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Diversity of glutamate transporter expression and function in the mammalian retina

T. Rauen

Max-Planck-Institut für Hirnforschung, Frankfurt, Federal Republic of Germany

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Summary. Glutamate is the major excitatory neurotransmitter of the mammalian retina and glutamate uptake is essential for normal transmission at glutamatergic synapses.

Between photoreceptors and second order neurons, increases in light intensity are signaled by decreases in the concentration of glutamate within the synaptic cleft. In such a system the precise control of glutamate in the synaptic cleft is thus essential and glutamate transporters are thought to contribute to this process. As demonstrated here, all neuronal and macroglial cells of the retina appear to express high-affinity glutamate transporters. GLAST1, GLT1, EAAC1 and EAAT5 are expressed in the retina and exhibit unique localisation and functional properties. In the present study we summarize retinal glutamate transporter expression, identify the major glutamate uptake site in the mammalian retina and discuss the possible functional roles of different glutamate transporter subtypes in glutamatergic neurotranmission in the retina.

Keywords: Amino acids – Glutamate – Transporter – Retina – Expression – Transmission

Introduction

Glutamate is the major excitatory neurotransmitter in the mammalian brain and retina, where it interacts with different types of glutamate receptors at synapses. In the retina, glutamate is the excitatory transmitter in the vertical signal transmission pathway from photoreceptors to ganglion cells (Massey and Miller, 1987, 1990). In the outer retina, glutamate is released continuously from photoreceptors in darkness, and this release is modulated by light (Trifonov, 1968; Dowling and Ripps, 1972; Cervetto and MacNichol Jr., 1972; Murakami et al., 1972; Copenhagen and Jahr, 1989). In the inner plexiform layer (IPL), glutamate is released from two basic types of bipolar cells: ON-bipolar cells release glutamate in the light, whereas OFF-bipolar cells release

glutamate in the dark. Photoreceptors and bipolar cells do not generate action potentials, but instead respond to light with graded potentials that in turn modulate the continuous release of glutamate. In such a system, it is critical to precisely control the glutamate concentration in the synaptic cleft, not only because of the neurotoxic effects of glutamate at high concentration, but also because the glutamate concentration represents the light signal (Copenhagen et al., 1983; Olney et al., 1986; Choi, 1992; Romano et al., 1995). Glutamate transporters are ultimately responsible for maintaining low extracellular glutamate concentrations and thus play a key role in regulating the balance between physiological signaling and pathological overactivation (Nicholls and Attwell, 1990; Rauen et al., 1998). Five related but distinct eukaryotic high-affinity glutamate transporters have been cloned to date: GLAST1 (Storck et al., 1992), GLT1 (Pines et al., 1992), EAAC1 (Kanai and Hediger, 1992), EAAT4 (Fairman et al., 1995), and EAAT5 (Arriza et al., 1997). Homologs of GLAST1 (EAAT1), GLT1 (EAAT2) and EAAC1 (EAAT3) have been identified in human brain (Arriza et al., 1994). In the mammalian retina, these transporter subtypes are differentially expressed and have specific distributions in different cell types (Rauen et al., 1996). In the present study we summarize retinal glutamate transporter expression, identify the major glutamate uptake site in the mammalian retina and discuss the possible functional roles of different glutamate transporter subtypes in glutamatergic neurotranmission in the retina.

Material and methods

Preparation of membrane vesicles from isolated rat retinae

Adult albino rats (Wistar) or pigmented rats (Brown Norway) of either sex were deeply anesthetized with halothane and decapitated, in accordance with the guidelines and policies approved by the Society for Neuroscience. Isolated retinae cells were homogenized on ice in 0.12 M potassium phosphate buffer, pH 6.8, containing 0.32 M mannitol, 1 mM $MgSO_4$, 0.5 mM K-EDTA and 1x Protease Inhibitor Cocktail (Boehringer Mannheim, Germany) (homogenization buffer) and centrifuged for 10 min at 800 × g to remove nuclei, cell debris and undisrupted cells (P1 fraction). The supernatant was centrifuged for 20 min at 27,000 × g at 4°C. The resulting pellet (P2 fraction) was suspended and diluted in 5 mM Tris/HCl and 1 mM K-EDTA, pH 7.0. After osmotic shock, the membranes were collected by centrifugation at 27,000 × g for 20 min at 4°C. The pellet was resuspended in homogenization buffer (1–2 mg protein/ml), divided into aliquots and frozen in liquid nitrogen. Under these conditions, transport activity of the membrane vesicles was stable for at least 2 months.

Volume determination of membrane vesicles derived from total rat retina was done essentially as described (Kanner and Sharon, 1978). The internal volume for L-[^{3}H]glutamate was found to be 3.4 ± 0.23 μl/mg of protein. When the determination was done with D-[^{3}H]glucose an internal volume of 4.1 ± 0.31 μl/mg protein was obtained.

L-[^{3}H]glutamate transport assay

Membrane vesicle preparations of isolated retinae were loaded with 0.32 M mannitol, 0.5 mM EDTA and 1 mM $MgSO_4$ in 120 mM potassium phosphate buffer, pH 6.8 (internal

medium) as described previously (Rauen et al., 1992; Rauen et al., 1998). Subsequently, 20 μl (1–2 mg protein/ml) of membrane vesicles were diluted at room temperature into 180 μl external solution supplemented with 1 μCi of L-[^{3}H]glutamic acid (46–56 Ci/mmol). The L-[^{3}H]glutamate uptake was terminated by addition of 2 ml of an ice-cold stopping solution (0.15 M NaCl, 0.32 M mannitol) and by rapid filtration through cellulose acetate filters, which were quickly rinsed with 4 ml of ice-cold stopping solution. Parallel incubations were carried out at 4°C to correct uptake rates for background and non-specific uptake. All concentration-dependent assays contained 1 μCi of L-[^{3}H]glutamate diluted as necessary with cold substrate. Affinity constants (K_m) were determined from uptake measurements at 13 different substrate concentrations. After filtration and washing, radioactivity was determined using liquid scintillation counting. Experiments were done at least in triplicate. All values reported are the mean ± SD of at least three independent observations.

Autoradiographic study of L-[^{3}H]glutamate uptake by rat retina

Isolated rat retinae were placed in 2 ml of oxygenated Hanks' Balanced Salt Solution (containing 1 g/l D-glucose) prewarmed to 37°C. After a preliminary incubation for10 min in the presence or absence of 10 mM DL-methionine-DL-sulfoximine (MSO) at 37°C, L-[^{3}H]glutamate was added to give a final concentration of 1 μM and the incubation was continued for various times (5, 15 and 30 min). The L-[^{3}H]glutamate uptake was terminated by 3 washes in 5 ml of ice-cold stop medium (50 mM Tris/HCl, pH 7.4; 150 mM LiCl and 1 mM L-glutamic acid). Then the tissue was fixed overnight with 4% paraformaldehyde and 0.1% glutaraldehyde in 50 mM phosphate buffer and 150 mM NaCl pH 7.4 (PBS). After several rinses in PBS containing 5% sucrose, the retinae were cryoprotected by immersion in ascending sucrose concentrations (10%, 20%, 30%). After cryoprotection, the retinae were cut into 12 μm thick vertical sections on a cryostat and collected onto slides coated with poly-D-Lysine (1 mg/ml). The sections were dehydrated in a graded series of ethanol, dipped in Ilford L-4 emulsion and stored with desiccant at 4°C for 2–4 weeks. After exposure, the slides were developed in Kodak D19, washed, fixed, coverslipped with Permount (Fisher Scientific, Fair Lawn, NJ) and examined with bright-field and Nomarski optics (Carl ZEISS, Axiomat).

Immunocytochemistry

Isolated retinae were immersion-fixed in 4% paraformaldehyde in 100 mM PBS for 30 min or 1 h at room temperature, respectively. After several washes in PBS, retinae were cryoprotected in 30% sucrose in PBS, cut into 15 μm thick vertical sections on a cryostat, collected onto slides coated with poly-D-lysine (1 mg/ml), air-dried, and stored at −20°C. For immunocytochemistry, retinal sections or Müller cell cultures were preincubated for 1 h at room temperature with 4% (v/v) normal goat serum (NGS) diluted in 0.1% (v/v) Triton X-100 and Tris-buffered saline, pH 7.2 (TBS). Subsequently the sections were incubated (24 h, 4°C) with affinity-purified transporter antibody (generously donated by J. D. Rothstein, Baltimore, MD, USA), at a concentration of 0.05 μg/ml for GLT1, 0.08 μg/ml for GLAST1 and 0.04 μg/ml for EAAC1 in TBS containing 0.1% Triton X-100 and 2% NGS.

Following primary antibody incubation, sections were rinsed (30 min) in TBS, incubated (1 h) with anti-rabbit IgG conjugated to Cy3 (1:1,000). The secondary antibody was diluted in TBS containing 2% (v/v) NGS and 0.1% (v/v) Triton X-100. After the final incubation, sections were rinsed (30 min) in TBS and coverslipped in Mowiol (Hoechst, Germany).

Controls consisted of omission of the primary antibody or substitution with nonimmune rabbit serum. No specific labelling was observed in any of the controls.

Results

Cellular localization of GLAST1, GLT1, EAAC1 and the major L-[^{3}H]glutamate uptake site in rat retina

Immunocytochemical studies in retinal tissue exhibited the quantitative differential expression of the three glutamate transporters and showed furthermore a characteristic cellular expression pattern for each subtype in the mammalian retina (Fig. 1A, B, C). GLAST1 was localized only in glial cells; whereas, GLT1 and EAAC1 were detected solely in neuronal cells.

GLT1 is preferentially expressed by different types of bipolar cells and possibly in photoreceptor cells. Several characteristic horizontal bands of labelling can be identified within the inner plexiform layer (IPL) (Fig. 1A). Two of these bands are more intense and they represent bipolar cell axon terminals as shown previously (Rauen et al., 1996). Using a different antiserum raised against the entire GLT1 protein (Danbolt et al., 1992), immunoreactivity for GLT1 has been detected in cone photoreceptors in addition to the staining of bipolar terminals reported here (Rauen and Kanner, 1994), suggesting that the two antisera recognize different epitopes and therefore possibly different isoforms of GLT1 in the rat retina.

EAAC1 expression is found in horizontal cells, amacrine cells, ganglion cells and faintly in cone bipolar cells (Fig. 1B). In the case of horizontal cells and amacrine cells this is surprising, because these neurons are not gluta-

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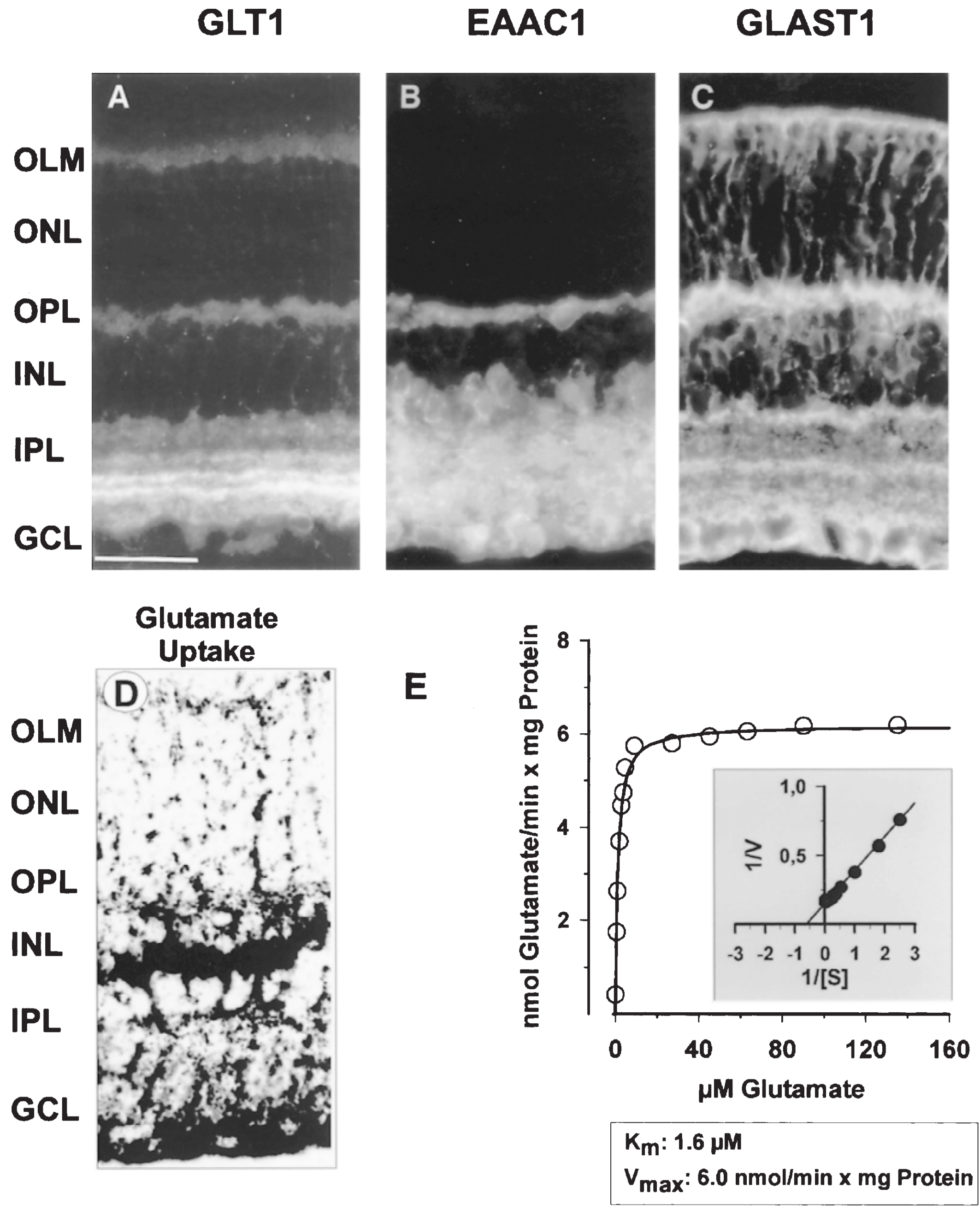
Fig. 1. Cellular localization of GLT1 (**A**), EAAC1 (**B**), GLAST1 (**C**), the major glutamate uptake site (**D**) in the rat retina and the kinetics of retinal glutamate uptake (**E**). Fluorescence micrographs of vertical cryostat sections through rat retinae that were immunolabeled for different glutamate transporters, as indicated. The retinal layering: *OLM* outer limiting membrane; *ONL* outer nuclear layer; *OPL* outer plexiform layer; *INL* inner nuclear layer; *IPL* inner plexiform layer; *GCL* ganglion cell layer is indicated on the left. Scale bar: 50 μm. **A.** GLT1 immunoreactivity is prominent in the inner plexiform layer (IPL), where several horizontal bands can be recognized. Two narrow bands in the inner half of the IPL are labeled most intensively. **B.** EAAC1 immunoreactivity is prominent in the OPL and in the IPL. The cell bodies of putative horizontal cells, cone bipolar cells, amacrine cells, displaced amacrine cells and ganglion cells express EAAC1. **C.** GLAST1 immunoreactivity is found throughout the retina, from the outer to the inner limiting membrane in Müller glial cells and astrocytes. **D.** Cellular localization of L-[^{3}H] glutamate uptake into intact rat retina. Isolated rat retinae were incubated with 1 μM L-[^{3}H] glutamate in the presence of 10 mM of the GS inhibitor MSO. Müller cells showed strong accumulation of L-[^{3}H]-glutamate. **E.** Kinetic properties of L-[^{3}H]glutamate uptake into retinal membrane vesicle preparations. Uptake velocity (in *nmol glutamate (min × mg protein)*) was measured by using 1 μCi of L-[^{3}H] glutamate and increasing concentrations of unlabelled L-glutamate at a membrane vesicle protein concentration of 40 μg and taken in the linear range of uptake (15 and 30 s). The data presented in the Michaelis-Menten plot are the mean of triplicate determinations from three representative experiments. The K_m and V_{max} values were obtained from the Lineweaver-Burk plot (*inset*) and represent the mean ± SEM of three independent experiments

matergic, but rather release GABA and/or glycine as their neurotransmitters (for review: (Massey and Redburn, 1987)). In the mammalian retina, horizontal and amacrine cells also express glutamic acid decarboxylase (GAD), an enzyme that catalyzes the conversion of glutamate to GABA (Vardi et al., 1994). Thus, in horizontal and amacrine cells, EAAC-1 might constitute a postsynaptic mechanism for supplying glutamate.



The most abundant retinal glutamate transporter, GLAST1, is expressed in Müller cells and their processes, with strongest immunoreactivity in the outer plexiform layer (OPL) and in several bands in the IPL (Fig. 1C). Müller cell perikarya in the outer part of the INL and their descending processes in the inner part of the INL were also labelled. Neuronal cell somata in the inner part of the INL and in the ganglion cell layer (GCL) remained unlabelled. Below the ganglion cells, close to the inner limiting membrane (ILM), GLAST1 immunolabelling appeared in cell bodies and processes that have been identified previously as astroglia (Derouiche and Rauen, 1995; Rauen et al., 1996). Comparison of GLAST1 immunoreactivity with that of the Müller cell marker glutamine synthetase (GS) (Riepe and Norenberg, 1977) revealed a very similar staining pattern (compare Fig. 6A and B in Rauen et al., 1998), but in contrast to the membranous staining of GLAST1 (Fig. 1C), the glutamine synthetase staining appeared to be cytoplasmic. The staining intensity of GLAST1 was not evenly distributed throughout Müller cells but appeared to be much more concentrated in the OPL and IPL where the lateral branches of Müller cell processes tightly ensheath glutamate-releasing photoreceptor and bipolar cell terminals.

To gain an insight into the function of the differently expressed glutamate transporter subtypes in retinal glutamate clearance, intact retinae were incubated with radiolabeled glutamate. After irreversible inhibition of glutamine synthetase (GS) by DL-methionine-DL-sulfoximine (MSO) (Meister, 1974), intense labelling was observed in perikarya and processes which span the entire retina from the inner to the outer limiting membrane (Fig. 1D). Comparing both the retinal expression pattern of GLAST1 (Fig. 1C) and the autoradiogram of retinal glutamate uptake (Fig. 1D) suggests that GLAST1 in Müller glial cells plays a major role in transmitter clearance in retinal tissue.

The retinal L-[^{3}H]glutamate uptake as a function of the glutamate concentration demonstrated 1st order kinetics with a K_m of $1.6 \pm 0.3 \mu M$ and a V_{max} of 6.0 ± 0.4 nmol/min $\times$ mg protein (Fig. 1E). This uptake was sodium-dependent and is consistent with the K_m value determined for heterologously expressed GLAST1 (Stoffel and Blau, 1995). Given a total intravesicular volume of approximately $4 \mu l$ per mg of protein (see methods), we calculated an average internal glutamate concentration of between 30 and $100 \mu M$, corresponding to a concentration gradient of 300–1,000 fold. This is probably a lower limit since not all membrane vesicles will be able to accumulate glutamate.

Discussion

In the vertical signal transmission pathway through the retina, synaptically released glutamate acts at different types of glutamate receptors on bipolar cells, horizontal cells, amacrine cells and ganglion cells (Brandstätter et al., 1998). The interaction between glutamate and its receptors determines the time course of the response in postsynaptic neurons and thus the responses to

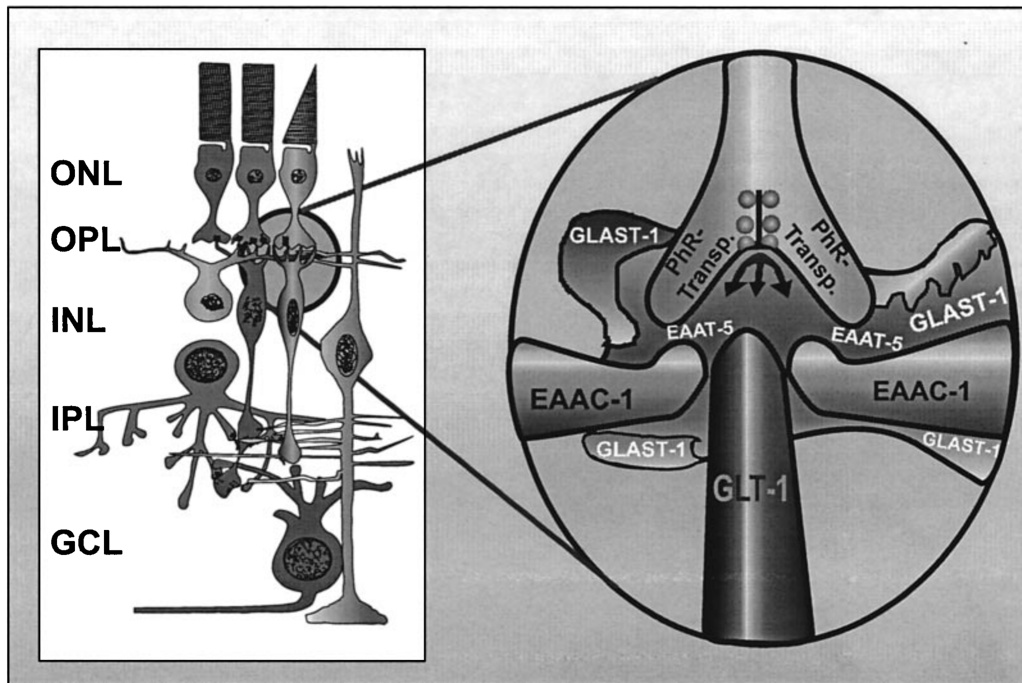


Fig. 2. Schematic diagram of glutamate transporter distribution at the photoreceptor synapse. *Left panel*: Schematic drawing of the cellular architecture of the mammalian retina. The retinal layering: Inner segments (IS); *ONL* outer nuclear layer; *OPL* outer plexiform layer; *INL* inner nuclear layer; *IPL* inner plexiform layer; *GCL* ganglion cell layer is indicated on the left. *Right panel*: The magnification of the first synapse in the retina illustrates that all components of the photoreceptor synapse contain glutamate transporters. In the postsynaptic area at the triad, GLT1 is expressed in bipolar cell dendrites. In the lateral elements, EAAC1 is found in horizontal cell processes. In the surrounding cell processes of Müller glial cells, GLAST1 is densely expressed and in lower concentrations also EAAT5 (unpublished observation). Finally, electrophysiological experiments suggest the presence of high-affinity glutamate transporter(s) in presynaptic photoreceptor terminals

light stimuli. Considering the tonic release of glutamate at glutamatergic synapses in the retina, it is crucial for their normal function to tightly control the extracellular glutamate concentration so that the receptors are not in the state of constant saturation. This is achieved essentially by high-affinity glutamate transporters localized in regions abutting the synapse or at the synapse itself. As demonstrated here and previously, all neuronal and macroglial cells of the retina appear to express high-affinity glutamate transporters (Rauen and Kanner, 1994; Derouiche and Rauen, 1995; Rauen et al., 1996; Lehre et al., 1997; Rauen et al., 1998; Eliasof et al., 1998). Moreover, all components of the photoreceptor synapse contain glutamate transporters (Fig. 2). In the postsynaptic side of the triad, GLT1 is expressed in bipolar cell dendrites. In the lateral elements, EAAC1 is found in horizontal cell processes. In the surrounding cell processes of Müller glial cells, GLAST1 is

densely expressed (Derouiche and Rauen, 1995) and also EAAT5 (Eliasof et al., 1998, Rauen, unpublished observation). Electrophysiological experiments suggest the presence of high-affinity glutamate transporter(s) in presynaptic photoreceptor terminals (Eliasof and Werblin, 1993; Picaud et al., 1995; Grant and Werblin, 1996). However, the molecular identity of the photoreceptor glutamate transporter(s) is still unclear. Nevertheless, one remarkable result is the observation that the activation of the photoreceptor transporter(s) is associated with a chloride conductance that can serve to mediate a hyperpolarizing response. This glutamate transporter-associated, chloride current is rapidly activated by glutamate, is dependent on Na^+ and is especially pronounced in EAAT4 and EAAT5 (Fairman et al., 1995; Wadiche et al., 1995; Arriza et al., 1997). These mixed characteristics of glutamate transporters – classical carrier properties and ligand-gated chloride channel properties – are of great physiological implication, because this would provide a potential mechanism to compensate for the depolarizing action of transmitter uptake (Wadiche et al., 1995).

Although no simple correlation between transporter subtype and retinal cell physiology can be made, the diverse population of glutamate transporter subtypes with unique localization and functional properties indicates that glutamate transporters play a wide variety of roles in retinal function. A characteristic of likely functional relevance is the chloride conductance with the different glutamate transporters; indeed, the size of the chloride conductance relative to transport capacity can vary dramatically between different transporter subtypes (Arriza et al., 1997). For example, the glutamate-elicited current in EAAT5 is carried largely by chloride ions, in contrast to the rather small but measurable chloride conductance associated with GLAST1, GLT1 and EAAC1 activity. Thus, EAAT5 may function more in providing positive feedback than in removing glutamate, and, through its hyperpolarzing action furthermore may increase the uptake capacity of GLAST1 in Müller cells. Both GLT1 and EAAC1 show neuronal localization. The presence of EAAC1 in GABAergic (non-glutamatergic) horizontal and amacrine cells, however, indicates that it may facilitate glutamate uptake for metabolic reasons. The synaptic terminal localization of GLT1 in glutamatergic neurons may represent a mechanism for ensuring a high signal-to-noise ratio for synaptic transmission in the retina. It is still too early to draw final conclusions for the specific function of different glutamate transporters in retinal synaptic transmission, considering that not all transporters have yet been identified and less is known about the specific properties of each transporter subtype. However, this study suggests a major role for the glial glutamate transporter GLAST1 in retinal transmitter clearance and emphasizes the importance of Müller glial cells in excitatory transmission in the retina.

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Author's address: Dr. Thomas Rauen, Max-Planck-Institut für Hirnforschung, Deutschordenstr. 46, D-60528 Frankfurt/M., Federal Republic of Germany,
Fax +49 (69) 96769 206; e-mail: Rauen@MPIH-Frankfurt.MPG.de

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