

Functions of the two glutamate transporters GLAST and GLT-1 in the retina

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ABSTRACT In the retina, the glutamate transporter GLAST is expressed in Müller cells, whereas the glutamate transporter GLT-1 is found only in cones and various types of bipolar cells. To investigate the functional role of this differential distribution of glutamate transporters, we have analyzed GLAST and GLT-1 mutant mice. In GLAST-deficient mice, the electroretinogram b-wave and oscillatory potentials are reduced and retinal damage after ischemia is exacerbated, whereas GLT-1-deficient mice show almost normal electroretinograms and mild increased retinal damage after ischemia. These results demonstrate that GLAST is required for normal signal transmission between photoreceptors and bipolar cells and that both GLAST and GLT-1 play a neuroprotective role during ischemia in the retina.

L-Glutamate is the major excitatory neurotransmitter in the mammalian retina (1). High-affinity glutamate transporters are believed to be essential for terminating synaptic transmission as well as for keeping the extracellular glutamate concentration below neurotoxic levels (1, 2). Five subtypes of glutamate transporter (GLAST, GLT-1, EAAC1, EAAT4, and EAAT5) (3–8) have been cloned, but the contributions of individual transporter subtypes to retinal function are poorly understood. Studies have been hampered by the lack of subtype-selective glutamate transporter drugs. As an alternative approach, we have analyzed GLAST- and GLT-1-deficient mice (9, 10). Our results demonstrate that GLAST is required in retinal signal transmission at the level of the photoreceptor and bipolar cell and that GLAST and GLT-1 are crucial for the protection of retinal cells from glutamate neurotoxicity.

MATERIALS AND METHODS

Immunohistochemistry. Mice were anesthetized with diethyl ether and perfused transcardially with saline, followed by 4% paraformaldehyde in 0.1 M sodium phosphate buffer containing 0.5% picric acid at room temperature. Eyes were removed and postfixed overnight in the same fixative, and 7- μ m-thick paraffin or frozen sections were cut and mounted onto gelatin- and poly-L[D]-lysine-coated slides. The sections were incubated overnight with an affinity-purified rabbit polyclonal antibody against the carboxyl-terminal sequence of the mouse GLAST (1.0 μ g/ml) (KKPYQLIAQDNEPEKPVAD-SETKM) (11, 12), an affinity-purified rabbit polyclonal antibody against the rat GLT-1 (0.2 μ g/ml) [anti-B12; gift from N. C. Danbolt] (13), or a mouse monoclonal antibody against glutamate synthetase (GS) (2.0 μ g/ml) (Chemicon) at room

temperature. The sections were then incubated with biotinylated goat anti-rabbit IgG (Nichirei, Tokyo) for GLAST and GLT-1 or biotinylated rabbit anti-mouse IgG (Nichirei) for GS for 1 hr, followed by further incubation with streptavidin-Texas red (NEN) for 30 min at room temperature. Sections were examined by a confocal laser scanning microscope (Molecular Dynamics).

Electroretinograms (ERGs). Mice (9–11 weeks old) were anesthetized by intraperitoneal injection of a mixture of xylazine (10 mg/kg) and ketamine (25 mg/kg). The pupils were dilated with 0.5% phenylephrine-hydrochloride and 0.5% tropicamide. A carbon fiber electrode was placed on the corneal surface, and a reference electrode was attached subcutaneously on the forehead. Single-flash ERGs were recorded after dark adaptation for more than 30 min. The animal's position was secured with a bite board and head holder to ensure a 30-cm distance between the photostimulator (SLS-3100, Nihon Kohden, Tokyo) and both eyes for all experiments. White test flashes of 10- μ s duration, with an intensity of 0.6 or 1.2 J, were presented. A bandpass frequency setting of 50–1000 Hz and 1–1000 Hz on the amplifier (Nihon Kohden, MEB-5304) was used to record the oscillatory potentials (OPs) and the a- and b-waves, respectively. The two responses were averaged with an averager (Nihon Kohden, MEB-5304). The a-wave amplitude was determined from the baseline to the bottom of the a-wave. The b-wave amplitude was determined from the baseline to the top of the b-wave. The OPs consisted of three to four wavelets (OP1–OP4). Because the third and fourth wavelets (OP3 and OP4) were missing in some instances, we limited the measurement to the constantly recordable OP1 and OP2 wavelets.

Induction of Retinal Ischemia. Adult mice (7–10 weeks old) were anesthetized with intraperitoneal injections of pentobarbital (60 mg/kg). Ischemia was achieved and the animals were treated essentially as described (14). Briefly, we instilled sterile saline into the anterior chamber of the right eye at 150 cm H₂O pressure for 60 min while the left eye served as nonischemic control. The animals were sacrificed 7 days after reperfusion, and eyes were enucleated for histological and morphometric study.

Histology and Morphometric Studies. The enucleated eyes were fixed in 4% paraformaldehyde and 1% glutaraldehyde buffered with 0.2 M sodium cacodylate (pH 7.4), followed by 10% paraformaldehyde in 0.1 M sodium phosphate buffer (pH 7.4) and embedded in paraffin. The posterior part of the eyes was sectioned sagittally at 7 μ m thickness through the optic

This paper was submitted directly (Track II) to the *Proceedings* office. Abbreviations: GS, glutamate synthetase; ERG, electroretinogram; INL, inner nuclear layer; IPL, inner plexiform layer; OPL, outer plexiform layer.

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nerve, mounted, and stained with hematoxylin and eosin. For the estimation of the thickness of the inner retinal layer, measurements were performed with a calibrated reticle at 80 \times magnification (Kontron Elektronik, Imaging System KS100). Four sections of each eye were used for measurements. Five animals were used in each group. Results are presented as mean \pm SEM and *n* is the number of eyes examined for each group. Student's *t* test was used to estimate the significance of the results.

RESULTS

Localization of Glutamate Transporters GLAST and GLT-1 in the Mouse Retina. In the brain, GLAST and GLT-1 are localized in the glia (13, 15). However, in the rat retina, GLAST is expressed in Müller cells (16, 17), whereas GLT-1 is expressed only in a specialized and restricted set of neuronal cells (mainly cone photoreceptors and cone bipolar cells) (17, 18). We verified that these localizations are also true for the

mouse. Fig. 1 presents vertical sections through a mouse retina immunostained with GLAST or GLT-1 antibody. GLAST immunoreactivity is present throughout the retina, from the inner limiting membrane to the outer limiting membrane (Fig. 1 *D* and *F*). Antibodies raised against GS, a specific marker for Müller cells (16), showed a very similar staining pattern (Fig. 1 *J–L*), indicating that GLAST is predominantly expressed in Müller cells. GLT-1 immunoreactivity is present in a small fraction of photoreceptors (Fig. 1, *G* and *H*). The sparse population of their cell bodies and the termination of their axons in pedicles suggests that they are cones. In the INL, bipolar cell bodies are labeled. The findings are in agreement with those reported by Rauen and Kanner for the rat retina (18).

Histological and Immunohistochemical Analyses of the Retinae of GLAST- and GLT-1-Deficient Mice. We next examined whether the absence of GLAST or GLT-1 affects the anatomical organization of the retina by histological and immunohistochemical analyses. Hematoxylin and eosin staining revealed the retinae of GLAST or GLT-1 mutant mice to be normally organized, consisting of several different cell layers (Fig. 1 *A–C*). In the GLAST or GLT-1 mutant animal, immunoreactivity for GLAST or GLT-1, respectively, observed in the wild-type animal was completely absent (Fig. 1 *E* and *J*). Müller cells in the two mutant mice appeared normal when they were analyzed with the GS antibody (Fig. 1, *J–L*). Thus, there are no gross anatomical changes in the retina of GLAST- and GLT-1-deficient mice.

ERGs of GLAST- and GLT-1-Deficient Mice. To examine the physiological function of GLAST and GLT-1 in visual synaptic transmission *in vivo*, we analyzed single-flash ERG patterns of GLAST and GLT-1 mutant mice. The ERG response to a light flash can be divided into three waves, named a-wave, b-wave, and OPs (19). The a-wave arises principally from photoreceptors, whereas the b-wave and OPs originate mainly in ON bipolar cells and the amacrine/interplexiform cells, respectively (19–21). Although the GLAST mutant mice had a normal a-wave amplitude, their b-waves and OPs were attenuated by more than 50% compared with the wild-type mice (Fig. 2*A* and Table 1). The ratio of b-wave to a-wave amplitude (b/a ratio) was significantly lower in GLAST mutant mice than in the wild-type mice (Table 1). In contrast, no apparent difference was found in the amplitudes of a-wave, b-wave, and OPs and b/a ratio between GLT-1 mutant and wild-type mice (Fig. 2*B* and Table 1). In both GLAST and GLT-1 mutant mice, the latency of a- and b-wave was slightly prolonged (Fig. 2). The same results were also obtained at a lower illumination intensity. These results suggest that GLAST is essential for proper neurotransmission of the light response from photoreceptors to bipolar cells.

Ischemia-Induced Retinal Damage in GLAST- and GLT-1-Deficient Mice. Glutamate transporters are thought to play a major role in keeping the extracellular glutamate concentration below neurotoxic levels (1, 2). To investigate this pathological role of GLAST and GLT-1 *in vivo*, we subjected GLAST and GLT-1 mutant mice to retinal ischemia. Retinal ischemia was induced by increasing intraocular pressure above systolic pressure for 60 min, and retinal injury was evaluated morphologically and morphometrically by measuring the thickness of the inner retinal layers (IRL, between the internal limiting membrane and the interface of the OPL and the outer nuclear layer). The representative qualitative pictures of the changes induced by ischemia in wild-type and GLAST and GLT-1 mutant mice are shown in Fig. 3. Seven days after pressure-induced ischemia, a decrease of the thickness in the inner plexiform layer (IPL), disorganization of the INL, and few and atypical ganglion cells were seen in the wild-type mice (Fig. 3 *A* and *B*). In GLAST mutant mice, the degenerative changes in IRL were more severe than those in the wild-type mice. Almost

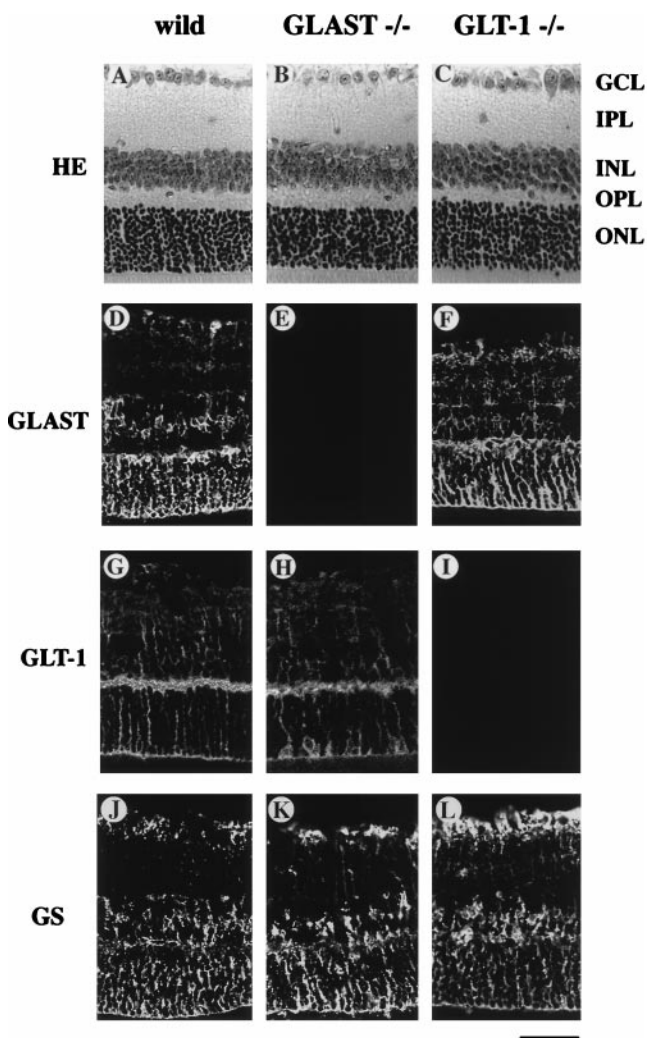


FIG. 1. Histological and immunohistochemical analyses of the retinae of the wild-type and GLAST and GLT-1 mutant mice. (*A–C*) Vertical sections of the adult mouse retinae from wild-type (*A*) and GLAST (*B*) and GLT-1 (*C*) mutant mice were stained with hematoxylin and eosin. GCL, ganglion cell layer; IPL, inner plexiform layer; INL, inner nuclear layer; OPL, outer plexiform layer; ONL, outer nuclear layer; HE, hematoxylin and eosin. (*D–L*) Sections of wild-type (*D*, *G*, and *J*) and GLAST (*E*, *H*, and *K*) and GLT-1 (*F*, *I*, and *L*) mutant mouse retinae were immunostained with the GLAST antibody (*D–F*), the GLT-1 antibody (*G–I*), and the GS antibody (*J–L*). (Scale bar = 50 μ m.)

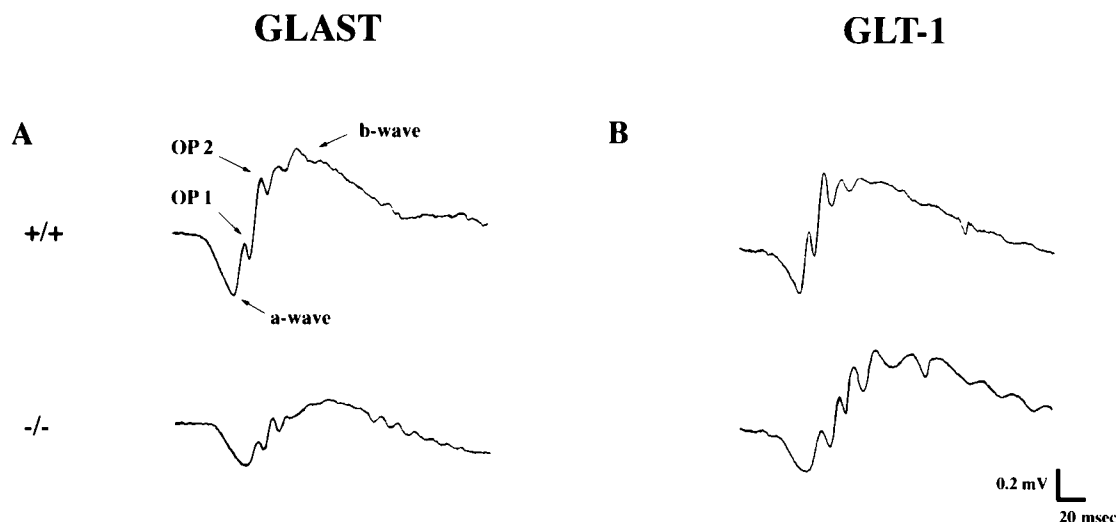


FIG. 2. ERGs of the wild-type and GLAST and GLT-1 mutant mice. (A) Dark-adapted ERG responses from the wild-type mouse (+/+) and GLAST mutant mouse (-/-). (B) Dark-adapted ERG responses from the wild-type mouse (+/+) and GLT-1 mutant mouse (-/-).

complete disappearance of the cells of the INL was observed in GLAST mutant mice. Furthermore, the nerve fiber layer, the IPL, and the OPL were thinner in GLAST mutant mice than in the wild-type siblings (Fig. 3D). In GLT-1 mutant mice, the ischemic degenerative changes were more severe than those in the wild-type mice, but clearly milder than those of the GLAST knockout mice (Fig. 3C).

Quantification of the effects of ischemia revealed that the thickness of IRL decreased from $101 \pm 3 \mu\text{m}$ in untreated controls ($n = 5$) to $71 \pm 4 \mu\text{m}$ in ischemic eyes of the wild-type mice ($n = 5$) (Fig. 3E). In GLAST mutant mice, a significant increase in ischemic damage was observed ($P < 0.05$). The IRL of ischemic eyes from GLAST mutant mice was $36 \pm 2 \mu\text{m}$ ($n = 5$). In contrast, GLT-1 mutant mice showed a significantly higher IRL ($53 \pm 3 \mu\text{m}$; $n = 5$; $P < 0.05$) than GLAST mutant mice. These data indicate that both GLAST and GLT-1 play an active role in preventing excitotoxic retinal damage after ischemia, but that GLAST participates more actively in the protection from ischemia than does GLT-1.

DISCUSSION

In the brain, GLAST is most abundantly expressed in the processes of Bergmann glia (15), cerebellar astrocytes associated with Purkinje cells (PCs). Bergmann glial processes surround the two distinct glutamatergic synapses onto PCs (i.e., parallel fiber and climbing fiber to PC synapses) (22). However, GLAST is not the dominant factor that determines the kinetics of excitatory postsynaptic synaptic currents at these synapses (10). In contrast, GLAST plays an essential role in the proper signal transmission in the retina. This finding is of interest because terminals of photoreceptors and bipolar cells have specific structural and functional features. They contain dense bars or ribbons anchored to the presynaptic membrane and covered with a layer of synaptic vesicles (23). In contrast to conventional synapses (i.e.,

parallel fiber and climbing fiber to PC synapses), which release neurotransmitter transiently, these ribbon synapses release neurotransmitter continuously (24). At the onset of a bright light, photoreceptors diminish their release of glutamate. Transmission of this effect to postsynaptic cells (bipolar cells) requires that the glutamate in the synaptic cleft be removed by either diffusion or reuptake. Therefore, GLAST is not required for proper synaptic transmission at conventional synapses but is essential for normal synaptic transmission at the photoreceptor synapse.

Our observation of the greater excitotoxic retinal damage in GLAST mutant mice than in GLT-1 mutant mice is compatible with the previous finding that accumulation of $[\text{D}]\text{-}[\text{H}]\text{glutamate}$ occurred predominantly in Müller cells (25). Furthermore, the occurrence of ischemic damage not only in IPL but also in OPL of GLAST mutant mice is in agreement with the previous finding that GLAST is expressed in Müller cell processes that ramify extensively in both IPL and OPL (16, 17). The results of our investigation indicate that both GLAST and GLT-1 play a neuroprotective role against ischemic retinal injury, but that GLAST participates more actively in the prevention of glutamate neurotoxicity after ischemia in the retina than does GLT-1. The neuroprotective role of GLAST and GLT-1 is in agreement with the previous finding that antisense knockout of GLAST and GLT-1 resulted in neurodegeneration characteristic of excitotoxicity (26). It has been suggested that under ischemia, glutamate neurotoxicity is exacerbated because of reversal of glutamate transport (27). However, our result indicates that GLAST and GLT-1 play a neuroprotective role during ischemia. Ischemic injury is implicated in a number of pathological states, such as central retinal artery occlusion, glaucoma, diabetic retinopathy, etc. Accordingly, the present results raise intriguing possibilities for the management of these pathological conditions with glutamate transporter activators, such as bromocryptine (28, 29).

Table 1. ERG responses

Mice	<i>n</i>	a-wave amplitude, μV	b-wave amplitude, μV	b/a amplitude ratio	OP1 amplitude, μV	OP2 amplitude, μV
Wild type	16	237.5 ± 26.2	461.9 ± 33.8	2.15 ± 0.19	173.8 ± 14.7	264.4 ± 17.7
GLAST -/-	8	253.8 ± 29.6	$217.5 \pm 30.9^*$	$0.98 \pm 0.21^*$	$72.8 \pm 7.6^*$	$114.3 \pm 12.4^*$
GLT-1 -/-	4	267.5 ± 66.9	475.0 ± 72.2	2.27 ± 0.78	137.5 ± 18.9	225.0 ± 23.6

All data are expressed as mean \pm SEM; *, $P < 0.005$ (two-tailed *t* test).

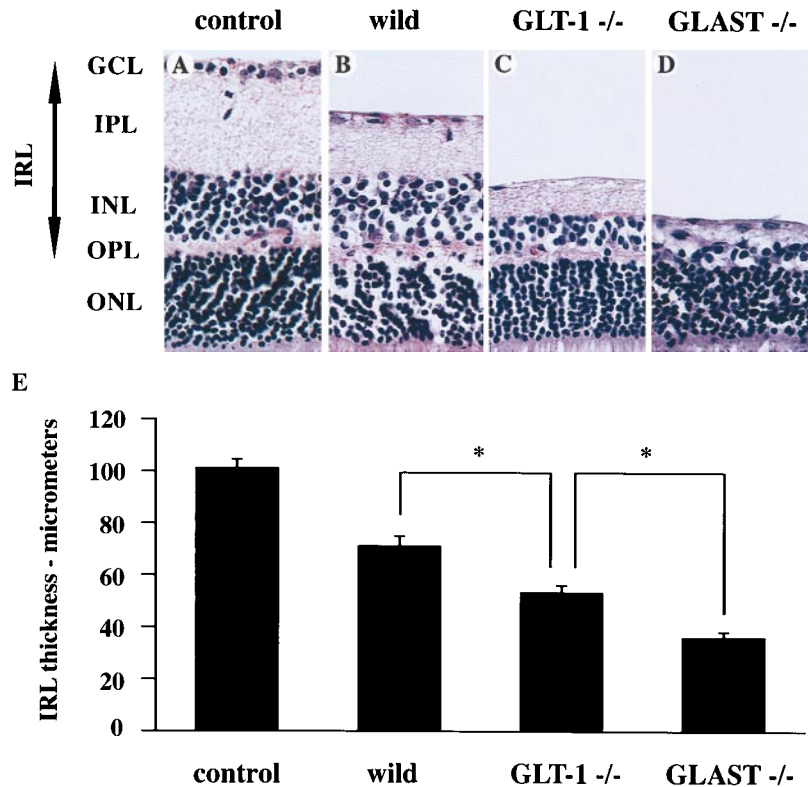


FIG. 3. The ischemic retinal changes in the wild-type and GLAST and GLT-1 mutant mice. (A) Micrograph of a section of a retina in the control eye taken from the wild-type mouse. (B–D) Ischemic retinæ from the wild-type (B) and GLT-1 (C) and GLAST (D) mutant mouse. IRL, inner retinal layer; other abbreviations as in Fig. 1. (E) Mean thickness of the inner retinal layers of the retinae in control eyes from the wild-type mice and in ischemic eyes from the wild-type and GLT-1 and GLAST mutant mice. Columns and error bars represent mean \pm SEM (*, $P < 0.05$).

To date, research on glutamate transporters in the retina has been conducted largely in isolated cells. Thus, GLAST and GLT-1 mutant mice will provide *in vivo* models for studying the role of glutamate transporters in retinal function.

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