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〔特集：統合失調症グルタミン酸系治療薬の臨床開発と基礎研究〕

NMDA受容体-D-セリン系を標的とした新規統合失調症治療薬の開発*

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要約：フェンサイクリジン（phencyclidine: PCP）を初めとするNMDA型グルタミン酸受容体遮断薬が，その力価に比例した統合失調症様の陽性・陰性症状と認知機能障害を引き起こすことから，NMDA受容体機能を促進する新規治療薬開発により，既存の抗精神病薬に反応する陽性症状だけでなく，難治性の症状も改善することが予測される．実際に，NMDA受容体グリシン調節部位を刺激し本受容体機能を促進する薬物が，NMDA受容体遮断薬を投与した動物の抗精神病薬抵抗性の異常行動を抑制し，抗精神病薬服用中の統合失調症患者の陰性症状や認知機能障害を改善することが報告されている．しかし，現在臨床試験が行われている，グリシン，D-セリン，D-アラニン，D-サイクロセリン，グリシン取込阻害薬などのグリシン調節部位の作動薬は，脳への移行性，選択性，腎毒性，あるいは部分作動薬としての作用などの問題があり，NMDA受容体機能賦活薬開発に向けた別の戦略が求められている．この点から注目される標的の1つとして，内在性D-セリンの代謝・機能システムが挙げられる．すなわち，D-セリンは，(1) グリシンとともにNMDA受容体グリシン調節部位を刺激する本受容体のコアゴニストであるが，グリシンとは異なりNMDA受容体への選択的性が高い，(2) 脳選択的でグリシン調節部位結合密度と酷似した分布を示す，(3) ニューロン-グリア間相互作用に関与する，等の点から，NMDA受容体機能を選択的・効率的に促進するためには，内在性D-セリンシグナルを増強する方法を開発するのが合理的と考えられ，細胞外D-セリン濃度を調節する，グリア細胞を含む分子細胞機構の解明が急がれる．この検討は，統合失調症の病態の理解にも新たな視点をもたらす可能性がある．

キーワード：統合失調症，抗精神病薬抵抗性症状，フェンサイクリジン，NMDA型グルタミン酸受容体，D-セリン

グルタミン酸（Glu）系が統合失調症の病態に関連している可能性は，本症の脳脊髄液中Glu濃度の減少を見いだしたKimら（1980）によって初めて提唱された．その後，筆者ら（1983）が統合失調症患者死後脳でカイニン酸型Glu受容体結合の上昇を検出したほか，脳におけるGlu伝達関連分子の変化が報告され，この仮説が支持された．同じ頃に，統合失調症様の陽性・陰性症状と認知機能障害を引き起こす薬物として1950年代から知られていた，フェンサイクリジン（phencyclidine: PCP）が，強力なNMDA型Glu受容体遮断作用をもつことが明らかにされ，次のような観察と合わせて，統合失調症では少なくとも本受容体機能が低下していると，広く考えられるようになった（西川，2005，2006）：①PCPのほか，ketamine，dizocilpineを初めとするNMDA受容体遮断薬は例外なく統合失調症様の陽性ならびに陰性症状を引き起こす（図1），②精神異常を誘発する力価はNMDA受容体遮断作用と相関しており，例えばketamine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時の血中濃度は，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．

以上の所見に基づいて，NMDA受容体は陰性症状・認知機能障害等の難治性統合失調症状の治療薬開発の標的として注目され，基礎的・臨床的研究が精力的に進められている（Kantrowitz and Javitt, 2010; Labrie and Roder, 2010; 西川，2005; 山本ら，2007）．本稿では，その現状と問題点を概観し，NMDA受容体の内在性調節因子の1つであるD-セリンの視点から，今後の戦略について述べてみたい．

I. 薬理学的に見た統合失調症の病態におけるNMDA受容体機能不全の位置づけ

従来，統合失調症の治療薬（抗精神病薬）が陽性症状を改善する力価とD2型ドーパミン（DA）受容体遮断作用が正比例し，DA作動薬が統合失調症様陽性症状を惹起するため，陽性症状には主にD2受容体を介するDA伝達の亢進が関与することが推測されてきた（西川，2006）．また，第二世代の抗精神病薬がセロトニン（5-

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略語 **DA**: dopamine（ドーパミン），**DAO**: D-amino acid oxidase, **Asc-1**: alanine-serine-cysteine transporter 1, **dsr-2**: D-serine responsive transcript-2, **dsm-1**: D-serine modulator-1, **Glu**: glutamate（グルタミン酸），**5-HT**: 5-hydroxytryptamine（セロトニン），**PAPST-1**: 3′-phosphoadenosine 5′-phosphosulfate transporter-1, **PCP**: phencyclidine（フェンサイクリジン），**NMDA**: N-methyl-D-aspartate
注）図2のみで使用した略語は図2の説明を参照．

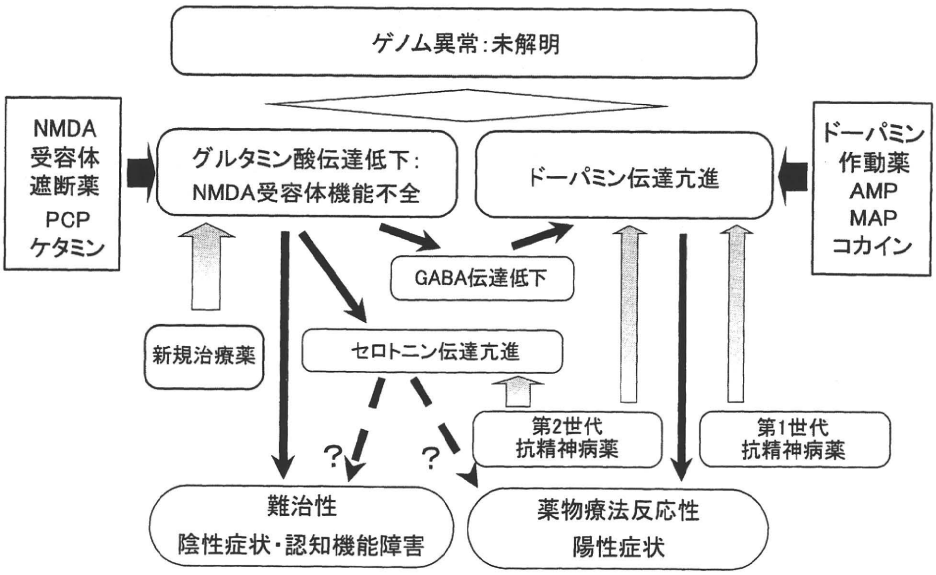

図1 薬理学的に見た統合失調症と神経伝達系との関連（仮説）．NMDA受容体，DA伝達系，セロトニン伝達系およびGABA伝達系などに作用する薬物の作用に基づいた，統合失調症状の発現機序と治療効果の標的を模式的に示した（説明は本文参照）．代謝型Glu受容体との関連性については本特集の茶木論文を参照．統合失調症には異なる原因をもつ複数の疾患が含まれると考えられることから（異質性），本図の仮説はすべての統合失調症患者にあてはまるものではないことに留意する必要がある．

hydroxytryptamine: 5-HT）伝達系にも強い抑制作用をもつことから（5-HT2受容体遮断作用，5-HT1A受容体刺激作用等），5-HT伝達系の亢進も統合失調症状の一部に関係する可能性がある．実際，5-HT2A受容体選択的な作動薬のN, N-dimethyltryptamineの二重盲検試験（Gouzoulis-Mayfrank et al, 2005）では，健常ボランティアに統合失調症様のformal though disorderや不適切な感情表出が認められた結果が報告されている．

近年の臨床的・基礎的研究は，NMDA受容体機能低下によって，統合失調症様の陽性・陰性症状と認知機能障害が出現することと，上述した，本症で推定されるDA伝達系・5-HT伝達系の障害に相互関係があることを示唆している．すなわち，PETを用いた研究において，統合失調症患者ではamphetamine誘発性の脳内DA遊離が健常者より多いことが指摘されてきたが，NMDA受容体遮断薬のketamineを投与された健常ボランティアでも，統合失調症様の精神変調が見られるだけでなく，このamphetamine投与後のDA遊離の上昇が増強される（Laruelle et al, 2005）．

さらに，実験動物では，NMDA受容体遮断薬を急性投与すると，大脳皮質を中心にDA伝達が亢進し（Kantrowitz and Javitt, 2010; Umino et al, 1998），前頭葉皮質・線条体・側坐核等の細胞外5-HT放出が増加する（Yan et al, 1997）．DA伝達系については，NMDA遮断薬を反復投与すると前頭葉皮質のDA代謝がかえって低下するという報告もあるが，amphetamineをチャレンジした時のDA遊離は，統合失調症患者に類似して，前頭葉皮質と線条体の双方で増強している（Kantrowitz and Javitt, 2010）．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（Mohn et al, 1999）も考え合わせると，NMDA受容体機能が低下した状態では，DA伝達が亢進しやすいと推測される．

これらのデータは，一群の統合失調症では，NMDA受容体を介するGlu伝達が減弱するため，DA伝達が過剰になった結果陽性症状が出現し，5-HT系を含むDA系以外の分子カスケードの異常により陰性症状や認知機能障害が引き起こされる可能性を示唆している（図1）（西川，2006）．すなわち，NMDA受容体機能促進薬は，単独でも，過剰なDAおよび5-HT伝達の抑制を通して，既存の抗精神病薬に反応する症状に効果を示し，その他の分子カスケードの障害を修復することによって抗精神病薬抵抗性症状を改善することが期待される．

II. NMDA受容体機能促進薬の統合失調症に対する臨床試験

NMDA受容体機能促進薬は，実験動物において統合失調症モデルと考えられる，種々の障害に対する改善効果が認められ（Contreras, 1990; Hashimoto et al, 1991; Kantrowitz and Javitt, 2010; Tanii et al, 1991, 1994），小規模ながら，臨床試験においても，従来の抗精神病薬が奏効しない陰性症状や認知機能障にも有効であることが示唆されている（Kantrowitz and Javitt, 2010; Kantrowitz et al, 2010; Labrie and Roder, 2010; Lane et al, 2010）．

初期の基礎研究として，筆者らは，①D-セリンやD-アラニンは，PCPが誘発する異常行動や前頭葉皮質のDA伝達亢進あるいはDA作動薬MAPによる移所運動量増加

を軽減するが，これらのアミノ酸のL体は無効なこと（Kanematsu et al, 2006; Tanii et al, 1991, 1994; 山本ら，2007），②NMDA受容体グリシン調節部位の選択的遮断薬により①の軽減効果は阻害されること（Tanii et al, 1994）などを明らかにし，ほぼ同時期にNMDA受容体遮断薬誘発性異常行動に対するD-セリンの拮抗作用を報告したContreras（1990）とともに，グリシン調節部位の作動薬（NMDA受容体コアゴニスト）が既存の薬物より優れた抗精神病薬になる可能性を実験データに基づいて提唱した．

臨床的には，その後，グリシン，D-セリン，D-アラニン，D-サイクロセリン，I型グリシントランスポーター阻害薬サルコシンなどのいずれかを，既存の抗精神病薬を服用中の患者に投与する「Add-on治療」の臨床試験が行われた．Risperidone, quetiapine, olannzapine, 第一世代抗精神病薬などと併用した場合は，陰性症状・認知機能障害のスコアの改善度が既存薬単独より有意に高かった（表1）（Kantrowitz et al, 2010; Labrie and Roder, 2010; Lane et al, 2010; 山本ら，2007）．メタ解析でも有効性が支持されているが（Tuominen et al, 2006），効果は強いとは言えず，長期投与試験では否定的な結果も報告されている（Labrie and Roder, 2010; 山本ら，2007）．また，clozapineとの併用では相加効果は認められないため（Labrie and Roder, 2010; 山本ら，2007），clozapineとNMDA受容体機能との相互作用の検討も重要と考えられる．一方，最近行われた高用量のD-セリン投与試験においては，齧歯類の実験から懸念されていた腎臓毒性が見られた患者が報告されており（Kantrowitz et al, 2010），臨床使用への警鐘と捉える必要がある．上記の臨床試験は海外で行われたものであるが，現在，我が国でも新規のI型グリシントランスポーター阻害薬やD-サイクロセリンの臨床試験が進行中である．

現在までに使用されている試験薬には，脳への低移行性，NMDA受容体への作用の非選択性（Labrie and Roder, 2010; 西川，2005）や，部分作動薬であるための低力価および治療域用量範囲の狭さ（Labrie and Roder, 2010; 西川，2005），既存の抗精神病薬とは異なる腎毒性等の副作用の恐れ（Kantrowitz et al, 2010）等の問題があり，効果が十分でないことや，長期使用の安全性が確保されていないことの原因となっていると推測される（表1）．また，NMDA受容体グリシン調節部位に直接結合するタイプの薬物は現状では技術的に開発が困難と判断されている．そこで，今後の開発研究では新たな分子標的を検討する必要がある．

III. D-セリンシステムの治療薬開発標的としての意義

統合失調症におけるNMDA受容体機能障害やその治療法の研究で，注目される生理活性物質の1つとして，本受容体の調節作用をもつ脳の内在性D-セリンが挙げられる．ここでは，D-セリンとその代謝や機能の分子機構について，新規治療薬開発における意義を考察する．

D-セリンは，グリシン，D-アラニンと同様に，単独ではNMDA受容体を介する神経伝達を生じないが，Gluが十分にNMDA受容体を活性化するためには不可欠であることが知られ，NMDA受容体のコアゴニスト（coagonist）と呼ばれている（図2）（西川，2005，2008）．筆者ら（Hashimoto et al, 1991; Tanii et al, 1991, 1994; 山本ら，2007）は，前項で述べたように，動物実験で抗精神病作用をもつ可能性を見いだしたが，この過程でD-アミノ酸としては例外的に，脳を中心に哺乳類の組織に一生の間高い濃度を保つことを発見した（西川，2005，2008）．さらに，NMDA受容体GRIN2B（NR2B）サブユニットと酷似した分布（成熟期では前脳部優位）を示すことを明らかにし，内在性のNMDA受容体制御因子であることを提唱した（西川，2008）．その後，前脳部組織のD-セリンを選択的に分解すると，グリシン調節部位に結合するもう1つの内在性物質のグリシンの濃度が変化しなくとも，NMDA受容体機能が低下するという実験結果が報告され，少なくとも前脳部ではD-セリンが，NMDA受容体の主要な内在性コアゴニストであると考えられている（西川，2008）．

さらに，①D-セリンの細胞外放出は，脱分極刺激よって増加せずにかえって減少し，神経インパルスの遮断により増加するのをはじめ，古典的な神経伝達物質とは著しく異なる機序で生ずる（西川，2008），②グリア選択的毒素によりグリア細胞の活性が低下した状態では細胞外D-セリン濃度が有意に低下する（Kanematsu et al, 2006），③NMDA受容体に作用するD-セリンがアストログリアから放出されることを示唆する所見がある（Henneberger et al, 2010; Panatier et al, 2008），④組織化学的にD-セリンはニューロンとグリアの双方に検出される（西川，2008），等の点から，D-セリンはシナプス-グリア相互作用に関与

表1 抗精神病効果が臨床的に検討されているNMDA受容体グリシン調節部位作動薬

アゴニスト（1日用量）	アゴニストとしての性質	選択性*	脳への移行	副作用
グリシン	Full agonist	非選択的	低い	けいれん閾値低下？
D-サイクロセリン	Partial agonist（治療用量域が狭い）	非選択的	高い	精神症状（高用量）
D-セリン	Full agonist	選択的	低い	腎毒性？
グリシントランスポーター阻害薬（Sarcosine）	Full agonist	非選択的？	低い？	けいれん閾値低下？
D-アラニン	Full agonist	選択的	低い	？

* NMDA受容体グリシン調節部位に対する選択性．

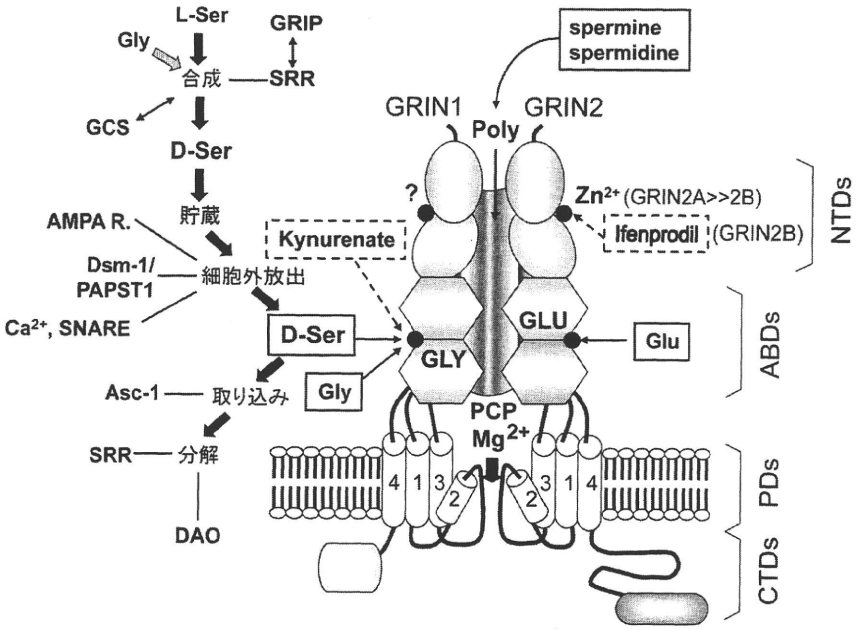

図2 D-セリンの代謝過程とGRIN1/GRIN2（NR1/NR2）型NMDA受容体イオンチャネル．D-セリンの代謝の分子機構は確立されていないため，現在推測されている過程と関連する候補分子を記載した．実線は直接の関係を示す実験データがあることを，両矢印は相互作用が示唆されることを表す．NMDA受容体は，細胞外からNa^+やCa^{2+}を流入させ，細胞内からK^+を透過させるイオンチャネルを構成しており，Glu結合部位（GLU），グリシン結合部位（GLY），マグネシウムイオン結合部位（Mg^{2+}），フェンサイクリジン結合部位（PCP），ポリアミン結合部位（Poly）などの，種々の調節部位をもつ．GRIN1サブユニット（多様なバリアントが存在）と4種のGRIN2サブユニットA～Dの少なくとも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ことが示唆されており，GLYはGRIN1上に，GLUはGRIN2上にあると考えられている．NMDA受容体の模式図はPaoletti & Neytonの総説（Curr Opin Pharmacol (2007) 7: 39-47）のFigure 1を，また図全体は筆者の総説（生化学 80: 267-276）の図1を改変．
ABDs: agonist binding domain（作動薬結合ドメイン），CTDs: C-terminal domains（C末端ドメイン），GCS: glycine cleavage system（グリシン開裂酵素系），NTDs: N-terminal domains（N末端ドメイン），PDs: pore domains（膜開口部ドメイン），SRR: serine racemase（セリンラセマーゼ），□ 作動薬，⬚ 遮断薬．

すると推測される．グリア細胞はシナプス伝達を修飾する働きをもつことが明らかになってきており，ニューロン間の伝達のファインチューニングを担っている可能性が指摘されている．

このようなD-セリンの特徴と，NMDA受容体グリシン調節部位の刺激が難治性統合失調症状を改善する点から見ると，D-セリンの細胞外濃度を調節する分子細胞機構を標的として，そのシグナルを高める操作が，NMDA受容体機能のファインチューニングを実現する治療薬創出に役立つと考えられる．

脳内では，内在性D-セリンの生合成，貯蔵，細胞外遊離，取り込み，分解などの代謝過程が存在することが明らかになっており，生理的な生合成，取り込みおよび分解に関与する候補分子として，それぞれ，L体のセリンをD体に変換するセリンラセマーゼ（SRR），アラニン-セリン-システイン中性アミノ酸トランスポーター（alanine-serine-cysteine transporter 1: Asc-1）およびD-アミノ酸酸化酵素（D-amino acid oxidase: DAO）の解析が進められている（西川，2008）．D-セリンの細胞外濃度を高める効果が想定されるのは，D-セリンの取り込みや分解の過程を阻害する物質であり，既に開発研究が始まっている．筆者がラットで*in vivo*ダイアリシス法を使って実験したところ，これを支持するデータとして，DAO，D-セリンを取り込むPAT1（proton-coupled amino acid transporter 1）等の阻害作用をもつD-サイクロセリン（Frecking and Hoeprich, 1966; Metzner et al, 2005）が，前頭葉皮質の細胞外D-セリン濃度を上昇させることがわかった（Fujihira et al, 2007）．さらに，Asc-1を阻害する物質も同様の効果をもつことが示唆された（西川ら，2008）．

一方，D-セリンの細胞外液中レベルがNMDA受容体GRIN2Bサブユニットと類似した分布を示すことや，D-セリンはNMDA受容体グリシン調節部位選択的な作用をもつ点より，D-セリン様の脳内分布が見られるD-セリン関連分子を標的とすることにより，NMDA受容体により選択性の高い治療薬が生まれることが期待できる．この観点から，筆者らがクローニングした，D-セリン選択的応答性をもつdsr-2（D-serine responsive transcript-2）(Taniguchi et al, 2005）や細胞内D-セリン濃度を変調させるdsm-1（D-serine modulator-1: ヒトPAPST-1（3′-phosphoadenosine 5′-phosphosulphate transporter-1）のラットorthologue）(Shimazu et al, 2006）は，D-セリンと共通の脳内分布を示す点で興味深く，D-セリンシグナル調節に関する機能の検

索を進めている．

IV. おわりに

本稿では，基礎的・臨床的研究から，DA受容体遮断薬より改善する症状のスペクトラムが広い新規統合失調症治療薬として，シナプス-グリア間相互作用を支えると推測されるD-セリンシステムを標的とすることにより，NMDA受容体機能のファインチューニングによる適度な活性化が得られる物質が注目されている点を中心に論じた．そのためには，NMDA受容体機能に直接影響する細胞外D-セリンシグナルを制御する分子細胞メカニズムが，詳細に解明されることが不可欠である．統合失調症では，D-セリンの基礎的なシグナルが低下することがNMDA受容体機能不全をもたらす可能性があることから，このような制御機構解明は，統合失調症におけるD-セリンシステムの分子細胞生物学的な病態分析や，そのモデル動物の開発を進展させ，より合理的で安全性の高いD-セリンシステム作用性治療薬創出を促進することが期待される．

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山本直樹，黒田安計，西川 徹（2007）ドーパミン・興奮性アミノ酸仮説．3. 原因と病態モデル．佐藤光源，丹羽真一，井上新平（編）統合失調症の治療：臨床と基礎．朝倉書店，東京，pp 38–54.
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Abstract: Toru NISHIKAWA (Division of Psychiatry and Behavioral Sciences, Graduate School of Medical and Dental Sciences, Tokyo Medical and Dental University) *Development of a novel pharmacotherapy targeted at the N-methyl-D-aspartate receptor-D-serine system for schizophrenia.* Jpn. J. Neuropsychopharmacol., 30: 201–206 (2010).

Based upon the evidence that N-methyl-D-aspartate (NMDA) type glutamate receptor antagonists including phencyclidine cause schizophrenia-like treatment-resistant negative symptoms as well as antipsychotic-responsive dopamine-related positive symptoms, the facilitation of the NMDA receptor function has been considered to be a rational therapeutic approach to ameliorate both of the above schizophrenic symptomatologies. However, the direct stimulation of the NMDA receptor glycine modulation site by glycine, D-serine, D-alanine and D-cycloserine to perform the facilitation appears to run into difficulties due to their poor permeability to the brain, lack of selectivity to the receptor, or side effects such as peripheral toxicity. Because D-serine is a selective endogenous co-agonist for the NMDA receptor acting at the glycine site with a NMDA receptor-like distribution in the brain, we have alternatively been trying to find suitable target molecules or cells that are involved in the metabolism and functioning system of glia-derived neuromodulator D-serine to increase its signal for the NMDA receptor. To this end, we have been investigating the molecular and cellular mechanisms controlling the extracellular contents of D-serine and have found that the substances that are able to inhibit the transport or degradation of D-serine moderately elevated the extracellular D-serine levels in the rat frontal cortex.

Key words: Schizophrenia, Antipsychotic-resistant symptoms, Phencyclidine, N-methyl-D-aspartate type glutamate receptor, D-Serine

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Case Report

A case of methamphetamine use disorder treated with the antibiotic drug minocycline☆

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Abstract

Methamphetamine (METH) use is one of the major public health concerns worldwide. Long-term use of METH induces not only dependence but also psychosis which is associated with METH-induced brain damage, including neuroinflammation produced by activated microglia. We report the case of a female patient whose psychotic symptoms in METH use disorder were successfully improved by anti-inflammatory drug minocycline therapy. Although the precise mechanism(s) underlying the efficacy of minocycline in METH use disorder are currently unclear, minocycline appears to be a good candidate for future investigation clinical trials for medication development in METH using populations.

1. Introduction

Methamphetamine use is a major public health concern worldwide. Long-term use of methamphetamine induces not only methamphetamine dependence but also adverse psychiatric symptoms, such as hallucinations, delusions, aggression, anxiety and insomnia. These psychiatric symptoms can persist for months or even years after the cessation of drug use [1,2]. Although such symptoms have been suggested to be associated with methamphetamine-induced brain damages, [3,4], several longitudinal studies have suggested that such brain damage is reversible and that methamphetamine-related psychotic symptoms are typically transient phenomena [5,6].

Activated microglia may produce neuroinflammation, which potentially hastens brain damage [7,8]. We previously reported that an antibiotic agent possessing anti-microglial activity, minocycline, attenuated behavioral abnormality and/or dopaminergic neurotoxicity after the administration of methamphetamine to animals [9,10]. In addition, a recent human PET study demonstrated that methamphetamine activated microglia in the brains of methamphetamine users [3]. Such findings, taken together with the present observations, led us to conclude that treatment with minocycline might ameliorate the severity of methamphetamine-related psychotic disorders. Here, we describe the course of illness of a patient with methamphetamine-related psychotic disorder who responded well to treatment with minocycline.

2. Case report

Ms. A was a 17-year-old Japanese female. At the age of 12, she started to use methamphetamine by daily intravenous injection. A few months after the onset of drug use, she experienced hallucinations (e.g., seeing insects and hearing voices) and exhibited unstable emotions and aggressive behaviors. This led the patient to stop using methamphet-

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amine for 5 months, after which the troubling symptoms disappeared (i.e., within several months). At the age of 15, the patient again started to use methamphetamine intravenously. The frequency of methamphetamine injection quickly increased up to four or five times a day. At the age of 17, the patient's hallucinations had substantially worsened, and she again stopped using methamphetamine. However, the hallucinations persisted after this second cessation of drug use. Under the influence of these hallucinations, the patient exhibited aggressive behaviors and withdrew socially. As a result, the patient's mother brought her to our clinic, 7 months after the patient had stopped using methamphetamine.

At her first visit, the patient expressed having a chronically depressed mood, suicidal ideation, appetite loss and insomnia, in addition to the psychiatric symptoms noted above. She had no history of psychiatric disorder prior to the use of methamphetamine. No abnormalities were identified on physiological or neurological examinations, nor were there any abnormal findings from routine laboratory examinations. The patient was diagnosed with methamphetamine-related psychotic disorder according to the *ICD-10* criteria. The total score on the Brief Psychiatric Rating Scale (BPRS) was 38. We started risperidone treatment at 3 mg/day, sulpiride at 150 mg/day and nitrazepam at 10 mg/day. However, this treatment protocol induced adverse reaction (e.g., akathisia) in the central nervous system (CNS), and therefore risperidone was switched to olanzapine at 10 mg/day. Furthermore, trazodone (50 mg/day) and lorazepam (3 mg/day) were added to treat the patient's unstable emotions and insomnia; this resulted in an improvement of the insomnia, but not of the remaining symptoms, in particular, the auditory hallucinations. We then attempted to increase the dose of olanzapine, but CNS adverse reactions once again appeared, and we were therefore unable to increase the olanzapine dosage. Although the patient tried taking sertraline, it was not an effective treatment for her symptoms. At this point, 2 months since the initial administration of olanzapine, the patient's BPRS score was 34. Therefore, we added minocycline to the treatment regimen (100 mg/day bid, morning and evening). Two weeks after the addition of minocycline, the patient's hallucinations had gradually improved. Moreover, with the amelioration of these symptoms, the patient's social withdrawal and aggressive behaviors also gradually disappeared. No new adverse reactions to minocycline were observed. Three months after the addition of minocycline to the patient's treatment regimen, her BPRS scores had decreased to 24.

3. Discussion

In this case study, we report that minocycline was an effective treatment for a Japanese female patient with methamphetamine-related psychotic disorder. To the best of our knowledge, this is the first report demonstrating that minocycline could be effective against methamphetamine-related psychotic disorder. In the present patient, the dominant symptoms were hallucinations that had appeared with methamphetamine use; these symptoms persisted long after the patient had stopped using methamphetamine. In spite of the administration of various antipsychotic drugs, the patient's symptoms did not improve. It should be noted that the antipsychotic dosages were limited due to adverse side-effects. However, minocycline was found to successfully treat the patient's symptoms over the course of several weeks.

The course of illness and symptomatic profile of the present case meet the *ICD-10* criteria for methamphetamine-related psychotic disorder, since the primary symptom, auditory hallucinations, appeared during and after cessation of the use of methamphetamine, and there were no apparent delusions. However, it should be noted that this patient could have schizophrenia. Furthermore, the alleviation of symptoms seen in this case might have been due to some coincidental event or to the concomitant use of antipsychotic medications, rather than to the direct effect of minocycline. It has also been shown that minocycline significantly reduces hallucinatory symptoms observed in patients with schizophrenia [11,12]. To clarify this important issue, larger studies will be needed to investigate the efficacy of minocycline monotherapy to treat the symptoms of methamphetamine-related psychotic disorders.

At present, the precise mechanism(s) remains obscure which might account for the efficacy of minocycline at treating methamphetamine-related psychotic disorder. However, the findings of the present case suggest that minocycline could serve as a candidate therapy in clinical trials to develop novel treatment regimens for methamphetamine-induced disorders [13].

4. Conclusion

Minocycline, a safe drug currently administered worldwide, could serve as a novel therapy for the treatment of methamphetamine-related psychotic disorder.

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Research Report

Characterization of [^{3}H]CHIBA-1001 binding to α7 nicotinic acetylcholine receptors in the brain from rat, monkey, and human

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ABSTRACT

Accumulating evidence suggests that the α7 subtype of nicotinic acetylcholine receptors (nAChRs) plays a role in the pathophysiology of neuropsychiatric diseases, including schizophrenia and Alzheimer's disease. Currently, there are no suitable small molecule radioligands for α7 nAChRs in the brain, although [^{125}I]α-bungarotoxin has been widely used as a radioligand for α7 nAChRs. In the present study, we characterized a new radioligand, 4-[^{3}H]methylphenyl 2,5-diazabicyclo[3.2.2]nonane-2-carboxylate ([^{3}H]CHIBA-1001), a derivative of the selective α7 nAChR agonist SSR180711, in brain membranes from rat, monkey, and human. Scatchard analysis revealed an apparent equilibrium dissociation constant (Kd) of 193.4 nM in rat brain membranes at 4 °C, and the maximal number of binding sites (Bmax) was 346.2 fmol/mg protein. The order of drugs for the inhibition of [^{3}H]CHIBA-1001 binding to rat brain membranes is SSR180711>A-844606>MG624>epibatidine>DMAB>A-582941, suggesting a similarity of α7 nAChR pharmacological profiles. In contrast, α-bungarotoxin, MLA, and nicotine were found to be very weak. The distribution of [^{3}H]CHIBA-1001 binding to crude membranes from dissected regions of rat, monkey, and human brain was different from that of [^{125}I]α-bungarotoxin binding, suggesting that [^{3}H]CHIBA-1001 binding sites may not be identical to [^{125}I]α-bungarotoxin binding in the brain. In summary, [^{3}H]CHIBA-1001 would be a useful radioligand for α7 nAChRs in the brains of rodents, non-human primates, and humans.

1. Introduction

One of the subtypes of nicotinic acetylcholine receptors (nAChRs) is composed of α7 subunits, which are pentameric; this subtype is distinguished from other nAChRs by its relatively high permeability to Ca^{2+}, which rapidly activates and desensitizes ligand-gated ion channels expressed in the mammalian central nervous system. Accumulating evidence suggests that α7 nAChRs play a role in the pathophysiology of a number of neuropsychiatric diseases such as schizophrenia and Alzheimer's disease (Freedman et al., 1995; Simosky et al., 2002; Hashimoto and Iyo, 2002; Martin et al., 2004; Hashimoto et al., 2005; Olincy and Stevens, 2007; Adams and Stevens, 2007; Dziewczapolski et al., 2009; Wang et al., 2009; Toyohara and Hashimoto, 2010). Some studies using postmortem human brain samples have demonstrated alterations in the

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levels of α7 nAChRs in the brains of patients with schizophrenia (Freedman et al., 1995; Marutle et al., 2001) and Alzheimer's disease (Hellström-Lindahl et al., 1999; Burghaus et al., 2000; Court et al., 2001; Wevers et al., 2000). In the brain, α7 nAChRs are widely expressed, with expression levels being particularly high in regions involved in cognitive processing, especially the hippocampus and cerebral cortex (Séguéla et al., 1993). Furthermore, animal studies using α7 nAChR knockout mice have demonstrated that α7 nAChRs might be involved in mediating the attentional effects of nicotine (Young et al., 2004, 2007). Moreover, a number of α7 nAChRs agonists such as PNU-282987 (Bodnar et al., 2005; Hajós et al., 2005), PHA-543613 (Wishka et al., 2006), AR-R17779 (Levin et al., 1999; Van Kampen et al., 2004), tropisetron (Hashimoto et al., 2006), SSR180711 (Pichat et al., 2007; Hashimoto et al., 2008a; Barak et al., 2009; Thomsen et al., 2009), and A-582941 (Bitner et al., 2007; Tietje et al., 2008) are reported to improve performance in animal models of cognitive deficits. It is therefore likely that α7 nAChR agonists are one of the potential therapeutic drugs for cognitive deficits in several neuropsychiatric diseases (Toyohara and Hashimoto, 2010).

It is, therefore, of great interest to determine whether α7 nAChRs are altered in the living brains of patients with these neuropsychiatric diseases. CHIBA-1001, 4-methylphenyl 2,5-diazabicyclo[3.2.2]nonane-2-carboxylate, is a derivative of the selective α7 nAChR agonist SSR180711 (Biton et al., 2007; Pichat et al., 2007) (Fig. 1). Previously, we reported that the IC_{50} values of SSR180711 and CHIBA-1001 for [^{125}I]α-bungarotoxin (0.5 nM) binding to the rat brain homogenates were 24.9 and 45.8 nM, respectively (Hashimoto et al., 2008b). Furthermore, CHIBA-1001 (1 μM) was found to be devoid of activity (inhibition lower than 50%) for a 28 standard receptor binding profile (Hashimoto et al., 2008b), indicating a high selectivity at α7 nAChRs. At present, it is not examined whether CHIBA-1001 is an agonist or an antagonist at α7 nAChRs. Recently, we have reported that [^{11}C]CHIBA-1001 might be a potential new positron emission tomography (PET) ligand for labeling in non-human primate (Hashimoto et al., 2008b) and human brain (Toyohara et al., 2009). Clinical PET studies using [^{11}C] CHIBA-1001 in patients with schizophrenia or Alzheimer's disease are currently underway (Toyohara et al., 2010). In addition, a number of new radioligands for α7 nAChRs, including [^{3}H]A-585539 (Anderson et al., 2008), [^{125}I]I-TSA (Ogawa et al., 2006), [^{11}C](R)-MeQAA (Ogawa et al., 2009), [^{11}C] GTS-21 (Kim et al., 2007), [^{18}F]NS10743 (Deuther-Conrad et al., 2009), [^{11}C]A-582941 and [^{11}C]A-844606 (Toyohara et al., 2010) have been reported (Toyohara et al., 2010). Although the radiolabeled peptide [^{125}I]α-bungarotoxin and the alkaloid [^{3}H]methyllycaconitine (MLA) have been widely used in *in vitro* receptor binding studies, these α7 nAChRs antagonists are substantially larger molecules than the endogenous agonists acetylcholine and choline. Detailed characterization of [^{3}H]CHIBA-1001 binding to brain membranes *in vitro* has not yet been reported, although a clinical study using [^{11}C] CHIBA-1001 has been started. In the present study, we performed a detailed characterization of [^{3}H]CHIBA-1001 binding to brain membranes from rat, non-human primate, and human. Furthermore, the regional distribution of [^{3}H] CHIBA-1001 binding in the brain was compared to that of [^{125}I]α-bungarotoxin binding.

2. Results

2.1. *Synthesis of [^{3}H]CHIBA-1001*

[^{3}H]CHIBA-1001 was synthesized by methylation of the precursor (Fig. 1). The radiochemical purity and specific activity of [^{3}H]CHIBA-1001 were approximately 99.2±0.4% (n=4) and 2960 GBq/mmol (based on the specific activity of [^{3}H]methyl iodide), respectively. The radiochemical yields of [^{3}H]CHIBA-1001 were 14.8±6.8% (n=4).

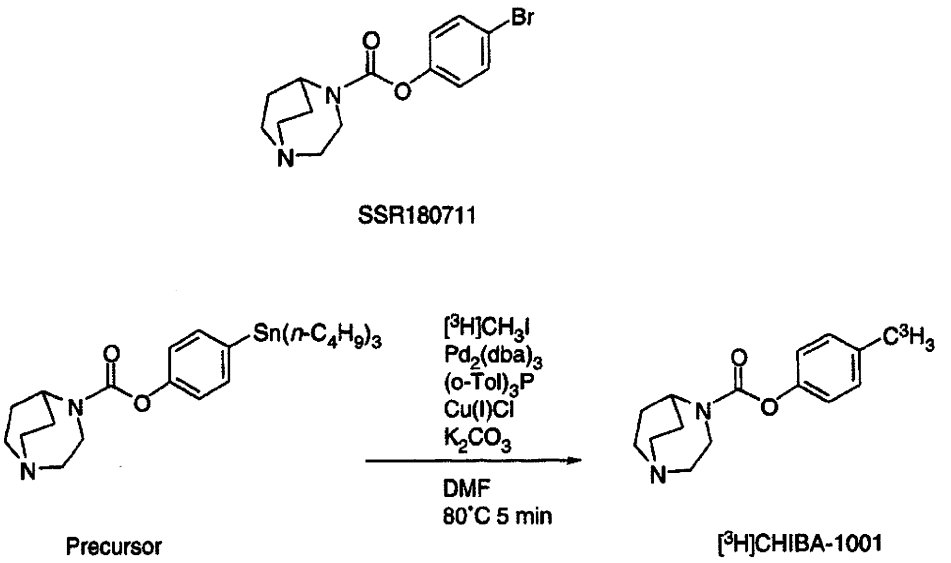

Fig. 1 – Synthesis of [^{3}H]CHIBA-1001. [^{3}H]CHIBA-1001 with a high specific activity was synthesized by *N*-methylation of the precursor, 4-(tributylstannyl)phenyl 2,5-diazabicyclo[3.2.2]nonane-2-carboxylate and [^{3}H]methyliodide.

2.2. *Equilibrium saturation binding of [^{3}H]CHIBA-1001 to rat brain membranes*

First, the kinetics of [^{3}H]CHIBA-1001 binding to rat brain membranes at 4 °C were studied. Specific binding reached equilibrium after 30 min (Supplemental Fig. 1). For saturation binding isotherms, six grade-diluted concentrations of [^{3}H] CHIBA-1001 (35–410 nM) were used. Specific binding of [^{3}H] CHIBA-1001 to rat brain membranes was saturable, and represented 40 to 50% of total binding over the concentration range examined (Fig. 2A). In saturation binding isotherms, nonlinear regression analysis of specific binding revealed an apparent Kd of 193.4±43.75 nM and a Bmax of 346.2±33.28 fmol/mg protein ($n=3$) at 4 °C (Fig. 2B). Nonspecific binding ranged from 15 to 50% of total binding for the range of [^{3}H]CHIBA-1001 concentrations.

2.3. *Pharmacological profiles of [^{3}H]CHIBA-1001 binding to rat brain membranes*

The pharmacological inhibition of specific [^{3}H]CHIBA-1001 (30 nM) binding to rat brain membranes was studied at 4 °C.

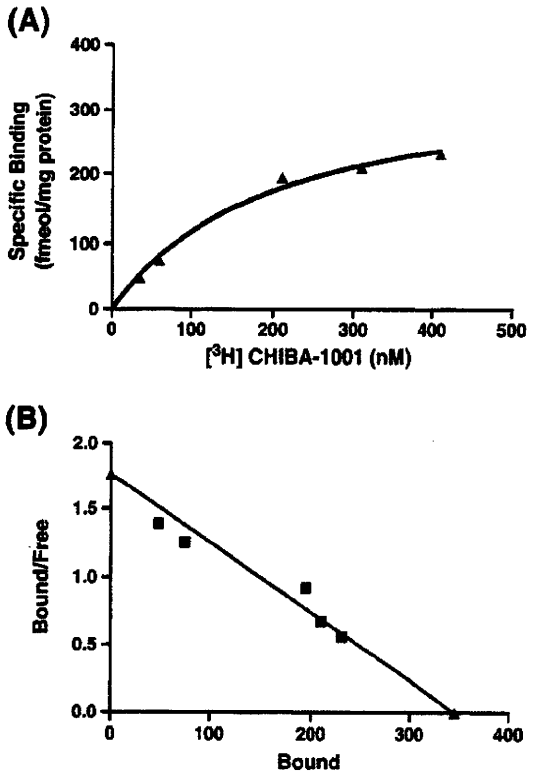

Fig. 2 – Specific binding of [^{3}H]CHIBA-1001 to rat brain membranes. Membranes were incubated with various concentrations of [^{3}H]CHIBA-1001 (35–410 nM) for 150 min at 4 °C. Nonspecific binding was estimated in the presence of 50 μM SSR180711. The results are from a typical experiment, and values are the average of duplicate determinations. (A) The saturation binding isotherm shows specific binding. (B) Scatchard plot analysis of [^{3}H]CHIBA-1001 binding gave a K_d of 193.4 nM and the B_{max} of 346.2 fmol/mg of protein.

Seven kinds of α7 nAChR compounds, including A-582941, PNU-282987, DMAB-anabaseine dihydrochloride, (±)-epibatidine, MG624, A-844606, and SSR180711, were found to displace [^{3}H] CHIBA-1001 binding to rat brain membranes (Fig. 3). The Ki value of SSR180711 was lowest, as expected, although epibatidine had low affinity at [^{3}H]CHIBA-1001 binding (Table 1). Other nAChR ligands such as (–)-nicotine, α-bungarotoxin, and MLA did not appear to inhibit [^{3}H]CHIBA-1001 binding to rat brain membranes.

2.4. *Regional distribution of [^{3}H]CHIBA-1001 binding in the rat brain*

Fig. 4 shows the regional distribution of specific bindings of [^{3}H] CHIBA-1001 in the rat brain, indicating no regional differences in [^{3}H]CHIBA-1001 binding. In contrast, regional distribution pattern of [^{125}I]α-bungarotoxin binding in the rat brain was similar to that reported previously (Davies et al., 1999). The binding of [^{125}I]α-bungarotoxin was relatively higher in the hippocampus and thalamus, while low binding density was shown in the cerebellum and striatum (Supplemental Figs. 2A and B). Furthermore, although the binding of [^{125}I]α-bungarotoxin to rat brain membranes was displaced by MLA, the binding of [^{3}H]CHIBA-1001 to rat brain membranes was not displaced by MLA (Table 1). In addition, specific binding of [^{125}I]α-bungarotoxin determined in the presence of SSR180711 and by MLA for nonspecific binding was very low in the striatum and cerebellum, although these data are preliminary (Supplemental Figs. 2A and B).

2.5. *Regional distribution of [^{3}H] CHIBA-1001 binding in the monkey brain*

The regional distribution of [^{3}H]CHIBA-1001 binding in the monkey brain was homogenous (Fig. 5). In contrast, regional

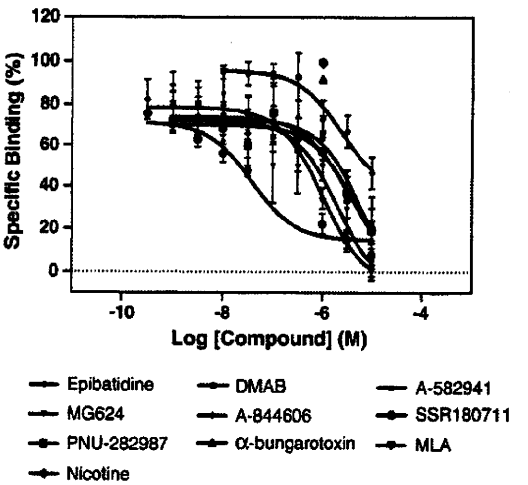

Fig. 3 – Competition curves of [^{3}H]CHIBA-1001 binding to rat brain membranes. Inhibition curves for the displacement of [^{3}H]CHIBA-1001 (30 nM) binding to rat brain cortex membranes by (±)-epibatidine, DMAB, A-58294, MG624, A-844606, SSR180711, PNU-282987, (–)-nicotine, α-bungarotoxin, and MLA. The K_i denotes the affinity constant for binding to a single state of binding sites. The results are means±S.E.M. of three separate experiments performed in duplicate.

Table 1 – Drug inhibition of [^{3}H]CHIBA-1001 binding to rat brain membranes.

Drugs	Ki (nM)
SSR180711	31.4
A844606	827
MG624	1625
Epibatidine	2195
DMAB	2506
PNU-282987	3529
A582941	5161
α-Bungarotoxin	>100,000
MLA	>100,000
Nicotine	>100,000

The inhibition of [^{3}H]CHIBA-1001 binding by various drugs was determined with [^{3}H]CHIBA-1001 (30 nM). Nine concentrations of the drugs were used for each determination. Ki values for the various drugs were determined as described in Materials and methods. The values represent the mean of three determinations done in duplicate.

distribution of [^{125}I]α-bungarotoxin binding in the monkey brain was not similar to that of [^{3}H]CHIBA-1001 binding (Figs. 5 and 6). The differences in binding between [^{3}H]CHIBA-1001 and [^{125}I]α-bungarotoxin were seen in the thalamus and striatum, both of which were found to have fewer binding sites of [^{125}I]α-bungarotoxin (Fig. 6).

2.6. *Regional distribution of [^{3}H]CHIBA-1001 binding in the human brain*

The regional distributions of [^{3}H]CHIBA-1001 binding and [^{125}I]α-bungarotoxin binding in the postmortem brain samples from 2 human subjects are shown in Fig. 7. The regional distribution of [^{3}H]CHIBA-1001 binding was homogenous (Fig. 7A), while low binding of [^{125}I]α-bungarotoxin was detected in the thalamus, cerebellum, and pons (Fig. 7B).

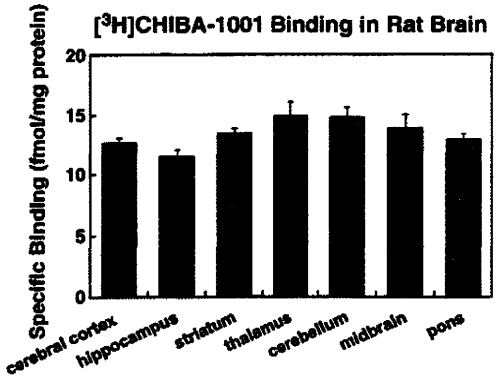

Fig. 4 – The regional distribution of [^{3}H]CHIBA-1001 binding in rat brain. Rat brains were dissected into seven regions, as described in Materials and methods. The regional distribution of [^{3}H]CHIBA-1001 (30 nM) binding in the rat brain was determined. The results are means ± S.E.M. of three separate experiments performed in duplicate.

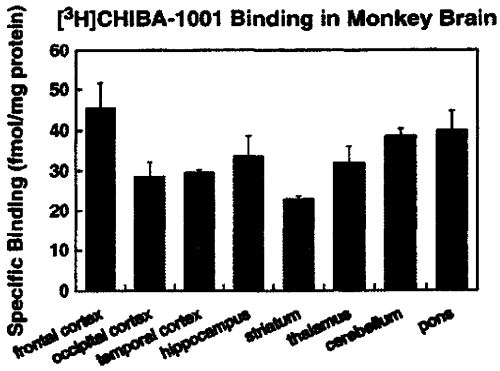

Fig. 5 – The regional distribution of [^{3}H]CHIBA-1001 binding in monkey brain. Monkey brains were dissected into eight regions, and the regional distribution of [^{3}H]CHIBA-1001 (30 nM) binding in the monkey brains was measured. The regional distribution of [^{3}H]CHIBA-1001 binding in the monkey brain was homogenous. The results are means ± S.E.M. of three separate experiments performed in duplicate.

3. Discussion

The present study suggests that [^{3}H]CHIBA-1001 would be a useful radioligand for labeling α7 nAChRs in brain membranes from rat, monkey, and human. We have previously reported that CHIBA-1001 (1 μM) is devoid of activity (inhibition lower than 50%) for a 28 standard receptor binding profiles, suggesting a high selectivity at α7 nAChRs (Hashimoto et al., 2008b). The saturation binding data of the present study indicate that [^{3}H]CHIBA-1001 binds with an affinity (Kd = 193.4 nM) to an apparently homogeneous population of receptors (Bmax = 346.2 fmol/mg protein) of rat cortex.

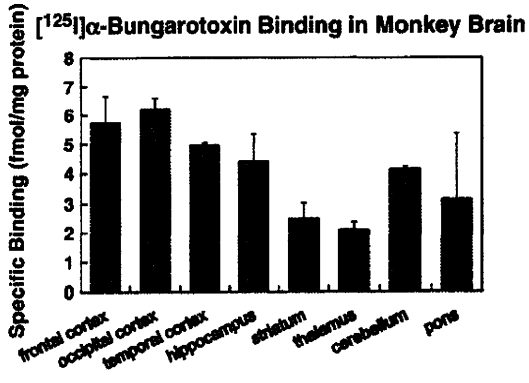

Fig. 6 – The regional distribution of [^{125}I]α-bungarotoxin binding in monkey brain. Monkey brains (n = 2) were dissected into eight regions, and the binding of [^{125}I]α-bungarotoxin (0.8 nM) was measured in each region performed in duplicate in the same way as [^{3}H]CHIBA-1001. Unlike [^{3}H]CHIBA-1001, the radioactivity of [^{125}I]α-bungarotoxin binding was in the order of (highest to lowest) thalamus > midbrain > hippocampus > frontal cortex > pons > striatum > cerebellum.

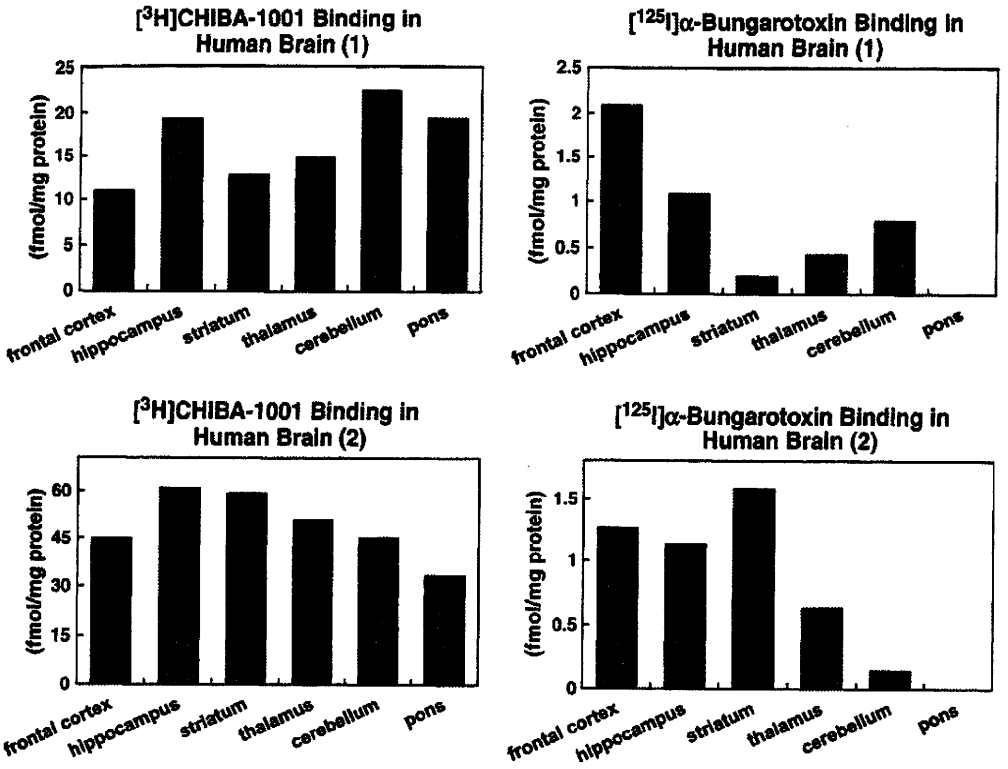

Fig. 7 – The regional distribution of [^{3}H]CHIBA-1001 binding and [^{125}I]α-bungarotoxin binding in the human brain. The regional distribution of [^{3}H]CHIBA-1001 (30 nM)(A) and [^{125}I]α-bungarotoxin (0.8 nM)(B) binding in the postmortem brain samples from 2 human subjects was measured. The regional distribution of [^{3}H]CHIBA-1001 binding was homogenous, whereas the distribution of [^{125}I]α-bungarotoxin binding was low in the cerebellum and pons. The results are the mean of data performed in duplicate.

The pharmacological inhibition study revealed some important points. Although the Ki values for the displacement of [^{3}H]CHIBA-1001 by a number of nAChR ligands were slightly higher than those obtained for other α7 nAChR ligands, as reported previously (Davies et al., 1999; Anderson et al., 2008), it is likely that the pharmacological specificity of [^{3}H]CHIBA-1001 could be related to α7 nAChRs because it was certainly inhibited by a number of α7 nAChRs ligands. In contrast, [^{3}H] CHIBA-1001 binding was not displaced by α-bungarotoxin and MLA, although [^{125}I]α-bungarotoxin binding was displaced by MLA as well as by SSR180711 and A-844606. It has been reported that Ki values for the displacement of [^{3}H]MLA binding by various α7 nAChR ligands correlate with those obtained in parallel for the displacement of [^{125}I]α-bungarotoxin binding (Davies et al., 1999), suggesting a similar pharmacology of these two radioligands. A recent study demonstrated that specific binding of [^{3}H]MLA to human frontal cortical membranes is not detectable due to high nonspecific binding levels, and that MLA shows considerably lower affinity at a new α7 nAChR radioligand, [^{3}H]A-585539, binding to human cortical membranes (Anderson et al., 2008), indicating a species difference (rat vs. human) for MLA's pharmacology. The reasons underlying the din of α-bungarotoxin or MLA inhibition on the [^{3}H]CHIBA-1001 binding and [^{125}I]α-bungarotoxin binding are currently unknown. It is unlikely that [^{3}H]CHIBA-1001 binding sites are identical to [^{125}I]α-bungarotoxin binding sites because α-bungarotoxin and MLA have high-molecular weight compounds as compared with the small size of α7 nAChR ligands such as SSR180711 and A-844606. It therefore seems that the [^{3}H] CHIBA-1001 binding characteristics may be different from those of [^{125}I]α-bungarotoxin binding in the brain although a further detailed study is necessary.

The α7 subunit of nAChRs is widely expressed in the brain, especially in regions associated with cognitive processing. In this study, we found that the regional distribution of [^{3}H]CHIBA-1001 binding was different from that of [^{125}I]α-bungarotoxin in the rat, monkey, and human brain. Our results show that high densities of α7 nAChRs can be found in the hippocampus, hypothalamus, and cortical areas in the rat brain. In this study, the high densities of [^{3}H]CHIBA-1001 binding sites in the hippocampus, thalamus, frontal cortex, and pons were similar to those of [^{125}I]α-bungarotoxin binding in the rat brain (Séguéla et al., 1993). The regional difference between these two ligands occurred in the cerebellum, where [^{3}H]CHIBA-1001 binding was high whereas [^{125}I]α-bungarotoxin binding was almost nonexistent. In adult rat cerebellum, α7 nAChRs were expressed in significant amounts, especially in the Purkinje cell layer P8–P15; they also appeared to play an important role in regulating calcium-dependent events, ultimately leading to developmental plasticity (Domínguez del Toro et al., 1997). It has been reported that a moderate density of α7 mRNA is present in

monkey and human cerebellum (Quik et al., 2000; Graham et al., 2002). The regional distribution of [^{3}H]CHIBA-1001 binding in the monkey brain seemed to be similar to the pattern in rat brain. It has been suggested that the distribution of [^{125}I]α-bungarotoxin binding is larger in the brains of rhesus monkeys than in rodent brain (Han et al., 2003). The reasons underlying the differences in the regional distribution of the [^{3}H]CHIBA-1001 binding and [^{125}I]α-bungarotoxin binding are currently unknown. The difference of molecular weight of two radioligands [^{3}H]CHIBA-1001 and [^{125}I]α-bungarotoxin may contribute to the regional differences in the brain although a further detailed study is needed.

In monkey brain, the region that appeared to make a difference in the binding of the two ligands was the thalamus, where [^{3}H]CHIBA-1001 binding was seen, as in other regions, while there was little [^{125}I]α-bungarotoxin binding. It has been reported that α7 nAChR mRNA is expressed in the thalamus, based on the use of *in situ* hybridization (Quik et al., 2000). Although the distribution of α7 nAChRs in primates is still not completely known, it has been suggested that the distribution of α7 nAChRs in the monkey brain is more similar to that in humans than in rodents (Quik et al., 2000). This difference between rodents and primates may reflect an increased need for thalamocortical modulation in the human neocortex (Breese et al., 1997), because activation of the α7 nAChRs by cholinergic afferents could modulate inhibitory activity and sensory processing within the corticothalamic axis. In this study, we found a regional difference in distribution between [^{3}H]CHIBA-1001 binding and [^{125}I]α-bungarotoxin binding in the human brain, although the reasons underlying this discrepancy are unknown.

Unlike [^{3}H]CHIBA-1001 binding, the density of [^{125}I]α-bungarotoxin binding was low in the cerebellum. In the human brain, α7 nAChRs have been shown to be distributed in regions related to cognitive function such as the nucleus accumbens, ventral hippocampus, amygdala, and frontal cortex (Levin et al., 2006; Nashmi and Lester, 2006). In addition, it has been suggested that the cerebellum is also involved in cognition, behavior, and emotion (Frings et al., 2007). For example, preliminary studies employing histoblots of cerebellar sections have suggested a higher concentration of α7 nAChR subunits in the molecular layer, and the immunohistochemistry in fixed tissue indicates that Purkinje cells strongly express α7 subunits (Court et al., 2000). Another report has reported the observation of α7 nAChRs mRNA and protein in various cells in the cerebellum (Graham et al., 2002; Hellström-Lindahl et al., 1998). For labeling α7 nAChRs in the cerebellum, it seems that [^{3}H]CHIBA-1001 may have a distinct advantage over [^{125}I]α-bungarotoxin. Thus, it is likely that [^{3}H]CHIBA-1001 could be a suitable radioligand for labeling α7 nAChRs in the cerebellum, although further study will be necessary.

The new radioligand [^{3}H]CHIBA-1001 may, however, have some limitations to its use. One is the relatively low affinity (Kd = 193.4 nM) of [^{3}H]CHIBA-1001 binding to crude brain membranes, suggesting that the practicality of this radioligand may be limited. Alternatively, a new radioligand, [^{3}H]A-585539, has demonstrated high-affinity binding consistent with α7 nAChRs pharmacology, a rapid association rate, and a relatively slow dissociation rate (Anderson et al., 2008). Another limitation is the high proportion (approximately 50%) of nonspecific binding of [^{3}H]CHIBA-1001 in the brain membranes, whereas nonspecific binding of [^{3}H]A-585539 has been found to be less than 10% of total binding (Anderson et al., 2008). As such, [^{3}H]A-585539 may be better than [^{3}H]CHIBA-1001 for labeling α7 nAChRs in the brain, although a further detailed study is necessary.

In conclusion, the present study suggests that [^{3}H]CHIBA-1001 would be a suitable radioligand for the *in vitro* labeling of α7 nAChRs in the brain. It is therefore likely that [^{3}H]CHIBA-1001 could be a useful radioligand for studying α7 nAChRs in postmortem brain samples from patients with neuropsychiatric diseases such as schizophrenia and Alzheimer's disease.

4. Experimental procedures

4.1. *Synthesis of [^{3}H]CHIBA-1001*

CHIBA-1001 and its precursor, 4-(tributylstannyl)phenyl 2,5-diazabicyclo[3.2.2]nonane -2-carboxylate (Fig. 1), were synthesized as described previously (Hashimoto et al., 2008b).

[^{3}H]CHIBA-1001 was synthesized by *N*-methylation of the precursor with [^{3}H]methyl iodide (Fig. 1). A 0.1-mL quantity of [^{3}H]methyl iodide toluene solution (370 MBq) (American Radiolabeled Chemicals, Inc., St. Louis, MO) was added to the ice-cold reaction vessel, which contained tris(dibenzylideneaceton) dipalladium (2.8 mg), tri-o-tolylphosphine (3.7 mg), the precursor (0.8 mg), copper (I) chloride (1.2 mg), and potassium carbonate (1.7 mg) in *N*,*N*-dimethylformamide DMF (0.3 ml). The reaction vessel was heated at 80 °C for 5 min. The mixture of reaction solution was transferred to the high-performance liquid chromatography (HPLC) injection unit through a glass filter to remove solid reagents, and injected into a preparative HPLC system using an YMC-Pack Pro C18 column (10 mm in inner diameter × 250 mm in length, YMC Co., Ltd., Kyoto, Japan). The radioactive peak fraction eluted by CH_3CN/50 mM CH_3COONH_4/CH_3COOH (250/750/3) at a flow rate of 4 ml/min was collected into an evaporation flask and evaporated; the residue was then re-dissolved with 2 ml of ethanol.

Chemical and radiochemical analyses of [^{3}H]CHIBA-1001 were performed by HPLC in a system consisting of a column (YMC-Pack Pro C18, 4.6 mm in inner diameter × 250 mm in length, YMC Co., Ltd., Kyoto, Japan) and the use of CH_3CN/50 mM CH_3COONH_4/CH_3COOH (250/750/3) as a mobile phase at a flow rate of 1 ml/min.

4.2. *Membrane preparation*

[^{3}H]CHIBA-1001 (2960 GBq/mmol) binding was performed using membrane-enriched fractions from rat, monkey, and human brain. For most of the experiments, male Crl:CD(SD) SPF/VAF rats (180–200 g) (Japan Charles River Inc., Tokyo, Japan) were used. The rats were killed by decapitation, and the brains were rapidly removed. The brains were dissected on ice into 7 sections, the frontal cortex, hippocampus, thalamus, striatum, cerebellum, midbrain, and pons (including medulla oblongata), by the method of Glowinski and Iversen (1966) and then stored at −80 °C until use. The brain was homogenized in 15 volumes of 0.32 M sucrose using a Teflon glass homogenizer and centrifuged at 1000 × *g* for 10 min (4 °C). The

supernatant was centrifuged at 48,000×g for 20 min (4 °C). The resultant pellets were homogenized in the buffer (120 mM NaCl, 5 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 50 mM Tris–HCl, pH 7.4 at 4 °C) with a Polytron and spun at 48,000×g for 20 min (4 °C). The membrane pellets were washed and re-suspended in ice-cold buffer and were then centrifuged two more times before storage at −80 °C. The final pellet was re-suspended in 10 volumes of the same buffer. Protein concentrations were measured according to the method of Lowry et al. (1951).

Brain samples of three male *Macaca fascicularis* (4560 g, 4890 g, and 4680 g) were provided by Hamamatsu photonics (Hamamatsu, Japan). The brains were dissected on ice into 8 sections, the frontal cortex, occipital cortex, temporal cortex, hippocampus, thalamus, striatum, cerebellum, and pons (including medulla oblongata), and then stored at −80 °C until use.

Postmortem human brain samples (n=2; one is male, age 77, Caucasian, liver cancer as cause of death, smoked 2–3 packs of cigarettes per day for 37 years — quit 24 years prior to death; the other is male, age 77, Caucasian, pulmonary fibrosis as cause of death, smoked 1 pack of cigarettes per day for 25 years — quit 5 years prior to death) were purchased from Analytical Biological Service Inc. (Cornell Business Park, Wilmington, DE) in the form of frozen 2–3 g blocks of each of 6 sections, the frontal cortex, hippocampus, thalamus, striatum, cerebellum, and pons. These samples were stored at −80 °C until use. The brain was further dissected on ice into 0.2–0.3 g for the binding assay, and membrane preparation was conducted in the same way as for rat brain, except for the process of homogenizing in 15 volumes of 0.32 M sucrose using a Teflon glass homogenizer and centrifugation at 1000×g for 10 min (4 °C); the pellet was then discarded.

The experimental procedures using rats, monkeys, and human postmortem brain samples were approved by the Animal Care and Use Committee and Ethics Committee of Chiba University.

4.3. [^{3}H]CHIBA-1001 binding assay

Aliquots of membrane suspension (200 μl) were added, in duplicate, to the reaction mixture containing [^{3}H]CHIBA-1001 and the indicated concentrations of test drug in a final volume of 0.5 ml. Nonspecific binding was estimated in the presence of 50 μM SSR180711. Binding was allowed to occur for 150 min at 4 °C. Bound radioactivity was isolated by rapid vacuum filtration onto Whatman GF/B glass filters pretreated with 0.5% polyethyleneimine (Sigma-Aldrich Corporation, St. Louis, MO) for 3–4 h using a 24-channel cell harvester (Brandell, Gaithersburg, MD). The filters were washed with 5 ml of ice-cold buffer 3 times. The radioactivity trapped by the filters was determined using a liquid scintillation counter (Beckman, LS-6500, Beckman Coulter K.K., Tokyo, Japan).

To examine the pharmacological profiles of [^{3}H]CHIBA-1001 binding, ten kinds of α7 nAChR compounds, including PNU-282987, MG624, (±)-epibatidine, MLA, α-bungarotokin, (−)-nicotine (Sigma-Aldrich, St. Louis, MO), DMAB-anabaseine dihydrochloride (Tocris, Bristol, UK), A-582941, A-844606, and SSR180711 (synthesized in our laboratory) were used.

Regional distribution of [^{3}H]CHIBA-1001 binding of each brain was compared to that of [^{125}I]α-bungarotoxin (5.92 TBq/mmol, PerkinElmer Life Sciences, Inc., Boston, MA) binding. The binding assay of [^{125}I]α-bungarotoxin was performed in the same way of the [^{3}H]CHIBA-1001 binding assay, except for the incubation condition (180 min at 37 °C) and filter pretreatment (0.5% polyethyleneimine with 0.1% bovine serum albumin).

4.4. Data analysis

The data show the mean±standard error of the mean (S.E.M.). Dissociation constant (Kd) and maximal binding (Bmax) values from saturation binding and IC_{50} values from binding displacement by some drugs were determined using GraphPad Prism (GraphPad Software, San Diego, CA). Ki values were calculated from the IC_{50} values using Microsoft Excel, where $Ki = IC_{50}/(1+[Ligand]/Kd)$(Cheng and Prusoff, 1973).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.brainres.2010.06.008.

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ORIGINAL ARTICLE

Enhanced Carbonyl Stress in a Subpopulation of Schizophrenia

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Context: Various factors are involved in the pathogenesis of schizophrenia. Accumulation of advanced glycation end products, including pentosidine, results from carbonyl stress, a state featuring an increase in reactive carbonyl compounds (RCOs) and their attendant protein modifications. Vitamin B_6 is known to detoxify RCOs, including advanced glycation end products. Glyoxalase I (GLO1) is one of the enzymes required for the cellular detoxification of RCOs.

Objectives: To examine whether plasma levels of pentosidine and serum vitamin B_6 are altered in patients with schizophrenia and to evaluate the functionality of *GLO1* variations linked to concomitant carbonyl stress.

Design: An observational biochemical and genetic analysis study.

Setting: Multiple centers in Japan.

Participants: One hundred six individuals (45 schizophrenic patients and 61 control subjects) were recruited for biochemical measurements. Deep resequencing of *GLO1* derived from peripheral blood or postmortem brain tissue was performed in 1761 patients with schizophrenia and 1921 control subjects.

Main Outcome Measures: Pentosidine and vitamin B_6 concentrations were determined by high-performance liquid chromatographic assay. Protein expression and enzymatic activity were quantified in red blood cells and lymphoblastoid cells using Western blot and spectrophotometric techniques.

Results: We found that a subpopulation of individuals with schizophrenia exhibit high plasma pentosidine and low serum pyridoxal (vitamin B_6) levels. We also detected genetic and functional alterations in *GLO1*. Marked reductions in enzymatic activity were associated with pentosidine accumulation and vitamin B_6 depletion, except in some healthy subjects. Most patients with schizophrenia who carried the genetic defects exhibited high pentosidine and low vitamin B_6 levels in contrast with control subjects with the genetic defects, suggesting the existence of compensatory mechanisms.

Conclusions: Our findings suggest that GLO1 deficits and carbonyl stress are linked to the development of a certain subtype of schizophrenia. Elevated plasma pentosidine and concomitant low vitamin B_6 levels could be the most cogent and easily measurable biomarkers in schizophrenia and should be helpful for classifying heterogeneous types of schizophrenia on the basis of their biological causes.

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SCHIZOPHRENIA IS A DEBILITATing and complex mental disorder with a prevalence of approximately 1% worldwide. Its pathophysiology remains unclear, despite extensive research.[1,2] Biochemical and pharmacological studies using human samples and animal models suggest that oxidative/carbonyl stress contributes to the pathophysiology of schizophrenia.[3-6] Oxidative stress is a central mediator of advanced glycation end product (AGE) formation, and pyridoxamine (vitamin B_6, biosynthesized from pyridoxal in vivo) is known to detoxify reactive carbonyl compounds via carbonyl-amine chemistry. Toxic reactive carbonyl compounds such as α-oxoaldehydes (eg, methylglyoxal, glyoxal, and 3-deoxyglucosone) are formed from sugars, lipids, and amino acids.[7-9] Accumulation of such reactive carbonyl compounds, referred to as carbonyl stress,[10] results in the modification of proteins and the eventual formation of AGEs such as pentosidine. Cellular removal of AGEs hinges largely on the activity of the zinc metalloenzyme glyoxalase I (GLO1).[11] The glyoxalase detoxifi-

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