FG-7142. This system may, at least, implicate the neocortex, because the brain region exhibits the most prominent alterations in the c-Fos induction pattern between the infant and adult periods. It is also postulated that the molecular cascade of the maturated system may contain the genes that show a stress-induced change in their neocortical expression during the adulthood, but not during the infant period. Therefore, we have performed in the neocortex of the male C57BL mice, a DNA microarray technique [the Mouse cDNA microarray (Agilent)] to isolate the candidate genes that are differentially expressed between postnatal days 8 and 56 at 1 h after the systemic administration of FG-7142 (20 mg/kg, i.p.) (Kurumaji et al. 2005).

Equal amounts of total RNA prepared from the neocortex (the dorsal part of the cerebral cortex divided along the rhinal fissure) of the individual rats of every experimental group using the Quiagen RNeasy Midi System (Quiagen, Valencia, CA, USA) were pooled. Twenty micrograms of the pooled total RNA was reverse transcribed using the oligo dT12-18 primer and aminoallyl-dUTP. The synthesized cDNA was labeled by reaction with dye, NHS-ester Cy3 (the vehicle-treated sample) or NHS-ester Cy5 (the FG 7142-treated sample) (Hughes et al. 2001). The labeled cDNA was applied to the DNA microarray (Mouse cDNA Microarray, Agilent). After washing, the microarray was scanned on a microarray scanner (ScanArray 5000, GSI Lumonics) and the image was analyzed using software (QuantArray, GSI Lumonics). The signal intensity of each spot was calibrated by subtraction of the intensity of the negative control, and was normalized to the global value of all the genes provided on the membranes. The expression of a gene on a specific spot was considered as relevant if the signal intensity was greater than twice the SD of the background.

In the DNA microarray experiments, we found eight developmentally regulated FG-7142-responsive genes, designated as axg 6~13 (anxiogenic responsive transcript 6~13), that met the following criteria for the candidate molecules associated with a matured stress system: (1) the signal intensity of spot labeled by Cy3 (vehicle-treated sample) was more than 500 and the calculated expression ratio of Cy5/Cy3 was greater than two or less than 0.5 in the adult animals, and (2) there were no differences in the hybridization signals between the vehicle-treated controls and the FG-7142-injected animals. No down-regulated genes by FC-7142, which exhibited a spot signal intensity less than 0.5, were observed in the adult neocortex. Further studies by the quantitative real-time RT-PCR method using the LightCycler system (Roche, Penzberg, Germany) revealed that FG 7142 induced a statistically significant increase in the rates of the mRNA levels of each of these eight genes to those of the housekeeping gene, glyceraldehyde-3-phospahte dehydrogenase (GAPDH) (Robbins and McKinney 1992), in the mouse neocortex on postnatal day 56, but not day 8. The neocortical expression of axg6, axg7, and axg8 were also upregulated by immobilization stress and another anxiogenic factor, yohimbine, in the 56-day-old mice.

The differential regulations of these eight transcripts by the anxiogenic drug between the stress hyporesponsive period and maturity suggest that mammalian brains might process the information from the stress stimuli by the distinct sets of molecules between the two stages of postnatal development. Moreover, the eight anxio-

genic-responsive transcripts could be novel members of the molecular cascades involved in the stress responses, because none of these transcripts have so far been reported to be responsive to any kind of stress.

4. Clinical Implications

Our studies presented here demonstrate the marked neuroatomical and molecular changes in stress responses during early life. The developmental changes suggest that stressor-specific neuron circuits and signaling pathways may maturate at the possible critical period between the stress hyporesponsive period and the adult period in mammalian brains and the acute and long-term effects of stresses on behavior should change across the critical period. It is proposed that a similar developmental mechanism could underlie the age-related transfigurations of the psychiatric symptoms and their courses of PTSD and other stress disorders in humans. This view also suggests the necessity of the development-based therapy for these mental dysfunctions. Therefore, the developmentally regulated FG-7142-responsive genes and/or their protein products would be implicated in the pathophysiology of a group of stress disorders and be suitable targets for the development of a novel and age-directed treatment or prophylaxis for these illnesses.

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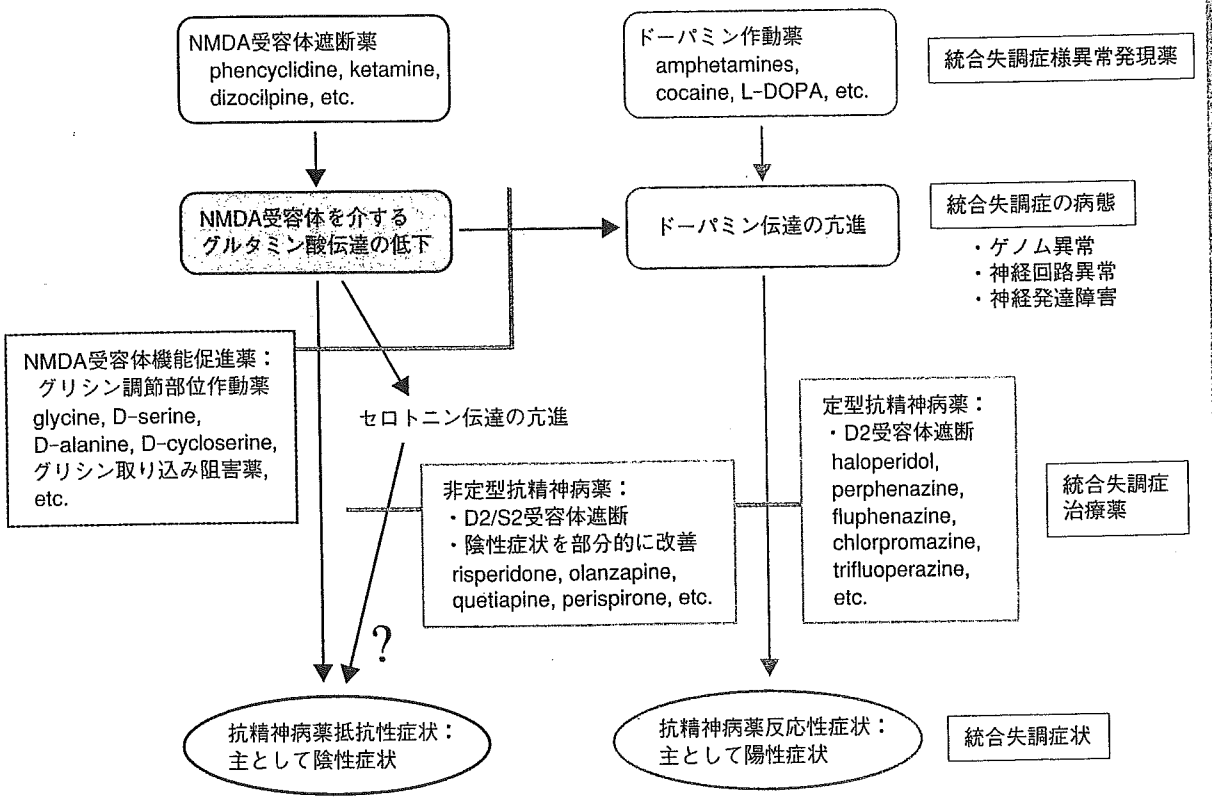
1 統合失調症

統合失調症は，思春期・青年期を中心に0.8%の高率で発症し，陽性症状と陰性症状に大別される多彩な精神症状を呈する．しかし，脳に明らかな神経病理学的変化は見出されず，原因となる分子異常は未だ不明であり，多因子性で原因の異なる複数の疾患からなる（異質性）と推測されている．治療薬や統合失調症様症状発現薬の作用からみると，脳内のドーパミン伝達亢進およびNMDA受容体を介するグルタミン酸伝達の低下が強く示唆される．分子遺伝学的解析では，dysbindinやneuregulin 1遺伝子との関連が最も注目されている．さらに，神経発達障害やオリゴデンドロサイトの異常を基盤とする可能性を支持する所見もあり，本症の分子病態を総合的に理解するため，遺伝子操作動物も取り入れた複合的なアプローチが試みられている．

概念図

薬理学的所見に基づいた統合失調症状の発現機序（仮説）



統合失調症では原因となる分子異常が未だ同定されていないが，薬理学的所見にもとづいて，図のような症状発現機構が考えられている．═は，治療薬の作用点を示す（図7-2および図7-3も併せて参照）．ドーパミン（DA）作動薬および遮断薬と，NMDA受容体の遮断薬およびグリシン調節部位に作用する機能促進薬の作用を比較すると，DA伝達の亢進は主として陽性症状に，NMDA受容体を介するグルタミン酸（Glu）伝達の低下は陽性・陰性双方の発現に関与すると推察される．NMDA受容体の機能低下は，二次的にDA伝達を亢進させるため，陽性症状が出現することと矛盾しない．NMDA受容体機能促進薬が陰性・陽性双方の症状を改善する可能性については，臨床的検証が不十分である．セロトニン伝達系の陰性症状への関与は，陰性症状を部分的に改善する薬物がS2型セロトニン受容体を強く遮断することや，NMDA受容体遮断薬投与後のセロトニン放出増加等により支持されるが，さらに検討が必要である（？マーク）．以上の仮説は，統合失調症の分子異常が，必ずしもDAやGluの伝達系内に限局することを意味せず，本症の異質性も考え合わせると，これらの制御系を含めて複数見出される可能性がある．これは，本症に関するゲノム，神経回路および神経発達障害の研究結果からも支持されている

表 7-1　DSM-Ⅳにおける統合失調症の診断基準

A. 特徴的症状
　（1）から（5）の症状のうち2つ以上，治療が成功した時を除き，おのおのは1ヶ月以上の期間ほとんどいつも存在
　　（1）妄想
　　（2）幻覚
　　（3）まとまりのない会話（例えば頻繁な脱線または滅裂）
　　（4）ひどくまとまりのないまたは緊張病性[5]の行動
　　（5）陰性症状：感情の平板化，思考の貧困または意欲の欠如
B. 社会的または職業的機能の低下
C. 期間：少なくとも6ヶ月持続
D. 失調感情障害と気分障害の除外
E. 物質や一般身体疾患が原因となるものを除外
F. 自閉性障害や他の広汎性発達障害の既往がある時は，顕著な幻覚や妄想が1ヶ月以上存在

DSM-Ⅳはアメリカ精神医学会が作成し，国際的に広く用いられている診断基準．
[5]緊張病性の行動：①カタレプシーまたは昏迷として示される無動症，②過剰な運動活動性，③極度の拒絶症，④奇妙な随意運動（常同運動，顕著なしかめ面ほか），⑤反響言語または反響動作

1 統合失調症の臨床的・生物学的特徴

統合失調症（schizophrenia）は，思春期から青年期前半を中心に0.8％の高率で発症し，慢性化しやすい重大な障害である．治療薬（抗精神病薬）に抵抗する症状のため，現在もわが国だけでも20万人以上が入院生活を余儀なくされている．長期的予後調査によると，抗精神病薬が導入された後も，症状がほとんど見られなく例は3割に満たない．

本症では，思考，知覚，感情，意欲などの脳機能が広汎に障害され，多彩な精神症状が出現する．これらの症状は一般に，陽性症状と陰性症状に分類される（概念図）．陽性症状は，妄想，幻覚，統制を欠いた行動・興奮など，発症すると新たに産出されたように見える異常をさす．陰性症状は，健常時の諸機能が減弱・脱落する異常を意味し，会話・思考内容の貧困化，感情鈍麻（感情表出の平板化と不調和），意欲の減退・引きこもり（目的指向的な行動の低下）等を含む．

このように特徴的な精神機能異常が認められるにもかかわらず，脳における明らかな変性，炎症，その他の神経病理学的変化は見出されていない．近年，脳機能画像所見，眼球運動，prepulse inhibition等の臨床生理学的検査における健常者との差異が注目されているが，生物学的マーカーは確立されておらず，臨床症状とその持続を指標とした国際的な基準によって診断されている（表7-1）．また，症状，経過，治療反応性等から見ると，原因の異なる複数の疾患から構成されていると推測される（異質性）．

統合失調症は孤発性だけでなく家族性に発症することがあり，遺伝子を多く共有するほど一致して発症する確率が高くなる現象が知られている（図7-1）．また，養子の研究からも，環境因よりも遺伝的背景の方が統合失調症の発症に大きな役割を果たすことが報告され，本症では，遺伝的要因の関与が確実視されている．しかし，一卵性双生児でも4～6割は一方の同胞は発症せず，メンデル遺伝に従う単一遺伝子疾患ではなく多因子疾患と推測されている．このため，分子遺伝学的解析は難航しており，統合失調症に特異的なゲノム異常は未発見である．

一方，精神神経疾患の病因・病態の解明には，死後脳の分析が欠かせないが，統合失調症では長期服薬の影響を除外し難い点が，疾患特異的な変化にアプローチするうえでの大きな問題となっている．

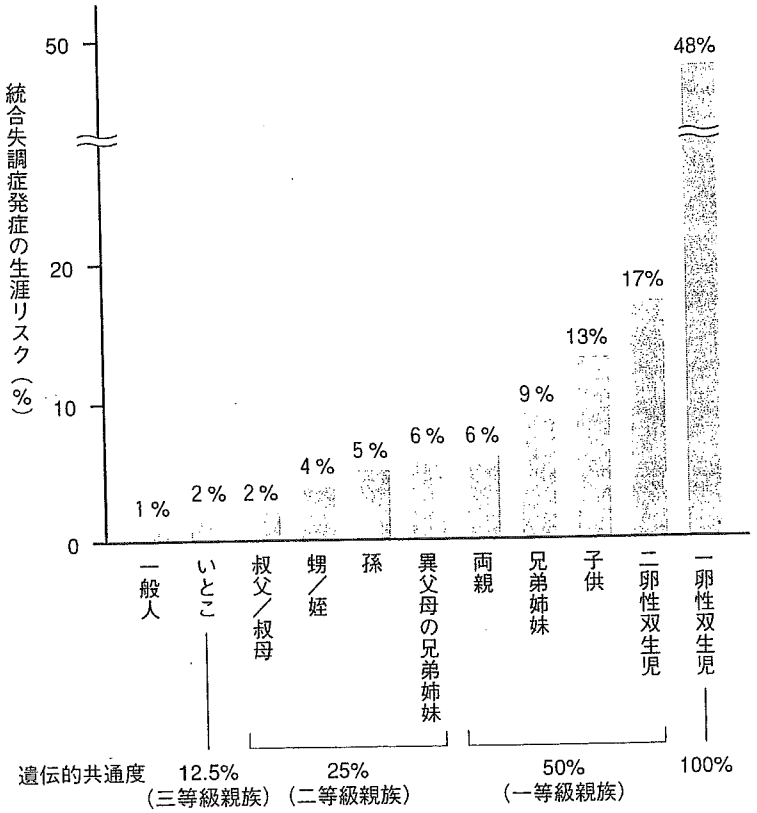


図7-1　遺伝的共通度と統合失調症発症の一致率との関係

Gottesmanの研究結果では（Schizophrenia Genetics, W. H. Freeman, New York, 1991），遺伝子を共有する割合が高い関係であるほど統合失調症の発症に関する一致率が高いことがわかる．「心の病気と分子生物学―生物学的精神医学の新展開」（Barondes, S. H. 著／石浦章一，丸山 敬 訳），日経サイエンス社，1994よりp.134の図を改変

2 神経伝達障害

統合失調症の分子病態として，現在，最も一般的に受け入れられているのは，本症の治療薬や統合失調症様異常発現薬に関する薬理学的研究に基づいた，脳内のドーパミン（DA：dopamine）およびグルタミン酸（Glu：glutamate）伝達系の障害の可能性である．

1）ドーパミン伝達の亢進

1952年に，クロルプロマジンの統合失調症状改善作用が発見されて以来，多くの異なった化学構造をもつ抗精神病薬が開発・導入されてきた．これらの薬物はいずれも，臨床力価に比例した強力なD2型DA受容体遮断作用をもつことが明らかにされた（図7-2）．また，amphetamine類（覚せい剤），cocaine，L-DOPA（L-3, 4-dihydroxyphenylalanine）その他のDA作動薬は，統合失調症ではないヒトにしばしば本症と区別が難しい幻覚・妄想状態を引き起こす（概念図，図7-2）．さらに，症状が目立たなくなった状態の統合失調症患者に，健常者には明らかな精神変調を惹起しない少量のDA作動薬を投与すると，幻覚妄想状態が再燃する．これらの事実から，統合失調症患者の脳では過剰なDA伝達あるいはそれが生じやすい異常が推測されるようになった．ただし，抗精神病薬は陰性症状にはほとんど効果を示さない点は，DA伝達の亢進が主として陽性症状の発現と関係することを示唆している．

死後脳の分析では，多くの研究グループがほぼ一致して，統合失調症における線条体D2受容体数の増加を報告しているのに対して，PETを用いた*in vivo*の測定のほとんどが，有意な差異を見出していない（D1受容体については双方の方法とも結果が一致していない）．最近，シナプス間隙のDA量に影響されるD2受容体リガンドを利用したPET研究が行われ（たとえばDA放

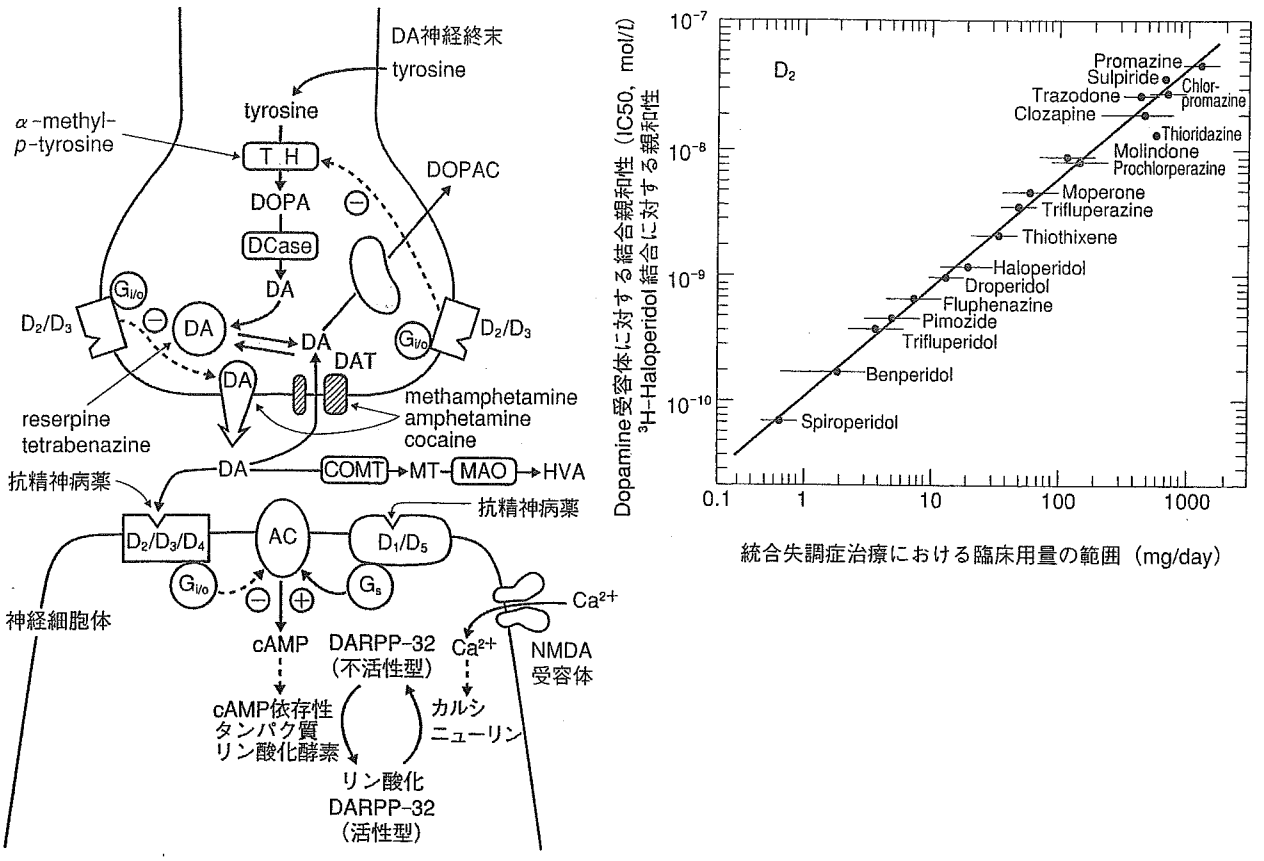


図 7-2　DA 神経伝達と統合失調症関連薬物

A）DA 性シナプスの模式図：統合失調症様異常発現薬（methamphetamine, amphetamine, cocaine），抗精神病薬および DA 代謝に作用する薬物の標的分子を示した．B）抗精神病薬は，幻覚・妄想状態を改善するのに要する標準的な一日使用量と，D2 型 DA 受容体遮断力価（受容体への親和性で表してある）がほぼ正比例する（Seeman, P. et al.：Nature, 261：717-719, 1976, Fig. 1 を改変）．こうした相関は，アデニル酸シクラーゼと負の共役をする D2，D3 および D4 受容体の遮断作用に共通して見られるが，本酵素と正の共役をする D1 および D5 受容体や，その他の神経伝達物質受容体では認められず，抗精神病作用は D2 ファミリー受容体の遮断作用を介して発揮されると考えられている．細胞内では DARPP-32 によって DA 受容体と NMDA 型グルタミン酸受容体（図 7-3）のシグナルが相互作用をもつと考えられている．AC：adenylate cyclase, COMT：catechol-*o*-methyltransferase, DARPP-32：dopamine- and cAMP- regulated phosphoprotein of 32kD, DAT：dopamine transporter, DCase：aromatic L-aminoacid decarboxylase, DOPA：L-3, 4-dihydroxyphenylalanine, G_s, $G_{i/o}$：G protein, HVA：homovanilic acid, MAO：monoamine oxidase, MT：3-methoxytyramine, TH：tyrosine hydroxylase

出の増加によりリガンド結合が減少），①少量の amphetamine で誘発される線条体の DA 放出は，健常者より統合失調症患者の方が多く，②α-methyl-*p*-tyrosine で DA 合成を一時的に阻害したときのリガンド結合の増加を指標とした基礎的な DA 放出量は，統合失調症患者の方が高いこと等がわかった．以上の所見は，統合失調症患者の脳内 DA 伝達が亢進していることを支持しているが，抗精神病薬の長期投与の代償的変化である可能性が完全に除外できない．

MEMO

統合失調症の症状は一般に幻覚・妄想等の陽性症状と感情平板化・意欲減退・思考の貧困化等の陰性症状に分類される．統合失調症治療薬である抗精神病薬は陽性症状を改善するが，陰性症状にはほとんど効果を示さない．抗精神病薬の治療効果は主として D2 ファミリーの DA 受容体遮断作用を介して発揮される．

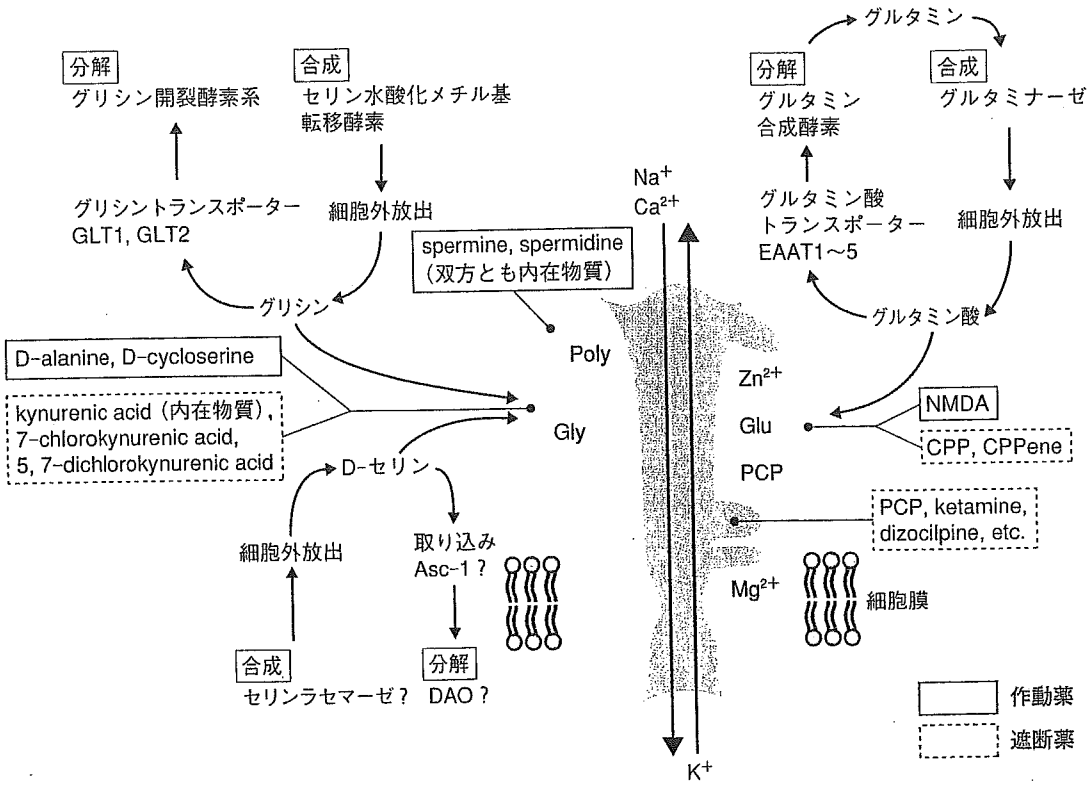


図7-3　NMDA受容体および主要な内在性リガンドの代謝経路と統合失調症関連薬物

NR1サブユニットと4種のNR2サブユニットA～Dの1種が組み合わさったヘテロメリック集合体を形成するタイプのNMDA受容体を模式的に示す．細胞外からNa^+やCa^{2+}を流入させ，細胞内からK^+を透過させるイオンチャネルを構成しており，グルタミン酸結合部位（Glu），グリシン調節部位（Gly）（概念図および本文図-2）を参照），マグネシウムイオン結合部位（Mg^{2+}），フェンサイクリジン結合部位（PCP），ポリアミン結合部位（Poly）等の調節部位をもつ．臨床的にPCP結合部位〔PCP，ketamine，dizocilpine (MK801)〕，Glu結合部位（CPP，CPPene）のいずれの遮断薬も統合失調症様異常を誘発することが知られている．NMDA受容体の調節に関わる内在性リガンドのうち，Glu，グリシンおよびD-セリンについては代謝経路の概略を図示した．後二者はコ・アゴニストと呼ばれ，単独では神経伝達を引き起こすことはないが，Gluが神経伝達を生じさせるにはその存在が不可欠である．このような生理機能の重要性から，Gluだけでなく D-セリンやグリシンの代謝・機能の分子機構またはその制御系と統合失調症との関連が注目されている

2）NMDA型グルタミン酸受容体機能の低下

次のような薬理学的根拠に基づいて，NMDA型Glu受容体（図7-3）を介するGlu伝達の低下が推測されている．

①phencyclidine〔1 (1-phenylcyclohexyl) -piperidine：PCP〕，ketamineをはじめとするNMDA受容体遮断薬が統合失調症様の陽性ならびに陰性症状を引き起こす（概念図）

②ketamineは，NMDA受容体遮断作用の強い立体異性体（S体>>R体）の方が精神異常を誘発しやすい

③統合失調症患者は健常者より，NMDA受容体遮断薬に感受性が高く精神障害が生じやすい

④PCPが精神障害のみを引き起こし，麻酔作用・意識障害を示さないときの血中濃度はnMオーダーで，NMDA受容体以外の神経伝達系には作用しない低レベルである

④統合失調症患者において，NMDA受容体機能を促進する本受容体グリシン調節部位（図7-3，概念図も参照）の作動薬（グリシン，D-セリン，D-サイクロセリン，D-アラニン，グリシントランスポーター阻害薬等）を既存の抗精神病薬と併用すると，抗精神病薬抵抗性の陰性症状や認知機能障害が改善される

死後脳の研究からは，NMDA受容体の結合・遺伝子を含む，さまざまなGlu伝達系の変化が報告されているが，研究者間で不一致の結果も多い．

3）ドーパミン-グルタミン酸相互作用の異常

PCP, ketamins等を急性投与した動物脳では，特に大脳皮質を中心にDA伝達が亢進する．これらのNMDA遮断薬を反復投与すると前頭葉皮質のDA代謝が低下するという報告もあるが，amphetamineをチャレンジしたときのDA遊離は前頭葉皮質と線条体の双方で増強している．NMDAR1サブユニットの発現低下マウスでは，DA作動薬への感受性が上昇していることも考え合わせると，NMDA受容体機能が低下した状態では，DA伝達が高まると推測される．したがって，少なくとも一群の統合失調症では，NMDA受容体を介するGlu伝達が減弱し，DA伝達が過剰になった結果陽性症状が出現し，DA以外の分子カスケードの異常により陰性症状が引き起こされる可能性がある（概念図）．

臨床的研究でも，こうした仮説を支持する所見が得られている．すなわち，katamineを健常ボランティアに投与した研究では，統合失調症様の精神変調が見られ，ketamine投与群は非投与の対照群に比較して，amphetamine誘発性のDA遊離の増加が亢進していた．

以上の相互作用より，NMDA受容体機能促進薬は陰性・陽性双方の症状を改善すると考えられる．ただし，臨床的には未だ十分な検証が行われていない．

MEMO

統合失調症様異常発現薬にはDA作動薬とNMDA受容体遮断薬があり，前者は陽性症状と，後者は陽性・陰性双方の症状と類似した異常を引き起こす．これらの異常は，しばしば統合失調症と誤診される．NMDA受容体の内在性コ・アゴニストでNMDA受容体グリシン調節部位を刺激するグリシンおよびD-セリンは，抗精神病薬抵抗性の統合失調症状を改善することが報告されている．このうちD-セリンは，NMDA受容体選択的な作用をもち，NR2Bサブユニットと類似した脳内分布を示す．

3 分子遺伝学的解析

これまでに単独の研究だけで，全ゲノム解析のレベルで統合失調症と有意な連鎖が認められた領域は，1q21-q22, 6p24-p22, 6q21-q25, 10q25-q26, 13q32-q34, 17p11-q25である．また，少なくとも独立した2つ以上の研究から連鎖が報告されているのは，1q21-q22, 1q42, 6p24-p22, 6q21-q25, 8p22-p21, 10p15-p11, 13q32-q34, 22q11-q22の領域である．最近の2つの大規模メタ解析を比較すると，双方から連鎖が支持されたのは8pと22qのみであり，1q, 2q, 3p, 5q, 6p, 11q, 13q, 14p, および20qはいずれか一方の研究から連鎖が示唆された．さらに，日本人の236家系についての新たな大規模解析では，1p, 14q, および20pと統合失調症の連鎖が検出されている．

連鎖が見られたゲノム領域から，統合失調症の感受性遺伝子として有意な関連が見出され，多くの研究が追認しているのは*DTNBP1***〔dytrobrevin binding-protein 1 (dysbindin)：6p22.3, 遺伝子名の右肩についている記号（*, **, #）については本節の第4パラグラフを参照〕および*NRG1*** (neuregulin 1：8p22-p21) である（ただし，*DTNBP1*では関連部位は研究者間で必ずしも一致せず，*NRG1*については関連を確認できなかった研究もある）．その他，D-amino-acid oxidase** (*DAO*：12q23-q24，図7-3参照)，D-amino-acid oxidase activator** (*DAOA*または*G72*：13q34，図7-3参照)，regulator of G-protein signalling 4** (*RGS4*：1q23.3),

carboxy-terminal PDZ domain ligand of neuronal nitric oxide synthase** (*CAPON*：1q22), protein phosphatase 3, catalytic subunit# (*PPP3CC*：8p21.3) and trace amine receptor 4* (TRAR4：6q23.2) 等も，十分ではないが，複数の報告があり注目されている．

染色体異常を手がかりとした検索も行われ，22q11領域の欠失が見られるVCFS (velo-cardio-facial syndrome) の患者では，妄想型統合失調症と類似の精神症状が出現する頻度が高いため，この領域の遺伝子の機能不全がある種の統合失調症を引き起こす可能性が考えられている．候補遺伝子としては，catechol-*o*-methyltransferase* (*COMT*，図7-2参照)，proline dehydrogenase# (*PRODH*)，zinc finger- and Asp-His-His-Cys (*DHHC*) domain-containing protein 8 (*ZDHHC8*) 等が注目されている．一方，スコットランドの家系解析から統合失調症と関連する1q42.1と11q14.3の相互転座が見出され，ブレークポイント領域にマップされる*DISC1* (disrupted in schizophrenia 1) またはその相互作用タンパク質の遺伝子と統合失調症の相関が報告されている．また，ブレークポイント近傍に位置する代謝型Glu受容体サブタイプ5 (*GRM5*) 遺伝子**の多型については，有意な相関が認められた．

以上の感受性遺伝子の中には，DA (*)，Glu伝達 (**) または双方 (#) に関係の深いものが含まれていることは注目に値する．ただし，個々の多型またはハプロタイプと統合失調症との関連は最も信頼できるレベルでもオッズ比が2.5に満たず，effect sizeは小さい．したがって，関連性は見かけ上である可能性も否定できないが，各遺伝子内または連鎖を示す遺伝子座内の複数の部位と本症の病態との関係を検討する必要がある．

4 神経回路異常と神経発達障害仮説

PETを用いた脳の血流や糖代謝の研究，fMRI (functional MRI) による研究等から，研究者間で比較的一致した所見として，統合失調症では，安静時または課題遂行時に，背外側前頭前野，内側前頭葉皮質，前部帯状回，左側頭葉，視床，海馬，小脳等における脳の活動性の変化が報告されている．このうち，前頭葉皮質各領域，視床，海馬等の体積については，メタ解析により，統合失調症における有意な減少が示唆された．したがって，以上の脳部位を含む，特定の神経回路が障害されている可能性がある．また，動物実験では，中脳から辺縁系領域に投射するDAニューロンの活動亢進と，大脳皮質から線条体，側坐核，中脳腹側被蓋野等の皮質下領域に投射するGluニューロンの機能低下が示唆されている．その原因として中脳皮質系，特に前頭葉皮質に投射するDAニューロンの低活動を想定する仮説が注目されている．ただし，DA作動薬により明らかに改善する統合失調症状が確認されない点は，この仮説を支持しない．

統合失調症の神経回路異常は，神経発達障害によってもたらされる可能性がある．すなわち，疫学的研究より，胎生期または周産期の栄養障害，薬物使用，ウイルス感染，神経発生過程の障害等と統合失調症の関連が指摘され，本症の一群では，特定の発達段階における何らかの侵襲，あるいは神経発達に重要な分子の異常が，正常な神経回路形成を阻害していることが示唆された．

神経発達障害を反映する所見として，当初，統合失調症患者死後脳における，ニューロンの配列，サイズ，樹状突起分枝等の変化が注目を集めたが，その後の研究では本症に対する特異性が確認されていない．最近は，①分子遺伝学的解析から統合失調症との関連が示唆されている候補遺伝子群が，発達期を通じてシナプスの構造や機能に影響する点や，②死後脳でオリゴデンドロサイトまたはミエリン形成に関連した分子群の発現が変化している点が，統合失調症における神経回路発達障害に関与する可能性が検討されている．

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Topics ―― 統合失調症の動物モデル

近年，従来から用いられてきた統合失調症様異常発現薬を投与した動物のほかに，遺伝子操作動物や，生後発達期に脳に侵襲を加えた動物も統合失調症モデルとして用いられるようになった．本症のモデルと判断する指標は，主に，①DA作動薬およびNMDA受容体遮断薬の異常行動惹起作用の亢進，②prepulse inhibitionの減弱（強い刺激への驚愕反応が，比較的弱い類似の刺激を先行させると抑制される現象で，知覚情報処理と密接に関係する：統合失調症において疾患特異的ではないが減弱がみられ，動物でも計測可能な生理学的指標として重視されている），③社会的行動の異常，④認知機能障害，⑤抗精神病薬の改善作用等である．これらの異常は，Dishevelled（*Dvl*），NR1サブユニット，calcineurin，*PRODH*等をはじめとした多くの遺伝子改変マウスや，新生仔期に海馬神経細胞の破壊またはサイトカイン（IL1-α）や成長因子（EGF：epidermal growth factor）の負荷を行ったラットで認められている．海馬破壊やIL1-α・EGF負荷では，統合失調症モデルの異常行動が思春期以降に発現する点も注目されている．

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Potential Role for Astroglial D-Amino Acid Oxidase in Extracellular D-Serine Metabolism and Cytotoxicity

Hwan Ki Park*, Yuji Shishido†, Sayaka Ichise-Shishido, Tomoya Kawazoe, Koji Ono, Sanae Iwana, Yumiko Tomita, Kazuko Yorita, Takashi Sakai and Kiyoshi Fukui‡

Department of Gene Regulatorics, The Institute for Enzyme Research, The University of Tokushima, Tokushima 770-8503

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D-Amino acid oxidase (DAO) is a flavoenzyme that catalyzes the oxidation of D-amino acids. In the brain, gene expression of DAO is detected in astrocytes. Among the possible substrates of DAO *in vivo*, D-serine is proposed to be a neuromodulator of the *N*-methyl-D-aspartate (NMDA) receptor. In a search for the physiological role of DAO in the brain, we investigated the metabolism of extracellular D-serine in glial cells. Here we show that after D-serine treatment, rat primary type-1 astrocytes exhibited increased cell death. In order to enhance the enzyme activity of DAO in cells, we established stable rat C6 glial cells overexpressing mouse DAO designated as C6/DAO. Treatment with a high dose of D-serine led to the production of hydrogen peroxide (H_2O_2) followed by apoptosis in C6/DAO cells. Among the amino acids tested, D-serine specifically exhibited a significant cell death–inducing effect. DAO inhibitors, *i.e.*, sodium benzoate and chlorpromazine, partially prevented the death of C6/DAO cells treated with D-serine, indicating the involvement of DAO activity in D-serine metabolism. Overall, we consider that extracellular D-serine can gain access to intracellular DAO, being metabolized to produce H_2O_2. These results support the proposal that astroglial DAO plays an important role in metabolizing a neuromodulator, D-serine.

Key words: D-amino acid oxidase, astrocytes, chlorpromazine, hydrogen peroxide, D-serine.

Abbreviations: 3-AT, 3-amino-1,2,4-triazole; CPZ, chlorpromazine; DAAO, D-amino acid oxidase; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NMDA, *N*-methyl-D-aspartate.

D-Amino acid oxidase (DAO; EC 1.4.3.3) is a flavoenzyme that catalyzes the oxidation of D-amino acids to the corresponding imino acids and hydrogen peroxide (H_2O_2) (*1*). The imino acid is nonenzymatically hydrolyzed to α-keto acid and ammonia. In mammals, DAO is found at the highest concentrations in the kidneys, liver and brain. Previously, we determined the primary structures of the porcine (*2*), human (*3*), rabbit (*4*), and mouse (*5*) kidney DAO mRNAs. We also carried out a series of molecular biological studies on the structure–function relationship of DAO for the porcine (*6*, *7*) and human enzymes (*8*). RNA blot hybridization analysis of porcine tissues showed that three mRNA species were expressed in the kidneys and liver, but only one was detected in the brain, indicating the active biosynthesis of DAO in the brain and the brain-specific regulation of the its expression (*9*). Moreover, we reported the structural organization of the human DAO gene and the regulation of its expression (*10*). We then mapped this gene to human chromosome 12, *i.e.*, to within 1-cM of the Spinocerebellar Ataxia 2 gene locus (*11*), suggesting a genetic link between DAO and neurologic disorders. Recently, we demonstrated the gene expression of DAO in type-1 astrocytes from rat cerebellum and cerebral cortex (*12*).

D-Serine occurs in the mammalian brain (*13*) and is an endogenous agonist of the NMDA receptor (*14*, *15*). The extracellular concentration of D-serine parallels or is higher than that of glycine in the prefrontal cortex and in the striatum, respectively (*16*). Several reports have indicated that D-serine may play a role in pathological conditions related to dysfunction of the NMDA receptor. Massive stimulation of NMDA receptors has been implicated in neural damage following stroke (*17*). Elevation of the extracellular concentration of D-serine was observed after transient cerebral ischemia in animal models (*18*), and drugs that block the glycine sites of NMDA receptors prevented stroke-induced damage (*19*). Hypofunction of the NMDA receptor has also been implicated in the pathology of schizophrenia. D-Serine greatly improved positive, negative and cognitive symptoms in schizophrenic patients (*20*). Mice expressing only 5% of the normal level of the NR1 subunit of the NMDA receptor exhibited behavioral abnormalities related to schizophrenia, including increased motor activity, stereotypy, and deficits in social and sexual interaction (*21*). It is notable that novel human gene G72 was recently implicated in schizophrenia, and the gene G72 product has been shown to bind with DAO

*Supported by a Japanese Government (Monbukagakusho) Scholarship.

†Present address: Brain Research Institute, Niigata University, Niigata 951-8122.

‡To whom correspondence should be addressed. Tel: +81-88-633-7430, Fax: +81-88-633-7431, E-mail: kiyo@ier.tokushima-u.ac.jp

and to enhance its catalytic activity. DAO is itself associated with schizophrenia, and the combination of the G72/DAO genotypes has a synergistic effect on disease risk (*22*). In this context, it is interesting to note that the inhibitory effect of chlorpromazine, a classical antipsychotic drug, on the activity of DAO through competition with its coenzyme, flavin adenine dinucleotide, was reported by Yagi *et al.* (*23*).

To investigate the potential role of DAO in D-serine metabolism, we established rat glial cell lines (C6) overexpressing mouse DAO and examined the effect of treatment with a high dose of D-serine. Here we show that treatment with a high dose of D-serine induced apoptosis followed by the production of H_2O_2 as a result of DAO's catalytic activity in C6 cells, suggesting that astroglial DAO is involved in regulation of the extracellular level of D-serine, a neuromodulator of the NMDA receptor.

MATERIALS AND METHODS

Reagents and Antibodies—3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and 3-amino-1,2,4-triazole (3-AT) were purchased from Sigma, St. Louis, MO, USA. The DAO inhibitors, *i.e.*, sodium benzoate and chlorpromazine hydrochloride (CPZ), were purchased from Sigma and Wako, Osaka, Japan, respectively. The anti–rat liver catalase IgG was a gift from Dr. S. Yokota (Biology Laboratory, Yamanashi Medical University, Japan). Texas Red–conjugated anti–rabbit IgG and FITC-conjugated anti–mouse IgG were purchased from ICN Pharmaceuticals, Inc., Aurora, OH, USA, and Zymed Laboratories, Inc., San Francisco, CA, USA, respectively. Monoclonal mouse anti–human catalase antibodies were obtained from Sigma. Rabbit polyclonal antibodies raised against cleaved caspase-3 were purchased from Santa Cruz Biotechnology, Santa Cruz, CA, USA.

Cell Culture—Rat C6 cells (Dainippon Pharmaceutical Co., Osaka, Japan) were maintained in DMEM/F12 (Invitrogen Life Technologies, Carlsbad, CA, USA) containing 15% (v/v) horse serum, 2.5% (v/v) fetal bovine serum (FBS), and 1% penicillin-streptomycin at 37°C under a humidified atmosphere containing 5% CO_2. Rat primary type-1 astrocytes were prepared as described previously (*12*). Briefly, mixed glial cultures were prepared from Sprague-Dawley rat cerebral cortex or cerebellum on postnatal days 1–2 in poly-D-lysine–coated culture flasks containing Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% (v/v) FBS. After 10–14 days of culture, astrocytes were isolated by shaking to dislodge microglia and subsequently purified by cytosine arabinoside treatment. The purified astrocytes were replated onto multi-well plates for assays.

Stable Cell Lines Overexpressing DAO—Rat C6 cells were transfected, using Effectene® Transfection Reagent (Qiagen, Valencia, CA, USA), with a plasmid encoding mouse DAO (pEF-BOSneo-mDAO). This plasmid was constructed by subcloning a cDNA fragment of mouse DAO (*5*) into the vector pEF-BOSneo (*24, 25*). Resistant clones were screened using G418 (400 μg/ml). Subsequently, several clones that overexpressed mouse DAO were selected by Western blotting using a rabbit polyclonal antibody against pig kidney DAO. One of the clones exhibiting the highest level of expression was designated as C6/DAO and analyzed in the present study. Cells were maintained in DMEM/F12 containing 15% (v/v) horse serum, and 2.5% (v/v) FBS supplemented with 250 μg/ml of G418.

Western Blot Analysis—For Western blot analysis, cells were collected by scraping in phosphate-buffered saline (PBS) and then treated with a lysis buffer (137 mM NaCl, 15 mM EGTA, 1 mM Na_3NO_4, 15 mM $MgCl_2$, 0.1% Triton X-100, and 25 mM MOPS) containing Complete™ protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). Cells were disrupted by sonication, extracted at 4°C for 30 min, and then centrifuged at 16,000 × *g* for 20 min. Protein samples resuspended in denaturating sample buffer were subjected to electrophoresis on 12.5% polyacrylamide gels (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan), followed by blotting onto Immobilon-P membranes (Millipore, Bedford, MA, USA). Detection of each protein was carried out with an ECL® Western blotting detection system (Amersham Biosciences, Little Chalfont, UK) according to the manufacturer's instructions.

Subcellular Fractionation—C6/DAO cells (2×10^6) were seeded into 100-mm dishes and incubated for 48 h. Cells were harvested by trypsinization, and washed twice in cold PBS. Then, they were divided into two tubes to prepare whole cell extracts and subcellular fractions. For subcellular fractionation, cells were first centrifuged at 200 × *g* for 10 min, and the resulting pellet was resuspended in 400 μl of cold hypotonic buffer (42 mM KCl, 10 mM HEPES, 5 mM $MgCl_2$, and Complete™ protease inhibitor cocktail) and then incubated for 30 min on ice. Cells were then broken by passing the cell suspension through a 30 G needle, followed by incubation for 30 min on ice. The intact nuclei, cell debris, and relatively heavy cellular membranes were initially removed by centrifugation at 1,000 × *g* for 10 min. The supernatants collected were centrifuged at 8,000 × *g* for 10 min to remove heavy membranes. Subsequently, the light membrane and cytosolic fractions were separated by centrifugation at 100,000 × *g* for 10 min. All pellets were resuspended in 400 μl of cold extraction buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and Complete™ protease inhibitor cocktail in 2× PBS). After vortexing and incubation for 30 min on ice, extracts were obtained as supernatants by centrifugation at 16,000 × *g* for 20 min.

Cell Viability—The cytotoxic effects of several amino acids including D-serine were examined by means of the MTT assay. Cells (1×10^4) were seeded onto 96-well plates. After incubation for 24 h, cells were treated with several concentrations of amino acids and H_2O_2, respectively. At 21 h after incubation, 50 μl of the MTT (1 mg/ml) solution was added, followed by incubation an additional 4 h. After centrifugation, the supernatant was removed from each well and 150 μl of dimethylsulfoxide was added to dissolve the insoluble formazan crystals. The absorbance was measured at 550 nm.

H_2O_2 Assay—The production of H_2O_2 from C6/DAO cells after D-serine treatment was measured with an Amplex® Red Kit according to the manufacturer's instructions (Molecular Probes, Inc., Eugene, OR, USA). Briefly, cells were seeded at a density of 1×10^4 cells/well onto 96-well plates. After 36 h incubation, the cells were washed once in 50 μl of Hank's balanced salt solution and then incubated

for 1 h with an Amplex Red reagent mixture containing several concentrations of D-serine. Fluorescence was measured with a fluorescence microplate reader with excitation at 530 nm and detection at 590 nm.

Immunocytochemistry—C6/DAO cells (3×10^5) were seeded onto poly-D-lysine–coated coverslips and then incubated for 24 h at 37°C. The cells were washed in 0.1 M PBS and fixed in cold methanol at –30°C. The organellar membranes were then permeabilized in 0.1 M PBS with 0.5% Triton X-100 plus 10% goat serum for 5 min. Subsequently, the cells were blocked for 30 min with 10% (v/v) goat serum, and then incubated with rabbit anti–pig kidney DAO antibodies (1:250) and monoclonal mouse anti-human catalase antibodies (1:2,000) overnight at 4°C. After being washed in cold PBS, the cells were treated with Texas Red–conjugated anti–rabbit IgG (1:100) and FITC-conjugated anti–mouse IgG (1:160). Immunofluorescent images were taken under a confocal laser scanning microscope.

TUNEL Assay—C6/DAO cells were seeded at a density of 2×10^4 cells/well on 8-well chamber slides. After 36 h incubation, the cells were treated with or without 30 mM D-serine for 24 h. They were then subjected to fluorescence-terminal dUTP nick-end labeling (TUNEL) using an *In Situ* Cell Death Detection Kit (Roche). Thereafter, the cells were mounted with propidium iodide (PI) on slides to label nuclei and then examined under a confocal laser scanning microscope.

Statistics—All data were expressed as the means ± SD (8 samples for each set of conditions) for three or more independent experiments. Statistical comparisons between different treatments were made using one-way ANOVA with the post-hoc Scheffé's test. Differences were considered significant if $P < 0.05$.

RESULTS

Incubation of Rat Primary Astrocytes with D-Serine—To clarify whether or not astroglial DAO is able to metabolize extracellular D-serine in the brain, we evaluated the cellular effect of D-serine on rat primary astrocytes. In this study, we hypothesized that extracelluar D-serine is metabolized through astroglial DAO activity, H_2O_2 thereby being produced, which may affect cell viability. Initially, the MTT assay was performed 21 h after D-serine (3 and 30 mM) treatment of rat cerebral cortex- and cerebellum-derived primary type-1 astrocytes. As shown in Fig. 1, cell viability decreased for both types of cells on D-serine treatment. In addition, the level of cell death for cerebellum-derived type-1 astrocytes was slightly higher than that for cerebral cortex–derived ones. This finding was in good accord with our previous report that the gene expression level of DAO is higher in type-1 astrocytes from the cerebellum than in those from the cerebral cortex (*12*). These results show that the application of a high dose of D-serine induce astroglial cell death, implicating cellular DAO activity in extracellular D-serine metabolism.

Overexpression and Subcellular Localization of DAO—In order to enhance the enzyme activity of DAO in cells, we established a stable transformant of rat C6 cells overexpressing DAO (C6/DAO). Western blot analysis revealed that DAO was highly expressed in C6/DAO cells but only weakly expressed in C6 ones (Fig. 2A). H_2O_2 is a ROS produced by oxidases, as well as through spontaneous or

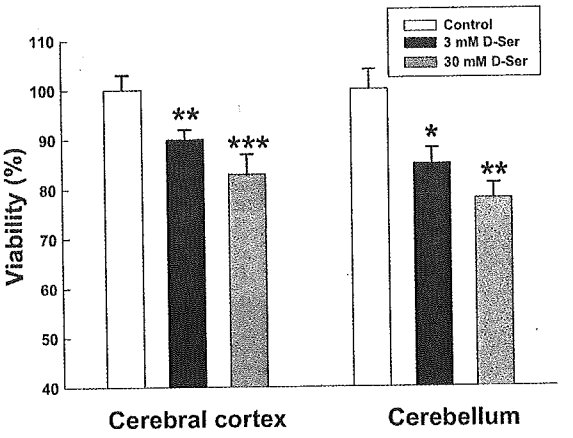

Fig. 1. **Effect of D-serine on viability of primary astrocytes.** The viability of primary astrocytes was examined by means of the MTT assay after treatment with 3 and 30 mM D-serine for 21 h. Type-1 astrocytes from cerebral cortex and cerebellum are shown on the left and right, respectively. The viability of untreated cells was taken as 100%, and the data are means ± SD of the percentage of the control values. The experiments are representative of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with control (ANOVA with post-hoc Scheffé's test).

enzymatic superoxide anion dismutation. A catalase that plays a role in detoxifying H_2O_2 was similarly detected in both types of cells, indicating that the DAO overexpressed in the cells had no influence on the expression of other peroxisomal enzymes.

The subcellular localization of the overexpressed DAO in C6 cells monitored with the peroxisomal marker catalase is shown in Fig. 2, B and C. Western blotting showed that most of the DAO was present in the heavy membrane fraction, which contained numerous peroxisomes, as judged on detection of a peroxisomal marker enzyme (Fig. 2B). This finding is well supported by the presence of a peroxisomal targeting signal at the C-terminus of mouse DAO and other mammalian DAOs (*2, 3, 5, 26*). Additional evidence of peroxisomal localization of overexpressed DAO in C6 cells was obtained by means of immunocytochemistry. As shown in Fig. 2C, co-localization of DAO and catalase was demonstrated on merging of the confocal images showing DAO (Red) and catalase (Green). Peroxisomes containing DAO in C6 cells appeared as yellow dots in the merged images. Virtually all the dots stained for the peroxisomal marker catalase were also positive for DAO. A similar punctate pattern of the intracellular distribution of peroxisomes was observed with the same monoclonal anti-catalase antibodies in HepG2 cells (*27*). Taken together, these results demonstrated that most of the DAO overexpressed in C6/DAO cells was recruited to peroxisomes containing catalase.

Cell Viability and Production of H_2O_2 in C6 Cells Treated with D-Serine—To confirm the effect of D-serine on primary astrocyte cultures, we also evaluated the viability of C6 and C6/DAO cells after D-serine treatment. The MTT assay was performed 21 h after treatment with 5, 10, 20 or 40 mM D-serine. As shown in Fig. 3A, the cell viability decreased in a dose-dependent manner. Based on

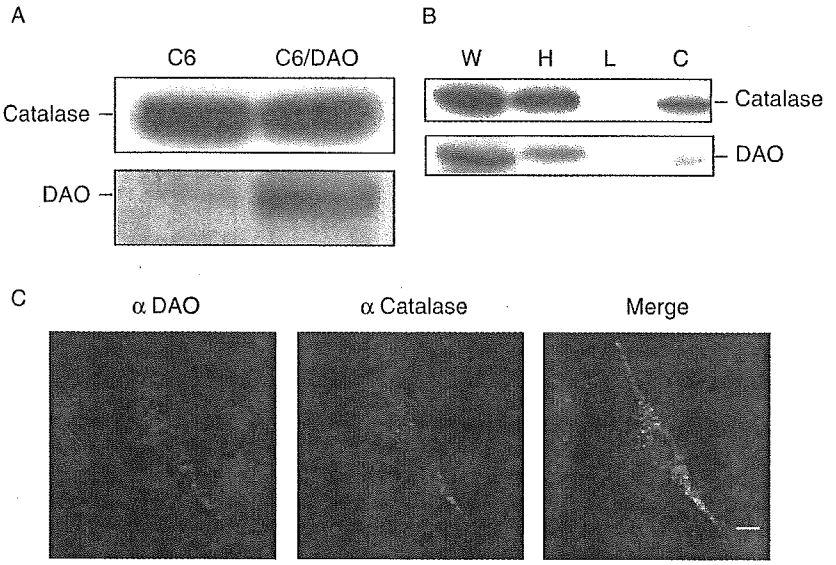

Fig. 2. **Expression and subcellular localization of DAO.** (A) Expression levels of DAO (lower) and catalase (upper) in C6 and C6/DAO cells, as determined on Western blotting. Each lane contains 15 μg of cell extract. The blots were probed with rabbit anti-pig kidney DAO antibodies and anti-rat liver catalase IgG, respectively. Membranes were then incubated with a horseradish peroxidase-conjugated secondary antibody. (B) Subcellular localization of DAO determined by Western blotting. Cellular proteins of C6/DAO were separated into whole cell lysate (W), heavy membrane (H), light membrane (L), and cytoplasmic (C) fractions. Protein samples (20 μl) were separated by electrophoresis on 12.5% polyacrylamide gels. Rabbit anti-pig kidney DAO antibodies (1:1,000) and anti-rat liver catalase antibodies (1:1,000) followed by a horseradish peroxidase-conjugated secondary antibody were used for Western blotting of DAO and catalase, respectively. Major components in the H fraction are mitochondria, lysosomes and peroxisomes, while those in the L fraction are microsomes, endoplasmic reticulum and Golgi apparatus. (C) Subcellular localization of DAO determined by immunocytochemistry. Rabbit anti-pig kidney DAO antibodies and monoclonal mouse anti-human catalase antibodies followed by Texas Red-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG were used for immunostaining of DAO and catalase, respectively. Scale bar, 10 μm.

the data shown in Fig. 3A, the viability of C6 cells after 30 mM D-serine treatment is expected to decrease to the level observed in primary astrocyte cultures. Therefore, we consider C6 cells comparable to primary astrocyte cultures in terms of D-serine metabolism. Moreover, the cytotoxic effect of D-serine on C6/DAO cells was greater than that on C6 cells, indicating that the cytotoxicity of D-serine in glial cells is dependent on the intracellular level of DAO.

We next examined the correlation between the production of H_2O_2 during the metabolism of D-serine and glial cell death. The production of H_2O_2 in C6/DAO cells was measured after treating the cells with various concentrations of D-serine for 1 h. As shown in Fig. 3B, C6/DAO cells treated with 20 and 40 mM D-serine exhibited an increase in H_2O_2 compared with vehicle-treated control cells. These results indicated that one of the possible causes of the astroglial cell death after D-serine treatment is H_2O_2. Although low doses of D-serine (5 and 10 mM) did not result in enhanced H_2O_2 production in this study, this apparent absence of H_2O_2 production might reflect the level of endogenous catalase, as can be seen in Fig. 2A.

Involvement of H_2O_2 in Cytotoxicity—To examine the cytotoxic effect of H_2O_2 on C6 and C6/DAO cells, we evaluated the cell viability at 21 h after H_2O_2 treatment. As shown in Fig. 4A, the cell viability decreased in a dose-dependent manner. To test the hypothesis that the cytotoxicity induced by D-serine in glial cells results from the production of H_2O_2, we evaluated cell viability after pretreatment with a catalase inhibitor, 3-amino-1,2,4-triazole (3-AT) (*28*), followed by D-serine treatment. As shown in Fig. 4B, 3-AT enhanced the cytotoxic effect of D-serine on both C6 and C6/DAO cells, indicating inhibition of cellular catalase activity. These results suggest that H_2O_2 is the causative agent of cell death induced by D-serine.

Effects of DAO Inhibitors Sodium Benzoate and Chlorpromazine on D-Serine-Induced Cytotoxicity—To verify that the cellular DAO activity is involved in the cell death induced by D-serine, sodium benzoate, a competitive inhibitor of DAO, was added to cells 30 min before exposure to 10 mM D-serine. In order to exclude a direct cytotoxic effect of sodium benzoate, but to obtain maximal inhibition, 20 mM sodium benzoate was used in the assay. Although the recovery of C6 cells pretreated with sodium benzoate was minimal, that of C6/DAO cells was significant (Fig. 5A). We did not observe a protective effect of sodium benzoate at 10 mM in C6/DAO cells treated with D-serine (data not shown).

We next examined another DAO inhibitor, chlorpromazine (CPZ), which is a classical antipsychotic drug and has been reported to inhibit DAO in competition with its coenzyme, flavin adenine dinucleotide (*23*). As shown in Fig. 5B, pretreatment with CPZ at a concentration of 1 μM significantly prevented the cell death induced by D-serine (10 mM) in C6/DAO cells. Taken together, these results indicated that astroglial DAO is involved in D-serine-induced cell death due to its D-serine metabolizing activity.

Amino Acid Specificity of Astroglial Cell Death—It has been reported that D-serine could be taken up by C6 cells

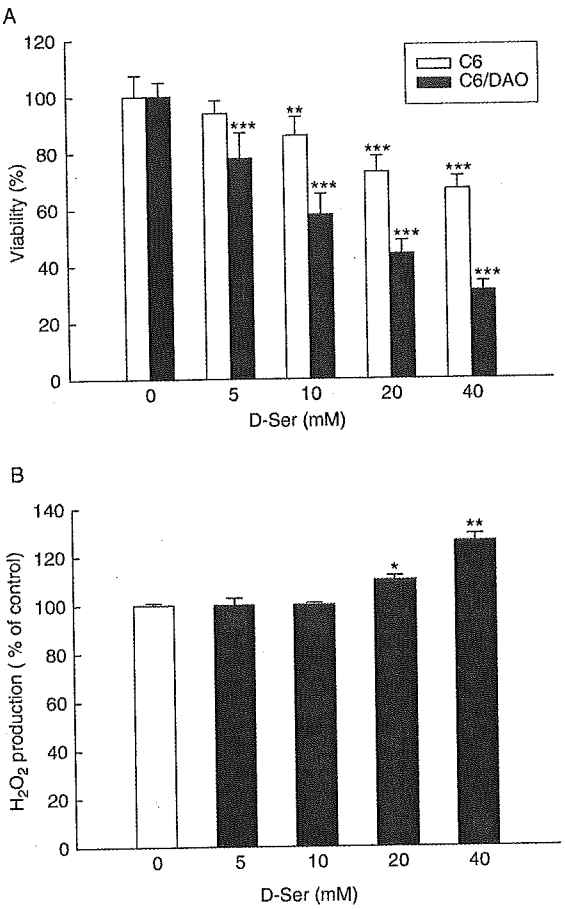

Fig. 3. **Effects of D-serine on cell viability and H_2O_2 production.** (A) The viability of C6 and C6/DAO cells was examined by means of the MTT assay after treatment with the indicated concentrations of D-serine for 21 h. (B) The production of H_2O_2 by C6/DAO cells was measured using a H_2O_2 detection kit after treatment with the indicated concentrations of D-serine for 1 h. The levels of viability and H_2O_2 production in untreated cells were taken as 100%, and the data are means ± SD of the percentage of the control values. The experiments are representative of three independent experiments. $^*P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with control (ANOVA with post-hoc Scheffé's test).

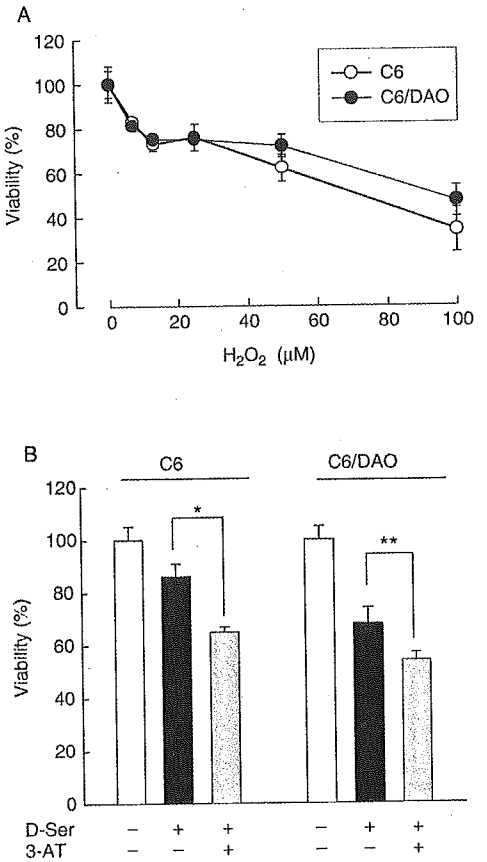

Fig. 4. **Effects of H_2O_2 and 3-AT on cell viability.** (A) The cytotoxic effect of H_2O_2 on glial cells was examined by means of the MTT assay. C6 and C6/DAO cells were treated with the indicated concentrations of H_2O_2 for 21 h. (B) Effects of catalase inhibition by 3-AT on C6 and C6/DAO cells were examined by means of the MTT assay. Both types of cells were pretreated with 20 mM 3-AT for 30 min before D-serine treatment. The cells were then treated with 10 mM D-serine for 21 h. The viability of untreated cells was taken as 100%, and the data are means ± SD of the percentage of the control values. The experiments are representative of three independent experiments. $^*P < 0.05$ and $^{**}P < 0.01$, compared with D-serine treatment (ANOVA with post-hoc Scheffé's test).

and that the properties of the uptake system resembled those of an ASCT2-like neutral amino acid transport system (*29*). Since this transport system showed broad substrate specificity and a higher affinity for L-serine than D-serine, we expected the cell death induced by D-serine to be common to other D- and L-amino acids. To examine this possibility, we determined the effects of various amino acids (3 and 30 mM) on C6 and C6/DAO cells. As shown in Fig. 6, the treatment of C6/DAO cells with 3 mM D-serine and D-serine plus glycine effectively decreased cell viability, whereas the treatment of C6 cells with other amino acids examined did not affect cell viability. In addition, the treatment of C6/DAO cells with 30 mM D-serine and glycine induced a greater increase in cell death compared with 3 mM treatment, whereas other amino acids examined did not exhibit a significant effect on cell viability. Although a previous *in vitro* experiment involving the purified pig DAO enzyme showed that D-alanine and D-proline were better substrates than D-serine (*30*), D-serine and glycine decreased cell viability most effectively in C6/DAO cells. It is of note that glycine, a poor substrate of DAO *in vitro*, exhibited a cytotoxic effect on C6/DAO cells and did not interfere with D-serine–induced cell death. These results suggest that astroglial cells possess an uptake system that preferentially transports D-serine among other D-amino acids, and that astroglial DAO can metabolize extracellular D-serine effectively.

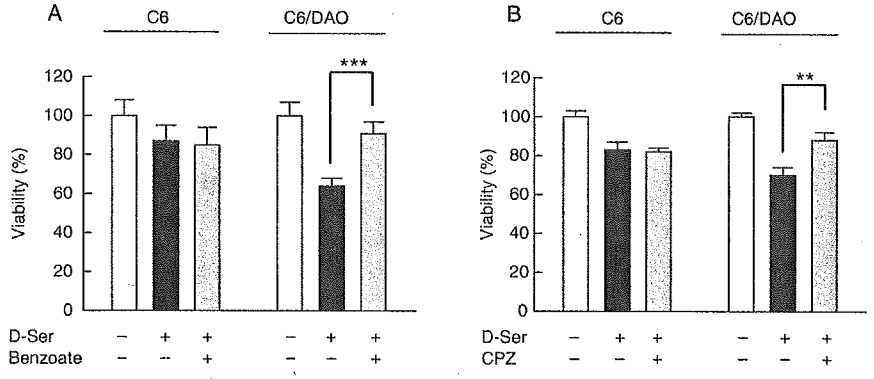

Fig. 5. **Effects of sodium benzoate and chlorpromazine on D-serine-induced cytotoxicity.** (A) C6 and C6/DAO cells were pretreated with 20 mM sodium benzoate for 30 min before D-serine treatment. (B) C6 and C6/DAO cells were pretreated with 1 μM chlorpromazine (CPZ) for 30 min before D-serine treatment. The cells were then treated with 10 mM D-serine for 21 h. Cell viability was measured by means of the MTT assay. The viability of untreated cells was taken as 100%, and the data are means ± SD of the percentage of the control values. The experiments are representative of three independent experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$, compared with D-serine treatment (ANOVA with post-hoc Scheffé's test).

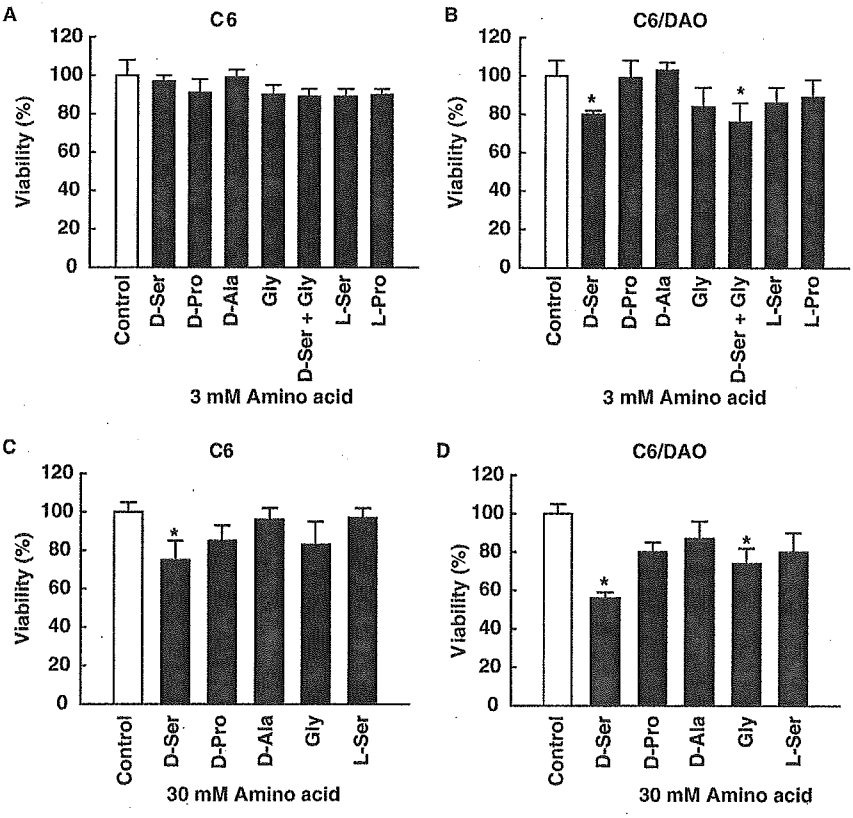

Fig. 6. **Effects of various amino acids on cell viability.** The MTT assay was performed after treatment with 3 mM (A, B) and 30 mM (C, D) amino acids in C6 (A, C) and C6/DAO cells (B, D) for 21 h. The viability of untreated cells was taken as 100%, and the data are means ± SD of the percentage of the control values. The experiments are representative of four independent experiments. $^{*}P < 0.05$, compared with control (ANOVA with post-hoc Scheffé's test).

Apoptosis in Astroglial Cells—We next examined whether or not the decrease in the number of viable cells after D-serine treatment was due to apoptosis, since H_2O_2 has been shown to induce programmed cell death. A TUNEL assay was performed 21 h after treatment with or without 30 mM D-serine. As shown in Fig. 7, D-serine–treated cells clearly showed TUNEL-positive nuclei compared with control cells. Arrows indicate strong TUNEL-positive nuclei.

To confirm the D-serine–induced apoptosis, we investigated the effect of D-serine on caspase-3 activation in C6/DAO cells by Western blot analysis, since the activation of caspase-3 by H_2O_2 has been reported in several cell types such as hippocampal neurons (*31*) and PC12 cells (*32*). As shown in Fig. 7G, treatment of C6/DAO cells with D-serine induced activation of caspase-3, producing an active subunit of about 20 kDa in size. Treatment of C6/DAO cells with H_2O_2 (50 μM) was used as a positive

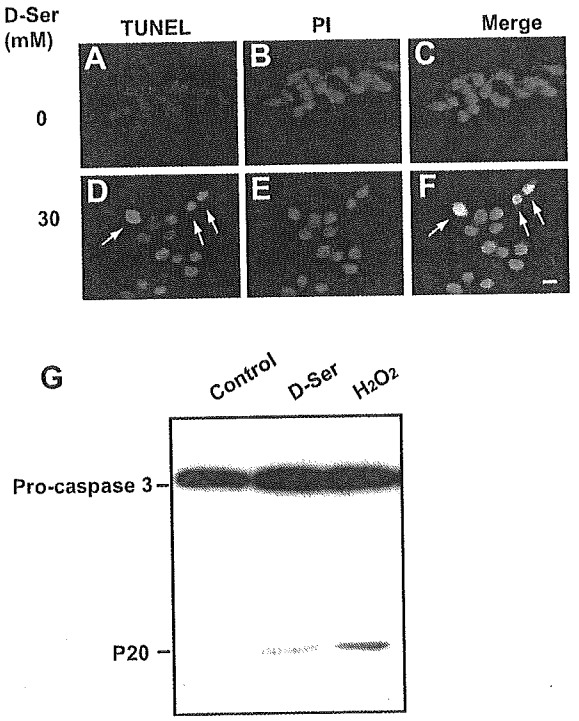

Fig. 7. **Apoptosis in astroglial cells.** (A–F) TUNEL-staining was performed after treatment with or without 30 mM D-serine for 21 h. (A–C) Vehicle-treated cells; (D–F) D-serine–treated cells. Cell nuclei were counterstained with PI (red). Arrows indicate strong TUNEL-positive cells. Scale bar, 10 μm. (G) Western blots show the effect of D-serine on caspase-3 activation. C6/DAO cells were incubated with D-serine (30 mM) and H_2O_2 (50 μM) for 21 h before assay. Cell lysates (20 μg) were subjected to electrophoresis on 15% polyacrylamide gels followed by transfer to Immobilon-P membranes and then incubation with rabbit anti-caspase-3 polyclonal antibodies (1:200). The secondary antibody reaction was performed as in Fig. 2.

control. Taken together, we consider that the astroglial cell death induced by D-serine treatment comprises apoptosis.

DISCUSSION

In the present study, we examined the effect of treatment with an artificially high dose of D-serine on glial cells to investigate the potential role of DAO in the brain system. We extended our studies from primary cultures of astrocytes to C6 cells, because C6 cells have frequently been used as a model of glial function and are considered to correspond to type-1 astrocytes, based on Ran-2 expression (*33*). The advantage of using a high dose of D-serine is that the involvement of astroglial DAO activity in the metabolism of D-serine can be detected through the cytotoxicity of the metabolite, H_2O_2. Previously, histochemical analysis revealed that DAO activity was absent in the forebrain, but present in the brain stem, cerebellum and spinal cord (*34*). However, we have reported that the gene expression of DAO was detected in C6 cells and in type-1 astrocytes from the cerebral cortex as well as the cerebellum (*12*). Moreover, we have observed that high-dose D-serine treatment induced the death of cerebral cortex–derived type-1 astrocytes (Fig. 1), suggesting that astroglial DAO from the cerebral cortex could be involved in the metabolism of D-serine.

Although the substrate of DAO *in vivo* was not known for many years, a substantial amount of free D-serine has been found in the mammalian brain (*13*). D-Serine is an agonist of the NMDA receptor (*15*) and is present at a low micromolar concentration in the mammalian brain (*16*). Moreover, D-serine has been implicated in several pathophysiological conditions related to NMDA receptor dysfunction. Therefore, it is considered that regulatory mechanisms governing the extracellular level of D-serine exist in the brain. Possible mechanisms are as follows.

First, in view of the production of D-serine, the extracellular concentration might be regulated by the activity of serine racemase, which is known to convert L-serine to D-serine. Serine racemase was found to be highly expressed in the brain and to be localized to astrocytes with a similar distribution to that of D-serine (*35*). The pharmacological inhibition of this enzyme decreased the level of D-serine (*36*), suggesting that this enzyme is involved in the regulation of the synaptic concentration of D-serine.

Second, it is possible that D-serine could be removed from the synaptic cleft by specific amino acid transporters. To date, the transport system most associated with serine has been reported to be system ASC, although uptake of D-serine may also occur through system L (*37*). In C6 cells, the D-serine uptake system showed broad substrate specificity and higher affinity for L-serine than for D-serine (*29*). In cortical astrocytes, D-serine has also been reported to be transported *via* a similar pathway (*38*). However, it is unlikely that these transport systems with broad substrate specificity act as regulators of the D-serine level, which apparently requires fine and specific regulation. NMDA receptors are regulated by the endogenous amino acids glycine and D-serine, which bind to an NMDA-associated glycine-binding site. In our experiment, D-serine and glycine decreased cell viability most effectively in C6/DAO cells (Fig. 6), although D-proline and D-alanine are better substrates than D-serine for the pig kidney DAO enzyme activity *in vitro* (*30*). Therefore, our data support the possibility that astroglial cells have a specific uptake system for D-serine with high affinity. Furthermore, it is interesting that D-serine plus glycine had a slightly increased cytotoxic effect on C6/DAO cells, although glycine is a poor substrate of DAO *in vitro*. In a previous uptake study, glycine reduced the accumulation of D- and L-serine in C6 cells (*29*). These observations suggested that glycine may also be transported and can be catalyzed by DAO.

Third, astroglial DAO may play a role in modulating the extracellular level of D-serine in the brain. In this study, we have shown that D-serine treatment induced the death of primary astrocytes and C6 cells. In addition, overexpression of DAO in C6 cells (C6/DAO cells) markedly increased the D-serine–induced cytotoxicity. These results indicate the possible involvement of astroglial DAO in the cytotoxic effect of a high dose of D-serine. Meanwhile, it is unlikely that the changes in cell viability observed with high doses of D-serine are due to non-specific effects such as high osmolality, because other amino acids did not have cytotoxic effects at the same doses (Fig. 6). However, it can not