

Here we show that functional glutamate transporters are expressed by IPCs in the mammalian cochlea. Glutamate transporter currents recorded from these cells exhibited a pharmacological profile consistent with GLAST and were absent in GLAST-deficient mice. Furthermore, analysis of GLAST promoter reporter transgenic mice revealed that GLAST promoter activity was elevated to supporting cells that surround IHCs in the organ of Corti. Transporter currents were not observed in IHCs or afferent dendrites, suggesting that transporter-mediated uptake at IHC afferent synapses is mediated by GLAST transporters in adjacent supporting cells.

Materials and Methods

Whole-mount preparation of cochleas. Apical turns of 5- to 15-d-old rat and mouse cochleas were isolated as described previously (Glowatzki and Fuchs, 2002) and placed in a superfusion chamber mounted on an upright microscope (Axioskop FS2; Zeiss, Oberkochen, Germany). The preparation was continuously superfused with artificial CSF (ACSF) containing the following (in mM): 119 NaCl, 2.5 KCl, 2.5 $CaCl_2$, 1.3 $MgCl_2$, 1 NaH_2PO_4, 26.2 $NaHCO_3$, and 11 glucose, pH 7.3 (solutions were saturated with 95% O_2/5% CO_2). For afferent recordings, the perfusion solution contained the following (in mM): 144 NaCl, 5.8 KCl, 1.3 $CaCl_2$, 0.9 $MgCl_2$, 0.7 NaH_2PO_4, 10 HEPES, and 5.6 glucose, pH 7.4. The temperature of the superfusing solution was increased above ambient by passing it through an in-line heater (Warner Instruments, Hamden, CT). Unless otherwise noted, experiments were done at 22–24°C.

Electrophysiological recordings. Cells were visualized through a 40× water immersion objective using infrared or green light, differential interference contrast (DIC) optics, and CCD (XC-73; Sony, Tokyo, Japan) or Newvicon (Dage-MTI, Michigan City, MI) cameras. Whole-cell recordings were made under visual control with an internal solution consisting of (in mM): 130 KA [in which A is SCN^-, NO_3^-, or $CH_3O_3S^-$ (MeS)], 10 EGTA, 20 HEPES, and 1 $MgCl_2$, pH 7.3. The puffer pipette contained 300–500 μM D-aspartate dissolved in ACSF and was connected to a Picospritzer (General Valve, Fairfield, NJ). Rapid solution exchange around outside-out patches was achieved with a flow pipe attached to a piezoelectric bimorph, as described previously (Bergles and Jahr, 1997). For afferent recordings, D-aspartate was applied via a 100-μm-diameter flow pipe positioned 100 μm from the recording site. UV photolysis was performed as described previously (Huang et al., 2005). Currents were recorded with an Axopatch 200B amplifier (Molecular Devices, Palo Alto, CA), digitized at 10–50 kHz, filtered at 2–10 kHz, and analyzed off-line using pClamp and Origin and Minianalysis (Synaptosoft, Decatur, GA) software. Data are expressed as mean ± SEM. Statistical significance was determined using Student's *t* test.

Generation of GLAST–Discosoma red mice. A mouse bacterial artificial chromosome (BAC) (RPCI-24-287G11) was obtained from BACPAC Resources (http://bacpac.chori.org) containing the entire GLAST gene plus 18 kb upstream of the first exon and 60 kb downstream of the last exon. This BAC was modified using a double homologous combination approach (Yang et al., 1997) to insert a cDNA encoding the fluorescent protein Discosoma red (DsRed) (DsRed Express; Clontech, Cambridge, UK) into exon 2 of the GLAST gene. This construct was injected into the pronuclei of fertilized zygotes to create transgenic mice.

Imaging. Cochleas from GLAST–DsRed BAC mice were fixed by perfusion with 4% paraformaldehyde in PBS and mounted on slides. DsRed fluorescence was imaged using a 4× air (Acroplan; Zeiss) or 60× water immersion objective (LUMPlanFL; Zeiss) on an LSM 510 NLO microscope (Zeiss) using a titanium:sapphire laser (Chameleon; Coherent, Santa Clara, CA) tuned to 930 nm for two-photon excitation of DsRed.

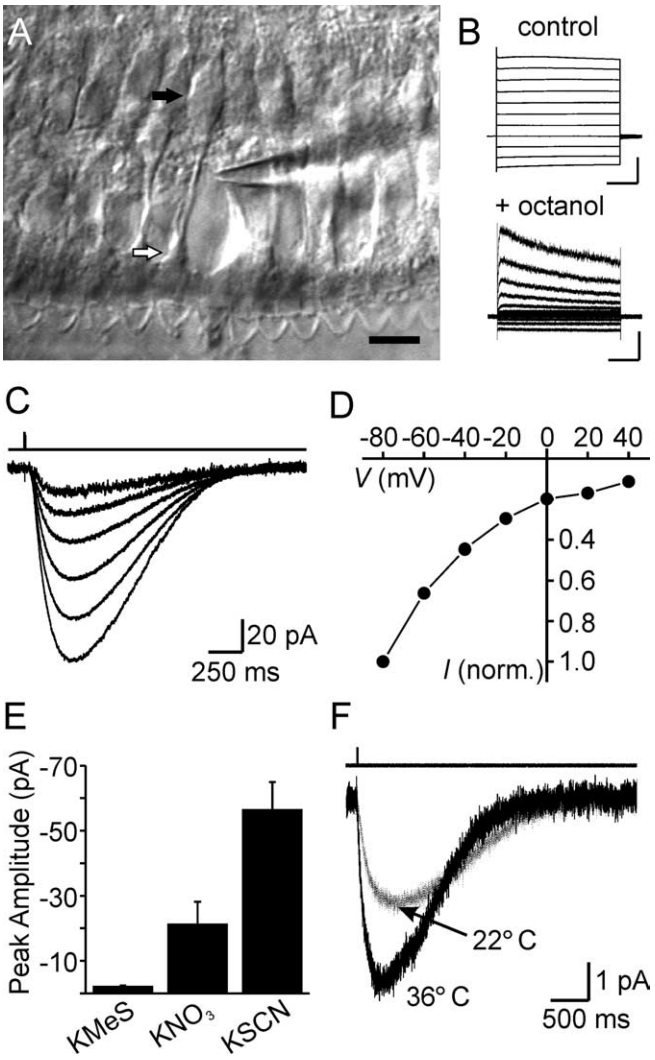

Figure 1. Glutamate transporter currents in cochlear IPCs. ***A***, Infrared-DIC image from a 5-d-old apical cochlear turn. Black arrow, IPC soma; white arrow, triangular-shaped end of IPC phalynx contacting the cuticular plate. Scale bar, 10 μm. ***B***, Responses of IPC to voltage steps (−60 to 140 mV), in control and in 1 mM octanol. $V_m = -90$ mV. Calibration: top, 4 nA, 50 ms; bottom, 100 pA, 50 ms. ***C***, Inward currents elicited in an IPC in response to 300 μM D-aspartate. The top trace shows the duration of application. ***D***, Current-to-voltage relationship of D-aspartate-evoked currents in IPCs ($n = 10$). Each current was normalized to the peak amplitude recorded at −80 mV. ***B–D*** were recorded with a KNO_3-based pipette solution. ***E***, Average amplitude of D-aspartate-evoked currents recorded at V_m of −80 mV with different intracellular anions. ***F***, D-Aspartate-evoked transporter currents recorded at room temperature (22°C) and at near-physiological temperature (36°C) ($V_m = -80$ mV, KMES-based pipette solution).

Results

Glutamate transporter expression by inner phalangeal cells in the cochlea

To determine the sites for glutamate uptake at IHC afferent synapses, we made whole-cell voltage-clamp recordings from IPCs, IHCs, and afferent dendrites, the three compartments closest to sites of glutamate release. IPCs were visible beneath IHCs as flask-shaped cells that extended processes along the lateral membrane of IHCs (Fig. 1*A*) and exhibited high resting potentials (−91 ± 4 mV) and low membrane resistances (12 ± 6 MΩ; $n = 15$) (Fig. 1*B*). To improve voltage control and reduce current loss, gap junctions were blocked with octanol (1 mM). This uncoupling

agent increased the membrane resistance of IPCs to >700 MΩ, and voltage-dependent outward currents became visible after depolarization (Fig. 1*B*). To test for functional glutamate transporters, we examined their response to focal application of D-aspartate, a substrate of glutamate transporters (Arriza et al., 1994). D-Aspartate was used for these experiments because it exhibits properties similar to glutamate at astroglial transporters (Arriza et al., 1994) but is not metabolized, and it is not an agonist of AMPA or metabotropic glutamate receptors (Huang et al., 2005). Brief application of D-aspartate (300 μM) to IPCs induced a transient inward current (−21.3 ± 6.8 pA; $n = 15$; KNO_3-based pipette solution) (Fig. 1*C*) that became smaller when the cell was held at more positive potentials but remained inward at 40 mV (Fig. 1*D*), similar to glutamate transporter currents recorded from astrocytes (Bergles and Jahr, 1997). All known glutamate transporters contain an anion channel that is opened by the binding and translocation of substrates and has a permeability that follows the chaotropic series (Wadiche et al., 1995). Consistent with this behavior, D-aspartate induced responses recorded with thiocyanate (SCN^-) in the pipette, which is highly permeant through this anion channel, were larger (−56.5 ± 8.5 pA; $n = 19$; $p < 0.01$) than responses recorded with nitrate (NO_3^-). Conversely, responses recorded with methanesulfonate, which does not permeate the transporter anion channel (Bergles et al., 2002), were much smaller (−2.2 ± 0.2 pA; $n = 4$; $p < 0.01$ for both NO_3^- and SCN^-) (Fig. 1*E*,*F*). Responses recorded under these conditions became larger and faster when the bath temperature was increased to 36°C (peak amplitude at 36°C; −4.8 ± 0.6 pA; $n = 4$; $p < 0.01$) (Fig. 1*F*), similar to the temperature dependence exhibited by glutamate transporter currents in astrocytes (Bergles and Jahr, 1998). D-Aspartate-induced currents were inhibited by 82 ± 8% ($n = 16$; KMES-based pipette solution) by 200 μM D,L-threo-β-benzyloxyaspartate (TBOA) (Fig. 2*A*), a selective antagonist of glutamate transporters (Shimamoto et al., 1998), but were not affected by the ionotropic glutamate receptor antagonists NBQX (2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoaline-7-sulfonamide disodium salt) (5 μM) or RS-CPP (*RS*-3-(2-carboxypiperazin-4-yl)propyl-1-phosphonic acid) (5 μM) ($n = 5$ of 5). Together, these results indicate that glutamate transporter activity can be recorded from IPCs in whole-mount preparations of the organ of Corti.

GLAST mediates glutamate uptake into IPCs

Astrocytes in the brain express GLT-1 (EAAT2) and GLAST (EAAT1) subtype glutamate transporters (Rothstein et al., 1994; Lehre et al., 1995). Notably, GLAST mRNA has been detected through PCR of cDNA libraries made from cochlear tissue (Li et al., 1994; Rebillard et al., 2003), and IPCs and other supporting cells that surround IHCs in the organ of Corti exhibit immunoreactivity to GLAST (Furness and Lehre, 1997; Hakuba et al., 2000). However, both approaches have yielded conflicting results regarding the expression of other transporter isoforms. To determine whether GLAST is responsible for IPC transporter currents, we examined their sensitivity to dihydrokainate (DHK), an antagonist that has a 100-fold higher affinity for GLT-1 (Arriza et al., 1994). D-Aspartate-induced transporter currents were inhibited only 15 ± 3% ($n = 10$; $p < 0.01$) by 300 μM DHK (Fig. 2*B*), in contrast to the ~ 83% inhibition observed for GLT-1 (Huang et al., 2004b).

Unlike GLT-1, transporter-associated anion currents mediated by GLAST (EAAT1) exhibit slower decay kinetics when transporting aspartate versus glutamate (Bergles et al., 1997; Wadiche and Kavanaugh, 1998). Application of a saturating dose

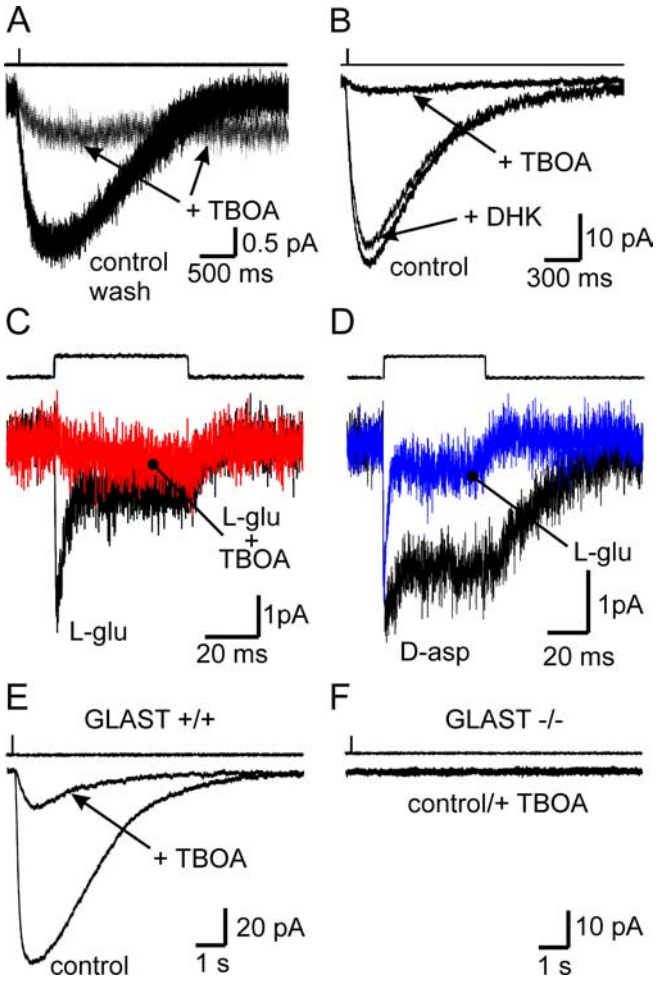

Figure 2. GLAST is the primary glutamate transporter in inner phalangeal cells. ***A***, Glutamate transporter currents recorded in an IPC in response to 500 μM D-aspartate, in the presence and absence of 200 μM TBOA (KMES-based pipette solution). $V_m = -80$ mV. ***B***, D-Aspartate-evoked transporter currents recorded from an IPC in control, DHK (300 μM), or TBOA (300 μM) ($V_m = -80$ mV, KNO_3-based pipette solution). ***C***, L-Glutamate (10 mM)-evoked transporter currents recorded from an outside-out patch from an IPC in control and with 300 μM TBOA. $V_m = -110$ mV. ***D***, Glutamate transporter currents recorded from an IPC outside-out patch in response to either 2 mM L-glutamate or 2 mM D-aspartate. $V_m = -110$ mV. In ***C*** and ***D***, the traces at the top show the duration of glutamate application. ***E***, ***F***, Glutamate transporter currents recorded from $GLAST^{+/+}$ and $GLAST^{-/-}$ mice IPCs in response to D-aspartate (500 μM), both with and without 300 μM TBOA. $V_m = -80$ mV. ***A–F***, KNO_3-based pipette solution.

of L-glutamate to outside-out patches from IPCs elicited inward currents (−1.8 ± 0.2 pA; $n = 7$) that rapidly decayed to a steady-state level in the continued presence of L-glutamate (Fig. 2*C*); these responses were inhibited 94 ± 0.5% ($n = 4$) by TBOA (300 μM), indicating that they were mediated by glutamate transporters. As expected for currents mediated by GLAST, D-aspartate-induced currents exhibited a slightly larger peak amplitude (−2.3 ± 0.2 pA; $p < 0.01$) and a much larger steady-state current (steady-state/peak, D-aspartate, 0.82 ± 0.04, $n = 4$; L-glutamate, 0.15 ± 0.02, $n = 4$; $p < 0.001$) (Fig. 2*D*). To confirm that IPC transporter currents were mediated exclusively by GLAST, we examined the response of IPCs to D-aspartate in cochleas from wild-type and $GLAST^{-/-}$ mice. Focal application of D-aspartate (500 μM) to IPCs in $GLAST^{+/+}$ cochleas induced a prominent inward current (−75.5 ± 7.7 pA, $n = 9$; KSCN-based pipette solution) that was inhibited 83 ± 2% by TBOA ($n = 4$) (Fig. 2*E*), whereas D-aspartate failed to elicit a response in IPCs from

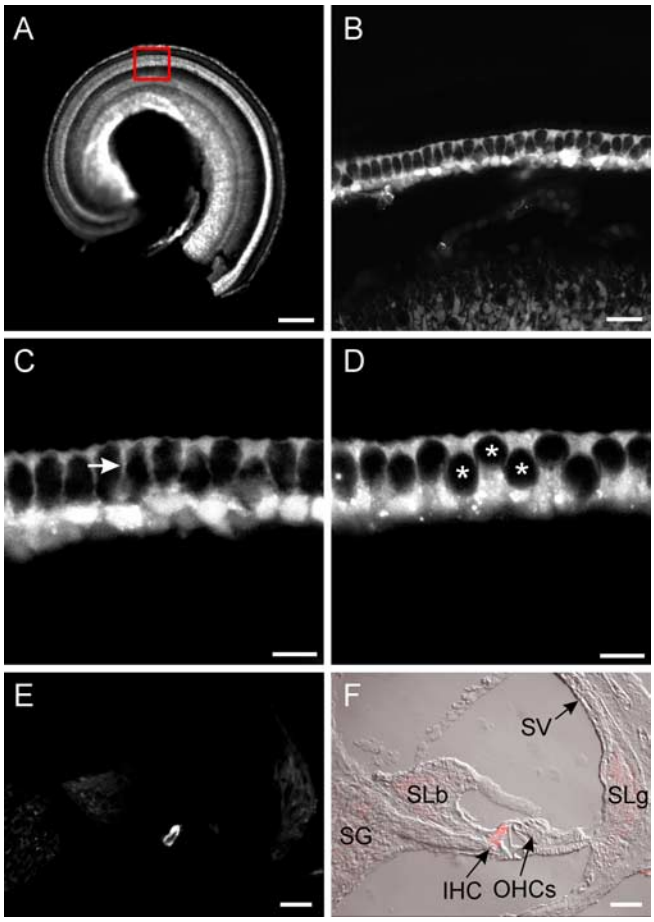

Figure 3. Visualization of GLAST promoter activity in GLAST–DsRed mice. ***A***, DsRed fluorescence in a whole-mount apical turn; a narrow band of labeling was observed in the IHC area (red box). Scale bar, 500 μm. ***B***, Region of the area outlined in ***A*** shown at higher magnification. Scale bar, 50 μm. ***C***, ***D***, Images taken at different focal planes of the IHC area. Arrow in ***C*** points to a labeled phalynx of an IPC. Asterisks in ***D*** mark unlabeled IHCs surrounded by DsRed$^+$ supporting cells. Scale bars, 25 μm. ***E***, DsRed fluorescence in a 14-μm-thick cochlear section. ***F***, Merged fluorescence and DIC image of the section shown in ***E***. In addition to intense labeling around the IHC, satellite cells in the spiral ganglion (SG) and cells in the spiral limbus (SLb) and spiral ligament (SLg) are labeled. SV, Stria vascularis. Scale bars, 50 μm. All images from apical turns of postnatal day 9 GLAST–DsRed BAC mouse cochleas.

GLAST$^{-/-}$ mice ($n = 6$ of 6 cells) (Fig. 2*F*). Together, these data indicate that GLAST is responsible for glutamate transporter currents in IPCs.

GLAST promoter activity is elevated in IPCs

Immunocytochemical studies suggest that GLAST is expressed by IPCs, border cells, and other small glial cells located near the basolateral membrane of IHCs (Furness and Lehre, 1997). To obtain a more complete picture of GLAST expression in the organ of Corti, we examined GLAST promoter reporter mice that were created by modifying a bacterial artificial chromosome containing the mouse GLAST gene. In the cochlea of transgenic GLAST–DsRed BAC mice, intense DsRed fluorescence was observed in a narrow band within the IHC region (Fig. 3*A*). At higher magnification, DsRed was found to be highly expressed by IPCs that effectively encapsulate IHCs (Fig. 3*B–E*). Much weaker DsRed fluorescence was observed in Deiters' cells beneath outer hair cells (OHCs), in accordance with the GLAST immunoreactivity exhibited by Deiters' cells (Furness et al., 2002). D-Aspartate elicited small inward currents in Deiters' cells (data not shown), supporting the hypothesis that outer hair cells secrete glutamate as a neurotransmitter (Ottersen et al., 1998). In addition, satellite cells in the spiral ganglion and cells within the spiral ligament and spiral limbus also expressed DsRed (Fig. 3*A*,*E*,*F*), consistent with the pattern of GLAST immunoreactivity in guinea pig and rat cochlea (Hakuba et al., 2000; Jin et al., 2003). The restricted pattern of DsRed expression in the cochlea of GLAST–DsRed BAC mice indicates that IPCs are specialized for removing glutamate released at IHC afferent synapses.

Glutamate transporter currents are not detected in IHCs or afferent dendrites

At some central synapses, transporters in presynaptic and postsynaptic membranes contribute to glutamate clearance (Otis et al., 1997; Diamond, 2001; Palmer et al., 2003). To assess whether glutamate is removed by transporters in IHCs, we measured the response of IHCs to focal application of D-aspartate. In contrast to the behavior of IPCs, D-aspartate failed to elicit a response in IHCs when recordings were performed with either NO_3^--based ($n = 4$ of 4 cells) (Fig. 4*A*) or SCN^--based ($n = 6$ of 6 cells) pipette solutions (Fig. 4*B*). As a more sensitive test of transporter activity, we measured the response of IHCs to UV-induced photolysis of 4-methoxy-7-nitroindolinyl (MNI)-D-aspartate, a caged analog of D-aspartate (Huang et al., 2005). With this approach, D-aspartate can be rapidly applied at a saturating concentration over the entire IHC. Although photorelease of D-aspartate induced a large inward current in IPCs (-178.6 ± 31.0 pA; $n = 5$) (Fig. 4*D*), it did not induce a detectible response in IHCs ($n = 5$ of 5 cells) (Fig. 4*C*). These data suggest that presynaptic uptake into IHCs is not a major mechanism for glutamate clearance.

In whole-mount preparations of the organ of Corti, it is possible to visualize and record from the afferent dendrites of the auditory nerve fibers at their point of contact with IHCs (Glowatzki and Fuchs, 2002). To determine whether functional transporters are present in these dendritic membranes, we measured the response of postsynaptic terminals to 500 μM D-aspartate. To minimize damage to the delicate terminals, D-aspartate was applied through a wide-bore pipette positioned $\sim$100 μm from the recording site. This method of D-aspartate application induced an inward current in IPCs (range, -20 to -75 pA; $n = 4$ of 4 cells) (Fig. 4*F*); however, D-aspartate did not induce an inward current in afferent terminals ($n = 3$ of 3 terminals) (Fig. 4*E*). The remote location of transporters at these synapses suggests that they may have limited effects on the time course of glutamate within the cleft. The mean time constant of decay of EPSCs recorded from afferent terminals increased slightly (range, 13–20%; $n = 3$) in 300 μM TBOA; for example, in one terminal, the time constant increased from 1.13 ± 0.04 ms ($n = 112$ EPSCs) in control to 1.36 ± 0.05 ms in TBOA ($n = 180$ EPSCs). However, this increase was not significantly greater than the progressive slowing of EPSCs observed in three control recordings. Although these results suggest that transporters do not shape cleft glutamate transients, it was necessary to remove the closely apposed phalangeal cell from the terminal to access the dendrite; thus, the microenvironment necessary for binding and uptake of glutamate may have been destroyed in these experiments. It is also possible that the low rate of spontaneous events (one to five per second) observed in this isolated preparation may artificially reduce the requirement for active uptake. Together, these data indicate that the primary mechanism for glutamate uptake near IHC afferent synapses is through GLAST-dependent transport into supporting cells adjacent to IHCs.

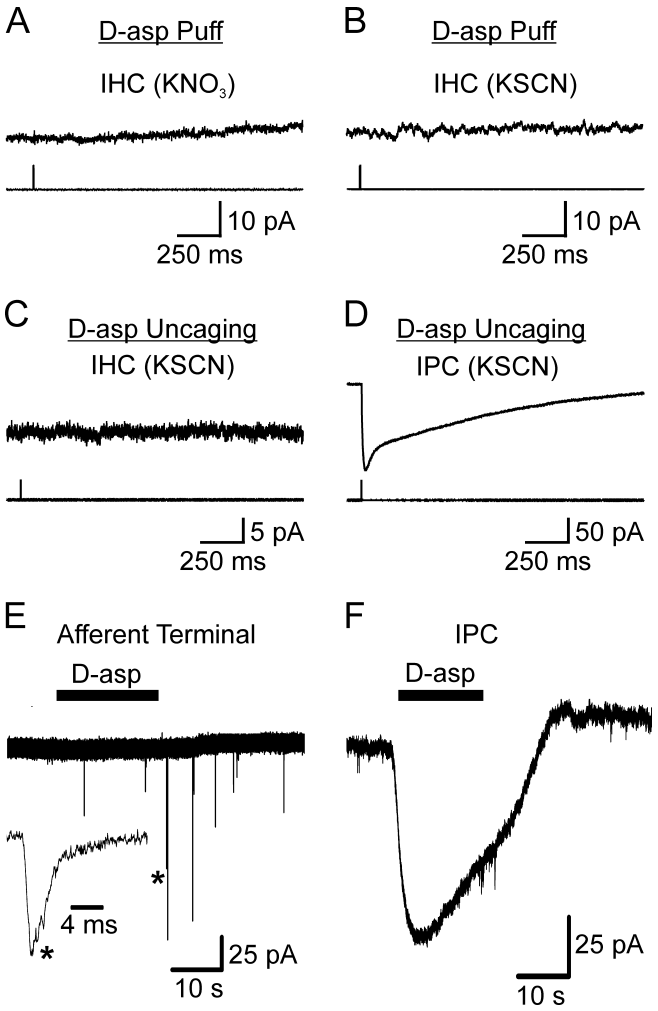

Figure 4. IHCs and their afferent fibers do not exhibit glutamate transporter currents. ***A***, ***B***, Responses of IHCs to 500 μM D-aspartate. ***A***, KNO_3-based pipette solution ($V_m = -88$ mV). ***B***, KSCN-based pipette solution ($V_m = -70$ mV). ***C***, ***D***, Comparison of IHC and IPC responses to UV photolysis of caged D-aspartate (MNI-D-aspartate, 500 μM). IHC, $V_m = -70$ mV; IPC, $V_m = -80$ mV. ***C***, ***D***, KSCN-based internal solution. ***E***, ***F***, Comparison of afferent fiber and IPC responses to 500 μM D-aspartate applied locally using a flow pipette. EPSCs appeared spontaneously in afferent fiber recordings; a single event (*) is shown on an expanded timescale. $V_m = -90$ mV. KSCN-based pipette solution.

Discussion

To enable accurate detection of sound, deflections of hair cell stereocilia must be translated into precise trains of action potentials in the afferent nerve. Glutamate release at IHC afferent synapses is multiquantal, with postsynaptic currents arising from the near simultaneous fusion of up to 20 vesicles (Glowatzki and Fuchs, 2002), yet afferent fibers can follow frequencies as high as 1 kHz (Trussell, 1999), suggesting that temporal overlap among individual synaptic events is minimized. Compared with central excitatory synapses, in which release is phasic and typically uniquantal, the persistent fusion of vesicles at IHC afferent synapses is expected to place greater demands on clearance mechanisms. However, our findings indicate that glutamate accumulation at these synapses is prevented by diffusion of glutamate away from receptors, dilution in the extracellular space, and uptake by transporters into surrounding glia. Because glutamate transporters bind glutamate rapidly and with high affinity but transport with only moderate efficiency (Wadiche and Kavanaugh, 1998; Bergles et al., 2002), this remote location of transporters may enable high-occupancy binding to receptors within the synaptic cleft and prevent transporters from slowing the decay of the glutamate transient through inefficient transport.

Previous studies showed that intracochlear perfusion of *trans*-pyrrolidine-2,4-dicarboxylate, a broadly selective glutamate transporter inhibitor, decreased the amplitude of the cochlear compound action potential and induced swelling of afferent terminals, whereas the GLT-1 selective antagonist DHK did not (Rebillard et al., 2003). These experiments are in accordance with our findings that GLAST rather than GLT-1 mediates transporter currents in IPCs. The conclusion that GLAST is the predominant, if not exclusive, transporter expressed by phalangeal cells is supported by not only pharmacological (i.e., DHK sensitivity) and kinetic (time course of currents in outside-out patches) data but by the fact that transporter currents were not present in *GLAST*$^{-/-}$ mice. Although the former results rule out GLT-1, the latter findings suggest that GLAST is the exclusive transporter expressed by these cells. Together, these results and the findings that GLAST-deficient mice experience pronounced swelling of afferent terminals and permanent hearing loss after noise exposure (Hakuba et al., 2000) support the hypothesis that glutamate uptake by IPCs at IHC afferent synapses is essential for normal hearing.

Ribbon synapses capable of sustaining high release rates are also formed between bipolar cells and ganglion cells in the retina. Unlike IHCs, bipolar cells express the glutamate transporter EAAT5 in the presynaptic terminal membrane (Palmer et al., 2003). This transporter exhibits unusually high anion permeability (Arriza et al., 1997), and glutamate release from bipolar terminals triggers a membrane hyperpolarization as a result of chloride influx through EAAT5. By simultaneously recording capacitance changes and transporter-associated charge movements, it was estimated that EAAT5 removes only a small fraction ($\ll$1%) of the glutamate released (Palmer et al., 2003). Thus, although presynaptic transporters are present at these ribbon synapses, they are used to limit membrane depolarization rather than clear glutamate; the majority of uptake at these synapses is presumably accomplished by Müller glial cells.

Whole-cell transporter currents recorded in response to photolysis of MNI-D-aspartate were small compared with transporter currents recorded from astrocytes under similar conditions (IPCs, -19 ± 6 pA, $n = 4$; astrocytes, -1357 ± 136 pA, $n = 7$; KMES internal, 500 μM MNI-D-aspartate), indicating that glutamate transporters are present at a much lower density in IPCs. However, whereas an astrocyte may contact as many as 140,000 synapses (Bushong et al., 2002), an IPC contacts approximately two dozen, suggesting that transporter expression is closely matched to the amount of glutamate released. Indeed, recent studies indicate that GLAST immunoreactivity is higher in the basal region of the cochlea, where synapses are more numerous, and on glial processes on the pillar-cell side of IHCs, where fibers exhibiting high spontaneous firing terminate (Furness and Lawton, 2003).

Intense sound induces swelling of afferent boutons, which will eventually degenerate if exposure to loud noise is prolonged (Robertson, 1983). Both sound-induced damage to boutons and destruction of hair cells by exposure to aminoglycoside antibiotics or ischemia are ameliorated by glutamate receptor antagonists (Basile et al., 1996; Hakuba et al., 2003), suggesting that glutamate-induced excitotoxicity may represent a common pathway in many aspects of cochlear damage. The ability to record transporter currents and monitor GLAST promoter activity directly from cochlear supporting cells provides new oppor-

tunities for understanding how transporter expression is regulated by sound and acoustic trauma.

References

Arriza JL, Fairman WA, Wadiche JI, Murdoch GH, Kavanaugh MP, Amara SG (1994) Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. J Neurosci 14:5559–5569.

Arriza JL, Eliasof S, Kavanaugh MP, Amara SG (1997) Excitatory amino acid transporter 5, a retinal glutamate transporter coupled to a chloride conductance. Proc Natl Acad Sci USA 94:4155–4160.

Basile AS, Huang JM, Xie C, Webster D, Berlin C, Skolnick P (1996) *N*-methyl-D-aspartate antagonists limit aminoglycoside antibiotic-induced hearing loss. Nat Med 2:1338–1343.

Bergles DE, Jahr CE (1997) Synaptic activation of glutamate transporters in hippocampal astrocytes. Neuron 19:1297–1308.

Bergles DE, Jahr CE (1998) Glial contribution to glutamate uptake at Schaffer collateral–commissural synapses in the hippocampus. J Neurosci 18:7709–7716.

Bergles DE, Dzubay JA, Jahr CE (1997) Glutamate transporter currents in bergmann glial cells follow the time course of extrasynaptic glutamate. Proc Natl Acad Sci USA 94:14821–14825.

Bergles DE, Diamond JS, Jahr CE (1999) Clearance of glutamate inside the synapse and beyond. Curr Opin Neurobiol 9:293–298.

Bergles DE, Tzingounis AV, Jahr CE (2002) Comparison of coupled and uncoupled currents during glutamate uptake by GLT-1 transporters. J Neurosci 22:10153–10162.

Brasnjo G, Otis TS (2004) Isolation of glutamate transport-coupled charge flux and estimation of glutamate uptake at the climbing fiber-Purkinje cell synapse. Proc Natl Acad Sci USA 101:6273–6278.

Bushong EA, Martone ME, Jones YZ, Ellisman MH (2002) Protoplasmic astrocytes in CA1 stratum radiatum occupy separate anatomical domains. J Neurosci 22:183–192.

Danbolt NC (2001) Glutamate uptake. Prog Neurobiol 65:1–105.

Diamond JS (2001) Neuronal glutamate transporters limit activation of NMDA receptors by neurotransmitter spillover on CA1 pyramidal cells. J Neurosci 21:8328–8338.

Fuchs PA, Glowatzki E, Moser T (2003) The afferent synapse of cochlear hair cells. Curr Opin Neurobiol 13:452–458.

Furness DN, Lawton DM (2003) Comparative distribution of glutamate transporters and receptors in relation to afferent innervation density in the mammalian cochlea. J Neurosci 23:11296–11304.

Furness DN, Lehre KP (1997) Immunocytochemical localization of a high-affinity glutamate-aspartate transporter, GLAST, in the rat and guinea-pig cochlea. Eur J Neurosci 9:1961–1969.

Furness DN, Hulme JA, Lawton DM, Hackney CM (2002) Distribution of the glutamate/aspartate transporter GLAST in relation to the afferent synapses of outer hair cells in the guinea pig cochlea. J Assoc Res Otolaryngol 3:234–247.

Glowatzki E, Fuchs PA (2002) Transmitter release at the hair cell ribbon synapse. Nat Neurosci 5:147–154.

Hakuba N, Koga K, Gyo K, Usami S-I, Tanaka K (2000) Exacerbation of noise-induced hearing loss in mice lacking the glutamate transporter GLAST. J Neurosci 20:8750–8753.

Hakuba N, Matsubara A, Hyodo J, Taniguchi M, Maetani T, Shimizu Y, Tsujiuchi Y, Shudou M, Gyo K (2003) AMPA/kainate-type glutamate receptor antagonist reduces progressive inner hair cell loss after transient cochlear ischemia. Brain Res 979:194–202.

Huang YH, Dykes-Hoberg M, Tanaka K, Rothstein JD, Bergles DE (2004a) Climbing fiber activation of EAAT4 transporters and kainate receptors in cerebellar Purkinje cells. J Neurosci 24:103–111.

Huang YH, Sinha SR, Tanaka K, Rothstein JD, Bergles DE (2004b) Astrocyte glutamate transporters regulate metabotropic glutamate receptor-mediated excitation of hippocampal interneurons. J Neurosci 24:4551–4559.

Huang YH, Sinha SR, Fedoryak OD, Ellis-Davies GC, Bergles DE (2005) Synthesis and characterization of 4-methoxy-7-nitroindolinyl-D-aspartate, a caged compound for selective activation of glutamate transporters and *N*-methyl-D-aspartate receptors in brain tissue. Biochemistry 44:3316–3326.

Jin ZH, Kikuchi T, Tanaka K, Kobayashi T (2003) Expression of glutamate transporter GLAST in the developing mouse cochlea. Tohoku J Exp Med 200:137–144.

Lehre KP, Danbolt NC (1998) The number of glutamate transporter subtype molecules at glutamatergic synapses: chemical and stereological quantification in young adult rat brain. J Neurosci 18:8751–8757.

Lehre KP, Levy LM, Ottersen OP, Storm-Mathisen J, Danbolt NC (1995) Differential expression of two glial glutamate transporters in the rat brain: quantitative and immunocytochemical observations. J Neurosci 15:1835–1853.

Li HS, Niedzielski AS, Beisel KW, Hiel H, Wenthold RJ, Morley BJ (1994) Identification of a glutamate/aspartate transporter in the rat cochlea. Hear Res 78:235–242.

Otis TS, Kavanaugh MP, Jahr CE (1997) Postsynaptic glutamate transport at the climbing fiber-Purkinje cell synapse. Science 277:1515–1518.

Ottersen OP, Takumi Y, Matsubara A, Landsend AS, Laake JH, Usami S (1998) Molecular organization of a type of peripheral glutamate synapse: the afferent synapses of hair cells in the inner ear. Prog Neurobiol 54:127–148.

Palmer MJ, Taschenberger H, Hull C, Tremere L, von Gersdorff H (2003) Synaptic activation of presynaptic glutamate transporter currents in nerve terminals. J Neurosci 23:4831–4841.

Rebillard G, Ruel J, Nouvian R, Saleh H, Pujol R, Dehnes Y, Raymond J, Puel JL, Devau G (2003) Glutamate transporters in the guinea-pig cochlea: partial mRNA sequences, cellular expression and functional implications. Eur J Neurosci 17:83–92.

Robertson D (1983) Functional significance of dendritic swelling after loud sounds in the guinea pig cochlea. Hear Res 9:263–278.

Rothstein JD, Martin L, Levey AI, Dykes-Hoberg M, Jin L, Wu D, Nash N, Kuncl RW (1994) Localization of neuronal and glial glutamate transporters. Neuron 13:713–725.

Shimamoto K, Lebrun B, Yasuda-Kamatani Y, Sakaitani M, Shigeri Y, Yumoto N, Nakajima T (1998) DL-threo-beta-benzyloxyaspartate, a potent blocker of excitatory amino acid transporters. Mol Pharmacol 53:195–201.

Trussell LO (1999) Synaptic mechanisms for coding timing in auditory neurons. Annu Rev Physiol 61:477–496.

Wadiche JI, Kavanaugh MP (1998) Macroscopic and microscopic properties of a cloned glutamate transporter/chloride channel. J Neurosci 18:7650–7661.

Wadiche JI, Amara SG, Kavanaugh MP (1995) Ion fluxes associated with excitatory amino acid transport. Neuron 15:721–728.

Yang XW, Model P, Heintz N (1997) Homologous recombination based modification in *Escherichia coli* and germline transmission in transgenic mice of a bacterial artificial chromosome. Nat Biotechnol 15:859–865.