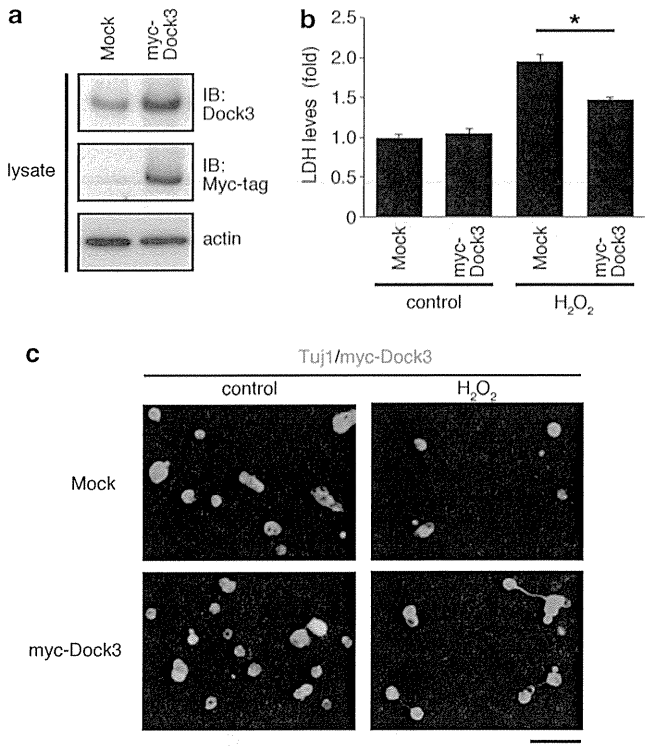

Figure 5 Effect of Dock3 on H_2O_2-induced RGC death *in vitro*. (**a**) Lysates from cultured RGCs transfected with empty vector (Mock) or myc-tagged Dock3 plasmids were subjected to immunoblot analysis using anti-Dock3 and anti-myc antibodies. (**b**) Cultured RGCs were stimulated with H_2O_2 for 16 h, and the extent of RGC death was quantified by examining extracellular LDH activities. Note the decrease in H_2O_2-induced death in RGCs transfected with Dock3. (**c**) Pictures of cultured RGCs before and 16 h after stimulation with H_2O_2. The data are presented as means ± S.E.M. of six samples for each experiment $^*P<0.05$

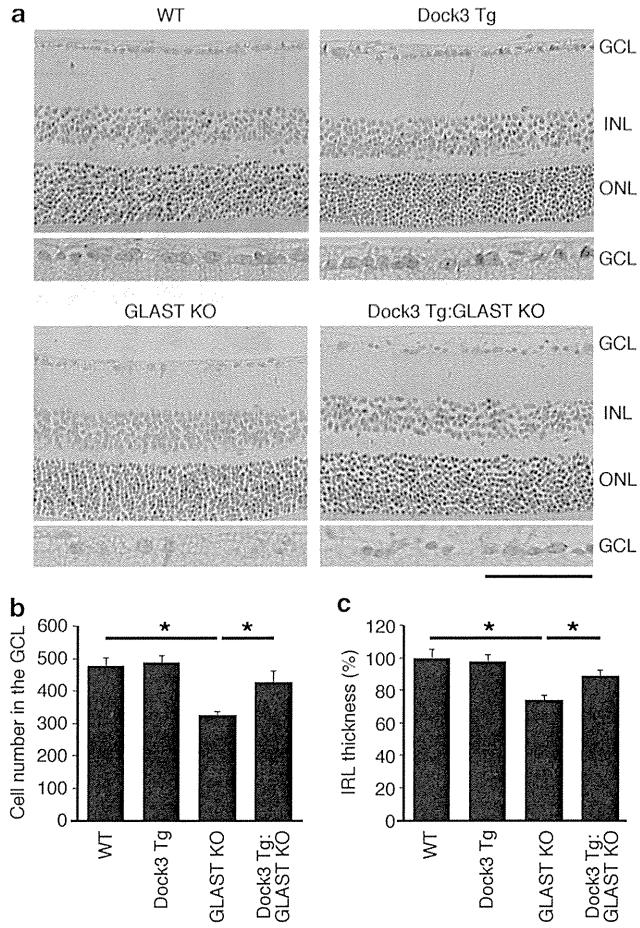

Figure 6 Effect of Dock3 on retinal degeneration in a mouse model of glaucoma. (**a**) H&E staining of retinal sections of WT, Dock3 Tg, GLAST KO and Dock3 Tg:GLAST KO mice at 3 months. WT and Dock3 Tg mice were littermates. GLAST KO and Dock3 Tg:GLAST KO mice were littermates. Scale bar: 100 μm and 50 μm in the upper and lower rows, respectively. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. (**b** and **c**) Quantification of cell number in the GCL (**b**) and thickness of the inner retinal layer (**c**) in WT, Dock3 Tg, GLAST KO and Dock3 Tg:GLAST KO mice. The data are presented as means ± S.E.M. of six samples for each experiment $^*P<0.05$

causes of retinal degeneration in GLAST KO mice, we generated Dock3 Tg:GLAST KO mice and examined the histopathology of the retina (Figure 6a). In 3-month-old Dock3 Tg:GLAST KO mice, cell number in the GCL (427 ± 26; $n=6$) and IRL thickness were significantly increased compared with those of GLAST KO mice (Figures 6b and c). We next examined total and phosphorylated (at pTyr1472) NR2B protein expressions in these mice. The ratio of phosphorylated/total NR2B in GLAST KO mice was significantly increased compared with that in WT mice, but such an increase was not detected in Dock3 Tg:GLAST KO mice (Figure 7). These results suggest that Dock3 overexpression prevents glaucomatous retinal degeneration in GLAST KO mice by suppressing both NR2B-mediated glutamate neurotoxicity and oxidative stress.

Discussion

Here we show that Dock3 directly binds to NR2B and stimulates NMDA-induced NR2B degradation in mouse retina, resulting in the protection of RGCs from excitotoxicity. We previously reported glaucomatous retinal degeneration due to glutamate neurotoxicity and oxidative stress in GLAST KO mice, the first animal model of normal tension glaucoma.[6,30] By generating Dock3 Tg:GLAST KO mice, we confirmed the neuroprotective effect of Dock3 *in vivo*. NR2B activation was increased in GLAST KO mice, but significantly suppressed in Dock3 Tg:GLAST KO mice. These results suggest that Dock3 exerts neuroprotective effects by regulating the activity of NR2B. It has been reported that GLAST (EAAT1 in humans) is downregulated in the retinas of human patients with glaucoma[31] and in fibroblasts from patients with Alzheimer's disease.[32] In addition, the accumulation of Aβ is also observed in apoptotic RGCs in a rat model of glaucoma.[27] Considering the high frequency of glaucoma in Alzheimer's disease patients,[28] common mechanisms such as GLAST dysfunction might contribute to both diseases. Taken together, these findings suggest that Dock3 overexpression may be a potential therapeutic target in treating various neurodegenerative disorders, including Alzheimer's disease and glaucoma.[21,22,33] Further studies are required to reveal the mechanism of NMDA-induced degradation of NR2B receptors, which may be caused by calpain and the ubiquitin–proteasome system.[34–37]

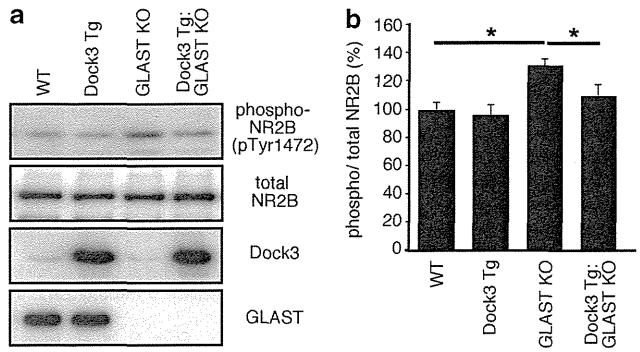

Figure 7 Effect of Dock3 on NR2B phosphorylation in a mouse model of glaucoma. (**a**) Immunoblot analysis of phosphorylated NR2B, total NR2B, Dock3 and GLAST in the retinas of WT, Dock3 Tg, GLAST KO and Dock3 Tg:GLAST KO mice. (**b**) Phosphorylation rate of NR2B in (**a**). The data are presented as means ± S.E.M. of six samples for each experiment $^*P<0.05$

In addition to glutamate neurotoxicity, oxidative stress is recognized as a common pathologic pathway in many neurodegenerative diseases, including glaucoma.[38] For example, the reduction in glutathione, a major antioxidant in the retina, was reported in the plasma of human glaucoma patients.[39] A similar reduction in glutathione concentration and increased oxidative stress were detected in GLAST KO mice.[6] We previously reported that apoptosis signal-regulating kinase 1 (ASK1), a member of mitogen-activated protein 3 kinase, is involved in RGC loss in GLAST KO mice.[30] ASK1 has key roles in human diseases closely related to the dysfunction of cellular responses to oxidative stress and endoplasmic reticulum stressors.[40,41] As Dock3 protects RGCs from oxidative stress, the combination of Dock3 overexpression and inhibition of ASK1 signaling by a specific inhibitor[42] may be further available for the management of glaucoma. One important point is that glaucoma is a complex disease resulting from the confluence of various factors.[27–32,43,44] To fill the gap between glaucoma patients and animal disease models,[45] we are currently investigating GLAST gene abnormalities in glaucoma patients.

We previously showed that Dock3 has important roles downstream of brain-derived neurotrophic factor (BDNF) signaling in the CNS, where it promotes axonal outgrowth by stimulating microtubule assembly through glycogen synthase kinase-3β (GSK-3β).[22] In addition to stimulating the outgrowth of optic nerve axons, BDNF protects RGCs after retinal and optic nerve injury.[46–48] Interestingly, GSK-3β is a target for lithium-induced neuroprotection against excitotoxicity in neuronal cultures and animal models of ischemic stroke.[49] GSK-3β activity has been associated with many psychiatric and neurodegenerative diseases, and it has become increasingly apparent that GSK-3β might be a common therapeutic target for different classes of psychiatric drugs.[50] Thus, Dock3 and GSK-3β could be potential targets for both neuroprotection and regeneration therapy. In the retina, there seems to be some cells that are positive for NR2B, but not Dock3 (Figure 2). This may mean that Dock3 decreases NR2B expression mainly in RGCs in normal retina, but overexpression of Dock3 in the whole retina may protect many types of retinal neurons. In addition, Dock3 expression in cells other than RGCs may have important roles for RGC protection and optic nerve regeneration in Dock3 Tg mice. For example, microglia that invade the inner retina during degeneration may release several trophic factors including BDNF, which stimulate neuroprotection.[51,52]

It has been estimated that glaucoma will affect more than 80 million individuals worldwide by 2020, with at least 6–8 million individuals becoming bilaterally blind.[53] Glaucoma is also the leading global cause of irreversible blindness, and is perhaps the most prevalent of all neurodegenerative diseases.[4,54] Although further *in vivo* studies are required, our findings raise intriguing possibilities for the management of glaucoma by Dock3 overexpression in combination with trophic factors, ASK1 inhibitors, GLAST overexpression and so on. We are currently investigating this possibility by using Dock3 virus vectors and GLAST Tg mice.

Materials and Methods

Cell culture and pull-down assay. His-tagged full-length Dock3[20] and the CTD of NR2B (NR2B-CTD)[15] were cotransfected to HEK293T cells using Lipofectamine Plus (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. After 24 h of transfection, the cell lysate was incubated with TALON resin (TAKARA, Shiga, Japan) for 20 min at 4 °C with gentle agitation.[20] After being washed, precipitated samples were separated on sodium dodecyl sulfate-polyacrylamide gels and subsequently electrotransferred to an Immobilon-P filter (Merck Millipore, Billerica, MA, USA). Membranes were incubated with antibodies against Dock3 (1 : 1000),[21] myc-tag (1 : 1000; Santa Cruz Biotechnology, Santa Cruz, CA, USA), NR2B (1 : 1000; BD Biosciences, San Diego, CA, USA) or actin (1 : 1000; BD Biosciences). Dock3 antibody was originally reported as an antibody against the modifier of cell adhesion protein.[20] Primary antibody binding was detected using horseradish peroxidase-labeled anti-mouse IgG secondary antibody (GE Health Care, Piscataway, NJ, USA) and visualized using the ECL Plus Western Blotting System (GE Health Care).

Mice. Experiments were performed using Dock3 Tg,[21,22] GLAST KO[6,30] and Dock3 Tg:GLAST KO mice in accordance with the Tokyo Metropolitan Institute for Neuroscience Guidelines for the Care and Use of Animals. C57BL/6J mice were obtained from CLEA Japan (Tokyo, Japan). Intraocular injection of NMDA (Wako, Osaka, Japan) or PBS was achieved essentially as previously described.[55] Briefly, a single 2-μl injection of 1 mM NMDA in 0.1 M PBS (pH 7.4) was administered intravitreally into the right eye of each mouse, thereby delivering a dose of 2 nmol of NMDA. The same volume of PBS was administered to the contralateral (left) eye as control. Animals were killed at 3 h and 1, 3, 5 and 7 days after NMDA injection.

Immunoprecipitation of the retina. The Dock3-NR2B complex was purified from the retina (1 mg) of WT and Dock3 Tg mice by immunoprecipitation with anti-Dock3 antibody (1 : 200). The immunoprecipitates were subjected to an immunoblot analysis with an antibody against Dock3 (1 : 1000) or NR2B (1 : 1000). Immunoblotting of retinas from WT, Dock3 Tg, GLAST KO and Dock3 Tg:GLAST KO were performed as reported previously.[55] Membranes were incubated with an antibody against NR2B (1 : 1000), phospho-NR2B at Tyr1472 (1 : 1000; BD Biosciences), Dock3 (1 : 1000), GLAST (1 : 1000)[56] or actin (1 : 1000).

Histological and morphometric studies. Paraffin-embedded retinal sections of 7 μm thickness were cut through the optic nerve and stained with hematoxylin and eosin (H&E). The extent of retinal degeneration was quantified in two ways.[52] First, the number of neurons in the GCL was counted from one ora serrata through the optic nerve to the other ora serrata. Second, in the same sections, thickness of the IRL (between the internal limiting membrane and the interface of the outer plexiform layer and the outer nuclear layer) was analyzed. Frozen retinal sections of 12 μm thickness were incubated using anti-Dock3 (1 : 200) and anti-NR2B (1 : 100) antibodies. Cy-3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA, USA) and Cy-2-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch) were used as secondary antibodies. Sections were examined by fluorescence microscopy (BX51; Olympus, Tokyo,

Japan) equipped with Plan Fluor objectives and connected to a DP70 camera (Olympus).

Assessment of H_2O_2-induced cell death in cultured RGCs. Primary culture of mouse RGCs was prepared according to Barres *et al.*[57] with *minor modifications.*[29] *Primary cultured RGCs were transfected with a myc-tagged* Dock3 plasmid by electroporation using the Amaxa Nucleofector Device (program O-005) with mouse neuron Nucleofector Kit (Amaxa Biosystems, Koeln, Germany). After 2 days, they were stimulated with 200 μM of H_2O_2 for 16 h, and the RGC death rate was analyzed using an LDH cytotoxic test kit (Wako) as previously reported.[29] For immunocytochemistry, RGCs were stained with antibodies against Tuj1 (1 : 1000; R&D, Minneapolis, MN, USA) and myc-tag (1 : 1000; MBL, Nagoya, Japan).

Statistics. For statistical comparison of two samples, we used a two-tailed Student's *t*-test. Data are presented as means ± S.E.M. $P < 0.05$ was regarded as *statistically significant.*

Conflict of Interest

The authors declare no conflict of interest.

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1. Christie MJ, Napier IA, Eckert A, Staufenbiel M, Hardeman E, Götz J. Dendritic function of tau mediates amyloid-β toxicity in Alzheimer's disease mouse models. *Cell* 2010; **142**: 387–397.
2. Shankar GM, Bloodgood BL, Townsend M, Walsh DM, Selkoe DJ, Sabatini BL. Natural oligomers of the Alzheimer amyloid-β protein induce reversible synapse loss by modulating an NMDA-type glutamate receptor-dependent signaling pathway. *J Neurosci* 2007; **27**: 2866–2875.
3. Milnerwood AJ, Gladding CM, Pouladi MA, Kaufman AM, Hines RM, Boyd JD *et al.* Early increase in extrasynaptic NMDA receptor signaling and expression contributes to phenotype onset in Huntington's disease mice. *Neuron* 2010; **65**: 178–190.
4. Zhang K, Zhang L, Weinerb RN. Ophthalmic drug discovery: novel targets and mechanisms for retinal diseases and glaucoma. *Nat Rev Drug Discov* 2012; **11**: 541–559.
5. Skolnick P, Popik P, Trullas R. Glutamate-based antidepressants: 20 years on. *Trends Pharmacol Sci* 2009; **30**: 563–569.
6. Harada T, Harada C, Nakamura K, Quah HM, Okumura A, Namekata K *et al.* The potential role of glutamate transporters in the pathogenesis of normal tension glaucoma. *J Clin Invest* 2007; **117**: 1763–1770.
7. Paoletti P, Bellone C, Zhou Q. NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease. *Nat Rev Neurosci* 2013; **14**: 383–400.
8. Kalbaugh TL, Zhang J, Diamond JS. Coagonist release modulates NMDA receptor subtype contributions at synaptic inputs to retinal ganglion cells. *J Neurosci* 2009; **29**: 1469–1479.
9. Brandstätter JH, Hartveit E, Sassoè-Pognetto M, Wässle H. Expression of NMDA and high-affinity kainate receptor subunit mRNAs in the adult rat retina. *Eur J Neurosci* 1994; **6**: 1100–1112.
10. Watanabe M, Mishina M, Inoue Y. Differential distributions of the NMDA receptor channel subunit mRNAs in the mouse retina. *Brain Res* 1994; **634**: 328–332.
11. Gründer T, Kohler K, Kaletta A, Guenther E. The distribution and developmental regulation of NMDA receptor subunit proteins in the outer and inner retina of the rat. *J Neurobiol* 2000; **44**: 333–342.
12. Sobczyk A, Scheuss V, Svoboda K. NMDA receptor subunit-dependent [Ca2 +] signaling in individual hippocampal dendritic spines. *J Neurosci* 2005 29; **25**: 6037–6046.
13. Yashiro K, Philpot BD. Regulation of NMDA receptor subunit expression and its implications for LTD, LTP, and metaplasticity. *Neuropharmacology* 2008; **55**: 1081–1094.
14. Roche KW, Standley S, McCallum J, Dune LyC, Ehlers MD, Wenthold RJ. Molecular determinants of NMDA receptor internalization. *Nat Neurosci* 2001; **4**: 794–802.
15. Nakazawa T, Komai S, Tezuka T, Hisatsune C, Umemori H, Semba K *et al.* Characterization of Fyn-mediated tyrosine phosphorylation sites on GluR12 (NR2B) subunit of the N-methyl-D-aspartate receptor. *J Biol Chem* 2001; **276**: 693–699.
16. Rong Y, Lu X, Bernard A, Khrestchatisky M, Baudry M. Tyrosine phosphorylation of ionotropic glutamate receptors by Fyn or Src differentially modulates their susceptibility to calpain and enhances their binding to spectrin and PSD-95. *J Neurochem* 2001; **79**: 382–390.
17. Salter MW, Kalia LV. Src kinases: a hub for NMDA receptor regulation. *Nat Rev Neurosci* 2004; **5**: 317–328.
18. Loftis JM, Janowsky A. The N-methyl-D-aspartate receptor subunit NR2B: localization, functional properties, regulation, and clinical implications. *Pharmacol Ther* 2003; **97**: 55–85.
19. Um JW, Nygaard HB, Heiss JK, Kostylev MA, Stagi M, Vortmeyer A *et al.* Alzheimer amyloid-β oligomer bound to postsynaptic prion protein activates Fyn to impair neurons. *Nat Neurosci* 2012; **15**: 1227–1235.
20. Namekata K, Enokido Y, Iwasawa K, Kimura H. MOCA induces membrane spreading by activating Rac1. *J Biol Chem* 2004; **279**: 14331–14337.
21. Namekata K, Harada C, Taya C, Guo X, Kimura H, Parada LF *et al.* Dock3 induces axonal outgrowth by stimulating membrane recruitment of the WAVE complex. *Proc Natl Acad Sci USA* 2010; **107**: 7586–7591.
22. Namekata K, Harada C, Guo X, Kimura A, Kittaka D, Watanabe H *et al.* Dock3 stimulates axonal outgrowth via GSK-3β-mediated microtubule assembly. *J Neurosci* 2012; **32**: 264–274.
23. Kashiwa A, Yoshida H, Lee S, Paladino T, Liu Y, Chen Q *et al.* Isolation and characterization of novel presenilin binding protein. *J Neurochem* 2000; **75**: 109–116.
24. Bertram L, Lill CM, Tanzi RE. The genetics of Alzheimer disease: back to the future. *Neuron* 2010; **68**: 270–281.
25. Chen Q, Yoshida H, Schubert D, Maher P, Mallory M, Masliah E. Presenilin binding protein is associated with neurofibrillary alterations in Alzheimer's disease and stimulates tau phosphorylation. *Am J Pathol* 2001; **159**: 1597–1602.
26. Sun L, Liu O, Desai J, Karbassi F, Sylvain MA, Shi A *et al.* CED-10/Rac1 regulates endocytic recycling through the RAB-5 GAP TBC-2. *PLoS Genet* 2012; **8**: e1002785.
27. Guo L, Salt TE, Luong V, Wood N, Cheung W, Maass A *et al.* Targeting amyloid-β in glaucoma treatment. *Proc Natl Acad Sci USA* 2007; **104**: 13444–13449.
28. Tamura H, Kawakami H, Kanamoto T, Kato T, Yokoyama T, Sasaki K *et al.* High frequency of open-angle glaucoma in Japanese patients with Alzheimer's disease. *J Neurol Sci* 2006; **246**: 79–83.
29. Harada C, Nakamura K, Namekata K, Okumura A, Mitamura Y, Iizuka Y *et al.* Role of apoptosis signal-regulating kinase 1 in stress-induced neural cell apoptosis *in vivo*. *Am J Pathol* 2006; **168**: 261–269.
30. Harada C, Namekata K, Guo X, Yoshida H, Mitamura Y, Matsumoto Y *et al.* ASK1 deficiency attenuates neural cell death in GLAST-deficient mice, a model of normal tension glaucoma. *Cell Death Differ* 2010; **17**: 1751–1759.
31. Naskar R, Vorwerk CK, Dreyer EB. Concurrent downregulation of a glutamate transporter and receptor in glaucoma. *Invest Ophthalmol Vis Sci* 2000; **41**: 1940–1944.
32. Zoia CP, Tagliabue E, Isella V, Begni B, Fumagalli L, Brighina L *et al.* Fibroblast glutamate transport in aging and in AD: correlations with disease severity. *Neurobiol Aging* 2005; **26**: 825–832.
33. Bai N, Hayashi H, Aida T, Namekata K, Harada T, Mishina M *et al.* Dock3 interaction with a glutamate-receptor NR2D subunit protects neurons from excitotoxicity. *Mol Brain* 2013; **6**: 22.
34. Hawasli AH, Benavides DR, Nguyen C, Kansy JW, Hayashi K, Chambon P *et al.* Cyclin-dependent kinase 5 governs learning and synaptic plasticity via control of NMDAR degradation. *Nat Neurosci* 2007; **10**: 880–886.
35. Wu HY, Hsu FC, Gleichman AJ, Baconguis I, Coulter DA, Lynch DR. Fyn-mediated phosphorylation of NR2B Tyr-1336 controls calpain-mediated NR2B cleavage in neurons and heterologous systems. *J Biol Chem* 2007; **282** 2007 5–20087.
36. Mao LM, Wang W, Chu XP, Zhang GC, Liu XY, Yang YJ *et al.* Stability of surface NMDA receptors controls synaptic and behavioral adaptations to amphetamine. *Nat Neurosci* 2009; **12**: 602–610.
37. Kurup P, Zhang Y, Xu J, Venkitaramani DV, Haroutunian V, Greengard P *et al.* Aβ-mediated NMDA receptor endocytosis in Alzheimer's disease involves ubiquitination of the tyrosine phosphatase STEP61. *J Neurosci* 2010; **30**: 5948–5957.
38. Osborne NN, del Olmo-Aguado S. Maintenance of retinal ganglion cell mitochondrial functions as a neuroprotective strategy in glaucoma. *Curr Opin Pharmacol* 2013; **13**: 16–22.
39. Gherghel D, Griffiths HR, Hilton EJ, Cunliffe IA, Hosking SL. Systemic reduction in glutathione levels occurs in patients with primary open-angle glaucoma. *Invest Ophthalmol Vis Sci* 2005; **46**: 877–883.
40. Nishitoh H, Kadowaki H, Nagai A, Maruyama T, Yokota T, Fukutomi H *et al.* ALS-linked mutant SOD1 induces ER stress- and ASK1-dependent motor neuron death by targeting Derlin-1. *Genes Dev* 2008; **22**: 1451–1464.
41. Hattori K, Naguro I, Runchel C, Ichijo H. The roles of ASK family proteins in stress responses and diseases. *Cell Commun Signal* 2009; **7**: 9.
42. Guo X, Harada C, Namekata K, Matsuzawa A, Camps M, Ji H *et al.* Regulation of the severity of neuroinflammation and demyelination by TLR-ASK1-p38 pathway. *EMBO Mol Med* 2010; **2**: 504–515.
43. Boland MV, Ervin AM, Friedman DS, Jampel HD, Hawkins BS, Vollenweider D *et al.* Comparative effectiveness of treatments for open-angle glaucoma: a systematic review for the U.S. Preventive Services Task Force. *Ann Intern Med* 2013; **158**: 271–279.
44. Hollands H, Johnson D, Hollands S, Simel DL, Jinapriya D, Sharma S. Do findings on routine examination identify patients at risk for primary open-angle glaucoma? The rational clinical examination systematic review. *JAMA* 2013; **309**: 2035–2042.
45. Bouhenni RA, Dunmire J, Sewell A, Edward DP. Animal models of glaucoma. *J Biomed Biotechnol* 2012; **2012**: 692609.

46. Harvey AR, Hellström M, Rodger J. Gene therapy and transplantation in the retinofugal pathway. *Prog Brain Res* 2009; **175**: 151–161.
47. Bessero AC, Clarke PG. Neuroprotection for optic nerve disorders. *Curr Opin Neurol* 2010; **23**: 10–15.
48. Harada C, Guo X, Namekata K, Kimura A, Nakamura K, Tanaka K *et al.* Glia- and neuronspecific functions of TrkB signalling during retinal degeneration and regeneration. *Nat Commun* 2011; **2**: 189.
49. Chuang DM, Wang Z, Chiu CT. GSK-3 as a target for lithium-induced neuroprotection against excitotoxicity in neuronal cultures and animal models of ischemic stroke. *Front Mol Neurosci* 2011; **4**: 15.
50. Hur EM, Zhou FQ. GSK3 signalling in neural development. *Nat Rev Neurosci* 2010; **11**: 539–551.
51. Harada T, Harada C, Kohsaka S, Wada E, Yoshida K, Ohno S *et al.* Microglia-Müller glia cell interactions control neurotrophic factor production during light-induced retinal degeneration. *J Neurosci* 2002; **22**: 9228–9236.
52. Katome T, Namekata K, Guo X, Semba K, Kittaka D, Kawamura K *et al.* Inhibition of ASK1-p38 pathway prevents neural cell death following optic nerve injury. *Cell Death Differ* 2013; **20**: 270–280.
53. Quigley HA. Glaucoma. *Lancet* 2011; **377**: 1367–1377.
54. Resnikoff S, Pascolini D, Etya'ale D, Kocur I, Pararajasegaram R, Pokharel GP *et al.* Global data on visual impairment in the year 2002. *Bull World Health Organ* 2004; **82**: 844–851.
55. Namekata K, Harada C, Kohyama K, Matsumoto Y, Harada T. Interleukin-1 stimulates glutamate uptake in glial cells by accelerating membrane trafficking of Na^+/K^+-ATPase via actin depolymerization. *Mol Cell Biol* 2008; **28**: 3273–3280.
56. Harada T, Harada C, Watanabe M, Inoue Y, Sakagawa T, Nakayama N *et al.* Functions of the two glutamate transporters GLAST and GLT-1 in the retina. *Proc Natl Acad Sci USA* 1998; **95**: 4663–4666.
57. Barres BA, Silverstein BE, Corey DP, Chun LL. Immunological, morphological, and electrophysiological variation among retinal ganglion cells purified by panning. *Neuron* 1988; **1**: 791–803.

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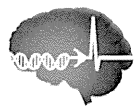

Molecular Brain

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Dock3 interaction with a glutamate-receptor NR2D subunit protects neurons from excitotoxicity

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Abstract

Background: N-methyl-D-aspartate receptors (NMDARs) are critical for neuronal development and synaptic plasticity. Dysregulation of NMDARs is implicated in neuropsychiatric disorders. Native NMDARs are heteromultimeric protein complexes consisting of NR1 and NR2 subunits. NR2 subunits (NR2A–D) are the major determinants of the functional properties of NMDARs. Most research has focused on NR2A- and/or NR2B-containing receptors. A recent study demonstrated that NR2C- and/or NR2D-containing NMDARs are the primary targets of memantine, a drug that is widely prescribed to treat Alzheimer's disease. Our laboratory demonstrated that memantine prevents the loss of retinal ganglion cells (RGCs) in GLAST glutamate transporter knockout mice, a model of normal tension glaucoma (NTG), suggesting that NR2D-containing receptors may be involved in RGC loss in NTG.

Results: Here we demonstrate that NR2D deficiency attenuates RGC loss in GLAST-deficient mice. Furthermore, Dock3, a guanine nucleotide exchange factor, binds to the NR2D C-terminal domain and reduces the surface expression of NR2D, thereby protecting RGCs from excitotoxicity.

Conclusions: These results suggest that NR2D is involved in the degeneration of RGCs induced by excitotoxicity, and that the interaction between NR2D and Dock3 may have a neuroprotective effect. These findings raise the possibility that NR2D and Dock3 might be potential therapeutic targets for treating neurodegenerative diseases such as Alzheimer's disease and NTG.

Keywords: NMDA receptor, NR2D, Dock3, Excitotoxicity, Glaucoma, Memantine, Glutamate transporter

Background

Glutamate is the major excitatory neurotransmitter in the mammalian central nervous system (CNS) and plays an essential role in neural development and information processing through a variety of ionotropic (ligand-gated) and metabotropic (G-protein–coupled) receptors [1]. However, increased levels of glutamate results in extensive stimulation of these receptors, which can eventually become neurotoxic [2,3]. Overstimulation of N-methyl-D-aspartate receptors (NMDARs) is implicated in many diseases, including epilepsy, schizophrenia, and various neurodegenerative disorders [4-7].

Molecular cloning methods have identified multiple NMDAR subunits, including NR1, a family of NR2 subunits (NR2A–NR2D), and two NR3 subunits (NR3A and NR3B). Native NMDARs are heteromultimeric protein complexes composed of NR1 and NR2 subunits, and in some cases NR3 subunits. NR2 subunits are major determinants of the functional properties of NMDARs, including characteristics such as agonist affinity, deactivation kinetics, single-channel conductance, Ca^{2+} permeability, and sensitivity to Mg^{2+}. Since NR2A- and NR2B-containing receptors are highly expressed in the cortex, and NR2C- and NR2D-containing receptors have low opening probabilities

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and low single-channel conductances, most previous research has focused on NR2A- and/or NR2B-containing receptors [8-10].

Recently, it was demonstrated that Mg^{2+} regulates the sensitivity of NMDARs to memantine, a drug that is widely prescribed for the treatment of Alzheimer's disease [11]. In a physiological concentration (1 mM) of extracellular Mg^{2+}, memantine exerts a more potent blocking effect at NR2C/D subunits than NR2A/B subunits. These findings suggest that NR2C- and/or NR2D-containing NMDARs are likely to be the main targets of memantine. In comparison with the NR2C subunit, NR2D is a particularly interesting NMDAR subunit because it mediates the mechanisms by which phencyclidine (PCP) induces locomotor hyperactivity in a novel environment, behaviors thought to model positive symptoms of schizophrenia [12]. NR2D subunits are broadly expressed in the adult mammalian brain, including the hippocampus, cortex and retina, all of which are regions of the CNS thought to be involved in Alzheimer's disease, schizophrenia, and glaucoma [8,13-15]. Previously, our laboratory demonstrated that memantine prevented the loss of retinal ganglion cells (RGCs) in glutamate aspartate transporter (GLAST) knockout mice, a model of NTG [16], suggesting that NR2D-containing receptors may be involved in RGC loss in NTG. In the present study, we first investigated whether NR2D is involved in the excitotoxic degeneration of RGCs. Using yeast two-hybrid screening we identified NR2D-interacting molecules that modulate the function or localization of NR2D-containing NMDARs.

Here, we report that NR2D deficiency protects RGCs from excitotoxicity. In addition, we identified dedicator of cytokinesis 3 (Dock3) as a novel NR2D subunit interacting protein, and showed that the interaction between NR2D and Dock3 protects RGCs from excitotoxicity by reducing the surface expression of NR2D.

Results

NR2D deficiency prevents RGC death in GLAST-deficient mice

To determine whether NR2D is involved in RGC degeneration in GLAST-deficient mice, we examined the histopathology of retinas from *GLAST*$^{-/-}$ and *NR2D*$^{-/-}$ mice. As shown in Figure 1, the retinas of *NR2D*$^{-/-}$ mice showed normal organization at 5 weeks. The cell number in the ganglion cell layer (GCL) of *NR2D*$^{-/-}$ mice (494 ± 26) was not significantly different from that in wild-type (WT) mice (514 ± 9), whereas the cell number in the GCL of *GLAST*$^{-/-}$ mice was significantly less (307 ± 6) than that in WT. However, in *GLAST*$^{-/-}$ *NR2D*$^{-/-}$ double-knockout mice, the number of GCL cells was significantly higher (401 ± 10) than that in *GLAST*$^{-/-}$ mice, although it was still lower than that in WT and *NR2D*$^{-/-}$ mice. These results suggest that NR2D deficiency protects against the loss of RGCs in GLAST-deficient mice.

NR2D deficiency prevents NMDA-induced-excitotoxic retinal cell death

In GLAST-deficient mice, both excitotoxicity and oxidative stress contribute to RGC degeneration [16]. To investigate

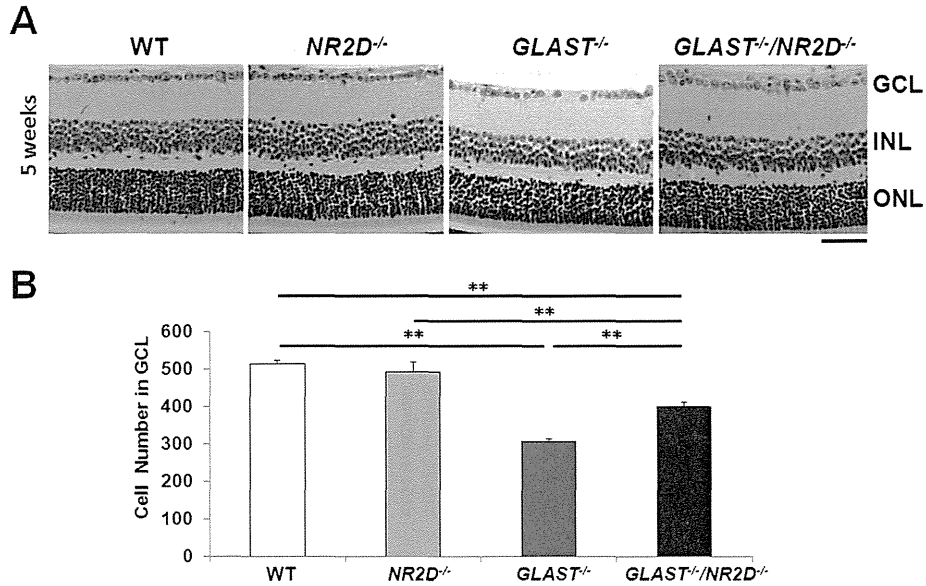

Figure 1 NR2D deficiency prevents the loss of RGCs in GLAST-deficient mice. (**A**) Hematoxylin and eosin staining of retinal sections at 5 weeks. Scale bar: 50 μm. (**B**) Quantification of RGC number in WT, *NR2D*$^{-/-}$, *GLAST*$^{-/-}$, and *GLAST*$^{-/-}$/*NR2D*$^{-/-}$ mice. The number of neurons in the GCL was counted in retinal sections from one ora serrata through the optic nerve to the other ora serrata. n = 5 per group. **p < 0.01.

whether NR2D deficiency reduces retinal cell death resulting from NMDA-induced excitotoxicity, we used TUNEL assay to examine the retinas of mice 24 h after an intravitreal injection of NMDA. No TUNEL-positive cells were detected in the controls or *NR2D*$^{-/-}$ mice after injection of phosphate-buffered saline (PBS) (Figure 2A), whereas a number of TUNEL-positive cells were observed in the GCL and inner nuclear layer (INL) after injection of NMDA (Figure 2B). NR2D deficiency significantly reduced the mean number of TUNEL-positive cells in the GCL, but not in the INL (Figure 2C, D). These results suggest that NR2D deficiency protects against excitotoxicity-induced retinal cell death in the GCL.

Identification of Dock3 as an NR2D-interacting molecule

Multiple studies show that protein-protein interactions involving the intracellular C-terminal domains of NMDARs control the function and localization of these receptors [17-20]. To identify novel binding partners of the NR2D subunit, we performed a yeast two-hybrid screening of a mouse brain cDNA library using the C-terminal domain of NR2D (residues 895–1323) as bait (Figure 3A). Two of the 77 clones initially identified as interacting proteins encoded the Dock homology region 2 (DHR-2) domain (Figure 3B) of dedicator of cytokinesis 3 (Dock3), also known as modifier of cell adhesion (MOCA), which is specifically expressed in the CNS [21]. To investigate whether NR2D and Dock3 also interact in mammalian cells, human embryonic kidney (HEK) 293 T cells were transfected with expression plasmids encoding a Myc-tagged Dock3 interaction domain (amino acids 796–1154) and/or the NR2D C-terminus (amino acids 895–1323) carrying an EGFP tag. Protein lysates were prepared from the transfected cells for co-immunoprecipitation analyses. Western blots of anti-EGFP immunoprecipitates with an anti-Myc antibody revealed that the Dock3 interaction domain co-precipitated with the EGFP-tagged NR2D C-terminus only when both proteins were expressed (Figure 3C, left panel). Conversely, the NR2D C-terminus was present in anti-Myc immunoprecipitates of Dock3 interaction domain (Figure 3C, right panel), indicating that Dock3 was associated with the NR2D C-terminus in heterologous HEK293T cells.

To determine whether Dock3 also interacted with NR2D in the retina, anti-NR2D immunoprecipitates from retina lysates were probed with an anti-Dock3 antibody. However, Dock3 was not detected in anti-NR2D immunoprecipitates, likely due to the low expression level of NR2D. Therefore, we performed co-immunoprecipitations using brain homogenates from mice at embryonic day 18, when the expression level of NR2D is high. The data clearly showed that Dock3 co-immunoprecipitated with NR2D (Figure 4A), and reciprocal co-immunoprecipitation experiments (Figure 4B) confirmed that Dock3 associated with NR2D in the embryonic brain.

Taken together, these results demonstrated that Dock3 effectively interacts with NR2D subunits in both cultured human cells and mouse embryonic brain.

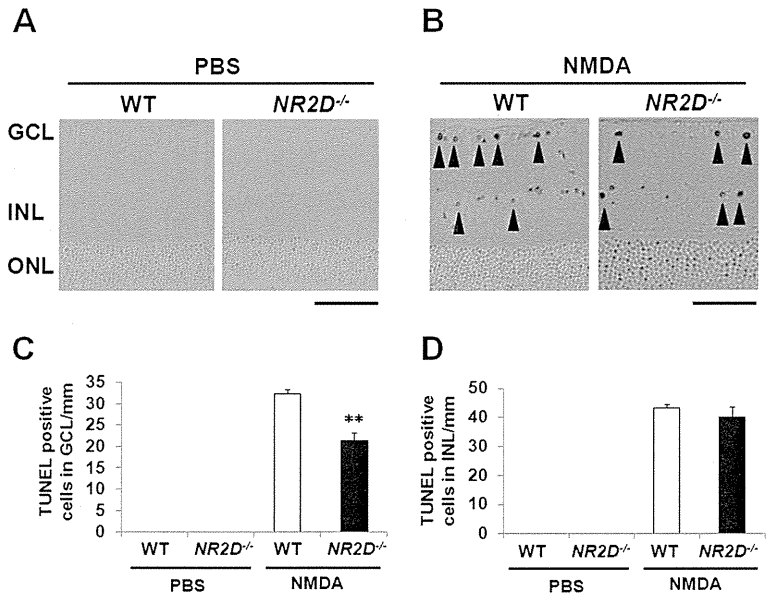

Figure 2 NR2D deficiency prevents NMDA-induced-excitotoxic retinal cell death. (**A** and **B**) TUNEL analysis of retinas of WT and *NR2D*$^{-/-}$ mice 24 hr after PBS and NMDA injection. Arrowheads in **B** indicate TUNEL-positive cells. Scale bar: 50 μm. (**C** and **D**) The number of TUNEL-positive cells in the GCL (**C**) and INL (**D**). n = 5 per group. **p < 0.01.

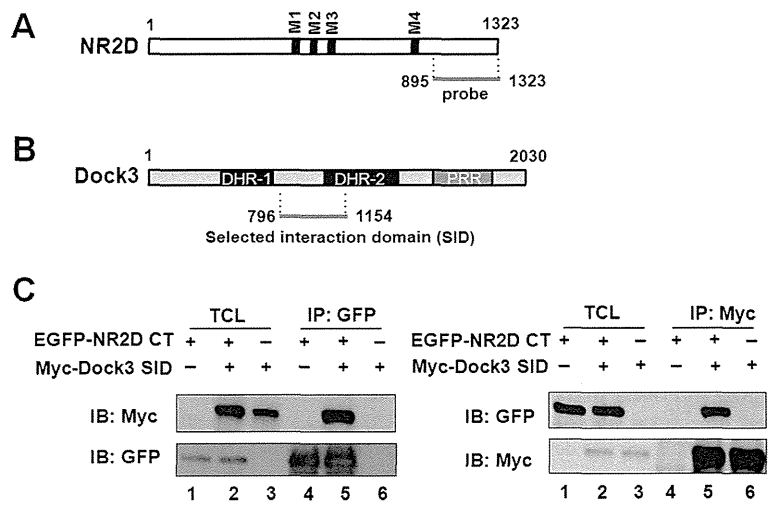

Figure 3 Identification of Dock3 as an NR2D-interacting molecule. (**A**) Schematic diagram of NR2D and a probe used for yeast two-hybrid screening. M1–M4 indicates the transmembrane regions. The numbers refer to the amino-acid positions of NR2D. (**B**) Schematic diagram of Dock3. The selected interaction domain (SID) corresponds to the minimal region common to all fragments identified by yeast two-hybrid screening. The numbers indicate the amino-acid positions within Dock3. DHR-1, Dock homology region 1; DHR-2, dock homology region 2; PRR, proline-rich region. (**C**) The interaction between NR2D and Dock3 in HEK293T cells. HEK293T cells were transfected with plasmids encoding the SID of Dock3 (Myc-Dock3 SID) and EGFP-tagged NR2D C-terminus (EGFP-NR2D CT). Lysates of transfected cells were immunoprecipitated (IP) with anti-GFP (left panel) or anti-Myc antibodies (right panel). Immune complexes were detected by Western blotting with anti-GFP or anti-Myc antibodies. In lanes 1–3, 1/10th volumes of the lysates used for immunoprecipitation were loaded for TCL samples. IB, Immunoblotting.

Dock3 and NR2D are co-expressed in the mouse retina

Because NR2D is involved in excitotoxic degeneration of RGCs, we examined the distribution of Dock3 and NR2D expression in the mouse retina. Immunohistochemical analysis showed that Dock3 is expressed in the RGCs (Figure 5A). In addition, co-labeling with anti-NR2D antibodies revealed that Dock3 colocalizes with NR2D in these cells (Figure 5B, C). These data suggest that co-expression of NR2D and Dock3 occurs not only in the embryonic brain, but also in the GCL of the retina.

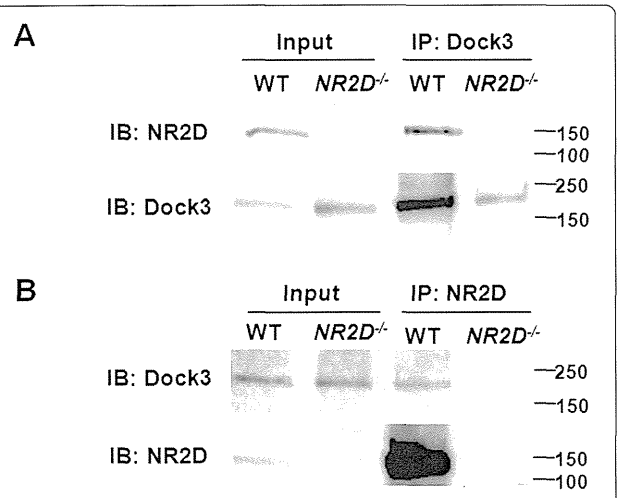

Figure 4 Interaction of Dock3 with NR2D in brain. (**A**) Identification of NR2D in Dock3 immunoprecipitates from WT, but not *NR2D*$^{-/-}$ embryonic-brain homogenates. (**B**) Identification of Dock3 in NR2D immunoprecipitates from WT, but not *NR2D*$^{-/-}$ embryonic-brain homogenates. The immunoprecipitates and brain lysates were subjected to immunoblotting with anti-NR2D and anti-Dock3 antibodies. IP, immunoprecipitation; IB, immunoblotting.

Overexpression of Dock3 inhibits glutamate-induced intracellular Ca^{2+} elevation and prevents glutamate-induced apoptosis in RGCs

Next, we examined the functional consequences of the interaction between NR2D and Dock3, using primary cultures of RGCs. Previously, we reported that glutamate-induced Ca^{2+} elevation and apoptosis in RGCs are mediated by NMDARs [22]. Therefore, we examined the effects of overexpressing Dock3 on glutamate-induced Ca^{2+} elevation and apoptosis in cultured RGCs. Fluorescence-ratio images, displayed in pseudocolor in Figure 6A, demonstrated that glutamate markedly increased the intracellular Ca^{2+} concentration in RGCs from WT mice. Overexpression of Dock3 significantly inhibited glutamate-induced intracellular Ca^{2+} elevation in RGCs (Figure 6A, B). Because an increase in intracellular Ca^{2+} in RGCs mediated by NMDARs is a key step in initiating apoptosis [22,23], we next examined the neuroprotective effect of Dock3 on RGCs. Whereas 300 μM glutamate induced apoptosis in RGCs from WT mice (Figure 6C), overexpression of Dock3 significantly inhibited glutamate-induced apoptosis (Figure 6C). Together, these findings demonstrate that overexpression of Dock3

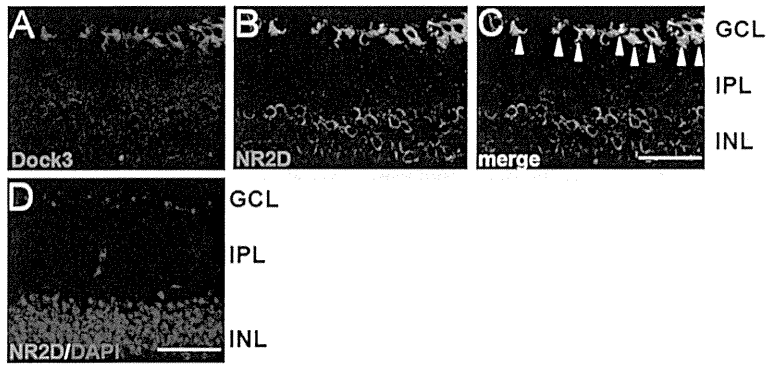

Figure 5 Co-localization of NR2D and Dock3 in the mouse retina. Double-labeling experiments were carried out on mouse retina sections (**A-C**). Dock3 (**A**) is red and NR2D (**B**) is green. Dock3 co-localized with NR2D in RGCs shown in the overlay panel (**C**) (arrowheads). Scale bar: 50 μm. (**D**) No NR2D immunoreactivity (green) was detected in retinas from *NR2D*$^{-/-}$ mice. Nuclei are counterstained with DAPI (blue). Scale bar: 50 μm. IPL, inner plexiform layer.

inhibits glutamate-induced intracellular Ca^{2+} elevation and prevents glutamate-induced apoptosis in RGCs, suggesting that the interaction between NR2D and Dock3 suppresses the function of NR2D-containing NMDARs.

Dock3 suppresses the surface expression of NR2D

Because NMDARs undergo regulated endocytosis [24-26], overexpression of Dock3 may reduce glutamate-induced Ca^{2+} elevation and apoptosis in RGCs by reducing the number of NMDARs on the cell surface. To investigate

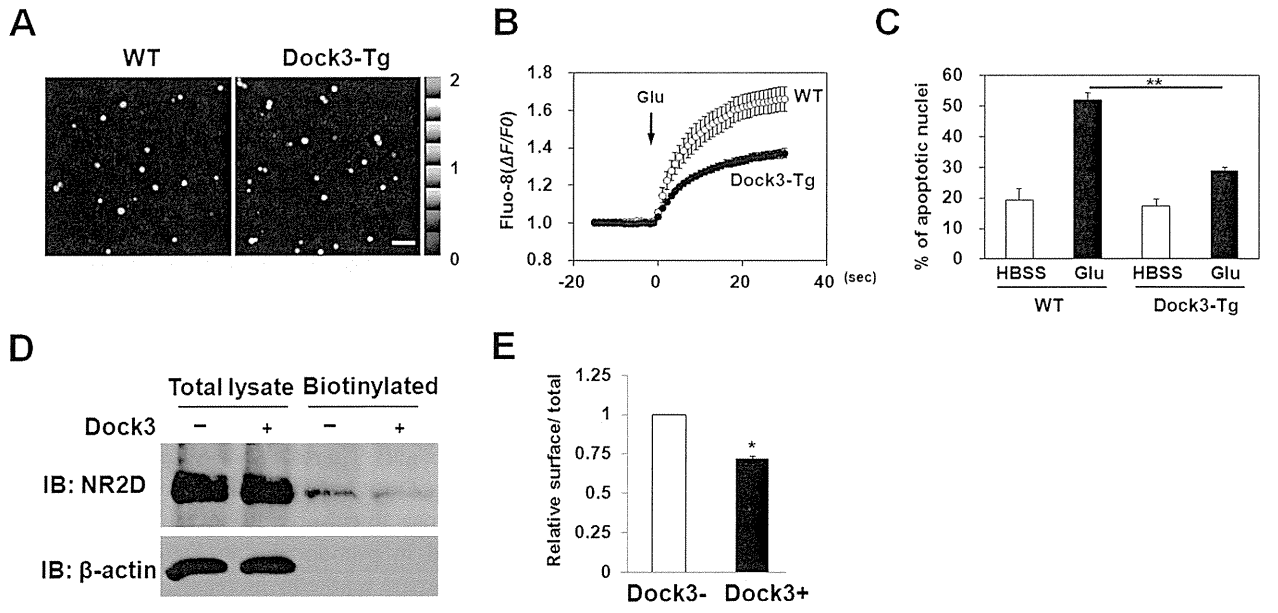

Figure 6 Overexpression of Dock3 inhibits glutamate-induced intracellular Ca^{2+} elevation and prevents glutamate-induced apoptosis in RGCs. (**A**) RGCs from WT or Dock3-transgenic (Tg) mice were labeled with Fluo-8 acetoxymethyl ester for 30 min, and then 300 μM glutamate (Glu) was added. Fluorescence-ratio images are displayed in pseudocolor, as indicated by the color bar on the right side of the images. Pseudocoloring represents ratios between 0 and 2, corresponding to 1, which is defined as the basal fluorescence intensities before Glu stimulation. Left and right panels show ratio images for WT and Dock3-Tg, respectively. Scale bar: 100 μm. (**B**) Changes in Fluo-8 fluorescence are expressed as ΔF/F0, where F0 is the basal fluorescence intensity before Glu stimulation. Glu was added as indicated. Data represent the mean ± SE from eight independent experiments. (**C**) Fragmented or shrunken nuclei were detected by Hoechst staining 24 hr after control (HBSS) or glutamate (Glu) treatment. **$p < 0.0001$ for WT Glu vs. Dock3-Tg Glu. WT, wild-type mice; Dock3-Tg, Dock3 transgenic mice. (**D**) N2A cells were transfected with NR1 and NR2D with (Dock3+) or without Dock3 (Dock3-). Surface proteins were biotinylated using sulfo-NHS-SS-biotin, immunoprecipitated with streptavidin beads and probed with NR2D antibodies. Data are from a single experiment, which is representative of five experiments that yielded similar results. (**E**) Relative ratio of biotinylated protein to total protein. *$p < 0.05$. Data represent the mean ± SE from five independent experiments.

whether Dock3 suppresses the surface expression of NR2D, we used N2A neuroblastoma cells. N2A cells were transfected with expression plasmids encoding NR1 and NR2D, with or without a plasmid harboring the full-length Dock3 cDNA. Cell-surface receptors were then quantified using a biotinylation assay. As shown in Figure 6D and E, the number of biotinylated NR2D subunits significantly decreased upon co-expression of Dock3, however, no changes was observed in the total amount of NR2D. These results suggest that Dock3 suppresses the surface expression of NR2D-containing NMDARs, thereby inhibiting glutamate-induced Ca^{2+} elevation and apoptosis in RGCs.

Discussion

In the present study, we show that NR2D is involved in the progressive loss of RGCs in GLAST-deficient mice, an animal model of NTG. In addition, we identify Dock3 as a novel NR2D-interacting protein and show that the interaction between NR2D and Dock3 protects RGCs from excitotxicity by reducing the surface expression of NR2D.

This is the first direct *in vivo* evidence of NR2D-NMDAR-mediated excitotoxicity in the context of glaucoma. In *GLAST*$^{-/-}$ mice, NR2D deficiency only partially prevented loss of RGCs, whereas inhibition of NMDARs with memantine almost completely prevented RGC loss [16]. This may be due to the involvement of other NR2 subunits in RGC degeneration, especially the NR2C subunit, given that NR2C-containing NMDARs are expressed in RGCs [15] and are the targets of therapeutic memantine activity [11]. NR2D deficiency prevented NMDA-induced-excitotoxic retinal cell death specifically in GCL, but not in INL. This can be explained by the lower expression level of NR2D in neurons of INL compared with those in GCL and by the contribution of other NR2 subunits to NMDA-induced cell death in INL.

Considering the high frequency of glaucoma in Alzheimer's disease patients [27], common mechanisms such as NR2D-NMDAR-mediated excitotoxicity might contribute to both diseases. In addition, NR2D mediates the capacity of PCP to induce locomotor hyperactivity in a novel environment, behavior that is thought to model positive symptoms in schizophrenia [12]. The genomic region that contains the NR2D gene locus has also been suggested to contribute to susceptibility to schizophrenia in a Japanese population [28]. NR2D is expressed in the adult mammalian brain, including the hippocampus, cortex, thalamus, and retina, all of which are CNS regions thought to be involved in Alzheimer's disease, schizophrenia, and glaucoma. Taken together, these findings suggest that NR2D may play roles in various neuropsychiatric diseases, including glaucoma, Alzheimer's disease, and schizophrenia, and may be a target for the development of novel drugs for the treatment of these neuropsychiatric diseases.

Multiple studies show that protein-protein interactions occurring at the intracellular C-terminal domains of NMDARs control the function and localization of these receptors. Whereas numerous proteins that interact with NR2A and NR2B have been identified [20,29,30], few directly interact with the NR2D subunit. The Abl tyrosine kinase is reported to interact directly with NR2D, but this interaction has no direct effect on NR2D function [31].

In the present study, we identified Dock3 as a novel NR2D-interacting protein. Dock3 was first identified as a presenilin (PS)-binding protein (PBP) [21] expressed in neurons and the testis, and is involved in cell adhesion and neurite outgrowth [32]. Dock3 may be linked to the pathogenesis of Alzheimer's disease: it is absent from the soluble fraction of samples taken from Alzheimer's disease brains [21], accumulates in neurofibrillary tangles [33], and regulates Aβ secretion [34]. Consistent with this hypothesis, deletion of Dock3 results in axonal degeneration and sensorimotor impairments [35]. Previously, we showed that overexpression of Dock3 induces optic nerve regeneration following injury [36]. In addition, we showed the role of Dock3 in glutamate-induced Ca^{2+} elevation and apoptosis of RGCs. In primary cultured RGCs, NMDARs contribute to Ca^{2+} elevation and apoptosis induced by 300 μM glutamate [22]. Overexpression of Dock3 significantly inhibited these glutamate-induced responses. Considering the direct interaction between Dock3 and NR2D, reflected in their co-localization, and the role of NR2D in NMDA-induced retinal cell death, these results suggest that Dock3 may play an important role in protecting RGCs from excitotoxicity through modulation of NR2D function.

Further mechanistic insights into the inhibition of glutamate-induced Ca^{2+} elevation and apoptosis of RGCs by overexpression of Dock3 were gained by conducting biochemical analyses of the surface expression of NR2D. Co-expression of Dock3 suppressed the expression of NR2D on the surface of the plasma membrane. Although further studies are required to clarify the mechanism by which Dock3 expression reduces the surface expression of NR2D, we can suggest two possible explanations. The first is that Dock3 could activate Rac1; activated Rac1 may then reduce the surface expression of NR2D by promoting endocytosis, similar to Kir2.1 channels [37]. The second possible explanation is that Dock3 interacts with both Fyn [36] and NR2D; Fyn may then regulate the surface expression of NR2D through tyrosine phosphorylation. The NR2D protein is developmentally regulated by tyrosine phosphorylation *in vivo*, suggesting that tyrosine phosphorylation may be important for regulating

the functions of this NMDAR subunit in the mammalian CNS [38].

Conclusions

We show here that NR2D is involved in excitotoxic degeneration of retinal cells and that Dock3 is a novel NR2D-interacting protein. Moreover, the interaction between NR2D and Dock3 protects RGCs from excitotoxicity by reducing the surface expression of NR2D. Identification of chemical compounds that can increase the expression of Dock3, or otherwise reduce the surface expression of NR2D, might have therapeutic benefit for the treatment of neurodegenerative disorders such as Alzheimer's disease and glaucoma.

Methods

Animals

GLAST and NR2D mutant mice were described previously [39,40]. Double-knockout mice ($GLAST^{-/-}/NR2D^{-/-}$) and homozygous GLAST-knockout mice ($GLAST^{-/-}$) were obtained by crossing double heterozygous mice ($GLAST^{+/-}/NR2D^{+/-}$). The homozygous NR2D-knockout mice ($NR2D^{-/-}$) were obtained by crossing heterozygous mice ($NR2D^{+/-}$). The genotypes of the mutant mice were determined as described previously [39,40]. Dock3 transgenic mice overexpress wild-type Dock3 under the control of the actin promoter [36]. In all experiments, age-matched WT and $GLAST^{-/-}$ littermate controls were used. All mice were of the C57BL/6 J genetic background, and all animal procedures were approved by the Animal Committee of Tokyo Medical and Dental University (0130166C).

Histological analysis

Mice were deeply anesthetized by diethyl ether. Eyes from mice at postnatal day 35 (P35) were enucleated, fixed in Davidson's solution fixative [41] overnight at 4°C, and dehydrated in 70% ethanol for three days at 4°C. The fixed eyes were then embedded in paraffin wax. Sections (7-μm thickness) of embedded retinal specimens were cut and stained with hematoxylin and eosin. The number of neurons in the GCL was counted from one ora serrate, through the optic nerve, to the other ora serrata. The average number of neurons in the GCL per eye was calculated from three sections of each retina.

Intravitreal injection of NMDA

WT and $NR2D^{-/-}$ mice (5 weeks old) were anesthetized by intraperitoneal injection of 50 mg/kg sodium pentobarbital, and their pupils were dilated with tropicamide. A single 2-μl injection of 20 mM NMDA in 0.1 M PBS (pH 7.40) was administered intravitreally into the right eye of each mouse, thereby delivering a dose of 40 nmol of NMDA. The same volume of PBS was administered to the contralateral (left) eye as control. To avoid lens injury, injections were performed under a stereomicroscope through a 32-gauge needle (Dentronics) connected to a 10-μl Hamilton syringe (Hamilton); the needle was inserted approximately 1 mm behind the corneal limbus.

TUNEL assay

Twenty-four hours after the NMDA or PBS injection, eyes were enucleated, fixed in Davidson fixative overnight at 4°C, embedded in paraffin, and sectioned (5-μm thickness). Apoptotic cells were labeled using the DeadEnd Fluorometric TUNEL (terminal deoxynucleotidyl transferase dUTP nick end labeling) System (Promega) according to the manufacturer's instructions. TUNEL-positive cells in GCL and INL were counted manually under a microscope. The average number of TUNEL-positive cells per eye was calculated for three sections of each retina.

Yeast two-hybrid screening

Yeast two-hybrid screening was performed by Hybrigenics Services, S.A.S., Paris, France. The coding sequence for the cytoplasmic region (aa 895–1323) of mouse NR2D (GenBank accession number gi: 144922605) was PCR-amplified and cloned into pB27 as a C-terminal fusion to LexA. The resulting plasmid, pB27 (N-LexA-NR2D-C), was used as a bait to screen a random-primed mouse adult brain cDNA library cloned into vector pP6. The bait strain/prey strain mating was spread on a medium lacking tryptophan, leucine, and histidine, and supplemented with 5 mM 3-aminotriazole to overcome bait auto-activation. A total of 357 positive clones were obtained out of 82 million interactions tested. The prey fragments of the positive clones were amplified by PCR and sequenced at their 5' and 3' junctions. The resulting sequences were used to identify the corresponding interacting proteins in the GenBank database (NCBI).

DNA constructs

Full-length mouse NR1 and NR2D cDNAs were amplified from vectors described previously [42,43] and subcloned into vector pEGFP C1 (Clontech), such that the NR2D protein was fused in frame with the C-terminal EGFP epitope. A similar plasmid was constructed for expression of the region containing the NR2D CT (aa 895–1323). The plasmid encoding mouse Dock3 cDNA (GenBank accession number gi: 148277095) was described previously [36]. The selected interacting domain (SID) of Dock3 was amplified by PCR and subcloned into vector pEF1/myc-His A (Invitrogen) such that the Dock3 protein was fused in frame with the N-terminal myc and His epitopes. All expression plasmids were confirmed by DNA sequencing.

Cell culture and transfection

HEK 293 T cells were cultured on 10-cm plates in Dulbecco's modified Eagle's medium (Sigma), supplemented with 10% fetal bovine serum (GIBCO) at 37°C in atmosphere containing 5% CO_2. When the cells reached 70% confluence, they were transiently transfected with cDNA constructs (up to 6 μg total) using the GeneJuice transfection reagent (Novagen).

Immunoprecipitation and immunoblotting

Cells were harvested 48 h after transfection and resuspended in cold lysis buffer (50 mM Tris–HCl, 1% Nonidet P-40, 5 mM EDTA, 150 mM NaCl, 0.5% Na-deoxycholate, 1 mM $MgCl_2$, 1 mM DTT, 1 mM Na_3VO_4, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride (PMSF), and Complete Protease Inhibitor Cocktail [Roche]). Samples were left on ice for 30 min and sonicated briefly. The insoluble fraction was removed by centrifugation at 15,000 rpm for 15 min. Protein concentration was determined using BCA Protein Assay kit (Sigma-Aldrich). Total cell lysates (TCLs) were boiled in the presence of 2× sample buffer. Immunoprecipitation was performed with 50 μl of anti-Myc magnetic beads (clone PL14; MBL), anti-GFP magnetic beads (clone RQ2; MBL), and 400 μg of cell homogenate according to the manufacturer's protocol. Immunoprecipitates and TCL were separated by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene difluoride (PVDF, Millipore) membranes. The membranes were blocked with 10% skim milk/PBST (PBS containing 0.05% Tween 20) solution for 1 h at room temperature, and then treated with primary antibodies. The following antibodies were used for the blots: anti–Myc-tag rabbit polyclonal antibody (1:1000; MBL) and anti-GFP rabbit polyclonal Living Colors Av peptide antibody (1:100; Clontech Laboratories). After 1 h at room temperature, the membrane was washed three times in PBST for 30 min and incubated for 1 h in horseradish peroxidase (HRP)-conjugated second antibody (1:10,000; Jackson ImmunoResearch Laboratories, Inc.).

For *in vivo* immunoprecipitation, 90 mg of brain tissue was collected from mice at embryonic day 18 and homogenized using a POLYTRON PT 1200E homogenizer (Kinematica AG) in 500 ul cold lysis buffer (300 mM NaCl, 5 mM Tris-Cl [pH 7.5], 0.5% Triton X-100, and Complete Protease Inhibitor Cocktail). Co-immunoprecipitation was performed according to the method previously described [44] with some modifications. Briefly, the sample was left on ice for 1 h, spun at 10,000 *g* for 20 min at 4°C, and the supernatant was collected, and the protein concentration was determined using BCA Protein Assay kit. NR2D antibody [45] (1 μg) and Dock3 antibody [46] (0.5 μg) were added to 500 μl of pre-cleared lysate and incubated at 4°C overnight. Protein G Sepharose (50 μl) was then added to collect the immunoprecipitate. Samples were incubated at 4°C for 2 h and centrifuged at 1,000 *g* for 1 min, and supernatant was removed. The precipitate was washed three times with 1 ml of lysis buffer. Each sample was diluted with 50 μl of 2× sample buffer and run on an SDS polyacrylamide gel (7.5%); an equal volume (20 μl) of each sample was loaded onto the gel. Separated proteins were transferred to PVDF membranes. The membranes were then incubated with an anti-NR2D guinea-pig polyclonal antibody (1:1000) and an anti-Dock3 rabbit polyclonal antibody (1:1000) at 4°C overnight. The membrane was washed three times in TBST (TBS containing 0.05% Tween 20) for 30 min and incubated for 1 h with an HRP-conjugated secondary antibody (1:5,000–1:10,000). SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was used to visualize the immunoreactive proteins.

Immunohistochemistry

Mice were deeply anesthetized by diethyl ether and perfused with 0.1 M PBS and then with 4% PFA in 0.1 M PB. Eyes were immediately enucleated and immersed in the same fixative for 2 hours at 4°C, followed by immersion in a sucrose solution (30% in PB) overnight at 4°C. Eyes were embedded in OCT compound (Sakura Finetechnical Co. Ltd) and frozen on dry ice. The eyes were sectioned (10-μm thickness) using a cryostat. After washing in PBS, the sections were blocked with 5% normal horse serum diluted in PBS with 0.2% Triton X-100 for 30 minutes at room temperature. Sections were incubated overnight at 4°C with a goat polyclonal antibody against NR2D (1:50; Santa Cruz Biotechnology, INC) and a rabbit polyclonal antibody against Dock3 (1:100). Sections were then incubated with secondary antibodies against goat and rabbit IgG (Alexa Furo 488, 1:1000; Alexa Furo 568, 1:500; both from Molecular Probes) for 1 h at room temperature. After a final rinse in PBS, the sections were cover-slipped with Fluoromount (Diagnostic BioSystems). Images were recorded with an LSM-510 META confocal laser microscope (Carl Zeiss).

Primary culture of mouse retinal ganglion cells

C57BL/6 J or Dock3-Tg mice (7–10 days old) were used for primary culture of RGCs according to Winzeler *et al.* [47] with some modifications. Briefly, retinae were digested with papain (16.5 units/ml) for 45 min at 37°C, triturated in Minimum Essential Medium (MEM; Invitrogen) containing 0.15% trypsin inhibitor (Roche Applied Science), and then triturated again in MEM containing 1% trypsin inhibitor. The cell suspension was incubated on a first panning plate (150 mm Petri dish) coated with Bandeiraea lectin I (L-1100; Vector Laboratories, Inc.) for 10 min at room temperature. Non-adherent cells were incubated for 45 min on a second panning plate (100 mm Petri dish) coated with goat anti-

mouse IgG + IgM (H + L) (Jackson ImmunoResearch Laboratories, Inc.) and mouse anti-mouse Thy1.2 IgM antibodies (MCA02R; AbD Serotec, Oxford, UK). The panning plate was washed with PBS, and adherent RGCs were released by treatment with 0.125% trypsin for 10 min at 37°C. The RGC suspension was mixed with 30% fetal bovine serum and centrifuged at 200 *g* for 10 min. RGCs were suspended in medium containing 1 mM glutamine, 5 μg/ml insulin, 60 μg/ml N-acetylcysteine, 62 ng/ml progesterone, 16 μg/ml putrescine, 40 ng/ml sodium selenite, 0.1 mg/ml BSA, 40 ng/ml triiodothyronine, 0.1 mg/ml transferrin, 1 mM sodium pyruvate, 2% B27 supplement (Invitrogen), 10 μM forskolin (Sigma), 50 ng/ml brain-derived neurotrophic factor (BDNF; PeproTech, Rocky Hill, NJ), 50 ng/ml ciliary neurotrophic factor (CNTF; PeproTech), and 50 ng/ml basic fibroblast growth factor (bFGF; PeproTech) in Neurobasal medium (Invitrogen). Ninety-six–well culture plates were coated with poly-D-lysine (Sigma) and laminin (Sigma) and mouse RGCs were plated at a density of 4,000 cells/well (or 4,000 cells/culture insert for μ-dishes (ibidi)) and cultured for at least 10 days before the experiments.

Induction and detection of apoptosis induced by glutamate

RGCs were washed twice (15-min incubation, ×2) with Hank's Balanced Salt Solution (HBSS; Invitrogen) containing 2.4 mM $CaCl_2$ and 20 mM HEPES without magnesium. Subsequently, RGCs were incubated for 2 h at 37°C with or without 300 μM glutamate and 10 μM glycine (a co-activator of NMDARs) in HBSS containing 2.4 mM $CaCl_2$ and 20 mM HEPES without magnesium. After HBSS or glutamate treatment, RGCs were cultured for 22 h at 37°C in the same medium to culture the RGCs, but without forskolin, BDNF, CNTF, and bFGF. To detect apoptosis using Hoechst 33342 (Dojindo), RGCs were washed once with PBS and incubated with 1 μg/ml Hoechst 33342 for 15 min at room temperature. Fluorescent images were randomly taken (four images/well) using an Olympus IX71 fluorescence microscope. For each treatment, at least eight images were taken from two wells of a 96-well plate. Fragmented or shrunken nuclei stained with Hoechst dye were deemed apoptotic, and neurons with round and smooth nuclei were counted as healthy. More than 200 neurons for each treatment were counted by a researcher blinded to the identity of the samples.

Measurement of intracellular calcium

Mouse RGCs were incubated for 30 min at 37°C in culture medium with 3 μM Fluo-8 acetoxymethyl ester (AAT Bioquest). Cells were washed twice (15-min incubation, ×2) with HBSS containing 2.4 mM $CaCl_2$ and 20 mM HEPES without magnesium, then stimulated with 300 μM glutamate and 10 μM glycine. Fluorescence images were acquired every 500 msec using an ORCA-R2 digital CCD camera (Hamamatsu Photonics) and analyzed using the MetaFluor fluorescence-ratio imaging software (Molecular Devices).

Surface-biotinylation assay

Neuro 2A cells were plated at a density of 2×10^5/well in 6-well plates and cultured in 95% air/5% CO_2 at 37°C. The cells were transiently co-transfected with the cDNAs encoding NR1 and NR2D with (Dock3+) or without (Dock3-) Dock3. Forty-eight hours after transfection, cells were incubated in PBS containing 1.5 mg/ml Sulfo-NHS-SS-biotin (Pierce) for 20 min at 4°C. Surface biotinylation was stopped by removing that solution and incubating the cells in 10 mM ice-cold glycine in PBS for 20 min. Cells were rinsed twice in PBS and then lysed in 200 μl PBS with Complete Protease Inhibitor Cocktail, 0.1% SDS, and 1% Triton X-100. A fraction (15%, 30 μl) of the cell lysate was removed to measure total protein concentration and for total input; the remaining 85% (170 μl) of the cell lysate was incubated with 70 μl of 50% avidin-agarose (Sigma) overnight at 4°C. After washing three times with lysis buffer, bound proteins were resuspended in 30 μl of 2× sample buffer and boiled. Samples were analyzed by SDS-PAGE followed by Western blotting using anti-NR2D guinea-pig polyclonal antibody (1:1000). The data were quantified by measuring the ratios between intensities of the biotinylated and total NR2D bands using the Image Lab software (Bio-Rad). Surface/total ratios from the Dock3- control were assigned a value of 1. Ratios of the Dock3+ groups were expressed relative to the controls and averaged.

Statistical analysis

All data are expressed as mean ± S.E. Statistical analyses were conducted using Student's *t*-test for comparison between two samples, or one-way ANOVA followed by Bonferroni's test for multiple comparisons, using the SPSS 17.0 software package. P values < 0.05 were considered statistically significant.

Abbreviations

CNS: Central nervous system; DHR-1: Dock homology region 1; DHR-2: Dock homology region 2; Dock3: Dedicator of cytokinesis 3; GCL: Ganglion cell layer; GLAST: Glutamate aspartate transporter; HEK: Human embryonic kidney; INL: Inner nuclear layer; MOCA: Modifier of cell adhesion protein; NMDAR: N-methyl-D-aspartate receptor; NTG: Normal tension glaucoma; PBP: Presenilin binding protein; PBS: Phosphate-buffered saline; PCP: Phenycyclidine; PMSF: Phenylmethylsulfonyl fluoride; PS: Presenilin; PVDF: Polyvinylidene difluoride; RGC: Retinal ganglion cell; TCL: Total cell lysate; WT: Wild-type.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KT, TA and NB conceived and designed the experiments. NB carried out all experiments except the experiments performed on primary cultured RGCs and analyzed the data. HH carried out the experiments performed on primary cultured RGCs and analyzed the data. KN, TH and MM contributed

reagents and materials. KT and NB wrote the paper. All authors have read and approved the manuscript for publication.

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References

1. Nakanishi S, Nakajima Y, Masu M, Ueda Y, Nakahara K, Watanabe D, Yamaguchi S, Kawabata S, Okada M: **Glutamate receptors: brain function and signal transduction.** *Brain Res Brain Res Rev* 1998, **26**:230–235.
2. Choi DW: **Glutamate neurotoxicity and diseases of the nervous system.** *Neuron* 1988, **1**:623–634.
3. Choi DW, Rothman SM: **The role of glutamate neurotoxicity in hypoxic-ischemic neuronal death.** *Annu Rev Neurosci* 1990, **13**:171–182.
4. Dingledine R, Borges K, Bowie D, Traynelis SF: **The glutamate receptor ion channels.** *Pharmacol Rev* 1999, **51**:7–61.
5. Chapman AG: **Glutamate and epilepsy.** *J Nutr* 2000, **130**(4S Suppl):1043S–1045S.
6. Moghaddam B, Jackson ME: **Glutamatergic animal models of schizophrenia.** *Ann N Y Acad Sci* 2003, **1003**:131–137.
7. Meldrum B: **Amino acids as dietary excitotoxins: a contribution to understanding neurodegenerative disorders.** *Brain Res Brain Res Rev* 1993, **18**:293–314.
8. Monyer H, Burnashev N, Laurie DJ, Sakmann B, Seeburg PH: **Developmental and regional expression in the rat brain and functional properties of four NMDA receptors.** *Neuron* 1994, **12**:529–540.
9. Cull-Candy SG, Leszkiewicz DN: **Role of distinct NMDA receptor subtypes at central synapses.** *Sci STKE* 2004, **2004**:re16.
10. Watanabe M, Inoue Y, Sakimura K, Mishina M: **Developmental changes in distribution of NMDA receptor channel subunit mRNAs.** *Neuroreport* 1992, **3**:1138–1140.
11. Kotermanski SE, Johnson JW: **Mg2+ imparts NMDA receptor subtype selectivity to the Alzheimer's drug memantine.** *J Neurosci* 2009, **29**:2774–2779.
12. Hagino Y, Kasai S, Han W, Yamamoto H, Nabeshima T, Mishina M, Ikeda K: **Essential role of NMDA receptor channel ε4 subunit (GluN2D) in the effects of phencyclidine, but not methamphetamine.** *PLoS One* 2010, **5**:e13722.
13. Ishii T, Moriyoshi K, Sugihara H, Sakurada K, Kadotani H, Yokoi M, Akazawa C, Shigemoto R, Mizuno N, Masu M: **Molecular characterization of the family of the N-methyl-D-aspartate receptor subunits.** *J Biol Chem* 1993, **268**:2836–2843.
14. Hrabetova S, Serrano P, Blace N, Tse HW, Skifter DA, Jane DE, Monaghan DT, Sacktor TC: **Distinct NMDA receptor subpopulations contribute to long-term potentiation and long-term depression induction.** *J Neurosci* 2000, **20**:RC81.
15. Jakobs TC, Ben Y, Masland RH: **Expression of mRNA for glutamate receptor subunits distinguishes the major classes of retinal neurons, but is less specific for individual cell types.** *Mol Vis* 2007, **13**:933–948.
16. Harada T, Harada C, Nakamura K, Quah HM, Okumura A, Namekata K, Saeki T, Aihara M, Yoshida H, Mitani A, *et al*: **The potential role of glutamate transporters in the pathogenesis of normal tension glaucoma.** *J Clin Invest* 2007, **117**:1763–1770.
17. Lin Y, Skeberdis VA, Francesconi A, Bennett MV, Zukin RS: **Postsynaptic density protein-95 regulates NMDA channel gating and surface expression.** *J Neurosci* 2004, **24**:10138–10148.
18. Sans N, Prybylowski K, Petralia RS, Chang K, Wang YX, Racca C, Vicini S, Wenthold RJ: **NMDA receptor trafficking through an interaction between PDZ proteins and the exocyst complex.** *Nat Cell Biol* 2003, **5**:520–530.
19. Chen BS, Roche KW: **Growth factor-dependent trafficking of cerebellar NMDA receptors via protein kinase B/Akt phosphorylation of NR2C.** *Neuron* 2009, **62**:471–478.
20. Yu XM, Askalan R, Keil GJ, Salter MW: **NMDA channel regulation by channel-associated protein tyrosine kinase Src.** *Science* 1997, **275**:674–678.
21. Kashiwa A, Yoshida H, Lee S, Paladino T, Liu Y, Chen Q, Dargusch R, Schubert D, Kimura H: **Isolation and characterization of novel presenilin binding protein.** *J Neurochem* 2000, **75**:109–116.
22. Hayashi H, Eguchi Y, Fukuchi-Nakaishi Y, Takeya M, Nakagata N, Tanaka K, Vance JE, Tanihara H: **A potential neuroprotective role of apolipoprotein E-containing lipoproteins through low density lipoprotein receptor-related protein 1 in normal tension glaucoma.** *J Biol Chem* 2012, **287**:25395–25406.
23. Sucher NJ, Lipton SA, Dreyer EB: **Molecular basis of glutamate toxicity in retinal ganglion cells.** *Vision Res* 1997, **37**:3483–3493.
24. Snyder EM, Philpot BD, Huber KM, Dong X, Fallon JR, Bear MF: **Internalization of ionotropic glutamate receptors in response to mGluR activation.** *Nat Neurosci* 2001, **4**(11):1079–1085.
25. Roche KW, Standley S, McCallum J, Dune Ly C, Ehlers MD, Wenthold RJ: **Molecular determinants of NMDA receptor internalization.** *Nat Neurosci* 2001, **4**:794–802.
26. Nong Y, Huang YQ, Ju W, Kalia LV, Ahmadian G, Wang YT, Salter MW: **Glycine binding primes NMDA receptor internalization.** *Nature* 2003, **422**:302–307.
27. Tamura H, Kawakami H, Kanamoto T, Kato T, Yokoyama T, Sasaki K, Izumi Y, Matsumoto M, Mishima HK: **High frequency of open-angle glaucoma in Japanese patients with Alzheimer's disease.** *J Neurol Sci* 2006, **246**:79–83.
28. Makino C, Shibata H, Ninomiya H, Tashiro N, Fukumaki Y: **Identification of single-nucleotide polymorphisms in the human N-methyl-D-aspartate receptor subunit NR2D gene, GRIN2D, and association study with schizophrenia.** *Psychiatr Genet* 2005, **15**:215–221.
29. Lau LF, Mammen A, Ehlers MD, Kindler S, Chung WJ, Garner CC, Huganir RL: **Interaction of the N-methyl-D-aspartate receptor complex with a novel synapse-associated protein, SAP102.** *J Biol Chem* 1996, **271**:21622–21628.
30. Kornau HC, Schenker LT, Kennedy MB, Seeburg PH: **Domain interaction between NMDA receptor subunits and the postsynaptic density protein PSD-95.** *Science* 1995, **269**:1737–1740.
31. Glover RT, Angiolieri M, Kelly S, Monaghan DT, Wang JY, Smithgall TE, Buller AL: **Interaction of the N-methyl-D-aspartic acid receptor NR2D subunit with the c-Abl tyrosine kinase.** *J Biol Chem* 2000, **275**:12725–12729.
32. Chen Q, Chen TJ, Letourneau PC, Costa La F, Schubert D: **Modifier of cell adhesion regulates N-cadherin-mediated cell-cell adhesion and neurite outgrowth.** *J Neurosci* 2005, **25**:281–290.
33. Chen Q, Yoshida H, Schubert D, Maher P, Mallory M, Masliah E: **Presenilin binding protein is associated with neurofibrillary alterations in Alzheimer's disease and stimulates tau phosphorylation.** *Am J Pathol* 2001, **159**:1597–1602.
34. Chen Q, Kimura H, Schubert D: **A novel mechanism for the regulation of amyloid precursor protein metabolism.** *J Cell Biol* 2002, **158**:79–89.
35. Chen Q, Peto CA, Shelton GD, Mizisin A, Sawchenko PE, Schubert D: **Loss of modifier of cell adhesion reveals a pathway leading to axonal degeneration.** *J Neurosci* 2009, **29**:118–130.
36. Namekata K, Harada C, Guo X, Kimura A, Kittaka D, Watanabe H, Harada T: **Dock3 stimulates axonal outgrowth via GSK-3β-mediated microtubule assembly.** *J Neurosci* 2012, **32**:264–274.
37. Boyer SB, Slesinger PA, Jones SV: **Regulation of Kir2.1 channels by the Rho-GTPase, Rac1.** *J Cell Physiol* 2009, **218**:385–393.
38. Dunah AW, Yasuda RP, Wolfe BB: **Developmental regulation of tyrosine phosphorylation of the NR2D NMDA glutamate receptor subunit in rat central nervous system.** *J Neurochem* 1998, **71**:1926–1934.
39. Ikeda K, Araki K, Takayama C, Inoue Y, Yagi T, Aizawa S, Mishina M: **Reduced spontaneous activity of mice defective in the epsilon 4 subunit of the NMDA receptor channel.** *Brain Res Mol Brain Res* 1995, **33**:61–71.
40. Watase K, Hashimoto K, Kano M, Yamada K, Watanabe M, Inoue Y, Okuyama S, Sakagawa T, Ogawa S, Kawashima N, *et al*: **Motor discoordination and increased susceptibility to cerebellar injury in GLAST mutant mice.** *Eur J Neurosci* 1998, **10**:976–988.

41. Chi ZL, Akahori M, Obazawa M, Minami M, Noda T, Nakaya N, Tomarev S, Kawase K, Yamamoto T, Noda S, *et al*: **Overexpression of optineurin E50K disrupts Rab8 interaction and leads to a progressive retinal degeneration in mice.** *Hum Mol Genet* 2010, **19**:2606–2615.
42. Mori H, Yamakura T, Masaki H, Mishina M: **Involvement of the carboxyl-terminal region in modulation by TPA of the NMDA receptor channel.** *Neuroreport* 1993, **4**:519–522.
43. Ikeda K, Nagasawa M, Mori H, Araki K, Sakimura K, Watanabe M, Inoue Y, Mishina M: **Cloning and expression of the epsilon 4 subunit of the NMDA receptor channel.** *FEBS Lett* 1992, **313**:34–38.
44. Khosravani H, Zhang Y, Tsutsui S, Hameed S, Altier C, Hamid J, Chen L, Villemaire M, Ali Z, Jirik FR, *et al*: **Prion protein attenuates excitotoxicity by inhibiting NMDA receptors.** *J Cell Biol* 2008, **181**:551–565.
45. Wu Y, Kawakami R, Shinohara Y, Fukaya M, Sakimura K, Mishina M, Watanabe M, Ito I, Shigemoto R: **Target-cell-specific left-right asymmetry of NMDA receptor content in schaffer collateral synapses in epsilon1/NR2A knock-out mice.** *J Neurosci* 2005, **25**:9213–9226.
46. Namekata K, Harada C, Taya C, Guo X, Kimura H, Parada LF, Harada T: **Dock3 induces axonal outgrowth by stimulating membrane recruitment of the WAVE complex.** *Proc Natl Acad Sci U S A* 2010, **107**:7586–7591.
47. Winzeler AM, Mandemakers WJ, Sun MZ, Stafford M, Phillips CT, Barres BA: **The lipid sulfatide is a novel myelin-associated inhibitor of CNS axon outgrowth.** *J Neurosci* 2011, **31**:6481–6492.

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特集2

中枢神経のトランスポーター・チャネル：新たな創薬標的として

精神神経疾患とグルタミン酸神経伝達：基礎医学的観点から

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SUMMARY

グルタミン酸は，中枢神経系において主要な興奮性神経伝達物質であり，記憶・学習などの脳高次機能に重要な役割を果たしている．しかし，その機能的な重要性の反面，興奮毒性という概念で表されるように，過剰なグルタミン酸は神経細胞障害作用を持ち，さまざまな精神神経疾患に関与すると考えられている．著者らは，グルタミン酸の細胞外濃度を制御するグリア型グルタミン酸トランスポーターの機能を阻害したマウスを作製し，そのマウスに神経細胞変性や社会行動の障害・強迫性行動・統合失調症様の行動異常が起こることを発見した．グリア型グルタミン酸トランスポーターを活性化する化合物は，新しい抗精神神経疾患薬として有用であると期待される．

KEY WORDS
グルタミン酸
トランスポーター
統合失調症
うつ病
神経変性疾患

はじめに

グルタミン酸は哺乳類の中枢神経系において記憶・学習などの高次機能を調節する主要な興奮性神経伝達物質として知られている[1]．一方で，細胞外グルタミン酸の上昇は，グルタミン酸受容体の過剰な活性化によりグルタミン酸興奮毒性とよばれる神経細胞障害作用を持ち，多くの精神疾患に関与している（**図1,2**）[2]．細胞外グルタミン酸濃度は，グルタミン酸トランスポーターにより厳密に制御されている．これまで，5種類のグルタミン酸トランスポーターサブタイプ，EAAT1（GLAST），EAAT2（GLT1），EAAT3（EAAC1），EAAT4，EAAT5が単離され，その分子的実態が明らかにされている．GLAST，GLT1は主にアストロサイトに，EAAC1とEAAT4は神経細胞に，EAAT5は網膜に発現している．シナプス間隙におけるグルタミン酸の除去は，主にアストロサイトに存在する2種類のグルタミン酸トランスポーターGLAST,GLT1の活性により制御されている．本稿では，GLAST,GLT1の機能障害に着目し，その機能障害がどのような精神神経疾患の発症に関与するかを概説する．

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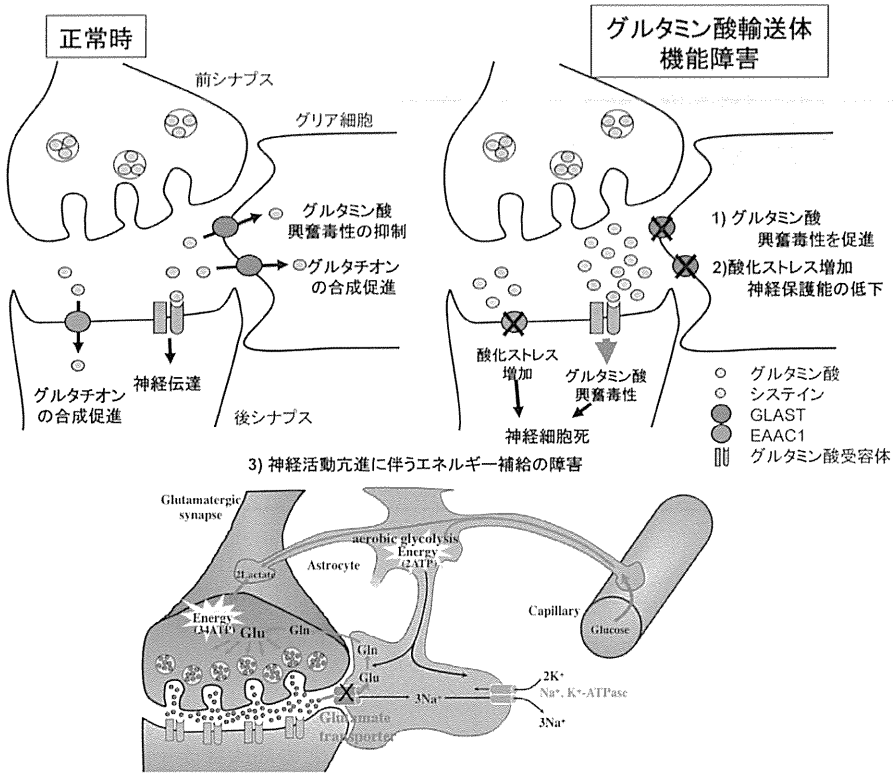

図1　グルタミン酸トランスポーターの機能障害が神経機能におよぼす影響
（p.5 カラー図参照）

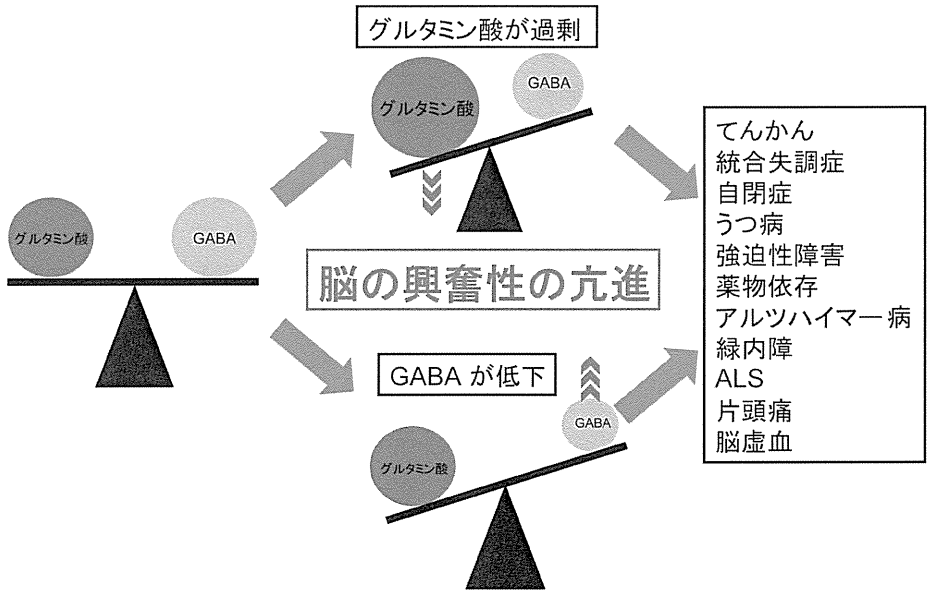

図2　グルタミン酸トランスポーターの機能異常による興奮と抑制のアンバランスがさまざまな精神神経疾患を引き起こす

I. グルタミン酸トランスポーターと精神疾患

1. 統合失調症における GLAST・GLT1 の関与

統合失調症は，幻覚・妄想などの陽性症状と，無為自閉，感情鈍麻，意欲の減退などの陰性症状，ワーキングメモリーなどの障害による認知障害を示し，世界中でおよそ 100 人に 1 人が発症する精神疾患である．NMDA 受容体欠損マウスや NMDA 受容体阻害剤を投与された動物が統合失調症様の症状を示すことから，グルタミン酸神経伝達の低下が統合失調症の有力な病態であると考えられている．しかし，最近の臨床試験結果から，グルタミン酸の放出を抑制する代謝型グルタミン酸受容体 mGluR2/3 のアゴニストが統合失調症の治療薬として有望であることが報告された[3]．この報告は，統合失調症では細胞外グルタミン酸濃度が上昇し，脳全体として興奮性優位となっている可能性を示唆している．さらに，統合失調症患者の遺伝子解析から，GLAST 遺伝子座の欠失や GLT1 のミスセンス変異がある症例が報告された[4,5]．そこで，まず GLAST 欠損マウスの行動解析を行った．GLAST 欠損マウスは，新規環境下に置かれると，野生型に比べ行動量が増加し（図 3），その増加はハロペリドールや mGluR2/3 のアゴニスト（LY379268）などの統合失調症治療薬で改善される[6]．さらに，グルタミン酸受容体阻害剤である MK-801 により誘発される行動量の増加が，GLAST 欠損マウスでは悪化する（図 3）[6]．これらの行動異常は，統合失調症の陽性症状に相当する．また，GLAST 欠損マウスは，新奇マウスに対する sniffing などの行動時間が減少し（図 3），巣作り行動も障害されており，社会行動に障害がみられた[7]．これらの行動異常は，統合失調症の陰性症状に相当する．さらに，GLAST 欠損マウスは pairwise discrimination task が障害され，統合失調症の認知障害に類似した症状を示す[7]．これらの結果は，GLAST 欠損マウスが統合失調症のモデル動物であることを示しており，「アストロサイトに発現するグルタミン酸トランスポーター GLAST の機能異常によるグルタミン酸機能亢進」が統合失調症の発症に重要な役割を果たすと考えられる．GLT1 に関しては，統合

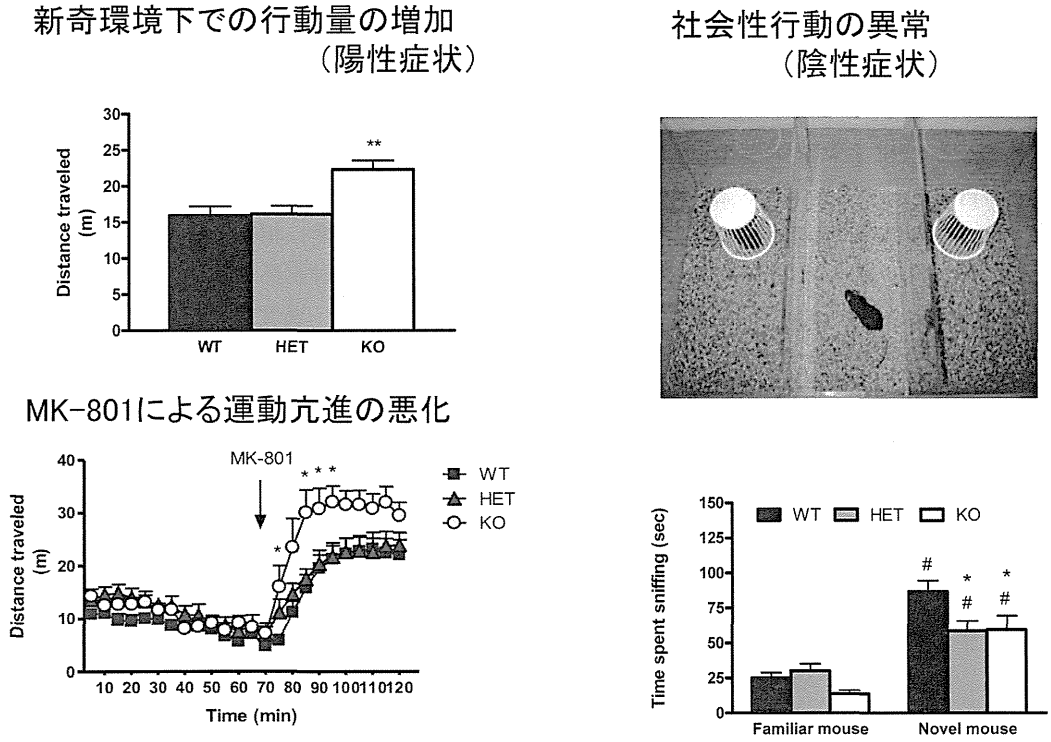

図 3　グルタミン酸輸送体 GLAST 欠損マウスは統合失調症に似た行動異常を示す

失調症の患者さんの約0.1％にArg106Hisのミスセンス変異がみられることが，最近報告された[5]．著者らは，このミスセンス変異によりGLT1のグルタミン酸取り込み活性が低下することを見出している．現在，GLT1の発現を低下させたマウスを用いて，統合失調症様行動異常が観察されるかどうか解析している．

統合失調症の主要な仮説の一つとして，「神経発達障害仮説」がある．統合失調症患者では，海馬・扁桃体の細胞構築の異常や萎縮，側脳室・第Ⅲ脳室の拡大が認められる．そこで著者は，グルタミン酸トランスポーターの機能障害が統合失調症に似た神経発達障害を引き起こすのではないかと考えた．統合失調症患者で遺伝子異常が報告されているGLASTおよびGLT1の両者を欠損させたダブル欠損マウス（DKマウス）を作製し，脳形成期におけるグルタミン酸機能亢進状態を再現した[8]．DKマウスは胎生17日以降に死亡し，大脳皮質・海馬の層形成障害，扁桃体の核形成障害，側脳室・第Ⅲ脳室の拡大など統合失調症に似た神経発達障害を示した．これらの脳発達障害は，グルタミン酸受容体NR1の欠損により正常化された[9]．さらに，DKマウスの脳では，視床に神経細胞死がみられた．統合失調症患者では，視床の体積減少，課題遂行中の血流低下，代謝異常が報告されている．以上の結果は，胎児期のグルタミン酸トランスポーターGLAST・GLT1の機能障害は，統合失調症に似た神経発達障害を起こすことを示している．また，統合失調症のリスク要因として報告されている胎児期・周産期の栄養障害・脳虚血・ウイルス感染は，グルタミン酸トランスポーターの機能を阻害することが知られている．これらのことは，アストロサイトに発現するグルタミン酸トランスポーターの機能障害は，統合失調症の発症に関与することを示している．

最近，統合失調症の発症危険状態を発症へと移行させる要因として，海馬における細胞外グルタミン酸濃度の上昇が報告された[10]．著者らは，グルタミン酸トランスポーターを一過性に欠損させるマウスを作製しており，そのマウスの解析は統合失調症の発症危険状態を発症へと移行させる機序を解明するのに貢献すると期待される．統合失調症の発症危険状態を発症へと移行させる機序の解明は，早期介入による統合失調症の発症予防法の開発に役立つと考えられる．

2. 強迫性障害・自閉症におけるGLT1の関与

強迫性障害は，強迫観念・強迫行為を特徴とする疾患である．多くは思春期過ぎから発症し，人口の2～3％が罹患歴を持つ．従来，セロトニン神経伝達の異常が強迫性障害に関与すると考えられてきた．しかし，セロトニン神経伝達を上昇させる抗うつ薬などの治療では，一部の患者さんにしか効果がなく，セロトニン神経伝達の障害だけでは強迫性障害の病態を説明できない．最近，グルタミン酸神経伝達の亢進が強迫性障害の発症にも重要な役割を果たすことが報告されている．主なものに，(1) 強迫性障害患者の脳内ではグルタミン酸量が増加し，これによる神経伝達が亢進している (2) グルタミン酸神経伝達に関わる遺伝子の一塩基多型頻度が強迫性障害患者では増加している (3) グルタミン酸神経伝達を抑制する薬剤に強迫性障害の治療効果がある，といったことがあげられる[11]．

自閉症は，社会性行動の喪失や言語発達の遅延を特徴とする脳高次機能の発達障害である．グルタミン酸神経伝達系の亢進は自閉症の重要なリスクであり，脳の形成にきわめて重要な役割を持つ．自閉症様の行動を示す脆弱X症候群や結節性硬化症の患者ではグルタミン酸神経伝達の亢進が報告されている．自閉症のゲノムワイドな連鎖解析により，11番染色体の11p12-13が自閉症に関連があることが明らかになった[12]．この領域はGLT1の遺伝子座である．

最近，著者らは誘導型Cre & loxPシステムを使って生後3週齢にGLT1遺伝子の欠損を誘導すると，マウスの思春期に相当する7週齢の時点で，GLT1蛋白質の量は，対照群と比べて約30％にまで減少した．このマウスは，過度な毛繕い行動を示すようになり，顔面には激しい裂傷が生じた．このマウスの痛覚や皮膚に異常がみられないことから，過度な毛繕い行動は中枢神経系の異常に起因すると考えられる．この行動異常は，強迫性障害と自閉症に共通する主要な症状である「繰り返し行動」に相当する．このモデルでは，不安の亢進や社会性行動の異常がみられず，純粋な「繰り返し行動」のみを再現しており，疾患モデルではなく症状モデルと考えられる．現在，このモデルを用いて，「繰り返し行動」の神経基盤や新規治療薬の開発を行っている．

II. グルタミン酸トランスポーターと神経疾患

1. アルツハイマー病における GLT1 の関与

アルツハイマー病は，神経変性による起こる認知症で，高齢化により患者数は増加し，超高齢化社会を迎える日本にとって根本的な治療法の確立が望まれている．アルツハイマー病の病態に過剰なグルタミン酸受容体の活性化が関与することは，グルタミン酸受容体阻害剤であるメマンチンが治療薬として用いられていることからも明らかである．グルタミン酸トランスポーターの障害がアルツハイマー病の発症に関与することを示す証拠として，(1) アルツハイマー病患者の脳では GLAST，GLT1，EAAC1 の発現量が減少している[13]，(2) アルツハイマー病モデルマウスの GLT1 の発現量を低下させると空間学習の障害が促進される[14]，(3) アルツハイマー病における神経変性の原因物質と考えられているβアミロイド蛋白により GLT1 の機能が障害される，などがある．GLT1 の活性化化合物が，新規アルツハイマー病の治療薬として有効かどうか，モデル動物による評価が期待される．

2. 正常眼圧緑内障における GLAST の関与

緑内障は，40 歳以上では約 5%が潜在的に罹患していると考えられており，日本人の中途失明原因の第 1 位である．さらに，高齢化により患者数は増加し，その治療は活力ある高齢化社会を作るためには必要不可欠である．わが国の緑内障の約 70%は正常眼圧緑内障であり，その病態は不明である．著者らは，グルタミン酸トランスポーター GLAST 欠損マウスが，正常眼圧緑内障と同じ症状を示すことを明らかにした（眼圧が正常であるにも関わらず，網膜神経節細胞が加齢に伴い選択的に変性し，視神経乳頭陥凹が拡大する）（**図 4**）[15]．さらに，緑内障患者の約 1%が，GLAST の機能障害を伴うミスセンス変異を持つことをみつけた．この結果は，GLAST の遺伝子異常によるグルタミン酸輸送活性の低下が，緑内障の発症に関与していることを示している．また，GLAST の発現を増加させる化合物をみつけ，その化合物が GLAST ヘテロマウスの緑内障様症状を改善することを見出した．

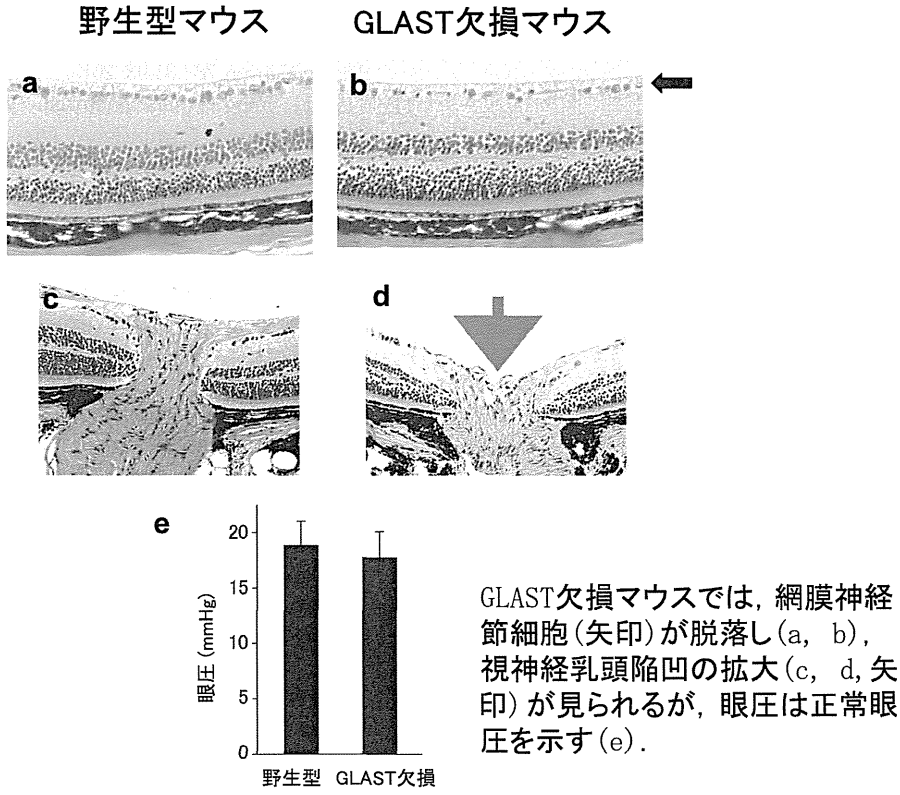

図 4　GLAST 欠損マウスはヒト正常眼圧緑内障と同様の異常を示す
（p.5 カラー図参照）