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べらどんな

炭酸脱水酵素

アセタゾラミド，いわゆるダイアモックスは1950年に合成された。最初は利尿薬としての効果が注目されていたが，その4年後に眼圧下降効果のあることをセント・ルイスのBeckerがはじめて報告した。

これが発見された動機には2説がある。ひとつは緑内障の患者に腎疾患があり，眼圧のコントロールが急に良くなった。不審に思って患者に訊いたら，ダイアモックスの服用をはじめていたというもの。

もうひとつは論理的な根拠からで，きれいな三段論法になっている。すなわち，①兎の房水には重炭酸塩が多く，血清中の濃度よりもはるかに高い。②重炭酸イオン HCO_3^- は炭酸ガス CO_2 と水 H_2O とから作られ，その際に炭酸脱水酵素が触媒として関係する。③ダイアモックスは炭酸脱水酵素阻害剤なので，毛様体上皮での房水産生を減らす筈である。

実際にダイアモックスを投与すると，緑内障眼の眼圧は見事に下がった。まことに結構なことだが，ヒトの房水では重炭酸塩の濃度は血清よりも低いのである。いわば「誤った理論から正しい結論が出た」ことになろう。

われわれ眼科医は「炭酸脱水酵素」と聞くと，反射的に房水産生を考えるが，進化論でははるかに重要な役割を演じているらしい。

46億年前に地球が誕生した。始生代，原生代と続いたあと，5億4千万年前に古生代がはじまる。その最初の4千万年がカンブリア紀であり，三葉虫などの動物が数も種類も爆発的に誕生した。動物が眼らしい眼を持つようになったのもこの時代からである。

大きな動物には骨格が必要である。カンブリア紀の動物は外骨格を採用した。蟹などの甲殻類や貝や珊瑚が現在でも使っている方法である。

外骨格の素材は炭酸カルシウム $CaCO_3$ である。これは炭酸 H_2CO_3 の塩で，炭酸ガスと水がその素材である。

「カンブリア紀に炭酸脱水酵素が利用できるようになったからこそ，地球上の動物が繁栄することになった」と言えそうだ。脊椎動物も例外ではない。

GEN

102. 正常眼圧緑内障とグルタミン酸輸送体

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正常眼圧緑内障（NTG）の原因には諸説あるが，確定には至っていない．またすでに存在する高眼圧モデル動物を利用できないことから，基礎研究の進展も十分とは言いがたい．筆者らはグルタミン酸輸送体の欠損マウスがNTGモデルとして利用可能なことを見いだしており，今後の治療研究に有用と思われる．

●網膜のグルタミン酸輸送体

グルタミン酸は中枢神経系の約80%の神経細胞で利用される興奮性神経伝達物質であり，網膜における主要な視覚伝達物質でもある．このグルタミン酸の濃度を調節する唯一の機構がグルタミン酸輸送体である．グルタミン酸輸送体はシナプス間隙のグルタミン酸を迅速に除去し，濃度を適切に保つことで，正常な視覚伝達を可能にしている[1]．網膜には4種類のグルタミン酸輸送体が存在するが，特に重要なのがMüller細胞に発現するGLASTである（**表1**）．Müller細胞にはグルタミン合成酵素が存在することから，GLASTによって取り込まれたグルタミン酸はグルタミンに変換され，細胞内グルタミン酸濃度は低く保たれる．このMüller細胞のグルタミン酸代謝機構が，GLASTによる強力なグルタミン酸取り込み能力を支えていると考えられる．

●グルタミン酸輸送体欠損マウス

緑内障の原因の一つとして，以前からグルタミン酸毒性の関与が指摘されている[2]．そこで筆者らが作製したGLASTの欠損マウスにおいて，網膜・視神経の詳細な検討を行ったところ，網膜神経節細胞数の減少に加えて，視神経乳頭の陥凹と視神経線維の減少が認められた（**図1**）．視機能を評価する目的で多局所網膜電位の二次核成分を測定したところ，網膜神経節細胞の減少にほぼ一致した電位の減弱が認められた．同様の変化は網膜神経節細胞に発現するグルタミン酸輸送体であるEAAC1の欠損マウスでも観察された．いずれのマウスも開放隅角であり，かつ眼圧は正常範囲内であったことから，これらは正常眼圧緑内障（NTG）様症状を示す疾患モデルと考えられた[3]．

表1　網膜に発現するグルタミン酸輸送体

	GLAST	GLT-1	EAAC1	EAAT4	EAAT5
神経節細胞層			○		
内顆粒層 グリア細胞	○				
内顆粒層 神経細胞		○	○		○
視細胞層		○			○
網膜色素上皮層					

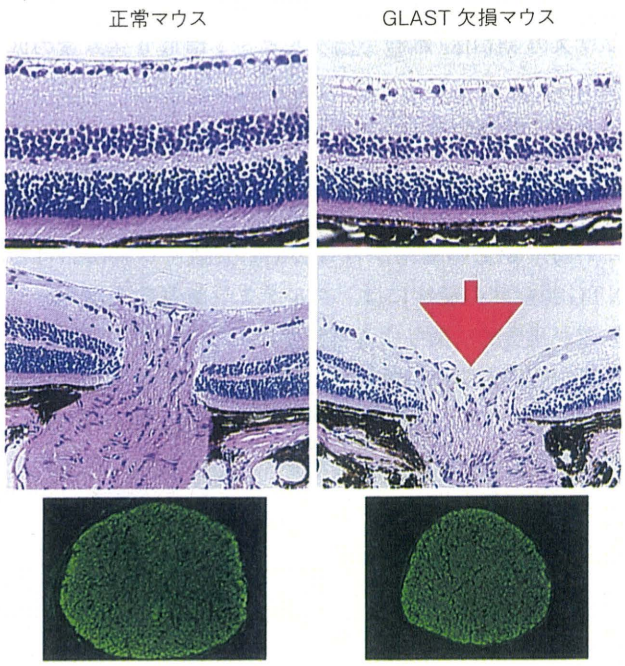

図1　GLAST欠損マウスで観察されたNTG様症状
生後8カ月齢のGLAST欠損マウスでは網膜神経節細胞の減少（**上段**），視神経乳頭陥凹（**中段**），視神経萎縮（**下段**）が確認された．（文献3より改変）

●グルタミン酸輸送体と酸化ストレス

GLAST欠損マウスでは硝子体中のグルタミン酸濃度に有意な上昇は認められなかった．しかし，グルタミン

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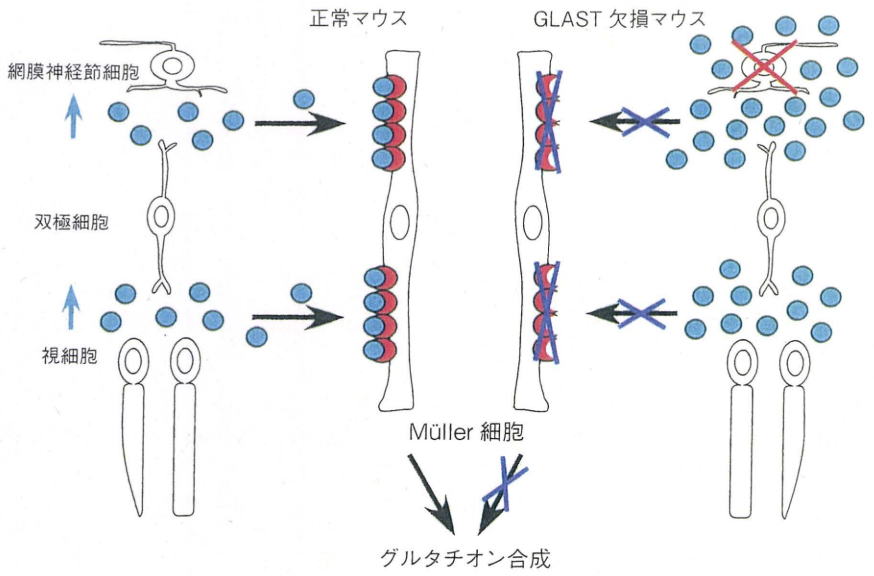

図 2　GLAST 欠損マウスにおいて予想される NTG 発症メカニズム
GLAST は Müller 細胞内に多くのグルタミン酸を取り込むことで，シナプス間隙のグルタミン酸濃度を調節し，適切な視覚伝達を可能にする．一方，GLAST 欠損マウスでは細胞外グルタミン酸濃度の慢性的な上昇によって，網膜神経節細胞死と視神経変性が起きると予想される．さらに Müller 細胞によるグルタミン酸取り込み量の減少は，グルタチオンの産生減少（酸化ストレスの増大）にもつながる可能性がある．

酸受容体阻害薬である memantine の連続投与により一部の網膜神経節細胞が保護されたことから，やはりグルタミン酸毒性の関与が考えられた．一方，GLAST 欠損マウスの Müller 細胞ではグルタミン酸取り込み量の低下に伴い，グルタミン酸・システイン・グリシンから合成される網膜の主要な抗酸化成分であるグルタチオンの産生が減少していた．さらに酸化ストレスの指標とされる脂質ヒドロペルオキシドの網膜内濃度が有意に上昇していた．以上の結果から GLAST 欠損マウスにおける NTG 様症状の発症には，グルタミン酸毒性と酸化ストレスが複合的に関与する可能性が示唆された（**図 2**）．

緑内障患者では網膜におけるグルタミン酸輸送体の発現量低下[2]や血中グルタチオン濃度の減少がすでに報告されている[4]が，GLAST 欠損マウスはこうした病態を再現しているとも考えられる．今後は緑内障患者における GLAST および EAAC1 の遺伝子変異検索とともに，両者の欠損マウスを用いた細胞保護療法に取り組む予定である．

☆　☆　☆

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基礎研究の新たな方向性を解く51

疾患解明Overview

グルタミン酸代謝異常疾患としての正常眼圧緑内障

相田知海，武田拓也，田中光一

疾患の概要

緑内障は視神経障害による視野欠損を特徴とする，わが国で最大の失明原因である．わが国の緑内障患者の約70％は眼圧が正常範囲内にある正常眼圧緑内障であるが，この疾患のモデル動物は存在せず，発症原因の解明や治療法の開発が遅れていた．われわれはグルタミン酸輸送体欠損マウスが，グルタミン酸興奮毒性と酸化ストレスを原因として，正常眼圧緑内障と同様の症状を再現することを発見した．このマウスは世界ではじめての正常眼圧緑内障のモデル動物であり，今後の病態解明や治療薬の開発に役立つことが期待される．

はじめに

緑内障は，視神経の障害による視野欠損を特徴とし，進行すると失明に至る疾患である．緑内障は，わが国の中途失明原因第１位で潜在患者数は約400万人，世界では第２位の失明原因で患者数は約7,000万人とも推定されている．さらに，高齢化に伴い，患者数の増加が予想される重要な疾患である．

以前は緑内障といえば，眼圧（眼球内の圧力）の上昇により視神経が圧迫され，網膜神経節細胞が細胞死を起こし，視野欠損が起きる疾患だと考えられてきた．最近，わが国での疫学調査（多治見スタディ）から，眼圧が正常範囲内にある正常眼圧緑内障が，緑内障の実に70％を占めることが明らかになった[1]．しかし，正常眼圧緑内障のモデル動物がなく，病態の解明や治療法の開発は困難であった．

研究の背景

正常眼圧緑内障は，家系内に緑内障患者がいる場合，発症リスクが高まることから，遺伝的要因が重要と考えられているが，その他にも血行動態因子，近視，内分泌異常，自己免疫障害，循環器疾患，特に高血圧・低血圧・血液粘性や，Ⅱ型糖尿病，片頭痛など多くの要因の関与が指摘されている[2)3)]．このように正常眼圧緑内障は，多因子疾患であり，従来の連鎖解析法では，疾患関連遺伝子の同定は困難である．このような手詰まりの状況において，正常眼圧緑内障の病態解明・治療法の開発を進めるためには，モデル動物を用いた研究が大きな役割を果たす．

遺伝子・分子レベルの知見

これまでの家系解析から，緑内障患者で変異が同定された遺伝子としては[4]，①原発開放隅角緑内障の３％を占めるミオシリン（房水，毛様体，線維柱帯，神経節細胞などに存在する分泌タンパク質），②遺伝性正常眼圧緑内障の16.7％（孤発性の12.3％）を占めるオプチニューリン（房水中または細胞内ではゴルジ体に存在

Normal tension glaucoma：a disorder of disrupted glutamate metabolisms

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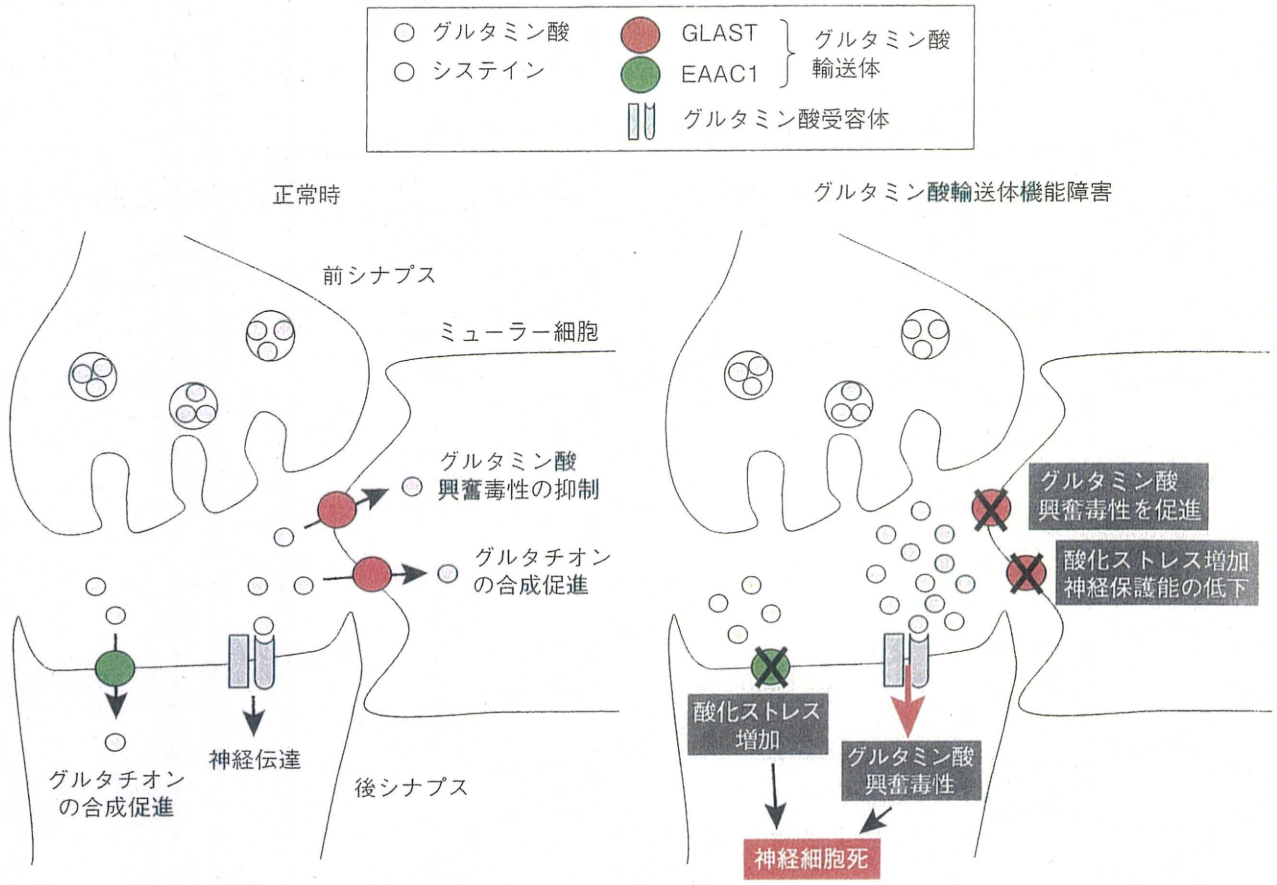
●概念図● グルタミン酸輸送体の機能障害による
グルタミン酸代謝異常が網膜神経節細胞死を引き起こす



する分泌タンパク質），③同じく５％を占めるWDR36（WD40-Repeat 36，WD40モチーフのリピートをもつタンパク質），④高リスクハプロタイプ（一塩基多型の組合わせ）が続発緑内障である落屑緑内障の発症リスクを700倍増加させ，患者の99％を占めるLOXL1（lysyl oxidase-like protein 1，細胞外マトリクスのエラスチン線維の形成にかかわる酵素）[5]，⑤発達緑内障（小児緑内障）の20％を占めるCYP1B1（cytochrome P450, family 1, subfamily B, polypeptide 1，薬剤代謝酵素）などがある．これらの変異は緑内障全体の一部でみられるに過ぎず，追試験や，マウスでの再現性に欠け，健常人でも変異があり，多くは機能的意義が不明である．

正常眼圧緑内障で傷害を受ける網膜神経節細胞の脆弱性には，グルタミン酸，虚血，軸索損傷，グリア細胞の活性化，酸化ストレス，自己免疫疾患などが関与すると示唆されている[4]．われわれは，正常眼圧緑内障の病態に関与する因子として，グルタミン酸に着目した．グルタミン酸は，網膜における光の情報処理に必要不可欠な神経伝達物質であり，常に細胞外に放出されている．しかし，過剰量の細胞外グルタミン酸はグルタミン酸受容体を過度に活性化して，神経細胞に損傷を引き起こす（グルタミン酸興奮毒性）．細胞外グルタミン酸濃度は，グルタミン酸の回収を担うグルタミン酸輸送体により厳密に制御されている．

われわれは，グルタミン酸輸送体の機能障害によるグルタミン酸興奮毒性が，正常眼圧緑内障における網膜神経節細胞の変性に関与しているのではないかと考えた（概念図）．グルタミン酸輸送体は網膜内にのみ存在し，その機能障害は眼圧には影響を与えないと考えられる．実際，緑内障患者の硝子体，高眼圧緑内障モデル動物の硝子体などでグルタミン酸濃度が上昇することが報告されている[6]．

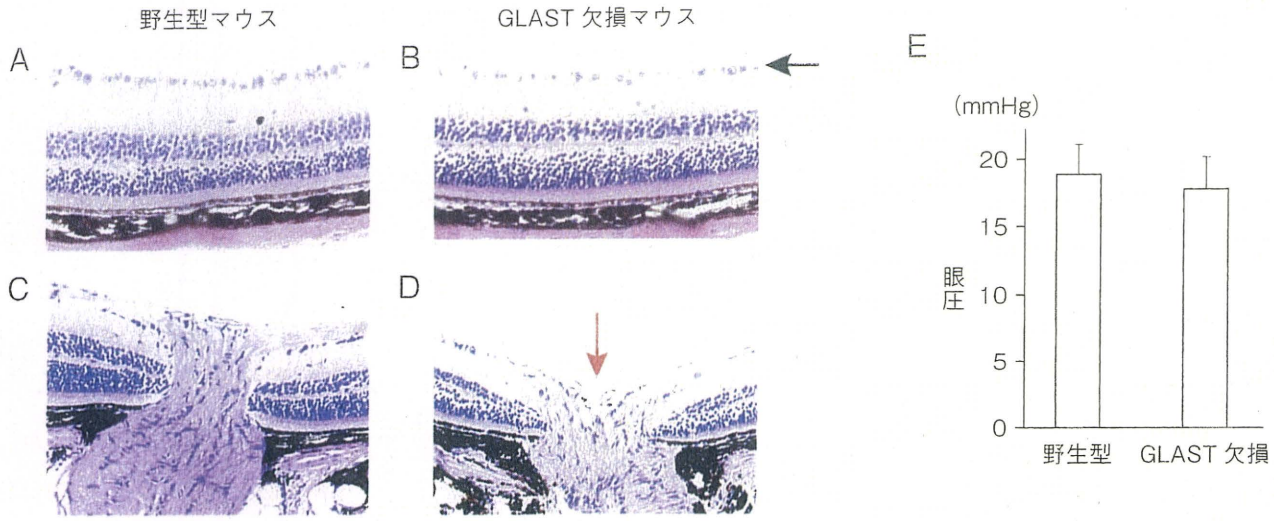


図1　グルタミン酸輸送体欠損マウスは，ヒト正常眼圧緑内障と同様の異常を示す
GLAST欠損マウスでは，網膜神経節細胞（←）が減少し（A，B），視神経乳頭陥凹の拡大（C，D，↓）がみられるが，眼圧は正常眼圧を示す（E）．EAAC1欠損マウスも同様の所見を示す（文献10より転載）

さらに，グルタミン酸輸送体は，抗酸化ストレス分子であるグルタチオンの合成原料であるグルタミン酸，システインの取り込みを通じ，神経細胞を酸化ストレスから保護することが知られている．したがって，グルタミン酸輸送体の機能障害は，グルタミン酸興奮毒性だけでなく，酸化ストレスにより，網膜神経節細胞の変性を促進する可能性もある．実際，緑内障患者房水の抗酸化能の低下，血漿でのグルタチオンの減少，緑内障とGSTM1（glutathione-S-transferase M1，グルタチオンを転移する酵素，解毒・抗酸化作用にかかわる）遺伝子多型との関連，GSTM1遺伝子欠損の頻度が緑内障患者で高く，酸化ストレスマーカーが亢進していることなど[7)8)]が報告されている．

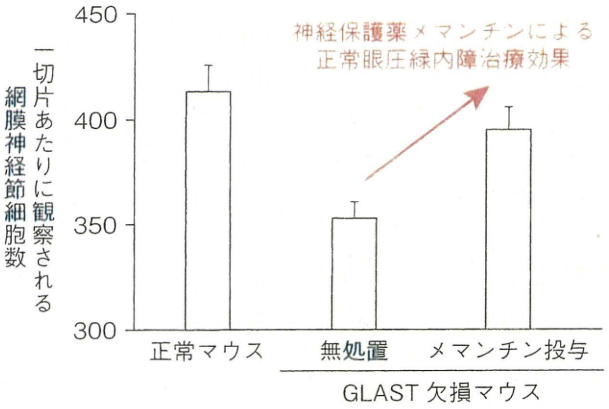


図2　アルツハイマー病治療薬は，グルタミン酸輸送体欠損マウスの病変を改善する
アルツハイマー病治療薬であるグルタミン酸受容体阻害薬メマンチンの継続投与により，GLAST欠損マウスの網膜神経節細胞死が抑制された

グルタミン酸輸送体と正常眼圧緑内障

正常眼圧緑内障で傷害を受ける網膜神経節細胞の周囲には，GLAST，EAAC1，GLT1の3種類のグルタミン酸輸送体が存在する[9)]．おのおのの遺伝子を欠損したマウスの網膜を調べたところ，GLASTおよびEAAC1欠損マウスにおいて，眼圧は正常であるにもかかわらず，網膜神経節細胞が加齢に伴い変性し，視神経乳頭陥凹の拡大，視覚機能異常が起こることを発見した（図1）[10)]．これらはヒトの正常眼圧緑内障において認められる所見とよく一致していた．さらに，GLAST欠損マウスにグルタミン酸受容体の阻害薬であるメマンチンを投与したところ，網膜神経節細胞の傷害が抑制できることを見出した（図2）．これらの結果より，グルタミン酸輸送体の機能障害によるグルタミン酸興奮毒性が，GLAST欠損マウスでみられる網膜神経節細胞死に関与していることが示された．

さらに，GLASTおよびEAAC1欠損マウスの網膜では過酸化脂質が増加していた（図3A）．過酸化脂質の増加は，酸化ストレスの増加を示しており，モデルマウスでみられる網膜神経節細胞の変性に酸化ストレスも関与している可能性がある．そこで，抗酸化物質であるグルタチオンの量を測定したところ，GLAST欠損マウスの網膜およびミューラー細胞（GLASTを発

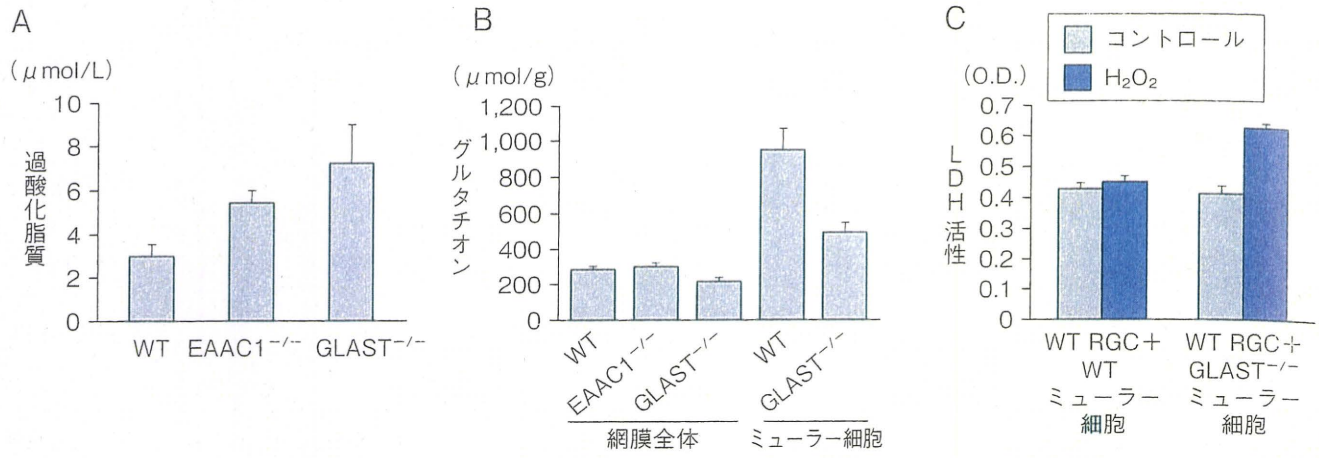


図3　グルタミン酸輸送体欠損マウスでは酸化ストレスが亢進する

A）GLAST欠損マウス，EAAC1欠損マウスの網膜では酸化ストレスマーカーが増加していた．B）GLAST欠損マウスのミューラー細胞ではグルタチオンが減少していた．C）このGLAST欠損ミューラー細胞を野生型の神経節細胞（retinal ganglion cell：RGC）と共培養すると，細胞死の指標であるLDH（lactate dehydrogenase）活性が増加した

現している細胞）においてグルタチオンは減少していた（図3B）．ミューラー細胞においては，GLASTにより細胞外から取り込まれるグルタミン酸がグルタチオン合成の律速段階なので，GLAST欠損マウスの網膜ではグルタチオンの減少が観察されたと考えられる．さらに野生型マウスの神経節細胞をGLAST欠損マウスのミューラー細胞と共培養したところ，酸化ストレス負荷による神経節細胞死が増加した（図3C）．このことは，GLAST欠損マウスでみられる神経節細胞死には，グルタミン酸興奮毒性の他に，ミューラー細胞でのグルタチオン減少による酸化ストレスの増加も関与していることを示している．これは，ミューラー細胞の神経保護作用の低下による可能性がある．

また，酸化ストレス負荷によりEAAC1欠損マウスの神経節細胞死が増加することから，EAAC1欠損マウスでみられる神経節細胞死にも酸化ストレスが関与している．EAAC1は神経節細胞に発現しており，グルタチオンの合成原料であるシステインを効率的に取り込み，グルタチオン合成を促進することにより，神経節細胞自身の酸化ストレスを抑制すると考えられる．

これまでの研究でも，緑内障患者ではGLASTが減少していることが報告されている[11]．さらに，高頻度で緑内障を併発するアルツハイマー病患者でもGLASTの減少が報告されており[12) 13)]，GLASTの機能異常が正常眼圧緑内障の病態に関与している可能性が高い．

本研究によりグルタミン酸輸送体の機能異常が，グルタミン酸興奮毒性および酸化ストレスにより網膜神経節細胞死を誘導し，正常眼圧緑内障を引き起こす重要な因子である可能性が示された（概念図）．本研究で用いられたマウスは世界ではじめての正常眼圧緑内障のモデル動物として，今後の病態解明や治療法の開発に役立つと期待される．

臨床応用・治療の実際

緑内障の治療は，高眼圧・正常眼圧にかかわらず眼圧降下を目的として，投薬，外科手術を行う．しかし，眼圧降下のみでは視野欠損の進行を遅らせることができない症例や進行の抑制が不十分な症例が多く存在する．

このため，眼圧降下以外の，網膜神経節細胞の新規保護法の開発が必要である．われわれの研究は，網膜神経節細胞をグルタミン酸興奮毒性および酸化ストレスから守る化合物が新規神経保護薬として有用であることを示している．グルタミン酸受容体阻害薬であるメマンチンは有力な候補で，われわれの正常眼圧緑内障モデルマウスに加え，高眼圧による緑内障モデルサルでも神経節細胞死を抑制する[14]．現在，緑内障に対するメマンチンの多施設第Ⅲ相臨床試験が進められている（http://www.glaucoma.org/treating/advances.php）．

緑内障の病態解明，緑内障に対する神経保護薬のスクリーニングやその治療効果の判定に，グルタミン酸輸送体欠損マウスはきわめて強力なツールである．またわれわれの研究からグルタミン酸輸送体自身が新た

な治療薬開発の標的となりうる可能性が示唆された．グルタミン酸輸送体の活性化剤は，副作用の少ない新たな緑内障治療薬となりうる[15]．

おわりに―今後の展望・残された課題

われわれは以前にC57BL/6Jと129の両系統の遺伝的背景をもつGLAST欠損マウスは神経節細胞変性がみられないことを報告したが[9]，C57BL/6J系統への純化をくり返すことで，網膜神経節細胞が変性することを見出した．このことは，C57BL/6Jと129の系統を比較することにより，新たな網膜神経節細胞の保護因子の同定が可能であることを示している[4]．

緑内障患者ではGLASTの発現量が減少するが，遺伝子自体の変異は未だ明らかでない．現在われわれは日本人の正常眼圧緑内障患者のDNAを用いて，GLASTとEAAC1の詳細な遺伝子解析を進めている．近年，多くの多因子疾患で大規模な全ゲノム解析が進み，新たなリスク遺伝子が次々と報告されている．緑内障は現在進行中である．また緑内障を含む35疾患での日本人のSNP（single nucleotide polymorphism：一塩基多型）頻度情報がオーダーメイド医療実現化プロジェクトから発表された．これら多くのゲノム解析により，緑内障のリスク遺伝子が同定され，SNPを用いた緑内障の早期発症予測が可能になるであろう．

グルタミン酸興奮毒性は，脳虚血，脳外傷，てんかんなどの急性神経疾患だけでなく，アルツハイマー病，筋萎縮性側索硬化症，エイズ脳症などの慢性神経疾患でみられる神経細胞死にも関与する．グルタミン酸輸送体欠損マウスを用いて開発される神経保護薬やグルタミン酸輸送体を活性化する薬剤は，多くの神経疾患における新しい治療薬としても大きく期待される．

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相田知海：東京医科歯科大学大学院疾患生命科学研究部分子神経科学分野助教．同大学院生命情報科学教育部博士課程卒業，疾患生命科学研究部分子神経科学分野特任助教を経て，2009年より現職．グルタミン酸輸送体を切り口に，個体におけるグルタミン酸代謝の統合的理解を目指している．

103. 緑内障における神経保護研究

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現在行われている緑内障治療は眼圧下降が中心だが，近年では多くの研究者や企業が，細胞死の分子メカニズムに注目した新薬の開発を目指している．神経変性疾患の分野ではグルタミン酸毒性や細胞死実行因子などをターゲットとした神経保護薬の開発が進められており，緑内障治療への応用も可能となるかもしれない．

●グルタミン酸毒性と神経保護

緑内障の原因として，以前からグルタミン酸毒性の関与が指摘されている．たとえば，高眼圧負荷を受けた眼球内ではグルタミン酸濃度が上昇するが，グルタミン酸受容体阻害薬はそれらが受容体と結合し，神経興奮毒性を発揮するのを抑制しようとするものである．最近注目されているグルタミン酸受容体阻害薬は，Alzheimer病治療薬として米国で認可されているmemantineであり，すでに緑内障に対する治験が開始されている．しかし，第三相試験においては有効性が確認されなかったとの情報もあり，実際に眼科臨床の現場で使用可能になるかどうかは，まだ不明である．

他の手法としては，たとえばグルタミン酸輸送体の機能を賦活化することによって，細胞外グルタミン酸濃度を低下させることが考えられる．筆者らは培養Müller細胞を用いた検討により，interleukin-1（IL-1）がGLASTによるグルタミン酸取り込み能を増大させることを示した[1]．さらにIL-1を前投与することで，グルタミン酸による網膜神経節細胞死を有意に抑制できることがわかった（**図1**）．その他にも筋萎縮性側索硬化症の治療薬であるriluzole[2]や脳梗塞の治療薬であるONO-2506（arundic acid）[3]は複数のグルタミン酸輸送体の発現量を上昇させることが報告されており，今後の研究の進展が期待される．

●細胞死実行遺伝子の抑制

これまでの細胞死研究のなかから，アポトーシス実行

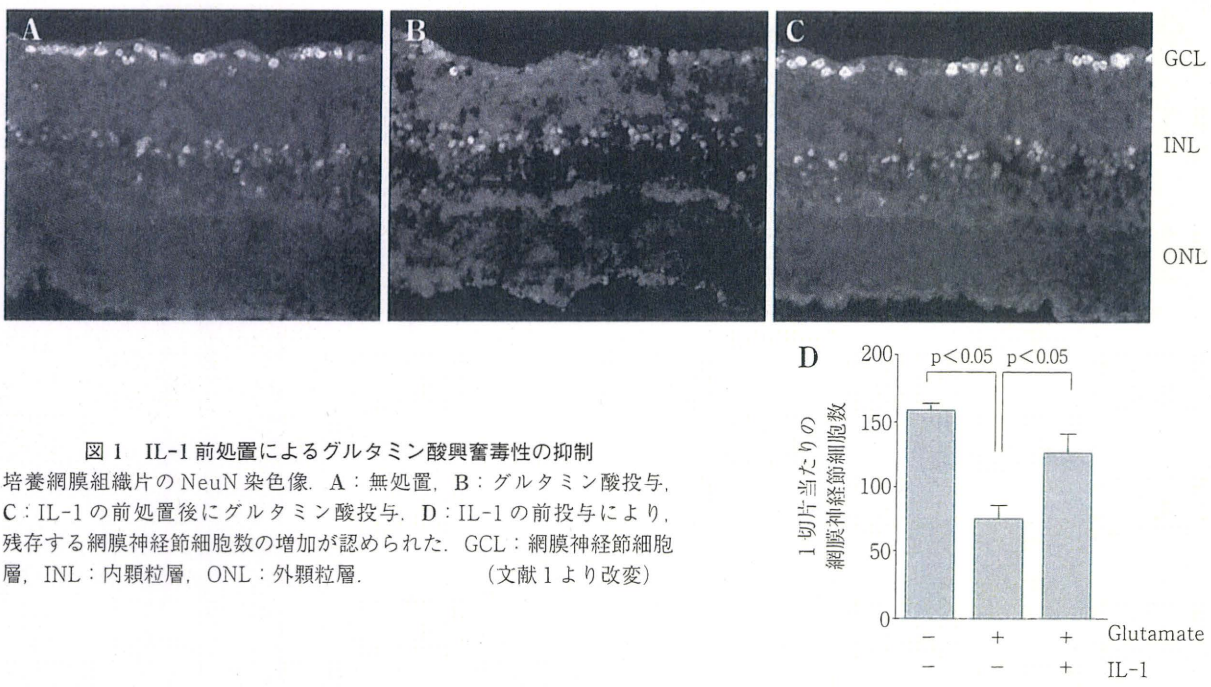

図1　IL-1前処置によるグルタミン酸興奮毒性の抑制
培養網膜組織片のNeuN染色像．A：無処置，B：グルタミン酸投与，C：IL-1の前処置後にグルタミン酸投与．D：IL-1の前投与により，残存する網膜神経節細胞数の増加が認められた．GCL：網膜神経節細胞層，INL：内顆粒層，ONL：外顆粒層．（文献1より改変）

0910-1810/09/¥100/頁/JCLS

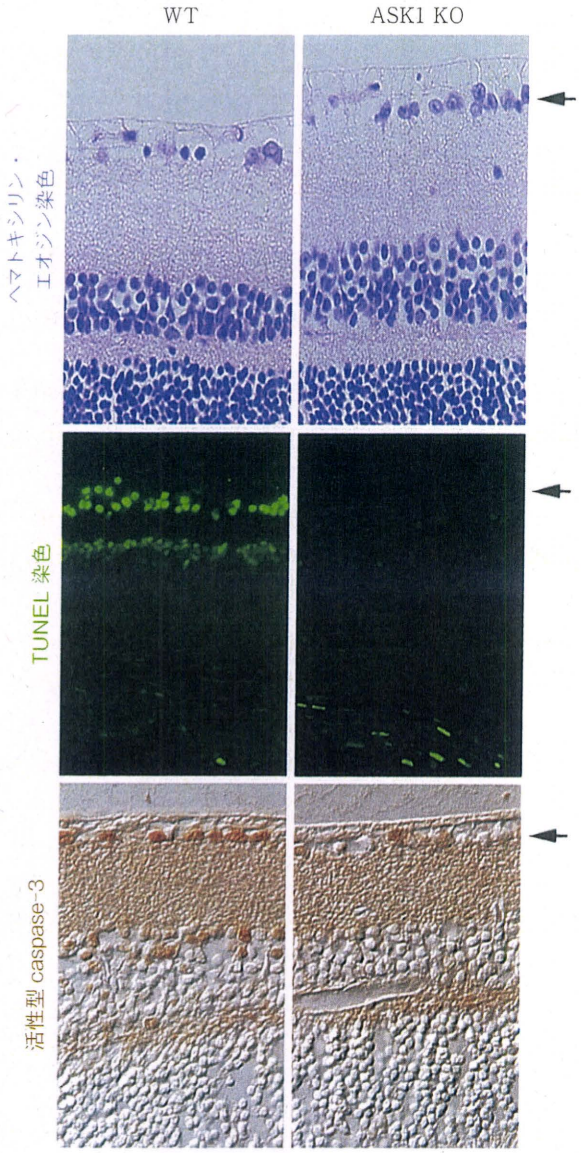

図 2　ASK1 欠損マウスの網膜における虚血耐性の解析
野生型マウス（WT）および同腹の ASK1 欠損マウス（ASK1 KO）を用いた虚血網膜の検討．**上段**：虚血負荷 7 日後のヘマトキシリン・エオジン染色．**中段**：虚血負荷 1 日後の TUNEL 染色．**下段**：虚血負荷 3 時間後の活性型 caspase-3 抗体による免疫染色．ASK1 欠損マウスでは，網膜神経節細胞層におけるアポトーシスの減少と残存細胞数の増加が認められた（矢印）．
（文献 5 より改変）

因子としての caspase family の重要性が明らかにされている．また，細胞死抑制因子についての研究も進展しており，たとえば Bcl-2 は caspase より上流で細胞死を抑制できると考えられている．Caspase 阻害薬は Alzheimer 病や脳虚血などにも有効な可能性があり，今後は網膜神経細胞を含めた神経保護薬としての臨床応用が期待される．

一方，筆者らは，さまざまな環境ストレスに応答して細胞の生死を制御する mitogen-activated protein kinase kinase kinase（MAPKKK）の 1 つである apoptosis signal-regulating kinase 1（ASK1）の機能に注目している[4]．ASK1 欠損マウスに対して高眼圧負荷を行ったところ，野生型マウス網膜と比較して，caspase-3 の活性低下と網膜神経節細胞死の軽減が確認された（**図 2**）．さらに ASK1 欠損マウス由来の培養網膜神経節細胞では，酸化ストレスに対する耐性も高まっていた[5]．つまり酸化ストレスの関与が示唆される緑内障の新たな治療標的として，ASK1～caspase-3 pathway を阻害することの有用性が示されたといえる．前号で紹介した GLAST 欠損マウスはグルタミン酸毒性に加えて，酸化ストレスが複合的に関与する正常眼圧緑内障モデル動物と考えられることから，現在は ASK1 欠損マウスとの交配によって，症状が軽快するかを検討中である．

☆　☆　☆

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Neuroscience Letters 465 (2009) 160–164

Contents lists available at ScienceDirect

Neuroscience Letters

journal homepage: www.elsevier.com/locate/neulet

Interleukin-1 attenuates normal tension glaucoma-like retinal degeneration in EAAC1-deficient mice

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ARTICLE INFO

Article history:
Received 11 June 2009
Received in revised form 2 September 2009
Accepted 13 September 2009

Keywords:
Interleukin-1
Glutamate transporter
Cell death
Neuroprotection
Glaucoma
Retina

ABSTRACT

Glaucoma, one of the leading causes of irreversible blindness, is characterized by progressive degeneration of retinal ganglion cells (RGCs) and optic nerves. Although glaucoma is often associated with elevated intraocular pressure, recent studies have shown a relatively high prevalence of normal tension glaucoma (NTG) in glaucoma patient populations. In the mammalian retina, glutamate/aspartate transporter (GLAST) is localized to Müller glial cells, whereas excitatory amino acid carrier 1 (EAAC1) is expressed in neural cells, including RGCs. Since the loss of GLAST or EAAC1 leads to retinal degeneration similar to that seen in NTG, we examined the effects of interleukin-1 (IL-1) on RGC death in GLAST- and EAAC1-deficient mice. IL-1 promoted increased glutamate uptake in Müller cells by suppressing intracellular Na^+ accumulation, which is necessary to counteract Na^+-glutamate cotransport. The observed trends for the glutamate uptake increase in the wild-type (WT), GLAST- and EAAC1-deficient mice were similar; however, the baseline glutamate uptake and intracellular Na^+ concentration in the GLAST-deficient mice were significantly lower than those in the wild-type mice. Consistently, pretreatment with IL-1 exhibited no beneficial effects on glutamate-induced RGC degeneration in the GLAST-deficient mice. In contrast, IL-1 significantly increased glutamate uptake by Müller cells and the number of surviving RGCs in the wild-type and EAAC1-deficient mice. Our findings suggest that the use of IL-1 for enhancing the function of glutamate transporters may be useful for neuroprotection in retinal degenerative disorders including NTG.

Glaucoma is characterized by a slow, progressive degeneration of retinal ganglion cells (RGCs) and their axons that are usually associated with elevated intraocular pressure (IOP). Recent studies have shown that glaucoma is affected by multiple genes and environmental factors [18,16], and there are several inherited and experimentally induced animal models of high IOP glaucoma [10]. There is, however, a subset of glaucoma – normal tension glaucoma (NTG) – that presents with statistically normal IOP. The number of NTG patients has been thought to be small relative to glaucoma patients as a whole, but recent studies have revealed an unexpectedly high prevalence of NTG [9]. The findings suggest that non-IOP-dependent factors may contribute to disease progression and that elucidating such factors is necessary to better understand the pathogenesis of glaucoma, especially in the context of NTG.

It is well known that an immoderate release of excitatory amino acids, such as glutamate, can cause neuronal cell death. Excessive extracellular concentrations of glutamate induce an uncontrolled elevation of intracellular calcium that enters the cell through chronically activated glutamate receptors. The glutamate transporter is the only mechanism for removal of glutamate from the extracellular fluid in the retina. In the inner plexiform layer where synapses exist across RGCs, three transporters are involved in this task: glutamate transporter 1 (GLT-1) located in the bipolar cell terminals; excitatory amino acid carrier 1 (EAAC1) in RGCs; and glutamate/aspartate transporter (GLAST) in Müller glial cells [5,7]. Previously, we found that GLAST and EAAC1 knockout (KO) mice showed spontaneous RGC death and optic nerve degeneration without elevated IOP [5]. Together with the downregulation of GLAST (human EAAT1) in glaucoma patients [12], these results suggest the involvement of glutamate neurotoxicity in the pathogenesis of glaucoma. Therefore, one possible treatment for glaucoma may involve increasing glutamate uptake by the glutamate transporters.

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doi:10.1016/j.neulet.2009.09.029

Table 1
Sequences of PCR primers used in this study.

		Product size (bp)
GLAST (accession no. NM_148938)		
Forward primer	5′-ATGACCAAAAGCAACGGAGA-3′ (1–20)	
Reverse primer	5′-GATTATGCCAATCACCACAG-3′ (400–420)	420
α Subunit of Na^+/K^+-ATPase (accession no. NM_144900)		
Forward primer	5′-CTGAGAACGGTTTCCTGCCC-3′ (2612–2640)	
Reverse primer	5′-CCTGATAATGAGCTTCCGCA-3′ (3011–3030)	419
β Subunit of Na^+/K^+-ATPase (accession no. NM_013415)		
Forward primer	5′-ATCCAAGCACAGAAGAATGA-3′ (361–380)	
Reverse primer	5′-GGTTTTGTTGATCCGGAGTT-3′ (851–870)	510
β-Actin (accession no. NM_007393)		
Forward primer	5′-ATGGATGACGATATCGCTGCGCT-3′ (1–23)	
Reverse primer	5′-TGTAGCCACGCTCGGTCAGGAT-3′ (577–599)	599

Glutamate transport is coupled with the cotransport of $3Na^+$, and the efficiency of glutamate uptake is influenced by both intracellular and extracellular Na^+ concentrations [17]. We recently showed that IL-1 increases glutamate uptake in mouse Müller glial cells by stimulating membrane trafficking of Na^+/K^+-ATPase and suppressing Na^+ accumulation [11]. Our observations suggest that IL-1 may prevent glutamate neurotoxicity by increasing the activity of glutamate transporters in Müller cells; however, retinal glia may express EAAC1 and GLT-1 as well as GLAST [8]. In this study, we investigated whether IL-1 protects retinal neurons from glutamate neurotoxicity in GLAST and EAAC1 KO mice and elucidated a role of glutamate transporters in the IL-1-mediated retinal neuroprotective effect.

Experiments were performed using GLAST-deficient (GLAST KO) and EAAC1-deficient (EAAC1 KO) mice [5] at 3 weeks of age in accordance with the Tokyo Metropolitan Institute for Neuroscience Guidelines for the Care and Use of Animals. Immunoblotting was performed as previously described [11]. Membranes were incubated with antibodies against GLAST (1:1000) [7] or EAAC1 (1:1000) [13]. Primary antibody binding was detected using horseradish peroxidase-labeled anti-mouse IgG secondary antibody (Amersham, Piscataway, NJ, USA) and visualized using an ECL Plus western blotting system (Amersham). For immunohistochemical analysis, 7-μm-thick retinal sections were incubated with one of the three sets of primary antibody mixes: rabbit anti-GLAST (0.5 μg/ml) and mouse anti-glutamine synthetase (1.0 μg/ml; Chemicon International, Temecula, CA, USA); rabbit anti-GLAST (0.5 μg/ml) and mouse anti-IL-1 receptor (0.5 μg/ml; BD Biosciences Pharmingen, San Jose, CA, USA); or rabbit anti-EAAC1 and mouse anti-calretinin (1.0 μg/ml; Chemicon International). Retinal explant cultures [11] were preincubated with or without 50 ng/ml IL-1 for 24 h and then stimulated with 5 mM glutamate for 1 h. After 72 h, retinal explants were immunostained with an antibody against NeuN (1.0 μg/ml; Chemicon International). RGC number was quantified by counting the cells in the ganglion cell layer from one ora serrata through the optic nerve to the other ora serrata, in three sections from three eyes.

Glutamate uptake assay in primary cultured Müller cells was performed as previously reported [6,11]. The effects of IL-1 on cell viability and ATP synthesis in Müller cells were analyzed using assay kits for lactate dehydrogenase (Wako, Osaka, Japan) and ATP (Toyo B-Net, Tokyo, Japan), respectively. Cultured Müller cells grown on glass-bottomed dishes were imaged live to record the dynamic intracellular ion state using the fluorescent dye CoroNa Green AM [11]. For RT-PCR analysis, total RNA was isolated from the cultured Müller cells using an Isogen reagent (Nippon Gene, Tokyo, Japan) and then reverse-transcribed with a Revertra ace (Toyobo, Osaka, Japan) to obtain cDNA. RT-PCR was carried out using primer pairs specific for mouse GLAST, α and β subunits of Na^+/K^+-ATPase, and β-actin (Table 1). Reactions were conducted under the following conditions: precycling at 94 °C for 2 min, followed by 24, 27 or 30 cycles consisting of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and polymerization at 72 °C for 30 s. The PCR products (10 μL) were size fractionated on agarose gels and detected by ethidium bromide staining. Data are presented as mean ± standard errors unless noted otherwise. Statistical analyses were performed using Student's *t*-test or one-way ANOVA followed by Dunnett's test using JMP7 software from SAS Institute (Cary, NC). Results were considered statistically significant if $p < 0.05$.

Immunohistochemical analysis was performed with retinas of wild-type (WT), GLAST KO and EAAC1 KO mice. As previously

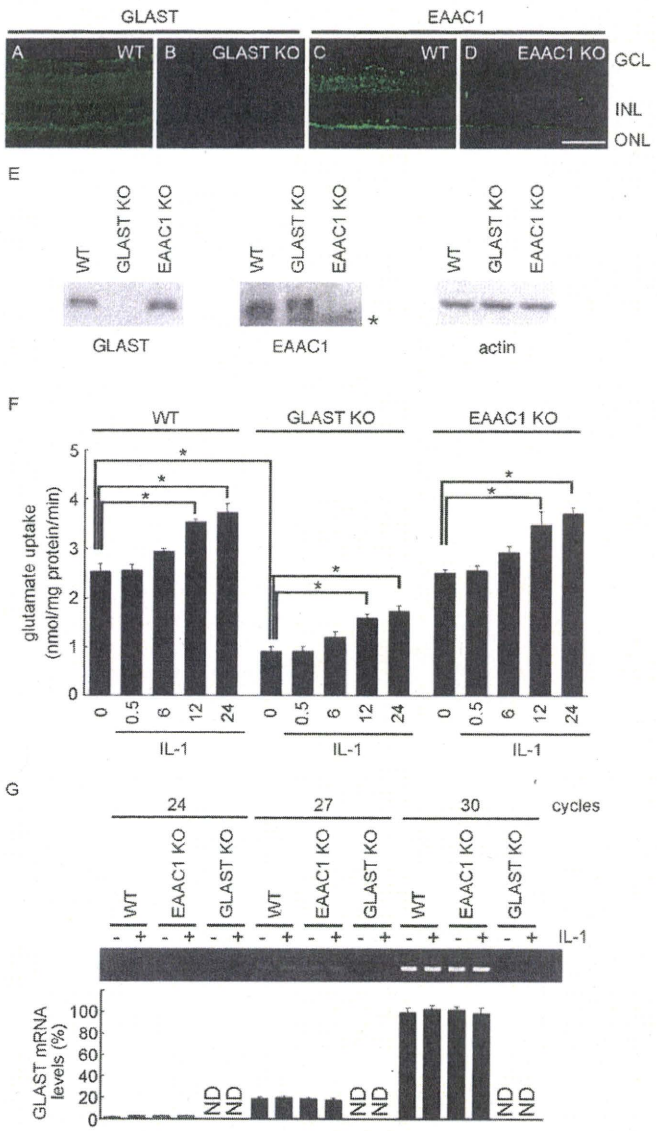

Fig. 1. Effect of IL-1 on glutamate uptake by Müller glial cells from WT, GLAST KO and EAAC1 KO mice. (A–D) Immunohistochemical analysis of GLAST (A and B) and EAAC1 (C and D) in retinas from WT (A and C), GLAST KO (B) and EAAC1 KO (D) mice. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar: 50 μm. (E) Immunoblot analysis of neural retinas from WT, GLAST KO and EAAC1 KO mice. (*) Non-specific bands. (F) Glutamate uptake activity in Müller cells from WT, GLAST KO and EAAC1 KO mice in the presence or absence of IL-1. Müller cells were pretreated with 50 ng/ml IL-1 for the indicated periods. The data are presented as means ± standard errors of three samples for each group. *$p < 0.01$. (G) RT-PCR analysis of GLAST expression levels in Müller cells treated with 50 ng/ml IL-1 for 24 h. Total RNA was isolated from cultured Müller cells, reverse-transcribed, and subjected to PCR analysis at indicated cycle numbers. The densitometric results are expressed as a percentage of the control values (non-treated WT at 30 cycles).

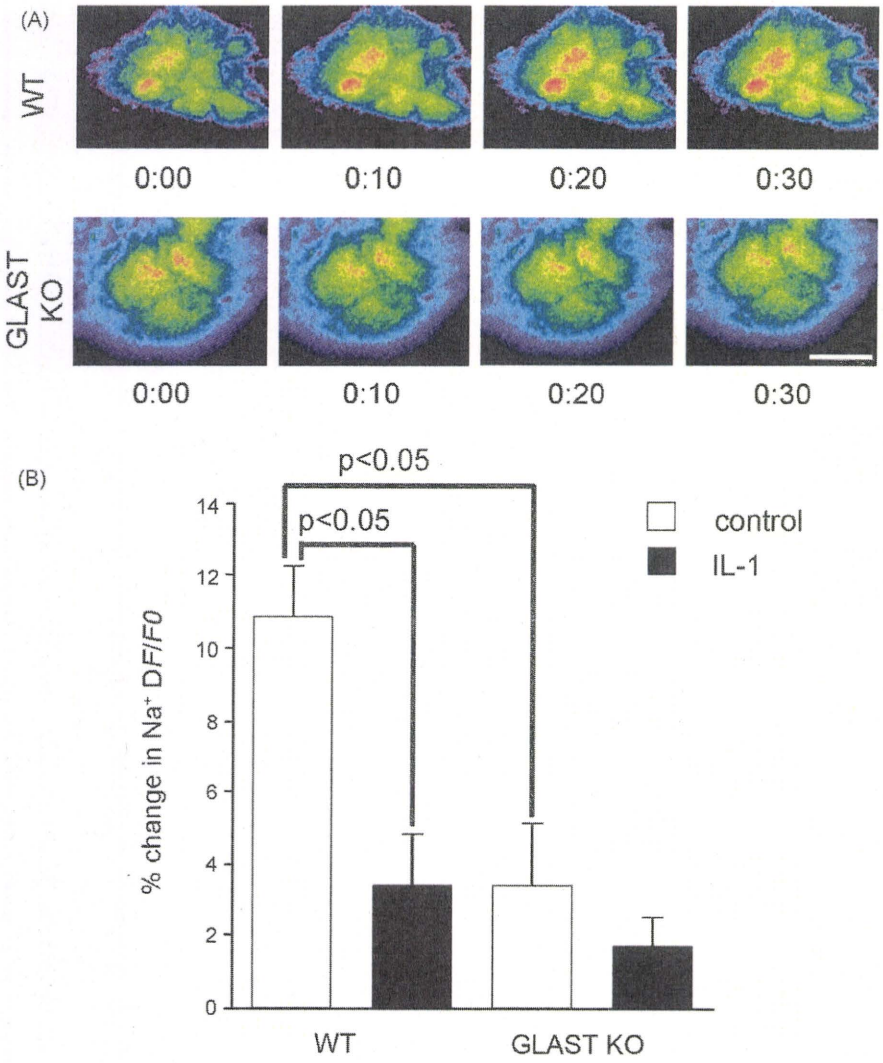

Fig. 2. Effect of GLAST on glutamate uptake activity and Na^+ accumulation in Müller glial cells. (A) Na^+ imaging of Müller cells treated with 3 mM glutamate. Fluorescence images are shown in pseudocolor, with blue and red representing the lowest and highest intensities, respectively. The indicated times represent the number of seconds after initial application of glutamate. Glutamate-induced Na^+ accumulation was increased in the WT Müller cells, but not in the GLAST KO Müller cells. Scale bar: 20 μm. (B) Quantification of Na^+ accumulation in response to the glutamate stimulation in Müller cells from WT and GLAST KO mice. Müller cells were loaded with the CoroNa Green Na^+ indicator and stimulated with a bath-application of 3 mM glutamate. Pretreatment with 50 ng/ml of IL-1 for 24 h significantly suppressed Na^+ accumulation in Müller cells from the WT, but not from the GLAST KO mice. The data are presented as means ± standard errors of 9–15 cells for each group from three independent cultures. $^*p < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

reported [5,7], GLAST is expressed in Müller glial cells (Fig. 1A) and EAAC1 is mainly expressed in RGCs and amacrine cells (Fig. 1C). These immunoreactivities were not observed in GLAST and EAAC1 KO mice (Fig. 1B and D). We next performed immunoblot analysis with neural retinas. GLAST and EAAC1 KO mice showed no compensatory upregulation of EAAC1 and GLAST, respectively (Fig. 1E).

The glutamate uptake by GLAST is important for retinal neuroprotection *in vivo* [5,7], and we recently reported that IL-1 increases glutamate uptake in Müller cells [11]. Consistent with the previous findings, pretreatment with IL-1 (50 ng/ml) for 12 and 24 h significantly increased the glutamate uptake activity in WT Müller cells, which we measured by monitoring the uptake of L-[^{3}H]glutamate (Fig. 1F). Since the IL-1 receptor in the mouse retina was co-expressed with GLAST in Müller cells [11], these results suggest strongly an interaction between GLAST and IL-1 receptor activation on glutamate uptake in Müller cells. However, retinal glia may express EAAC1 and GLT-1 as well as GLAST [8]. Therefore, we explored the relationship between IL-1 stimulation and the activation of glutamate transporters using Müller cells prepared from the GLAST and EAAC1 KO mice. The capacity of glutamate uptake was severely impaired in the GLAST KO Müller cells (0.9 ± 0.1 nmol/mg protein/min), but was normal in the EAAC1 KO Müller cells (2.6 ± 0.1 nmol/mg protein/min), compared with the WT Müller cells (2.6 ± 0.2 nmol/mg protein/min; Fig. 1F). We next stimulated these Müller cells with 50 ng/ml of IL-1 for the indicated time periods prior to the detection of glutamate uptake. Pretreatment with IL-1 for 12 and 24 h significantly increased glutamate uptake in the WT (3.6 ± 0.1 and 3.8 ± 0.2 nmol/mg protein/min) and EAAC1 KO Müller cells (3.5 ± 0.3 and 3.8 ± 0.1 nmol/mg protein/min). Interestingly, IL-1 also stimulated glutamate uptake in the GLAST KO Müller cells, though modestly (1.7 ± 0.1 and 1.8 ± 0.1 nmol/mg protein/min), where the basal glutamate uptake in the GLAST KO Müller cells was significantly lower than in the WT Müller cells. These results indicate that IL-1 increases glutamate uptake in Müller cells by stimulating multiple glutamate transporters including GLAST.

We next examined whether IL-1 alters GLAST expression levels in Müller cells. The RT-PCR analysis amplified the expected 420 bp product using primers specific for GLAST, and IL-1 had no effect on GLAST expression levels (Fig. 1G). The reaction did not result in a product if reverse transcriptase was absent (data not shown). In addition, we evaluated the effects of IL-1 on Müller cell death

by examining extracellular lactate dehydrogenase (LDH) activities. However, IL-1 had no effect on Müller cell viability (103 ± 4% compared with non-treated controls).

Since glutamate transport is coupled with the cotransport of $3Na^+$, the efficiency of glutamate uptake is influenced by both intracellular and extracellular Na^+ concentrations [17]. Accordingly, we next evaluated the effect of GLAST activity and IL-1 on Na^+ concentrations in cultured Müller cells. Müller cells were loaded with the CoroNa Green Na^+ indicator and stimulated with a bath-application of 3 mM glutamate. In the WT Müller cells, the intracellular Na^+ concentration clearly increased in response to glutamate, and IL-1 pretreatment for 24 h significantly suppressed this upregulation (Fig. 2A and B). In contrast, in the GLAST KO Müller cells, neither glutamate stimulation nor IL-1 had a significant effect on the intracellular Na^+ concentration (Fig. 2A and B). These results are consistent with impaired glutamate uptake in GLAST KO Müller cells (Fig. 1F). Elevated intracellular Na^+ concentration is decreased by Na^+/K^+-ATPase, which is dependent on ATP levels [2–4]. Consequently, we further examined whether IL-1 increases ATP synthesis in Müller cells. However, we found that stimulation with IL-1 for 24 h had no effect on ATP synthesis in Müller cells (105 ± 5% compared with non-treated controls). In addition, IL-1 had no effect on the expression levels of Na^+/K^+-ATPase subunits (103 ± 3% in α subunit and 97 ± 4% in β subunit compared with non-treated controls).

Our results suggested that IL-1 may protect retinal neurons from glutamate neurotoxicity by increasing the glutamate uptake through GLAST in Müller cells. In order to confirm this hypothesis, we prepared retinal explants from WT, GLAST KO and EAAC1 KO mice. The WT retinal explants stimulated with 5 mM glutamate for 1 h (Fig. 3B and E) showed a clear decrease in the number of NeuN-positive neurons in the GCL, compared with the non-treated controls (Fig. 3A and D). However, pretreatment of the explants with IL-1 (50 ng/ml) significantly increased the number of surviving RGCs (Fig. 3C, F and S). We carried out similar experiments using the GLAST KO mice. The number of RGCs was significantly decreased in these mice at 3 weeks after birth (Fig. 3G and J), as previously reported [5]. The glutamate stimulation slightly decreased the number of RGCs, but IL-1 failed to protect them (Fig. 3H, I, K, L and S). Finally, we applied glutamate to retinal explants from the EAAC1 KO mice. The number of RGCs was normal in the non-treated controls (Fig. 3M and P) and IL-1 pretreatment significantly prevented glutamate-induced RGC death (Fig. 3N, O, Q, R and S).

GLAST is a major, glial-type glutamate transporter expressed in Müller cells [7], and glutamate-induced electrogenic currents are absent in Müller cells that lack GLAST [15]. However, a recent study demonstrated that GLT-1 and EAAC1, as well as GLAST, are expressed in rat retinal glial cells [8]. The authors reported that GLAST expression levels were increased in the context of glutamate and potassium loading, whereas GLT-1 expression increased during hypoxia. These observations suggest that various subtypes of glutamate transporters in Müller cells are involved in retinal physiology and pathology. In the present study, we found that glutamate uptake activity and glutamate-induced intracellular Na^+ accumulation were both severely impaired in Müller cells of GLAST KO mice. We previously proposed a model in which IL-1 increases glutamate uptake in Müller cells by accelerating membrane trafficking rates of Na^+/K^+-ATPase and hence suppressing Na^+ accumulation, which is required for counteracting the Na^+-glutamate cotransport by GLAST [11]. These findings suggest that GLAST plays a key role in the IL-1-mediated glutamate uptake increase in Müller cells.

Glutamate excitotoxicity is associated with various eye diseases, including diabetic retinopathy and glaucoma [5,7,16]. Indeed, we recently reported that our GLAST and EAAC1 KO mice were the first animal models of NTG [5]. Oxidative stress is also involved in glaucoma and other retinal degeneration [1]. Glutathione, a tripeptide

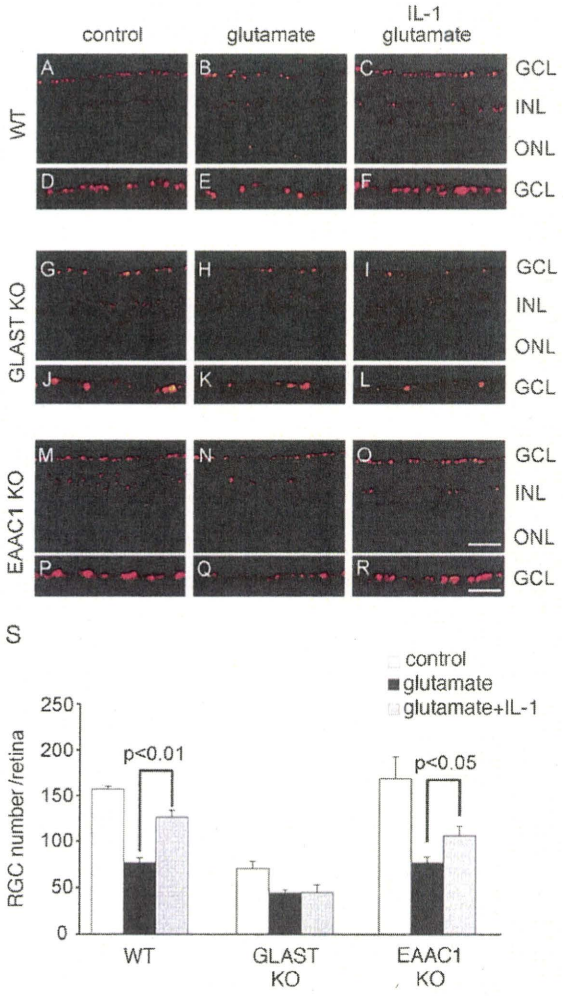

Fig. 3. IL-1 protects retinal neurons from glutamate neurotoxicity in WT and EAAC1 KO, but not GLAST KO mice. (A–R) Immunohistochemical analysis of mouse retinal explants from WT (A–F), GLAST KO (G–L) and EAAC1 KO (M–R) mice, stained with an anti-NeuN antibody. Explants were untreated (A, D, G, J, M, P), treated with 5 mM glutamate alone (B, E, H, K, N, Q), or treated with both 50 ng/ml IL-1 and 5 mM glutamate (C, F, I, L, O, R). (S) Quantification of NeuN-positive cells in the GCL. The data are presented as means ± standard errors of three samples for each group. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer. Scale bar: 50 μm (A–C, G–I, M–O) and 25 μm (D–F, J–L, P–R).

of glutamate, cysteine and glycine, plays a key role in protecting RGCs against oxidative stress. Since glutamate uptake is the rate-limiting step in glial glutathione synthesis, activation of glutamate transporters in Müller cells by IL-1 may be an effective strategy for protecting RGCs. In order to explore this possibility, we administered IL-1 to retinal explants from GLAST and EAAC1 KO mice. In the GLAST KO mice, IL-1 failed to protect RGCs from glutamate neurotoxicity. This result is consistent with the severely impaired glutamate uptake in the GLAST KO Müller cells compared with the WT and EAAC1 KO Müller cells. On the other hand, IL-1 partially prevented glutamate neurotoxicity and increased the number of surviving RGCs in the EAAC1 KO mice. Our present results confirm that GLAST is indeed the key transporter that is involved in the IL-1-mediated retinal neuroprotection. However, since IL-1 was still able to increase glutamate uptake in the GLAST KO Müller cells, IL-1 may also stimulate other subtypes of glutamate transporters, such as GLT-1 [8] and currently unidentified glutamate transporters [15].

Our findings suggest that IL-1 may be a viable option for elevating glutamate uptake in the case where GLAST is normally expressed in Müller cells. Accordingly, we have started the genetic analysis of GLAST and EAAC1 in human glaucoma, especially in

NTG patients. To determine whether overexpression of GLAST and IL-1 stimulation may synergistically exert retinal neuroprotective effects, we are currently generating mice that overexpress GLAST. Another important point is that we examined the chronic effect of IL-1 using *ex vivo* culture system. This is because repeated injections of IL-1 to small mouse eyes are difficult and often wound normal retinal tissue. IL-1 is an important mediator of brain injury induced by ischemia or trauma, and has been implicated in chronic brain diseases including Alzheimer's disease, Parkinson's disease, and multiple sclerosis [1,14]. Thus, *in vivo* study to evaluate the long-term effect of IL-1 will be required as the next step to avoid its possible side effects. Further efforts to discover new compounds that can enhance glutamate uptake for a prolonged period may lead to the development of novel strategies for the management of various forms of retinal degeneration, including glaucoma.

Acknowledgements

This work was supported by the Ministry of Education, Culture, Sports, Science and Technology of Japan (K.N., K.T., T.H.), the Ministry of Health, Labour, and Welfare of Japan (Y.M., K.T., T.H.), the Japan Society for the Promotion of Science for Young Scientists (C.H.), the Welfare and Health Funds from Tokyo Metropolitan Government (Y.M.), the Uehara Memorial Foundation, the Naito Foundation, the Suzuken Memorial Foundation, the Daiwa Securities Health Foundation, the Takeda Science Foundation, and the Japan Medical Association (T.H.).

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Cell Death and Differentiation (2010) 17, 1751–1759

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ASK1 deficiency attenuates neural cell death in GLAST-deficient mice, a model of normal tension glaucoma

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Apoptosis signal-regulating kinase 1 (ASK1) is an evolutionarily conserved mitogen-activated protein kinase (MAPK) kinase kinase and has an important role in stress-induced retinal ganglion cell (RGC) apoptosis. In the mammalian retina, glutamate/aspartate transporter (GLAST) is a major glutamate transporter, and the loss of GLAST leads to optic nerve degeneration similar to normal tension glaucoma (NTG). In GLAST$^{-/-}$ mice, the glutathione level in the retina is decreased, suggesting the involvement of oxidative stress in NTG pathogenesis. To test this hypothesis, we examined the histology and visual function of GLAST$^{+/-}$:ASK1$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice by multifocal electroretinograms. ASK1 deficiency protected RGCs and decreased the number of degenerating axons in the optic nerve. Consistent with this finding, visual function was significantly improved in GLAST$^{+/-}$:ASK1$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice compared with GLAST$^{+/-}$ and GLAST$^{-/-}$ mice, respectively. The loss of ASK1 had no effects on the production of glutathione or malondialdehyde in the retina or on the intraocular pressure. Tumor necrosis factor (TNF)-induced activation of p38 MAPK and the production of inducible nitric oxide synthase were suppressed in ASK1-deficient Müller glial cells. In addition, TNF-induced cell death was suppressed in ASK1-deficient RGCs. These results suggest that ASK1 activation is involved in NTG-like pathology in both neural and glial cells and that interrupting ASK1-dependent pathways could be beneficial in the treatment of glaucoma, including NTG.
Cell Death and Differentiation (2010) **17,** 1751–1759; doi:10.1038/cdd.2010.62; published online 21 May 2010

It is estimated that glaucoma affects nearly 70 million individuals worldwide, including at least 6.8 million who are bilaterally blind.[1] The disease is characterized by the slow progressive degeneration of the retinal ganglion cells (RGCs) and their axons, which are usually associated with elevated intraocular pressure (IOP). Recent studies have shown that glaucoma is affected by multiple genes and environmental factors,[2,3] and there are several inherited and experimentally induced animal models of high IOP glaucoma, including DBA/2J mice and laser-induced chronic ocular hypertension model.[4–6] There is a subtype of glaucoma termed normal tension glaucoma (NTG), however, that presents with statistically normal IOP. The number of NTG patients has been thought to be small relative to the total number of glaucoma patients, but recent studies have revealed an unexpectedly high prevalence of NTG.[7] These findings suggest that non-IOP-dependent factors may contribute to disease progression, and elucidating these factors is necessary to better understand the pathogenesis of glaucoma, especially in the context of NTG. For this purpose, an animal model representing disease characteristics of NTG would be extremely useful. To date, some animal models have been introduced, for example, the optic nerve ligation model shows RGC loss with normal IOP,[8] but this is more suitable as a model of ischemia or optic nerve injury. In addition, preparation of these artificial models requires a high level of technical skills, but unfortunately, long-term reproducibility seems to be somewhat limited. Thus, there has been a great demand to create suitable animal models of NTG.

In addition to more extensively studied factors such as reduced ocular blood flow and systemic blood pressure changes, excessive stimulation of the glutamatergic system has been proposed to contribute to the death of RGCs in glaucoma. Excessive extracellular concentrations

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Keywords: glaucoma; ASK1; glutamate transporter; glia; neural cell death

Abbreviations: ASK1, apoptosis signal-regulating kinase 1; GLAST, glutamate/aspartate transporter; NTG, normal tension glaucoma; Aβ, amyloid beta; GDNF, glial cell line-derived neurotrophic factor; GLT-1, glutamate transporter 1; iNOS, inducible nitric oxide synthase; IL-1, interleukin-1; IOP, intraocular pressure; MAPK, mitogen-activated protein kinase; mfERGs, multifocal electroretinograms; NO, nitric oxide; NTN, neurturin; RGC, retinal ganglion cell; ROS, reactive oxygen species; TLR4, Toll-like receptor 4; TNF, tumor necrosis factor

Received 28.12.09; revised 22.3.10; accepted 15.4.10; Edited by N Bazan; published online 21.5.10

of glutamate induce uncontrolled elevation of intracellular calcium, which enters through chronically activated glutamate receptors. Glutamate uptake by the glial cells is a well-known mechanism to maintain low extracellular levels of glutamate and promote efficient interneuronal signaling in the central nervous system (CNS). Furthermore, the same process is considered to be neuroprotective during neurodegeneration. Clearance of glutamate from the extracellular space is accomplished primarily by the action of glutamate transporters.[9] In the CNS, the glutamate/aspartate transporter (GLAST) and glutamate transporter 1 (GLT-1) are Na^+-dependent glutamate transporters found in astrocytes. Genetic deletion of GLAST and/or GLT-1 causes abnormal brain development and neurological symptoms such as motor deficits.[9–11] We have previously reported that GLAST, located in Müller glial cells, is the only glial-type glutamate transporter in the retina, whereas GLT-1 is expressed in neurons, including bipolar cells and photoreceptors.[12] Not surprisingly, GLAST is more active than GLT-1 in preventing glutamate neurotoxicity after ischemia.[12] In addition, we recently found that GLAST-deficient ($GLAST^{-/-}$ and $GLAST^{+/-}$) mice show spontaneous RGC death and optic nerve degeneration without elevated IOP.[13] Interestingly, GLAST is essential not only to keep the extracellular glutamate concentration below a neurotoxic level but also to maintain glutathione levels by transporting glutamate, which is a substrate for glutathione synthesis, into Müller cells. As retinal concentration of glutathione, a major cellular antioxidant in the retina, was decreased in GLAST-deficient mice, both glutamate neurotoxicity and oxidative stress may be involved in NTG-like pathology.[13] Together with the evidence that downregulation of GLAST (human EAAT1) in the retina and of glutathione level in the plasma are found in human glaucoma patients,[14,15] it is appropriate to consider GLAST-deficient mice as a valid and adequate model that offer a powerful system to determine the mechanisms of and evaluate new treatments for NTG.

Apoptosis signal-regulating kinase 1 (ASK1) has key roles in human diseases closely related to the dysfunction of cellular responses to oxidative stress and endoplasmic reticulum stressors, including neurodegenerative diseases.[16,17] We have previously reported that ASK1 is primarily expressed in RGCs, and $ASK1^{-/-}$ mice are less susceptible to ischemic injury.[18] The role of ASK1 in glaucoma, however, is unknown. In an attempt to identify the apoptotic signals regulating RGC death in GLAST-deficient mice, we generated

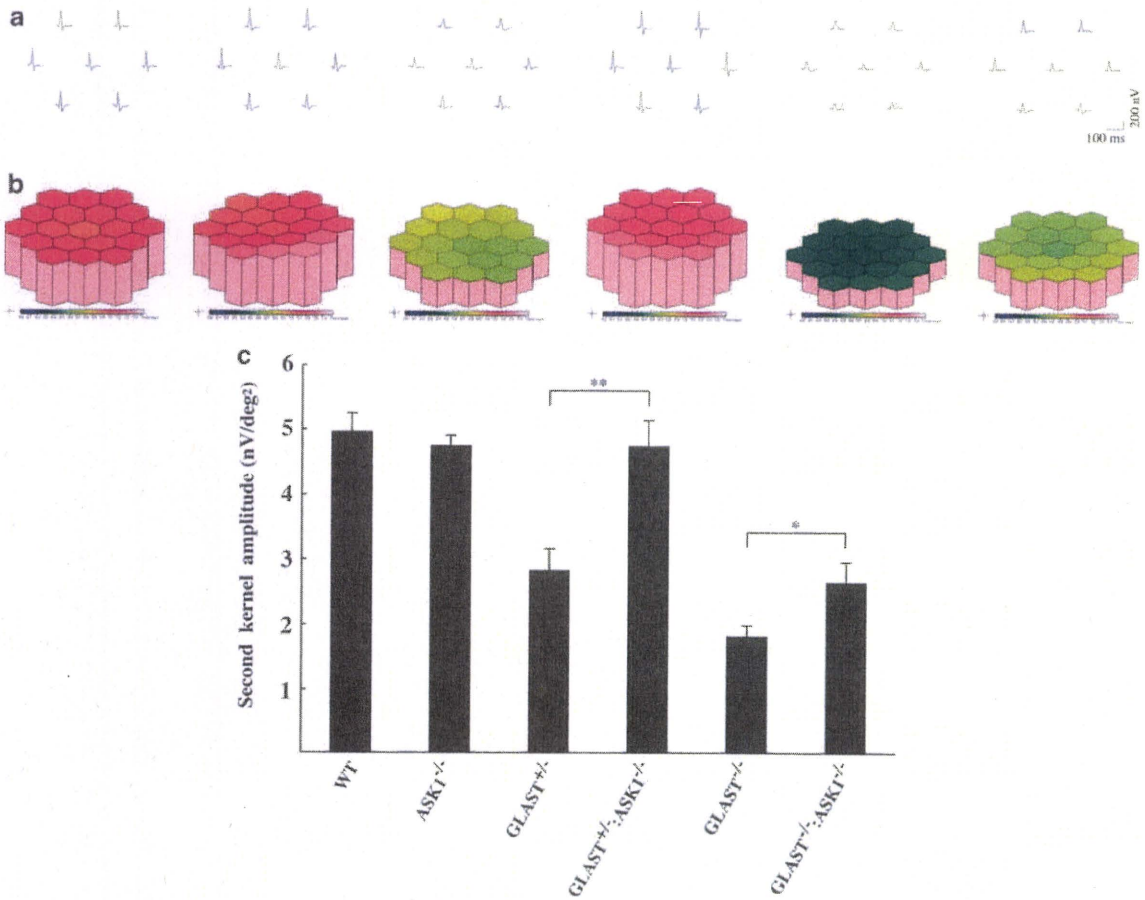

Figure 1 Effect of ASK1 on visual response in $GLAST^{+/-}$ and $GLAST^{-/-}$ mice. (**a**) Summed responses of the second-order kernel examined using multifocal electroretinograms. (**b**) Averaged responses of the second-order kernel are demonstrated using three-dimensional plots. The degree of retinal function is presented in the color bar. A higher score (red) indicates highly sensitive visual function and a lower score (green) indicates retinal dysfunction. (**c**) Quantitative analysis of the visual response amplitude. Note the improved visual function of $GLAST^{+/-}$:$ASK1^{-/-}$ and $GLAST^{-/-}$:$ASK1^{-/-}$ mice compared with $GLAST^{+/-}$ and $GLAST^{-/-}$ mice, respectively. Values are given in nV per square degree (nV/deg^2). $^{**}P<0.01$, $^{*}P<0.05$

GLAST$^{+/-}$:ASK1$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice and determined the effect of ASK1 deficiency on the NTG-like phenotype.

Results

ASK1 deficiency protects visual function in GLAST-deficient mice. To determine whether ASK1 deficiency is capable of preventing the NTG-like phenotype in GLAST-deficient mice,[13] GLAST$^{+/-}$:ASK1$^{+/-}$ mice were interbred and genotyped at weaning. GLAST$^{+/-}$:ASK1$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice were born in accordance with Mendelian inheritance ratios, survived into adulthood and were fertile. We first examined the visual function of these mice at 3 months of age (3 M) using multifocal electroretinograms (mfERGs), an established noninvasive method.[13] Figure 1a and b show the averaged responses of the second-order kernel in each group. The visual function of WT and ASK1$^{-/-}$ mice was indistinguishable (Figure 1c). As we have previously reported, visual function in the GLAST$^{+/-}$ and GLAST$^{-/-}$ mice was impaired in all visual fields, but was clearly improved by ASK1 deficiency (Figure 1a–c). In particular, the amplitude of the secondary kernel in GLAST$^{+/-}$:ASK1$^{-/-}$ mice (4.7 ± 0.4 nV/deg^2; $n=8$) was not significantly different compared with WT mice (5.0 ± 0.3 nV/deg^2; $n=9$) ($P=0.67$, Figure 1c). These results suggest that ASK1 deficiency has no harmful effects during development and prevents visual disturbances in GLAST-deficient mice.

ASK1 deficiency protects retinal neurons in GLAST-deficient mice. We next analyzed the histopathology of the retina. Consistent with the results of the mfERGs, the retina of ASK1$^{-/-}$ mice showed normal organization at 3 weeks (3 W), 3 M and 6 M (Figure 2). Cell number in the ganglion cell layer (GCL) was significantly decreased after 3 M in GLAST$^{+/-}$ mice and after 3 W in GLAST$^{-/-}$ mice (Figures 2 and 3a). In addition, the thickness of the inner retinal layer (IRL) was decreased after 3 M in both strains (Figure 3b). In GLAST$^{+/-}$:ASK1$^{-/-}$ mice, however, GCL cell number was significantly increased at 3 M and 6 M compared with GLAST$^{+/-}$ mice (Figure 3a). IRL thickness was increased to a normal level ($105 \pm 11\%$ at 3 M and $95 \pm 11\%$ at 6 M; $n=6$) in GLAST$^{+/-}$:ASK1$^{-/-}$ mice (Figure 3b). In GLAST$^{-/-}$:ASK1$^{-/-}$ mice, IRL thickness was significantly increased at 3 M and 6 M compared with GLAST$^{-/-}$ mice (Figure 3b). In addition, GCL cell number was increased at 3 W and 3 M, but not at 6 M (Figure 3a). These results suggest that ASK1 deficiency prevents the loss of RGCs and secondary retinal degeneration in GLAST-deficient mice.

ASK1 deficiency prevents optic nerve degeneration in GLAST-deficient mice. As nearly half of the cells in the rodent GCL are displaced amacrine cells, we needed to distinguish RGCs from displaced amacrine cells by retrograde labeling.[18] As ASK1 deficiency was most effective in 3 M GLAST$^{+/-}$ mice, we examined RGC number in WT, ASK1$^{-/-}$, GLAST$^{+/-}$ and GLAST$^{+/-}$:ASK1$^{-/-}$ mice at 3 M (Figure 4a–h). RGC number per square millimeter in ASK1$^{-/-}$ mice (4200 ± 238; $n=3$) was normal compared with WT mice (4050 ± 170; $n=3$) ($P=0.64$, Figure 4m). In GLAST$^{+/-}$ mice, RGC number (3358 ± 180; $n=3$) was significantly reduced compared with WT mice ($P<0.05$). However, RGC number in GLAST$^{+/-}$:ASK1$^{-/-}$ mice (4067 ± 121; $n=3$) was clearly increased compared with

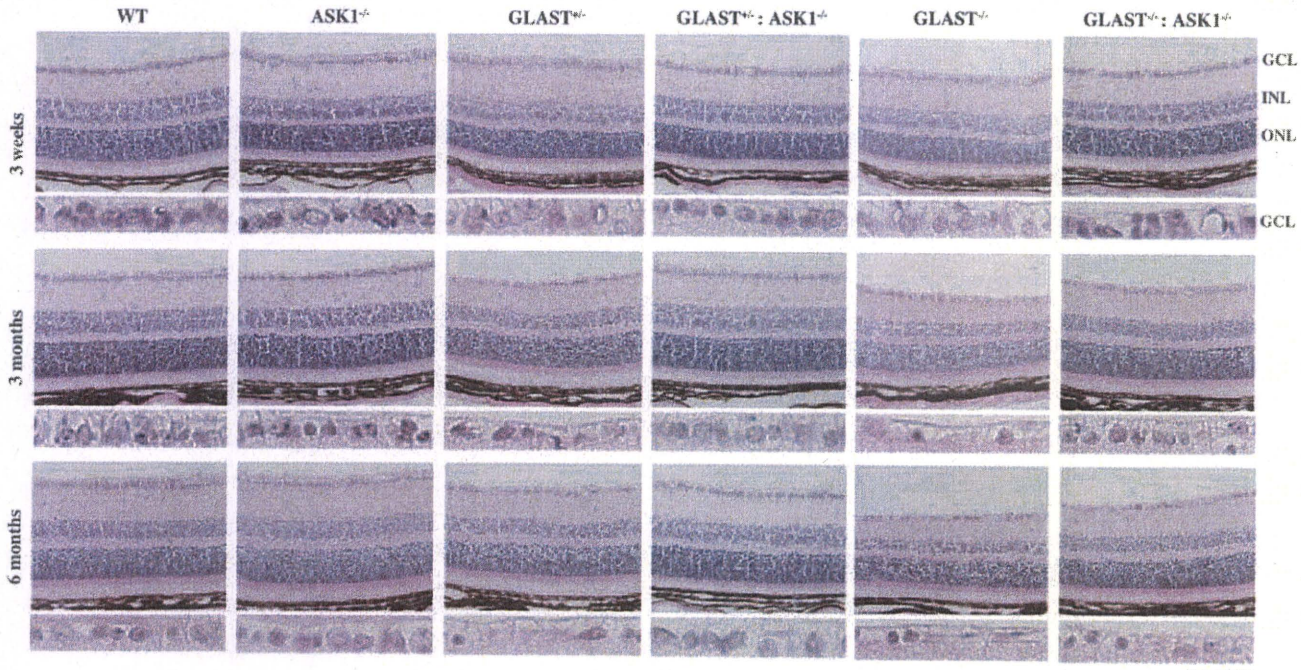

Figure 2 Effect of ASK1 on the progress of glaucoma. H&E staining of retinal sections at 3 weeks, 3 months and 8 months. WT, GLAST$^{+/-}$ and GLAST$^{-/-}$ mice were littermates. GLAST$^{+/-}$:ASK1$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice were littermates. Scale bar: 100 and 400 μm in the upper and lower rows, respectively. GCL, ganglion cell layer; INL, inner nuclear layer; ONL, outer nuclear layer

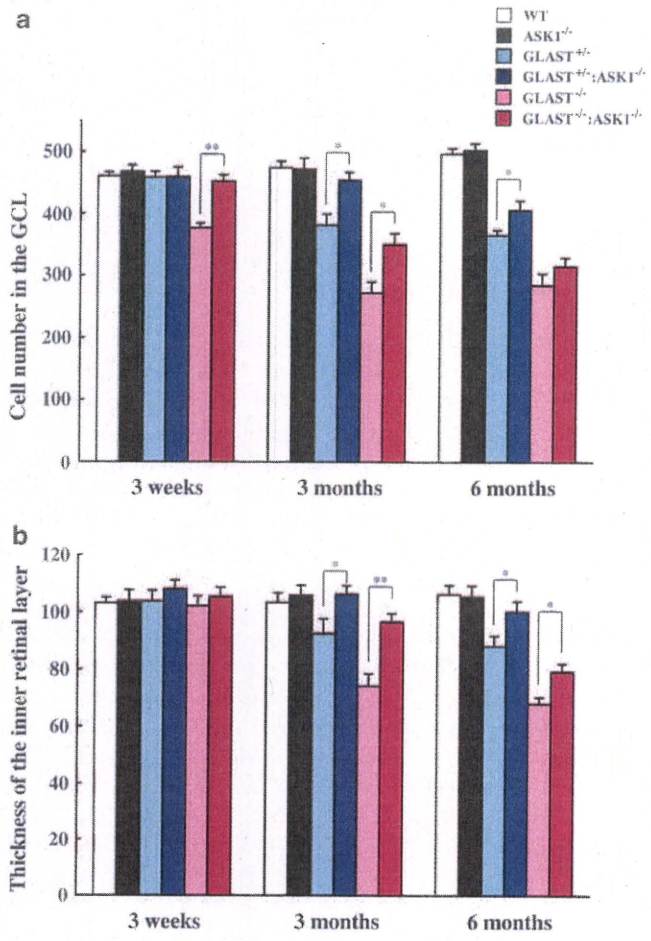

Figure 3 Quantification of the severity of glaucoma. (**a**) RGC number in WT, ASK1$^{-/-}$, GLAST$^{+/-}$, GLAST$^{+/-}$:ASK1$^{-/-}$, GLAST$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice. The number of neurons in the GCL was counted in retinal sections from one ora serrata through the optic nerve to the other ora serrata. (**b**) Thickness of the inner retinal layer in WT, ASK1$^{-/-}$, GLAST$^{+/-}$, GLAST$^{+/-}$:ASK1$^{-/-}$, GLAST$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice. $^{**}P<0.01$, $^{*}P<0.05$

GLAST$^{+/-}$ mice ($P<0.05$) and in normal range compared with WT mice ($P=0.94$, Figure 4m). Similarly, RGC number in GLAST$^{-/-}$:ASK1$^{-/-}$ mice (3392 ± 102; $n=3$) was increased compared with GLAST$^{-/-}$ mice (2592 ± 269; $n=3$) ($P<0.05$,Figure 4m).

Degeneration of the optic nerve is one of the hallmarks of glaucoma. To analyze morphological changes in the optic nerve, semi-thin transverse sections were cut and stained with toluidine blue (Figure 4i–l). Consistent with severe RGC loss, the degenerating axons in 3 M GLAST$^{+/-}$ mice had abnormally dark axonal profiles (arrowheads in Figure 4k). Such degenerating axons, however, were almost absent in GLAST$^{+/-}$:ASK1$^{-/-}$ mice (Figure 4l). Taken together, these results demonstrate that ASK1 deficiency protects against RGC loss and optic nerve degeneration in GLAST-deficient mice, which leads to improved visual function as detected by mfERG (Figure 1).

IOP measurement in GLAST/ASK1 double-deficient mice. We have previously reported that GLAST-deficient mice show normal IOP compared with WT mice.[13] To determine the effect of ASK1 on IOP, we examined the IOP of ASK1$^{-/-}$, GLAST$^{+/-}$:ASK1$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice. IOP measurements were carried out at around 2100 hours, when IOP is highest in mouse eyes.[19] The IOP values of ASK1$^{-/-}$, GLAST$^{+/-}$:ASK1$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice were not significantly decreased compared with WT and GLAST$^{-/-}$ mice (Figure 5). These results suggest that the recovery of NTG-like pathology in GLAST$^{+/-}$:ASK1$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice is IOP independent.

Role of oxidative stress and glutamate neurotoxicity in GLAST/ASK1 double-deficient mice. Oxidative stress has been proposed to contribute to RGC death in glaucoma, and a reduction in glutathione levels was reported in the plasma of human glaucoma patients.[15] Consistent with these findings, we have previously reported a decreased glutathione concentration in the retina of GLAST$^{-/-}$ mice.[13] To determine the effect of ASK1 on glutathione synthesis, we examined the glutathione concentration in the retina of 6 M ASK1$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice and found that it was not significantly increased compared with WT and GLAST$^{-/-}$ mice, respectively (Figure 6a). In addition, the malondialdehyde concentration in the retina of ASK1$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice was indistinguishable from that of WT and GLAST$^{-/-}$ mice, respectively (Figure 6b).

We have previously reported that intravitreal glutamate concentration is normal, but memantine, *N*-methyl-D-aspartate receptor antagonist, partially protected RGCs in GLAST$^{-/-}$ mice.[13] In addition, we showed that GLAST has a major role in glutamate uptake into Müller glial cells.[20] To explore the possibility that ASK1 is involved in glutamate transport, we examined glutamate uptake activity in Müller glial cells prepared from ASK1$^{-/-}$ and GLAST$^{+/-}$:ASK1$^{-/-}$ mice, and found that it was not significantly increased compared with WT and GLAST$^{+/-}$ mice, respectively (Figure 7). These findings suggest that ASK1 deficiency attenuates NTG-like degeneration without affecting the conditions of oxidative stress and glutamate neurotoxicity in GLAST-deficient mice.

Effect of ASK1-p38 mitogen-activated protein kinase (MAPK) signaling in Müller glial cells and RGCs. ASK1 is activated in response to cytotoxic stresses, including reactive oxygen species (ROS) and tumor necrosis factor (TNF), and relays these signals to p38 MAPK.[16,17] To determine whether this pathway is active in Müller glial cells, we first examined the effects of TNF on cultured Müller cells from WT and ASK1$^{-/-}$ mice. Western blot analysis demonstrated that stimulation of WT Müller cells with TNF leads to strong phosphorylation of p38 in a dose-dependent manner (Figure 8a). The activation of p38, however, was significantly suppressed in ASK1-deficient Müller cells (Figure 8a). Nitric oxide (NO) generated by inducible nitric oxide synthase (iNOS) is involved in retinal neuronal cell death,[21,22] and a previous study has reported that TNF-induced iNOS expression and NO release are suppressed by a specific inhibitor of p38 in mouse astrocytes.[23] These results suggest that the ASK1-p38 pathway regulates TNF-induced iNOS expression in Müller cells. To evaluate this possibility, we next examined iNOS

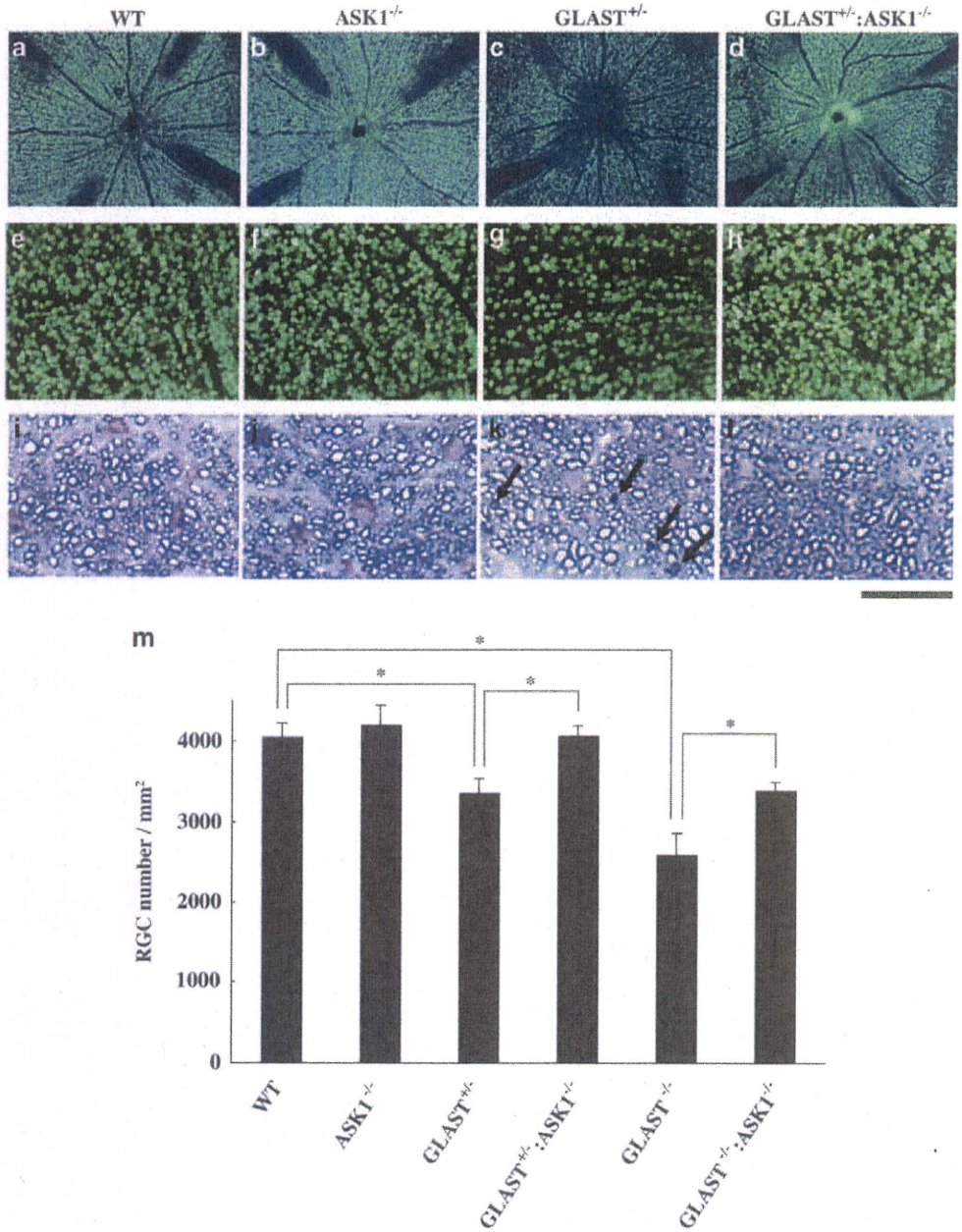

Figure 4 Effect of ASK1 on RGC and optic nerve degeneration. (**a–h**) Retrogradely labeled RGCs in WT, ASK1$^{-/-}$, GLAST$^{+/-}$ and GLAST$^{+/-}$:ASK1$^{-/-}$ mice. (**e–h**) Magnified images of (**a–d**), respectively. (**i–l**) Staining of semi-thin sections with toluidine blue revealed the presence of abnormally dark axonal profiles (arrowheads) and reduced axons in GLAST$^{+/-}$ mice (**k**), which was ameliorated in GLAST$^{+/-}$:ASK1$^{-/-}$ mice (**l**). (**m**) Quantification of labeled RGC number in WT, ASK1$^{-/-}$, GLAST$^{+/-}$, GLAST$^{+/-}$:ASK1$^{-/-}$, GLAST$^{-/-}$ and GLAST$^{-/-}$:ASK1$^{-/-}$ mice. Scale bar: 1 mm (**a–d**); 200 μm (**e–h**); 20 μm (**i–l**). *$P<0.05$

protein levels in cultured Müller cells. In untreated Müller cells, iNOS protein was almost absent, but TNF clearly increased iNOS expression levels (Figure 8b). Similar iNOS induction was detected in GLAST-deficient Müller cells (Figure 8b). However, TNF-induced iNOS expression was completely suppressed in ASK1-deficient Müller cells (Figure 8b). These results suggest that the ASK1-p38 pathway is required in Müller cells for the TNF-induced iNOS production, which may lead to the death of retinal neurons including RGCs. We further examined the direct effect of TNF on cultured RGCs.[18] TNF-induced cell death in cultured RGCs from ASK1-deficient mice was significantly decreased (41 ± 9%; $n=6$) compared with that from WT mice ($P<0.05$, Figure 8c). Taken together, loss of ASK1 prevents TNF-induced RGC death through both the direct pathway and the indirect pathway through Müller cells that is independent of GLAST.

Discussion

In this study, we show that ASK1 is associated with progressive RGC loss, glaucomatous optic nerve degeneration and visual disturbances in GLAST-deficient mice. We previously suggested the possibility that dysfunction of