Glaucoma, a progressive optic neuropathy due to retinal ganglion cell (RGC) degeneration, is one of the leading causes of irreversible blindness. Although glaucoma is often associated with elevated intraocular pressure (IOP), IOP elevation is not detected in a significant subset of glaucomas, such as normal tension glaucoma (NTG). Moreover, in some glaucoma patients, significant IOP reduction does not prevent progression of the disease. Thus, understanding IOP-independent mechanisms of RGC loss is important. Here, we show that mice deficient in the glutamate transporters GLAST or EAAC1 demonstrate spontaneous RGC and optic nerve degeneration without elevated IOP. In GLAST-deficient mice, the glutathione level in Müller glia was decreased; administration of glutamate receptor blocker prevented RGC loss. In EAAC1-deficient mice, RGCs were more vulnerable to oxidative stress. These findings suggest that glutamate transporters are necessary both to prevent excitotoxic retinal damage and to synthesize glutathione, a major cellular antioxidant and tripeptide of glutamate, cysteine, and glycine. We believe these mice are the first animal models of NTG that offer a powerful system for investigating mechanisms of neurodegeneration in NTG and developing therapies directed at IOP-independent mechanisms of RGC loss.
The potential role of glutamate transporters in the pathogenesis of normal tension glaucoma

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Glaucoma, a progressive optic neuropathy due to retinal ganglion cell (RGC) degeneration, is one of the leading causes of irreversible blindness. Although glaucoma is often associated with elevated intraocular pressure (IOP), IOP elevation is not detected in a significant subset of glaucomas, such as normal tension glaucoma (NTG). Moreover, in some glaucoma patients, significant IOP reduction does not prevent progression of the disease. Thus, understanding IOP-independent mechanisms of RGC loss is important. Here, we show that mice deficient in the glutamate transporters GLAST or EAAC1 demonstrate spontaneous RGC and optic nerve degeneration without elevated IOP. In GLAST-deficient mice, the glutathione level in Müller glia was decreased; administration of glutamate receptor blocker prevented RGC loss. In EAAC1-deficient mice, RGCs were more vulnerable to oxidative stress. These findings suggest that glutamate transporters are necessary both to prevent excitotoxic retinal damage and to synthesize glutathione, a major cellular antioxidant and tripeptide of glutamate, cysteine, and glycine. We believe these mice are the first animal models of NTG that offer a powerful system for investigating mechanisms of neurodegeneration in NTG and developing therapies directed at IOP-independent mechanisms of RGC loss.

Introduction

It is estimated that glaucoma affects nearly 70 million individuals worldwide, with at least 6.8 million bilaterally blind (1). The disease is characterized by a slow progressive degeneration of retinal output neurons (retinal ganglion cells [RGCs]) and their axons, which is usually associated with elevated intraocular pressure (IOP). The common adult-onset glaucoma is primary open-angle glaucoma (POAG), which is probably caused by a reduction in outflow of aqueous humor through the trabecular outflow pathways (2). Normal tension glaucoma (NTG), a subset of POAG that indicates statistically normal IOP, also shows glaucomatous optic neuropathy and relevant visual field defect. Several population studies have suggested that NTG represents 20%-90% of all POAG, with percentages seeming to vary according to race (3–5). Interestingly, IOP still seems to play a role in NTG because a substantial number of patients with NTG as well as other forms of POAG benefit from lowering of IOP (6). Thus, NTG may be caused by the vulnerability of optic nerves to normal range of IOP. However, it should be noted that some of NTG patients are still progressive in spite of sufficient IOP reduction. All these observations suggest a possibility that factors not dependent on IOP may contribute to disease progress and that elucidating such factors would be necessary to understand the pathogenesis of glaucoma and guide efforts toward improved therapeutics.

Recent studies have shown that glaucoma is affected by multiple genetic and environmental factors (7, 8), and this complexity can make it difficult to reach definitive conclusions (especially in human studies, in which many factors cannot be controlled). Animal studies complement human studies and can provide important insights into genetic etiology and molecular mechanisms for further assessment in people. There are now several published mouse models of pressure-induced RGC death. These include inherited and experimentally induced models (9). However, no animal model for NTG is currently known and available for research.

Besides more extensively investigated factors, such as reduced ocular blood flow, ocular vascular dysregulation, and systemic blood pressure alterations, excessive stimulation of the glutamatergic system, specifically the NMDA subtypes, has been proposed to contribute to death of RGCs in glaucoma. Glutamate transporter is the only mechanism for the removal of glutamate from the extracellular fluid in the retina (10). Therefore, it is hypothesized that the increase in glutamate may result from a failure of the glutamate transporters adjacent to RGCs. In the inner plexiform layer where synapses on RGCs exist, there are 3 transporters involved in this task: glutamate transporter 1 (GLT-1), located in the bipolar cell terminals; excitatory amino acid carrier 1 (EAAC1) in retinal neurons including RGCs; and glutamate/aspartate transporter (GLAST) in Müller cells (11). However, there is still debate on whether excitotoxic damage is involved in the pathophysiology of glaucoma (12). Here, we utilized mice in which 1 of each of these 3 glutamate transporters has been knocked out.

Nonstandard abbreviations used: EAAC1, excitatory amino acid carrier 1; GCL, ganglion cell layer; GLAST, glutamate/aspartate transporter; GLT-1, glutamate transporter 1; GS, glutamine synthetase; INL, inner nuclear layer; IOP, intraocular pressure; 2K, second-order kernel; mfERG, multifocal electroretinogram; NTG, normal tension glaucoma; ONL, outer nuclear layer; POMC, primary open-angle glaucoma; RGC, retinal ganglion cell.

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and examined the long-term effect of retinal morphology during postnatal development on RGC survival in vivo. We found that GLAST- and EAAC1-knockout mice showed spontaneously occurring RGC death and typical glaucomatous damage of the optic nerve without elevated IOP. To our knowledge, these glutamate transporter knockout mice are the first animal models of NTG, which offer a powerful system for determining mechanisms and evaluating new treatments of NTG.

Results
Degeneration of RGCs in GLAST- and EAAC1-deficient mice. Immunohistochemical studies demonstrated the presence of 3 subtypes of glutamate transporters in the inner plexiform layer of the rat retina. GLAST is expressed on Müller cells, GLT-1 on the bipolar cell terminals, and EAAC1 on ganglion cells (11). We verified that these localizations are also true for the mouse. As we previously reported (13), GLAST immunoreactivity is present throughout the retina and double-labeled by glutamine synthetase (GS), a specific marker for Müller glial cells (Figure 1A). EAAC1 and GLT-1 are neural-type glutamate transporters; however, their distribution is different. EAAC1 is mainly localized to the inner retinal layer and double-labeled by calretinin, a specific marker for RGCs and amacrine cells (Figure 1B). On the other hand, GLT-1 immunoreactivity is present in a small fraction of photoreceptors in the outer nuclear layer (ONL) in addition to bipolar cells but not in RGCs (Figure 1C). The findings are in agreement with those reported by Rauen for rat retina (11). The retinae of GLAST (14) and EAAC1 (15) mutant mice have normal organization at birth (Figure 2A). However, we noticed severe retinal degeneration in 8-month-old GLAST<sup>−/−</sup> mice (see also Figure 3, A and B), in
Degeneration of optic nerve in GLAST- and EAAC1-deficient mice. Degeneration of the optic nerve is one of the hallmarks of glaucoma. Consistent with severe RGC loss, optic nerve cupping (Figure 3B) and thinning of the optic nerve (Figure 5D) were apparent in 8-month-old GLAST−/− mutants. Quantitative analysis revealed that optic nerve area in GLAST−/− mice (0.074 ± 0.002 mm²; n = 6) was significantly decreased compared with that in WT mice (0.096 ± 0.005 mm²; n = 6, P = 0.0019). To analyze morphological changes in the optic nerve, semithin transverse sections were cut and stained with toluidine blue. The degenerating axons in 5-week-old GLAST−/− mutants had abnormally dark axonal profiles (Figure 5F). In addition, compared with WT mice (Figure 5E), the density of axons through the optic nerve was clearly declined in GLAST−/− mice (Figure 5F). We next examined the iridocorneal angle in GLAST−/− mutants and found that it was well formed with an obvious Schlemm canal and a normal extent of trabecular meshwork (Figure 5H). EAAC1−/− mutants also showed excavation of the optic nerve and normal iridocorneal angle morphology (data not shown).

IOP measurement in GLAST- and EAAC1-deficient mice. GLAST and EAAC1 mutant mice showed POAG-like phenotype, which suggests the involvement of increased IOP. Therefore, we examined the IOPs of WT, EAAC1−/−, and GLAST−/− mice. IOP measurement was carried out for both young (4 week) and adult (9 to 11 month) mice around 9 pm, when IOP is highest in mouse eyes (18). The IOPs of EAAC1−/− and GLAST−/− mice were not significantly increased compared with WT at both time points (Figure 5I). These results suggest that RGC loss and glaucomatous-like changes of the optic nerve in these mutants are IOP-independent.

Impaired visual function in GLAST-deficient mice. To determine whether the observed degeneration of RGCs and the optic nerve is functionally significant, we analyzed multifocal electoretinograms (mERGs) of GLAST−/− mouse retina. RGCs contribute to the human mERG response, and the second-order kernel (2K), which appears to be a sensitive indicator of inner retinal dysfunction (19), is impaired in glaucoma patients (20). The averaged 2K responses of 10 mice are shown in Figure 6A. The response topography demonstrated that the 2K component derived from GLAST−/− mice tended to be shorter in all visual fields compared with that of WT mice (Figure 6B). Quantitative analysis revealed that the 2K amplitude was reduced by about half in GLAST−/− mice compared with that in WT mice (P < 0.005) (Figure 6C), suggesting that RGC loss is functionally significant in GLAST−/− mice.

Role of glutamate neurotoxicity in RGC degeneration in GLAST-deficient mice. To determine the contribution of excitotoxicity to RGC death in these models, we first measured the intravitreal glutamate concentration in WT and mutant mice. Glutamate concentration was not significantly increased in GLAST or EAAC1 mutant mice (Figure 7A). Because an increase in glutamate concentration in the extracellular space just adjacent to RGCs might be obscured due to dilution within the vitreous (21), failure to detect glutamate elevation in the vitreous fluid does not necessarily exclude glutamate involvement in RGC degeneration in the mutants. To assess glu-
tamate involvement in RGC degeneration, we next examined the effect of the NMDA receptor antagonist memantine (22) on RGC degeneration in GLAST mutants. Memantine (10 mg/kg injected daily) prevented RGC loss when applied from P7 to P13 (Figure 7B), and the RGC number in GLAST−/− mice was normal at P14 (Figure 7C). The protective effect of glutamate receptor antagonists suggests that glutamate neurotoxicity is partly involved in RGC loss in GLAST mutant mice. However, since glutamate concentration in the vitreous fluid was normal, other factors, in addition to glutamate neurotoxicity, might contribute to RGC degeneration of GLAST-deficient mice.

Role of oxidative stress in RGC degeneration of GLAST- and EAAC1-deficient mice. In addition to excitotoxicity, oxidative stress has also been proposed to contribute to RGC death in glaucoma (23, 24). In adult GLAST−/− and EAAC1−/− mice, lipid hydroperoxides were increased in retinas, suggesting the involvement of oxidative stress in RGC loss (Figure 8A). Previous studies demonstrated that glutathione, a tripeptide of glutamate, cysteine, and glycine, has a central role in protecting RGCs against oxidative stress and that glutamate uptake is a rate-limiting step in glial glutathione synthesis (25, 26). Because retinal glutathione was specifically distributed in Müller cells (Figure 8B), we hypothesized that oxidative stress may result from impaired glial glutathione synthesis in GLAST−/− mice. In agreement with this hypothesis, glutathione content in the retina and in cultured Müller cells was decreased in GLAST−/− mice (Figure 8C). A similar reduction in glutathione level was recently reported in the plasma of human POAG patients (27). To confirm that the reduction in Müller cell glutathione levels makes RGCs more vulnerable to oxidative stress, we examined the effect of H2O2 on WT RGCs in a mixed culture with Müller cells from GLAST−/− or WT mice. We used a concentration of H2O2 that does not induce Müller cell death. RGC loss was significantly higher when RGCs were mixed with Müller cells from GLAST−/− mice than when mixed with Müller cells from WT mice (Figure 8D). These results indicate that GLAST deficiency leads to glaucomatous-like RGC loss in 2 ways: first, by excessive stimulation of NMDA receptors and second, by reducing the glutathione levels in Müller cells, thus making RGCs more vulnerable to oxidative stress.

Unlike glial GLAST, neuronal EAAC1 does not play a major role in clearing glutamate from the extracellular space (15, 28). Instead, EAAC1 can transport cysteine, an obligate precursor for neuronal glutathione synthesis, far more effectively than GLAST (29). Aoyama et al. recently reported that EAAC1 deficiency leads to impaired neuronal glutathione metabolism and age-dependent brain atrophy (29). In EAAC1−/− mutant mice, retinal glutathione content was not decreased (Figure 8C). These measurements represent bulk glutathione content in retinal homogenates from

Figure 5
Optic nerve degeneration and normal IOP in glutamate transporter mutant mice. (A and B) Optic nerve atrophy in 8-month-old GLAST−/− mice. In WT mice (A), the nasal and temporal nerve fibers (arrows) are beneath the internal limiting membrane (arrowhead) and enter a well-formed optic nerve (N). In GLAST−/− mice (B), the nerve fiber layer has become thin and almost absent as it enters the nerve (bold arrows). Cupping extends to the posterior aspect of the inner retinal layer (arrowheads). (C and D) Thinning of optic nerve is apparent in GLAST−/− (D) compared with WT (C) mice. (E and F) Staining of semithin sections with toluidine blue reveals the presence of abnormally dark axonal profiles (arrowheads) and decline of axons in GLAST−/− (F) compared with WT (E) mice. (G and H) Aqueous humor drainage structures in WT (G) and GLAST−/− (H) mice. Iridocorneal angle in GLAST−/− mice is normal with an obvious Schlemm’s canal (arrow) and trabecular meshwork (asterisk) compared with those in WT mice. The angle recess between the cornea (C) and iris is wide open. (I) IOP of young (4 weeks old) and adult (9–11 months old) mice. Sample numbers are indicated in parentheses. Scale bar: 200 μm (A, B, G, and H); 130 μm (C and D); 9 μm (E and F).
EAAC1−/− and WT mice. Thus, glutathione deficiency in RGCs of EAAC1−/− mice may not be apparent because a large share of retinal glutathione is localized to Müller cells (26), which do not express EAAC1. To directly determine the vulnerability of EAAC1−/− RGCs to oxidants, primary cultures of mutant and WT RGCs were treated with H2O2. Cultured RGCs from EAAC1−/− mice were susceptible to H2O2 compared with those from WT mice (Figure 8E). These findings suggest that EAAC1 deficiency makes RGCs more vulnerable to oxidative stress.

Discussion
Here, we show that GLAST- and EAAC1-knockout mice show progressive RGC loss and glaucomatous optic nerve degeneration without elevated IOP. Our study raises the possibility that dysfunction of the glutamate transporters GLAST (EAAT1 in humans) and EAAC1 (EAAT3 in humans) plays a role in RGC death in human NTG. It is reported that EAAT1 is downregulated in human POAG and NTG, show extensive RGC loss (33). Thus, the C57BL/6J strain may have a potentially pathogenic allele of Optn. Although more studies are needed to determine whether the NTG-like phenotype in GLAST−/− mice on a C57BL/6 background may be apparent because a large share of retinal glutathione is localized to Müller cells (26), which do not express EAAC1. To directly determine the vulnerability of EAAC1−/− RGCs to oxidants, primary cultures of mutant and WT RGCs were treated with H2O2. Cultured RGCs from EAAC1−/− mice were susceptible to H2O2 compared with those from WT mice (Figure 8E). These findings suggest that EAAC1 deficiency makes RGCs more vulnerable to oxidative stress.

Figure 6
Impaired multifocal electoretinogram in GLAST−/− mice. (A) Averaged responses of 2K from 10 mice. The visual stimulus was applied to 7 different areas in the retina. The 7 individual traces demonstrate the average responses to the visual stimulus at the corresponding stimulus area. (B) Three-dimensional plots showing the amplitude variation across the arrays in A. (C) Quantitative analysis of 2K amplitude. The response amplitudes for each stimulus element were added and the result was divided by the total area of the visual stimulus. n = 10 per group; *P < 0.005. Values in B and C are given in nV per square degree (nV/deg2).
cocultured with Müller cells from GLAST<sup>−/−</sup> mice are susceptible to free radical stimulation (Figure 8D). We previously reported that neurotrophins alter the production of some trophic factors in Müller cells, which indirectly leads to neural cell survival during photoreceptor degeneration (43, 44). We suggest that such a glia-neuron network is functional in various forms of neurodegenerative diseases, and the messengers between glia and neurons may be different according to the situation. In addition, each single Müller cell extends from the outer to the inner surface of the retina. This allows the Müller cells to form elaborate intimate contacts with the somata of all types of retinal neurons as well as with the fibers and synapses in the neuropile of the 2 plexiform layers. Their ubiquitous presence makes the Müller cells a suitable target for drug delivery and gene therapy in retinal degenerative diseases. Furthermore, recent studies have shown that Müller cells could proliferate after neurotoxic damage and produce bipolar cells and rod photoreceptors in the adult mammalian retina (45, 46). Therefore, Müller cells may be a new therapeutic target for both neuroprotection and regeneration in retinal degenerative diseases (47, 48).

Methods

Mice. Experiments were performed using EAAC1<sup>+/−</sup>, EAAC1<sup>−/−</sup> (15), GLT-1<sup>−/−</sup> (17), GLAST<sup>+/−</sup>, and GLAST<sup>−/−</sup> (14) mice with approval from the Institutional Animal Care and Use Committee of Tokyo Medical and Dental University and the Tokyo Metropolitan Institute for Neuroscience. All the mice used in this study were backcrossed with C57BL/6 more than 8 times.

Immunohistochemistry. Frozen 12-μm-thick retinal sections were incubated with 1 of 4 sets of primary antibody mix. Primary antibodies used were mouse anti-GS (Chemicon International) and rabbit anti-GLAST (13) or anti-glutathione (Signature Immunologics); and mouse anti-calretinin (Chemicon International) and rabbit anti-EAAC1 (49) or anti–GLT-1 (13). Fluorescence immunohistochemistry was performed using Cy2-conjugated donkey anti-mouse IgG and Cy3-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories Inc.). Sections were examined by fluorescence microscopy (Olympus).

Histological and morphometric studies. Paraffin sections (7-μm thick) of retinal specimens were cut through the optic nerve and stained with H&E. RGC number and the extent of retinal degeneration were quantified in 3 ways. First, the cell density of each layer was analyzed (50). Second, in the same sections, the number of neurons in the GCL was counted from one ora serrata through the optic nerve to the other ora serrata. Third, RGCs were retrogradely labeled from the superior colliculus with Fluoro-Gold (Fluorochrome) (16). Seven days after Fluoro-Gold application, eyes were enucleated and retinas were detached and prepared as flattened whole mounts in 4% paraformaldehyde in 0.1 M PBS solution. GCL was examined in whole-mounted retinae with fluorescence microscopy to determine the RGC density. Four standard areas (0.04 mm<sup>2</sup>) of each retina at the point of 0.1 mm from the optic disc were randomly chosen, labeled cells were counted by observers.
Figure 8
Increased oxidative stress in glutamate transporter mutant mice. (A) Lipid hydroperoxide concentration in whole retina of WT, EAAC1−/−, and GLAST−/− mice. Sample numbers are indicated in parentheses. (B) Immunohistochemical analysis of mouse retina double-stained with glutathione and GS. Glutathione-like immunoreactivity was observed in Müller glial cells (arrowheads). Scale bar: 20 μm. (C) Mean glutathione concentration in whole retina and cultured Müller cells. Sample numbers are indicated in parentheses. (D) Lactate dehydrogenase (LDH) release from H2O2-treated RGCs cocultured with WT or GLAST−/− Müller cells. n = 3 per group. (E) Lactate dehydrogenase release from H2O2-treated RGCs from WT or EAAC1−/− mice, n = 4 per group. *P < 0.05. **P < 0.005.

blinded to the identity of the mice, and the average number of RGCs/mm² was calculated. The changes in RGC number were expressed as percentages of the WT control eyes. In some experiments, memantine (10 mg/kg; Merz) was injected i.p. into GLAST−/− mice and their littermates daily from P7 to P13. These mice were sacrificed on P14 and processed for RGC count.

For the analysis of the optic nerve, frozen 10-μm-thick sections were cut at 2.5 mm from the eyeball and stained with H&E. Quantitative analysis of the optic nerve area was carried out using a computerized image analysis program (Scion Image Beta 4.0.3; Scion Corporation). For detailed morphological analysis, optic nerves were fixed in 2% glutaraldehyde and 2% paraformaldehyde in 0.1 M phosphate buffer overnight at 4°C. After dissection, the pieces of tissue were placed in 1% osmium tetroxide. After dehydration, the pieces were embedded in EPON (Nisshin EM). Transversal semithin (1 μm) sections were stained with 0.2% toluidine blue in 1.0% sodium borate.

**IOP measurement.** IOP was directly measured by a microneedle method in anesthetized mice as described previously (18, 51). The microneedle was connected to a pressure transducer, which relayed its signal to a bridge amplifier and then to an analog-to-digital converter and a computer (World Precision Instruments). The microneedle tip was inserted through the cornea and the data were automatically collected online into a computer database. To minimize variation, data were collected during a time window 4–6 minutes after injection of anesthetic, during which time IOP plateaued (51). Animal age was 4 weeks or 9–11 months, and body weight ranges were 13–24 g or 28–36 g at the time of IOP measurement, respectively. Since 24-hour IOP pattern in mouse eyes is biphasic and IOP is highest around 9:00 pm (18), we examined IOP between 8:00 pm and 11:00 pm.

**mfERGs.** Mice (12–16 weeks old) were anesthetized by i.p. injection of a mixture of xylazine (10 mg/kg) and ketamine (25 mg/kg). Pupils were dilated with 0.5% phenylephrine hydrochloride and 0.5% tropicamide. mfERGs were recorded using a VERIS 5.1 system (Electro-Diagnostic Imaging Inc.). The visual stimulus consisted of 7 hexagonal areas scaled with eccentricity. The stimulus array was displayed on a high-resolution black and white monitor driven at a frame rate of 100 Hz. The 2K, which is impaired in patients with glaucoma (20), was analyzed.

**Glutamate assay.** Vitreous samples (5–10 μl) were surgically extracted from adult mice, and glutamate was analyzed by serial enzymatic reactions as previously reported (52).

**Cell culture.** RGCs (16) and Müller cells (44) were prepared from P7 mice. In some experiments, WT RGCs were cocultured with Müller cells from GLAST−/− mice or their WT littermates. These culture cells were stimulated with 200 μM H2O2 for 16 hours, and RGC death rate was analyzed using a lactate dehydrogenase cytotoxic test kit (Wako) as previously reported (16).

**Lipid peroxidation and glutathione assay.** The concentrations of lipid peroxides and glutathione were measured using Bioxytech LPO-560 (Oxis Health Products Inc.) and Glutathione assay kit (Cayman Chemical) per the manufacturers’ protocols.

**Statistics.** For statistical comparison of 2 samples, we used a 2-tailed Student’s t test or Mann-Whitney U test. In other cases, we also used 1-factor ANOVA and Tukey-Kramer test. Data are presented as the mean ± SEM. P < 0.05 was regarded as statistically significant.

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