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Glutamate Transport by Retinal Müller Cells in Glutamate/Aspartate Transporter-Knockout Mice

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ABSTRACT Glutamate transporters are involved in maintaining extracellular glutamate at a low level to ensure a high signal-to-noise ratio for glutamatergic neurotransmission and to protect neurons from excitotoxic damage. The mammalian retina is known to express the excitatory amino acid transporters, EAAT1–5; however, their specific role in glutamate homeostasis is poorly understood. To examine the role of the glial glutamate/aspartate transporter (GLAST) in the retina, we have studied glutamate transport by Müller cells in GLAST$^{-/-}$ mice, using biochemical, electrophysiological, and immunocytochemical techniques. Glutamate uptake assays indicated that the K_m value for glutamate uptake was similar in wild-type and GLAST$^{-/-}$ mouse retinas, but the V_{max} was ~50% lower in the mutant. In Na^+-free medium, the V_{max} was further reduced by 40%. In patch-clamp recordings of dissociated Müller cells from GLAST$^{-/-}$ mice, application of 0.1 mM glutamate evoked no current showing that the cells lacked functional electrogenic glutamate transporters. The result also indicated that there was no compensatory upregulation of EAATs in Müller cells. [^{3}H]D-Aspartate uptake autoradiography, however, showed that Na^+-dependent, high-affinity transporters account for most of the glutamate uptake by Müller cells, and that Na^+-independent glutamate transport is negligible. Additional experiments showed that the residual glutamate uptake in Müller cells in the GLAST$^{-/-}$ mouse retina is not due to known glutamate transporters—cystine-glutamate exchanger, ASCT-1, AGT-1, or other heteroexchangers. The present study shows that while several known glutamate transporters are expressed by mammalian Müller cells, new Na^+-dependent, high-affinity glutamate transporters remain to be identified.

INTRODUCTION

Neurons and glial cells express a family of Na^+-dependent, excitatory amino acid transporters, EAAT1–5, which function to maintain extracellular glutamate at a low level, thereby ensuring a high signal-to-noise ratio for glutamatergic neurotransmission and protecting neurons from excitotoxic damage in the CNS (Attwell et al., 1993; Seal and Amara, 1999). EAAT1 (GLAST) and EAAT2 (GLT-1) are expressed predominantly by glial cells, while EAAT3 (EAAC1) is localized primarily to neurons (Rothstein et al., 1994).

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EAAT4 is found primarily in the cerebellum (Fairman et al., 1995) and EAAT5 appears to be exclusive to the retina (Arriza et al., 1997; Eliasof et al., 1998a,b).

The mammalian retina expresses EAAT1–5, and provides an excellent system to study the function of individual EAATs (Pow, 2001a). Immunocytochemical studies indicate that EAAT1 (GLAST) is localized exclusively to Müller cells (Rauen et al., 1996; Lehre et al., 1997; Pow and Barnett, 1999), whereas EAAT2 is surprisingly absent in the retinal glial cells, but is expressed by cone photoreceptors and bipolar cells (Rauen and Kanner, 1994). EAAC1 (EAAT3) is present in neurons in the inner retina (Rauen et al., 1996; Schultz and Stell, 1996). EAAT4 has been reported in retinal astrocytes (Ward et al., 2004), while EAAT5 is localized to photoreceptors, bipolar cells, and possibly some Müller cells (Arriza et al., 1997; Eliasof et al., 1998a; Barnett and Pow, 2000).

The biochemical properties and subcellular localization of glutamate transporters suggest that they are well positioned to regulate the extracellular glutamate level (Danbolt, 2001). However, there is some controversy as to whether the transporters play an active role in determining the magnitude and time course of synaptic transmission at glutamatergic synapses (Bergles et al., 1999). In contrast, there is general consensus that glutamate transporters play an important role in regulating excitotoxic damage (Rothstein et al., 1996; Jabaudon et al., 1999; Li et al., 1999; Barnett and Pow, 2000; Rao et al., 2002; Izumi et al., 2002). Unfortunately, the contribution of individual EAAT subtypes to excitotoxicity is unknown because selective and potent inhibitors for the different EAAT subtypes are not available (Seal and Amara, 1999). Although antisense technology has been used to address this issue in some cases (Rothstein et al., 1996; Jabaudon et al., 1999; Li et al., 1999; Barnett and Pow, 2000; Rao et al., 2002), the availability of mice with the targeted disruption of GLT1, GLAST, and EAAC1 has provided a more powerful approach (Tanaka et al., 1997; Watase et al., 1998; Peghini et al., 1997).

The GLAST$^{-/-}$ and GLT1$^{-/-}$ mice have been particularly useful in analyzing the role of glial glutamate transporters in excitotoxicity (Harada et al., 1998; Mitani and Tanaka, 2003). Because GLAST is a major glutamate transporter in the mammalian retina, loss of GLAST is expected to render the retina highly susceptible to excitotoxic damage. However, the GLAST$^{-/-}$ mouse retina shows a benign phenotype, although it is more sensitive to prolonged ischemia than the normal retina (Harada et al., 1998). The reason for the lack of excitotoxic damage is unknown. It could be due to a compensatory upregulation of other EAATs by Müller cells, or Müller cells might express other glutamate transporters that can adequately handle the elevated extracellular glutamate level in the absence of GLAST. In the present study, we have examined these possibilities. Our results suggest that Müller cells in the GLAST$^{-/-}$ mouse retina readily take up glutamate, and that the uptake is not mediated by known glutamate transporters—EAATs, cystine-glutamate exchanger, ASCT-1, AGT-1 or other heteroexchangers—but is likely to be due to one or more Na^+-dependent, high-affinity glutamate transporters that remain to be identified. Thus, glial cells appear to express multiple glutamate transporters that are involved in regulating the extracellular glutamate level, and thereby modulating the extent of excitotoxic damage to neurons.

MATERIALS AND METHODS

Animals

C57B6/6J mice were purchased from Harlan-Sprague-Dawley (Indianapolis, IN), and GLAST knockout (GLAST$^{-/-}$) mice were generated by mating GLAST$^{+/-}$ (heterozygote) mice (Harada et al., 1998). GLAST$^{-/-}$ mice were identified by polymerase chain reaction (PCR) of tail DNA, using GLAST-specific oligonucleotide primers. All animals were maintained in clear plastic cages with standard light cycles (12 light/12 dark) and handled according to approved protocols. Experimental procedures were designed to conform to the National Institutes of Health (NIH) guidelines for the use of animals in biomedical research.

Histology

Mice were killed by an overdose of CO_2 and enucleated rapidly. Eyes were fixed in 2.5% glutaraldehyde/1% paraformaldehyde/0.1 M phosphate buffer, pH 7.4/3% sucrose for 30 min, dehydrated, embedded in epoxy resin, sectioned (250 nm), and processed for light microscopy as described by Marc and Liu (1985). For immunocytochemistry, eyes were fixed in 4% paraformaldehyde, and immersed in 15% sucrose overnight. Cryostat sections were cut and processed for immunocytochemistry as described in detail elsewhere (Kuzmanovic et al., 2003).

Glutamate Uptake Kinetics

Retinas were quickly removed and gently homogenized in ice-cold buffer (30 ml/g tissue) containing 20 mM HEPES, 118 mM NaCl, 5 mM KCl, 1.18 mM $MgSO_4$, 2.5 mM $CaCl_2$ and 10 mM glucose, adjusted to pH 7.4 with Tris base. After centrifugation at 1,000*g* for 15 min, the pellet was resuspended and recentrifuged. The final pellet was suspended in the same buffer, placed on ice, and used immediately for uptake assays (Alan and Harris, 1987). For experiments in Na^+-free medium, NaCl was replaced by equimolar concentration of LiCl.

Aliquots (180 μl) of the membrane vesicle preparation (~70–100μg protein) were preincubated for 10 min at 37°C. Uptake was initiated by adding 20 μl of glutamate mixture containing 1 μCi L-[G-^{3}H]glutamic acid (56 Ci/mmol, Amersham Biosciences, Piscataway,

NJ) and varying concentrations of L-glutamate (0.3–500 μM). The reaction was terminated after 3 min by the addition of 5 ml ice-cold buffer followed by rapid filtration through glass fiber filters (Schleicher & Schuell glass fiber filters #30, Keene, NH) pre-soaked in 0.05% polyethyleneimine. The tubes and filters were rinsed with 5 ml cold buffer and run through the filter. Subsequently, the filters were dried and their radioactivity was determined by liquid scintillation counting.

Preliminary experiments showed that glutamate uptake is linear for 5 min in the concentration range used. Parallel incubations were carried out at 0°C to correct for nonspecific uptake. Data are expressed as the mean ± SEM of net glutamate uptake (uptake minus uptake at 0°C) in nmol/mg protein. min for four independent experiments. Protein was determined with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories, Hercules, CA), with bovine serum albumin (BSA) as the standard.

Nonlinear regression analysis was used to calculate V_{max} and K_m using GraphPad Prism, V3.0 (GraphPad Software, San Diego, CA). Eadie-Hofstee plots were used to analyze the results of glutamate uptake experiments. Statistical comparisons were performed by unpaired *t*-test using GraphPad Prism, V3.0.

Patch-Clamp Studies

Müller cells were dissociated from mouse retina as described previously (Bringmann et al., 1999). Briefly, isolated retinas were incubated for 30 min at 37°C in Ca^{2+}/Mg^{2+}-free phosphate-buffered saline (PBS) containing 0.2–0.6 mg/ml papain (Boehringer, Mannheim, Germany). After washing with PBS containing DNase I (150 U/ml; Sigma, Deisenhofen, Germany), retinas were triturated with a pipette until single cells were dissociated. The cell suspension was stored on ice until use. Single Müller cells were identified on the basis of their morphology and selected for recording.

Dissociated cells were suspended in an extracellular solution and added to a recording chamber mounted on a microscope stage (Axioskop, Zeiss, Germany). The extracellular solution contained (in mM): NaCl, 110; KCl, 3; $CaCl_2$, 2; $MgCl_2$, 1; Na_2HPO_4, 1; glucose, 11; HEPES-Tris, 10; $NaHCO_3$, 25; and was equilibrated to pH 7.4 by bubbling with 95% O_2/5% CO_2. During application of neurotransmitters, the dominant K^+ conductance of Müller cells was blocked by 1 mM Ba^{2+}. Recordings were performed in the whole-cell configuration at 20–24°C. The recording chamber was perfused continuously (2 ml/min) after establishing the whole-cell configuration. Recording electrodes were made from borosilicate glass (Science Products, Hofheim, Germany) and had resistances of 4–6 MΩ when filled with a solution containing (mM): KCl, 130; NaCl, 10; $MgCl_2$, 2; $CaCl_2$, 1; EGTA, 10; HEPES-Tris, 10; pH 7.1.

For current and voltage recordings, the patch-clamp amplifier Axopatch 200A (Axon Instruments, Foster City, CA) was used. Currents were low-pass filtered at 1 kHz and digitized at 5 kHz, using a 12 bit-A/D converter. Transporter-mediated currents were recorded according to protocols described in detail earlier (Pannicke et al., 2002; Biedermann et al., 2002). Voltage command protocols were generated, and data analysis was performed with the software ISO 2 (MFK, Niedernhausen, Germany) and SigmaPlot (Jandel Scientific, San Rafael, CA). Mean values with standard deviations (SD) are given.

Glutamate Uptake: D-Aspartate Uptake Immunocytochemistry

Mouse retinas were incubated for 10 min in Ames medium containing either 1 mM or 5 mM D-aspartate, fixed with 2.5% glutaraldehyde, 1% formaldehyde in 0.1 M, pH 7.4, phosphate buffer supplemented with 3% sucrose and 1 mM $MgSO_4$, dehydrated in graded methanols, and embedded in epoxy resin (Marc, 1999). Serial thin 250-nm sections were fully etched in sodium methoxide and probed for D-aspartate, L-glutamate, L-glutamine, taurine, L-aspartate, glutathione using anti-hapten IgGs described in previous work (e.g., Marc et al., 1995; Marc and Cameron, 2002). Signals were visualized with calibrated silver-intensified 1-nm immunogold secondary IgGs, channels registered with <200-nm precision with PCI Geomatica® (Richmond Hill, Canada) remote sensing code (for details, see Marc et al., 1995) and analyzed with in-house multivariate analysis code (CellKit V1.0.6) developed under IDL VM™ from RSI (Boulder, CO). Signal histograms were calibrated as intracellular concentration by remapping pixel values to known concentrations in ovalbumin-lysine-amino acid standard stacks similar to those fabricated in previous studies (Ottersen et al., 1992; Crook and Pow, 1997; Marc and Jones, 2002).

Glutamate Uptake: [^{3}H]D-Asp Autoradiography

Retinas were incubated in mouse bicarbonate medium containing 16 μCi [^{3}H]D-Asp (1 μM) for 15 min at room temperature, with or without 1 mM D-aspartate as a competitor (n = 4 for each group; 16 retinas were processed in all). In some experiments, retinas were preincubated for 5 min with CPG, (S)-4-carboxyphenylglycine, an inhibitor of the cystine-glutamate exchanger (100 μM) before adding [^{3}H]D-Asp (1 μM). Retinas were fixed in 2.5% glutaraldehyde/1% paraformaldehyde, dehydrated, embedded in epoxy resin, sectioned (250 nm), and processed for light microscope autoradiography as described by Marc and Liu (1985). In detail, 250-nm sections were placed at 25 mm from the end of a glass slide, dipped in 1:1 aqueous Kodak NTB-3 at 40°C and dried at an angle of 70°C, were exposed for 15 days, developed in 1:1 aqueous Kodak Dektol® at 16°C, fixed, washed, dried, and stained with toluidine blue as specified in Marc et al. (1978). This

protocol allows for precise, quantitative comparisons of labeling across specimens. Images were captured with a Zeiss 40× oil planapochromatic objective at a resolution of 221 nm/pixel at 8 bits. Fields subtending 100 μm in width from each specimen were captured and the integrated grain density computed for each layer (outer nuclear layer, inner nuclear layer [INL], inner plexiform layer). In no case could retinas from the knockout and wild-type mice be distinguished morphologically.

For quantitation of [^{3}H]D-Asp transport by Müller cells, the signal was expressed as the fractional area (F) of the INL occluded by grains: F = G/A, where G = grain pixels and A = total sample area pixels. F was corrected for background signals, which were negligible (mean background F over all samples = 0.00097) and the signal-to-noise ratio ranged from 10 to 300. Light, moderate, and heavy labeling correspond to F-values of roughly 0.01, 0.1, and 0.5, respectively. F was calculated from 8-bit autoradiographic images segmented by two standard algorithms, with F = N(255)/N(total), N(255) = white pixels, N(total) = sample area in pixels:

Method 1. Accurate, robust sampling for light labeling composed of isolated grains: The image (I) was segmented (S) into white grains with pixel value (PV) 255 and black nongrains (PV = 0) as follows: U = unsharp mask of I (1.5-pixel radius), isolates grains via a high pass function; 2M = 2 serial median filters of U (3-pixel radius), erases high-frequency grains; D = U-2M; difference image with isolated grains on a black background; S = threshold at PV 45 of D, converts grains to PV 255.

Method 2. Underestimate sampling for moderate labeling with overlapping grains, as median filtering cannot be used: U = unsharp mask of I (1.5-pixel radius), isolates grains via a high pass function; V = U-255, inverted U; X = max-min stretch of V, converts grains to the brightest values in the image; S = threshold a PV 170 of X; converts 50–100% of grains to 255.

These two methods are necessary, as method 1, although accurate and precise for low signals, cannot be used when grains overlap: the median filter will not extract them properly. Method 2 effectively captures grain clumps, but loses some single grains and always underestimates labeling as the max-min stretch produces a bimodal intensity histogram where grain signals overlap the tissue signals. Thus, some are arbitrarily cut off by the thresholding step. Our threshold value always captures at least 50% of the signal. As a result, differences between low and high label densities are always underestimated.

RESULTS

Retinal Histology

Morphological studies have previously shown that retinas from GLAST$^{-/-}$ mice appear completely normal and show no signs of neuronal degeneration (Harada et al., 1998) (Fig. 1A). Because early ischemic damage may not accompanied by histological changes (Penn et al., 1988), we examined GFAP expression by Müller cells, which is a better indicator of neuronal loss in the retina (Sarthy and Ripps, 2001). For this purpose, cryostat sections of retina from GLAST$^{-/-}$ mice were treated with anti-GFAP and the immunostaining pattern was determined using a confocal microscope. As shown in Figure 1B, GFAP-immunolabeling in the mutant mouse retina was limited to astrocytes (at the vitread border of the tissue) and was not seen in the radial processes of Müller cells. These data are consistent with the absence of neuronal degeneration in the GLAST$^{-/-}$ mouse retina.

Glutamate Uptake: Contribution of GLAST

Glutamate uptake studies carried out with isolated retina and membrane vesicle preparations clearly show that glutamate uptake in this tissue is mediated by a high-affinity, sodium-dependent process that is driven by the transmembrane sodium ion gradient (White and Neal, 1976; Sarthy et al., 1986; Rauen et al., 1998). The mammalian retina expresses five different excitatory amino acid transporters (Pow, 2001a), and among these transporters, GLAST (EAAT1), appears to be the major glutamate transporter expressed by Müller cells (Rauen et al., 1998). Although it is generally assumed that GLAST plays a key role in glutamate uptake, its specific contribution to overall glutamate uptake in the retina is unknown. To address this issue, we have studied glutamate uptake by retinas from wild-type and GLAST$^{-/-}$ mice. Uptake of [^{3}H]L-glutamate was measured in the presence of increasing concentrations of unlabeled L-glutamate, and the results obtained are presented in Figure 2. In retinas from normal mice, glutamate was taken up by a high-affinity uptake system with a K_m 22.9 ± 2.7 μM and V_{max} 0.35 ± 0.02 nmol/min · mg protein. The K_m for uptake in retinas from GLAST$^{-/-}$ mice was similar (K_m = 24.4 ± 1.9 μM). The V_{max}, however, was found to be 0.15 ± 0.01 nmol/min · mg protein ($P < 0.005$ by unpaired *t*-test). This result suggests that GLAST accounts for at least 50% glutamate transport in the normal retina.

To characterize further the glutamate transporters in the GLAST$^{-/-}$ mouse retina, we examined the Na^+-dependence of glutamate uptake. Retinal homogenates were incubated with [^{3}H]L-glutamate in Na^+-free medium and glutamate accumulated was determined. As shown in Figure 2., there was significant glutamate uptake by retina, and the K_m for uptake was 56.15 ± 1.5 μM and the V_{max} was 0.11 ± 0.01 nmol/min · mg

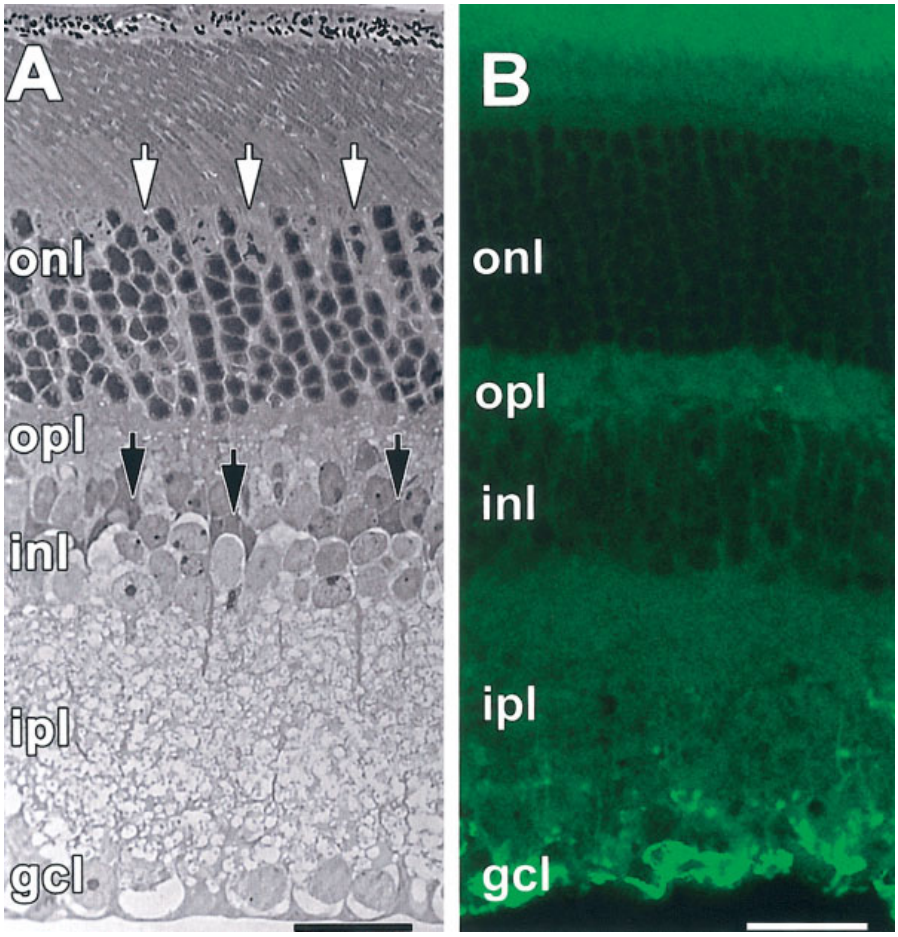


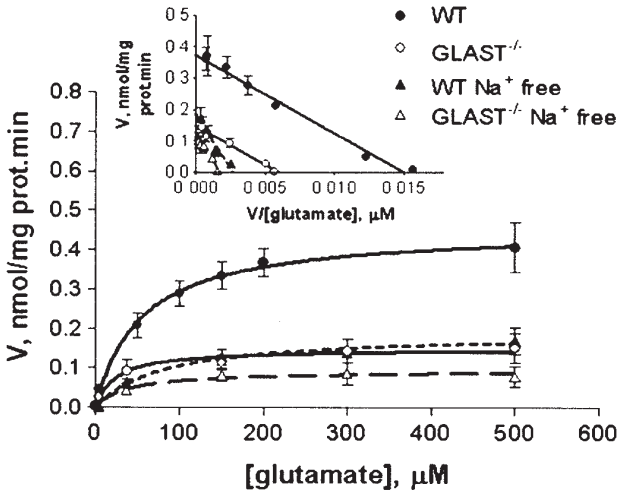
Fig. 1. Histology of the glial glutamate/aspartate transporter $(GLAST)^{-/-}$ mouse retina. **A:** Toluidine blue-stained phase contrast image of a 250-nm section of retina from a $GLAST^{-/-}$ mouse displayed at maximum optical resolution. The histology is completely indistinguishable from a wild-type retina, including such fine details as the segmented chromatin of cone nuclei (white arrows) and the basophilic smooth chromatin of Müller cells (black arrows). **B:** Immunofluorescence micrograph of a cryostat section stained with anti-glial fibrillary acidic protein (GFAP). Note the presence of GFAP in retinal astrocytes, and its absence from the radial processes of Müller cells. Abbreviations: onl, outer nuclear layer; opl, outer plexiform layer; inl, inner nuclear layer; ipl, inner plexiform layer; gcl, ganglion cell layer. Scale bars = 20 μm in A; 25 μm in B.

protein ($P < 0.005$ by unpaired t-test). The result shows that Na^+-independent transport accounts for ~70% of glutamate uptake in the $GLAST^{-/-}$ retina. Because these data were obtained from retinal homogenates, we do not know whether the Na^+-independent transport is glial or neuronal in origin.

Electrogenic Glutamate Transporter Activity

To examine directly whether Müller cells upregulate other EAATs to compensate for the loss of GLAST in $GLAST^{-/-}$ mouse retina, we have determined the presence of glutamate-induced membrane currents by patch-clamp recording. Müller cells were dissociated from papain-treated retina, and used for patch-clamp recording in the whole cell configuration (Fig. 3). In Müller cells from $GLAST^{-/-}$ mice, the membrane potential recorded under current-clamp conditions (I = 0) was −78 ± 5 mV (n = 22). This value is similar to that recorded for isolated Müller cells from wild-type mice (Pannicke et al., 2002: −84 ± 6 mV, n = 85). The membrane resistance, recorded at a voltage step from −80 to −90 mV, was 46 ± 12 MΩ (n = 22 cells) and 47 ± 32 MΩ (n = 85 cells) for Müller cells from the mutant and wild-type mice, respectively.

Next, we applied glutamate to the extracellular solution to activate Na^+/glutamate transporters. At a holding potential of −80 mV, application of 0.1 or 1 mM glutamate to Müller cells from $GLAST^{-/-}$ mice evoked no current (n = 9 and n = 6 cells, respectively; Fig. 3A). In Müller cells from wild-type mice, however, glutamate evoked inward currents of 25 ± 9 pA (0.1 mM, n = 12 cells; Fig. 3B) or 28 ± 15 pA (1 mM, n = 9 cells).



	WT	GLAST-/-	WT Na+ free	GLAST-/- Na+ free
Vmax (nmol/mg prot.min)	0.35±0.02	0.15±0.01	0.15±0.02	0.11±0.01
Km (uM)	22.90±2.70	24.40±1.97	50.22±3.75	56.15±151

Fig. 2. Kinetics of L-glutamate uptake. L-glutamate uptake was measured at increasing concentrations (0.3–500 μM) in the linear range in normal or Na^+-free medium. Data are mean ± SEM of duplicate determinations of net glutamate uptake from four independent experiments. The uptake was saturable and fit a Michaelis-Menten curve. Inset: Eadie-Hofstee plot of the data. The tabulation presents K_m and V_{max} values for glutamate uptake by retina homogenates from wild-type (WT) and glial glutamate/aspartate transporter $(GLAST)^{-/-}$ mice.

Therefore, Müller cells from $GLAST^{-/-}$ mice appear to lack functional electrogenic glutamate transporters. Moreover, application of 1 mM γ-aminobutyric acid (GABA) evoked inward currents of 17.5 ± 5 pA (n = 6, holding potential −80 mV; Fig. 3C) in cells from $GLAST^{-/-}$ mice, clearly demonstrating that GABA electrogenic transporters were not impaired (Biedermann et al., 2002).

It has been demonstrated that in addition to the uptake of glutamate, EAATs are coupled to an anion conductance that allows for larger currents when anions other than Cl^- are used (Eliasof and Jahr, 1996; Billups et al., 1996). Therefore, we examined Müller cell responses to 1mM glutamate when extracellular Cl^- was replaced by SCN^-. Under these conditions no currents were evoked at a holding potential of −80 mV in cells from $GLAST^{-/-}$ mice (n = 6 cells). However, when the cells were voltage clamped at +50 mV, outward currents of 46 ± 28 pA were evoked by 1 mM glutamate in 5 of 7 cells tested (Fig. 3D). This is similar to the outward currents (35 ± 20 pA, n = 3, and 46 ± 19 pA, n = 4, respectively) evoked under the same conditions in control cells (Fig. 3E). In contrast to cells from GLAST knockout mice, inward currents (15 ± 10 pA, n = 7; not shown) were evoked in cells from control mice by 1 mM glutamate in SCN^--containing solution at a holding potential of −80 mV. A possible explanation for the occurrence of the outward currents is the existence of another EAAT. For example, Arriza et al. (1997) demonstrated that a large part of the glutamate-evoked current mediated by EAAT5 is due to its anion conductance. Müller cells of some species have been reported to express EAAT5 (Eliasof et al., 1998a). However, the existence of EAAT5 in Müller cells from a mammalian retina remains to be demonstrated (Pow, 2001a).

D-Aspartate Uptake by Müller Cells

Although the patch-clamp studies showed that electrogenic glutamate transporters were not present in Müller cells, it is still possible that other nonelectrogenic, glutamate transporters could mediate glutamate uptake by Müller cells in the $GLAST^{-/-}$ mouse retina. Therefore, we used uptake studies to examine the ability of retinal Müller cells to accumulate exogenous glutamate. Because glutamate is rapidly degraded to glutamine in Müller cells, we used D-aspartate as a substrate to determine glutamate uptake. Also, D-Asp was localized by immunocytochemistry using a specific

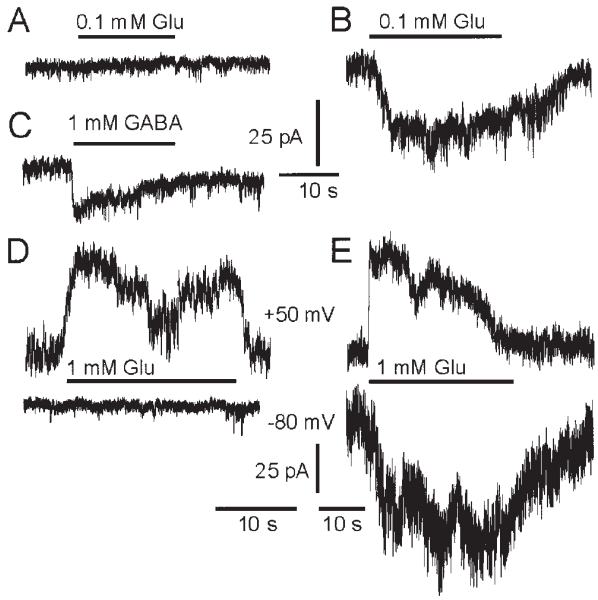


Fig. 3. Patch-clamp recordings from dissociated retinal Müller cells. Retinas from glial glutamate/aspartate transporter (GLAST)$^{-/-}$ or wild-type mice were treated with papain and Müller cells were dissociated by trituration. Patch-clamp recording were performed in the whole cell configuration as described under methods. **A:** Application of 0.1 mM glutamate (Glu) had no effect on a Müller cell from a GLAST$^{-/-}$ mouse when the cell was voltage clamped at −80 mV. **B:** In a Müller cell from wild-type mouse, glutamate-induced inward currents could be easily detected. **C:** γ-Aminobutyric acid (GABA) evoked an inward current, probably mediated by the GABA transporter, in a Müller cell from GLAST $^{-/-}$ retina. **D,E:** 1 mM Glu was applied after replacement of extracellular Cl^- by SCN^-. Whereas the cell behavior at a holding potential of −80 mV was similar to the experiments in Cl^--containing solution (lower traces in D,E), outward currents were evoked at a holding potential of +50 mV (upper traces in D,E).

antibody (Marc et al., 1995). Results of the uptake experiment are presented in Figure 4A,B. In retinas from wild-type mice, there was a high level of D-Asp uptake in the cell bodies in the INL, where Müller cells are located (Fig. 4A). The radial processes of Müller cells were also labeled. This result is in agreement with D-Asp uptake pattern seen in other mammalian retinas (Kalloniatis et al., 1996). When retinas from GLAST$^{-/-}$ mice were examined, there was also strong D-Asp uptake by Müller cell bodies and radial processes (Fig. 4B).

Next, we used computational molecular phenotyping to estimate glutamate levels in retinal cells. One advantage of the method (Marc and Jones, 2002; Jones et al., 2003) is that it allows for concurrent quantitative measures of multiple intracellular markers in the same cells. Calibrated small molecular immunocytochemistry can accurately detect concentrations in the range of 0.1 –10 mM (Marc et al., 1990, 1995; Marc and Jones, 2002). It is also possible to use pattern recognition methods to completely segment images into discrete cellular compartments (Marc et al., 1995), each of which can be individually sampled. The same specimens illustrated in Figure 4A,B were segmented by pattern recognition and calibrated signal histograms acquired from 42 GLAST$^{-/-}$ Müller cells and 46 wild-type Müller cells and their processes (Fig. 4C,D). The breadth of each histogram is variation in intracellular partitioning, with higher signal levels generally observed in the end feet. The modal levels of endogenous markers that comprise the definitive Müller cell signature of high taurine, high L-glutamine and low L-glutamate (Marc et al., 1995; Kalloniatis et al., 1996) are very similar between Müller cells from GLAST$^{-/-}$ and wild-type mice, with slightly higher levels in the former (median and mean values can be similarly compared with the same conclusions). However, in the very same sets of cells, the accumulation of D-aspartate in GLAST$^{-/-}$ Müller cells is clearly 50% that achieved in wild-type cells. The result cannot be attributed to methodological differences as all samples were contained in the same wells and were concurrently probed (for details, see Marc et al., 1995).

[^{3}H]D-Aspartate Autoradiography

Because the uptake immunocytochemistry data indicated that Müller cells in GLAST$^{-/-}$ retinas could take up glutamate efficiently, we decided to compare glutamate uptake between Müller cells from mutant and normal retinas, using quantitative [^{3}H]autoradiography. In these experiments, isolated retinas were incubated with [^{3}H]D-Asp at 1 μM concentration and processed for autoradiography by standard techniques. Results of the experiment are presented in Figure 5. The autoradiograms showed heavy labeling of rods, as well as some cell bodies in the INL (Fig. 5A). Based on their location and morphological features, many of the [^{3}H]-labeled cell bodies in the INL were identified as belonging to Müller cells (M, black arrows). Furthermore, in GLAST $^{-/-}$ mice, there was only a slight decrease in [^{3}H]D-Asp uptake by the entire retina, as well as by Müller cells (Fig. 5B). As is typical of all vertebrates, amacrine cells (A, white arrows) showed no [^{3}H]D-Asp accumulation. Finally, the pattern of [^{3}H]D-Asp labeling was identical to that seen in other mammalian retinas (Kalloniatis et al., 1996). Quantitation of [^{3}H]D-Asp transport by Müller cells was carried out as described under Materials and Methods. The results showed that Müller cells in the GLAST$^{-/-}$ mouse retina are capable of accumulating glutamate through a high-affinity uptake system and that D-aspartate uptake was quantitatively similar to that observed in the normal retina (Fig. 5C).

To examine whether glutamate uptake was Na^+-dependent, we carried out [^{3}H]D-Asp uptake in Na^+-free medium (Marc and Jones, 2002). In addition, the contribution of the cystine-glutamate exchanger toward glutamate uptake was determined by performing uptake in the presence of (S)-4-carboxyphenylglycine (CPG), a known inhibitor of the cystine-glutamate exchanger (Ye et al., 1999). Results of the experiment are shown in Figure 5C. In the wild-type retina, glutamate

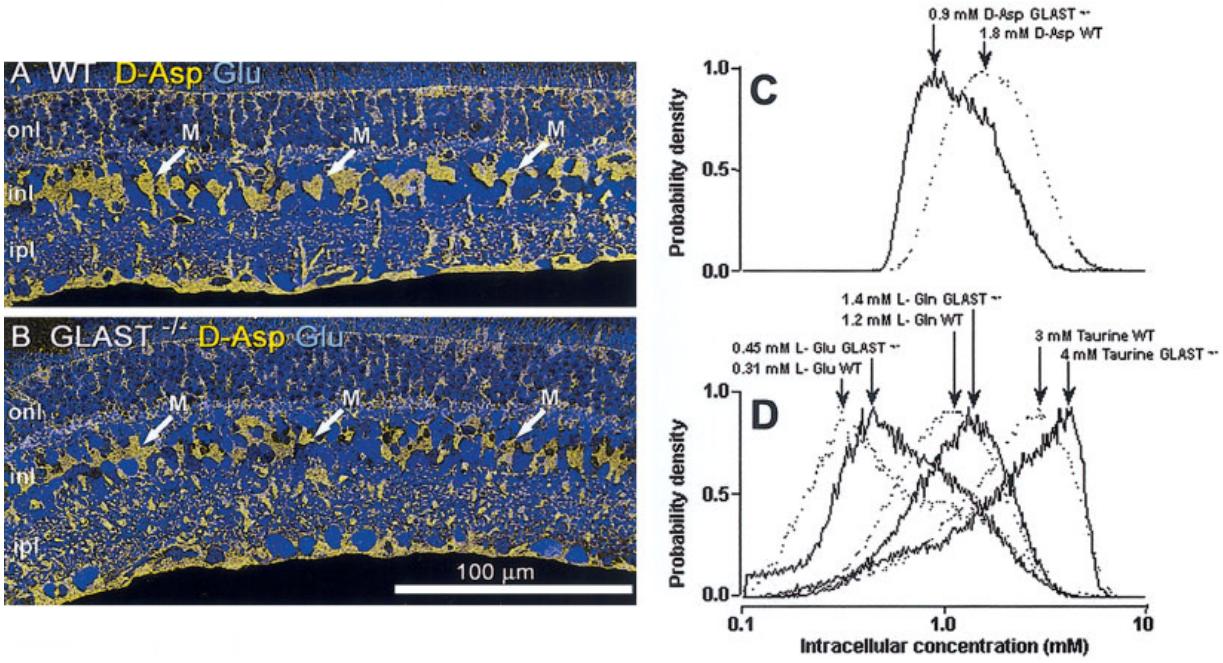


Fig. 4. D-Aspartate uptake in retina. **A,B:** Isolated retinas were incubated with D-Asp and processed for D-aspartate immunocytochemistry as described in the Materials and Methods. Micrographs show D-aspartate accumulation in (A) wild-type and (B) glial glutamate/aspartate transporter (GLAST) $^{-/-}$ mice. Retinal sections (250 nm) were processed concurrently by probing with anti-D-Asp (1:10,000 dilution) and visualized with silver-intensified immunogold. The distribution and content of D-Asp (D-Asp; yellow) were similar in Müller cells (arrows) of wild-type and GLAST $^{-/-}$ mice. Endogenous retinal glutamate levels (Glu; blue) were also similar between the mutant and wild-type animals. **C,D:** Pairs of quantitative probability density histograms for D-aspartate (C) and endogenous L-glutamate, L-glutamine, and taurine (D) signals in Müller cells from GLAST$^{-/-}$ (solid lines) and wild-type (WT) (dashed lines) animals detected concurrently with quantitative, calibrated immunocytochemistry. All samples were 250-nm sections, probed simultaneously, and represent the intracellular signals from 42 Müller cells in GLAST$^{-/-}$ and 46 Müller cells in wild-type mice, and their distal and proximal processes. All endogenous signals (lower curves) were slightly higher in the GLAST$^{-/-}$ Müller cells by a factor of 1.2–1.5, which is likely true inter-sample variation and not due to differences in probing or detection. As each curve set (e.g., all dashed curves) represent exactly the same cell, the lower amount of D-aspartate detected in GLAST$^{-/-}$ Müller cells after loading with a saturating level of D-aspartate (upper curves) represents a 50% decrease in V_{max}, even though the intracellular signatures are completely normal. onl, outer nuclear layer; inl, inner nuclear layer and ipl, inner plexiform layer.

uptake by Müller cells was reduced by ~70%, in the absence of Na^+. In contrast, glutamate uptake by Müller cells was totally blocked by Na^+-removal in the GLAST$^{-/-}$ mouse retina. Furthermore, CPG did not significantly affect glutamate uptake in either case. These results show that Na^+-dependent, high-affinity transporters account for most of the glutamate uptake by Müller cells, and that Na^+-independent glutamate transport is negligible in the GLAST$^{-/-}$ mouse retina.

Other Glutamate Transporters

In addition to EAATs, several other transporters are capable of taking up glutamate. These include three Na^+-dependent, zwitterionic amino acid transporters—ASCT1, ASCT2, and ATB° (Palacin et al., 1998), glutamate-GABA and glutamate-glycine heteroexchangers (Levi et al., 1976; Bonanno et al., 1993, 1994), as well as the Na^+-independent systems, the cystine-glutamate exchanger (Bannai and Tateishi, 1986) and AGT-1 (Matsuo et al., 2002).

Because ASCT1 is electroneutral and can transport aspartate and glutamate (Arriza et al., 1993; Shafqat et al., 1993; Zerangue and Kavanaugh, 1996), and is the best-studied zwitterionic amino acid transporter, we examined ASCT1 expression in retinal sections from GLAST$^{-/-}$ and wild-type mice using a highly specific antibody (Sakai et al., 2003). ASCT1 was found to be widely distributed in the retina, suggesting that it was expressed by most retinal cells, including Müller cells (Fig. 6A,B).

Although ASCT1 is expressed by Müller cells, we do not think this system is involved in glutamate uptake because the ASCT1 system (also the ASCT2) has a very low affinity for aspartate and glutamate (Zerangue and Kavanaugh, 1996); and therefore, may not be suitable for scavenging glutamate at low extracellular concentrations. Moreover, D-Asp is not taken up by or ASCT-1 or ASCT-2 (Utsunomiya-Tate et al., 1996; Tetsuka et al., 2003). We did not examine the involvement of the glutamate-GABA and glutamate-glycine heteroexchangers because these transporters have been described in synaptosomal

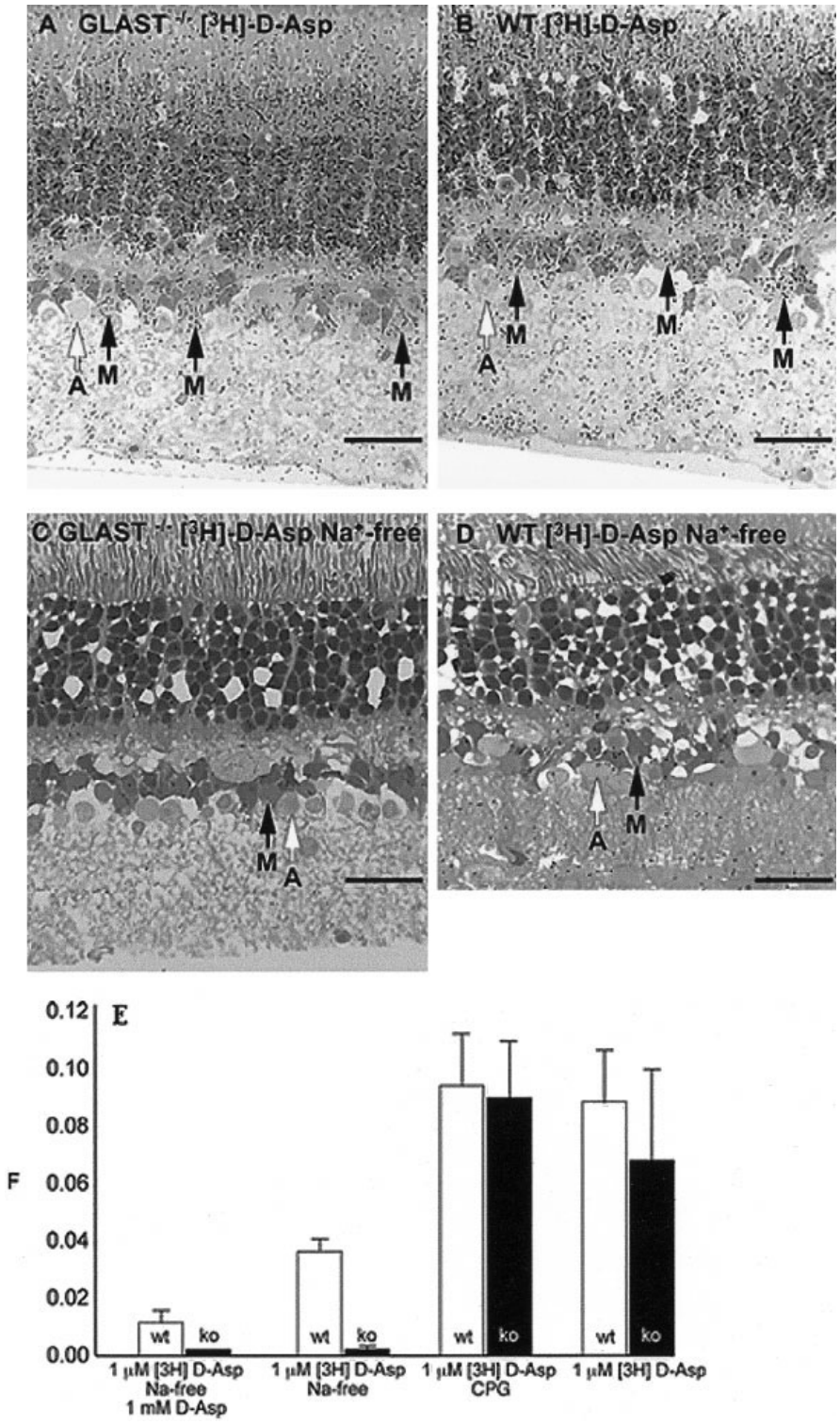

A GLAST -/- [3H]-D-Asp
B WT [3H]-D-Asp
C GLAST -/- [3H]-D-Asp Na+-free
D WT [3H]-D-Asp Na+-free
A
M
E
F
0.12
0.10
0.08
0.06
0.04
0.02
0.00
wt
ko
1 μM [3H] D-Asp Na-free 1 mM D-Asp
1 μM [3H] D-Asp Na-free
1 μM [3H] D-Asp CPG
1 μM [3H] D-Asp

Figure 5.

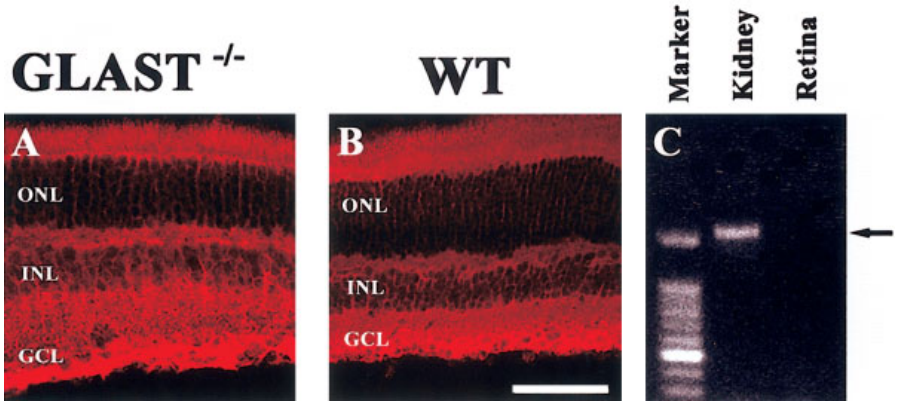


Fig. 6. Localization of ASCT1 and AGT1. Cryostat sections of paraformaldehyde-fixed retinas were treated with anti-ASCT1 and the antigen localized by indirect immunofluorescence. **A:** Glial glutamate/aspartate transporter (GLAST)$^{-/-}$ mouse retina treated with anti ASCT1. **B:** Wild-type mouse retina treated with anti-ASCT1. Note the heavy labeling throughout the retina indicating the presence of ASCT1 in most of the retinal cells. **C:** Reverse transcription-polymerase chain reaction (RT-PCR) analysis of AGT1 transcripts in mouse kidney and retina. The primer design and PCR conditions were as described by Matsuo et al. (2002). The PCR product had a size of ~1,400 bp (arrow). Note that the AGT1 mRNA is absent in the retina but is present in the kidney. Scale bar = 50 mm.

preparations and are not well characterized in other systems.

Another potential transporter that could be responsible for glutamate uptake by Müller cells in GLAST$^{-/-}$ mouse retina is the glutamate-cystine exchanger, which is an electroneutral, Na^+-independent transporter that normally transports cystine using the transmembrane gradient of glutamate as the driving force (Bannai and Tateishi, 1986). However, If the extracellular concentration of glutamate is high, the exchanger can operate in the reverse. Because a large fraction (~70%) of glutamate uptake in GLAST$^{-/-}$ mouse retina is Na^+-independent (Fig. 2) and Müller cells have been shown to express the glutamate-cystine exchanger (Kato et al., 1993; Pow, 2001b; Tomi et al., 2002, 2003), we examined the involvement of the exchanger in glutamate uptake. For this study, [^{3}H]D-Asp uptake was carried out in Na^+-free medium in the presence of a known inhibitor of cystine-glutamate exchanger, CPG and the retinas were processed for autoradiography. As shown in Figure 5, there was significant [^{3}H]D-Asp uptake by Müller cells under these conditions, which suggests that the glutamate-cystine exchanger cannot account for the glutamate uptake seen in Müller cells in GLAST$^{-/-}$ mouse retina.

Although the [^{3}H]D-Asp autoradiography data suggested that glutamate uptake by Müller cells was largely Na^+-dependent, it is still possible that an electroneutral, sodium-independent glutamate transport system could account for some glutamate uptake by Müller cells lacking GLAST. Such a system has been recently cloned and characterized (Matsuo et al., 2002). It is termed, system AGT1, and transports aspartate and glutamate in a sodium-independent manner, but with high affinity. It is an exchanger and therefore glutamate from the extracellular medium can be taken up into cells very effectively in exchange for intracellular aspartate or other acceptable amino acids (Matsuo et al., 2002). To examine whether mouse retina expresses this transporter, we have used RT-PCR to detect the presence of AGT1 transcripts in the retina. The experimental data presented in Figure 6C show that the retina from GLAST$^{-/-}$ mice does not express AGT1, ruling out the involvement of this transporter in glutamate uptake by Müller cells. The transporter responsible for glutamate uptake by retinal Müller cells in GLAST$^{-/-}$ mice remains to be identified.

Fig. 5. Light microscopic autoradiography of [^{3}H]D-aspartate transport in isolated retinas from (**A,C**) glial glutamate/aspartate transporter (GLAST)$^{-/-}$ and (**B,D**) wild-type mice in the presence of normal Na^+ concentration (**A,B**) or Na^+-free media (**C,D**). The pattern of labeling is identical to that of all other mammals and characteristic of Müller cell transport. There is no detectable difference between transport in GLAST knockout and wild-type mice (n = 4 for each group). As is typical of all vertebrates, amacrine cells (A, white arrows) show no D-aspartate accumulation, while Müller cells (M, black arrows) are the dominant transporters in the inner nuclear layer (A,B). In both wild-type and GLAST$^{-/-}$ mice, absence of Na^+ yielded massive blockade of transport (C,D). Scale, 20 μm. (E). Quantitation of [^{3}H]D-Asp transport by Müller cells. The histograms illustrate the mean fractional signals (F) from four retinas of either wild-type (wt) or GLAST$^{-/-}$ (ko) for three different conditions. CPG, (S)-4-carboxyphenylglycine, an inhibitor of the cystine-glutamate exchanger. F is the fractional area of the INL occluded by grains: F = G/A, where G = grain pixels and A = total sample area pixels. Light, moderate and heavy labeling correspond to F-values of roughly 0.01, 0.1, and 0.5, respectively. See Materials and Methods for the calculation methods for F.

DISCUSSION

The mammalian retina expresses all the excitatory amino acid transporters, GLAST, GLT-1, EAAC1, EAAT4, and EAAT 5 (Seal and Amara, 1998; Pow, 2001a; Ward et al., 2004). Although it is known the transporters are expressed by different retinal cell types (Pow, 2001a), the relative contribution of the

transporters toward retinal glutamate uptake has remained unresolved. The present study shows that GLAST is the most dominant glutamate transporter, accounting for at least 50%of glutamate uptake, in the mammalian retina. Furthermore, the experimental data show that glutamate is taken up by a high-affinity system with a K_m ~20–25 μM. This value is in good agreement with the K_m reported for glutamate uptake in other mammalian retinas (White and Neal, 1976; Sarthy et al., 1986; Rauen et al., 1998). Comparison of the V_{max} values, an indicator of the number of uptake sites, suggests that the GLAST$^{-/-}$ mouse retina has less than 50% of glutamate uptake capacity compared to the wild-type. Uptake experiments carried in Na^+-free, medium further showed that glutamate uptake by normal Müller cells was reduced by ~30% in the absence of Na^+, which is in agreement with the biochemical data on glutamate uptake (Fig. 2). Surprisingly, Na^+-removal completely suppressed glutamate uptake by Müller cells in the GLAST$^{-/-}$ mouse retina. These results show that Na^+-dependent, high-affinity transporters account for most of the glutamate uptake by Müller cells, and that Na^+-independent glutamate transport is negligible.

The differential photoreceptor labeling patterns seen in autoradiographic and immunocytochemical experiments is noteworthy. Figures 4 and 5 clearly show the persistence of D-Asp transport by Müller cells in GLAST$^{-/-}$ mice. However, it is also evident that rod photoreceptor transport is apparently detected by autoradiography but not by immunocytochemistry. This phenomenon is consistent with other comparisons of D-Asp transport by autoradiography (Marc and Lam, 1981) and immunocytochemistry (Marc et al., 1995). This is attributable to two key quantitative differences in such experiments: (1) the mathematics of transport kinetics at high (immunocytochemical) and low (autoradiographic) substrate levels; and (2) the differential transport geometries of Müller cells and rods that impact the measurement of K_m.

A simplistic explanation of kinetic differences shows how radically the parameters of transport influence uptake patterns. For immunocytochemistry, use of substrate concentrations $C \gg K_m$ reduces the net time integral solution of the transport differential equation to

$$C = V_m t$$

where K_m is the half-saturation constant, V_m is the maximal velocity, t is the incubation time, and C is the net transported concentration. Thus, in these studies, the ratio between rod and Müller cell transport is directly proportional to V_m alone and independent of K_m and C. Since V_m is known to be a partial function of surface area, Müller cell transport measured by immunocytochemistry must be greater than photoreceptor transport by some ratio Ri = (Müller cell V_m/rodV_m). Immunocytochemistry provides a unique estimate of R and for the data reported here, R is likely >10. Our images are nearly identical to those of Barnett and Pow (2000), who also show no significant D-Asp transport by normal rat rods using immunocytochemistry.

For autoradiography, use of substrate concentrations $C \ll K_m$ yields a different net time integral solution of the transport differential equation:

$$C = C(0)\exp(t/\tau)$$

where C(0) is the initial concentration, t is the incubation time, and τ is the transport time constant K_m/V_m. Thus, transport signals in autoradiography are dependent on all three factors: substrate level, V_m and K_m. The autoradiographic transport ratio is Ra = exp[(Müller cell t) − (rod t)] and the consistent presence of rod labeling argues that the K_m value for D-Asp transport in rods is lower than that of Müller cells. Autoradiography is uniquely sensitive to K_m where as immunocytochemistry is not. Thus, the differences in labeling pattern of rods implies that they have a higher in situ affinity (lower K_m) for D-Asp than Müller cells. Immunocytochemistry is blind to this difference, but autoradiography is not.

Transport in tissue slices or intact retinas yields different kinetic parameters based on geometry and require methods that either mathematically recognize layering and tortuosity effects (Wood and Sidhu, 1986) or biochemically abrogate them (Müller and Marc, 1990). Put simply, thicker slices artifactually increase measured K_m values and cells that are distributed across slices will have higher K_m values than those will at the margins. Our measured transport K_m of 23–24 μM likely reflects the in situ Müller cell K_m and our autoradiography suggests that the ratio of Müller cell and rod in situ K_m values is actually on the order of 0.1, completely consistent with the gauntlet effect described by Wood and Sidhu (1986). Thus, both immunocytochemistry and autoradiography accurately reflect different kinetic parameters of D-Asp transport.

Because GLAST is a major glutamate transporter in the mammalian retina, loss of GLAST would have been expected to render the retina susceptible to excitotoxic damage. However, the GLAST$^{-/-}$ mouse retina normally shows a benign phenotype (Harada et al., 1998). What factors account for glutamate homeostasis in the absence of GLAST? One possibility is that GLT-1 and EAAC1 are able to remove efficiently the increased extracellular glutamate that is expected to accumulate in the absence of GLAST. GLT-1 is expressed by some photoreceptors and bipolar cells, and EAAC1 is found in the inner retina (Rauen et al., 1996). It is possible that these EAATs can clear glutamate quite efficiently, in the absence of GLAST. EAAT4, expressed by astrocytes may also play a role in glutamate clearance in the nerve fiber layer. In contrast, EAAT5, which is known to be expressed by photoreceptors is unlikely to play a significant role since the transporter is not present at synaptic regions but is located elsewhere (Pow and Barnett, 2000). Our uptake data in Na^+-free conditions further indicate that the EAATs together account for

less than 40% of glutamate uptake in the absence of GLAST. Therefore, it is not clear how these transporters can handle the large amounts of glutamate continuously released from rods in the dark.

A second possibility is that Müller cells may express other EAATs to compensate for the loss of GLAST. This situation can be ruled out because the patch-clamp studies clearly show that retinal Müller cells from GLAST$^{-/-}$ mice lack functional electrogenic glutamate transporters; thus there is no compensatory upregulation of EAATs in the Müller cells. Moreover, this observation is in accord with the results reported by Voutsinos-Porche et al. (2003), who found no changes in the levels of GLT-1 or EAAC1 in cortical membrane fractions isolated from GLAST$^{-/-}$ and wild-type mice.

Perhaps the most likely explanation is that other glutamate transporters expressed by Müller cells can take over for GLAST function under normal conditions. This idea is supported by our experiments on D-Asp uptake, which show that Müller cells devoid of GLAST can accumulate exogenous glutamate at a level comparable to that seen in the normal retina. In the present work, we have examined the involvement of three potential candidates: (1) ASCT-1 an electroneutral, Na^+-dependent, zwitterionic amino acid transporter that can transport aspartate and glutamate; (2) AGT-1, high-affinity, Na^+-independent glutamate transporter; and (3) the Na^+-independent, cystine-glutamate exchanger (Palacin et al., 1999). Although we found ASCT1 in Müller cells, it is not a likely candidate because aspartate or glutamate is a poor substrate for ASCT-1 (Arriza et al., 1993; Shafqat et al., 1993). ASCT-2 can also be ruled out because its K_m for glutamate is 1.6 mM (Utsunomiya-Tate et al., 1996).

Because Müller cells express high levels of cystine-glutamate exchanger (Tomi et al., 2003), we thought this transporter would be good candidate for glutamate uptake. The uptake autoradiography experiment, however, showed that glutamate uptake by Müller cells was not affected by CPG, a well-known inhibitor of cystine-glutamate exchanger. Also, glutamate uptake by Müller cells was completely Na^+-dependent in the GLAST$^{-/-}$ mouse retina. Finally, the involvement of AGT-1 could be ruled out because we did not find AGT1 transcripts in retinas from either the wild-type or the GLAST$^{-/-}$ mice. These considerations lead us to conclude that, in addition to GLAST and ASCT1, retinal Müller cells must express unidentified Na^+-dependent, nonelectrogenic glutamate transporters that are important for regulating glutamate levels in the retina. Finally, one outcome of these studies is that glial cells express multiple glutamate transporters whose relative roles in regulating glutamate excitotoxicity remains to be explored.

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