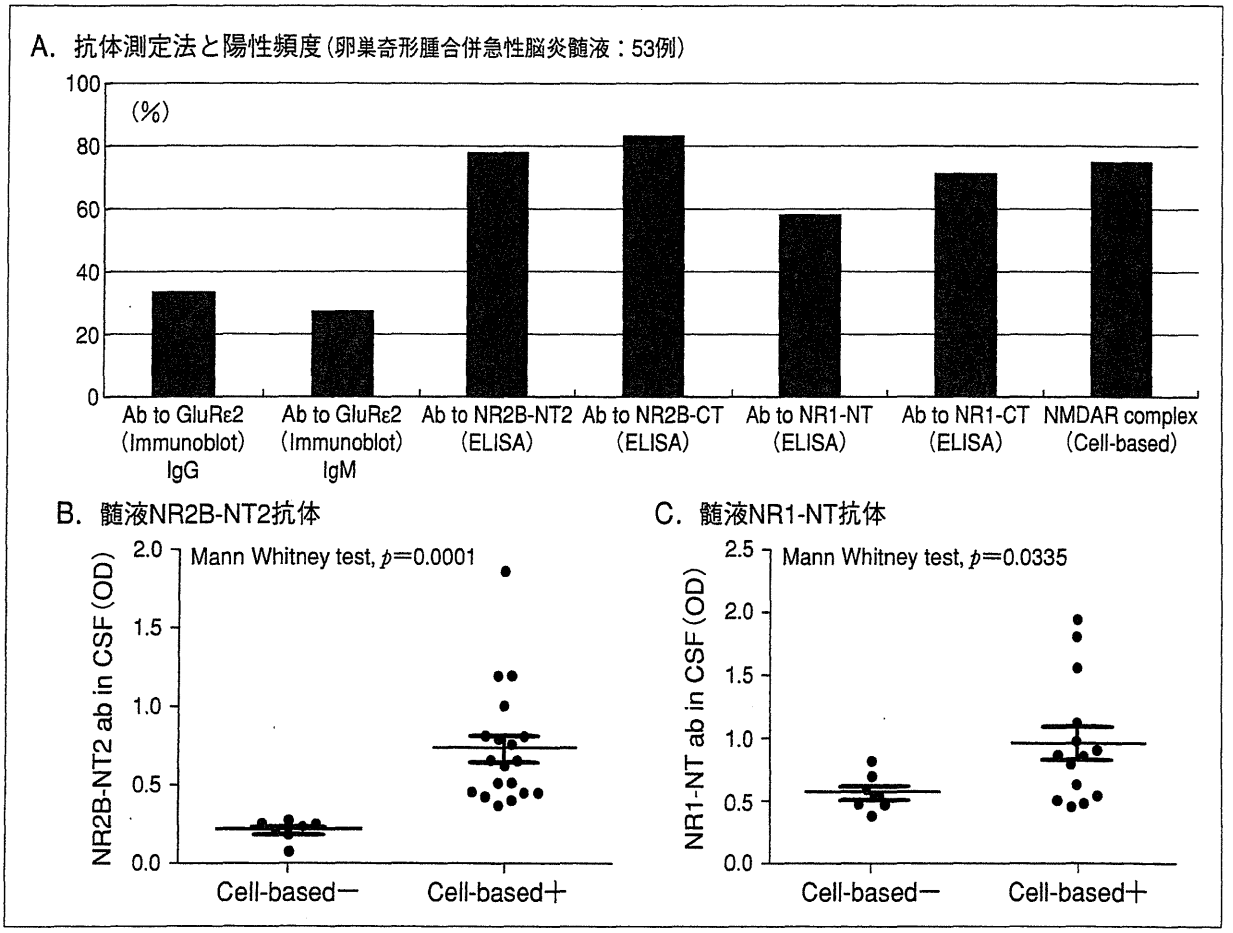

図3 NMDA型グルタミン酸受容体抗体測定法と感度比較

A：イムノブロット，ELISA, cell-based assay(Dalmau法)による卵巣奇形腫合併急性脳炎53例髄液での陽性率を示す．イムノブロット法での陽性頻度は30%，ELISAで疾病対照の平均＋2SD以上を陽性とすると，GluN2BのN末に対する抗体(NR2B-NT2抗体)は80%程度，GluN1のN末に対する抗体(NR1-NT抗体)は60%程度の陽性頻度で，cell-based assayでは陽性頻度は70%程度である．B, C：cell-based assay＋：Dalmauらの開発したcell-based assay(図2-A)でNMDA型GluR抗体陽性の髄液検体，cell-based assay－：Dalmauらの開発した方法(図2-A)でNMDA型GluR抗体陰性の髄液検体．

を発現するベクターをtransient transfectionしたHEK細胞の細胞外ドメイン立体構造を抗原とするcell-based assayによる抗体(Dalmau抗体)測定を国内で初めて可能にした(図2-A)．

2009年に森らは，GluN1とGluN2Bを発現するベクターをstable transfectionしたHEK細胞の細胞外ドメイン立体構造を抗原とするcell-based assayを開発，安定した培養とDalmau抗体測定を実現した(図2-A)[12]．このstable transfectionしたHEK細胞を用いたDalmau抗体測定は，森らの好意により静岡てんかん・神経医療センターにおいても2013年から可能になっている．

Dalmau抗体以外のNMDA型GluRサブユニットを認識する抗体もNMDA型GluRを認識する抗体であり，広義のNMDAR抗体である．本稿では図2のA)～C)あわせて「広義のNMDAR抗体」と呼び，Dalmau抗体は狭義のNMDAR抗体と呼ぶことにする．

抗体測定法の比較・validation

イムノブロット法によるGluRε2抗体の「卵巣奇形腫を合併する急性脳炎(抗NMDAR脳炎-OT)」症例の髄液での陽性率は，30%程度と低かった(図3-A)．cell-based assayによるDalmau抗体は，前述の髄液検体での陽性率は70%程度であった．次にわれわれが開発した半定量可能なELISAでは[10]，抗NMDAR脳炎-OTの髄液検体でのGluN2BのN末に対する抗体(NR2B-NT2抗体)は80%程度，GluN1のN末に対する抗体(NR1-NT抗体)は60%程度の陽性率であり，cell-based assayの

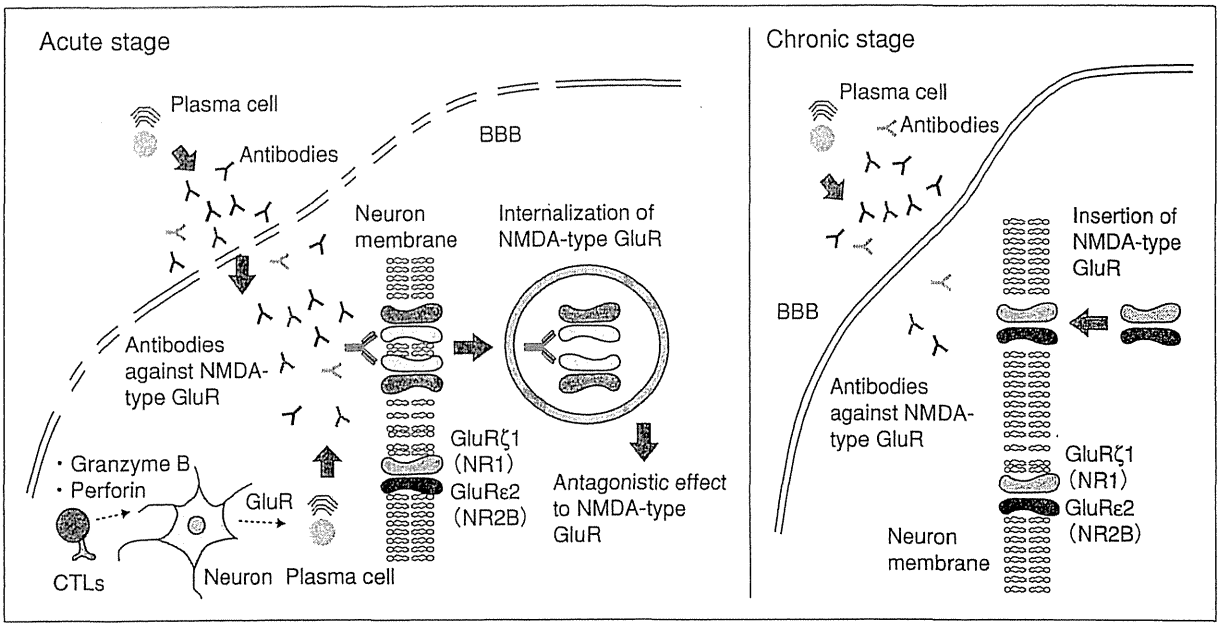

図4　NMDA型グルタミン酸受容体抗体と非ヘルペス性急性辺縁系脳炎の病態仮説
血液中にできたN末エピトープを有するNMDA型GluR抗体は，血液脳関門の破綻などにより中枢神経系に至り，中枢神経系内で産生された抗体とともに，急性期脳炎症状に寄与するが，回復期・慢性期になると血液脳関門の回復により髄液中で低下する病態を考えている．

Dalmau抗体とELISAによるNMDA受容体抗体とは感度に大きな違いはなかった．

Dalmau抗体陽性19検体，陰性7検体の髄液について，われわれのELISAでNR2B-NT2抗体を測定すると，Dalmau抗体陽性群は有意に陰性群に比べてELISA値が高値であり(図3-B)，NR1-NT抗体についてもDalmau抗体陽性群は有意に陰性群に比べてELISA値が高値であった(図3-C)[11]．以上よりELISAのNR2B-NT2抗体，NR1-NT抗体は，十分にNMDA型GluRに対する髄液中の抗体を検出できていると考えられる．NR1-NT抗体はDalmau抗体陽性群の5/14検体でDalmau抗体陰性群と同等の値であったが，これらの検体ではNR2B-NT2抗体は明らかに高値であり，NR2B-NT2抗体のみを有する可能性がある．

NMDAR抗体の病的機能

1．NMDA型GluRのinternalization

脳炎患者血清中のNMDAR抗体は，細胞表面のNMDA型GluRを架橋することによりinternalization(細胞内取りこみ)させることが報告されている[9][13]．抗体によるinternalizationは，NMDA型GluRの拮抗作用＝機能低下をひき起こし，脳炎における意識障害や行動異常などの辺縁系症状に関連している可能性がある(図4)．NHALEに高頻度に出現する幻覚，不安，不眠などの症状は，NMDA受容体拮抗薬である塩酸ケタミン，塩酸メマンチンなどの副作用としても知られていて，自己抗体によるNMDA型GluRの拮抗作用がNHALEの臨床症状となっていると考えられる．われわれの研究グループのTakanoらは，このNMDA型GluR内在化が温度感受性を有することを見出していて，低体温療法が内在化を防ぐ可能性が示唆される[12]．

われわれは，NHALE患者髄液のIgG分画を用いて培養ラット胎児神経細胞に対する影響を評価し，NMDAR抗体によるNMDA型GluRの内在化がシナプス外のNMDA型GluRに主に起こり，シナプスNMDA型GluRでは軽度であると推定している[11]．NMDAR抗体はシナプス外のNMDA型GluRの内在化を主に起こすために，興奮毒性が抑制され，アポトーシスが軽減し，比較的予後が良いものと推測している(図5)．

Mikasovaらは，脳炎のNMDAR抗体がGluN2Aをシナプスに繋ぎ止めているEphrin-B2 receptorとの結合を切り，シナプスNMDA型GluRがシナプス外に移動し，シナプス外で抗体により内在化する機序を考えている[14]．

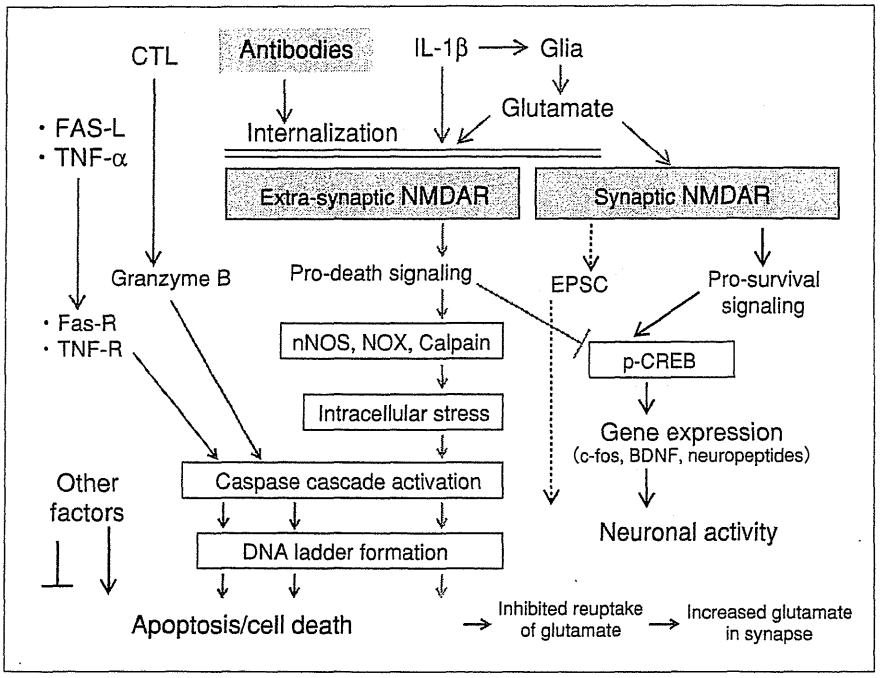

図5　NMDA型グルタミン酸受容体抗体の作用
p-CREB：phosphorylated cAMP-response-element-binding-protein，CTL：cytotoxic T cell，EPSC：excitatory postsynaptic current，NMDAR：NMDA-type GluR.

2．アポトーシス誘導作用

GluN2BなどのN末細胞外ドメインの283～287番目のアミノ酸配列(Asp/Glu-Trp-Asp/Glu-Tyr-Ser/Gly)とds-DNAに分子相同性があるため，全身性エリテマトーデス(SLE)患者のds-DNA抗体は，中枢神経系でNMDA型GluR(NR2A/2B)と交叉反応し，アポトーシスなどを起こすこと，Asp/Glu-Trp-Asp/Glu-Tyr-Ser/Glyで免疫した動物で，LPSやエピネフリンで血液脳関門(BBB)の透過性を高めると，この細胞外ドメイン抗体が中枢神経系に至り，行動や認知機能に影響を及ぼすことが動物実験で示されている[15)~17)]．

3．NMDA型GluRチャネル機能への作用

われわれはラット海馬スライス標本を用いて，GluRε2のN末側に対するウサギ抗体，抗GluRε2抗体陽性のRasmussen症候群患者血清のIgG分画の興奮性シナプス後電流(EPSC)への影響を検討したが，現在までのところGluR3抗体とは異なり，NMDAR抗体には明らかな電気生理学的作用を見出せていない[18)]．

広義のNMDAR抗体の神経疾患における特徴・意義

Dalmau抗体は当初，卵巣奇形種のある脳炎(抗NMDAR脳炎-OT)に特異的と考えられていた[8)]．しかし最近では，奇形腫を合併しない脳炎や，原因不明のてんかん[19)]や，Creutzfeldt-Jakob病[10)]でもDalmau抗体陽性例が報告されている．一方，イムノブロット法によるGluRε2抗体やELISAによるNR2B-NT2抗体も多くの神経疾患で高値となることがわかっている．個々の疾患における特徴，意義について以下に述べる．

1．非ヘルペス性急性辺縁系脳炎(NHALE)

辺縁系脳炎は海馬・扁桃体などの辺縁系を主座とする脳炎で，辺縁系症状と呼ばれる特徴的な症状が診断のポイントとなる．髄液NMDAR抗体陽性の症例の初発神経症状では，行動異常(74%)，記憶障害(7%)，幻覚(5%)などが多かった[20)]．非傍腫瘍性NHALEの病因としては，NMDA型GluR，電位依存性カリウムチャネル(voltage-gated potassium channel：VGKC)複合体などに対する神経自己抗体が知られている(表2，3)．髄液NMDAR抗体は，血液中で産生され血液脳関門の破綻を介して血液中から中枢神経系に至るものと，中枢神経系で形質細胞から産生されるものがあると推定している(図4)．NHALEではNR2B抗体，Dalmau抗体ともに75%程度に陽性となり，エピトープとしては，NR2B-NT2，NR2B-M3-4，

表2　神経細胞表面抗原に対する抗体と脳炎

	NMDAR抗体	LGI1抗体	Caspr2抗体	GABA$_B$R抗体	AMPAR抗体	GAD抗体	GlyR抗体	NAE抗体
好発年齢(歳)	6～39 (Mean 26.5)	30～80 (Median 60)	46～77 (Median 60)	24～75 (Median 62)	38～87 (Median 60)	若い成人女性		28～85 (平均58)
性	小児：男＞女 成人：男＜女	65％男	85％男	男＝女	90％女性	女性		81％女性
臨床症状	急性に，言動異常，記憶障害，ほか)	亜急性，急性に，記憶障害，てんかん発作	Morvan症候群	記憶障害，てんかん発作	記憶障害	てんかん，軽度認知障害	筋強剛，ミオクローヌス，驚愕反応	急性脳症＞精神病型＞小脳失調型
検査	50％MRI病変	84％MRI病変，60％低Na血症	40％MRI病変	66％MRI病変	90％MRI病変	抗GAD抗体＞1,000 U/ml		100％TPO抗体，MRI正常
腫瘍合併	卵巣奇形腫	稀	胸腺腫など	60％肺小細胞がん	70％肺がんなど	稀	報告なし	
抗体	IgG1主体	IgG4＞IgG1	IgG4＞IgG1	IgG1主体	?	IgG1主体	IgG1主体	
予後	比較的良好，再発あり	単相性	腫瘍による	不良	再発	慢性経過	免疫治療有効例	ステロイド有効

NMDAR：NMDA型glutamate receptor，LGI1：leucine-rich glioma-inactivated 1，Caspr2：contactin-associated protein 2，GABABR：r-aminobutyric acid type B receptor，AMPAR：α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor，GAD：glutamic acid decarboxylase，GlyR：glycine receptor，NAE, N-terminal α-enolase.（Lancaster E, et al. Neurology 2011；77：179-89，Vincent A, et al. Lancet Neurol 2011；10：759-72，米田　誠．Bio Clinica 2009；24：1199-205より改変引用）

NR2B-CTなど，幅広い領域に対する抗体が存在，N末に対する抗体が中枢神経系内でNMDA型GluRの内在化を起すが，GluR興奮毒性を抑制することができるため予後を比較的良くしていると，われわれは推定している(図5)．

2．卵巣奇形腫合併脳炎(抗NMDAR脳炎-OT)

卵巣奇形腫あるいは正常卵胞にはGluN2B(NR2B)およびGluN1(NR1)などのNMDA型GluRをはじめとして種々の神経分子が発現していると推定されている[21]．奇形腫を合併する急性脳炎では，奇形腫あるいは卵巣に発現するNMDA型GluRなどが抗原となってNMDAR抗体がつくられる．NR2B抗体，Dalmau抗体ともに88％程度に陽性となり，エピトープとしては，NR2B-NT2, NR2B-M3-4, NR2B-CTなど，幅広い領域に対する抗体が存在する(表3)．GluN2BおよびGluN1に対する抗体の優位性は，急性期では症例ごとにさまざまであったが，回復期以降はGluN2Bに対する抗体が優位となっていく．

3．Rasmussen症候群

Rasmussen症候群は，神経症状のない健常者になんらかの先行感染症(上気道炎など)やワクチン接種があった後に限局性に細胞傷害性T細胞(CTL)を主役とした自己免疫性炎症が起こり，てんかん発作が発症，難治に経過，しだいに片麻痺・知的障害などが出現し，半球性の萎縮が明らかとなり，適切な治療がないと「寝たきり」となる慢性進行性の疾患とされている[22)23)]．感染により感作を受けたnaïve T cellsが活性化してeffector T cellsになり，BBBを超えて中枢神経系に進入し，CTLは交差反応可能な神経分子を発現した神経細胞などにGranzyme Bなどを分泌して，アポトーシスを誘導する．その結果，神経分子の断片化が起こり，中枢神経系でNR2B抗体などの種々の自己抗体産生などが起こり，さらには補体のうちのC5bからC9までの5つの蛋白が集合したMACが形成され，中枢神経系病変が進行すると考えている．髄液NMDAR抗体などの自己抗体は必ずしも全経過中認められるわけではなく，発病からしばらくしててんかん発作が増加する時期に陽性化することが多い(表3)．

4．脳炎後てんかん

われわれは199症例の急性脳炎脳症症例の後遺症を調査し，知的・運動障害は発病年齢が若いほど強く，てんかん発作・知的障害は慢性期に進行する経過を示すことを明らかにした[24]．てん

表3 自己免疫介在神経疾患と自己抗体

疾患	NMDAR抗体			VGKC抗体		NAE抗体
	GluRε2抗体（イムノブロット法）	Dalmau抗体（cell-based assay）	NR2B-NT2抗体（ELISA）	LGI1抗体*1	Caspr2抗体*1	
非ヘルペス性急性辺縁系脳炎	髄液（急性期）：IgG-31.8％陽性，IgM-27.9％陽性	髄液（急性期）：75.0％陽性	髄液（急性期）：78.0％陽性	VGKC抗体血清：21.1％強陽性，壮年期発症例に陽性が多い*2		NAE抗体陽性橋本脳症の9％を占める*3
卵巣奇形腫合併脳炎	髄液（急性期）：IgG-37.7％陽性，IgM-15.1％陽性	髄液：88.5％陽性	髄液：88.2％陽性	髄液：0/2検体陽性	髄液：0/2検体陽性	
Rasmussen症候群	髄液（発病後6～23カ月）陽性率：IgG-57.1％，IgM-28.6％		髄液：91.7％陽性			
HSV脳炎		13/44（30％）陽性				
脳炎後てんかん			髄液：41.4％陽性	髄液：2/3検体弱陽性	髄液：2/3検体弱陽性	
てんかん		髄液5/19陽性				
Creutzfeldt-Jakob病		70歳女性髄液陽性	髄液：11/12検体陽性	VGKC抗体陽性例：1例		
再発性多発軟骨炎			脳炎合併髄液：陽性1例報告			
MELAS		42歳男性髄液陽性	髄液：1/3陽性			
脳腫瘍	髄液：陽性1例報告		髄液：陽性1例報告			

NMDAR：NMDA型glutamate receptor，LGI1：leucine-rich glioma-inactivated 1，Caspr2：contactin-associated protein 2，NR2B-NT2：n-terminal of GluN2B，HSV：herpes simplex virus，*1 Autoimmune Encephalitis Mosaic 1キット（Euroimmune）による抗体判定，感度はかなり低い，*2 厚生科研高橋班研究報告書，*3 厚生科研高橋班研究報告書．

かん発作頻度・記憶障害の程度は髄液NR2B抗体陽性例で有意に重度であった．脳炎後てんかんではmatrix metalloproteinase-9（MMP-9）が高値で，tissue inhibitor of metalloproteinase-1（TIMP-1）が脳炎後5年程度かけて漸減するため，徐々に血液脳関門破綻が進行，髄液内のNR2B-NT2抗体が増加する可能性をわれわれは報告した[25]．脳炎後てんかん症例の慢性期のNR2B抗体は，補体依存性の作用などでニューロンに障害をもたらしていると推定している．髄液NR2B抗体は40％程度に陽性となり，エピトープとしては，NR2B-NT2，NR2B-M3-4，NR2B-CTなど，幅広い領域に対する抗体が存在する（表3）．

5．Creutzfeldt-Jakob病（CJD）

Fujitaらは，CJD患者の血清が海馬に反応する抗体を有し，血清髄液にNR2B抗体が高頻度に存在すること，血清にVGKC抗体を有する症例があることを報告している（表3）[10][26]．自己免疫介在性辺縁系脳炎とCJDには精神症状や認知機能低下といった臨床特徴に類似性があり，CJDにおいてもNMDAR抗体が病態に関与している可能性を示唆している．

6．再発性多発軟骨炎

自己免疫性の慢性炎症性疾患で，主に耳介，鼻，気道軟骨が障害されるが，頭痛，髄膜炎，脳炎などを合併する．本症に合併した辺縁系脳炎で髄液NR2B抗体が陽性の症例報告がある[27]．

7．脳腫瘍

てんかん発作で発病し，MRI所見，生検によりglioblastomaと診断された症例で，髄液GluRε2抗体，NR2B-NT2抗体が陽性で，Dalmau抗体が陰性の症例報告がある[28]．脳腫瘍により破壊された神経細胞からのGluN2Bが抗原となって抗体が産生されたものと推定している．

NMDAR抗体は，Dalmau抗体であれ，NR2B抗体であれ，複数の疾患で陽性となり，疾患マーカーというよりは辺縁系症状や認知機能障害をもたらす病態のマーカーとして診断に有用で，免疫修飾治療の適応を示唆する治療マーカーとなる可能性があると考えている．

文　献

1) 森　寿．グルタミン酸受容体チャンネルの構造と機能．生化学 2005；77：619-29.
2) Groc L, Heine M, Cousins SL, et al. NMDA receptor surface mobility depends on NR2A-2B subunits. Proc Natl Acad Sci USA 2006；103：18769-74.
3) 高橋幸利，最上友紀子，高山留美子，ほか．免疫性神経疾患：最近の進歩，NMDA型グルタミン酸受容体と神経疾患．Neuroimmunology 2009；17：245-55.
4) 高橋幸利，植田佑樹，保立麻美子．病因．辻　貞敏・編．新しい診断と治療のABC74/神経5てんかん．大阪：最新医学社；2012. p. 60-71.
5) Rogers SW, Andrews PI, Gahring LC, et al. Autoantibodies to glutamate receptor GluR3 in Rasmussen's encephalitis. Science 1994；265：648-51.
6) Takahashi Y, Mori H, Mishina M, et al. Autoantibodies to NMDA receptor in patients with chronic forms of epilepsia partialis continua. Neurology 2003；61：891-6.
7) 高橋幸利．小児期の中枢神経系感染症による難治てんかんにおける抗GluRε2自己抗体の存在．日本小児科学会誌 2002；106：1402-11.
8) Dalmau J, Tüzün E, Wu H, et al. Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. Ann Neurol 2007；61：25-36.
9) Dalmau J, Gleichman AJ, Hughes EG, et al. Anti-NMDA-receptor encephalitis：case series and analysis of the effects of antibodies. Lancet Neurol 2008；7：1091-8.
10) Fujita K, Yuasa T, Takahashi Y, et al. Antibodies to N-methyl-D-aspartate glutamate receptors in Creutzfeldt-Jakob disease patients. J Neuroimmunol 2012；251：90-3.
11) 高橋幸利．グルタミン酸受容体抗体の意義．脳と発達 2013；45：99-105.
12) Takano S, Takahashi Y, Kishi H, et al. Detection of autoantibody against extracellular epitopes of N-methyl-D-aspartate receptor by cell-based assay, Neurosci Res 2011；71：294-302.
13) Hughes EG, Peng X, Gleichman AJ, et al. Cellular and synaptic mechanisms of anti-NMDA receptor. J Neurosci 2010；30：5866-75.
14) Mikasova L, De Rossi P, Bouchet D, et al. Disrupted surface cross-talk between NMDA and Ephrin-B2 receptors in anti-NMDA encephalitis. Brain 2012：135；1606-21.
15) DeGiorgio LA, Konstantinov KN, Lee SC, et al. A subset of lupus anti-DNA antibodies cross-reacts with the NR2 glutamate receptor in systemic lupus erythematosus. Nat Med 2001；7：1189-93.
16) Kowal C, Degiorgio LA, Lee JY, et al. Human lupus autoantibodies against NMDA receptors mediate cognitive impairment. Proc Natl Acad Sci USA 2006；103：19854-9.
17) Huerta PT, Kowal C, DeGiorgio LA, et al. Immunity and behavior：antibodies alter emotion. Proc Natl Acad Sci USA 2006；103：678-83.
18) 高橋幸利，高木佐知子，西村成子，角替央野．てんかんと抗NMDA受容体抗体．Clinical Neuroscience 2006；24：219-21.
19) Niehusmann P, Dalmau J, Rudlowski C, et al. Diagnostic value of N-methyl-D-aspartate receptor antibodies in women with new-onset epilepsy. Arch Neurol 2009；66：458-64.
20) 高橋幸利，植田佑樹，保立麻美子，ほか．非ヘルペス性急性辺縁系脳炎．小児内科 2013；45：376-80.
21) Tachibana N, Shirakawa T, Ishii K, et al. Expression of various glutamate receptors including N-methyl-D-aspartate receptor (NMDAR) in an ovarian teratoma removed from a young female with anti-NMDAR encephalitis. Intern Med 2010；49：2167-73.
22) 高橋幸利，植田佑樹，保立麻美子，ほか．Rasmussen症候群．小児内科 2013；45：416-21.
23) Takahashi Y, Mine J, Kubota Y, et al. A substantial number of Rasmussen syndrome patients have increased IgG, CD4＋T cells, TNFα, and Granzyme B in CSF. Epilepsia 2009；50：1419-31.
24) 高橋幸利，山崎悦子，長尾雅悦，ほか．急性脳炎の後遺症に関する調査―ADL・てんかん発作・知的障害・精神障害・記憶障害・運動障害―．Neuroinfection 2009；14：106-12.
25) Suriadi M, Takahashi Y, Nishimura S, et al. Dysfunction of blood-brain barrier in epileptic patients after acute encephalitis. Epileptologia 2012；20：51-61.
26) Fujita K, Yuasa T, Takahashi Y, et al. Voltage-gated potassium channel complex antibodies in Creutzfeldt-Jakob disease. J Neurol 2012；259：2249-50.
27) Kashihara K, Kawada S, Takahashi Y. Autoantibodies to glutamate receptor GluRepsilon2 in a patient with limbic encephalitis associated with relapsing polychondritis. J Neurol Sci 2009；287：275-7.
28) 六反田　拓，稲富雄一郎，米原敏郎，ほか．血清・髄液中抗グルタミン酸受容体抗体陽性が診断を混乱させたglioblastomaの1例．臨床神経 2008；48：497-500.

短　報

卵巣奇形腫を合併し抗NMDA受容体抗体陽性のglioblastomaの1例

藤井　裕樹[1)4)]　久保　智司[1)4)]　柚木　太淳[1)5)]　佐藤　恒太[1)5)]
高松　和弘[1)]　田中　惠子[2)]　高橋　幸利[3)]　栗山　勝[1)*]

要旨：症例は54歳女性である．痙攣発作をともなう意識障害を呈し，頭部MRI T_2，FLAIR強調像にて右側頭葉内側に高信号をみとめ，卵巣奇形腫を合併していた．グルタミン酸受容体（GluR）ε2，δ2に対する抗体が陽性で，NMDARヘテロマー（NR1＋NR2）抗体も陽性であり，抗NMDA受容体脳炎をうたがった．卵巣奇形腫の摘出をおこない，経過をみていたところ，発症約6ヵ月後に右側頭葉にglioblastomaが明らかとなった．卵巣奇形腫を合併し，GluR抗体および複合体抗体陽性でglioblastomaの症例の報告はない．本抗体が陽性であっても，経時的に注意深く経過をみることが重要である貴重な教訓的症例であった．

（臨床神経 2013;53:712-715）
Key words：卵巣奇形腫，抗NMDA受容体抗体，glioblastoma，limbic encephalitis

はじめに

痙攣発作を呈し，頭部MRIで右側頭葉内側に異常信号をみとめた症例に対して，ヘルペス脳炎として治療を開始した．しかし，卵巣奇形腫を合併し，GluR複合体抗体陽性であったため，抗NMDA（N-methyl-D-aspartate）受容体脳炎の可能性を考え，卵巣摘出を施行し，慎重に経過観察をおこなっていたところ，発症約6ヵ月後に病変はglioblastomaであることが判明した．卵巣奇形腫を合併し，GluR複合体抗体陽性のglioblastomaの症例は過去に報告はなく，臨床的に重要であり報告する．

症　例

症例：54歳，女性

主訴：痙攣発作

現病歴：2011年1月X日朝6時頃，睡眠中に左顔面と両上肢の強直間代性の痙攣が出現した．数回の両上肢の強直性痙攣が出現し，当院に緊急入院した．

既往歴，家族歴：特記事項なし．

入院時現症：血圧150/80 mmHg，脈拍112/min，頸部・胸腹部異常所見なし．神経学的には，意識レベルJCS I-3．来院後も左顔面の痙攣が出現した．脳神経に特記所見なし．四肢運動系，感覚系異常なし，髄膜刺激症状なし．腱反射正常，病的反射なし．小脳性運動失調なし，膀胱直腸障害なし．

検査所見：白血球8,960/mm^3，LDL-コレステロール209 mg/dlと軽度上昇以外には，末梢血・生化学に異常なし．脳脊髄液（CSF）検査では，細胞数（単核球）2/mm^3，蛋白30 mg/dl，糖77 mg/dlで正常範囲であった．脳波は右大脳半球に徐波をみとめ，右側頭葉から鋭波をみとめた．血清の水痘帯状ヘルペス・単純ヘルペス・EBウイルス・サイトメガロウイルス・ムンプスウイルス抗体価に有意な増加はみとめなかった．CSF中の水痘帯状ヘルペス・単純ヘルペス・サイトメガロウイルス抗体価の有意な増加はなく，ヘルペスウイルスのPCRは陰性であった．頭部MRI T_2，FLAIR強調像にて右側頭葉内側に高信号をみとめ，高信号は扁桃体，海馬と海馬周囲に広がっており，軽度の腫脹を呈し，海馬頭部に軽度の造影効果をみとめた（Fig. 1A, B）．

経過：辺縁系脳炎を示唆する画像所見をみとめたことから，当初ヘルペス脳炎の可能性を考え直ちにアシクロビル1,500 mg/日の点滴を開始し，抗てんかん薬としてアレビアチンを投与した．入院翌日には，意識障害は改善し，以後痙攣発作の出現はなかった．骨盤画像検査で右卵巣に石灰化をともなう奇形腫をみとめた（Fig. 1E, F）．卵巣奇形腫を有した辺縁系脳炎がうたがわれ，早期の摘出術をC病院婦人科に依頼し，第17病日に卵巣腫瘍摘出術を施行した．病理組織検査では良性成熟性奇形腫であり，脳組織を含んでいた．

*Corresponding author: 脳神経センター大田記念病院脳神経内科〔〒720-0825　広島県福山市沖野上町3-6-28〕
1) 脳神経センター大田記念病院脳神経内科
2) 金沢医科大学神経内科
3) 静岡てんかん神経医療センター小児科
4) 現：広島大学大学院脳神経内科学
5) 現：岡山大学大学院・医学部脳神経内科学
（受付日：2012年9月7日）

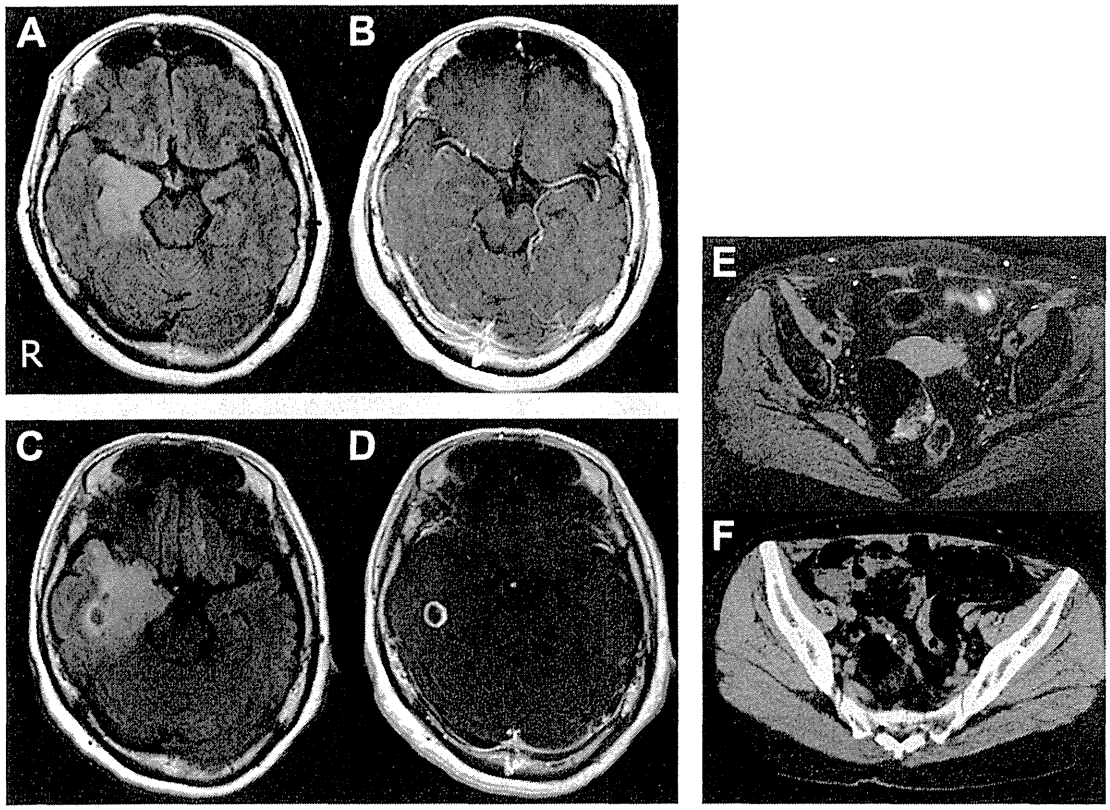

Fig. 1　MRI findings.

A, B (Brain MRI on admission): (A) Axial MRI of the brain reveals a high-intensity area on a FLAIR image (Axial, TR 8,500 ms, TE 91 ms) in the right medial temporal lobe. (B) Gadolinium-enhanced T_1-weighted image (Axial, 1.5 T; TR 650 ms, TE 17 ms) shows slight enhancement of the right hippocampus. C, D (Course of brain MRI): (C) FLAIR image shows an enlarged lesion, and (E) gadolinium-enhanced T_1-weighted image shows ring enhancement in the right temporal lobe 6 months after discharge. E, F (Pelvic MRI and CT on admission): (E) T_1-weighted image with fat suppression (Axial, 1.5 T; TR 532 ms, TE 11 ms) shows an apparently circumscribed right ovarian tumor containing fat. (F) Computed tomography shows calcification and fat in the ovarian tumor, which is consistent with ovarian teratoma.

その後の検査で，CSF 中の GluRε2, δ2 に対する抗体が陽性で，GluR 複合体（NR1＋NR2）抗体も陽性であった．以上の結果から，臨床的には卵巣奇形腫に合併した抗 NMDA 受容体脳炎が考えられたが，神経学的には典型的な症状や神経学的所見も示さず，第 56 病日に自宅退院した．発症 2 ヵ月後の外来での経過観察中の頭部 MRI では著変はなかったが，4 ヵ月後の MRI で右側頭葉内側の病変は軽度拡大し，同部位に結節状の造影部分が出現した．この頃から嗅覚異常の後に左手が熱くなる 2 分程度の発作（鉤発作）がみられるようになった．ステロイドパルス療法を施行したが効果はみとめなかった．6 ヵ月後の頭部 MRI で右側頭葉内側，海馬・側脳室下角の下外側（紡錘状回）の病変が増悪し，造影 T_1 強調像でリング状増強効果がみられた（Fig. 1C, D）．開頭摘出術を施行し，病理組織検査で glioblastoma と診断された（Fig. 2）．その後，拡大局所照射，テモゾロフトによる追加加療をおこない，現在，経過観察中である．

考　察

本例は，痙攣発作をともなう意識障害で発症し，頭部 MRI で右側頭葉内側に異常信号をみとめ，卵巣腫瘍を合併，髄液 GluR 複合体抗体陽性であったことより，抗 NMDA 受容体脳炎がうたがわれたが，6 ヵ月後に右側頭葉の glioblastom が判明した症例である．

非ヘルペス性辺縁系脳炎のうち，卵巣奇形腫の合併例では，抗NMDA受容体抗体が高率に陽性であり[1]，Dalmau らにより，NMDA 型 GluR 複合体抗体陽性の急性辺縁系脳炎に対して抗 NMDA 受容体脳炎という名称が提唱されている[1)2)]．本例は典型的な抗 NMDA 受容体脳炎とくらべてことなる点が指摘できる．第 1 に発症がやや高齢であった．抗 NMDA 受容体脳炎では 40 歳代の発症も報告されてはいるが[3]，20～34 歳が多い．症状も痙攣をともなう意識障害以外，重篤な神経症状は生じなかったが，本例が典型的な臨床を示さなかった原因は，卵巣腫瘍摘出もふくめ早期に治療を開始し，進行抑制

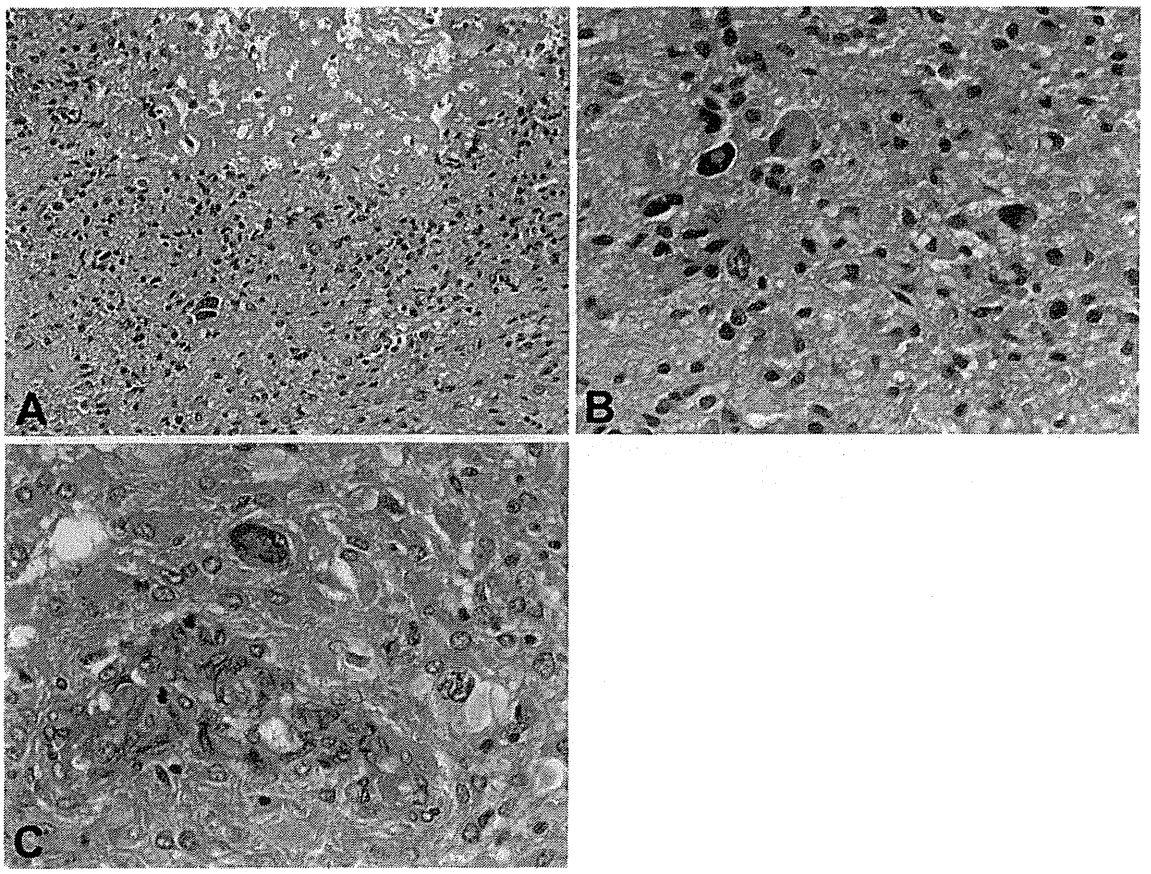

Fig. 2　Pathological findings of brain biopsy from the right temporal lobe.
Photomicrographs of specimen reveal the tumor tissue with polymorphic cells and necrosis [H–E stain, magnification 10 × (A), 20 × (B)], and with microvascular proliferation of endothelial cells [H–E stain, magnification 20 × (C)], suggesting of glioblastoma. The immuno-staining were negative for CAM5.2 and positive for GFAP. The positive percentage of MIB-1 were from 6.7% to 59.2% (Data were not shown).

できたためではないかと推測していた．また本疾患ではてんかんのみの症例[4]も報告はされている．本例の画像は軽度造影を示し一側に限局していたが，本疾患では片側病変の症例，造影効果を示す症例も少なくなく，抗NMDA受容体脳炎としても矛盾しなかった．まれではあるが，glioblastomaをふくむ原発性あるいは転移性脳腫瘍が急性脳炎あるいは脳症に類似した臨床症状や画像所見を呈した症例が報告されており[5]，glioblastomaの診断や治療の遅れにつながることが指摘されている．

抗GluRε2抗体（NR2B）は細胞表面側ドメイン（NT1）を認識する抗体であるが，Rasmussen脳炎，急性脳炎など多くの疾患で陽性となり，近年，疾患特異性が問題となっている[6]．卵巣腫瘍の合併に関しては，抗NMDA受容体脳炎症例の約40％には卵巣腫瘍はみとめられておらず[2)7)]，これも当初強調されたほどには特異的な合併ではない．glioblastomaの症例で抗GluRε2抗体が陽性であった症例は，検索しえたかぎりでは，1例が報告されている[8]．しかし，卵巣奇形腫を合併し，GluR抗体および複合体抗体が陽性であったglioblastomaの症例は過去に報告はなかった．抗NMDA受容体脳炎では卵巣奇形腫の神経組織細胞膜上に発現している抗原に対して，抗NMDA受容体抗体が産生されていると推測されているが，glioblastomaの細胞でもNMDA受容体を発現すると報告されている[9]．近年，5種のヒトglioblastomaの培養細胞株と5例のglioblastoma患者組織から培養されたglioblastoma細胞株を検討し，NMDA受容体陽性細胞がそれぞれ平均6.56％，2.15％発現していると報告された．Glioblastoma腫瘍細胞の増殖制御因子とされるearly growth response-1（EGR-1）の活性化が，NMDA受容体の刺激によって介在される可能性が指摘されている[9]．Glioblastoma細胞表面上にNMDA受容体が発現し，その抗体が産生されているならば，腫瘍の増大に影響を与える可能性も考えられる．今後，glioblastomaと抗NMDA受容体抗体との関連に関してはさらに検討が必要である．

抗NMDA受容体抗体は，抗NMDA受容体脳炎の診断には必須である．しかしながら，本抗体は疾患特異的ではなく，陽性であっても経時的に経過観察することはきわめて重要で

あることを強調したい．今後，卵巣奇形腫や各種脳腫瘍における抗NMDA受容体抗体陽性率の検証も重要と思われた．

本論文の要旨は第91回日本神経学会中国・四国地方会（2011年12月10日，高知）にて発表した．

謝辞：本例の脳外科治療に関して脳神経センター大田記念病院脳神経外科佐藤倫由先生，卵巣腫瘍摘出術を施行していただきました福山中央病院婦人科寺澤晃司先生，病理学的助言をいただきました福山市医師会診断病理学センター元井信先生に深謝致します．

※本論文に関連し，開示すべきCOI状態にある企業，組織，団体はいずれも有りません．

文　献

1) Dalmau J, Tüzün E, Wu HY, et al. Paraneoplastic anti-N-methyl-D-aspartate receptor encephalitis associated with ovarian teratoma. Ann Neurol 2007;61:25-36.
2) Dalmau J, Gleichman AJ, Hughes EG, et al. Anti-NMDA-receptor encephalitis: case series and analysis of the effects of antibodies. Lancet Neurol 2008;7:1091-1098.
3) 高橋幸利，山崎悦子，西村成子ら．急性辺縁系脳炎・脳症とNMDA型グルタミン酸受容体．臨床神経 2008;48:926-929.
4) Irani SR, Bera K, Waters P, et al. N-methyl-D-aspartate antibody encephalitis: temporal progression of clinical and paraclinical observations in a predominantly non-paraneoplastic disorder of both sexes. Brain 2010;133:1655-1667.
5) Nam TS, Choi KH, Kim MK, et al. Glioblastoma mimicking herpes simplex encephalitis. J Korean Neurosurg Soc 2011;50:119-122.
6) Takahashi Y, Mori H, Mishina M, et al. Autoantibodies to NMDA receptor in patients with chronic forms of epilepsia partialis continua. Neurology 2003;61:891-896.
7) Dalmau J, Lancaster E, Martinez-Hemandez E, et al. Clinical experience and laboratory investigations in patients with anti-NMDAR encephalitis. Lancet Neurol 2011;10:63-74.
8) 六反田拓，稲富雄一郎，米原敏郎ら．血清・髄液中抗グルタミン酸受容体抗体陽性が診断を混乱させたglioblastomaの1例．臨床神経 2008;48:497-500.
9) Mittelbronn M, Harter P, Warth A, et al. EGR-1 is regulated by N-methyl-D-aspartate-receptor stimulation and associated with patient survival in human high grade astrocytomas. Brain Pathol 2009;19:195-204.

Abstract

Glioblastoma with ovarian teratoma having N-methyl-D-aspartate receptor (NMDAR) antibody in CSF—A case report

Hiroki Fujii, M.D.[1)4)], Satoshi Kubo, M.D.[1)4)], Taijun Yunoki, M.D.[1)5)], Kouta Sato, M.D.[1)5)], Kazuhiro Takamatsu, M.D.[1)], Keiko Tanaka, M.D. Ph.D.[2)], Yukitoshi Takahashi, M.D., Ph.D.[3)] and Masaru Kuriyama, M.D., Ph.D.[1)]

[1)]Department of Neurology, Ota Memorial Hospital.
[2)]Department of Neurology, Kanazawa Medical University
[3)]National Epilepsy Center, Shizuoka Institute of Epilepsy and Neurological Disorders
[4)]Present address: Department of Clinical Neuroscience and Therapeutics, Hiroshima University
[5)]Present address: Department of Neurology and Neuroscience, Okayama University

A 54-year-old woman presented with complex partial seizure with impaired consciousness. Brain MRI revealed a high intensity lesion on T_2-weighted and FLAIR images in the left temporal lobe, indicating limbic encephalitis. CT and MRI of the pelvis showed right ovarian teratoma. The cerebrospinal fluid (CSF) were positive for antibodies against the GluRε2, GluRδ2, and antibodies against NR1 + NR2B heteromers. On the basis of these data, anti-N-methyl-D-aspartate receptor (NMDAR) encephalitis associated with ovarian teratoma was suspected, and the right ovariectomy was performed. Six months after onset, brain biopsy from the right temporal lobe led to a diagnosed of glioblastoma. This is the first glioblastoma case with ovarian teratoma having autoantibodies against GluR and NR1 + NR2B heteromers in CSF. We suggest that patients with NMDAR antibodies should be carefully diagnosed with anti-NMDAR encephalitis.

(Clin Neurol 2013;53:712-715)

Key words: ovarian teratoma, anti-N-methyl-D-aspartate receptor antibody, glioblastoma, limbic encephalitis

Cellular/Molecular

Neuronal D-Serine and Glycine Release Via the Asc-1 Transporter Regulates NMDA Receptor-Dependent Synaptic Activity

Dina Rosenberg,[1] Samar Artoul,[1] Adi C. Segal,[1] Goren Kolodney,[1] Inna Radzishevsky,[1] Elena Dikopoltsev,[1] Veronika N. Foltyn,[1] Ran Inoue,[2] Hisashi Mori,[2] Jean-Marie Billard,[3] and Herman Wolosker[1]

[1]Department of Biochemistry, The Rappaport Faculty of Medicine and Research Institute, Technion–Israel Institute of Technology, Haifa 31096, Israel, [2]Department of Molecular Neuroscience, Graduate School of Medicine and Pharmaceutical Sciences, University of Toyama, Toyama 930-0194, Japan, and [3]Center of Psychiatry and Neuroscience, University Paris Descartes, Sorbonne Paris City, UMR 894, 75014 Paris, France

D-Serine and glycine are coagonists of NMDA receptors (NMDARs), but their relative contributions for several NMDAR-dependent processes are unclear. We now report that the alanine–serine–cysteine transporter-1 (Asc-1) mediates release of both D-serine and glycine from neurons, and, in turn, this modulates NMDAR synaptic activity. Asc-1 antiporter activity is enhanced by D-isoleucine (D-Ile), which releases D-serine and glycine from Asc-1-transfected cells, primary neuronal cultures, and hippocampal slices. D-Ile has no effect on astrocytes, which do not express Asc-1. We show that D-Ile enhances the long-term potentiation (LTP) in rat and mouse hippocampal CA1 by stimulating Asc-1-mediated endogenous D-serine release. D-Ile effects on synaptic plasticity are abolished by enzymatically depleting D-serine or by using serine racemase knock-out (SR-KO) mice, confirming its specificity and supporting the notion that LTP depends mostly on D-serine release. Conversely, our data also disclose a role of glycine in activating synaptic NMDARs. Although acute enzymatic depletion of D-serine also drastically decreases the isolated NMDAR synaptic potentials, these responses are still enhanced by D-Ile. Furthermore, NMDAR synaptic potentials are preserved in SR-KO mice and are also enhanced by D-Ile, indicating that glycine overlaps with D-serine binding at synaptic NMDARs. Altogether, our results disclose a novel role of Asc-1 in regulating NMDAR-dependent synaptic activity by mediating concurrent non-vesicular release of D-serine and glycine. Our data also highlight an important role of neuron-derived D-serine and glycine, indicating that astrocytic D-serine is not solely responsible for activating synaptic NMDARs.

Introduction

NMDA receptors (NMDARs) are key to several physiological and pathological processes, including learning and memory and neurotoxicity (Traynelis et al., 2010). In addition to glutamate, NMDARs require the binding of a coagonist (glycine or D-serine) for channel opening (Johnson and Ascher, 1987; McBain et al., 1989). Several studies indicate that endogenous D-serine is the main coagonist for NMDARs (Mothet et al., 2000; Shleper et al., 2005; Junjaud et al., 2006; Inoue et al., 2008; Basu et al., 2009; Henneberger et al., 2010). D-Serine is present in astrocytes (Schell et al., 1995) and is synthesized by the enzyme serine racemase (SR) (Wolosker et al., 1999).

The roles of glia and neurons in D-serine dynamics are yet to be determined. Several reports suggest that D-serine is a gliotransmitter (Mothet et al., 2005; Panatier et al., 2006; Papouin et al., 2012). Conversely, recent data indicate that SR is predominantly expressed in glutamatergic neurons, challenging the notion that D-serine is exclusively released from astrocytes (Kartvelishvily et al., 2006; Miya et al., 2008; Balu and Coyle, 2012; Benneyworth et al., 2012).

D-Serine and glycine appear to target different types of NMDARs (Papouin et al., 2012) and are thought to be regulated by distinct uptake and release pathways. Glycine levels are regulated by glycine transporters GlyT1 and GlyT2 (Tsai et al., 2004). GlyT1 is widely expressed in the forebrain, whereas GlyT2 is more restricted to glycinergic terminals (Betz et al., 2006). D-Serine levels are regulated by two types of antiporters: the Na^+-dependent ASCT1 and ASCT2 (Ribeiro et al., 2002) and the Na^+-independent alanine–serine–cysteine transporter-1 (Asc-1) (Fukasawa et al., 2000). These antiporters catalyze amino acid hetero-exchange, in which the uptake or release of D-serine is coupled to the transport of a neutral amino acid in the opposite direction. ASCT1 and ASCT2 antiporters are widely expressed but exhibit low affinity for D-serine (Ribeiro et al., 2002). Conversely, Asc-1 is restricted to neurons and display high affinity for

Received Aug. 11, 2012; revised Nov. 13, 2012; accepted Dec. 21, 2012.

Author contributions: D.R., J.-M.B., and H.W. designed research; D.R., S.A., A.C.S., I.R., E.D., J.-M.B., and H.W. performed research; G.K., I.R., V.N.F., R.I., and H.M. contributed unpublished reagents/analytic tools; D.R., H.M., J.-M.B., and H.W. analyzed data; J.-M.B. and H.W. wrote the paper.

This work was supported by grants from the Israel Science Foundation, Rappaport Institute of Medical Research, Jessie Kaplan Research Fund, IMHRO, and Albert Goodstein Research Fund (H.W.), Ministry of Education, Culture, Sports, Science, and Technology of Japan Grant 22150003 (H.M.), and by the National Institute of Health and Medical Research (J.M.B.). The authors thank Prof. M. Gavish for help with the binding experiments.

Correspondence should be addressed to either of the following: Jean-Marie Billard, Center of Psychiatry and Neuroscience, University Paris Descartes, Sorbonne Paris City, UMR 894, 75014 Paris, France, E-mail: jean-marie.billard@inserm.fr; or Herman Wolosker, Department of Biochemistry, The Rappaport Faculty of Medicine and Research Institute, Technion–Israel Institute of Technology, Haifa 31096, Israel, E-mail: hwolosker@tx.technion.ac.il.

DOI:10.1523/JNEUROSCI.3836-12.2013

 0270-6474/13/333533-12$15.00/0

both D-serine and glycine (Fukasawa et al., 2000; Helboe et al., 2003). Data obtained with Asc-1 knock-out (KO) mice indicate that Asc-1 is the main D-serine transporter in the brain (Rutter et al., 2007). These mice exhibit a 70–80% reduction in the synaptosomal uptake of D-serine (Rutter et al., 2007), along with seizures that result in early postnatal death (Xie et al., 2005).

In the present report, we investigated the role of Asc-1 in controlling the levels of D-serine in the context of NMDAR-dependent synaptic activity. We identify D-isoleucine (D-Ile) as a selective activator of Asc-1 antiporter activity, which enhances long-term potentiation (LTP) at the hippocampal CA1–CA3 via release of endogenous D-serine. Moreover, we found that Asc-1 regulates NMDAR potentials, which involves glycine release as well. Our data indicate that D-serine and glycine share a common release pathway via Asc-1 to regulate NMDARs. This pathway highlights the role of neurons in releasing D-serine and glycine and may underlie their partial overlapping roles in modulating NMDARs. In this framework, Asc-1 can be a useful pharmacological target to modulate NMDAR activity and counteract neurotoxicity.

Materials and Methods

Materials. L-Serine and D-Ile were purchased from Bachem. D-[^{3}H]Serine (lots 110818 and 111107) and D-[^{14}C]isoleucine (lot 110628) were obtained from American Radiolabeled Chemicals. [^{3}H]Glycine (lot 3632835), [^{3}H]glutamic acid (lot 674819), [^{3}H]MDL 105,519 [(*E*)-3-(2-phenyl-2-carboxyethenyl)-4,6-dichloro-1[3*H*]-indole-2-carboxylic acid] (lot 643251), [^{3}H]dopamine (lot 677942), and [^{3}H]GABA (lot 675800) were purchased from PerkinElmer Life and Analytical Sciences. D-and L-Alanine, catalase, choline chloride, dopamine, ((*R*)-*N*-[3-(4′-fluorophenyl)-3-(4′phenylphenoxy)) sarcosine hydrochloride (NFPS), GABA, glycine, glutamate, 3-methyl-2-oxopentanoic acid (2-keto 3-methylvaleric acid), and pyruvate were obtained from Sigma-Aldrich. D-2-Amino-5-phosphonovalerate (D-APV) and 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo[*f*]quinoxaline-2,3-dione (NBQX) were purchased from Tocris Bioscience. D-Serine was provided by Bachem or Sigma-Aldrich. All pharmacological agents were bath applied from appropriate stock solutions stored at −20°C.

Primary cultures. Animals were killed by decapitation after isoflurane anesthesia. All animal procedures were in accordance with the Committee for the Supervision of Animal Experiments (Technion–Israel Institute of Technology). Primary neuronal cultures from the hippocampus were prepared from E18 Sprague Dawley rat embryos as described previously (Kartvelishvily et al., 2006). The neurons were cultured in Neurobasal medium supplemented with 2% B27. Such cultures typically contain <2% contaminant astrocytes (Kartvelishvily et al., 2006). Primary astrocyte cultures were obtained from the hippocampus of P0–P2 Sprague Dawley rats as described previously (Kartvelishvily et al., 2006). The neuronal and astrocytic cultures were used 14–20 d after plating.

Cell transfection. HEK293 cells were cultured in DMEM supplemented with 10% FBS and antibiotics. For transfection, cells were seeded at 70–90% confluence and then transfected using Lipofectamine 2000 (Invitrogen) with rat Asc-1 (SLC7A10)–pExpress-1 (Open Biosystems), rat 4F2 heavy chain (4F2hc)–pCMV–SPORT6 (Open Biosystems), rat GlyT1b–pExpress-1 (Open Biosystems), rat GlyT2–pRc/RSV (provided by Prof. N. Nelson, Tel Aviv University, Tel Aviv, Israel), human ASCT1–pCMV5 (provided by Prof. S. G. Amara, University of Pittsburgh, Pittsburgh, PA), and rat ASCT2–pRK5–KS (provided by Prof. S. Bröer, Australian National University, Canberra, Australian Capital Territory, Australia). Cells were used 48 h after transfection.

Amino acid uptake and release by cell cultures. Primary cultures or transfected HEK293 cells were cultured in 96-well plates and loaded with 100 nM to 5 μM D-[^{3}H]serine in HEPES-buffered saline (HBS) (in mM: 137 NaCl, 5.4 KCl, 0.34 K_2HPO_4, 0.44 KH_2PO_4, 0.41 $MgSO_4$, 0.49 $MgCl_2$, 1.07 $CaCl_2$, 5.6 D-glucose, and 10 HEPES, pH 7.4) at 25°C. D-[^{3}H]Serine uptake was terminated by washing the cells four times with ice-cold HBS. The radioactivity was monitored after lysing the cells with 100 μl of water per well for 10 min. For uptake in the absence of Na^+, we used an Na^+-free HBS medium in which cholineCl was substituted for Na^+, and the pH was adjusted with Tris base. Blanks were performed by incubating the primary or transfected cells with ice-cold uptake medium, and they typically accounted for <10% of the total radioactivity. The values were corrected for protein content in each well. When monitoring D-[^{3}H]serine uptake in HEK293 transfected cells, specific uptake was determined by subtracting the basal D-[^{3}H]serine uptake from cells transfected with a suitable control plasmid (GFP or 4F2hc gene alone). Kinetic constants were calculated by nonlinear regression curve fit using GraphPad Prism 5 program. Competitive inhibition was analyzed using the following equation: $K_{mapp} = K_m \times (1 + [I]/K_i)$, where K_{mapp} is the K_m in the presence of the inhibitor.

For D-[^{3}H]serine release experiments, the cells were preloaded with 5 μM D-[^{3}H]serine for 20–40 min. Subsequently, the wells were washed three times with cold HBS and exposed for 1–5 min to release medium (HBS with or without Na^+) supplemented with different drugs at room temperature. Released D-[^{3}H]serine (D-serine$_{out}$) was monitored in the medium by scintillation counting. Remaining intracellular D-[^{3}H]serine (D-serine$_{in}$) was determined after lysing the cells with 100 μl of water per well. Fractional D-serine release was calculated by using the formula: (D-serine$_{out}$ × 100)/(D-serine$_{out}$ + D-serine$_{in}$). To avoid any D-serine reuptake in some experiments, we included 10 μg/ml recombinant *Escherichia coli* D-serine dehydratase (DsdA) along with 4 mM pyruvate in the release media. This enzyme converts any released D-[^{3}H]serine into [^{3}H]pyruvate that is further diluted by the excess unlabeled pyruvate included in the release media (Rosenberg et al., 2010). Typically, no more than 7–10% D-serine was released during stimulation.

Endogenous amino acid release from slices. Two- to 3-month-old male Sprague Dawley rats were anesthetized with isoflurane and killed by decapitation. Hippocampi were dissected, chopped into strips measuring 400 μm by 400 μm using a McIlwain tissue chopper (Gonzalez-Alvear and Werling, 1994), and washed with oxygenated modified Krebs–HEPES buffer (MKB) (in mM: 127 NaCl, 1.3 NaH_2PO_4, 15 HEPES, 10 glucose, 5 KCl, and 2.5 $CaCl_2$, pH 7.4 adjusted with NaOH). Subsequently, the slices were equilibrated by a 30 min perfusion with oxygenated MKB in 0.3 ml chambers at a flow rate of 0.6 ml/min in a Suprafusion 1000 (SF-6) apparatus (Brandel) at 37°C. After equilibration in perfusion medium, samples were collected at 1.6 min intervals, and the endogenous D-serine peak was monitored by HPLC as described previously (Rosenberg et al., 2010). To estimate the total D-serine content, the slices were incubated for 20 min with 0.2 M HCl to release all intracellular endogenous D-serine. The results were expressed as percentage D-serine release/time unit. Endogenous glutamate release was monitored by HPLC using the same methodology described above for D-serine.

D-[^{3}H]Serine release from slices. Hippocampal slices were loaded with 5 μM D-[^{3}H]serine by 20 min incubation in oxygenated MKB at 37°C. The slices were then washed three times with oxygenated MKB and transferred to the superfusion apparatus. D-[^{3}H]Serine release was monitored by liquid scintillation counting. Total amino acid loading was estimated as described above for endogenous D-serine release. In some experiments, the slices were preloaded with D-[^{3}H]serine using sodium-free MKB (in mM: 127 cholineCl, 1.3 KH_2PO_4, 15 HEPES, 10 glucose, 3.7 KCl, and 2.5 $CaCl_2$, pH 7.4). To monitor subsequent D-[^{3}H]serine release, regular MKB was returned during the equilibration step at the perfusion apparatus. D-[^{3}H]Serine is not significantly metabolized in forebrain slices (Rosenberg et al., 2010).

Glycine release from slices. Hippocampal slices were loaded with 2 μM [^{3}H]glycine essentially as described above for D-serine. To ensure that the released radioactivity correspond to authentic glycine, the amino acids were separated by HPLC (Rosenberg et al., 2010), and the peak corresponding to glycine was manually collected and monitored by scintillation counting.

Ligand binding. [^{3}H]MDL 105,519 binding to isolated rat brain membranes was performed at 4°C in 10 mM Tris-acetate buffer, pH 7.4, and monitored by filtration assay as described previously (Baron et al., 1996).

Ex vivo *electrophysiology.* Experiments were performed in accordance with the European Council Directive (86/809/EEC) regarding the care and use of animals for experimental procedures and approved by the local ethics committee. Transverse hippocampal slices (400 μm) were

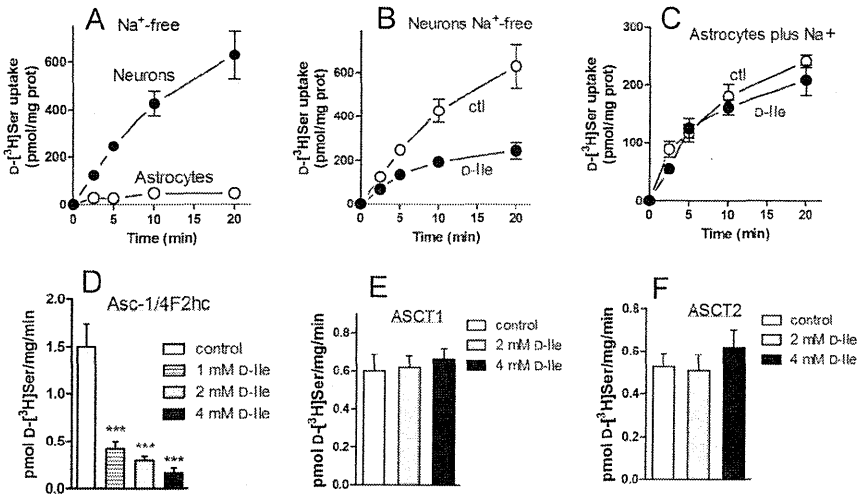

Figure 1. D-Ile inhibits D-serine transport by Asc-1. ***A***, Comparison of D-serine uptake by primary cultures of rat hippocampal neurons (●) and astrocytes (○) in the absence of Na^+. The uptake was performed with 5 μM D-[^{3}H]serine in a Na^+-free medium, in which NaCl was substituted by cholineCl. ***B***, D-Ile inhibits D-serine uptake by neurons. D-[^{3}H]Serine uptake was performed in Na^+-free medium, in either the absence (○) or presence (●) of 1 mM D-Ile. ctl, Control. ***C***, Na^+-dependent D-serine uptake by glia is insensitive to D-Ile. D-[^{3}H]Serine uptake in primary astrocytes was monitored in Na^+-containing medium, in either the absence (○) or presence (●) of 1 mM D-Ile. ***D***, D-Ile inhibits Asc-1 in transfected HEK293 cells. D-Serine uptake in Asc-1 and 4F2hc transfected cells was assayed in medium lacking Na^+ and supplemented with 100 nM D-[^{3}H]serine. The values were calculated by subtracting blanks consisting of cells transfected with 4F2hc only, which accounted for <10% of the total D-[^{3}H]serine accumulation. ***E***, D-Ile does not inhibit ASCT1 in HEK293 transfected cells. Uptake of D-[^{3}H]serine (100 nM) was assayed in Na^+-containing media. The values were calculated by subtracting blanks consisting of cells transfected with GFP, which accounted for ~30% of the total D-[^{3}H]serine accumulation. ***F***, D-Ile does not inhibit ASCT2 in HEK293 transfected cells. Uptake of D-[^{3}H]serine (100 nM) was assayed in Na^+-containing media. The values were calculated by subtracting blanks consisting of cells transfected with GFP, which accounted for ~35% of the total D-[^{3}H]serine accumulation. The values are the mean ± SEM of four to six experiments with different cultures. ***$p < 0.001$, different from control.

obtained from Sprague Dawley rats or C57BL/6 wild-type (WT) and SR-KO mice (3- to 4-month-old animals). The animals were anesthetized with halothane before decapitation. Slices were prepared in ice-cold artificial CSF (aCSF) and placed in a holding chamber for at least 1 h. The composition of aCSF was as follows (in mM): 124 NaCl, 3.5 KCl, 1.5 $MgSO_4$, 2.3 $CaCl_2$, 26.2 $NaHCO_3$, 1.2 NaH_2PO_4, and 11 glucose, pH 7.4. A single slice was transferred to the recording chamber at a time and continuously submerged with aCSF pregassed with 95% O_2/5% CO_2.

Extracellular recordings were obtained at 25–28°C from the apical dendritic layer of the CA1 area using micropipettes filled with 2 M NaCl. Presynaptic fiber volleys (PFVs) and field EPSPs (fEPSPs) were evoked by electrical stimulation of Schaffer collaterals and commissural fibers located in the stratum radiatum. NMDAR-mediated fEPSPs were isolated in slices perfused with low-Mg^{2+} (0.1 mM) aCSF supplemented with NBQX (10 μM). The averaged slope of three PFVs and fEPSPs was measured using Win LTP software (Anderson and Collingridge, 2001). To evaluate the level of receptor activation, the fEPSP/PFV ratio was plotted against stimulus intensity (300, 400, and 500 μA). The effects of exogenous D-Ile (1 or 2 mM) and D-serine (100 μM) were assessed by determining the fEPSP/PFV ratio 15 min after the addition of the amino acid to the aCSF.

To investigate LTP of synaptic transmission, a test stimulus was applied every 10 s in control medium and adjusted to get an fEPSP with a baseline slope of 0.1 V/s. In experiments performed on rat slices, the averaged slope of three fEPSPs was measured for 15 min before theta-burst stimulation (TBS), consisting of five trains of four 100 Hz pulses each, separated by 200 ms and delivered at the test intensity. This sequence was repeated three times with an interburst interval of 10 s. In experiments done on hippocampal slices from mice, the conditioning stimulation consisted on one high-frequency train at 100 Hz for 1 s. In recordings from both rats and mice, testing with a single pulse was then resumed for 60 min to determine the level of LTP. In pharmacological experiments, D-APV (80 μM), D-Ile (1 mM), or D-serine (100 μM) were added to the aCSF 10 min before the establishment of the baseline and maintained throughout recording.

In some experiments, NMDAR-mediated synaptic potentials as well as LTP were monitored in slices preincubated for at least 90 min with 20 μg/ml purified recombinant DsdA to deplete D-serine contents. The purified recombinant enzyme was prepared as described previously (Shleper et al., 2005).

Statistical analyses. This was performed by repeated-measures ANOVAs, followed by Tukey's *post hoc* test or by paired and unpaired *t* tests.

Results

Asc-1 as a D-serine release pathway from neurons

The plasma membrane Asc-1 (SLC7A10) is exclusively found in neurons *in vivo* and is the main transporter that mediates neuronal D-serine uptake (Fukasawa et al., 2000; Helboe et al., 2003; Matsuo et al., 2004; Rutter et al., 2007; Rosenberg et al., 2010). In contrast to astrocytic D-serine transporters, neuronal Asc-1 displays high affinity for D-serine and does not require Na^+ for activity (Fukasawa et al., 2000; Helboe et al., 2003; Matsuo et al., 2004; Rutter et al., 2007; Rosenberg et al., 2010). Accordingly, we found that our hippocampal neuronal cultures take up D-serine in the absence of Na^+, whereas D-serine transport in primary astrocyte cultures is strictly Na^+ dependent (Fig. 1*A*).

In light of the recent data indicating the existence of a neuronal pool of D-serine (Kartvelishvily et al., 2006; Rosenberg et al., 2010; Benneyworth et al., 2012) and the unique Na^+-independent uptake of D-serine in neurons, we performed a low-throughput screening for compounds that affect D-serine transport in neurons by Asc-1, focusing on D-amino acids. We found that D-Ile specifically inhibits neuronal D-serine uptake without affecting astrocytic transport (Fig. 1*B*,*C*).

To confirm that D-Ile acts on Asc-1, we first analyzed its specificity in HEK293 cells transfected with different transporters. We found that D-Ile inhibits the uptake of D-serine in cells expressing Asc-1 and its ancillary subunit 4F2hc that is essential for Asc-1 activity (Fig. 1*D*). Conversely, D-Ile had no effect in cultures transfected with the Na^+-dependent transporters ASCT1 (Fig. 1*E*) or ASCT2 (Fig. 1*F*), which are present in glia but also presumably in neurons (Bröer et al., 1999; Weiss et al., 2001; Sakai et al., 2003; Gliddon et al., 2009).

We wondered whether D-Ile is a transportable competitive substrate of Asc-1. We found that the inhibition of the neuronal D-serine transport by D-Ile was more pronounced at lower D-serine concentrations, indicating that D-Ile may compete with D-serine (Fig. 2*D*). Dixon plot analysis suggests a competitive inhibitory pattern (Fig. 2*E*). Further kinetic analysis revealed a K_m of 43 ± 4 μM for D-serine, which is almost the same as that reported previously for Asc-1 (Fukasawa et al., 2000). D-Ile increased the K_m to 110 ± 15 μM without changing the V_{max} and exhibited an apparent K_i of 0.98 ± 0.1 mM (Fig. 2*F*). Double-reciprocal plot analysis confirmed a competitive inhibition (Fig. 2*G*).

As an additional indication that D-Ile is a competitive substrate, we found that Asc-1/4F2hc transfected cells take up

D-[^{14}C]Ile, and this was prevented by adding D-serine (Fig. 2*A*). Preincubation of D-[^{14}C]Ile with D-amino acid oxidase (DAO), an enzyme that converts D-Ile into 3-methyl valeric acid, abolished the specific uptake, indicating no contamination of the D-[^{14}C]Ile by L-isomers (Fig. 2*A*). Primary neuronal cultures also transported D-[^{14}C]Ile (Fig. 2*B*), and this was prevented by D-serine or by excess unlabeled D-Ile. Likewise, D-alanine, a highly selective Asc-1 substrate (Rutter et al., 2007), inhibited neuronal uptake of D-[^{14}C]Ile (Fig. 2*B*), confirming the role of D-Ile as a transportable Asc-1 ligand.

Conceivably, D-Ile uptake is coupled to D-serine release via Asc-1 antiporter activity. Using primary neuronal cultures previously loaded with D-serine, we found that D-Ile induces robust release of D-serine, reflecting a D-Ile/D-serine exchange (Fig. 2*C*). In contrast, D-Ile slightly inhibited D-[^{3}H]serine release from astrocytes (Fig. 2*C*), confirming that these cells do not release D-serine via Asc-1.

D-Ile-induced D-serine release by Asc-1 was confirmed in Asc-1/4F2hc transfected HEK293 cells, whereas cells transfected with the 4F2hc subunit alone were insensitive to D-Ile (Fig. 3*A*,*B*). Furthermore, because Asc-1 mediates L-alanine efflux by facilitated diffusion from *Xenopus* oocytes (Fukasawa et al., 2000), we investigated whether D-serine leaks through this transporter by a similar mechanism. Accordingly, Asc-1/4F2hc transfection doubled the rate of D-serine efflux even in the absence of extracellular Asc-1 substrates, reflecting net release of D-serine uncoupled from the antiporter activity (Fig. 3*A*). In contrast to Asc-1, transfection of the obligatory exchanger ASCT1 did not release any D-serine when compared with GFP in the absence of extracellular amino acid substrates (Fig. 3*C*). ASCT1 was also insensitive to D-Ile, whereas L-alanine promoted significant D-serine release by ASCT1 when compared with GFP, indicating that ASCT1 is functional when expressed in HEK293 cells (Fig. 3*C*,*D*). Thus, D-serine leakage resulting from Asc-1 transfection was probably not attributable to activation of hetero-exchange by extracellular amino acids in the unstirred layer around the cells. The data are consistent with the notion that Asc-1 mediates D-serine release from cells by both antiporter and facilitated diffusion mechanisms.

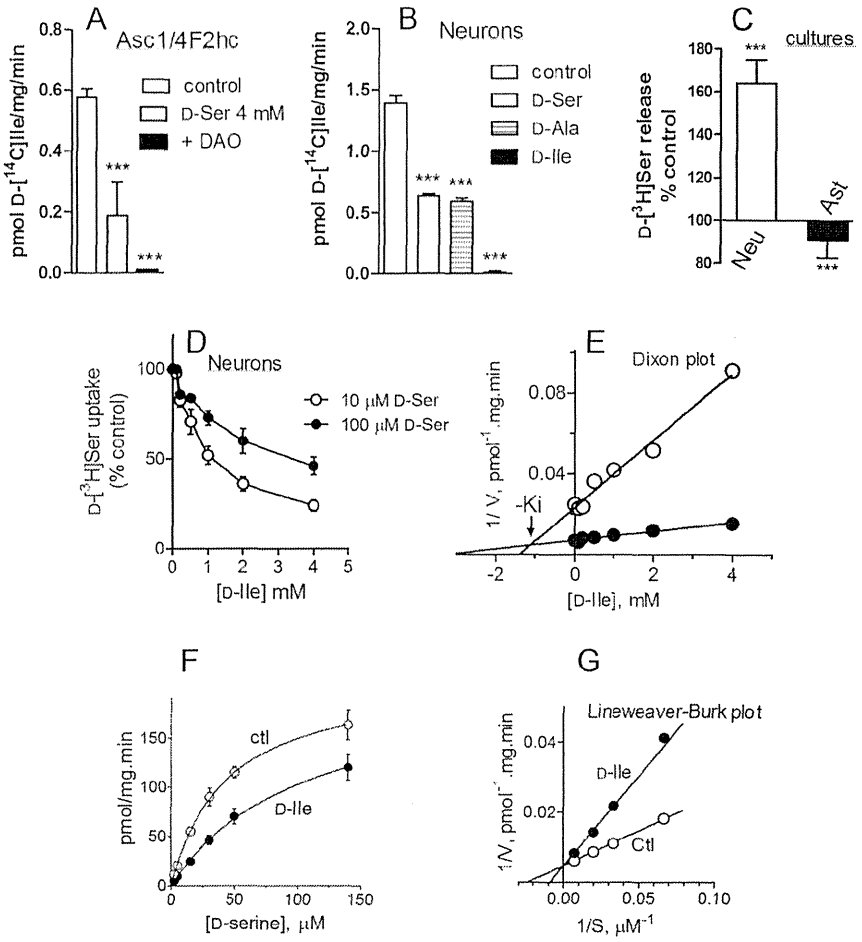

Figure 2. D-Ile is a competitive Asc-1 substrate that promotes D-serine release from neurons. ***A***, D-Ile uptake in HEK293 cells transfected with Asc-1/4F2hc. D-Ile transport was assayed in a Na^{+}-free medium containing 2 µM D-[^{14}C]Ile and was inhibited by adding 4 mM D-serine (gray bar). Incubation for 30 min with 20 µg/ml DAO and 2 µg/ml catalase completely degraded D-[^{14}C]Ile and abolished its transport (black bar). The medium contained 10 mM unlabeled 3-methyl valeric acid to block the reuptake of the corresponding keto acid generated by DAO activity. ***B***, D-Ile uptake in primary neuronal cultures. D-[^{14}C]Ile uptake in neurons was prevented by 2 mM D-serine, 2 mM D-alanine, or 4 mM D-Ile. ***C***, D-Ile elicits D-serine release from neurons but not astrocytes. Primary cultures were preloaded for 30 min with 5 µM D-[^{3}H]serine and, after washing, further incubated for 2–5 min in the absence or presence of 1 mM D-Ile. The results are expressed as the percentage of fractional D-[^{3}H]serine release observed in the absence of D-Ile. Neu, Primary neuronal culture; Ast, primary astrocytic culture. ***D***, Increasing the concentration of D-serine protects from D-Ile inhibition. D-[^{3}H]Serine transport into primary neuronal cultures was performed in the presence of 10 µM (○) or 100 µM (●) D-serine as described in the legend to Figure 1. ***E***, Dixon plot of the data from ***D*** suggests a competitive-type inhibition by D-Ile. ***F***, Initial rate of D-[^{3}H]serine transport as function of D-[^{3}H]serine concentration in the presence (●) and absence (○) of 1.5 mM D-Ile. ctl, Control. ***G***, Lineweaver–Burk plot of the data from ***F*** reveals a competitive inhibition of D-[^{3}H]serine transport by D-Ile. The values are the mean ± SEM of three to six experiments with different culture preparations. ***$p < 0.001$, different from control.

D-Serine and glycine dynamics are linked

To investigate whether D-Ile can be used as a tool to study the role of Asc-1 in more physiological preparations, we monitored the rate of endogenous D-serine release from acute hippocampal slices. Perfusion of slices with D-Ile more than doubled the rate of endogenous D-serine release monitored by HPLC (Fig. 4*A*). Conversely, D-Ile did not affect the rate of endogenous glutamate release, indicating that it does not interfere with glutamate transporters (Fig. 4*B*). These data are consistent with endogenous D-serine release from slices through Asc-1.

Despite its high affinity for glycine (Fukasawa et al., 2000; Helboe et al., 2003), a possible role of Asc-1 in glycine dynamics has not been investigated previously. We also found that D-Ile promotes glycine release from slices through activation of Asc-1 antiporter activity (Fig. 4*C*). To confirm that the radioactivity in the perfusate consisted of authentic [^{3}H]glycine, we validated its identity by HPLC analysis (Fig. 4*D*), ruling out significant metabolism of [^{3}H]glycine throughout the experiment. The data are consistent with the notion that Asc-1 antiporter activity promotes a concurrent release of neuronal D-serine and glycine from acute brain slices.

To confirm that D-Ile directly affects glycine fluxes through Asc-1, we characterized its actions in primary cultures and trans-

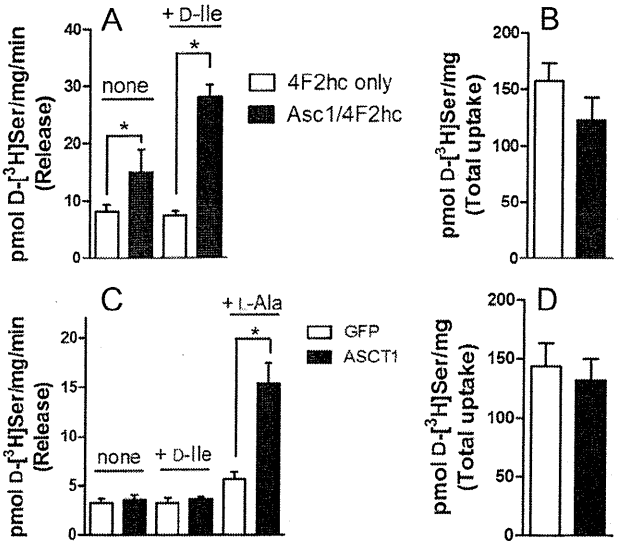

Figure 3. Characteristics of D-serine release from cells expressing Asc-1 and ASCT1 transporters. ***A***, Transfection of HEK293 cells with Asc-1/4F2hc (filled bars) accelerates the rate of D-serine release when compared with 4F2hc alone (open bars). D-Ile induces D-serine release from cells transfected with Asc-1/4F2hc but not from those transfected with 4F2hc alone. Total levels of D-[^{3}H]serine loading are shown in ***B*** and were attained by preloading the cells with 2 μM D-[^{3}H]serine for 30 min (4F2hc only, open bars) or 6 min (Asc-1/4F2hc, filled bars). ***C***, Transfection of HEK293 cells with ASCT1 (filled bars) does not affect basal D-serine release when compared with GFP control (open bars). D-Ile does not stimulate release from ASCT1 or GFP transfected cells, whereas addition of the substrate L-alanine (1 mM) increases the rate of D-[^{3}H]serine release from ASCT1 transfected cells when compared with GFP. Total levels D-[^{3}H]serine loading are shown in ***D*** and were attained by incubating the cells with 2 μM D-[^{3}H]serine for 30 min (GFP) or 10 min (ASCT1). The values are the mean ± SEM of three to six experiments with different culture preparations. *$p < 0.050$, different from control.

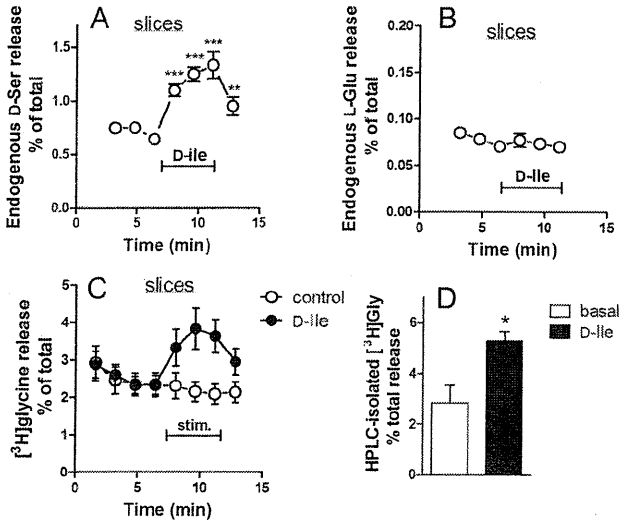

Figure 4. Asc-1-mediates release of D-serine and glycine from hippocampal slices without affecting glutamate dynamics. ***A***, D-Ile induces endogenous D-serine release from slices. Perfusion of acute hippocampal slices with oxygenated MKB supplemented with D-Ile (1 mM) at the indicated times (horizontal bar) induced release of endogenous D-serine. ***B***, D-Ile has no effect on endogenous L-glutamate release. ***C***, D-Ile elicits release of glycine from slices. Hippocampal slices were prelabeled for 20 min with 2 μM [^{3}H]glycine. The slices were then perfused with either none (○) or 1 mM D-Ile (●) at the indicated times. ***D***, Quantification of authentic [^{3}H]glycine release by HPLC analysis from basal (open bar) or D-Ile-stimulated fractions (filled bar). The results are mean ± SEM of 4–10 experiments with different preparations. * $p < 0.05$, ***$p < 0.001$, different from control.

fected cells. We found that D-Ile inhibits glycine uptake by neuronal cultures (Fig. 5*A*) but does not affect glycine transport in astrocytes, which lack Asc-1 activity (Fig. 5*B*). As expected, the uptake of glycine in astrocytes was decreased by the GlyT1 transporter inhibitor NFPS (Fig. 5*B*). The role of Asc-1 in mediating glycine transport was also confirmed in transfected cells. D-Ile inhibited glycine uptake by Asc-1/4F2hc transfected HEK293 cells (Fig. 5*C*) but had no effect in cells transfected with ASCT1 or ASCT2 (Fig. 5*D*,*E*). Furthermore, D-Ile does not affect the glycine transporters GlyT1b and GlyT2 (Fig. 5*F*,*G*).

Selectivity of D-Ile

We further evaluated D-Ile selectivity by investigating its effect on additional neurotransmitter systems. D-Ile does not affect neuronal or astrocytic glutamate uptake (Fig. 6*A*,*B*), nor does it change neuronal GABA or dopamine transport (Fig. 6*C*,*D*). In addition, D-Ile does not displace the binding of the selective NMDAR coagonist site ligand MDL 105,519 to washed brain membranes, indicating that it does not interact with the NMDAR coagonist binding site (Fig. 6*E*). The latter is in agreement with previous data showing that D-Ile does not affect the activity of recombinant NMDARs expressed in *Xenopus* oocytes (McBain et al., 1989).

The Na^+-independent transport of D-serine is mostly abolished in Asc-1 KO mice, indicating that Asc-1 accounts for >90% of the transport of D-serine in the absence of Na^+ (Rutter et al., 2007). To confirm that D-Ile is indeed acting on Asc-1 in slices, we inhibited all other transporters that are Na^+ dependent by monitoring D-serine release in an Na^+-free medium. Although the basal rate of D-serine release increases by omission of Na^+, we found that the effect of D-Ile was not attenuated (Fig. 7*A*). Quantitative analysis of the maximal net D-serine release after subtracting the basal rate shows that the D-Ile-stimulated D-serine release in the absence of Na^+ is at least as high as that observed in normal Na^+ (Fig. 7*B*). D-Alanine transport is also disrupted in Asc-1-KO mice, indicating that it is mediated by Asc-1 (Rutter et al., 2007). Thus, as an additional control, we applied D-alanine during preloading with D[^{3}H]-serine and found that it significantly decreased D-Ile-induced D-serine release (Fig. 7*C*). The data support the notion that Asc-1 is the main target for D-Ile.

D-Ile mimics the activation of Asc-1 by the endogenous substrates

The extracellular concentrations of the main Asc-1 substrates L-alanine, L-serine, and L-cysteine in the brain are 14, 36, and 2 μM, respectively (Lindroth et al., 1985). Altogether, these concentrations are higher than the apparent K_m of Asc-1 for these amino acids (11, 23, and 24 μM for L-alanine, L-serine, and L-cysteine, respectively) (Fukasawa et al., 2000). Thus, it is likely that Asc-1 antiporter activity is operational *in vivo*. However, we found that the concentrations of the main Asc-1 substrates in the hippocampal slice perfusates measured by HPLC were only 0.3 ± 0.05 and 0.5 ± 0.01 μM for L-alanine and L-serine, respectively. These concentrations are ~40-fold lower than their physiologic extracellular values, suggesting that the Asc-1 antiporter activity may be limited by the washout of extracellular substrates in the *ex vivo* perfused slices. In agreement, we found that perfusing the slices with physiological-like extracellular concentrations of L-alanine, L-serine, and L-cysteine enhances the rate of D-serine release to values similar to those obtained with D-Ile, suggesting that they restore the antiporter activity of Asc-1 (Fig. 7*D*). Thus, D-Ile mimics the physiologic activation of Asc-1 by typical extracellular endogenous substrates.

Effects of D-Ile on synaptic plasticity

Does D-serine or glycine release by Asc-1 play a role in synaptic plasticity? To answer this question, we monitored the effects of D-Ile on NMDAR-dependent LTP in the Schaffer collateral–CA1 synapse in adult rats. We found that D-Ile significantly increases the magnitude of NMDAR-dependent LTP induced by TBS (D-Ile, 132 ± 4.3% of baseline when averaged for the last 15 min of recordings vs control, 118.9 ± 4.1%, $p = 0.02$) (Fig. 8*A*). D-APV blocked the enhancement of LTP by D-Ile (99.9 ± 2.5% of baseline), confirming that it depends on NMDARs (Fig. 8*A*). D-Ile did not change the AMPAR-mediated fEPSPs evoked at different stimulus intensities, indicating that it does not affect basal glutamatergic transmission (Fig. 8*B*). Furthermore, D-Ile effect is not attributable to an increase in presynaptic release of glutamate, because it does not affect paired-pulse facilitation, an electrophysiological paradigm used to determine whether the drug affects the mechanisms of glutamatergic release (Fig. 8*C*).

Because D-serine seems to be the dominant coagonist for LTP in acute slices (Mothet et al., 2006; Henneberger et al., 2010), we wondered whether the enhancement of LTP by D-Ile is attributable to acceleration of endogenous D-serine release via Asc-1. For this purpose, we incubated slices with the recombinant enzyme DsdA. This enzyme selectively destroys endogenous D-serine and prevents the NMDAR-dependent neurotoxicity in organotypic slices and NMDAR potentials in the retina (Shleper et al., 2005; Gustafson et al., 2007). We found that treatment of the hippocampal slices with DsdA abolished TBS-induced LTP (DsdA, 106.3 ± 3.2% of baseline vs control, 120.9 ± 5.7%, $p = 0.0001$), confirming the critical role of D-serine in synaptic plasticity (Fig. 8*D*). Most importantly, D-Ile had no effect on LTP in slices treated with DsdA (D-Ile, 102.4 ± 2.4%) (Fig. 8*E*). These results indicate that D-Ile-mediated LTP enhancement is attributable to a specific increase in synaptic D-serine via Asc-1.

To confirm whether D-Ile effects on synaptic plasticity can be ascribed to a specific change in D-serine dynamics, we monitored LTP in mice with deletion of the SR gene (SR-KO). We used a single tetanus of 100 Hz for 1 s to induce reproducible NMDAR-dependent LTP in these animals. As expected, we found that D-Ile enhances LTP in WT mice (D-Ile, 124.4 ± 3.3% of baseline vs control, 113.6 ± 4.3%, $p = 0.02$), and this was blocked by D-APV (93.3 ± 2.7% of baseline) (Fig. 8*F*). In contrast, D-Ile did not enhance LTP in SR-KO mice (D-Ile, 107.8 ± 4.1% of baseline vs control, 110.3 ± 4.7%), confirming its specificity toward D-serine (Fig. 8*G*). Conversely, addition of exogenous D-serine enhanced LTP in WT and SR-KO mice by 10.7 and 18.6%, respectively (Fig. 8*H,I*), indicating that SR-KO mice respond to exogenous D-serine at least as well as WT.

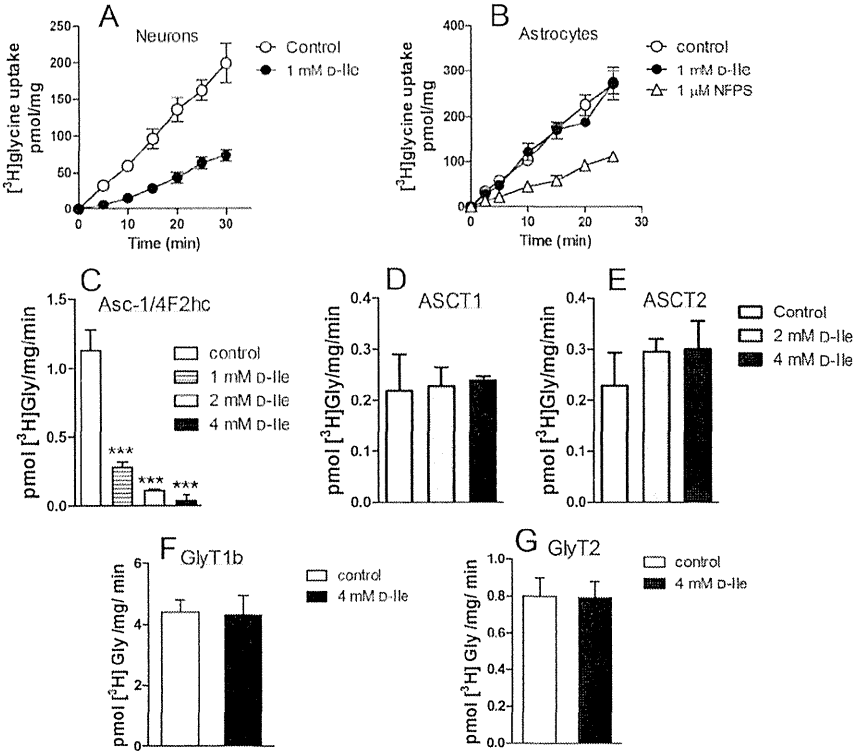

Figure 5. D-Ile inhibits glycine transport by Asc-1. ***A***, D-Ile inhibits glycine uptake in primary neuronal cultures. The assay was performed with 2 μM [^{3}H]glycine in a Na^+-free medium, in either the absence (○) or presence (●) of 1 mM D-Ile. ***B***, D-Ile has no effect on glycine uptake by rat primary astrocyte cultures. The uptake was performed with 2 μM [^{3}H]glycine in an Na^+-containing medium with none (○), 1 mM D-Ile (●), or 1 μM of the GlyT1 inhibitor NFPS (△). ***C***, D-Ile inhibits glycine uptake by Asc-1 in transfected HEK293 cells. Glycine uptake was monitored in Na^+-free medium containing 100 nM [^{3}H]glycine. The values were calculated by subtracting blanks consisting of cells transfected with 4F2hc only, which accounted for <10% of the total D-[^{3}H]serine accumulation. ***D–G***, D-Ile has no effect on glycine uptake by ASCT1 (***D***), ASCT2 (***E***), GlyT1b (***F***), or GlyT2 (***G***) transfected HEK 293 cells. These transport activities were monitored in Na^+-containing media, in the presence of 100 nM [^{3}H]glycine. The values were calculated by subtracting blanks consisting of cells transfected with GFP alone, which accounted for ~10–30% of the total [^{3}H]glycine accumulation. The values are the mean ± SEM of four to six experiments with different cultures. ***$p < 0.001$, different from control.

We also found that the LTP in the absence of D-Ile was the same in 2- to 3-month-old WT and SR-KO mice (Fig. 8, compare *H, I*). This contrasts with previous data that found LTP deficits in SR-KO using juvenile mice (21–28 d old) induced by a pairing protocol (Basu et al., 2009) or the striking decrease in LTP promoted by acute depletion of endogenous D-serine during treatment with DsdA (Fig. 8*D*) or DAO enzymes (Mothet et al., 2006; Henneberger et al., 2010). Altogether, the data support the notion that endogenous D-serine release mediates D-Ile effects on LTP, but that adult SR-KO mice may have adaptations on the LTP mechanisms to cope with the lower D-serine availability.

Role of Asc-1 in NMDAR-dependent synaptic activity

Does Asc-1 modulate synaptic NMDAR responses? We found that D-Ile also significantly increased the isolated NMDAR-dependent fEPSPs in rat hippocampal CA1 slices recorded in the presence of the AMPA antagonist NBQX and low magnesium concentration. D-Ile increased NMDAR fEPSPs by 25% whatever the intensity of stimulation in 11 of 16 tested slices (Fig. 9*A*). By comparison, addition of exogenous D-serine at saturating concentrations increased NMDAR-mediated fEPSPs by 45% in 12 of 15 slices (Fig. 9*B*). It is noteworthy that NMDAR fEPSPs were drastically decreased by depleting endogenous D-serine by DsdA treatment (between 60 and 70% reduction depending on

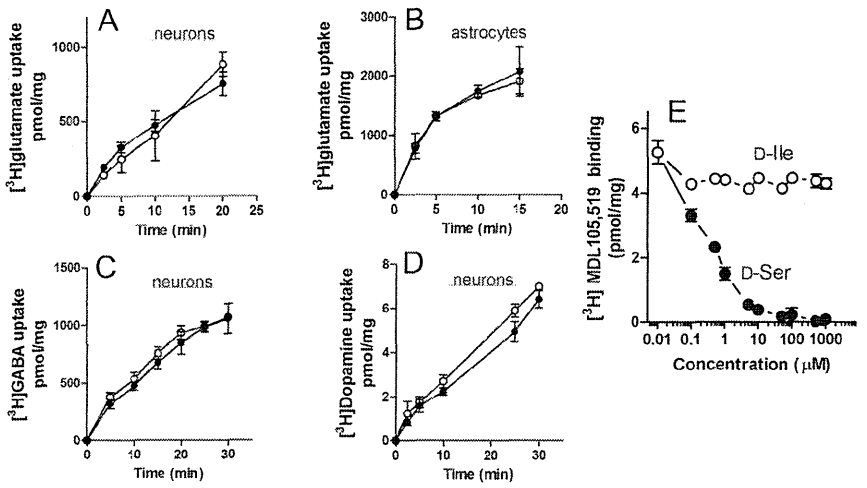

Figure 6. D-Ile has no effect on other neurotransmitter transporters and does not bind to the NMDAR coagonist site. ***A***, Glutamate uptake in rat primary neuronal cultures assayed with 1 μM [^{3}H]glutamate in Na^+-containing medium, in either the absence (○) or presence (●) of 1 mM D-Ile. ***B***, Glutamate uptake in rat primary astrocytic cultures in either the absence (○) or presence (●) of 1 mM D-Ile. ***C***, GABA uptake in rat primary neuronal cultures in the presence of 1 μM [^{3}H]GABA and in the absence (○) or presence (●) of 1 mM D-Ile. ***D***, Dopamine uptake in rat primary neuronal cultures performed in the presence of 1 μM [^{3}H]dopamine and in either the absence (○) or presence (●) of 1 mM D-Ile. ***E***, D-Ile does not affect the binding of the selective NMDAR coagonist site ligand [^{3}H]MDL 105,519 (100 nM) to washed rat brain membranes. Binding was abolished by D-serine (IC_{50} ~300 nM). The values are the mean ± SEM of three to four experiments.

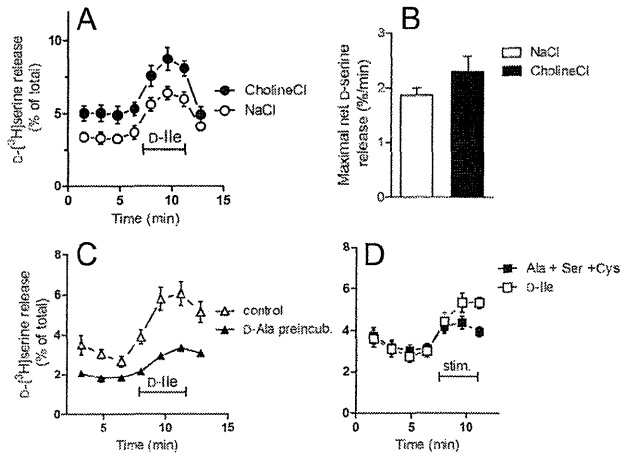

Figure 7. D-Ile elicits D-serine release from an Asc-1-sensitive pool and mimics the effects of typical extracellular Asc-1 substrates. ***A***, D-Ile-induced D-serine release from acute hippocampal slices in media containing NaCl (○) or cholineCl (●). Slices were preloaded with 5 μM D-[^{3}H]serine pre-equilibrated as described in Materials and Methods. Removal of Na^+ (●) was attained by a 15 min perfusion with cholineCl-containing medium before stimulation with 1 mM D-Ile. ***B***, Maximal net release of D-serine by D-Ile calculated from the data of ***A*** was similar in slices perfused with normal Na^+ (open bar) and Na^+-free (filled bar) media. ***C***, Preincubation with 1 mM D-alanine during loading with D-[^{3}H]serine decreases D-Ile-induced D-serine release. ***D***, Comparison between D-serine release promoted by D-Ile with that induced by perfusing the slices with L-alanine (14 μM), L-serine (36 μM), and L-cysteine (2 μM). The values are mean ± SEM of four to six experiments. ***$p < 0.001$, different from control.

stimulus intensity), demonstrating that D-serine is required for synaptic NMDAR activation (Fig. 9*C*). Nevertheless, depletion of endogenous D-serine by DsdA treatment did not abolish the stimulatory effect of D-Ile on NMDAR fEPSPs, regardless of the stimulus intensity (30% in average in 10 of 15 tested slices) (Fig. 9*D*). This suggests that a significant fraction of the D-Ile-mediated enhancement of NMDAR-mediated potentials could reflect the release of endogenous glycine as well. The data are consistent with the notion that Asc-1 also releases glycine from acute slices by antiporter activity (Fig. 4*C*).

Additional evidence that Asc-1 contributes to glycine dynamics comes from experiments with SR-KO mice (Fig. 10). We found that these mice exhibit normal NMDAR fEPSPs, suggesting a possible compensatory mechanism by endogenous glycine, because their D-serine levels are only 10% of the WT (Fig. 10*A*). Furthermore, we found that D-Ile significantly stimulated NMDAR fEPSPs by ~40% in SR-KO mice (10 of 12 tested slices), strengthening the notion that Asc-1 also releases glycine (Fig. 10*B*). Exogenously added D-serine stimulated NMDAR fEPSPs by 40%, in both WT (9 of 10 tested slices) and SR-KO (8 of 10 tested slices) mice, indicating similar occupancy of the coagonist binding site in the two groups of animals (Fig. 10*C*). These effects were specific for NMDARs, because the AMPAR fEPSPs were similar in WT and SR-KO (Fig. 11*A*) mice and unaffected by D-Ile (Fig. 11*B*,*C*).

Discussion

The relative contribution of neurons versus astrocytes in D-serine signaling has been unclear. Recent reports have demonstrated that SR is predominantly expressed by glutamatergic neurons (Kartvelishvily et al., 2006; Ito et al., 2007; Yoshikawa et al., 2007; Miya et al., 2008; Balu and Coyle, 2012; Benneyworth et al., 2012). Our study now demonstrates that Asc-1, a neuronal specific transporter, releases D-serine to regulate NMDAR-dependent synaptic activity. Furthermore, our data disclose a role of Asc-1 in mediating concurrent release of neuronal glycine, which also plays a role in activating NMDARs, especially at low-frequency stimulation.

To investigate the role of neuronal D-serine, we looked for compounds that specifically affect neuronal D-serine dynamics without acting on D-serine release by astrocytes. We found that D-Ile interacts with Asc-1 in transfected cells, primary neuronal cultures, and acute hippocampal slices, enhancing the neuronal release of D-serine via D-Ile/D-serine exchange.

Because the K_i of D-Ile for Asc-1 is quite high, a key issue was to evaluate its selectivity against additional targets that may affect D-serine or glycine dynamics. We found that D-Ile induces D-serine release from neurons but not astrocytes, which is in agreement with Asc-1 localization in neuronal structures (Helboe et al., 2003; Matsuo et al., 2004). In addition, D-Ile interacts with Asc-1 in transfected cells but does not affect Na^+-dependent D-serine transporters such as ASCT1 and ASCT2. We also found that D-Ile does not bind to the coagonist site of NMDARs, nor does it affect GlyT1, GlyT2, glutamate, GABA, and dopamine transporters.

Targeted deletion of Asc-1 gene in mice indicates that Asc-1 is responsible for >90% of the Na^+-independent transport of D-serine (Rutter et al., 2007). To investigate whether Asc-1 is the main target of D-Ile, we monitored its effects in the absence of Na^+, which allows near complete isolation of the Asc-1 component of D-serine transport. We found that D-Ile effects in both neuronal cultures and slices were independent on Na^+. Thus,

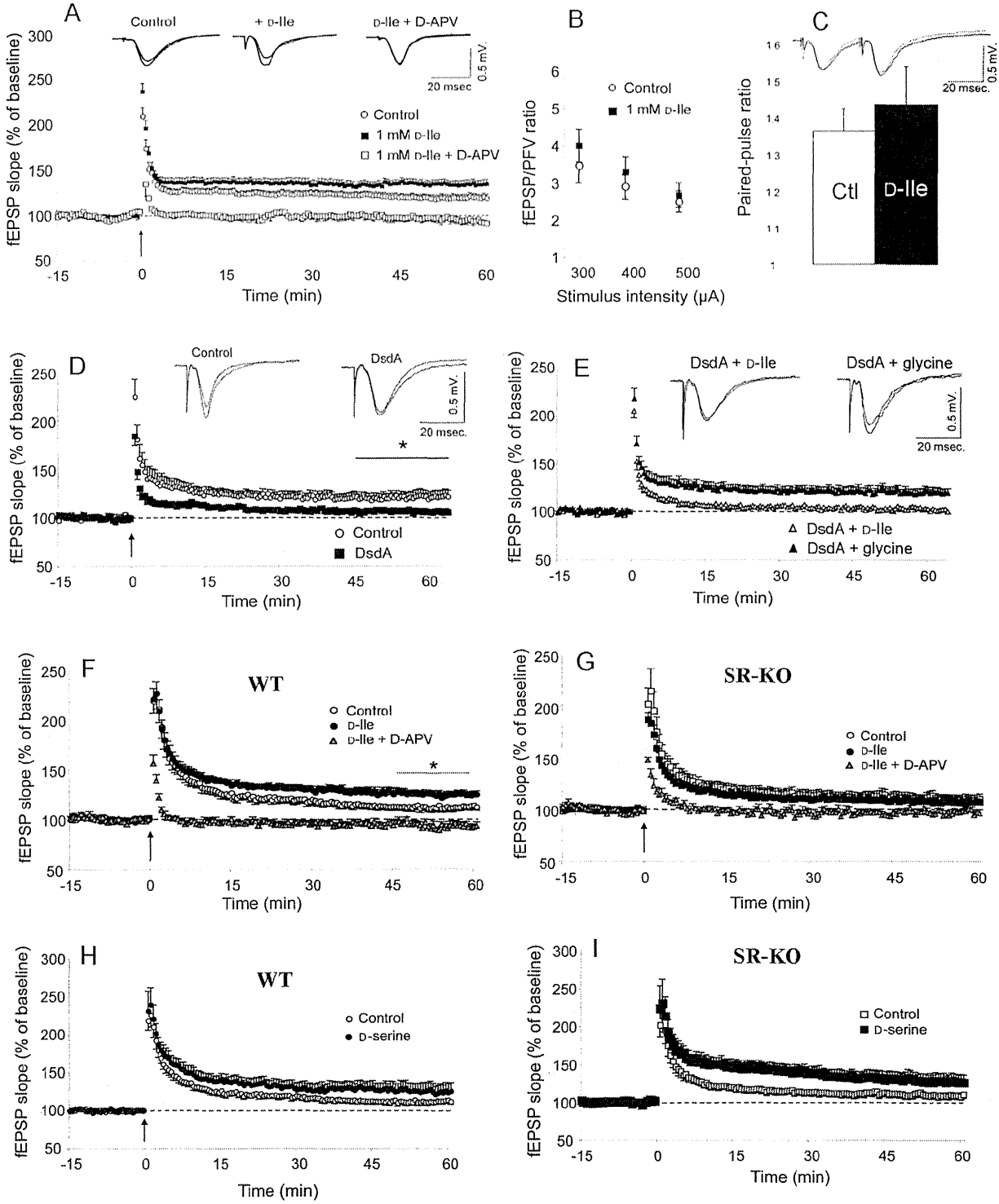

Figure 8. D-Ile enhances NMDAR-dependent LTP in hippocampal slices through D-serine release. ***A***, LTP induced by TBS in the hippocampal CA1 area of young adult rats is significantly enhanced by 1 mM D-Ile (■, $n = 13$) when compared with control slices (○, $n = 12$). LTP mediated by D-Ile is dependent on NMDARs, because it is abolished in the presence of 80 μM D-APV (□, $n = 10$). The inset depicts representative traces of fEPSPs recorded before and 60 min after TBS in a control slice and in the presence of D-Ile with or without D-APV. ***B***, Basal synaptic transmission is not affected by D-Ile. AMPAR fEPSP/PFV ratio was calculated at increasing stimuli intensity before (open symbols) and 15 min after application of 1 mM D-Ile (filled symbols). The values are mean ± SEM of recordings from 12 slices. Ctl, Control. ***C***, D-Ile does not affect the paired-pulse facilitation determined at 30 ms stimulation interval in slices from adult Sprague Dawley rats. The values are mean ± SEM of eight experiments. The inset depicts representative traces of PPF recordings before (solid line) and after (dashed line) D-Ile application. ***D***, D-serine is critical for the expression of synaptic plasticity. TBS-induced LTP as shown in control slices ($n = 11$) was abolished in slices incubated for at least 90 min with recombinant DsdA enzyme to deplete endogenous D-serine ($n = 13$). ***E***, D-Ile-mediated LTP increase requires endogenous D-serine. LTP expression in DsdA-pretreated slices is not enhanced by adding 1 mM D-Ile ($n = 13$ slices) but is rescued by exogenous 500 μM glycine ($n = 12$). ***F***, D-Ile also enhances LTP expression in WT mice. Effects of 1 mM D-Ile ($n = 14$ slices) on D-APV-sensitive LTP induced by one train of 100 Hz for 1 s in CA1 area compared with control conditions ($n = 17$). ***G***, D-Ile improvement of LTP requires D-serine. TBS-induced LTP generated in slices from SR-KO mice ($n = 17$) is not enhanced by D-Ile ($n = 14$). ***H***, ***I***, LTP expression is enhanced by saturating the NMDAR binding sites with 100 μM D-serine in both WT (***H***) and SR-KO (***I***) mice. *$p < 0.05$, different from control.

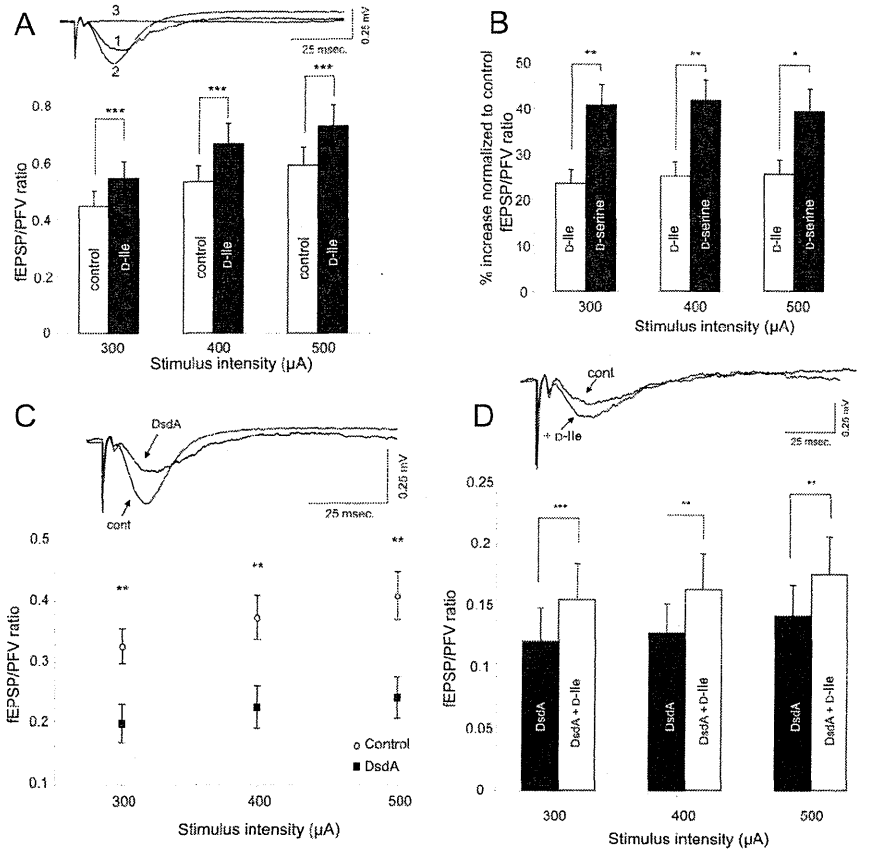

Figure 9. D-Ile enhances NMDAR synaptic potentials in hippocampal slices via D-serine and glycine release. ***A***, D-Ile increases NMDAR potentials isolated in low-magnesium medium supplemented with NBQX. fEPSP/PFV ratios were monitored at increasing stimuli intensity before (open bars) and after (filled bars, $n = 11$) addition of 1 mM D-Ile. The inset depicts representative traces of NMDAR fEPSPs recorded before (***1***) and after (***2***) the addition of D-Ile or D-Ile plus 30 μM D-APV (***3***). ***B***, Percentage increase in NMDAR fEPSP/PFV ratio induced by either 1 mM D-Ile or 100 μM D-serine ($n = 12$) at different stimuli intensity. ***C***, Endogenous D-serine is required for NMDAR potentials. When compared with control slices (○, $n = 32$), the fEPSP/PFV ratio is significantly decreased in slices pretreated for at least 90 min with 20 μg/ml DsdA (■, $n = 18$) to deplete endogenous D-serine. ***D***, D-Ile still increases NMDAR potentials in D-serine-depleted slices. fEPSP/PFV ratio in slices incubated with DsdA before (filled bars) and after (open bars, $n = 10$ slices) 1 mM D-Ile. $^*p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, different from control.

D-Ile-induced D-serine release from slices perfused without Na^+ was at least as high as that observed under normal Na^+ concentration. Furthermore, D-Ile effects were also inhibited by D-alanine, an Asc-1 substrate whose transport is disrupted in Asc-1 KO mice (Rutter et al., 2007). We found that D-alanine blocks D-[^{14}C]Ile transport in neurons and inhibits D-Ile-induced D-serine release from slices when added before D-Ile.

Together, these results suggest that D-Ile is quite selective for Asc-1. However, they do not completely rule out the involvement of additional transporters. Indeed, it is possible that a small fraction of D-serine can also be released by another carrier whose kinetics is indistinguishable from Asc-1. Alternatively, Asc-1 antiporter activity may trigger D-serine release from other pathways via the release of an unidentified mediator.

We also considered the possibility that D-Ile directly opens channels that release both glutamate and D-serine, such as volume-regulated channels (Takano et al., 2005; Rosenberg et al., 2010) or connexin 43 hemichannels (Stehberg et al., 2012). However, D-Ile does not affect endogenous glutamate release from slices, nor does it change the paired-pulse facilitation, an indicator of presynaptic glutamate release. In addition, D-Ile does not affect AMPAR transmission, indicating that it does not activate nonselective channels that are expected to release glutamate as well.

We demonstrate that D-Ile affects synaptic plasticity in CA1 neuronal networks via enhancement of NMDAR-dependent LTP, suggesting a role for neuronal D-serine release. However, it is conceivable that D-Ile, especially at high concentrations, enhances LTP by mechanisms unrelated to D-serine release. To investigate this possibility, we used several controls using slices from SR-KO mice or slices in which endogenous D-serine was depleted by treating with DsdA, an enzyme that selectively destroys D-serine (Shleper et al., 2005). We found that D-Ile does not enhance LTP in either of the D-serine depletion settings, supporting the notion that D-Ile effects are mediated by endogenous neuronal D-serine.

We also considered the possibility that Asc-1 hetero-exchange by D-Ile does not reflect a physiological condition. However, in normal brain, the extracellular concentrations of Asc-1 amino acid substrates are enough to saturate Asc-1 antiporter activity (Lindroth et al., 1985; Fukasawa et al., 2000). This contrasts with the very low extracellular concentration of the Asc-1 substrates detected in the slice perfusates of our *in vitro* experiments, presumably caused by the washout of the extracellular medium during perfusion. Supplementing the perfusion buffer with extracellular Asc-1 substrates at physiological levels increases the rate of D-serine release to levels similar to those observed with D-Ile alone. Thus, D-Ile appears to restore the physiologic activity of Asc-1.

Although we describe a role of Asc-1 in mediating D-serine release, its relative contribution for physiological NMDAR activation is not warranted by the effects of D-Ile. Indeed, Mothet et al. (2005) demonstrated vesicular D-serine release from astrocytes, and this pathway is required for hippocampal LTP (Henneberger et al., 2010). A major role of glia in D-serine release has also been demonstrated in the hypothalamus and prefrontal cortex, as well (Panatier et al., 2006; Fossat et al., 2012). In addition, our data do not discard a role for ASCT1 and ASCT2 in mediating D-serine release. Although these transporters are insensitive to D-Ile, they likely play a role in D-serine dynamics, especially in the retina, which lacks Asc-1-mediated D-serine transport (O'Brien et al., 2005). These pathways are presumably regulated by different mechanisms and operate at different timescales in distinct synaptic and extrasynaptic microenvironments.

Another aspect of our study concerns the role of Asc-1 in glycine dynamics. The glycine transporters GlyT1 and GlyT2 are generally considered the sole regulators of extracellular glycine concentrations, whereas Asc-1 was not previously thought to affect glycine levels. We found that Asc-1 also plays a role as a pathway for glycine release along with D-serine. This provides a mechanism to release NMDAR coagonists from neurons, indicating that the dynamics of these two coagonists are connected.

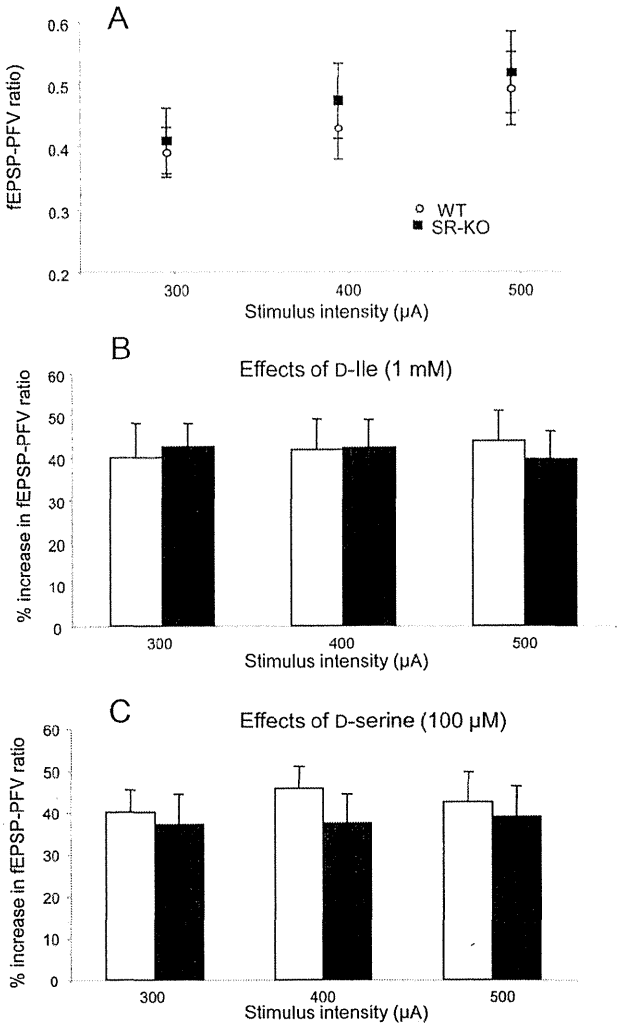

Figure 10. D-Ile increases NMDAR potentials in hippocampal slices from WT and SR-KO mice. ***A***, NMDAR synaptic transmission is preserved in SR-KO adult mice. fEPSP/PFV ratios of isolated NMDAR potentials determined with increasing stimuli intensities in slices from WT (○, $n = 22$) and SR-KO (■, $n = 22$) mice. ***B***, NMDAR fEPSPs are equally enhanced by D-Ile in slices from WT and SR-KO mice. Bars depict a 40% increase in the NMDAR potentials during application of 1 mM D-Ile onto slices from both WT (open bars, $n = 12$) and SR-KO (filled bars, $n = 10$) mice. ***C***, D-serine equally stimulates NMDAR in both groups of mice. Effect of exogenous application of 100 μM D-serine in slices from WT (open bars, $n = 9$) and SR-KO (filled bars, $n = 8$) mice.

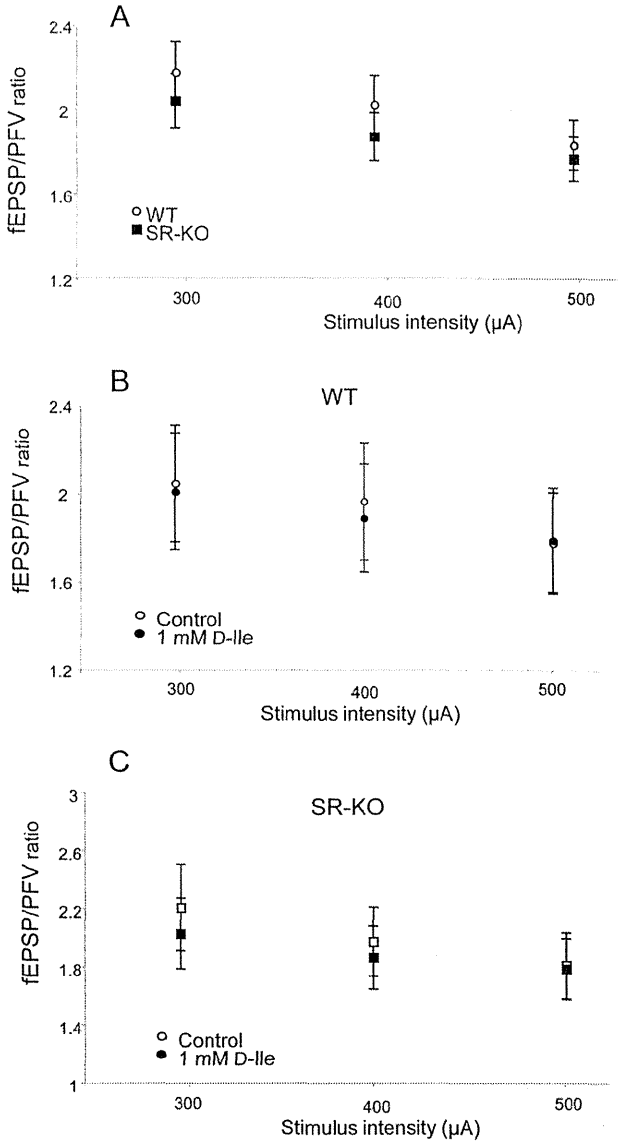

Figure 11. D-Ile does not affect basal glutamatergic transmission. ***A***, AMPAR synaptic transmission in hippocampal CA1 is unchanged in slices from SR-KO compared with WT mice. fEPSP/PFV ratios of AMPAR potentials were determined with increasing stimuli intensities in slices from WT (○, $n = 59$) and SR-KO (■, $n = 59$) mice. ***B***, AMPAR fEPSPs are not altered by D-Ile. fEPSP/PFV ratios of AMPAR potentials induced at CA3/CA1 synapses showing the absence of significant effects of 1 mM D-Ile in slices from WT mice ($n = 17$). ***C***, D-Ile has no effect on AMPAR potentials in slices from SR-KO mice ($n = 15$).

The role of Asc-1 in glycine release was further defined by the electrophysiological experiments. We found that D-serine was not the sole coagonist for low-frequency-activated NMDAR potentials, contrasting with its critical role in the high-frequency NMDAR activation required for LTP. Thus, although depletion of endogenous D-serine by DsdA drastically decreased the NMDAR fEPSPs confirming the major role of D-serine in modulating NMDARs, it did not affect the stimulatory effect of D-Ile. This suggests an effect of D-Ile on glycine release as also revealed in our biochemical experiments. Furthermore, D-Ile robustly increased the NMDAR fEPSPs in adult SR-KO mice, likely reflecting glycine release through Asc-1. These results are compatible with the notion that glycine compensates for D-serine deficits, at least under moderate NMDAR activation, such as those induced by low-frequency stimulation of glutamatergic afferents.

Papouin et al. (2012) recently reported that D-serine is the sole coagonist at the synaptic NMDARs, whereas glycine is only required for extrasynaptic NMDAR activation. Conversely, our observation that the synaptic NMDAR responses are essentially unaltered in adult SR-KOs suggests that D-serine is not the sole coagonist at the synapse. Our SR-KO mice do not exhibit changes in the expression of NMDAR subunits (Inoue et al., 2008), and their extracellular levels of glycine, glutamate, and glutamine monitored by *in vivo* hippocampal microdialysis are the same as the WT mice (Horio et al., 2011). Like Papouin et al. (2012), we observed that acute enzymatic depletion of D-serine abolishes LTP and strongly diminishes the synaptic NMDAR responses by 60–70%. A parsimonious explanation for these findings is that D-serine is the main synaptic NMDAR coagonist under normal conditions, but there is still substantial overlap with glycine at synaptic NMDARs. Glycine effects become more evident when its release is enhanced by D-Ile or by deleting SR gene.