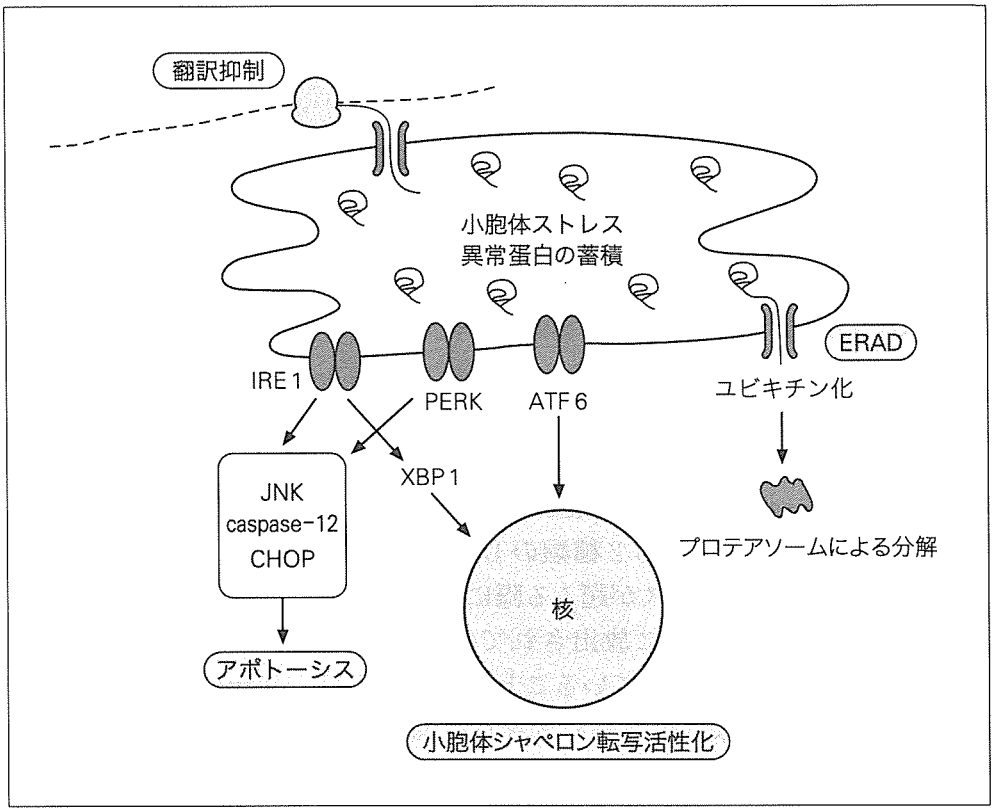

図2　小胞体ストレス応答とアポトーシスの経路

外因性経路に加えて内因性経路が利用されることになる．

アポトーシスのシグナル伝達経路(3)：小胞体経路(ER pathway)

小胞体は膜蛋白質や分泌性蛋白質の品質管理を行う細胞内小器官であり，新生した分泌系蛋白質が翻訳と共役して常に小胞体内腔に送り込まれ，小胞体シャペロンがそのような分子の折り畳み(folding)を助けている．小胞体内腔に折り畳みが完了していない(unfolded protein)または折り畳みに失敗した蛋白質(misfolded protein)が小胞体に過剰に蓄積した状態を小胞体ストレスと呼ぶ[5,6]．小胞体ストレスはストレス応答として，小胞体センサー分子IRE 1，ATF 6，PERKなどを介して，小胞体シャペロンの転写活性化，翻訳の全般的抑制などを引き起こし，ストレスを回避しようとするが，これらの手段で間に合わない場合にはアポトーシスを起こす(図2)[6]．

アポトーシスを起こす経路には諸説あるが，次の3種類の経路が有力といわれている．第1はセンサー分子IRE 1がアダプター分子TRAF 2と結合し，TRAF 2がASK 1を活性化して向細胞死的に働く蛋白質キナーゼJNKを活性化する経路である[7]．第2はATF 6経路とPERK経路の両方で転写が誘導される転写因子CHOPによる経路である[8]．CHOPの下流のシグナルはまだよくわかっていない．第3はカスパーゼ-12を介する経路である[9]．カスパーゼ-12は小胞体膜の細胞質側に存在し，小胞体ストレスの際は切断され，活性化されて小胞体から遊離し，カスパーゼ-9を活性化するらしい．カスパーゼ-12のノックアウト細胞では小胞体ストレスによる細胞死は抑制される．ヒトではカスパーゼ-12に相当するカスパーゼは配列上活性を失っているが，ヒトではカスパーゼ-4が小胞体ストレス誘起性細胞死の担い手とする報告がある[10]．

アポトーシス阻害因子とその制御

上記のようなアポトーシスはアポトーシス阻害因子(inhibitor of apoptosis proteins：IAP)と呼ばれる内因性のカスパーゼ阻害因子によって制御されることが知られている[11]．IAPはBIR(Baculovirus IAP repeat)ドメインと呼ばれる金属結

合モチーフを持つことが構造上の特徴で，8種類知られるヒトのIAPのうち，最も細胞死抑制効果が強いとされるXIAPはカスパーゼ-3,-7,-9を阻害する．ショウジョウバエのIAPは発生過程の細胞死を制御するうえで中心的な役割を果たしているが，マウスのIAPはノックアウトしても著しい表現型はなく，これが生理的役割が大きくないことを示しているのか，類似分子により補償されているためなのか，明らかではない．ただ，ヒトのミトコンドリア膜間スペースにはsecond mitochondria-derived activator of caspase(Smac)とhigh-temperature-requirement potein A2(HtrA 2)/Omiと呼ばれる2種類のIAP阻害因子が存在し，アポトーシスが起こる際にはともにMOMPによって細胞質に放出されてIAPの機能を抑制することが知られていることから，哺乳類においてもIAPはアポトーシスを常に抑制する構成的因子として一定の役割を果たしているものと考えるのが妥当である[12,13]．

カスパーゼ非依存性細胞死

アポトーシスは以上に述べてきたようにカスパーゼ依存的な細胞死を起こすが，細胞はカスパーゼの活性を抑制した状態でアポトーシス刺激を受けても死んでしまうことがある．これをカスパーゼ非依存性細胞死(caspase-independent cell death：CICD)と呼んでいる[3]．線虫のプログラム細胞死はカスパーゼに依存的であり，CICDが存在するという積極的な証拠はない．しかし脊椎動物ではカスパーゼ阻害剤の存在下やApaf-1，カスパーゼ-9，カスパーゼ-3などのミトコンドリアの下流のアポトーシスシグナル分子を欠損した細胞で，ミトコンドリアクリステの膨化や細胞質の空胞形成などを特徴とする，形態的にアポトーシスとは大きく異なる細胞死が観察される．外因性経路においても，FADDやreceptor-interacting protein(RIP)依存性にカスパーゼ活性を阻害した条件下でCICDが観察される．このシグナル経路の詳細は不明であるが，アポトーシス時にミトコンドリアから細胞質に放出されるapotposis-inducing factor(AIF)，endonuclease G，HtrA 2/Omiが関与しているとの考えもある．また，本来は生存維持の方向に働くとされるautophagyが細胞死を誘導する可能性も提示され，注目されている[14]．

呼吸器疾患とアポトーシス

呼吸器科疾患では，最近，acute respiratory distress syndrome(ARDS)，慢性閉塞性肺疾患(COPD)に伴う肺気腫，喘息，肺線維症におけるアポトーシスの関与が注目されている[15]．これらのトピックスに関しては本特集号でそれぞれの領域の専門家が論文を寄せられているので，以下にごく簡単に紹介する．ARDSでは多核白血球のアポトーシスの遅延と内皮・上皮細胞のアポトーシス増加が病因に関与していることが示唆されている[16,17]．前者には生存促進作用を持つgranulocyte colony-stimulating factor(GCSF)やgranulocye/macrophage colony-stimulating factor(GMCSF)の関与が，後者には外因性経路(Fas/Fas ligand system)，内因性経路(ストレス)，nitric oxideなどの関与が疑われている．COPDに伴う肺気腫に関しては，プロテアーゼおよびその阻害因子の不均衡，酸化的ストレス，喫煙，マクロファージ，白血球，CD8陽性T細胞による慢性炎症などが病因として挙げられている一方，肺気腫における肺胞壁の破壊には肺上皮および内皮細胞のアポトーシスが伴うことが組織学的に示されている[17]．COPDにおけるアポトーシスの誘因として直接的，間接的に喫煙と酸化的ストレスが働いているものと思われる．喘息の原因は不明だが，気道のリモデリングと気道・肺の好酸球，CD4陽性T細胞，マスト細胞の増加を伴う慢性炎症が原因に関わっていると思われる[18]．*ex vivo*の研究では，喘息患者における末梢のCD4陽性T細胞や好酸球のアポトーシスの減少が観察され，炎症を惹起しているのかもしれない．副腎皮質ステロイドの効果は，一部は炎症に関与する細胞のアポトーシスを誘導するところにあるものと思われるが，*in vivo*の研究では，この効果はβ2アドレナリン受容体アゴニストによって拮抗されるとの注目すべき報告がなされている[19]．肺線維症における線維化は，肺胞上皮細胞のアポトーシスに二次的に生じる可能性が指摘

されている[20]．肺胞上皮細胞のアポトーシスの増加は，患者でも，げっ歯類におけるbleomycin誘発性肺線維症でもみられ，後者に関してはアポトーシスも線維化もカスパーゼ阻害剤によって抑制される．肺胞上皮細胞のアポトーシスにはFasによる外因性経路，アンジオテンシン，活性化T細胞が産生するperforin，interleukin-13刺激，transforming growth factor β1の活性化などの関与が示唆されている[21]．

おわりに

アポトーシスと関連する細胞死の基本的なシグナル経路が解明され，様々な呼吸器疾患との関わりも最近明らかになってきた．一般的に言って，肺構造の破壊は肺上皮および内皮細胞の細胞死増加と炎症細胞のアポトーシス阻害によって引き起こされるようである．したがって，アポトーシスの阻害は強力な治療法になりうるが，タイミングよく，細胞特異的に効果が得られるように行わないと重大な副作用を来す恐れがある．今後は疾患におけるアポトーシスの役割の詳細をさらに明らかにし，分子メカニズムの正確な理解に基づいた治療法を開発する必要がある．

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グルタミン酸受容体と神経疾患

AMPA受容体と変異SOD1タンパク質異常

舘野 美成子　高橋 良輔

はじめに

筋萎縮性側索硬化症 amyotrophic lateral sclerosis (ALS)は，上位および下位運動ニューロンが選択的かつ系統的に障害される代表的な進行性神経変性疾患である[1]．有病率は人口10万人当たり2〜7人で，患者の多くは孤発性だが約10%は家族性を示す．1993年に家族性ALSの最初の原因遺伝子としてスーパーオキシドジスムターゼ1 superoxide dismutase 1(SOD1)が同定されて以来，変異SOD1はALS発症分子機構を解く鍵として精力的に研究されてきた[1,2]．本稿では，家族性ALSのモデルマウスである変異SOD1トランスジェニックマウスにおいて，下位運動ニューロンで特異的に発現しているカルシウム透過型AMPA受容体が，変異SOD1タンパクの構造変換を促進しALS発症を促す因子であることを紹介する．

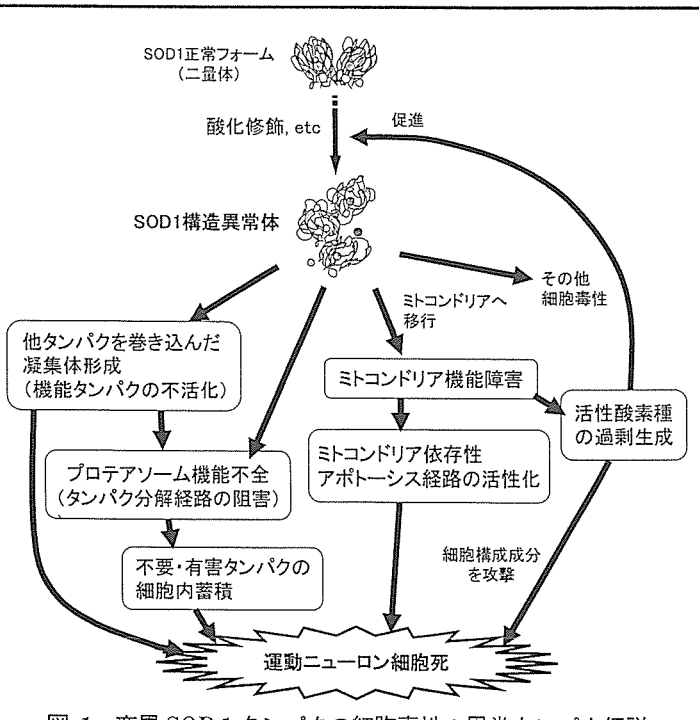

図1　変異SOD1タンパクの細胞毒性：異常タンパク仮説
変異SOD1タンパクは立体構造が不安定で，酸化修飾を受けると凝集化が促進される[3,4]．このような構造異常体は，主にミトコンドリアやプロテアソームの機能障害を引き起こすことで運動ニューロン変性を導くと考えられている[2]．

変異SOD1タンパクの細胞毒性：異常タンパク仮説

SOD1は真核細胞の細胞質で主力となる活性酸素除去酵素で，酸素呼吸の副産物として生成されるスーパーオキシド($\cdot O_2^-$)の過酸化水素への変換を触媒する[1]．このSOD1遺伝子の変異が家族性ALSの原因として発表された当時は，誰もがSOD1活性の低下がALSの発症要因と考えたことであろう．しかしその予想は見事に打ち砕かれた．これまでの研究より，ALSを引き起こすSOD1変異は機能獲得型(gain-of-function)であること，即ち，変異によってSOD1タンパクが新たに獲得した(未知の)毒性によることが示されている[1,2]．

図1に変異SOD1タンパクの毒性として最も支持されている仮説：異常タンパク仮説を紹介する[2,3]．これは変異SOD1タンパクの立体構造が変化して凝集化したもの，または凝集途上の中間体(構造異常体)が細胞毒性を有し，運動ニューロン変性が引き起こされる，という考え方である．SOD1はわずか153個のアミノ酸からなる小さなタンパクだが，ALS患者から同定された変異は100種類以上に及び，変異箇所はタンパクのほぼ全域に散在している．変異SOD1タンパクの多くは立体構造が不安定化で凝集しやすく，酸化修飾を受けると巨大な凝集塊を形成しうることが示されている[3,4]．SOD1構造異常体が有する細胞毒性の実態はまだ明らかにされていないが，SOD1変異をもつ家族性ALS患者やトランスジェニックマウスの残存脊髄運動ニューロン内には抗SOD1抗体陽性の凝集体が観察されており[5,6]，このことからも異常タンパク仮説が強く支持

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カルシウム透過型 AMPA 受容体の減少による ALS 発症遅延と延命効果

chat-GluR2-Tg ライン	Transgene コピー数	GluR 2 発現量[a]		*chat-GluR2*-Tg x *hSOD1*^{G93A}-Tg で得られた littermates 間での発症時期[b]・生存期間の比較(日齢, Mean±SEM)			
				hSOD1^{G93A}/+	*chat-GluR2*/+ ; *hSOD1*^{G93A}/+	GluR 2 増加による遅延効果	*p* value[c]
Tg 7	10	0.96	発症時期	219.7±3.0	262.2±2.9	+42.5(=19.3%)	0.0001
			生存期間	262.5±4.5	300.1±3.9	+37.6(=14.3%)	0.0001
Tg 10	16	4.78	発症時期	219.8±2.6	238.5±2.7	+18.7(=8.6%)	0.0001
			生存期間	264.5±2.2	279.7±3.1	+15.2(=5.8%)	0.0005
Tg 3	2	1.58	発症時期	225.6±1.0	230.4±1.0	not significant	>0.05
			生存期間	267.1±5.1	273.3±5.2		

[a] 脊髄運動ニューロンにおける *GluR2* mRNA 総量を定量化し, non-transgenic littermates における発現量(=1.0)に対する相対値として表記.

[b] Rotarod test におけるスコア(運動能力)が急低下する時期として判定.

[c] 各遺伝子型 littermates 10〜15 匹における値を ANOVA+*post hoc* Fisher's PLSD 法で検定.

脊髄においてほぼ運動ニューロン特異的に GluR 2 遺伝子を過剰発現するトランスジェニックマウス;*chat-GluR2*-Tg と家族性 ALS モデルマウス;*hSOD1*^{G93A}-Tg とのダブルトランスジェニックマウスでは, GluR 2 発現量に応じて発症時期と生存期間の延長が認められた[9].

されている.

カルシウム透過型 AMPA 受容体による ALS 発症促進効果

しかし, なぜ病変部位でのみ変異 SOD 1 タンパクの凝集化が認められるのだろうか. SOD 1 変異をもつ ALS 患者やトランスジェニックマウスでは, 変異タンパクはもちろん全身の細胞で発現している. にも関わらず, 変異 SOD 1 が病変部位特異的に構造変換・蓄積するならば, 細胞種特異的な促進要因があるはずである. この問題に対して我々は, 孤発性 ALS に対して以前から提唱されていたグルタミン酸仮説に注目した[7,8]. シナプス間隙に放出されたグルタミン酸はシナプス後細胞膜上のグルタミン酸受容体に結合・活性化することで興奮性刺激を伝えるが, グルタミン酸受容体の過度の刺激は神経細胞死を引き起こす(神経興奮毒性). グルタミン酸に対する脆弱性は神経細胞の種類によって異なるが, ALS で障害される脊髄運動ニューロンはグルタミン酸に対して極めて脆弱であること, 孤発性 ALS 患者の脳脊髄液中ではグルタミン酸濃度が上昇していることなどから, ALS における運動ニューロン変性にグルタミン酸毒性が関与している可能性が指摘されていた. さらに薬理学的解析から, 脊髄運動ニューロンの高いグルタミン酸脆弱性はカルシウム透過型の AMPA 受容体を介していることが示唆された. AMPA 受容体は通常カルシウム非透過型だが, 脊髄運動ニューロンを含む, ごく限られたニューロンではカルシウム透過型も発現しているのである.

そこで我々は, このカルシウム透過型 AMPA 受容体と ALS における運動ニューロン変性との関係を追及することにした. AMPA 受容体は 4 種類のサブユニット GluR 1-4 がランダムに会合した 4 量体であり, カルシウム透過性は通常 GluR 2 サブユニットの有無で決定される. 即ち GluR 2 を含む受容体は非透過型, 含まずに会合すると透過型となる. 我々は脊髄運動ニューロンの GluR 2 発現量を特異的に上げることにより AMPA 受容体のカルシウム透過性が低下したトランスジェニックマウス(*chat-GluR2*-Tg)を作成した[9]. そして代表的な ALS モデルマウスである *hSOD1*^{G93A}-Tg(93 番目のグリシンをアラニンに置換したヒト変異 SOD 1 遺伝子を導入)と交配し, ALS 発症時期・生存期間について littermates 間で比較した(表). GluR 2 発現量が最も増大したライン(Tg 7:野生型マウスの約 5 倍)では, 脊髄運動ニューロンにおける AMPA 受容体の大半がカルシウム非透過型を示し, 発症時期が 42.5 日(=19.3%), 生存期間が 37.8 日(=14.3%)も遅延することがわかった. GluR 2 発現量の低いラインでは発症遅延・延命効果も低く, AMPA 受容体のカルシウム透過性と発症までの期間には相関関係が認められた. 一方, AMPA 受容体をカルシウム透過性にする変異(RNA 編集部位のアミノ酸をアスパラギンに置換)をもつ GluR 2 遺伝子を導入したトランスジェニックマウスでは, 中年以降に ALS 様の運動能力低下を示すこと, さらにこの Tg マウスや GluR 2 ノックアウトマウスを *hSOD1*^{G93A}-Tg と交配すると発症が早まり, 生存期間が短縮されることが報告された[10,11]. これらの結果より, カルシウム透過型 AMPA 受容

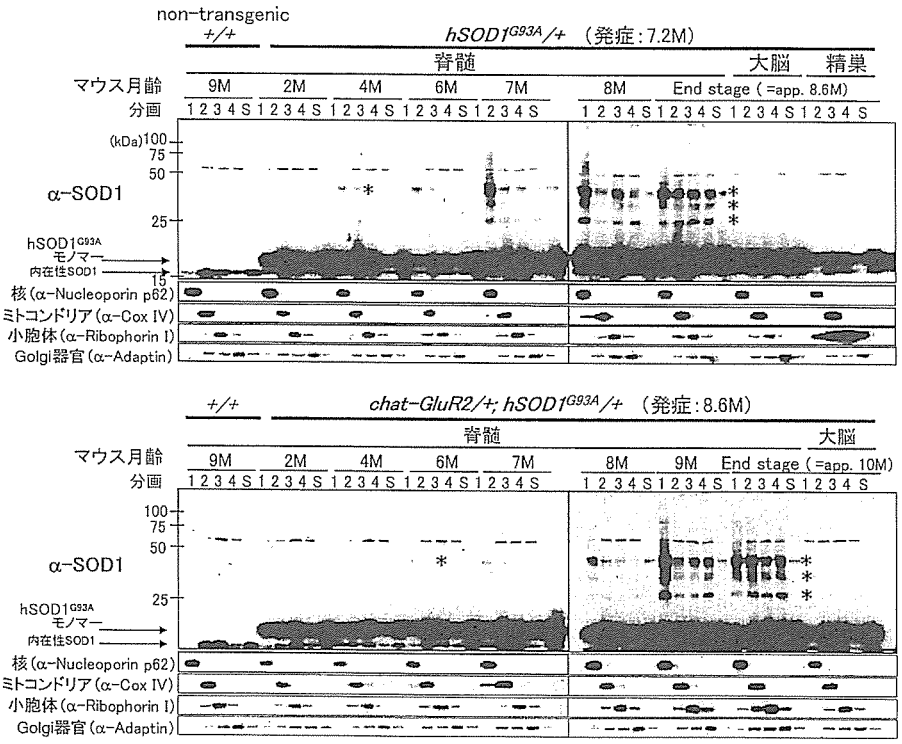

図 2　カルシウム透過型 AMPA 受容体の減少による SOD 1 構造異常体形成遅延

マウス脊髄を等張液中で破砕後遠心分離し，1：核，2：ミトコンドリア，3：ミクロソーム(小胞体膜断片等)，4：Golgi 器官，S：細胞質可溶性タンパク，を主に含む 5 つの分画に分け，ウェスタンブロット法により各分画における SOD 1 タンパクのサイズ変化を調べた．*hSOD1*^{G93A}-Tg では発症に大幅に先行して 4ヵ月齢から分画 1・2 に SOD 1 構造異常体(＊)の蓄積が認められるのに対し，*chat-GluR2*-Tg 7 とのダブルトランスジェニックマウスでは，構造異常体形成が 2ヵ月近く遅れているのがわかる[9]．

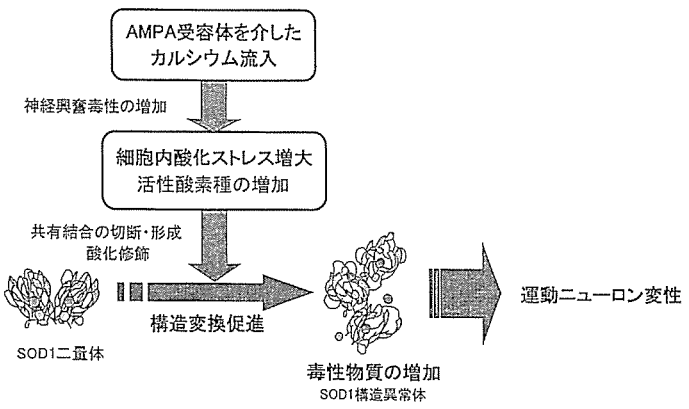

図 3　カルシウム透過型 AMPA 受容体を介した運動ニューロン変性(仮説)

脊髄運動ニューロンではグルタミン酸刺激によりカルシウム透過型 AMPA 受容体が活性化され，ミトコンドリアにおける活性酸素種の生成が促進される[12]．活性酸素種の増加により変異型 SOD 1 タンパクの構造変換が促進され，構造異常体の細胞毒性により運動ニューロン変性が引き起こされると考えられる．

体は運動ニューロン変性の促進因子であり，変異 SOD 1 の存在下では変異 SOD 1 タンパクの毒性を促進して ALS 発症を早める危険因子であることが示された．

カルシウム透過型 AMPA 受容体による SOD 1 構造異常体形成促進

次に我々は，カルシウム透過型 AMPA 受容体が変異 SOD 1 毒性を促進するメカニズムについて解析した．異常タンパク仮説を踏まえ，マウス脊髄の細胞分画を行い，毒性を有するとされる構造異常体の細胞内分布・出現時期に及ぼす影響を調べた(図 2)[9]．*hSOD1*^{G93A}-Tg では発症の 3ヵ月前(4ヵ月齢)には核およびミトコンドリア分画に SOD 1 構造異常体が検出され，発症期には他分画にも広がり，病気の進行とともに飛躍的に蓄積していく．これに対し *chat-GluR2*-Tg 7 とのダブルトランスジェニックマウスでは，構造異常体の出現パターンは変わらないが出現時期が 2ヵ月近く遅れていた．この結果は AMPA 受容体のカルシウム透過性が下がると SOD 1 構造異常体の形成が遅れることを示している．変異 SOD 1 タンパクの構造変換は酸化修飾によって著しく促進されるので[3,4]，脊髄抽出液中のタンパクの酸化修飾レベルを代表的な酸化修飾であるカルボニル化を指標に定量化した[9]．*hSOD1*^{G93A}-Tg では

構造異常体の出現と同調してカルボニル化されたタンパク量が上昇するが，Tg 7とのダブルトランスジェニックマウスでは構造異常体の遅延と同様に約2ヵ月遅れていた．よってカルシウム透過型AMPA受容体の発現は運動ニューロン内における酸化修飾反応を促進し，その結果，変異SOD 1タンパクの構造変換が促進されている可能性が強く示唆された．培養下の脊髄運動ニューロンでは，グルタミン酸刺激後，AMPA受容体を介して流入したカルシウムが一過的にミトコンドリアに移行して，ミトコンドリアにおける活性酸素種の生成増大をもたらすことが報告されている[12]．これらの結果を総合すると，脊髄運動ニューロンではグルタミン酸に曝されるたびにカルシウム透過型AMPA受容体を介して細胞内酸化ストレスが上昇し，過剰に生成された活性酸素種が変異SOD 1タンパクの構造変換を促進する，という反応が繰り返されていることが予想される(図3)[13]．

むすび

本稿では家族性ALSモデルマウスでの知見をもとに，脊髄運動ニューロンが特異的に発現しているAMPA受容体のサブタイプ(カルシウム透過型)が，細胞内酸化ストレスの上昇を介して原因遺伝子産物の毒性型への変換を促進している可能性を紹介した．紙面の都合上割愛したが，孤発性ALSにおいてもカルシウム透過型AMPA受容体の増加と発症との関係が注目されている[14]．GluR 2サブユニットはRNA編集による厳密な制御を受けており，この編集機構が破綻した場合にも受容体はカルシウム透過性となる．孤発性ALS患者の脊髄運動ニューロンではGluR 2の編集効率が有意に低下しており，よって家族性ALSにおける変異SOD 1タンパクと同様に，ある種のタンパクの酸化修飾・構造変換が亢進されて運動ニューロン変性が引き起こされている可能性が考えられる．したがって，この受容体サブタイプを特異的に阻害または減少させる手段が開発されれば，現在有効な治療法のないALS患者一般を救うことができるかもしれない．

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Research Report

Tonic–clonic seizures induce division of neuronal progenitor cells with concomitant changes in expression of neurotrophic factors in the brain of pilocarpine–treated mice

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Abstract

Epileptic seizures cause severe and long-lasting events on the architecture of the brain, including neuronal cell death, accompanied neurogenesis, reactive gliosis, and mossy fiber sprouting. However, it remains uncertain whether these functional and anatomical alterations are associated with the development of hyperexcitability, or as inhibitory processes. Neurotrophic factors are probable mediators of these pathophysiological events. The present study was designed to clarify the role of various neurotrophic factors on the pilocarpine model of seizures. At 4 h following pilocarpine-induced seizures, expression of NGF, BDNF, HB-EGF, and FGF-2 increased only in the mice manifesting tonic–clonic convulsions and not in mice without seizures. NT-3 expression decreased in pilocarpine-treated mice experiencing seizures, tonic–clonic or not, compared to mice with no seizures. Neuronal cell damage, which was evident by Fluoro-Jade B staining, was observed within 24 h in the mice exhibiting tonic–clonic seizures, followed by an increase in the number of BrdU-positive cells and glial cells, which were evident after 2 days. None of these pathophysiological changes occurred in the mice which showed no seizures, although they were injected with pilocarpine, nor in the activated epilepsy-prone EL mice, which experienced repeated severe seizures. Together, these results suggest that neuronal damage occurring in the brain of the mice manifesting tonic–clonic seizures is accompanied by neurogenesis. This sequence of events may be regulated through changes in expression of neurotrophic factors such as NGF, BDNF, HB-FGF, and NT-3.

Theme: Disorders of the nervous system
Topic: Epilepsy, basic mechanisms

Keywords: Epilepsy; EL mouse; NGF; BDNF; NT-3; HB-EGF; FGF-2 neuronal progenitor cell; Seizure

1. Introduction

Severe or repeated seizures cause various pathophysiological changes, including neuronal cell death, accompanied neurogenesis, reactive gliosis, and mossy fiber sprouting. Enhanced neurogenesis was demonstrated in various models of epilepsy, including chemical [2,15,18] and electrical kindling [15,19,24]. Hippocampal sclerosis, including activation of astrocytes and microglia [28,30], is often observed in temporal lobe epilepsy with a concomitant marked loss of hippocampal neurons [5,15]. Mossy fiber synaptic reorganization is the commonly encountered change in human epileptic hippocampus [8], and in an animal model of epilepsy [27]. However, the relationship of these events remains unclear. Furthermore, it is still controversial whether these functional and anatomical alterations may be associated

Abbreviations: NGF, nerve growth factor; BDNF, brain-derived neurotrophic factor; EGF, epidermal growth factor; HB-EGF, heparin-binding epidermal growth factor-like growth factor; NT-3, neurotrophin 3

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with causal mechanisms underlying the development of hyperexcitability, or as inhibitory processes.

Neurotrophic factors appear to play a key role in these changes, since their expression changes during the pathophysiology of the seizures [9]. However, it remains uncertain whether they act as promoting factors of epileptogenesis or act as endogenous anti-epileptogenic substances. The expression of nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) is rapidly up-regulated following seizures [9]. In addition, their expression is enhanced in the hippocampus of temporal lobe epilepsy patients [14], suggesting that these neurotrophic factors play pivotal roles in the epileptogenesis or anti-epileptogenesis [9]. Indeed, several studies indicate that NGF promotes kindling epileptogenesis [1,29]. In addition, antibodies to NGF attenuate cholinergic sprouting and increase the size of basal forebrain cholinergic neurons that are associated with seizure-induced injury caused by pilocarpine [7]. In contrast, the role of BDNF remains controversial. A chronic infusion of BDNF in the hippocampus delays [20] and accelerates [21] epileptogenesis. Decreased BDNF signaling in transgenic mice reduces epileptogenesis [10], and expression of BDNF increased in pilocarpine seizures [22]. In contrast to these neurotrophic factors, NT-3 mRNA undergoes a delayed down-regulation, suggesting that the neurotrophic factor has a different function in epileptogenesis. NT-3 inhibits seizure development and related synaptic reorganization [31]. Deletion of the NT-3 gene retards the development of kindling epileptogenesis [3]. Up-regulation of basic fibroblast growth factor (b-FGF, FGF-2) follows seizure induced by chemical [6,9] and electrical kindling [9,25]. Long-term, low-dose infusion of bFGF prevents kainate-induced hippocampal cell loss, although it has no effect on seizure latency or duration [12]. Thus, chronic elevation of bFGF levels after seizures may prevent hippocampal cell damage. There is a significant elevation of heparin-binding epidermal growth factor-like growth factor (HB-EGF) mRNA and its protein [17] after kainate injection. The present study was designed to examine the role of these neurotrophic factors in the cellular changes that occur following the seizure-induced damage. The roles of these neurotrophic factors were evaluated by estimating the changes in their expression in a pilocarpine model of seizures. For comparison, the effects of repeated seizures were studied on the pathophysiological changes which occurred in the epilepsy-prone EL mice [16] after repeated vestibular stimulation. The results obtained suggest that NGF, BDNF, NT-3, and HB-EGF are involved in the cellular changes that occur following seizure-induced damage.

2. Materials and methods

2.1. Animals

The epilepsy-prone EL mice were propagated in our laboratory. The C57BL/6 (B6) mice and ddY mice, parent strain of EL mice, were purchased from Shizuoka Experimental Animals Co., Hamamatsu, Japan. All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee at Nagoya University. The mice were housed in a polystyrene cage in a temperature-regulated (22 ± 2 °C), light-controlled (lighted from 0700 to 1900 h) room and were fed ad libitum with a commercial stock diet (CE-2, Japan CLEA Co., Ltd., Urawa). Both sexes of the mice were used when they are 5 weeks old.

2.2. Vestibular stimulation of EL mice

Some EL mice were subjected to 30 'tosses', 15 cm high, once a week, starting at 5 weeks old, for 3, 7, and 15 weeks, respectively. The mice were killed 4 h after the final vestibular stimulation.

2.3. Chemical convulsant treatment

Pilocarpine hydrochloride (Sigma-Aldrich, P6503) was dissolved in 0.85% NaCl (saline, 15 mg/ml) and filtered through a 0.45-μm membrane filter (Advantec Co., Ltd.) and injected i.p. to some groups of B6 mice at a dose of 300 mg/kg body weight. Fifteen minutes before the injection of pilocarpine, the mice were injected i.p. with atropine methyl bromide dissolved in saline (0.25 mg/ml) at a dose of 5 mg/kg. Two hours thereafter, the mice were injected i.p. with diazepam (Cercine, Takeda Pharmaceutical Co., Ltd., 0.5 mg/ml) at a dose of 10 mg/kg. The control mice were injected with saline instead of pilocarpine. For assay of neurotrophic factor expression in the brain, the mice were killed 4 h after the pilocarpine injection. Four hours and 1, 2, 28, and 90 days after the pilocarpine injection, the mice were anesthetized and underwent intracardiac infusion of phosphate-buffered saline (PBS) followed by 4% paraformaldehyde in PBS for histochemical analysis. None of the pilocarpine-treated EL mice were subjected to vestibular stimulations.

2.4. BrdU-labeling

Division of hippocampal cells was evaluated using the bromodeoxyuridine (BrdU, Sigma, B5002) labeling method. They were killed 4 h and 1 and 2 days after pilocarpine treatment and received i.p. injection of 50 mg/kg of BrdU 2 h before sacrifice. Some other B6 mice were injected with BrdU twice daily on the 2nd and 3rd day to examine the fate of BrdU-labeled cells and killed 28 and 90 days after the pilocarpine treatment.

2.5. Northern blot analysis

For assays of the expression of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), basic fibroblast growth factor (bFGF, FGF-2), epidermal growth

factor (EGF), heparin-binding epidermal growth factor-like growth factor (HB-EGF), and neurotrophin 3 (NT-3), the mice were killed 4 h after the vestibular stimulation and pilocarpine injection, and the brains were quickly removed, placed in 1 ml of ISOGENE (Nippon Gene Co., Ltd., Tokyo) and homogenized (HG30, Hitachi, Tokyo). The total RNA was extracted and Northern blot analyses were performed as previously described [16] using the following radiolabeled cDNA fragments as probes, which were prepared by RT-PCR screening of the mouse library using the following oligonucleotides as primers: [NGF] forward: 5′-TCCTAGTGAAGATGCTGTGC-3′, reverse: 5′-ACTCTCAACAGGATTGGAGG-3′; [HB-EGF] forward: 5′-ATGCTGAAGCTCTTTCTGGC-3′, reverse: 5′-ACGCCCAACTTCACTTTCTC-3′; [FGF-2] forward: 5′-AAGCGGCTCTACTGCAAGAA-3′, reverse: 5′-AACAGTATGGCCTTCTGTCC-3′; [NT-3] forward: 5′-ATCAAGCTGATCCAGGCGGA-3′, reverse: 5′-GTCAGTGCTCGGACATAGGT-3′. 1.1 kb *Eco*RI and *Bam*HI fragment of BDNF cDNA and 1.2 kb *Pst*I fragment of β-actin cDNA were used, respectively, as probes for Northern blot analysis of these proteins. The expression of each mRNA was measured using a BAS 2000 bioimaging analyzer (Fuji Film, Tokyo) and normalized to the levels of β-actin mRNA.

2.6. Histochemistry

Mice were anesthetized before undergoing intracardiac perfusion of sodium sulfide followed by 4% paraformaldehyde in phosphate-buffered saline (PBS). The mice were killed 4 h and 1, 2, 3, 7, 28, and 90 days after the pilocarpine treatment. For BrdU analysis, the drug was injected i.p. at a dose of 50 mg/kg 2 h before intracardiac perfusion of sodium sulfoxide and 4% paraformaldehyde. The brains were isolated and immersed overnight in 4% paraformaldehyde at 4 °C and then dehydrated by treating with 20% sucrose. The tissues were embedded in O.C.T. compound (Tissue-Tek; Mikes, Elkhart, IN) and frozen. Frozen sections (10 μm) were cut using a cryostat microtome (Leica), then transferred to MAS-coated slides (MATSUNAMI, S-9441) and air-dried. Coronal cryosections of 10 μm thickness, including hippocampus, were processed for histological staining.

2.7. BrdU analysis

Counting of BrdU-labeled cells was performed on 3–6 sections per animal. The sections were sampled at an interval of 100 μm beginning at a random point close to the front of the hippocampus in order to avoid counting the same cell in 2 sections. BrdU- and NeuN-double positive cells were confirmed with Apo Tome microscope (Carl Zeiss).

2.8. Antibodies

To avoid nonspecific staining, M.O.M. (mouse on mouse) Immunodetection Kit (VECTOR, BMK-2202) was used throughout. The following antibodies were used: a monoclonal anti-BrdU [mouse immunoglobulin G (IgG); COSMOBIO, BU1/75] at a dilution of 1:100, a monoclonal anti-NeuN [mouse IgG; CHEMICON,] at a dilution of 1:400, a monoclonal anti-glial fibrillary acidic protein (GFAP, an astrocytric marker; rabbit IgG; IMMUNON) at a dilution of 1:100, and a monoclonal anti-CD11 (rat IgG, DSHB, M1/70) at a dilution of 1:1. To detect neuronal damage after seizures, Fluoro-Jade B (FJB, Histo-Chem., Inc.) staining method was used. FJB is an anionic fluorescein and has a specific affinity for degenerating neurons [23].

2.9. Statistical analysis

Welch's test or Student's *t* test after two-way ANOVA was used to determine the significance of differences of the data in each experiment [26].

3. Results

All EL mice, which were subjected to the vestibular stimulation once a week, manifested repeated severe seizures within 3 weeks. However, they did not exhibit status epilepticus throughout the whole experimental period of 20 weeks. Upon pilocarpine injection, some (23/54) B6 mice exhibited severe tonic–clonic convulsions within 30 min and were grouped as 'pilocarpine 2'. Some other mice (21/54), which did not manifest seizures were grouped as 'pilocarpine 1'. In contrast, none of the pilocarpine-treated EL mice exhibited tonic–clonic seizure although they were given a same dose of the drug. None of the EL mice exhibited seizures even when they were injected with pilocarpine at its dose which caused the response in some B6 mice nor by repeated vestibular stimulations.

3.1. Neurotrophin expression

Figs. 1A–F illustrate the expression of various neurotrophic factors in the brain of ddY, EL, and B6 mice, which were subjected to vestibular stimulation and treated with the chemical convulsant, pilocarpine, respectively. As shown, expression of NGF, BDNF, and HB-EGF was markedly increased only in the brain of the pilocarpine-treated mice, which manifested tonic–clonic convulsions (Figs. 1A–C, E, F). In contrast, expression of NT-3 decreased significantly in both ddY mice and B6 mice (Fig. 1D). It is noteworthy that there was no meaningful change in expression of these neurotrophic factors in the brains of B6 mice which showed no sign of convulsions, even though they were treated with the same dose of pilocarpine (Fig. 1, pilocarpine 1). It should be noted also that expression of these neurotrophic factors did not change significantly in the EL mice, although they manifested repeatedly severe convulsions after periodical vestibular stimulations.

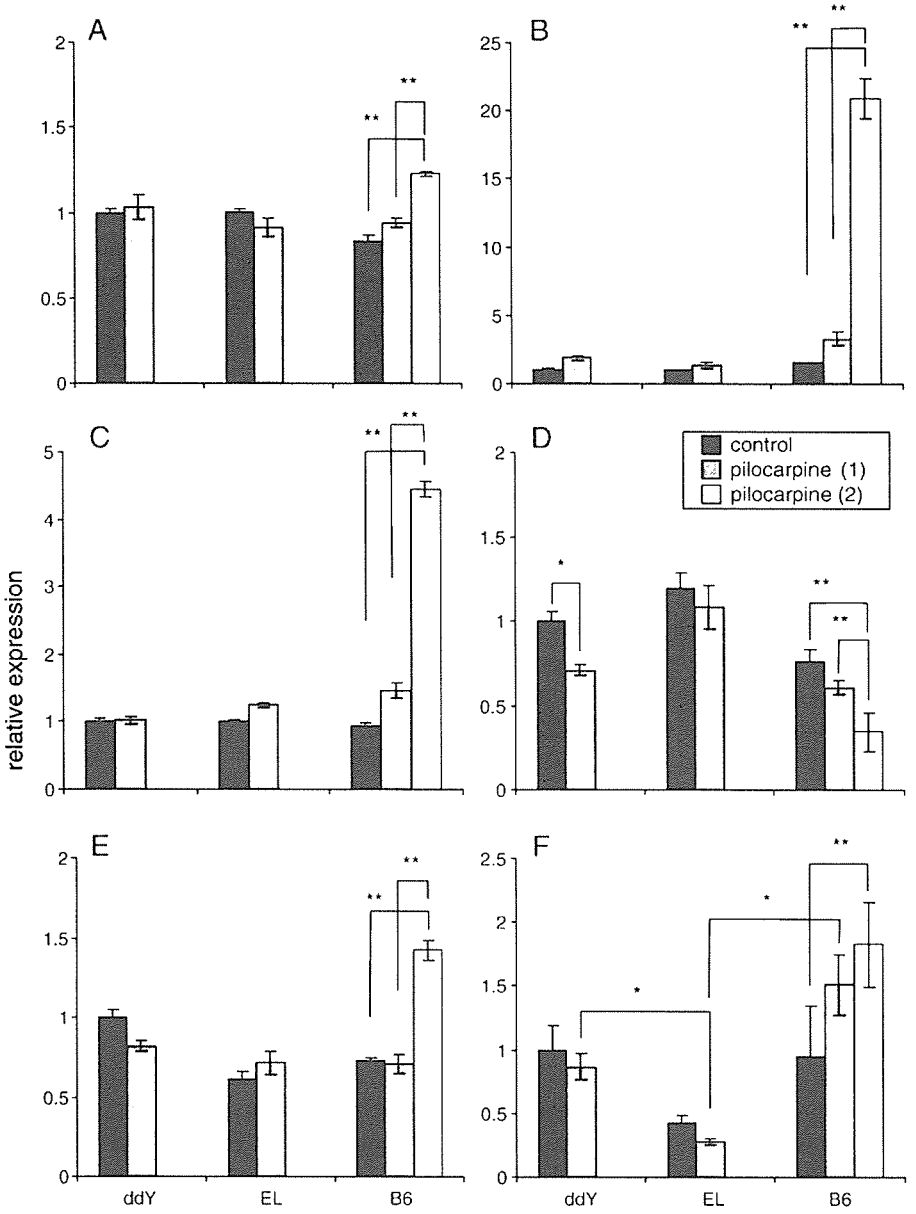


Fig. 1. The relative expression of NGF (A), 4.2 kb BDNF (B 1.5 kb BDNF (C), NT-3 (D), HB-EGF (E), and FGF-2 (F), respectively, in the brains of the ddY mice, EL mice, and C57BL/6 (B6) mice. Some of these mice were injected i.p. with pilocarpine hydrochloride (Sigma, P6503, 300 mg/kg, hatched bars). Fifteen minutes before the injection of pilocarpine, the mice were injected i.p. with atropine methyl bromide (5 mg/kg). Two hours after the injection of pilocarpine, the mice were injected i.p. with diazepam (10 mg/kg). The mice exhibiting tonic–clonic seizures after injection of pilocarpine were grouped as pilicarpine 2 and the remaining mice, which did not exhibit seizures, were grouped as pilocarpine 1. Control mice were injected with 0.85% NaCl instead of pilocarpine. Expression of the neurotrophic factors were measured 4 h after pilocarpine injection. None of the mice were subjected to vestibular stimulation in the study. For details, see Materials and methods. Mean ± SEM of 4–5 animals per group. Statistically different from the control mice: $*P < 0.05$, $**P < 0.01$.

3.2. Histochemistry

Fig. 2 illustrates the histochemical analysis of BrdU-positive cells in the dentate gyrus of control and pilocarpine-treated B6 mice. As shown, the BrdU-positive cells in the control mice were confined to the subgranular zone. The B6 mice which manifested tonic–clonic seizures had about 9.7 and 15.1 BrdU-positive cells, in average, per 10-μm cryosection 2 days and 28 days, respectively, after treatment of pilocarpine (Fig. 2C). In contrast, there were 1.5 and 2.1 BrdU-positive cells on 2 days and 28 days, respectively, in the control mice (Fig. 2B). The double-staining analysis for BrdU and NeuN, a marker of neuronal cells, showed enhanced neurogenesis in the B6 mice which manifested tonic–clonic seizures (Fig. 2). The number of NeuN- and BrdU-double positive cells increased from 0.03, on average, on 2 days to 1.2 on 28 days in the control mouse, and from 1.2 on 2 days to 1.9 cells per cryosection on 28 days after pilocarpine injection. In contrast, there was no appreciable change in the number of BrdU-positive cells in

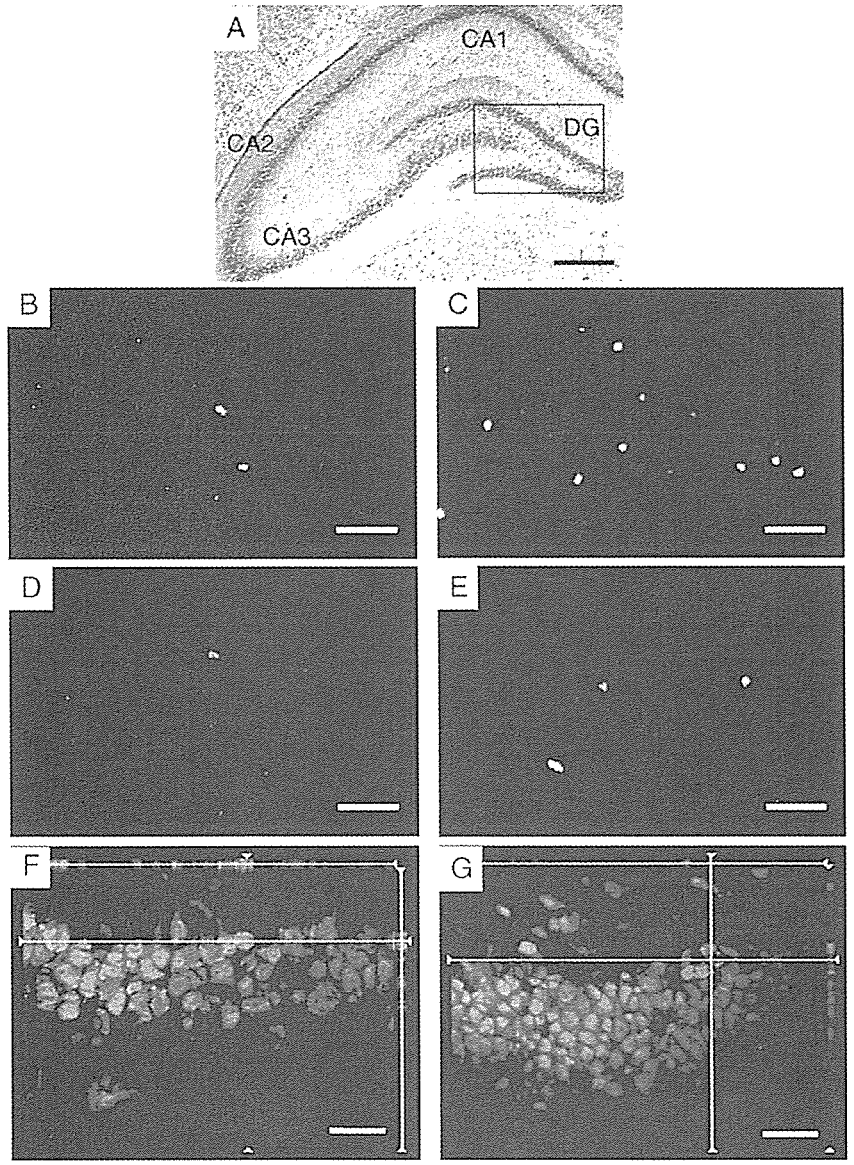


Fig. 2. Structure of mouse hippocampus showed by Nissl staining (A) and BrdU-positive cells in the dentate gyrus of C57BL/6 (B6) mice (B–D) and EL mouse (E). (B) Baseline division of hippocampal cells of a saline-treated control mouse. (C) Increased BrdU–positive cells observed after 2 days in the hippocampus of a B6 mouse treated with pilocarpine and manifested tonic–clonic seizures. Note the clustering of BrdU–positive nuclei in the subgranular proliferative zone at the border of the hilus and granule cell layer. (D) The B6 mouse which manifested no seizures in spite of pilocarpine injection showed BrdU incorporating as same level as saline-treated control. (E) EL mouse experienced repeatedly seizures also did not show increased BrdU-positive cells. (F, G) BrdU (green)- and NeuN (red)-double labeling for control (F) and pilocarpine-seized B6 mouse observed after 2 days (G). Note that the BrdU (green)- and NeuN (red)-double positive cells were noted in the pilocarpine-seized B6 mouse (G), whereas these cells were rarely observed in the saline-treated mice (F). CA1-3, pyramidal cell layer CA1-3 region; DG, dentate gyrus. Scale bar: panel A, 300 μm; panels B–G, 50 μm.

the mice, which exhibited no sign of tonic–clonic convulsions (Fig. 2D). Furthermore, there was no change in the number of BrdU-positive cells in the brain of EL mice, which experienced repeatedly severe seizures after consecutive vestibular stimulations (Fig. 2E). BrdU (green)- and NeuN (red)-double positive cells were noted after 2 days in the pilocarpine-seized B6 mouse (Fig. 2G), whereas these cells were rarely observed in the saline-treated mice (Fig. 2F).

Fig. 3 shows the cells stained with Fluoro-Jade B, a marker of damaged neurons, in the brain of mice, which manifested tonic–clonic convulsions after pilocarpine injection. As shown, a number of Fluoro-Jade B-positive cells were observed after 2 days in the hilus and CA1 pyramidal cell layer of the pilocarpine-treated B6 mice which exhibited tonic–clonic convulsions (Figs. 3C, D). No Fluoro-Jade B-positive cells were noted in the corresponding region of the saline-injected B6 mice (Fig. 3A) and in the B6 mice which manifested no seizures, although they were injected with pilocarpine (data not shown). A marked neuronal cell loss was observed after 90 days in the CA1 and CA3 region of the B6 mice which exhibited tonic–

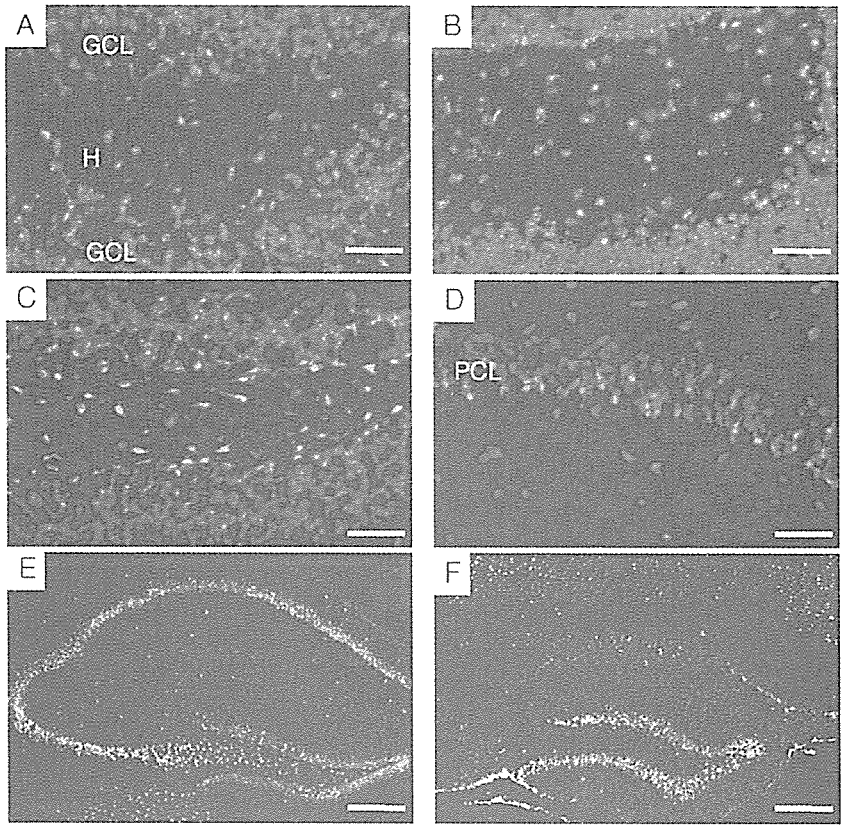
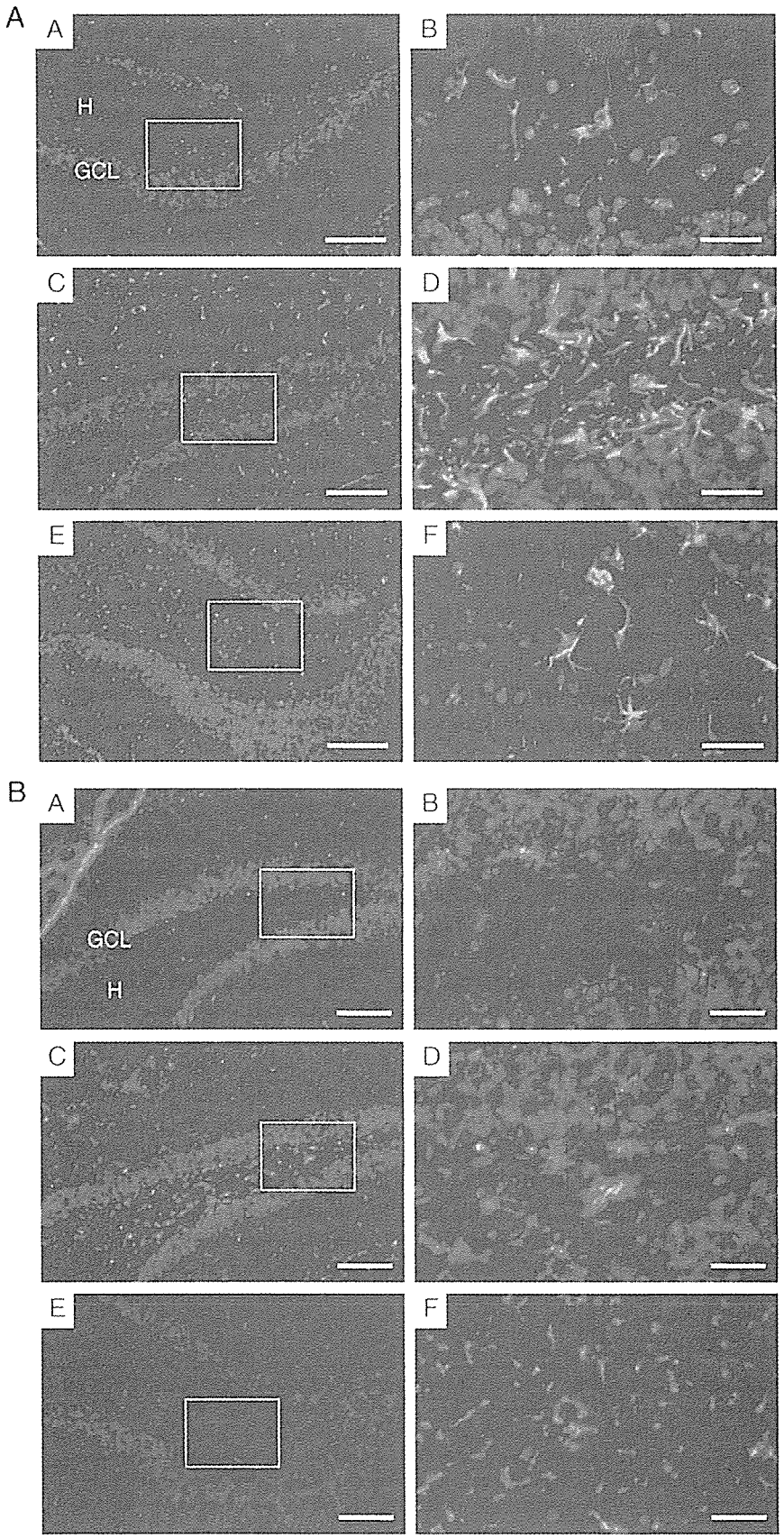


Fig. 3. FJB-positive damaged neurons and loss of NeuN-positive neurons in the hippocampus. FJB-positive cells of the saline-treated C57BL/6j (B6) mouse (A), and the EL mouse which experienced convulsions repeatedly by vestibular stimulations (B). (C) Increased FJB-positive cells observed after 2 days in the B6 mouse which exhibited tonic–clonic seizures after pilocarpine treatment. (D) FJB-positive cells are also detected in the CA1 region of the B6 mouse, which manifested tonic–clonic seizures. (E) Image of NeuN staining in the control B6 mouse. (F) Loss of pyramidal NeuN-positive cells observed after 90 days in the B6 mouse, which manifested tonic–clonic seizures after pilocarpine treatment. GCL, granule cell layer; H, hilus; PCL, pyramidal cell layer (CA1 region). Scale bars: panels A–D, 50 μm; panels E and F, 200 μm.

clonic seizures (Figs. 3E, F). There were no Fluoro-Jade B-positive cells in the brains of the EL mice, which experienced severe convulsions repeatedly as shown in Fig. 3B. Figs. 4A and B illustrate changes in the number of astrocytes, and microglia as evidenced by staining with anti-GFAP and anti-CD11b antibodies, respectively. As shown, the number of these cells was apparently increased after 2 days in the various regions of the hippocampus of the mice, which exhibited tonic–clonic seizures (Figs. 4AC, D and BC, D). Here again, there was no change in the number of these cells in the brain of the B6 mice, which did not manifest convulsions after pilocarpine injection (data not shown), nor in the stimulated EL mice, even though they experienced severe seizures repeatedly (Figs. 4AE, F and BE, F).

4. Discussion

The results obtained here suggest that the neurotrophic factors such as NGF, BDNF, HB-FGF, and NT-3 may be involved in the cellular changes that occur following seizure-induced damage in the mice manifesting tonic–clonic seizures induced by pilocarpine injection. It was evidenced by the findings that their expression changes during seizures, although we show here only the changes in mRNA levels and not of proteins of the neurotrophic factors. First, it was shown here that NGF and BDNF are up-regulated in the mice manifesting tonic–clonic seizures after pilocarpine injection (Figs. 1A, B), confirming those of previous animal experiments [9,13] and in the hippocampus of temporal lobe epilepsy patients [14]. Several studies indicate that NGF promotes epileptogenesis [1,7,29]. In contrast, the roles of BDNF and b-FGF remain controversial [10,20,21]. It was also shown here that HB-EGF was up-regulated in the mice exhibiting tonic–clonic seizures caused by pilocarpine injection (Fig. 1E). These results are consistent with those reported previously showing that HB-EGF expression is significantly up-regulated in kainate-induced seizures [17]. To our knowledge, there is no previous report on the effects of HB-EGF on seizure severity. NT-3 mRNA undergoes a down-regulation in the mice manifesting tonic–clonic seizures after injection of pilocarpine (Fig. 1D), confirming the previous reports, which demonstrated its decreased expression in the kainate-induced [11] and kindling-dependent [4]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seizure models, suggesting that down-regulation of NT-3 was performed in order to prevent further development of pathological events. These results are consistent with the previous reports showing that NT-3 has a facilitatory effect on kindling and sprouting [3,31]. Expression of b-FGF (FGF-2) was enhanced in the pilocarpine-injected mice which exhibited tonic–clonic seizures (Fig. 1F). These results are consistent with those of the previous reports, showing that its expression follows seizure induced by chemical [6,9] and electrical kindling [9,25]. Long-term, low-dose infusion of bFGF prevents kainate-induced hippocampal cell loss, although it has no effect on seizure latency or duration [12]. It should be noted that these changes in neurotrophic factor expression were measured, in the present study, in the entire brain, and not in isolated regions such as hippocampus. Thus, it is possible that we could have missed changes in expression in more limited areas of the brain.

It should be noted that all histological and biochemical changes examined here were observed only in the mice, which elicited tonic–clonic seizures. First, the cells stained with Fluoro-Jade B, a marker of damaged neurons, were noted only in the mice that experienced tonic–clonic seizures after injection of pilocarpine. In contrast, Fluoro-Jade B-positive cells were not observed in the mice that showed no sign of seizures even though they were treated with the same dose of pilocarpine. In addition, there was no cell stained with Fluoro-Jade B in the activated EL mice although they exhibited severe seizures repeatedly. Second, the number of BrdU-positive cells, a marker of division of dividing cells, increased significantly only in the mice, which exhibited tonic–clonic seizures after injection of pilocarpine, whereas no appreciable change in the number of BrdU-stained cells was observed in the mice that showed no seizures, although they were treated with pilocarpine. Furthermore, there was no significant change in the number of BrdU-positive cells in the activated EL mice, which experienced severe seizures repeatedly. It is noteworthy that an increase in the number of BrdU-positive cells was evident as late as 2 days after the pilocarpine injection and not after 1 day (data not shown). Third, activation of astrocytes and microglia were noted mainly in the mice that showed tonic–clonic seizures after pilocarpine treatment. Fourth, the changes of expression of NGF, BDNF, HB-FGF, and NT-3 occurred, here again, only in the mice that exhibited tonic–clonic convulsions after pilocarpine injection. Furthermore, no significant change was observed in expression of these neurotrophic factors in the activated EL mice, which manifested severe seizures. There may simply be strain differences, and therefore severe seizures in B6 mice may result in severe damage, whereas severe seizures in EL mice may not elicit damage, regardless of whether the seizures are tonic–clonic or not. This is clearly correlated with whether neurogenesis and glial activation occur as well, although the correlation cannot be made with neurotrophic factors for the reasons given above. These results together suggest that neuronal damage occurring in the brain of the mice manifesting tonic–clonic seizures is accompanied by neurogenesis.

It remains uncertain whether seizure-induced neuronal degeneration is responsible for enhancement of progenitor cell division. Nakagawa et al. have shown that pyramidal neuronal degeneration is associated with enhancement of progenitor cell division in kainic acid-induced seizures but not in a kindling model [15]. As shown in Figs. 2 and 3, the number of Fluoro-Jade B-stained cells and BrdU-positive cells was increased only in the mice which exhibited tonic–clonic seizures after pilocarpine injection. Hence, it is possible that neurogenesis occurs when the degree of seizures was severe enough to cause neuronal damage.

It is noteworthy that none of the EL mice manifested tonic–clonic seizure even when they were treated with the pilocarpine. In addition, any of the histological changes examined was not observed in these mice, nor in the EL mice which experienced repeatedly seizure episodes after periodical vestibular stimulations. This may indicate that the EL mice are resistant to various epileptogenic stimuli. None of the pilocarpine-treated EL mice and their control animals was subjected to vestibular stimulations. Hence, one might not expect up-regulation of neurotrophic factors, nor in labeling of BrdU, FJB, GFAP, or CD11b already in the control animals. In fact, there was no difference in expression of these factors between the control EL mice and the control B6 mice.

Mounting evidence indicates that neurotrophic factors such as NGF, BDNF, and NT-3 have functional roles in the regulation of pathophysiological changes that associated with seizure-induced brain injury. For example, NGF is known to have important roles in the remodeling of networks that follow repetitive seizures [7]. Granule cell mRNA levels for NGF, BDNF, and NT-3 correlate with neuronal loss and mossy fiber sprouting in the epileptic human hippocampus [14]. Together, these growth factors may regulate the downstream events that follow severe seizure induced by pilocarpine injection.

Fig. 4. (A) A, B: GFAP, a marker of astrocyte-positive cells in the hippocampus of the saline-treated control C57BL/6j (B6) mouse. C, D: Increased GFAP-positive cells after 2 days in the mouse exhibited tonic–clonic seizures caused by pilocarpine injection. E, F: GFAP-positive cells in the EL mouse, which experienced seizures repeatedly after vestibular stimulation. GCL, granule cell layer; H, hilus. Scale bars: panels A, C, and E, 100 μm; panels B, D, and F, 25 μm. (B) A, B: CD11b, a marker of activated microglia-positive cells in the hippocampus of the saline-treated B6 mouse. C, D: Increased activated microglial cells observed after 2 days in the B6 mouse, which exhibited tonic–clonic seizures after pilocarpine treatment. E, F: CD11b-positive cells in the EL mouse, which experienced seizures repeatedly by periodical vestibular stimulations. H, hilus; GCL, granule cell layer. Scale bars: panels A, C, and E, 100 μm; panels B, D, and F, 25 μm.

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REGULAR PAPER

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Cytokine production of activated microglia and decrease in neurotrophic factors of neurons in the hippocampus of Lewy body disease brains

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Abstract Dementia is a frequent complication of Parkinson's disease (PD) and usually occurs late in the protracted course of the illness. We have already reported numerous MHC class II-positive microglia in the hippocampus in PD patients, and that this phenomenon may be responsible for functional changes in the neurons and the cognitive decline in PD patients. In this study, we have investigated the distribution of activated microglia and the immunohistochemical and the mRNA expression of several cytokines and neurotrophic factors of the hippocampus in PD and dementia with Lewy bodies (DLB). The brains from five cases of PD and five cases of DLB that were clinically and neuropathologically diagnosed, and those from four normal controls (NC) were evaluated by immunohistochemistry using anti-HLA-DP, -DQ, -DR (CR3/43), anti-α-synuclein, anti-brain-derived neurotrophic factor (BDNF), and anti-glial fibrillary acidic protein antibodies. In addition, the mRNA expressions of cytokines (IL-1α, IL-1β, TNF-α, IL-6, TGF-β) and neurotrophic factors (BDNF, GDNF, NGF, NT-3) of these brains were evaluated by the reverse transcription-PCR method. MHC class II-positive microglia were distributed diffusely in the hippocampus of PD and DLB brains. Although the cytoplasm of pyramidal and granular cells of the hippocampus in NC brains was strongly stained by anti-BDNF antibodies, it was only weakly stained in PD and DLB brains. The mRNA expression of IL-6 was significantly increased in the hippocampus of PD and DLB brains, and that of BDNF was significantly decreased in the hippocampus of DLB brains. The increased number of activated microglia and the production of neurotrophic cytokines such as IL-6, together with the decreased expression of the neurotrophic factors of neurons in the hippocampus of PD and DLB brains, may be related to functional cellular changes associated with dementia.

Keywords Lewy body disease · Microglia · Major histocompatibility complex class II antigen · Interleukin-6 · Brain-derived neurotrophic factor

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Introduction

Microglia, which are now widely accepted as being of a mononuclear, phagocyte lineage, qualify as immunocompetent cells in the central nervous system (CNS) by virtue of their ability to express major histocompatibility complex (MHC) class II antigens [24, 29, 57]. Microglia with resting or ramified morphology seldom express those antigens, and the up-regulation of MHC class II antigen is an early consequence of activation,

the threshold of detection being reached prior to the onset of visible morphological changes. MHC class II expression on microglia is also up-regulated in pathological situations where neurons degenerate [38, 45, 46]. Parkinson's disease (PD) and dementia with Lewy bodies (DLB) are two such conditions [30, 41, 44]. The salient pathological features of PD are selective neuronal loss, presumably by apoptosis, and the presence of Lewy bodies (LB) in the affected regions [32, 54]. The presence of activated microglia and the absence of reactive astrocytosis in the substantia nigra (SN) of patients with PD suggest microglial involvement in the pathological process of dopaminergic neurons [49]. DLB is a recently recognized cause of neurodegenerative dementia and is clinically characterized by fluctuating but progressive cognitive impairment, parkinsonism, and psychosis with recurrent hallucinations. The neuropathological hallmarks are widely distributed LB throughout the paralimbic and neocortical regions as well as SN [48]. We have already demonstrated that MHC class II-positive activated microglia were widely distributed in the affected regions, including the hippocampus, frequently in association with α-synuclein-positive Lewy neurites or monoaminergic neurites in PD brains [30]. These activation of microglia may be related to reduction of neurotransmitters or neurotrophic factors of adjacent neurons. Nowadays glial cells are known to possess neurotrophic properties that are essential to the survival of dopaminergic and cholinergic neurons [52]. In the present study, we report an increased number of activated microglia, the production of neurotrophic cytokines such as interleukin (IL)-6, and a decreased expression of the neurotrophic factors of neurons in the hippocampus of PD and DLB brains.

Materials and methods

Subjects

Autopsy brains from five clinically and neuropathologically confirmed cases of PD (ages at death 71–81 years, mean 74.8 years), five cases of DLB (ages at death 65–79 years, mean 74.6 years), and four age-matched individuals (ages at death 73–81 years, mean 75.8 years) were examined in this study. All PD patients had presented clinically with resting tremor, rigidity and akinesia, and had responded to levodopa during the course of their disease. Neuropathologically, the brain specimens showed neuronal loss in the SN, locus ceruleus and dorsal vagal nuclei, and Lewy bodies appeared in the SN, locus ceruleus, dorsal vagal nuclei and neocortex [17]. The diagnosis of DLB was made according to the consensus criteria for its pathological diagnosis [48]. Neuropathological findings of PD and DLB are summarized in Table 1. PD-related pathological staging was evaluated according to the Braak stages [7].

Conventional histopathology

All brains were removed within 12 h of death and immersed in 20% neutral-buffered formalin and fixation periods of all brains were within 3 weeks and there was no significant difference between PD, DLB and controls. Each brain region was sliced into 5-mm-thick sections along various planes: cerebrum in the frontal plane, brainstem and spinal cord in the horizontal plane, and cerebellum in the sagittal plane. The tissues were embedded in paraffin and sectioned at a 10-μm thick-

Table 1 Neuropathological findings of PD, DLB and control cases. For NFT, the stage (I–V) is shown; for Aβ, stages of cortical Aβ deposition (A–C) are shown, PD stages are given as 1–6 (*0*, no PD lesions). The table lists the predisposed induction sites of the PD-related pathology. The degree of pathology is assessed semiquantitatively and indicated by: −, absent; +, slight; + +, moderate; + + +, severe (*PD* Parkinson's disease, *DLB* dementia with Lewy bodies, *NFT* neurofibrillary tangle, *aβ* β-amyloid, *ol* olfactory bulb, *dm* dorsal IX/X motor nucleus, *rm* nucleus raphes magnus and/or gigantocellular reticular nucleus. *co* coeruleus-subcoeruleus complex, *sn* posterior portion of substantia nigra , *db* interstitial nucleus of the diagonal band and/or basal nucleus. of Meynert, *CA2*. second sector of the Ammon's horn, *mc* transentorhinal region and/or ectorhinal region, *hc* high-order sensory assosiation areas and prefrontal areas of the neocortex, *fc* first-order sensory assosiation areas and premotor areas and/or primary sensory and motor fields of the neocortex)

	Age	Sex	Dulation (years)	NFT	aβ	PD	ol	dm	rm	co	sn	db	CA2	mc	hc	fc
1	81	M	13	II	A	3	+ +	+ + +	+ +	+ +	+	+	-	-	-	-
2	74	F	13	II	A	3	+ + +	+ +	+ +	+ +	+ +	+	-	-	-	-
3	74	M	11	II	A	4	+ + +	+ + +	+ +	+ + +	+ +	+	+	+	-	-
4	74	M	11	II	A	4	+ + +	+ + +	+ + +	+ +	+ +	+	+	+	-	-
5	71	M	8	II	A	4	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+ +	+	-	-
6	77	M	9	II	A	6	+ + +	+ + +	+ +	+ +	+ + +	+ + +	+ +	+ + +	+ +	+
7	65	M	5	II	A	6	+ + +	+ + +	+ +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+
8	75	M	4	II	A	6	+ + +	+ + +	+ + +	+ +	+ + +	+ + +	+ + +	+ + +	+ +	+
9	79	M	13	II	A	6	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+
10	77	M	11	II	A	6	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ + +	+ +	+
11	73	F	-	I		0	-	-	-	-	-	-	-	-	-	-
12	74	M	-			0	-	-	-	-	-	-	-	-	-	-
13	75	M	-			0	-	-	-	-	-	-	-	-	-	-
14	81	M	-			0	-	-	-	-	-	-	-	-	-	-

ness. For routine histological examinations, each section was stained with hematoxylin and eosin (H-E), and the Klüver-Barrera (K-B) method was used.

Immunohistochemical staining

The 10-μm-thick sections were deparaffinized and rehydrated according to the standard procedures for immunohistochemistry. They were then subjected to microwave treatment for 30 min in 0.01 M citrate buffer at pH 6.0, removed from the buffer to cool down to room temperature, and treated for 20 min with 0.3% H_2O_2 solution in 0.01 M phosphate-buffered saline at pH 7.4. After blocking, they were incubated for 74 h at 4°C with primary antibodies, treated with biotinylated second antibodies (DAKO, Carpinteria, CA) for 1 h at room temperature, and then incubated with avidin-labeled horseradish peroxidase (DAKO) for 1 h at room temperature. Peroxidase labeling was visualized by brief incubation in 0.01% 3,3'-diaminobenzidine and 0.1% H_2O_2 in 0.05 M TRIS-HCl buffer at pH 7.6. Nuclei were counterstained with hematoxylin. Double immunostaining was also performed. The first cycle was carried out as mentioned above, and stained sections were again subjected to a microwave treatment for 30 min in 0.01 M citrate buffer at pH 6.0. The second immunohistochemical cycle was carried out similarly to the first one, except that it was incubated with avidin-labeled alkaline phosphatase (DAKO), and immunolabeling was visualized by incubation in fast red. The primary antibodies used in this study were monoclonal antibody to human HLA-DP, DQ, DR (clone CR3/43) at a dilution of 1:100 (DAKO A/S, Glostrup, Denmark), monoclonal antibody to human GFAP (clone 6F2) at a dilution of 1:400 (DAKO A/S), monoclonal antibody to human brain-derived neurotrophic factor (BDNF; clone 35928.11) at a dilution of 1:100 (R&B Systems Inc., USA), and polyclonal goat antibody to human α-synuclein (N-19) at a dilution of 1:100 (Santa Cruz, CA).

Reverse transcription-PCR method

Ribonucleic acid (average 21.86 mg/punched tissue) extracted from punched samples using a modified acid phenol-guanidine method was used as a template for first-strand cDNA synthesis as follows. A random primer (0.1 mg) was incubated at 95°C for 10 min with the RNA (1 μg) at a volume of 30 μl, and then placed on ice for 5 min. Next, the mixture was incubated at 37°C for 90 min with a mixture of 100 U M-MLV reverse transcriptase (GIBCO BML), 1× reverse transcription (RT) buffer, 10 mM dithiothreitol, 40 U RNasin, and 0.56 mM each of dATP, dGTP, dCTP and dTTP in a volume of 50 μl, then repeated at 95°C for 10 min. The cDNA was amplified with Taq DNA polymerase (Takara, Tokyo) using primer pairs specific to NGFβ (sense primer: AGTTTTACCAAGGGAGCA, antisense primer: GGCAGTGTCAAGGGAATG), BDNF (sense primer: AAGAAAGCCCTAACCAGT, antisense primer: CGAAAGTGTCAGCCAATG), NT-3 (sense primer: GCTTATCTCCGTGGCATC, antisense primer: TGTTGTCGCAGCAGTTCG), glial cell line-derived neurotrophic factor (GDNF; sense primer: GCCAGAGGATTATCCTGA, antisense primer: CCCAGACCCAAGTCAGTG), IL-6 (sense primer: TCAATGAGG AGACTTGCC, antisense primer: TGAGTTGTCATGTCCTGC), TGFβ (sense primer: AGCTGTACATTGACTTCC, antisense primer: GGACAGCTGCTC CACCTT), TNFα (sense primer: CCCAGGCAGTCAGATCAT, antisense primer: GGCAGAGAGGTTGAC), IL-1α (sense primer: AGGAAGAAATCATC AAGC, antisense primer: TGGGCAGTCACATACAAT) or IL-1β (sense primer: TGGCTTATTACAG TGGCA, antisense primer: AAGAAGGTGCTCAG GTCA) for 40 cycles (94°C for 1 min, 55°C for 1 min, and 72°C for 2 min), and GAPDH (sense primer: GAAGGTGAAGGTCGGAGTC, antisense primer: GAAGATGGTGATGGGATTTC) for 30 cycles. The 195-bp (NGFβ), 260-bp (BDNF), 257-bp (NT-3), 240-bp (GDNF), 259-bp (IL-6), 251-bp (TGFβ), 296-bp (TNFα), 274-bp (IL-1α), 247-bp (IL-1β) and 228-bp (GAPDH) PCR products were resolved by electrophoresis in 2% agarose gels, stained with ethidium bromide, and photographed.

Quantification

Average numbers of HLA-DP, DQ, DR (CR3/43)-positive cell counts in the hippocampus, amygdala and transentorhinal cortex were calculated as the sum of reactive microglia in five ×200 fields of five different sections. Stat View (Abacus Co., Cary, NC) was used for statistical analysis. Differences were analyzed by the Mann-Whitney test. Statistical significance was confirmed using backward elimination at a probability value of 0.05.

Results

Immunohistochemical study

An immunohistochemical study using anti-α-synuclein showed that a few α-synuclein-positive presynaptic terminals were stained in the hippocampus CA2/3 region and dentate gyrus of NC brains (Fig. 1A, Fig. 2A). However, there were also some α-synuclein-positive Lewy neurites in the hippocampus CA2/3 region (Fig. 1B) and mossy fibers in the dentate gyrus (Fig. 2B) of PD brains. In DLB brains, Lewy neurites (Fig. 1C) and mossy fibers (Fig. 2C) were more numerous.

An immunohistochemical study using anti-HLA-DP, DQ, DR (CR3/43) showed that CR3/43-positive activated microglia were not seen in the hippocampus CA2/3 region and dentate gyrus of NC brains (Fig. 1D,

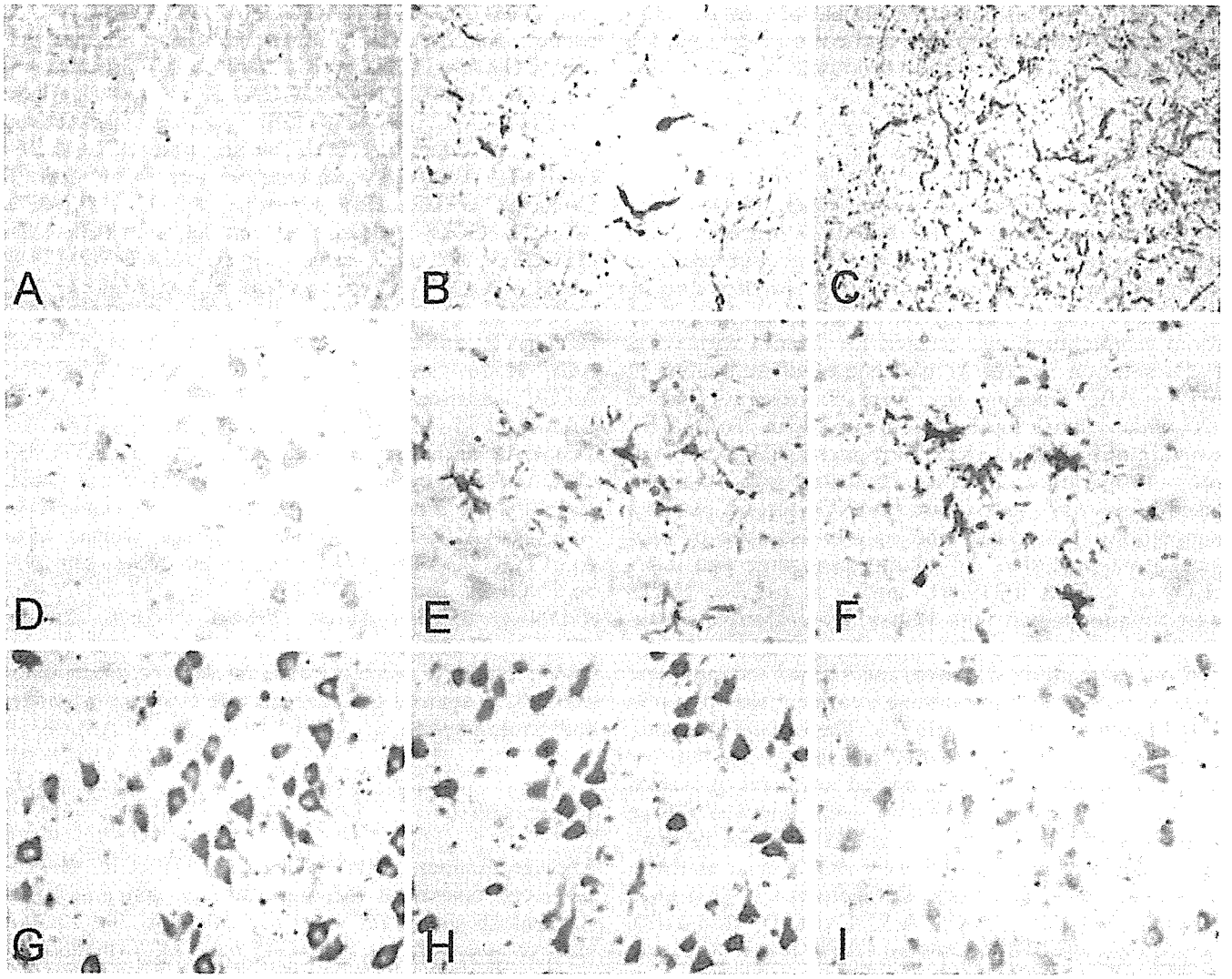

Fig. 1 Immunohistochemical studies of the hippocampus CA2/3 region in NC (**A**, **D**, **G**), PD (**B**, **E**, **H**) and DLB (**C**, **F**, **I**). Immunohistochemical study was carried out with anti-α-synuclein (**A–C**). **A** A few α-synuclein-positive presynaptic terminals are seen in NC. **B** There are some α-synuclein-positive Lewy neurites in PD. **C** In DLB, Lewy neurites are more numerous. Immunohistochemical study was also carried out with anti-HLA-DP, DQ, DR (CR3/43) (**D–F**). **D** CR3/43-positive microglia are not seen in NC. **E**, **F** In PD and DLB, many CR3/43-positive ramified-shaped microglia are seen. Another immunohistochemical study was carried out with anti-BDNF (**G–I**). **G** Almost all neuronal cytoplasm is stained in NC. **H** Almost all neuronal cytoplasm is weakly stained, and nuclei are strongly stained in PD. **I** In DLB, almost all neuronal cytoplasm and nuclei are weakly stained (*NC* normal control, *PD* Parkinson's disease, *DLB* dementia with Lewy bodies, *BDNF* brain-derived neurotrophic factor). ×380

Fig. 2D). In PD and DLB brains, many CR3/43-positive ramified-shaped microglia were seen in the hippocampus CA2/3 region (Fig. 1E, F) and dentate gyrus (Fig. 2E, F).

An immunohistochemical study using anti-BDNF showed that almost all neurons were strongly stained in the cytoplasm of NC brains (Fig. 1G, Fig. 2G). In PD brains, almost all neuronal cytoplasm was weakly stained, and their nuclei were strongly stained (Fig. 1H, Fig. 2H). In DLB, almost all neuronal cytoplasm and nuclei were weakly stained (Fig. 1I, Fig. 2I).

Double immunohistochemical staining of the hippocampus of PD patients showed many CR3/43-positive microglia in the CA2/3 region and dentate gyrus, whereas only a few GFAP-positive astrocytes were seen in both regions (Fig. 3A, B). Some of CR3/43-positive microglia were associated with α-synuclein-positive Lewy neurites in the CA2/3 region (Fig. 3C) and Lewy body-containing neurons in the dentate gyrus (Fig. 3D). Some CR3/43-positive microglia were associated with weakly BDNF-positive neurons in the CA2/3 region (Fig. 3E) and the dentate gyrus (Fig. 3F).

Distribution of activated microglia

Compared to NC patients, brains from PD and DLB patients had a significantly higher number of CR3/43-positive microglia in all areas of the limbic system, especially the CA2/3 region and the dentate gyrus of the hippocampus. However, there was no statistical differ-