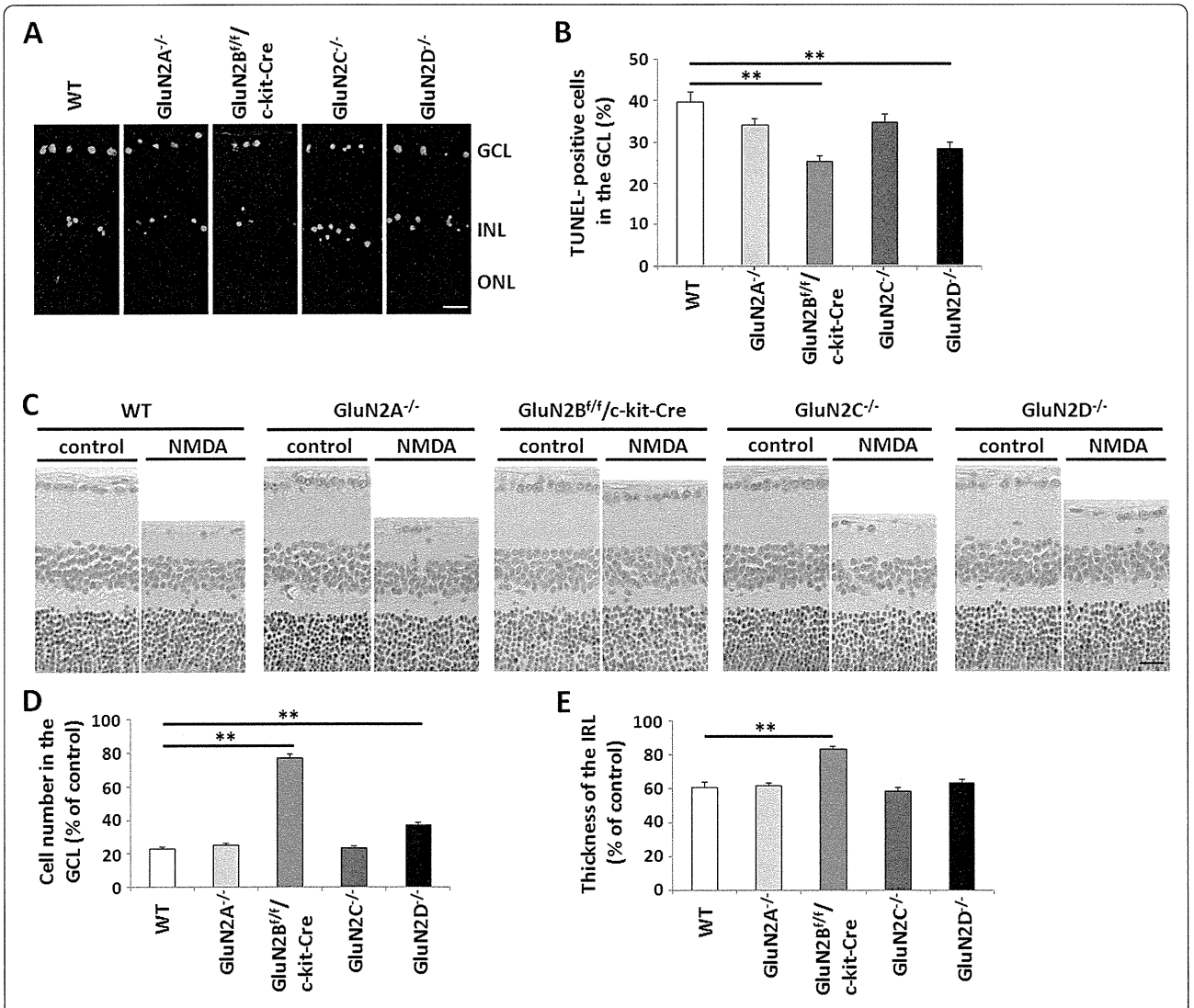

Figure 4 TUNEL staining and morphometric analysis following NMDA treatment. (A) Representative photos of TUNEL staining at 1 day after NMDA treatment from WT and GluN2 mutant mice. Scale bar, 20 μm. **(B)** Quantification of TUNEL-positive cells in the GCL. The data are presented as mean ± S.E.M. of 5 samples for each experiment. $^{**}P$ <0.01 **(C)** Representative photos of HE staining at 7 days after NMDA and PBS treatment from WT and GluN2 mutant mice. Scale bar, 20 μm. **(D-E)** Quantification of cell number in the GCL **(D)** and thickness of IRL **(E)**. The data are presented as mean ± S.E.M. of 5 samples for each experiment. $^{**}P$ <0.01. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer; IRL, inner retinal layer.

findings may provide a novel therapeutic strategy for various forms of retinopathy that are mediated by E-containing lipoproteins through LRP-1.

The failure of GluN2C deficiency to protect RGCs from NMDA-induced excitotoxicity can be explained by the data showing that only a small number of RGCs expressed GluN2C [32]. However, almost RGCs express GluN2A [32]. The failure of GluN2A deficiency to protect RGCs from NMDA-induced excitotoxicity may be explained by the distinct functional properties conferred by GluN2 subunits on the receptors, and the different signaling pathway couplings [13,33]. This variety is due to the large and divergent cytoplasmic C-terminal domains of GluN2 subunits [34]. A previous report showed that C-terminal domains of GluN2B subunits were more lethal than GluN2A subunits, and different coupling to PSD-95/nNOS signaling cassette may contribute to differential susceptibility of GluN2 subunits to excitotoxic injury [33]. Another possible explanation is that the location of NMDARs at synaptic or extrasynaptic sites determines the neuroprotective or neurotoxic effects of glutamate. A high level of synaptic NMDAR activity promotes neuronal survival, whereas extrasynaptic NMDAR activity promotes cell death [35]. In the retina, GluN2B is

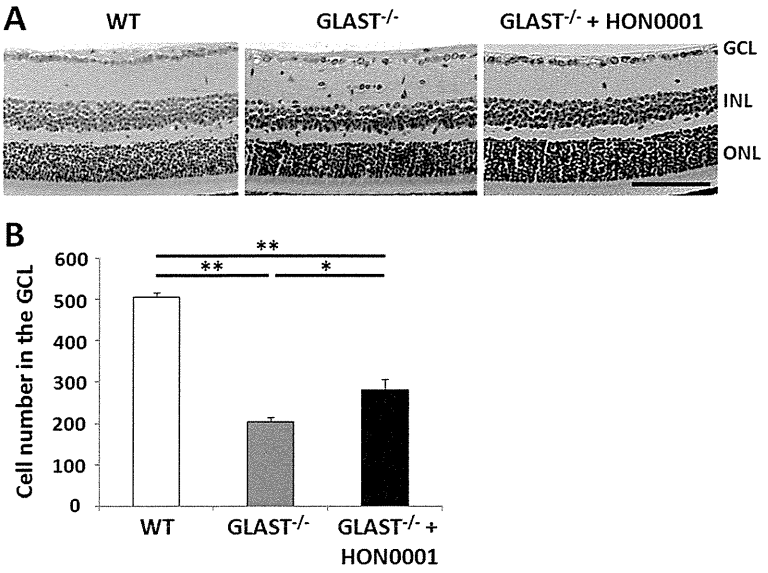

Figure 5 GluN2B antagonist HON0001 rescues RGCs death in GLAST-deficient mice. (A) Representative photos of wild-type (WT), saline-($GLAST^{-/-}$) or HON0001- ($GLAST^{-/-}$ + HON0001) treated retinas. HON0001 (10 mg/kg) or saline were injected orally (p.o.) into $GLAST^{-/-}$ mice from P21 to P35. The animals were killed at P35 after HON0001/saline treatment. Scale bar, 100 μm. **(B)** Quantification of the cell number in the GCL. The data are presented as mean ± S.E.M. of 4 (WT and $GLAST^{-/-}$) and 6 ($GLAST^{-/-}$ + HON0001) samples for each experiment. *P <0.05, **P <0.01. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer.

enriched at the perisynaptic site, whereas synaptic NMDARs primarily contain GluN2A [36].

The number of cells in the GCL of $GluN2B^{f/f}$/c-kit-Cre mice was significantly decreased at 5 weeks. This finding is consistent with a previous study showing that NMDAR hypofunction increased neuronal death in the developing brain [26,37]. GluN2B is a major GluN2 subunit in the immature retina [38]; therefore, ablation of GluN2B in the developing retina can cause excessive neuronal apoptosis, resulting in a reduction in the cell number in the GCL of $GluN2B^{f/f}$/c-kit-Cre mice. Thus, loss of GluN2B can increase RGC death in the immature retina, but protect RGCs from glutamate excitotoxicity in the adult.

Conclusions

We showed that GluN2B- and GluN2D-containing NMDARs played a critical role in NMDA-induced excitotoxic retinal cell death and RGC degeneration in GLAST KO mice. Inhibition of GluN2B and GluN2D activity is a potential therapeutic strategy for the treatment of several retinal diseases, including retinal ischemia, diabetic retinopathy, and glaucoma.

Methods

Animals

B6.Cg-TgN(Thy1-CFP)23Jrs/J transgenic mice (thy1-CFP mice) and c-kit-Cre transgenic mice have been described previously [17,24]. c-kit-Cre transgenic mice were bred with ROSA-tdTomato mice [25] to examine Cre activity. c-kit-Cre mice were bred with $GluN2B^{flox/flox}$ ($GluN2B^{f/f}$) mice [23] to generate GluN2B conditional knockout mice ($GluN2B^{f/f}$/*c-kit-Cre*). The homozygous GluN2A KO ($GluN2A^{-/-}$) [19], GluN2C KO mice ($GluN2C^{-/-}$) [20] and GluN2D KO mice ($GluN2D^{-/-}$) [21] were obtained by crossing heterozygous $GluN2A^{+/-}$, $GluN2C^{+/-}$ and $GluN2D^{+/-}$ mice, respectively. GLAST KO mice have been described previously [39,40]. In all experiments, age matched WT and $GluN2B^{f/f}$ 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).

Isolation of single ganglion cells from mouse retina and RT-PCR

5 week old Thy1-CFP mice were deeply anesthetized by diethyl ether and retinas were dissociated by using the Papain Dissociation System (Worthington Biochemical Corporation) at 37°C for 30 min. Single-CFP-expressing cell was aspirated by glass microcapillaries and placed into the PCR-tube containing 10 μl of resuspention buffer. Single-cell RT-PCR was performed using the SuperScript III CellsDirect cDNA Synthesis System (Invitrogen). Total RNA (5 μg) from whole retina were used to synthesize first-strand cDNA by using SuperScript III First-Strand Synthesis System (Invitrogen). The retina cDNA served as positive controls. The following primers were used for cDNA detection: GluN2A FWD: 5′ GTG TGC GAC CTC ATG TCC G 3′; REV: 5′ GCC TCT TGG TCC GTA TCA TCT C 3′; GluN2B FWD: 5′

CAG CAA AGC TCG TTC CCA AAA 3′; REV: 5′ GTC AGT CTC GTT CAT GGC TAC 3′; GluN2C FWD: ATC CCC GAC GGC TGA GA 3′; REV: 5′ TTC CTA GTC CAA GCA CAA AAC G 3′; GluN2D FWD: 5′ TGT GTG GGT GAT GAT GTT CGT 3′; REV: 5′ CCA CAG GAC TGA GGT ACT CAA AGA 3′; GluN1 FWD: 5′ GCC GAT TTA AGG TGA ACA GC 3′; REV: 5′ AAT TGT GCT TCT CCA TGT GC 3′; Brn3 FWD: 5′ GCA GTC TCC ACT TGG TGC TTA CTC 3′; REV: 5′ TTC CCC CTA CAA ACA AAC CTC C 3′; β-actin FWD: 5′ ATA TCG CTG CGC TGG TCG TC 3′; REV: 5′ TCA CTT ACC TGG TGC CTA GGG 3′. The thermal cycler conditions were 5 min at 94°C and then 40 cycles of 30 s at 94°C, 30 s at 60°C, and 30 s at 72°C, followed by 7 min at 72°C.

Western blot analysis

Retinas were quickly removed and homogenized in 100 μl of 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]). Protein concentration was determined by BCA Protein Assay kit (Sigma-Aldrich). Thirty microgram of the protein was loaded per lane. Primary antibodies used were GluN2A (1:500, Covance), GluN2B (1:500) [41], GluN2C (1:100, Invitrogen), GluN2D (1:500) [42], β-actin (1:1000, Santa Cruz). They were then incubated with anti-rabbit, guinea pig or mouse IgG-HRP-conjugated secondary antibody (1:5000, Jackson ImmunoResearch). SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was used to visualize the immunoreactive proteins.

Immunohistochemistry

Sections were prepared as previously described [15]. Frozen retinal sections of 12 μm thickness were incubated using anti-Brn3 (1:50, Santa Cruz), anti-calretinin (1:500, Swant) and anti-GluN2B (1:100) antibodies. For Brn3 and calretinin detection, Cy-3-conjugated donkey anti-goat IgG (1:500, Jackson ImmunoResearch) and goat anti-rabbit IgG Alexa 488 (1:1000, Molecular Probes) were used as secondary antibodies. For GluN2B detection, peroxidase labelled polymer conjugated to goat anti-rabbit IgG (DAKO) was used as secondary antibody. Images were recorded with an LSM-510 META confocal laser microscope (Carl Zeiss).

Histology and morphometric analysis

Eyes from mice at postnatal day 35 (P35) were enucleated and fixed in Davidson's solution fixative [43], then embedded in paraffin wax. In some experiments, HON0001 (10 mg/kg, a gift from T. Honda at Hoshi University) [16] or saline was injected orally (p.o.) into GLAST KO mice daily from P21 to P35. These mice were sacrificed on P35 and processed for RGC count. Paraffin sections (7 μm thick) were cut though the optic nerve and stained with hematoxylin and eosin (H&E). The number of neurons in the GCL was counted as previously described [15]. The thickness of the IRL (from GCL to INL) was measured at a distance of 0.5 to 1.0 mm from optic disc.

Animal models of NMDA-induecd retinal neuronal death and morphometric analysis

Intravitreal injection of NMDA (Sigma) was conducted as previously described [15]. Briefly, a single 2-μl injection of 20 mM NMDA in 0.1 M PBS (pH 7.4) was administered intravitreally into the right eye of each mouse, the same volume of PBS was administered to the contralateral (left) eye as control. The animals were sacrificed at 1 day or 7 days after injection, and eyes were enucleated for morphometric and TUNEL analysis. Paraffin sections (5 μm thick) were cut though the optic nerve and stained with H&E. The extent of NMDA-induced retinal cell death after 7 days was quantified by counts of neurons in the GCL and the thickness of the IRL. The changes of the number of ganglion cells and thickness of IRL after NMDA injection were expressed as percentages of the control eyes.

TUNEL staining

At 1 day after the NMDA or PBS injection, TUNEL staining was performed with paraffin sections (5 μm thick) according to the manufacturer's instructions (Promega). Fluorescence detection was carried out using Alexa-fluor -568-conjugated streptavidin (Molecular Probes). TUNEL-positive cells in the GCL were counted and expressed as percentages of total DAPI stained cells in the GCL.

Statistics

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. Data are expressed as mean ± S.E.M. P values < 0.05 were considered statistically significant.

Abbreviations

GLAST: Glutamate aspartate transporter; INL: Inner nuclear layer; IPL: inner plexiform layer; IRL: Inner retina layer; LRP: lipoprotein receptor-related protein; NMDAR: N-methyl-D-aspartate receptor; PBS: Phosphate-buffered saline; PMSF: Phenylmethylsulfonyl fluoride; RGC: Retinal ganglion cell; RT-PCR: Reverse transcriptase polymerase chain reaction; WT: Wild-type.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

KT, NB, and TA conceived and designed the experiments. NB, SK and MY carried out experiments and analyzed the data. KS 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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Dock3 attenuates neural cell death due to NMDA neurotoxicity and oxidative stress in a mouse model of normal tension glaucoma

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Dedicator of cytokinesis 3 (Dock3), a new member of the guanine nucleotide exchange factors for the small GTPase Rac1, promotes axon regeneration following optic nerve injury. In the present study, we found that Dock3 directly binds to the intracellular C-terminus domain of NR2B, an *N*-methyl-D-aspartate (NMDA) receptor subunit. In transgenic mice overexpressing Dock3 (Dock3 Tg), NR2B expression in the retina was significantly decreased and NMDA-induced retinal degeneration was ameliorated. In addition, overexpression of Dock3 protected retinal ganglion cells (RGCs) from oxidative stress. We previously reported that glutamate/aspartate transporter (GLAST) is a major glutamate transporter in the retina, and RGC degeneration due to glutamate neurotoxicity and oxidative stress is observed in GLAST-deficient (KO) mice. In GLAST KO mice, the NR2B phosphorylation rate in the retina was significantly higher compared with Dock3 Tg:GLAST KO mice. Consistently, glaucomatous retinal degeneration was significantly improved in GLAST KO:Dock3 Tg mice compared with GLAST KO mice. These results suggest that Dock3 overexpression prevents glaucomatous retinal degeneration by suppressing both NR2B-mediated glutamate neurotoxicity and oxidative stress, and identifies Dock3 signaling as a potential therapeutic target for both neuroprotection and axonal regeneration.
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Excitatory neurotransmission mediated by *N*-methyl-D-aspartate receptors (NMDARs), one of the ionotropic glutamate receptors, has fundamental roles in both physiological and pathological processes in the mammalian central nervous system (CNS). Overactivation of NMDA receptors is thought to be a key contributing factor in the pathophysiology of many CNS disorders, such as Alzheimer's disease[1,2] and Huntington disease.[3] Glutamate excitotoxicity is also implicated in the degeneration of the retinal ganglion cells (RGCs) and optic nerves observed under pathological conditions including glaucoma.[4,5] Consistently, we previously reported that loss of glutamate/aspartate transporter (GLAST) induces optic neuropathy without affecting intraocular pressure and exhibits many features similar to human normal tension glaucoma.[6]

NMDA receptors are tetramers that form functional receptor channels. Two NR1 subunits, which bind its co-agonist glycine, must combine with at least two NR2 (A–D) subunits, which bind glutamate.[7] In the retina, NMDARs on RGCs are activated by glutamate released from cone bipolar cells.[8] All four NR2 subunits are expressed in vertebrate retina, and both NR2A and NR2B are prevalent in the inner plexiform layer, where NMDARs are localized on RGC dendrites.[9–11] Among them, NR2B-containing receptors are shown to exhibit relatively high affinity both for glutamate and for the co-agonist glycine, prolonged channel opening and greater overall Ca^{2+} current per event.[12,13] It is well known that the intracellular C-terminus domain (CTD) of NR2B regulates the internalization and degradation of NR2B-containing receptors.[14–17] At postsynaptic sites, NR2B-CTD is phosphorylated at Tyr1472 by Fyn, thereby mediating complex formation of NMDA receptors with the postsynaptic density protein 95 (PSD95). This NMDAR-PSD95 interaction is required for excitotoxic downstream signaling.[17] Functionally, NR2B has been implicated in many forms of synaptic plasticity related to the physiology of striatal neurons and the pathogenesis of various neurological disorders.[18] Indeed, amyloid-β (Aβ) and tau protein promote Fyn-mediated NR2B stabilization at the plasma membrane, resulting in the enhancement of NMDAR-mediated neurodegeneration in the brain of a mouse model of Alzheimer's disease.[1,2,19]

Dedicator of cytokinesis 3 (Dock3), a new member of the guanine nucleotide exchange factors, is specifically expressed in neural cells and causes cellular morphological changes by activating the small GTPase Rac1.[20] We previously reported that Dock3 is primarily expressed in RGCs in the retina, and that Dock3 overexpression stimulates axonal regeneration after optic nerve injury *in vivo*.[21,22] Dock3 was initially identified as a binding protein to presenilin1,

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Keywords: Dock3; glutamate; oxidative stress; neuroprotection; glaucoma
Abbreviations: Aβ, amyloid-beta; ASK1, apoptosis signal-regulating kinase 1; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CTD, intracellular C-terminus domain; Dock3, Dedicator of cytokinesis 3; GLAST, glutamate/aspartate transporter; GSK-3β, glycogen synthase kinase-3β; LDH, lactate dehydrogenase; NMDA, *N*-methyl-D-aspartate; PSD95, postsynaptic density protein 95; RGC, retinal ganglion cell
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a major causative gene of early-onset familial Alzheimer's disease.[23,24] Although the critical role of Dock3 in Alzheimer's disease is still unknown, it is reported that Dock3 is deposited and insolubilized in Alzheimer's disease brain.[23,25] In addition, a recent study reported that Rac1 activation mediated by Dock1 (Dock180), a homolog protein of Dock3, regulates endocytic membrane trafficking.[26] These observations suggest a possible interaction between Dock3 and NR2B, which may regulate NR2B expression in neural tissues. In addition, recent studies suggested a possibility that there is a causal relationship between Alzheimer's disease and glaucoma.[27,28] These observations prompted us to examine the interaction between Dock3 and NR2B in the retina, and the effects of Dock3 on GLAST-deficient (GLAST KO) mice that suffer from glaucoma-like RGC degeneration.[6]

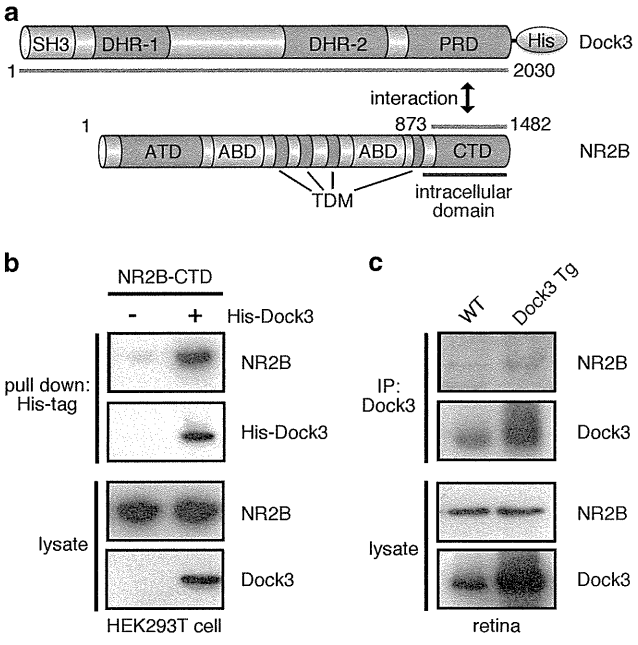

Figure 1 Dock3 interacts with the CTD of NR2B. (**a**) Schematic representation showing the His-tagged WT Dock3 and NR2B-CTD used in the pull-down assay. SH3, Src homology 3 domain; DHR-1, Dock homology region 1; DHR-2, dock homology region 2; PRD, proline-rich domain; ATD, amino-terminal domain; ABD, agonist binding domain; TDM, transmembrane domain; CTD, intracellular C-terminal domain. (**b**) Lysates from HEK293T cells transfected with His-tagged full-length Dock3 and NR2B-CTD were subjected to a His-tag pull-down assay. (**c**) Lysates from retinas of WT and Dock3 Tg mice were subjected to immunoprecipitation assay using an anti-Dock3 antibody

Results

Dock3 interacts with NR2B in the retina. We first examined whether Dock3 binds to NR2B in neural cells. For this experiment, we transfected His-tagged full-length Dock3 and CTD of NR2B (NR2B-CTD) (Figure 1a) to HEK293T cells and found that Dock3 binds to NR2B-CTD using His-tag pull-down assay (Figure 1b). We next examined whether Dock3 and NR2B are bound in neural tissues *in vivo*. The interaction between endogenous Dock3 and NR2B in wild-type (WT) mouse retina was confirmed by a co-immunoprecipitation assay (Figure 1c). We also found that the binding between Dock3 and NR2B was increased in Dock3 Tg mice, in which Dock3 is overexpressed in the retina (Figure 1c).[21]

We previously showed that Dock3 is abundantly expressed in retinal ganglion cells (RGCs) and stimulates axon outgrowth in adult mice.[21,22] Double-labeling immunohistochemistry showed that NR2B immunoreactivity was co-localized with Dock3 mainly in the ganglion cell layer (GCL) (Figure 2). These data support the binding of Dock3 and NR2B in RGCs of the adult retina *in vivo*.

Dock3 increases NMDA-mediated NR2B degradation in the retina. NMDA receptors undergo endocytosis and lysosomal sorting for receptor degradation.[14–18] As Dock3 and NR2B are bound in the retina, we investigated whether Dock3 affects NR2B degradation in the retina after NMDA stimulation. For this purpose, NR2B protein levels in the retinas from WT and Dock3 Tg mice were determined by immunoblot analysis at 3 and 24 h after the intraocular injection of phosphate-buffered saline (PBS; control) or NMDA. In the control retinas, NR2B expression levels in Dock3 Tg mice were not different compared with those in WT mice (Figure 3). NMDA injection had no effects on NR2B expression levels in the retina of WT mice, whereas NR2B expression levels were significantly decreased in Dock3 Tg mice (Figure 3). These results suggest that Dock3 enhances NR2B degradation due to glutamate neurotoxicity in the retina.

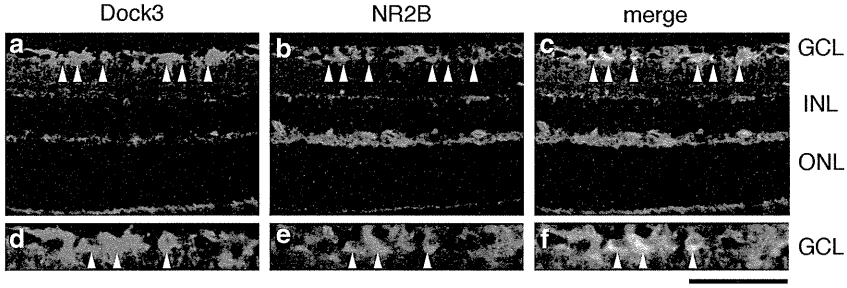

Figure 2 Expression of Dock3 and NR2B in the mouse retina. (**a–c**) Immunohistochemical analysis of mouse retina double-labeled (**c**) with antibodies against Dock3 (**a**) and NR2B (**b**). (**d–f**) Enlarged images of the GCL in (**a–c**), respectively. Arrow heads indicate double-labeled cells. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar: 100 μm (**a–c**) and 50 μm (**d–f**)

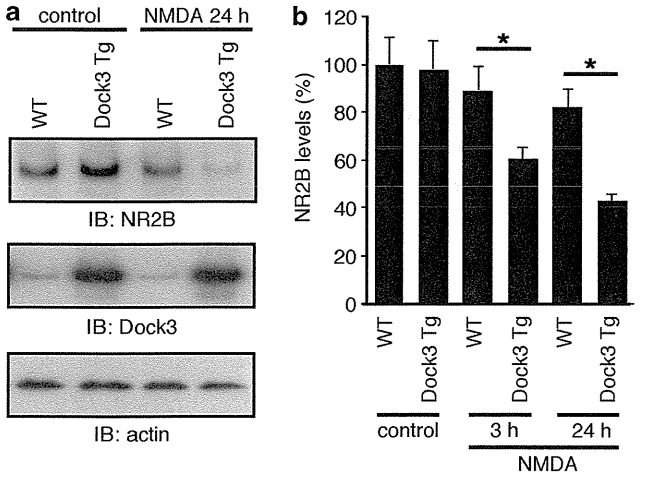

Figure 3 Effect of Dock3 on NR2B expression levels in the retina. (**a**) Immunoblot analysis of NR2B and Dock3 in the retinas of WT and Dock3 Tg mice 24 h after the injection of PBS (control) or NMDA. (**b**) Quantitative analysis of NR2B expression at 3 and 24 h after the injection of PBS (control) or NMDA. The data are presented as means ± S.E.M. of six samples for each experiment $^*P<0.05$

Dock3 overexpression protects retinal neurons from glutamate neurotoxicity. Our present results suggest that overexpression of Dock3 in the retina may ameliorate glutamate neurotoxicity via NR2B degradation. To determine this possibility, we examined the effect of intraocular injection of NMDA on retinal cell death in WT and Dock3 Tg mice. The retinal structure of adult Dock3 Tg mice was normal, and the cell number in the GCL (497 ± 26; $n=6$) was comparable to WT mice (488 ± 31; $n=6$) (Figures 4a and b). In addition, the thickness of the inner retinal layer (IRL, between the internal limiting membrane and the interface of the outer plexiform layer and the outer nuclear layer) was similar in both strains (Figure 4c). Intraocular administration of NMDA induced cell death in the GCL in both WT and Dock3 Tg mice, but the number of surviving neurons in Dock3 Tg mice was significantly higher than that in WT mice at 5 and 7 days after NMDA injection (Figures 4a and b). Additionally, IRL thickness in Dock3 Tg mice was significantly increased at 7 days after NMDA injection compared with that in WT mice (Figure 4c). These results suggest that Dock3 overexpression prevents retinal cell death from glutamate neurotoxicity *in vivo*.

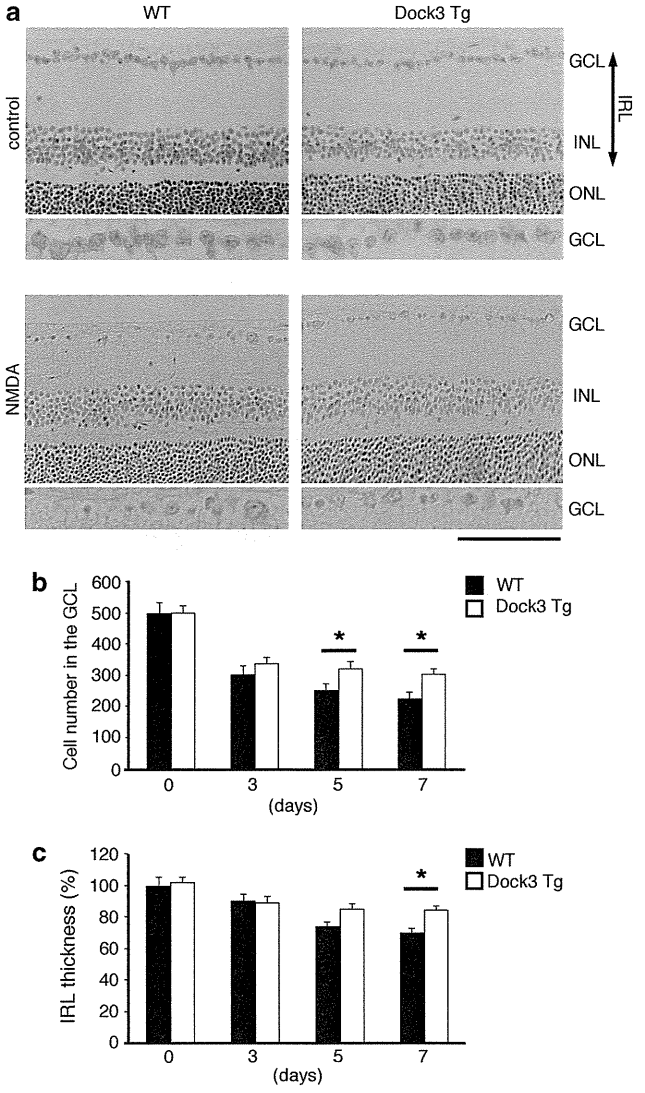

Figure 4 Dock3 protects retinal neurons from glutamate neurotoxicity. (**a**) H&E staining of retinal sections in WT and Dock3 Tg mice 7 days after intraocular treatment of PBS (control) or NMDA. Scale bar: 100 μm and 50 μm in the upper and lower panels, respectively. GCL, ganglion cell layer; INL, inner 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**) at 0, 3, 5 and 7 days after NMDA treatment. The data are presented as means ± S.E.M. of six samples for each experiment $^*P<0.05$

Dock3 overexpression protects RGCs from oxidative stress. Our previous study showed that oxidative stress, as well as glutamate neurotoxicity, induces RGC death.[29] To assess whether Dock3 also prevents oxidative stress-induced RGC death, cultured RGCs from WT mice were transfected with myc-tagged Dock3 plasmid by electroporation, and hydrogen peroxide (H_2O_2)-induced RGC death was assessed through the lactate dehydrogenase(LDH) leakage assays. Immunoblot analysis demonstrated that electroporation increased Dock3 expression levels in cultured RGCs (Figure 5a). The LDH leakage assay revealed that H_2O_2-induced cell death was significantly reduced in RGCs transfected with Dock3 plasmid compared with mock plasmid (Figures 5b and c). These results suggest that Dock3 overexpression protects RGCs from both glutamate neurotoxicity and oxidative stress.

Dock3 overexpression prevents glaucomatous retinal degeneration. We previously reported that glutamate neurotoxicity and oxidative stress are involved in RGC degeneration in GLAST KO mice, which is a mouse model of normal tension glaucoma.[6] In 3-month-old GLAST KO mice, the cell number in the GCL (325 ± 10; $n=6$) was significantly reduced compared with that in WT (479 ± 23; $n=6$) and Dock3 Tg (488 ± 21; $n=6$) mice (Figures 6a and b). Consistently, the thickness of the IRL was significantly reduced in GLAST KO mice compared with WT and Dock3 Tg mice (Figure 6c). As Dock3 may inhibit the two main

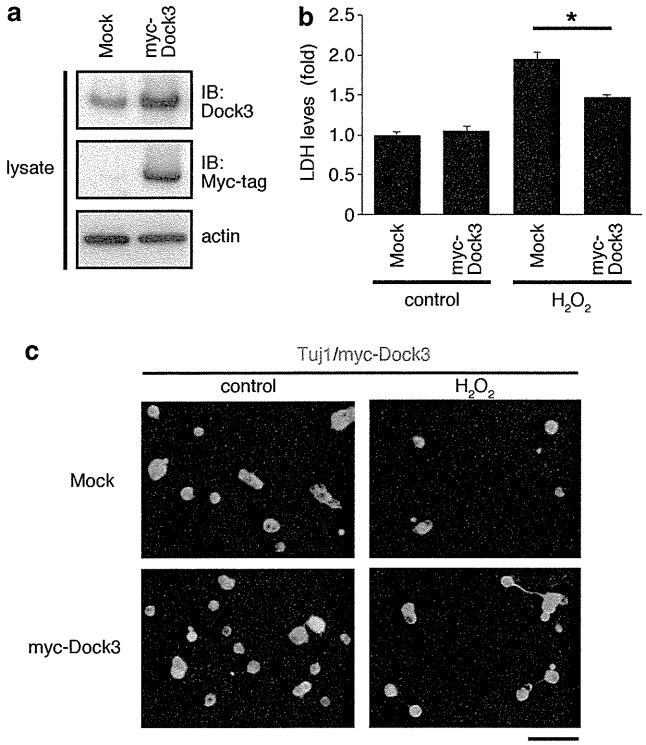

Figure 5 Effect of Dock3 on H_2O_2-induced RGC death *in vitro*. (**a**) Lysates from cultured RGCs transfected with empty vector (Mock) or myc-tagged Dock3 plasmids were subjected to immunoblot analysis using anti-Dock3 and anti-myc antibodies. (**b**) Cultured RGCs were stimulated with H_2O_2 for 16 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 $^*P<0.05$

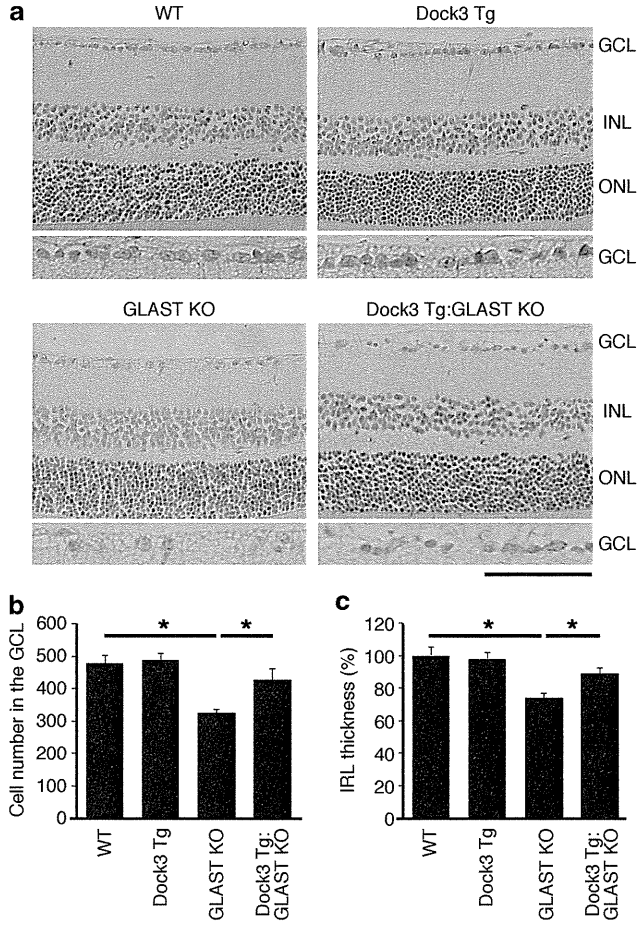

Figure 6 Effect of Dock3 on retinal degeneration in a mouse model of glaucoma. (**a**) H&E staining of retinal sections of WT, Dock3 Tg, GLAST KO and Dock3 Tg:GLAST KO mice at 3 months. WT and Dock3 Tg mice were littermates. GLAST KO and Dock3 Tg:GLAST KO mice were littermates. Scale bar: 100 μm and 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]

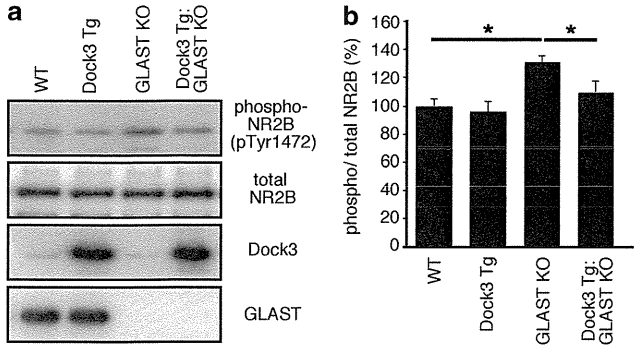

Figure 7 Effect of Dock3 on NR2B phosphorylation in a mouse model of glaucoma. (**a**) Immunoblot analysis of phosphorylated NR2B, total NR2B, Dock3 and GLAST in the retinas of WT, Dock3 Tg, GLAST KO and Dock3 Tg:GLAST KO mice. (**b**) Phosphorylation rate of NR2B in (**a**). The data are presented as 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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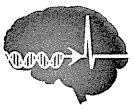

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Dock3 interaction with a glutamate-receptor NR2D subunit protects neurons from excitotoxicity

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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

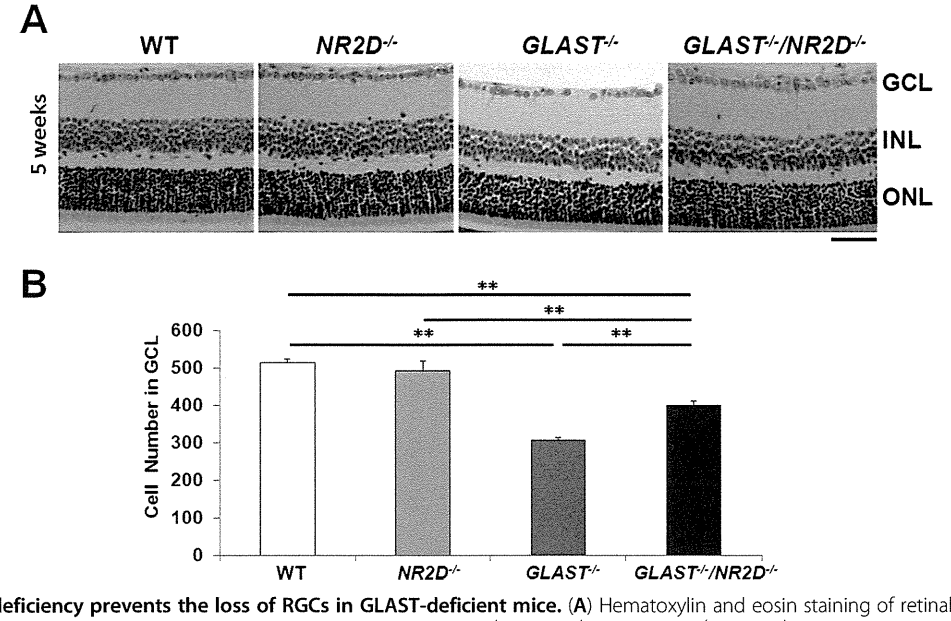

Figure 1 NR2D deficiency prevents the loss of RGCs in GLAST-deficient mice. (**A**) Hematoxylin and eosin staining of retinal sections at 5 weeks. Scale bar: 50 μm. (**B**) Quantification of RGC number in WT, $NR2D^{-/-}$, $GLAST^{-/-}$, and $GLAST^{-/-}/NR2D^{-/-}$ mice. The number of neurons in the 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.

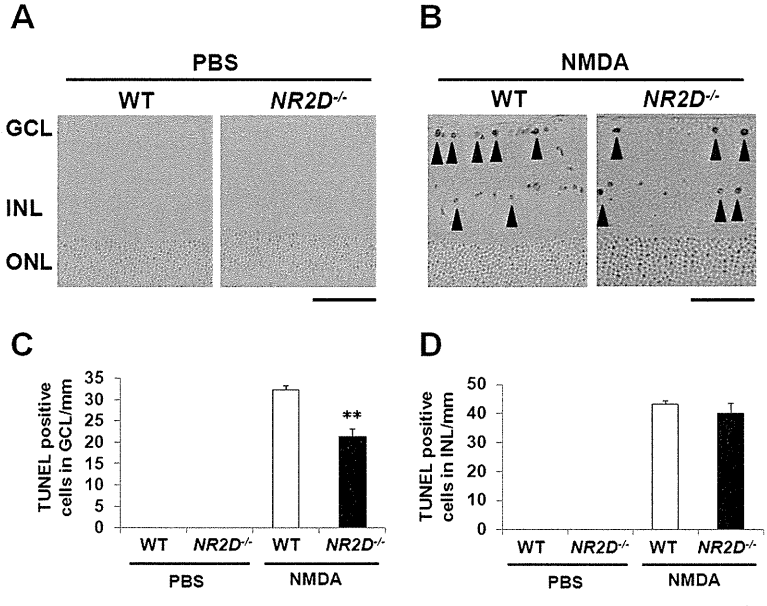

Figure 2 NR2D deficiency prevents NMDA-induced-excitotoxic retinal cell death. (**A** and **B**) TUNEL analysis of retinas of WT and *NR2D*$^{-/-}$ mice 24 hr after PBS and NMDA injection. Arrowheads in **B** indicate TUNEL-positive cells. Scale bar: 50 μm. (**C** and **D**) The number of TUNEL-positive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

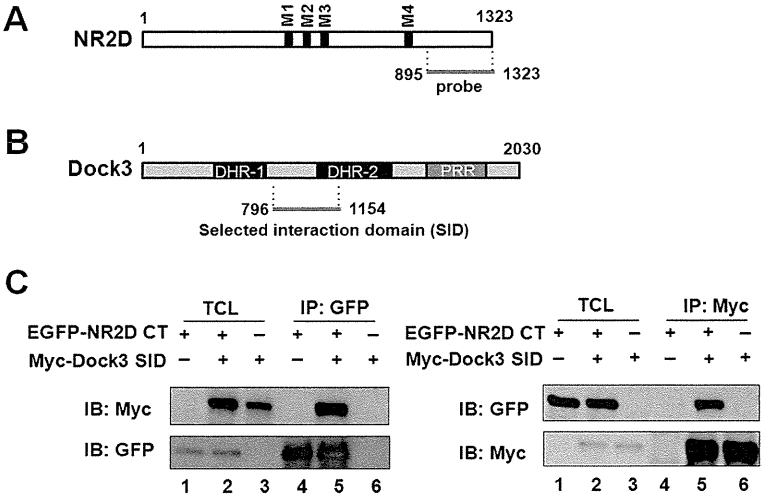

Figure 3 Identification of Dock3 as an NR2D-interacting molecule. (**A**) Schematic diagram of NR2D and a probe used for yeast two-hybrid screening. M1–M4 indicates the transmembrane regions. The numbers refer to the amino-acid positions of NR2D. (**B**) Schematic diagram of Dock3. The selected interaction 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.

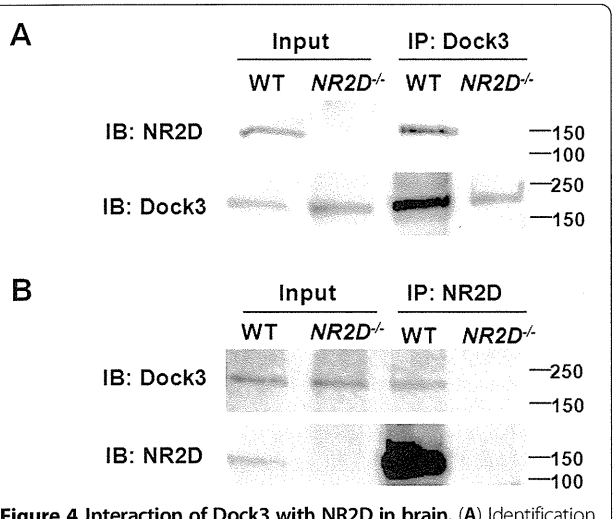

Figure 4 Interaction of Dock3 with NR2D in brain. (**A**) Identification of NR2D in Dock3 immunoprecipitates from WT, but not *NR2D*$^{-/-}$ embryonic-brain homogenates. (**B**) Identification of Dock3 in NR2D immunoprecipitates from WT, but not *NR2D*$^{-/-}$ embryonic-brain homogenates. The immunoprecipitates 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

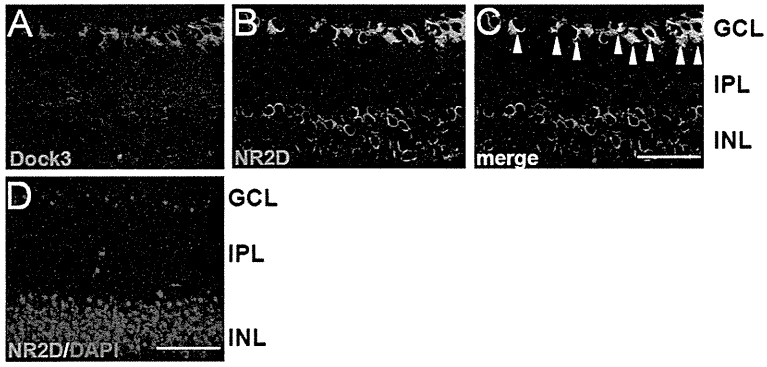

Figure 5 Co-localization of NR2D and Dock3 in the mouse retina. Double-labeling experiments were carried out on mouse retina sections (**A**-**C**). Dock3 (**A**) is red and NR2D (**B**) is green. Dock3 co-localized with NR2D in RGCs shown in the overlay panel (**C**) (arrowheads). Scale bar: 50 μm. (**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

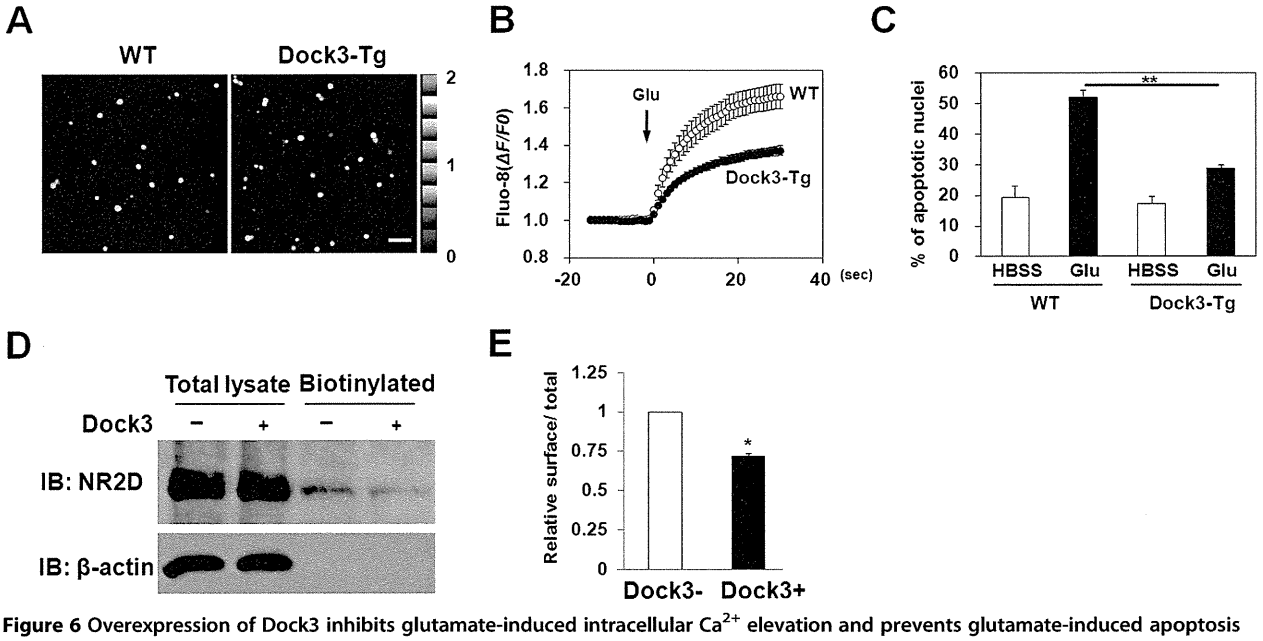

Figure 6 Overexpression of Dock3 inhibits glutamate-induced intracellular Ca^{2+} elevation and prevents glutamate-induced apoptosis in RGCs. (**A**) RGCs from WT or Dock3-transgenic (Tg) mice were labeled with Fluo-8 acetoxymethyl ester for 30 min, and then 300 μM glutamate (Glu) was added. Fluorescence-ratio 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-