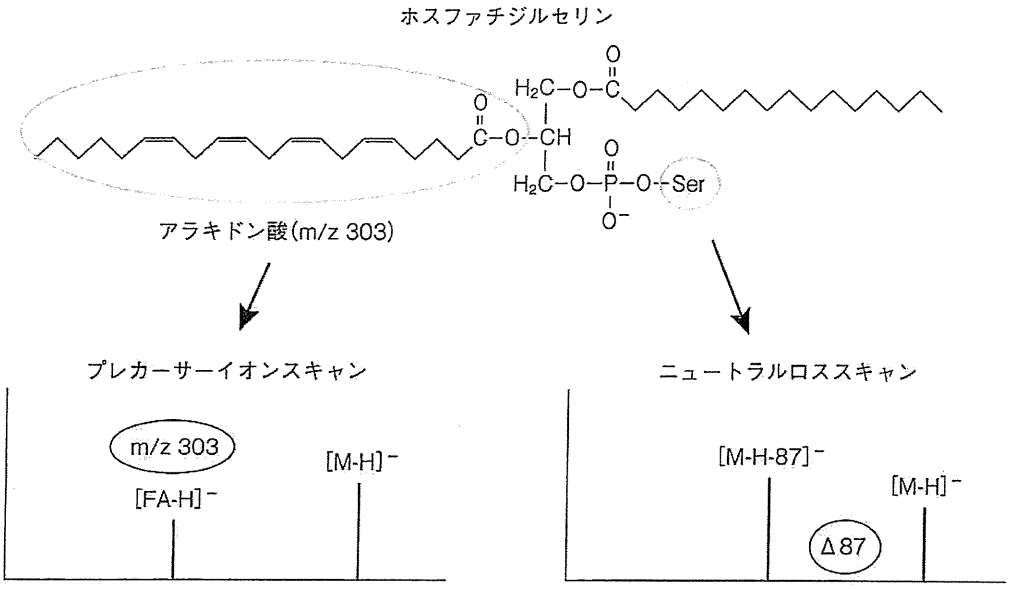


図2　リン脂質の分子種解析
アラキドン酸の分子量である303のプレカーサーイオンスキャンを行うことで，アラキドン酸含有リン脂質を選択的に検出できる．また，セリンの分子量である87のニュートラルロススキャンを行うと，ホスファチジルセリンを特異的に検出できる

でき，TriVersa NanoMate®等の定速注入とプレカーサーイオンスキャンやニュートラルロススキャンを組合わせた方法は，多検体を対象にしたリン脂質分子種の大まかな測定に適している[5]．また，例えばリン脂質以外にもトリグリセリドなどアラキドン酸を有する脂質分子を網羅的に測定することも可能であり，測定試料中の大まかな傾向を簡便に把握することが目的の初期解析には有用である．一方で分離操作を行わないため，イオン化抑制が起きやすく定量性に欠けることに注意が必要である．最近では，前処理や，誘導体化，付加イオンを工夫することにより，より高感度な選択的手法としての利用が広がってきている．

2 リン脂質分子種の局在解析

❶ 質量顕微鏡法の原理

リン脂質は生体膜の主要な構成成分として，膜ドメインの形成や膜輸送，シグナル伝達などさまざまな細胞機能にかかわっている．そこで，生体におけるリン脂質の機能をさらに理解するためには，組織や細胞におけるリン脂質分子種の局在を可視化することが望まれる．このような生体分子のイメージングを目指す技術として質量顕微鏡法（imaging mass spectrometry：IMS）が挙げられる（図3）[6]．IMSは，スライドガラスに乗せた組織切片上で質量分析を二次元的に行うことで，「どのような分子がどこに存在するか」を解析するための技術である．サンプルのイオン化法には，マトリクス支援レーザー脱離イオン化法（matrix-assisted laser desorption/ionization：MALDI）が用いられる．MALDI法とは，試料をあらかじめマトリクス（イオン化補助物質）と混合・共結晶化した後にレーザーを照射することで，従来イオン化されにくかった生体高分子の効率的なイオン化を可能にした手法である．IMSでは，組織の凍結切片上にマトリクス溶液を均一に塗布し，組織内での位置情報を失わずに生体分子とマトリクスを共結晶化させる．この試料に対して微小径のUVレーザーを二次元的に照射し，微小領域に存在する分子のイオン化を行い，イオン化された分子のMSスペクトルを測定する．こうして得られた，切片上の座標に対応したMSスペクトルから任意のピーク情報を抽出し，イオン強度に基づいて二次元情報の再構築を行ない，分子の局在情報を点描画のように画

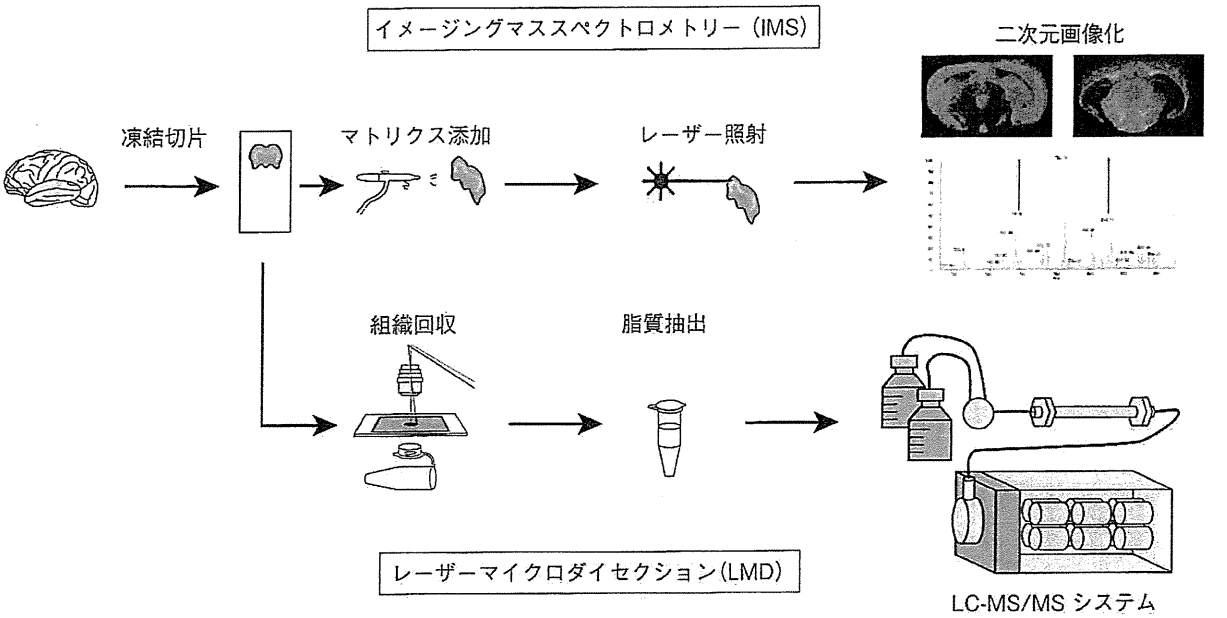


図3　イメージングマススペクトロメトリーとレーザーマイクロダイセクションの手順
詳細は本文参照

像化する．とくに組織切片上で形成されるマトリクス結晶がいかに均一であるかが画像の質に大きく影響することから，測定対象に応じて最適化されたさまざまなマトリクス塗布法が開発されている．

❷ IMSの可能性と課題

IMSを用いたリン脂質分子種の解析例として，マウス脳内のリン脂質組成が脳の成長，加齢にともない大きく変動することが報告されている[7]．また，疾患組織をIMSで画像化することによって病理診断法として応用できる可能性が示唆されている．例えば，大腸癌肝転移の病理切片では，パルミチン酸をもつスフィンゴミエリンが有意に上昇していることが報告されている[8]．このようにIMSを使うことで，単に病理切片から病変部位を特定するのみならず，病変部位の由来や進行度を局所の生化学的変化として捉えられることが期待される．

前述のIMSの手法は，分子の局在が可視化できるという利点があるものの，定量性および量的に少ない分子種の測定にやや難がある．この点を補うものとして，組織切片から特定の部位をレーザーマイクロダイセクション（laser microdissection：LMD）で回収し，LC-MS/MSで分析する手法がある（図2）．この手法はIMSに比べてサンプル抽出操作とLC分離のために測定時間が長くなるが，微量分子種を含めたプロファイル解析が可能であり，また微小領域（直径5 μm程度）の組織を回収できるため，ある程度の位置情報も得られる．すなわち，LMD-LC-MS/MSによる解析を行うことで，IMSで得られた結果を補完することができる．

さらに最近，溶媒抽出表面分析（liquid extraction surface analysis：LESA）という，NanoMate®のナノESIと組合わせて，組織表面局所1 mm径の領域から直接溶媒抽出して自動測定する手法が開発された[9]．この手法の空間分解能はIMS等に比べ劣るが，組織レベルの領域ごとの違いや，病変局所と正常部の違いなどを調べるうえで有効な手段である．また，この手法は抽出過程を省いた，体液の直接分析にも応用可能であり，今後，臨床分析等の分野での利用が期待できる．

3 リゾリン脂質の分析

近年，リゾリン脂質に対する特異的な受容体や産生酵素が同定され，それらの生理機能が注目されている．例えば，スフィンゴシン1リン酸（S1P），リゾホス

ファチジン酸（LPA），リゾホスファチジルセリン（LPS）などは，それぞれ必要なときに必要な場所で酵素的に産生され，細胞膜上の受容体を介して血管形成や着床，炎症・免疫反応の制御など多彩な生理機能を発揮することが知られている[10]．作用後は再アシル化，脱リン酸化，脱アシル化などを受けて速やかに代謝，不活性化される．これらの受容体はリゾリン脂質の極性基だけではなく，グリセロール骨格に結合している脂肪酸鎖によっても親和性が左右される場合がある．例えばLPA受容体の1つLPA3は，不飽和脂肪酸を有するLPAを選択的に認識する．したがって，どのような脂肪酸鎖が結合したリゾリン脂質がいつ，どこで，どれだけ生成しているかを解析することは重要である．

LPAやLPSなどのグリセロリゾリン脂質を構成する脂肪酸鎖には多様性が認められ，逆相系のLCとオンラインのESIによるイオン化，MS/MSによる分子種特異的なフラグメントイオンを検出することで解析可能である．逆相系のLCでグリセロリゾリン脂質を展開した場合，一般的に1-アシル型リゾリン脂質よりも2-アシル型リゾリン脂質の方が先に溶出される．S1Pはスフィンゴシン骨格にリン酸基を結合した構造をもち，遊離アミノ基とリン酸基を有する双性イオンである．S1Pは物性がグリセロリゾリン脂質と近いため，グリセロリゾリン脂質と同様のLC-MS条件で分離，同定が可能である．

4 酸化リン脂質の分析

生体膜リン脂質を構成する多価不飽和脂肪酸鎖は，生体内で生じた活性酸素種によりラジカル連鎖反応が起こり，脂質ヒドロペルオキシド，ヒドロキシ体やアルデヒドなどの酸化生成物になる．これらの酸化リン脂質はいくつかの受容体により認識される[11]．その1つはマクロファージ／単球で発現するCD36スカベンジャー受容体であり，酸化LDL中に含まれる酸化リン脂質を認識して貪食する．この酸化LDLの取り込みは，マクロファージの泡沫化と動脈硬化の進展の引き金となる．また，酸化ホスファチジルコリンは血小板活性化因子（PAF）受容体に結合し，炎症を促進する作用を有することも知られている．このように酸化リン脂質の生成は，肥満や糖尿病などのメタボリックシンドローム，および心筋梗塞や動脈硬化症などの酸化ストレスによる病態の発症や進行に関係していると考えられている．また，虚血再環流時には活性酸素種によるラジカル生成が局所的に起こり，酸化リン脂質の生成が亢進すると考えられている．酸化リン脂質の高感度測定を行うことにより，このような酸化ストレスに起因する病態形成の分子メカニズムの研究や，新たな疾患バイオマーカーの同定につながる可能性が期待される．

一般的に生体内で生じた酸化リン脂質は，生成した後に速やかに消去される機構が存在している．したがって，組織中に存在する酸化リン脂質の量は，前駆体であるリン脂質の量に比べて極少量である．酸化リン脂質の測定において最も大きな問題となるのが，量的に多いリン脂質の混入によるイオン化抑制である．このため，質量分析を行う前にLCを用いて生体内で量的に多いリン脂質と量的に少ない酸化リン脂質を分離することが重要である．これまでに酸化リン脂質については，逆相LCと三連四重極型MS/MSを用いた包括的な測定系が報告されている[12]．このような酸化リン脂質の包括的測定系をさまざまな生体試料に適用することにより，今後，酸化ストレスが関与すると考えられるさまざまな生命現象や疾患に対する理解が進み，新たな分子標的が見出される可能性が期待される．

また，血漿リポタンパク質や脂肪組織中には，リン脂質と同様にトリグリセリドにもこのような酸化体が検出される[13]．リン脂質に加え，トリグリセリドやコレステリルエステル等の脂肪酸エステルの酸化体も，肥満や炎症に関連する疾患の解析に重要になってくる可能性がある．

5 多価不飽和脂肪酸の代謝とメタボロミクス

多価不飽和脂肪酸には，分子内の二重結合の位置により分類されるn-3系，n-6系とよばれる脂肪酸が存在しており，それらは哺乳動物の体内において相互変換されることはなく，代謝的に質の異なる脂肪酸である．これらの多価不飽和脂肪酸は，シクロオキシゲナー

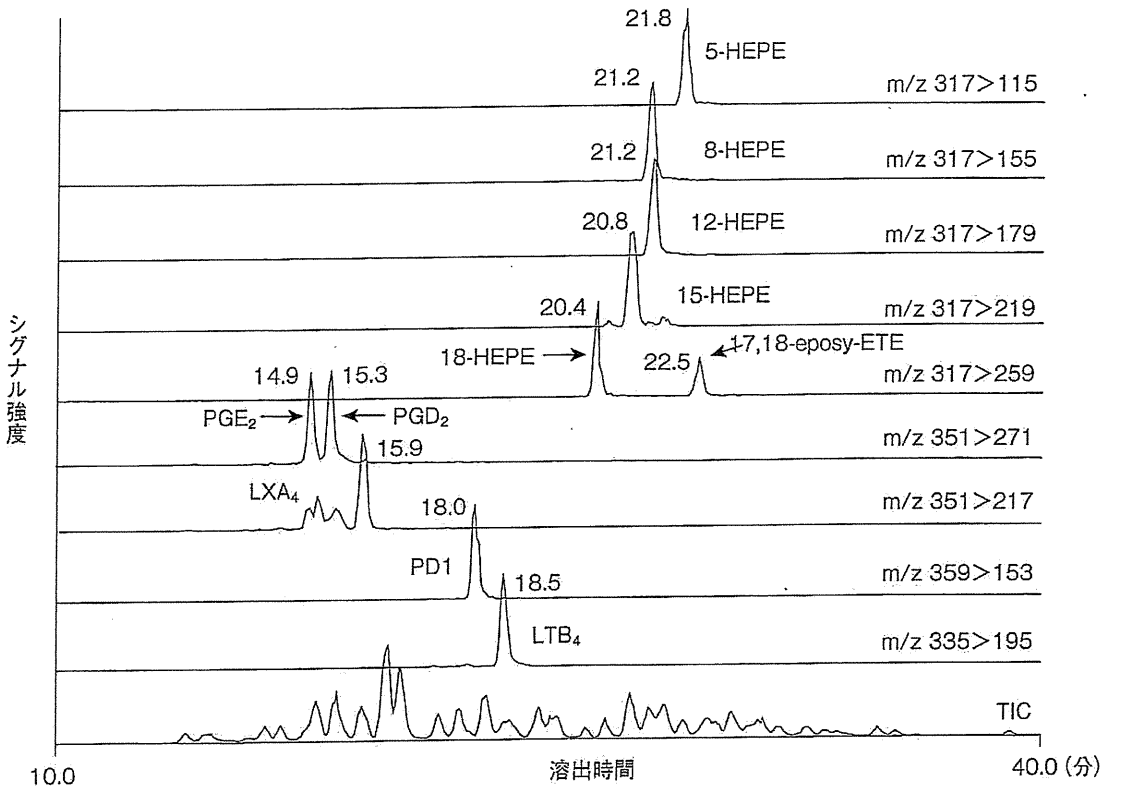


図4 LC-MS/MSによる脂肪酸代謝物の分析
化合物の構造に特異的なプレカーサーイオン，プロダクトイオンの組合わせを用いたMRMにより，それぞれの脂肪酸代謝物の構造に特異的な検出ができる．また，18-HEPEと17,18-epoxy-ETEや，PGE_2とPGD_2のように，MRM条件が同じ場合でも，LCの溶出時間が異なることで区別することができる

ゼ，リポキシゲナーゼ，シトクロムP450など，酵素的な酸化反応によって生理活性を獲得し，炎症をはじめとするさまざまな生体調節機能を担っている．例えばn-6系脂肪酸のアラキドン酸由来のプロスタグランジンやロイコトリエンは，炎症の初期症状である発熱，発赤，浮腫，疼痛にかかわっている[14]．一方，魚油などに多く含まれるエイコサペンタエン酸（EPA）やドコサヘキサエン酸（DHA）などのn-3系脂肪酸には，古くから抗炎症作用や心血管保護作用があることが知られている．n-3系脂肪酸は主にアラキドン酸代謝系と競合することで炎症を抑制すると考えられてきたが，最近新たにn-3系脂肪酸から生成する抗炎症性代謝物（レゾルビン，プロテクチン）が見出されている[15]．そこで，これらの一連の脂肪酸代謝物がいつ，どこで，どれだけ生成しているのかを全体像として把握することが，互いに質の異なる多価不飽和脂肪酸による生体の調節機能を理解するうえで重要である．

LC-ESI-MS/MSを用いることにより，脂肪酸代謝物を一斉定量分析するシステムが確立されている[16)17)]．一般に脂肪酸およびその代謝物は，分子の疎水的性質から逆相LCでよく分離される．また末端にカルボキシル基をもち，負イオンになりやすいため，ESIによるソフトイオン化が適している．さらに脂肪酸酸化物の場合，水酸基が付加した部位の近傍で特異的なフラグメントイオンを生成しやすいことから，測定には三連四重極型MS/MSを用いた，プレカーサーイオンとプロダクトイオンの組合わせで分子構造特異的なシグナルを検出するMRM（multiple reaction monitoring）が適している．このようなMRMチャネルを，保持時間の違いを加味しながら複数組合わせることで，数百種類以上の脂肪酸代謝物の一斉定量分析が可能になる（図4）．このシステムを用いることで，最近われわれ

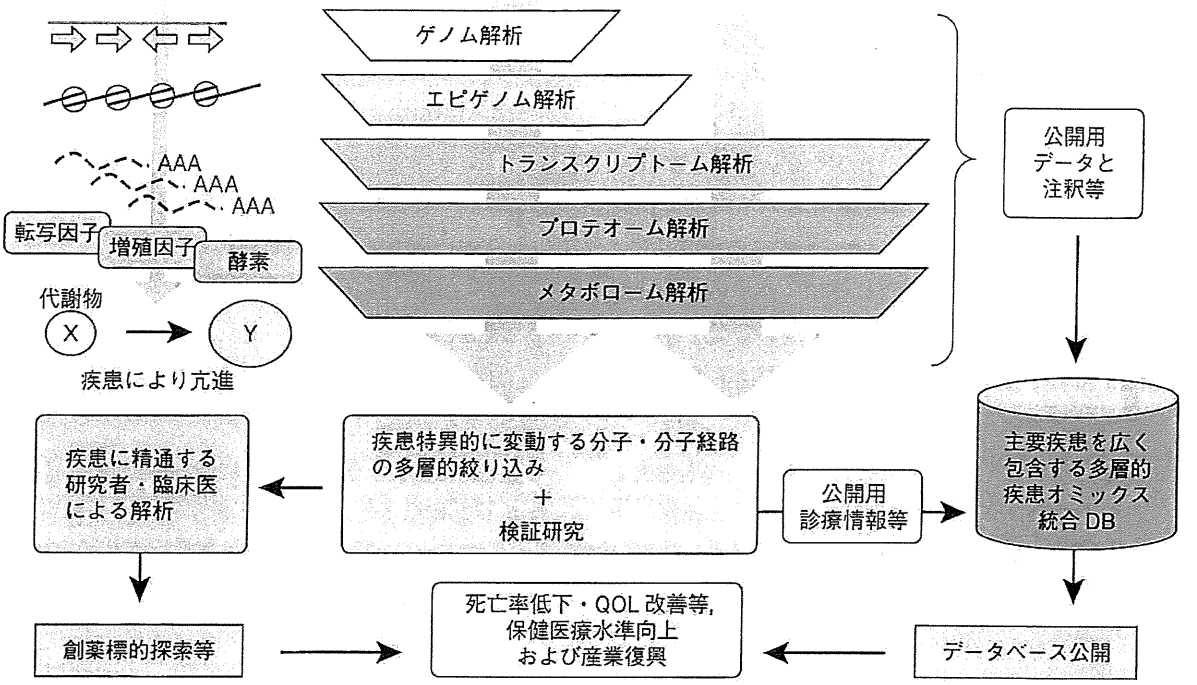


図5　多層的オミックス解析による創薬標的の網羅的探索を目指した研究
ゲノム（遺伝子多型やコピー数多型），エピゲノム（メチル化修飾），トランスクリプトーム（mRNAやmicroRNA），プロテオーム（タンパク質），メタボローム（低分子代謝物）に関し，それぞれの測定結果を3層または5層で解析し，各疾患で多層的に変化している生体内パスウェイを同定する．この解析は，測定を行った研究者と当該疾患に精通する臨床医がチームを組んで行う．これにより創薬標的を探索する．また各測定データおよび診療情報は，疾患オミックス統合データベースとして公開される予定である

は急性炎症の収束にともなう脂質メディエーター代謝系のダイナミックな変化を明らかにし，その機能的役割について報告した[18]．

6 メタボロミクスから多層的オミックスへの展開

そもそもオミックスとは生体の有する分子情報を網羅的に測定・解析する研究領域である．予見なしに分子の状態およびそのレベルを測定するための方法論として，ゲノム（遺伝子多型やコピー数多型），エピゲノム（メチル化修飾），トランスクリプトーム（mRNAやmicroRNA），プロテオーム（タンパク質），メタボローム（低分子代謝物）などのさまざまな階層でさまざまな解析系が確立され，その技術は今もなお進歩し続けている．そのなかでもとくに技術基盤が確立しているゲノムおよびトランスクリプトーム領域では，多くの疾患に関連する遺伝子多型や遺伝子発現パターンについて情報が蓄積されている．一方で，近年のプロテオームやメタボローム解析技術の進歩にも目覚ましいものがあり，精度の高い測定方法により得られたメタボローム情報は，ゲノム解析やプロテオーム解析と組合わせることで，より確度の高い「多層的オミックス」情報となる．このような多層的オミックス解析は，その病態をもたらす原因がどのオミックス階層に存在するかなど，発症機序の解明へとつながる可能性が期待される[19]．

独立法人医薬基盤研究所は，平成22年度から「多層的オミックス解析による創薬標的の網羅的探索を目指した研究」を公募し，国立がん研究センター研究所を中心とした5年間のプロジェクトが採択された（図5）．本プロジェクトでは，死亡率が高い，または国民罹患

率が高く経済的な損失をもたらしている主要11疾患，すなわち，てんかん，統合失調症，成人固形腫瘍（肺癌，腎癌，乳癌），肥満症，非アルコール性脂肪性肝炎（NASH），拡張型心筋症，大動脈瘤，小児白血病，アレルギー疾患，アルツハイマー病，脊柱管狭窄症を対象とし，国立高度専門医療センター由来の臨床試料（病変組織等）を用いて，3～5層の多層的にオミックス解析を行う．最終的な目標は，疾患の予防・診断・治療法開発に利用しうる確度の高い新規の創薬標的候補となる分子を見出すこと，また得られたデータを統合的に解析・発信するための疾患横断的な多層的オミックスデータベースを構築すること，である．

このうちメタボローム領域は，病態などの表現形質の発現に直結する生体内代謝物の変動を指標に，新規の創薬標的候補および診断マーカーを探索する．1つの臨床試料（病変組織等）に関し，MSを用いたイオン性代謝物分析・疎水性（脂質）代謝物分析，およびNMR法による分析という3種のアプローチで生体内代謝物を網羅的に解析している．また拡張型心筋症など，数種の疾患に関しては，食餌の影響がコントロール可能な疾患モデル動物の解析も行っている．とくに脂質メタボローム解析に関しては，リン脂質，トリグリセリド，コレステロールエステル，酸化脂肪酸等を幅広く対象としており，今後イオン性代謝物，NMRによる解析結果とともに，多層的に疾患との関連解析が行われる予定である．また，平成22年度より発足した新学術領域研究「生命応答を制御する脂質マシナリー」においても，よりクオリティーの高い脂質メタボローム解析を行うための質量分析センターが設置され，モデル生物センター，ヒトSNPセンターなどと連動して，今後わが国の脂質バイオロジー研究をリードする拠点となることが期待されている．

おわりに

多くの生物のゲノム配列が明らかとなり，ゲノミクスやトランスクリプトミクスが進展した現在，残された課題の1つは，表現型に最も近い低分子化合物の代謝動態を網羅的かつ定量的に解析し，生物機能との関連を明らかにすることである．このような代謝物の網羅的解析（メタボロミクス）への関心の高まりに伴い，近年，本稿で述べたLC-MS以外にも，GC-MS，キャピラリー電気泳動マススペクトロメトリー（CE-MS）などを用いた微量分析法が進歩し，それぞれ分析対象となる化合物の物性の違い（水溶性，脂溶性など）に応じて最適化されたシステムが応用されつつある．そこから期待される成果としては，新しい代謝経路または代謝調節機構の解明，新規活性代謝物とその生合成遺伝子の同定，新しい診断バイオマーカーの同定，医薬品の薬効や副作用のメカニズム解明，など多岐にわたっている．

本稿ではとくに脂肪酸の質の違いを明らかにすることができるメタボローム解析技術について述べたが，このように精度の高い測定方法により得られた情報は，ゲノム解析やプロテオーム解析と組合わせることで，より確度の高い「多層的オミックス」情報となる．これらは，さまざまなバイオロジーや病態の背後に潜む分子メカニズムを「脂肪酸の質の違い」という観点から明らかにし，これまでにない新しい脂質バイオロジー研究へと展開していく可能性がある．また，炎症や癌など個別の組織で特徴的な代謝系を捉えることによって，各種疾患に対する新しいバイオマーカーの同定，さらに代謝機能の個人差を加味した個別化医療に貢献する可能性もある．網羅的なオミックス解析の成果を基に，個別の脂質分子種に関する機能解明（質の解明）を行うことにより，今後さらに多くの基礎，臨床研究への応用が期待されるだろう．

文献

1）Murakami, M. et al.：Prog. Lipid Res., 50：152-192, 2011
2）Shindou, H. et al.：J. Lipid Res., 50：S46-S51, 2009
3）河野 望 他：生化学, 83：462-474, 2011
4）Harkewicz, R. & Dennis, E. A.：Annu. Rev. Biochem., 80：301-325, 2011
5）Yang, K. et al.：Anal Chem., 81：4356-4368, 2009
6）Zemski Berry, K. A. et al.：Chem. Rev., 111：6491-6512, 2011
7）Sugiura, Y. et al.：J. Lipid Res., 50：1776-1788, 2009
8）Shimma, S. et al.：J. Chromatogr., 855：98-103, 2007
9）Eikel, D. & Henion, J.：Rapid Commun. Mass Spectrom., 25：2345-2354, 2011
10）井上飛鳥，青木淳賢：実験医学, 27：2059-2067, 2009
11）Hazen, S. L.：J. Biol. Chem., 283, 15527-15531, 2008

12) Morgan, A. H. et al.：Nat. Protoc., 5：1919-1931, 2010
13) Ikeda, K. et al.：Cancer Sci., 102：79-87, 2011
14) Funk, C. D.：Science, 294：1871-1875, 2001
15) Serhan, C. N. et al.：Nat. Rev. Immunol., 8：349-361, 2008
16) 有田 誠：メタボロミクス：その解析技術と臨床・創薬応用研究の最前線（田口 良／編），pp179-184, メディカルドゥ，2010
17) Masoodi, M. et al.：Rapid Commun. Mass Spectrom., 22：75-83, 2008
18) Yamada, T. et al.：FASEB J., 25：561-568, 2011
19) 大保木啓介，松本健治，斎藤博久：医療，65：135-145, 2011

参考図書

『メタボロミクス：その解析技術と臨床・創薬応用研究の最前線』（田口 良／編），遺伝子医学MOOK16，メディカルドゥ，2010

『脂質生物学がわかる』（清水孝雄／編），わかる実験医学シリーズ，羊土社，2004

Profile　筆頭著者プロフィール

有田　誠：概論プロフィールを参照．

ACS Chemical Neuroscience

Research Article

pubs.acs.org/acschemicalneuroscience

Discovery of a Tamoxifen-Related Compound that Suppresses Glial L-Glutamate Transport Activity without Interaction with Estrogen Receptors

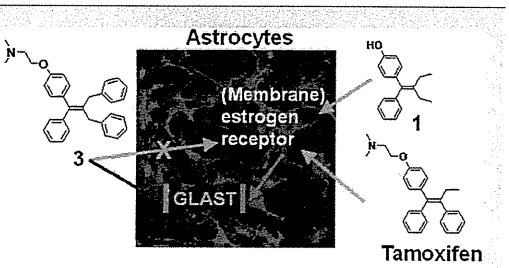
Kaoru Sato,*[,‡,†] Jun-ichi Kuriwaki,[‡,†] Kanako Takahashi,[‡] Yoshihiko Saito,[§] Jun-ichiro Oka,[§] Yuko Otani,[∥] Yu Sha,[∥] Ken Nakazawa,[‡] Yuko Sekino,[‡] and Tomohiko Ohwada*[,∥]

[‡]Laboratory of Neuropharmacology, Division of Pharmacology, National Institute of Health Sciences, 1-18-1 Kamiyoga, Setagaya-ku, Tokyo 158-8501, Japan

[§]Laboratory of Pharmacology, Faculty of Pharmaceutical Sciences, Tokyo University of Science, 2541 Yamazaki, Noda-city, Chiba 278-8510, Japan

[∥]Laboratory of Organic and Medicinal Chemistry, Graduate School of Pharmaceutical Sciences, University of Tokyo, 7-3-1, Hongo, Bunkyo-ku, Tokyo 113-0033, Japan

ABSTRACT: We recently found that tamoxifen suppresses L-glutamate transport activity of cultured astrocytes. Here, in an attempt to separate the L-glutamate transporter-inhibitory activity from the estrogen receptor-mediated genomic effects, we synthesized several compounds structurally related to tamoxifen. Among them, we identified two compounds, **1** (YAK01) and **3** (YAK037), which potently inhibited L-glutamate transporter activity. The inhibitory effect of **1** was found to be mediated through estrogen receptors and the mitogen-activated protein kinase (MAPK)/phosphatidylinositol 3-kinase (PI3K) pathway, though **1** showed greatly reduced transactivation activity compared with that of 17β-estradiol. On the other hand, compound **3** exerted its inhibitory effect through an estrogen receptor-independent and MAPK-independent, but PI3K-dependent pathway, and showed no transactivation activity. Compound **3** may represent a new platform for developing novel L-glutamate transporter inhibitors with higher brain transfer rates and reduced adverse effects.



KEYWORDS: *Tamoxifen, astrocyte, L-glutamate transporter, ERα, tetrasubstituted ethylene, nongenomic pathway*

L-Glutamate (L-Glu) is one of the major excitatory neurotransmitters in the central nervous system (CNS), but high concentrations of extracellular L-Glu cause excessive stimulation of L-Glu receptors in the CNS, leading to neurotoxicity.[1,2] Astrocyte L-Glu transporters are the only machinery available to remove L-Glu from extracellular fluid and to maintain a low and nontoxic concentration of L-Glu.[3] Consequently, dysfunction of astrocyte L-Glu transporters is considered to be implicated in the pathology of neurodegenerative conditions.[4] Therefore, exogenous compounds that can regulate the function of L-Glu transporters may provide chemical tools to investigate the regulatory mechanisms of these transporters at the molecular level, and would also be candidate therapeutic agents.

There is growing evidence that estrogen receptor (ER) α, which is a nuclear ER (nER) that mediates genomic effects, can also be translocated to plasma membranes and mediate acute nongenomic effects in some cases. We have clarified that 17β-estradiol (E2) inhibits L-Glu transporters via a nongenomic pathway involving membrane-associated ERα (mERα).[5] Tamoxifen (Tam), a synthetic estrogen analogue that is clinically used in the treatment of breast cancer to block the proliferative action of estrogens,[6] also inhibited astrocyte L-Glu transporters at picomolar concentration, probably through the same nongenomic pathway as E2.[7] Because overexpression of astrocyte L-Glu transporters is often associated with neuropsychiatric disorders,[4] inhibitors of L-Glu transporters may be clinically useful to ameliorate these disorders.[8] However, Tam also acts on genomic pathways involving nuclear estrogen receptors (nERs) α and β, depending on the cell type and promoter context,[9] and so may cause adverse effects including endometrial changes, depression and weight gain.[10,11] Therefore, Tam-inspired compounds that retain the inhibitory effect on L-Glu transporters, but lack the nER-mediated genomic effects, would be useful tools for biological research, as well as candidate therapeutic agents.

Tam is a tetrasubstituted triphenylethylene derivative, in which the four substituents on the olefinic carbon atoms are different. This structural complexity makes the stereospecific synthesis of Tam-related derivatives difficult. We thus focused on Tam-inspired compounds bearing identical substituents on at least one of the olefinic carbon atoms.[12] It is well-known that the *N,N*-dimethylaminoethyl substituent on the phenolic oxygen atom and the regiochemistry of the tetrasubstituted

Received: September 29, 2011
Accepted: November 14, 2011
Published: November 14, 2011

olefin of Tam are crucial for ER binding activity.[13] So, we considered that more symmetrical derivatives of Tam might show reduced ER-binding ability.

Among our synthesized compounds, we found two, compounds **1** (YAK01) and **3** (YAK037), with potent L-Glu transporter-inhibitory activity. Studies of their mechanisms of action indicated that, unlike Tam, compound **3** acts through an ER-independent and MAPK-independent, but PI3K-dependent pathway and shows no transactivation activity for nERs. We believe this compound may represent a new platform for developing novel L-Glu transporter inhibitors with higher brain transfer rates and reduced adverse effects.

RESULTS AND DISCUSSION

We synthesized several Tam-inspired compounds bearing identical substituents on one carbon atom of the olefin,[12] and found that two of them were potent inhibitors of astrocyte L-Glu transporters. The diethyl-substituted derivative **1** inhibited L-Glu transporters in the picomolar range (62.7 ± 7.48% of control at 1 pM; Figure 2A). The dose–response curve for the inhibitory activity was not linear, but followed an inverted U-shaped curve; however, such a non-monotonic dose dependence is rather common for hormones and their mimetics.[14] On the other hand, when the symmetrical substituent was changed from ethyl to benzyl (**2**), the inhibitory effect was lost (Figure 2B). However, when the phenolic oxygen atom of **1** was substituted with a *N*,*N*-dimethylaminoethyl group (Figure 1C), we found that the resulting compound **3** showed dose-dependent L-Glu transporter inhibition in the picomolar range (63.8 ± 5.49% of control at 1 pM; Figure 2C). The dose-dependency of the effect of **3** suggested that the underlying mechanism might be different from that in the case of **1**. Compound **4** was inactive (Figure 2D).

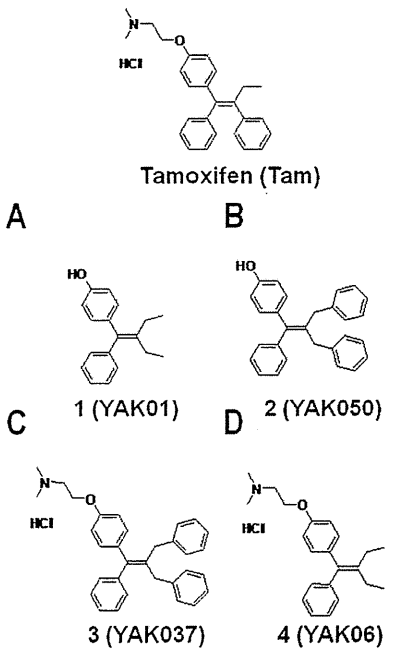


Figure 1. Chemical structures of the newly synthesized tamoxifen-related compounds.

We next examined the effects of **1** and **3** on cell viability by means of MTT reduction assay and LDH leakage assay, using the same cultured sample. Neither of the compounds was cytotoxic at concentrations below 1 μM (Figure 3), though 100 μM **1** and 10 μM **3** caused severe cell damage. These results exclude the possibility that the L-Glu clearance-inhibitory effects of these compounds at concentrations below 1 μM were caused by cell damage.

In order to confirm the involvement of L-Glu transporters in the inhibition of L-Glu uptake by our compounds, and to rule out the possibility that **1** and **3** act by inducing L-Glu release from astrocytes, we next examined the effect of **1** and **3** on L-Glu clearance when the L-Glu transporter activity was blocked with TBOA, a potent nonselective L-Glu transporter inhibitor (IC_{50}: 48 μM for GLAST/EAAT1, 7 μM for GLT1/EAAT2). We confirmed that application of 1 mM TBOA potently inhibited L-Glu transporter activity; that is, TBOA caused reversible chemical knock-down of L-Glu transporter activity.[7] When either **1** or **3** was coapplied with 1 mM TBOA, these compounds no longer influenced L-Glu clearance (Figure 4), indicating that the actions of these compounds are indeed mediated by L-Glu transporters, and do not involve L-Glu release from astrocytes.

Our cultured astrocytes predominantly expressed ERα, and little or no expression of ERβ was detected.[5] Tam is known to be a partial agonist of ERs,[9] raising the possibility that the compounds exerted their inhibitory effects via interaction with ERα. Therefore, we examined the involvement of ERα by coapplication of ICI182,780, a high-affinity antagonist of ERs. ICI182,780 dose-dependently blocked the inhibition of L-Glu uptake caused by **1** (Figure 5A) at 0.01, 0.1, and 1 μM, at which the effects of Tam were reported to be completely suppressed.[7] In contrast, ICI182,780 had no effect on the inhibition by **3** (Figure 5B), suggesting that the mechanism of the inhibition by **3** is independent of ERs. We further examined the signal transduction pathways mediating the effects of **1** and **3**. When coapplied with U0126, which inhibits mitogen-activated protein kinase/extracellular signal-regulated kinase 1 (MEK1, IC50: 70 nM) and MEK2 (IC50: 60 nM), the inhibitory effect by **1** was blocked, whereas that of **3** was not (Figure 6A). On the other hand, when coapplied with LY294002, a specific phosphoinositide 3-kinase (PI3K) inhibitor (IC50: 70 nM), the inhibitory effects of both compounds were completely blocked (Figure 6B). These results suggest that PI3K is a common mediator of the effects of both compounds, whereas mitogen-activated protein kinase (MAPK) is involved only in the mechanism of inhibition by **1**.

Finally we examined the ER-agonist potency of **1** and **3**, i.e., the transcriptional effects of these compounds via human ERα and ERβ, using HEK293/hERα and HEK293/hERβ reporter cells (Figure 7). Compound **1** showed agonist activity in both of 293/hERα and 293/hERβ reporter cells, though the binding affinities were much weaker than that of E2. The EC50 values of **1** for ERα and ERβ are 30.8 nM and 10.4 nM, respectively (1.25 nM and 0.864 nM, respectively, for E2). The relative agonist activity of **1** was 66.8% of that of E2 in HEK293/hERα and 122.0% of that of E2 in HEK293/hERβ. Strikingly, **3** showed no agonist potency for ERα or ERβ. These findings strongly suggest that **3** can inhibit L-Glu transporters without interaction with ERs.

In this study, we examined the potential of Tam-related compounds to inhibit GLAST/EAAT1 and GLT1/EAAT2, which are major astrocytic L-Glu transporters in the rat

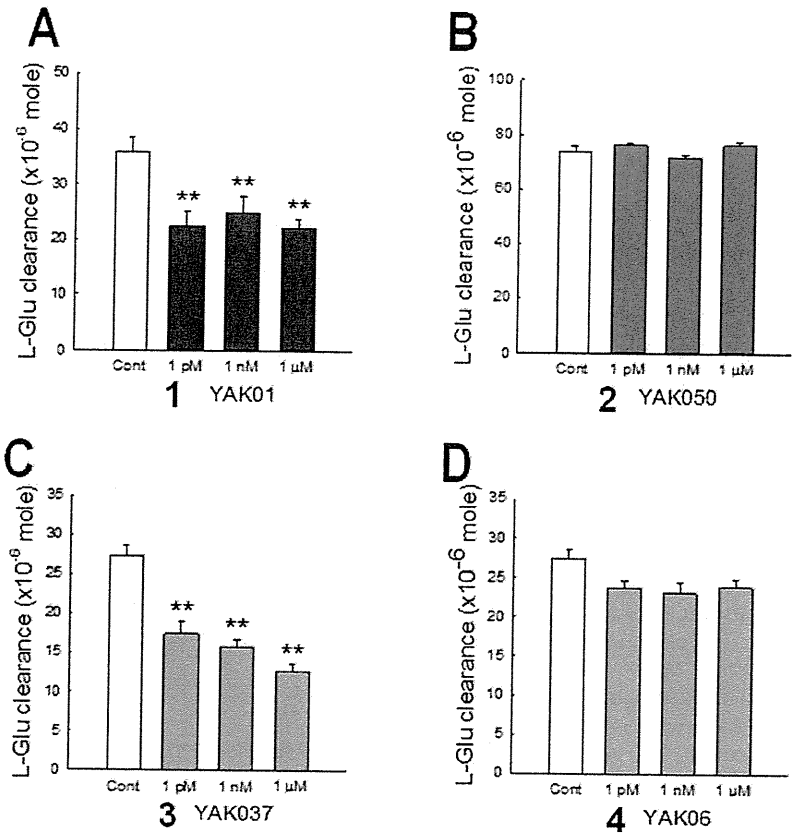


Figure 2. Compounds **1** and **3** inhibited L-Glu clearance in cultured astrocytes. The open column shows the control clearance, and colored columns show the clearance in the presence of various concentrations of compounds **1** (A), **2** (B), **3** (C), and **4** (D). $^{**}p < 0.01$ vs control group ($N = 6$), Tukey's test following ANOVA.

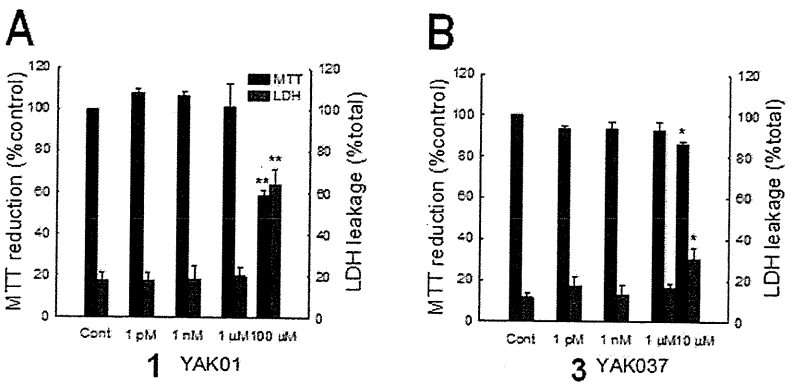


Figure 3. Effects of compounds **1** and **3** on cell viability. The results of MTT reduction and LDH leakage assays of **1** (A) and **3** (B) are shown. $^{*}p < 0.05$, $^{**}p < 0.01$ vs control group ($N = 6$), Tukey's test following ANOVA.

forebrain. Although GLT-1 is the main regulator of synaptically released L-Glu in vivo, the predominant subtype changes to GLAST in cultured astrocytes, possibly owing to the lack of interaction of astrocytes with neurons.[15] We confirmed that GLAST is the main functional L-Glu transporter in our primary-cultured astrocytes by Western blotting and pharmacological experiments (data not shown), in accordance with a previous report.[16] Therefore, the effects of the compounds observed here can be interpreted as being due to modulation of GLAST functional activity.

There is growing evidence that ERα, which is a nER that mediates genomic effects, can also be translocated to plasma membranes and mediate acute nongenomic effects in some cases. Transfection of CHO cells with nERs was reported to result in ER expression in both nuclei and membranes.[17] ERs on the plasma membranes of tumor cells were demonstrated to be structurally similar to nERs.[18] Further, mERα activated metabotropic glutamate receptor 5 (mGluR5) in striatal neurons in the CNS.[19] In our previous study, we clarified that the predominant ER subtype in cultured astrocytes was ERα, and

dx.doi.org/10.1021/cn200091w | ACS Chem. Neurosci. 2012, 3, 105–113

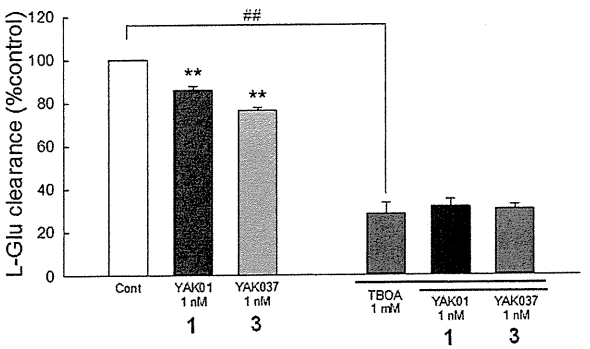


Figure 4. Compounds **1** and **3** suppressed L-Glu clearance in astrocyte culture by decreasing the functional activity of L-Glu transporter. L-Glu clearance in the presence and absence of compounds **1** and **3** is shown, together with their effects in the copresence of the potent nonselective L-Glu transporter inhibitor TBOA. $^{**}p < 0.01$ vs control group ($N = 6$), Tukey's test following ANOVA.

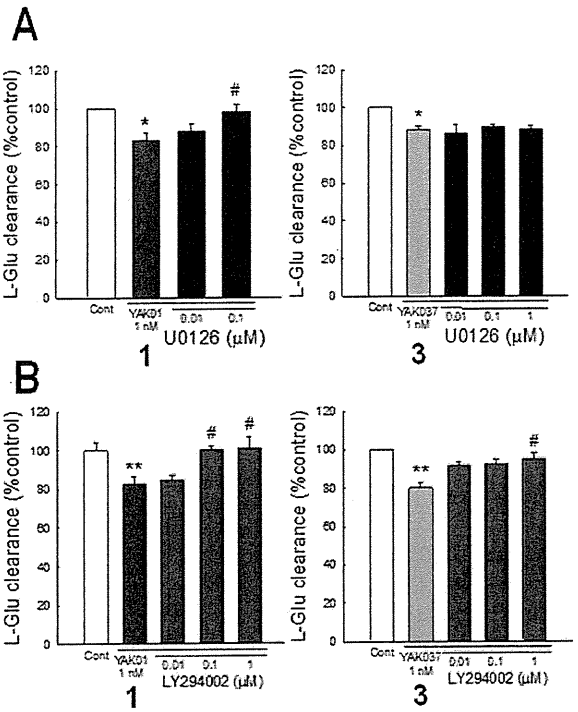


Figure 6. Involvement of MAPK and PI3K in the L-Glu transporter-inhibitory activity of compounds **1** (A) and **3** (B). Effects of compounds **1** (left panels) and **3** (right panels) on L-Glu clearance in the presence and absence of various concentrations of U0126, an inhibitor of MAPK/ERKs (A) or LY294002, a specific inhibitor of PI3K (B). $^{*}P < 0.05$, $^{**}p < 0.01$ vs control group, $\#p < 0.05$ vs compound-treated group ($N = 6$), Tukey's test following ANOVA.

estrogens (such as E2 and Tam) inhibited L-Glu transporter activity via the activation of mERα.[5] We found that the effects of **1** were blocked by ICI182,780, suggesting an interaction of **1** with ERα. In addition, our pharmacological experiments showed that activation of both of MAPK and PI3K is necessary for the L-Glu transporter-inhibitory activity of **1**. There are many reports indicating that nongenomic effects involving mERα are mediated via MAPK[19−21] and PI3K.[20,22] Taken together, the effects of **1** may be mediated by mERα in a similar manner to E2 and Tam. E2 was reported to activate MAPK via both PI3K-dependent and independent pathways in a single neuron.[20] Whether or not the same signaling pathways also exist in astrocytes is not yet known. It is of interest that other studies have found that estrogens also inhibit dopamine transporter (DAT) through the activation of mERα.[23,24]

On the other hand, the effect of **3** was ER-independent and MAPK-independent, but PI3K-dependent. Our binding assay revealed that **1** binds with ERs, but **3** does not. Based on these results, we propose that the mechanisms of the L-Glu transporter-inhibitory effects of **1** and **3** are different, as illustrated in Figure 8. The effect of **3** was possibly mediated by GPR30, a newly found ER, which is suggested to mediate the rapid nongenomic effects of estrogens.[25,26] In the case of GPR30, ICI182,780 acts as agonist, leading to activation of

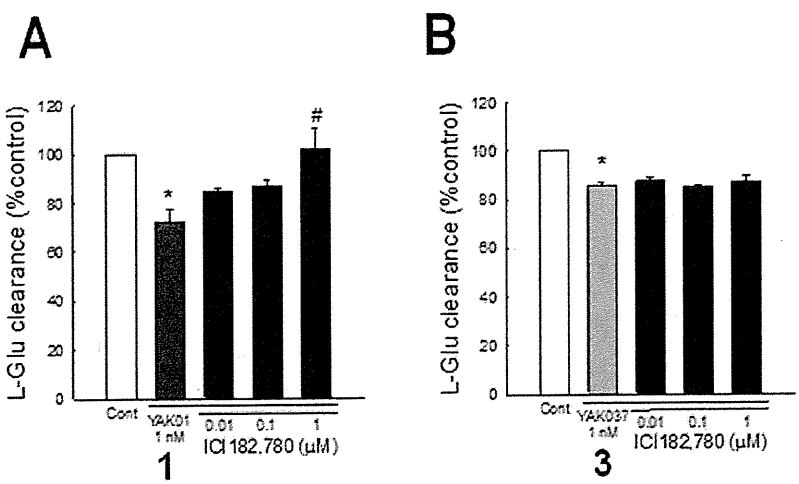


Figure 5. Involvement of ERs in the L-Glu transporter-inhibitory effects of compounds **1** and **3**. Effects of compounds **1** (A) and **3** (B) on L-Glu clearance in the presence and absence of various concentrations of ICI182,780, a high-affinity antagonist of ERs. $^{*}P < 0.05$ vs control group, $\#p < 0.05$ vs compound-treated group ($N = 6$), Tukey's test following ANOVA.

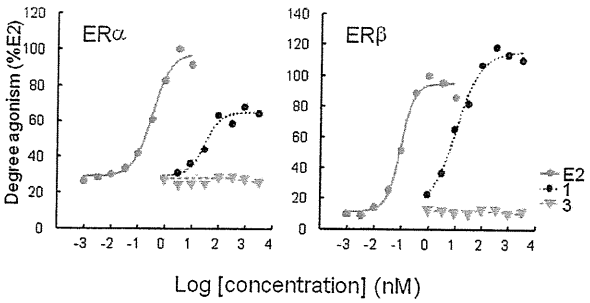


Figure 7. ER agonist potency of compounds **1** and **3** to nERs: dose dependence of binding of compounds **1** and **3** in HEK293/hERα cells (left) or HEK293/hERβ cells (right). Compound 1 showed dose-dependent agonist activity in both of HEK293/hERα cells (left) and HEK293/hERβ cells (right), though 3 showed no agonist potency for ERα or ERβ.

signal transduction pathways in a similar manner to estrogens.[27,28] However, we could not detect any effects of ICI182,780 alone on L-Glu transporter in our experiments (data not shown). In addition, Kuo et al. reported that GPR30 in astrocytes is detected not in the cell membranes but in the smooth endoplasmic reticulum,[29] while the cellular localization of GPR30 has been still controversially argued. In these contexts, GPR30 is an unlikely mediator to block the L-Glu transporters by the action of **3**.

According to Kisanga et al., the concentration of Tam in serum during conventional treatment for breast cancer (1–20 mg daily) is in the range from 20 to 225 nM.[30] Because **3** is more hydrophobic than Tam (the values of clogP for Tam and **3** are 7.56 and 9.70, respectively), it should exhibit greater permeability into the brain. Although other L-Glu transporter inhibitors, mainly L-Glu/aspartate analogues, are known, few of them have high brain transfer rates. Therefore, **3** is expected to be useful for biological research, and is also considered to be a promising candidate or lead compound for pharmacological application.

In conclusion, examination of several Tam-inspired compounds led to the discovery of two compounds that inhibited astrocytic L-Glu transporters at picomolar concentration. The inhibitory activity of compound **1** was mediated through the ER-MAPK/PI3K pathway, like that of Tam, though its transactivation activity was drastically reduced as compared with E2. In contrast, the inhibitory effect of **3** was manifested through an ER-independent and MAPK-independent, but PI3K-dependent pathway, and **3** showed no transactivation activity. These results suggest that **3** may represent a new platform for the development of novel L-Glu transporter inhibitors with higher brain transfer rates and reduced adverse effects.

■ METHODS

Chemistry. *General Procedures.* All reagents were commercial products and were used without further purification, unless otherwise noted. NMR data were recorded on a JEOL-400 or a Bruker Avance 400 NMR spectrometer (400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR). *d*-$CDCl_3$ was used as a solvent, unless otherwise noted. Chemical shifts (δ) are reported in ppm with respect to internal tetramethylsilane (δ = 0 ppm) or undeuterated residual solvent (i.e., $CHCl_3$ (δ = 7.265 ppm)). Coupling constants are given in hertz. Coupling patterns are indicated as follows: m = multiplet, d = doublet, s = singlet, br = broad. High-resolution mass spectrometry (HRMS) was conducted in the electron spray ionization (ESI)-time-of-flight

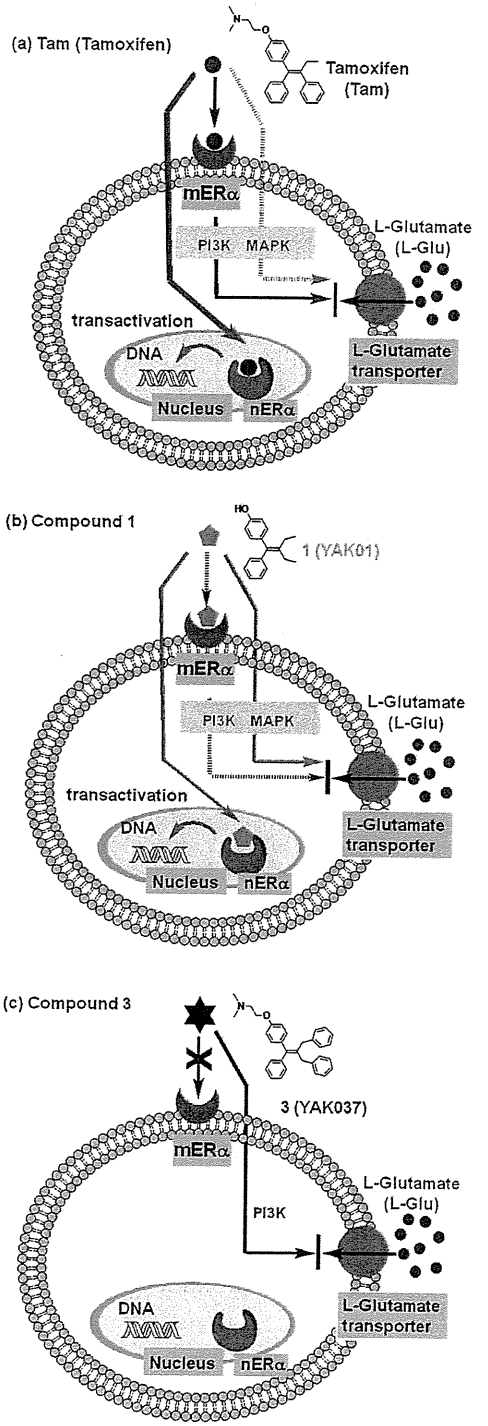


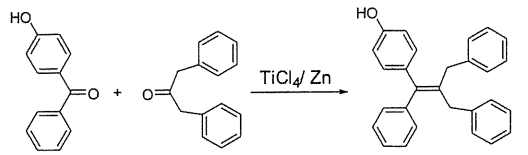
Figure 8. Schematic illustration of the proposed mechanisms of the effects of tamoxifen (a) and compounds **1** (b) and **3** (c).

(TOF) detection mode on a Bruker micrOTOF-05. FAB-MS and high-resolution FAB-MS were obtained on a JMS700-MSTATION (JEOL, Japan). Column chromatography was carried out on silica gel (silica gel 60N (100–210 μm), Kanto Chemicals, Japan). Flash column chromatography was performed on silica gel H (Merck, Germany). Analytical thin-layer chromatography (TLC) was performed on

dx.doi.org/10.1021/cn200091w | ACS Chem. Neurosci. 2012, 3, 105–113

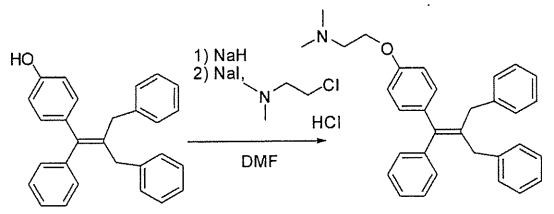
precoated plates of silica gel HF_{254} (Merck, Germany). All the melting points were measured with a Yanaco Micro Melting Point apparatus and are uncorrected. Combustion analyses were carried out in the microanalysis laboratory of this faculty.

Synthesis of Compounds. Compounds **1** and **2** were synthesized from 4-hydroxybenzophenone and butyl-3-one or dibenzylacetone by using $TiCl_4$ in the presence of Zn. Introduction of the *N,N*-dimethylaminoethyl moiety at the phenolic hydroxyl group of **1** and **2** was carried out by base treatment, followed by addition of 2-dimethylaminoethyl chloride hydrochloride.

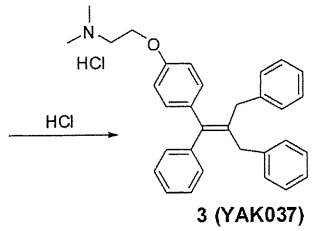


2 (YAK050)

Synthesis of Tamoxifen-Related Compounds. Compound 2 (YAK050). To a suspension of Zn powder (916.6 mg; 6.9 equiv with respect to 4-hydroxybenzophenone) in dry THF (30 mL) in a 200 mL three-necked flask, $TiCl_4$ (0.61 mL, 2.8 equiv) was added dropwise under an argon atmosphere at −20 °C (in an ice-salt bath) over 2 min. The resulting light green-yellow mixture was stirred at −20 °C for 20 min and then the cooling bath was removed. After 20 min, the flask was immersed in a preheated oil bath at 100 °C and refluxed at 100 °C with stirring for 2.5 h. To the resulting deep blue mixture was added in one portion a solution of 4-hydroxybenzophenone (401.3 mg, 2.02 mmol) and dibenzyl ketone (1.2735 g, 3 equiv) in 50 mL of dry THF. The resultant mixture was heated at reflux at 100 °C with stirring for 2 h, then allowed to cool to rt, and poured into 400 mL of 0.5 N aqueous NaOH solution. The whole was extracted with ethyl acetate (500 mL). The organic layer was washed with water, dried over $MgSO_4$ and evaporated to give a pale yellow oil (1.5172 g), which was column-chromatographed (silica gel, acetone/*n*-hexane (1:7)) to give 365.0 mg (48% yield) of the olefin **2** as a white amorphous solid. Mp: 57–60 °C. ^1H NMR ($CDCl_3$): δ: 7.287–7.079 (m, 17H), 6.760 (d, 2H, J = 8.8 Hz), 4.792 (s, 1H), 3.413 (s, 2H), 3.377 (s, 2H). ^{13}C NMR ($CDCl_3$): δ: 154.1, 143.0, 140.7, 140.4, 135.8, 135.4, 130.7, 129.4, 128.8, 128.3, 128.3, 128.2, 126.5, 125.9, 115.1, 37.4, 37.2. HRMS (ESI^-): Calcd. for $C_{28}H_{23}O$ ($[M - H]^-$), 375.1754. Found: 375.1744. Anal. Calcd for $C_{28}H_{24}O{\cdot}0.2H_2O$: C, 88.48; H, 6.47; N, 0.00. Found: C, 88.36; H, 6.63; N, 0.00.



2 (YAK050)

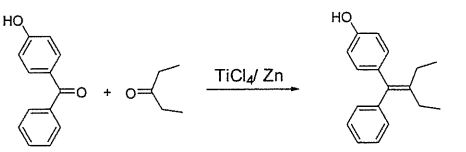


3 (YAK037)

Compound 3 (YAK037). To a suspension of NaH (60%, 42 mg, 1.05 mmol) in DMF (3 mL) at 0 °C was added a solution of the phenol **2** (158.2 mg, 0.420 mmol) in DMF (3 mL). The reaction mixture was stirred for 30 min at 0 °C, and then a solution of 2-dimethylaminoethyl chloride hydrochloride (181.0 mg, 1.256 mmol, 3.0 equiv) and NaI (94.0 mg, 0.627 mmol, 1.5 equiv) in DMF (3 mL) was added. The reaction mixture was stirred at 50 °C for 30 min, and then saturated aqueous NH_4Cl was added to quench the reaction. The mixture was extracted with Et_2O. The organic layer was washed with brine, dried over Na_2SO_4 and evaporated to afford a residue, which was column-chromatographed (ethyl acetate/Et_3N = 100/1) to give the intermediate amine (83.0 mg, 44% yield). The HCl salt of the resultant amine was prepared by repeated addition of a solution of 2 N HCl in Et_2O to a solution of the amine in ethyl acetate, followed by evaporation of the organic solvent to give **3**.

3: White solid. Mp. 169–170 °C. ^1H NMR ($CDCl_3$): δ: 13.073 (brs, 1H), 7.306–7.195 (m, 13H), 7.102–7.074 (m, 4H), 6.832 (d, 2H, J = 8.8 Hz), 4.481–4.459 (m, 2H), 3.425–3.390 (m, 6H), 2.893 (s, 6H). ^{13}C NMR ($CDCl_3$) δ: 155.7, 142.8, 140.4, 140.3, 140.2, 136.8, 136.2, 130.9, 129.4, 128.8, 128.7, 128.4, 128.3, 128.3, 126.6, 126.0, 125.9, 114.3, 62.8, 56.5, 43.6, 37.4, 37.2. HRMS (ESI^+, $[M + H]^+$): Calcd. for $C_{32}H_{34}NO$, 448.26349. Found: 448.26092. Anal. Calcd for $C_{32}H_{34}ClNO{\cdot}1/4H_2O$: C, 78.67; H, 7.12; N, 2.87. Found: C, 78.64; H, 7.30; N, 2.87.

Compound 1 (YAK01).



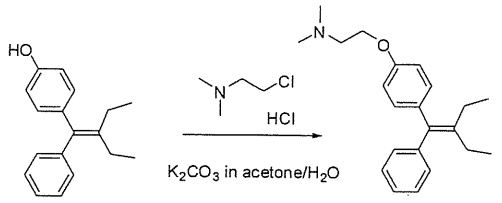
1 (YAK01)

To a suspension of Zn (0.86 g, 13.2 mmol) in 30 mL of dry THF at −5 °C was added dropwise $TiCl_4$ (0.72 mL, 6.6 mmol) under an argon atmosphere. The mixture was heated at reflux for 2 h. A solution of 4-hydroxybenzophenone (341.1 mg, 1.7 mmol) and 3-pentanone (0.50 mL, 5.0 mmol) in 50 mL of dry THF was added in one portion, and heating was continued at reflux for 6 h. Then the reaction mixture was cooled to rt, quenched with 10% aqueous K_2CO_3 (100 mL) and extracted with ethyl acetate (3 × 80 mL). The combined organic phase was washed with brine (50 mL), dried over Na_2SO_4, and evaporated to give a residue, which was flash column-chromatographed (3:1 hexane/ethyl acetate) to afford **1** (383.4 mg, 88.3%) as a white solid.

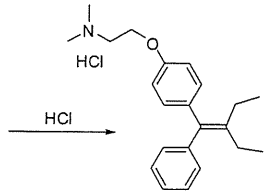
1: Mp. 76.0–76.5 °C (colorless needles, recrystallized from *n*-hexane). ^1H NMR ($CDCl_3$) δ: 7.261 (2H, t, J = 8.0 Hz), 7.173 (1H, d, J = 7.2 Hz), 7.128 (2H, d, J = 7.6 Hz), 7.009 (2H, d, J = 8.8 Hz), 6.726 (2H, d, J = 8.8 Hz), 4.763 (1H, s), 2.152 (2H, quartet, J = 7.6 Hz), 2.115 (2H, quartet, J = 6.0 Hz), 1.007 (3H, t, J = 7.6 Hz), 0.994 (3H, t, J = 7.6 Hz). ^{13}C NMR ($CDCl_3$) δ: 153.7, 143.7, 142.0, 136.5, 136.2, 130.5, 129.2, 127.9, 125.9, 114.8, 24.4, 24.3, 13.3. HRMS (ESI^-, $[M - H]^-$): Calcd. for $C_{18}H_{19}O^-$, 251.14414. Found: 251.14730. HRMS (FAB-MS, $[M]^+$) Calcd. for $C_{18}H_{20}O$, 252.1514. Found: 252.1528. Anal. Calcd. for $C_{18}H_{20}O$: C, 85.67; H, 7.99; N, 0.00. Found: C, 85.38; H, 8.13; N, 0.00.

Compound 4 (YAK06).

2-Dimethylaminoethyl chloride hydrochloride (282.4 mg, 2.0 mmol) and K_2CO_3 (1.5734 g, 11.4 mmol) were stirred in acetone/H_2O (18 mL/2 mL) at 0 °C for 30 min, then compound **1** (139.1 mg, 0.55 mmol) and K_2CO_3 (421.1 mg, 3.1 mmol) were added, and the whole was heated at reflux for 24 h, then cooled to rt. Inorganic materials were removed by filtration, and the filtrate was evaporated. The residue was flash column-chromatographed (100:1 ethyl acetate/Et_3N) to afford the amine as a white solid (88.0 mg). To a solution of the amine in ethyl acetate, a solution of HCl in ether was added to give a precipitate, which was collected and recrystallized from ethanol/ethyl acetate to give **4** (95.0 mg, 48%) as a white powder. **4**: Mp. 129.5–130.2 °C. ^1H NMR ($CDCl_3$) δ 726–6.90 (9H, m), 4.07 (2H, t, J = 6.0 Hz), 2.75 (2H, t, J = 6.0 Hz), 2.40 (6H, s), 2.15 (4H, d, J = 7.2 Hz), 1.00 (6H, t, J = 7.2 Hz). HRMS (FAB-MS, $[M\text{-}Cl]^+$): Calcd. for $C_{22}H_{30}NO^+$: 324.2322. Found: 324.2321.



1 (YAK01)



4 (YAK06)

Biology. All procedures using live animals in this study were conducted in accordance with the guidelines of the National Institute of Health Sciences, Japan.

Materials. Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were purchased from GIBCO (CA, USA). Glutamate dehydrogenase (GLD) was purchased from Roche (Mannheim, Germany). β-Nicotinamide adenine dinucleotide (βNAD), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 1-methoxy-5-methylphenazinium methyl sulfate (MPMS), lactate lithium salt and LY294002 were purchased from Sigma (MO, USA). DL-threo-β-benzyloxyaspartic acid (TBOA) and ICI182,780 were purchased from Tocris (MO, USA). U0126 was purchased from Promega (WI, USA). Assay kits for hormonal effects on HEK293/hERα and HEK293/hERβ reporter cells were purchased from Clontech (CA, USA).

Cell Culture. Primary cultures of astrocytes were prepared from the cerebral cortices of 3-day-old neonates of Wistar rats, as described previously.[31] Briefly, dissociated cortical cells were suspended in modified DMEM containing 30 mM glucose, 2 mM glutamine, 1 mM pyruvate and 10% FBS, and plated on uncoated 75 cm^2 flasks at the density of 600 000 cells/cm^2. A monolayer of type I astrocytes was obtained 12–14 days after plating. Nonastrocytes such as microglia were detached from the flasks by shaking and removed by changing the medium. Astrocytes in the flasks were dissociated by trypsinization, reseeded on uncoated 96-well microtiter plates at 20 000 cells/cm^2, and incubated until the cells became confluent (approximately 9–10 days after reseeding). In this culture, >98% of the cells were identified as type I astrocytes on the basis of positivity for GFAP and flattened, polygonal appearance.

Measurement of Extracellular L-Glu Concentration. Extracellular L-Glu concentration was measured by means of a colorimetric method according to Abe et al.[32] Briefly, 50 μL of culture supernatant was transferred to each well of a 96-well microtiter plate and mixed with 50 μL of substrate mixture consisting of 20 U/mL GLD, 2.5 mg/mL β-NAD, 0.25 mg/mL MTT, 100 μM MPMS and 0.1% (v/v) Triton X-100 in 0.2 M Tris-HCl buffer (pH 8.2). After 10 min incubation at 37 °C, the reaction was stopped by adding 100 μL of solution containing 50% (v/v) dimethylformamide and 20% (wt/vol) SDS (pH 4.7). In this reaction, MTT (yellow) is converted into MTT formazan (purple) in proportion to the L-Glu concentration. The amount of MTT formazan was determined by measuring the absorbance at 570 nm (test wavelength) and 655 nm (reference wavelength) with a microplate reader. The concentration of L-Glu was estimated from a standard curve, which was constructed in each assay using cell-free medium containing known concentrations of L-Glu. L-Glu clearance was shown as the amount of L-Glu taken up by astrocytes, which was calculated from the concentration difference in the medium.

Treatment with Test Compounds. L-Glu was dissolved at 1 mM in phosphate-buffered saline and diluted to 100 μM with the culture medium. Compounds **1**, **2**, **3**, and **4** were dissolved at 100, 100, 100, and 10 mM, respectively, in dimethyl sulfoxide (DMSO) and diluted to the required final concentrations with the culture medium. The concentration of DMSO in the medium was controlled to be below 0.1%, because we had already confirmed that 0.1% DMSO has no effect on L-Glu transport activity or cell viability (data not shown). Cells were incubated with test compounds for 24 h. TBOA (IC50 = 48 μM for GLAST, 7 μM for GLT1) was freshly dissolved at 1 mM in culture medium for each experiment. ICI182,780 (IC50 = 0.29 nM for ERs), U0126 (IC50 = 72 nM for MEK1, 58 nM for MEK2), and LY294002 (IC50 = 1 μM for class 1 PI3K, 19 μM for class 2 PI3K) were dissolved at 1, 5, and 5 mM, respectively, in DMSO, and the solutions were diluted with culture medium to yield the required final concentrations. These inhibitors were coapplied with 1 nM test compounds (**1**–**4**) for 24 h.

Assay Procedure for Hormonal Effects on HEK293/hERα and HEK293/hERβ Reporter Cells. Human embryo kidney 293 cells (HEK293) were grown in FBS (+) DMEM in 100 mm dishes. Cells were subcultured once or twice a week at about 80% confluence. A solution of 12.4 μL of 2 M calcium ion, 100 ng/well reporter or negative control vector (pERE-TA-SEAP or pTA-SEAP, Clontech), 50 ng/well expression vector (pcDNA3 ERα or pcDNA3 ERβ, generous gift from Dr. Shige-aki Kato, University of Tokyo, Japan), and 100 ng/well positive control vector (pSV-β-galactosidase, Promega) was diluted to a final volume of 10 μL/well. This mixture was carefully added dropwise to the same volume of HEPES solution with slow vortexing, and the mixture was incubated at rt for 20 min to obtain a precipitate. Cells from the exponential growth phase were seeded (3.0×10^4 cells/ml) into 96-well plates the day before transfection. The cells were incubated with fresh medium for 1 h, then 1/10 volume of precipitate was added to each well and incubation was continued for 24 h at 37 °C in an atmosphere of 5% CO_2 in air. The medium was replaced with fresh FBS (-) medium and incubation was continued for a further 24 h. Then the cells were incubated with test compounds for 24 h at 37 °C in an atmosphere of 5% CO_2 in air. SEAP activity (Great EscapeTM SEAP chemiluminescence kit 2.0, Clontech) and β-galactosidase activity (β-Galactosidase Enzyme Assay System with Reporter Lysis Buffer, Promega) were measured with a Spectramax M5 microplate reader (Molecular Devices Japan, Tokyo, Japan). All transfections were performed in triplicate.

Statistical Analysis. Data were obtained from four independent experiments (averaged values of six wells for each) unless otherwise noted. Data are expressed as means ± SEM of these data. Tests of homogeneity of variance, normality, and distribution were performed to ensure that the assumptions required for standard parametric ANOVA were satisfied. Statistical analysis was performed by one-way repeated-measures ANOVA with post hoc Tukey's test for multiple pairwise comparisons.

■ AUTHOR INFORMATION

Corresponding Author

*(K.S.) Telephone and Fax: +81-3-3700-9698. E-mail: kasato@nihs.go.jp. (T.O.) Telephone: +81-3-5840-4730. Fax: +81-3-5840-4735. E-mail: ohwada@mol.f.u-tokyo.ac.jp.

Author Contributions

†These two authors equally contributed to this Article. Individual author contributions: K.S. designed the biological experimental plan, performed biological experiments, data analysis, manuscript writing and preparation. J.K. and Y.S. performed experimental work. K.T. contributed to the data analysis. J.O., K.N. and Y.S. provided advice on the experimental direction. Y.O. carried out organic synthesis, data analysis and wrote portions of the manuscript. Y.S. carried out organic synthesis. T.O. designed and oversaw all organic chemistry studies, carried out organic synthesis and also performed data analysis and manuscript writing and preparation.

Funding

This work was partly supported by a Grant-in-Aid from the Food Safety Commission, Japan (No. 1003), a Grant-in-Aid for Young Scientists from MEXT, Japan (KAKENHI 21700422), the Program for Promotion of Fundamental Studies in Health Sciences of NIBIO, Japan, a Health and Labor Science Research Grant for Research on Risks of Chemicals, a Health and Labor Science Research Grant for Research on New Drug Development from MHLW, Japan, awarded to K.S., and a Health and Labor Science Research Grant for Research on Risks of Chemicals from MHLW, Japan, awarded to K.N. and T.O.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank Dr. Shige-aki Kato for providing pcDNA3 hERα and pcDNA3 hERβ.

■ ABBREVIATIONS

βNAD; β-nicotinamide adenine dinucleotide; CNS; central nervous system; DMEM; Dulbecco's modified Eagle's medium; DMSO; dimethyl sulfoxide; E2; 17β-estradiol; ESI; electron spray ionization; FBS; fetal bovine serum; GLD; glutamate dehydrogenase; HEK-293; Human embryo kidney 293 cells; HRMS; high-resolution mass spectrometry; L-Glu; L-glutamate; MAPK; mitogen-activated protein kinase; MEK; mitogen-activated protein kinase/extracellular signal-regulated kinase; mERα; membrane-associated estrogen receptor α; mGluR5; metabotropic glutamate receptor 5; MPMS; 1-methoxy-5-methylphenazinium methyl sulfate; MTT; 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide; nERs; nuclear estrogen receptors; PI3K; phosphatidylinositol 3-kinase; Tam; tamoxifen; TBOA; DL-threo-β-benzyloxyaspartic acid; TLC; thin-layer chromatography; TOF; time-of-flight

■ REFERENCES

(1) Kumar, A., Singh, R. L., and Babu, G. N. (2010) Cell death mechanisms in the early stages of acute glutamate neurotoxicity. *Neurosci. Res. 66*, 271–278.

(2) Choi, D. W. (1988) Glutamate neurotoxicity and diseases of the nervous system. *Neuron 1*, 623–634.

(3) Logan, W. J., and Snyder, S. H. (1971) Unique high affinity uptake systems for glycine, glutamic and aspartic acids in central nervous tissue of the rat. *Nature 234*, 297–299.

(4) Beart, P. M., and O'Shea, R. D. (2007) Transporters for L-glutamate: an update on their molecular pharmacology and pathological involvement. *Br. J. Pharmacol. 150*, 5–17.

(5) Sato, K., Matsuki, N., Ohno, Y., and Nakazawa, K. (2003) Estrogens inhibit L-glutamate uptake activity of astrocytes via membrane estrogen receptor alpha. *J. Neurochem. 86*, 1498–1505.

(6) Olivier, S., Close, P., Castermans, E., de Leval, L., Tabruyn, S., Chariot, A., Malaise, M., Merville, M. P., Bours, V., and Franchimont, N. (2006) Raloxifene-induced myeloma cell apoptosis: a study of nuclear factor-kappaB inhibition and gene expression signature. *Mol. Pharmacol. 69*, 1615–1623.

(7) Sato, K., Saito, Y., Oka, J., Ohwada, T., and Nakazawa, K. (2008) Effects of tamoxifen on L-glutamate transporters of astrocytes. *J. Pharmacol. Sci. 107*, 226–230.

(8) Bunch, L., Erichsen, M. N., and Jensen, A. A. (2009) Excitatory amino acid transporters as potential drug targets. *Expert Opin. Ther. Targets 13*, 719–731.

(9) Margueron, R., Duong, V., Bonnet, S., Escande, A., Vignon, F., Balaguer, P., and Cavailles, V. (2004) Histone deacetylase inhibition and estrogen receptor alpha levels modulate the transcriptional activity of partial antiestrogens. *J. Mol. Endocrinol. 32*, 583–594.

(10) Thompson, D. S., Spanier, C. A., and Vogel, V. G. (1999) The relationship between tamoxifen, estrogen, and depressive symptoms. *Breast J. 5*, 375–382.

(11) Grilli, S. (2006) Tamoxifen (TAM): the dispute goes on. *Ann. Ist. Super. Sanita 42*, 170–173.

(12) Sha, Y., Tashima, T., Mochizuki, Y., Toriumi, Y., Adachi-Akahane, S., Nonomura, T., Cheng, M., and Ohwada, T. (2005) Compounds structurally related to tamoxifen as openers of large-conductance calcium-activated K^+ channel. *Chem. Pharm. Bull. (Tokyo) 53*, 1372–1373.

(13) Robertson, D. W., Katzenellenbogen, J. A., Long, D. J., Rorke, E. A., and Katzenellenbogen, B. S. (1982) Tamoxifen antiestrogens. A comparison of the activity, pharmacokinetics, and metabolic activation of the cis and trans isomers of tamoxifen. *J. Steroid Biochem. 16*, 1–13.

(14) Weltje, L., vom Saal, F. S., and Oehlmann, J. (2005) Reproductive stimulation by low doses of xenoestrogens contrasts with the view of hormesis as an adaptive response. *Hum. Exp. Toxicol. 24* (9), 431–437.

(15) Perego, C., Vanoni, C., Bossi, M., Massari, S., Basudev, H., Longhi, R., and Pietrini, G. (2000) The GLT-1 and GLAST glutamate transporters are expressed on morphologically distinct astrocytes and regulated by neuronal activity in primary hippocampal co-cultures. *J. Neurochem. 75*, 1076–1084.

(16) Guillet, B., Lortet, S., Masmejean, F., Samuel, D., Nieoullon, A., and Pisano, P. (2002) Developmental expression and activity of high affinity glutamate transporters in rat cortical primary cultures. *Neurochem. Int. 40*, 661–671.

(17) Razandi, M., Pedram, A., Greene, G. L., and Levin, E. R. (1999) Cell membrane and nuclear estrogen receptors (ERs) originate from a single transcript: studies of ERalpha and ERbeta expressed in Chinese hamster ovary cells. *Mol. Endocrinol. 13*, 307–319.

(18) Pappas, T. C., Gametchu, B., and Watson, C. S. (1995) Membrane estrogen receptors identified by multiple antibody labeling and impeded-ligand binding. *FASEB J. 9*, 404–410.

(19) Grove-Strawser, D., Boulware, M. I., and Mermelstein, P. G. (2010) Membrane estrogen receptors activate the metabotropic glutamate receptors mGluR5 and mGluR3 to bidirectionally regulate CREB phosphorylation in female rat striatal neurons. *Neuroscience 170*, 1045–1055.

(20) Mannella, P., and Brinton, R. D. (2006) Estrogen receptor protein interaction with phosphatidylinositol 3-kinase leads to activation of phosphorylated Akt and extracellular signal-regulated kinase 1/2 in the same population of cortical neurons: a unified mechanism of estrogen action. *J. Neurosci. 26*, 9439–9447.

(21) Szego, E. M., Barabas, K., Balog, J., Szilagyi, N., Korach, K. S., Juhasz, G., and Abraham, I. M. (2006) Estrogen induces estrogen receptor alpha-dependent cAMP response element-binding protein phosphorylation via mitogen activated protein kinase pathway in basal forebrain cholinergic neurons in vivo. *J. Neurosci. 26*, 4104–4110.

(22) Vasudevan, N., Kow, L. M., and Pfaff, D. (2005) Integration of steroid hormone initiated membrane action to genomic function in the brain. *Steroids 70*, 388–396.

(23) Alyea, R. A., Laurence, S. E., Kim, S. H., Katzenellenbogen, B. S., Katzenellenbogen, J. A., and Watson, C. S. (2008) The roles of membrane estrogen receptor subtypes in modulating dopamine transporters in PC-12 cells. *J. Neurochem. 106* (4), 1525–1533.

(24) Watson, C. S., Alyea, R. A., Hawkins, B. E., Thomas, M. L., Cunningham, K. A., and Jakubas, A. A. (2006) Estradiol effects on the dopamine transporter - protein levels, subcellular location, and function. *J. Mol. Signaling 1*, 5.

(25) Filardo, E. J., and Thomas, P. (2005) GPR30: a seven-transmembrane-spanning estrogen receptor that triggers EGF release. *Trends. Endocrinol. Metab. 16* (8), 362–367.

(26) Revankar, C. M., Cimino, D. F., Sklar, L. A., Arterburn, J. B., and Prossnitz, E. R. (2005) A transmembrane intracellular estrogen receptor mediates rapid cell signaling. *Science 11;307* (5715), 1625–1630.

(27) Filardo, E. J., Quinn, J. A., Bland, K. I., and Frackelton, A. R. Jr. (2000) Estrogen-induced activation of Erk-1 and Erk-2 requires the G

protein-coupled receptor homolog, GPR30, and occurs via transactivation of the epidermal growth factor receptor through release of HB-EGF. *Mol. Endocrinol. 14* (10), 1649–1660.

(28) Thomas, P., Pang, Y., Filardo, E. J., and Dong, J. (2005) Identity of an estrogen membrane receptor coupled to a G protein in human breast cancer cells. *Endocrinology 146* (2), 624–632.

(29) Kuo, J., Hamid, N., Bondar, G., Prossnitz, E. R., and Micevych, P. (2010) Membrane estrogen receptors stimulate intracellular calcium release and progesterone synthesis in hypothalamic astrocytes. *J. Neurosci. 30* (39), 12950–12957.

(30) Kisanga, E. R., Gjerde, J., Guerrieri-Gonzaga, A., Pigatto, F., Pesci-Feltri, A., Robertson, C., Serrano, D., Pelosi, G., Decensi, A., and Lien, E. A. (2004) Tamoxifen and metabolite concentrations in serum and breast cancer tissue during three dose regimens in a randomized preoperative trial. *Clin. Cancer Res. 10*, 2336–2343.

(31) Suzuki, K., Ikegaya, Y., Matsuura, S., Kanai, Y., Endou, H., and Matsuki, N. (2001) Transient upregulation of the glial L-glutamate transporter GLAST in response to fibroblast growth factor, insulin-like growth factor and epidermal growth factor in cultured astrocytes. *J. Cell Sci. 114*, 3717–3725.

(32) Abe, K., Abe, Y., and Saito, H. (2000) Evaluation of L-glutamate clearance capacity of cultured rat cortical astrocytes. *Biol. Pharm. Bull. 23*, 204–207.

Cell Mol Neurobiol
DOI 10.1007/s10571-012-9829-z

SHORT COMMUNICATION

Cell-Autonomous Enhancement of Glutamate-Uptake by Female Astrocytes

Yosuke Morizawa · Kaoru Sato · Junpei Takaki · Asami Kawasaki · Keisuke Shibata · Takeshi Suzuki · Shigeru Ohta · Schuichi Koizumi

Received: 27 February 2012 / Accepted: 8 March 2012

Abstract Since gonadal female hormones act on and protect neurons, it is well known that the female brain is less vulnerable to stroke or other brain insults than the male brain. Although glial functions have been shown to affect the vulnerability of the brain, little is known if such a sex difference exists in glia, much less the mechanism that might cause gender-dependent differences in glial functions. In this study, we show that in vitro astrocytes obtained from either female or male pups show a gonadal hormone-independent phenotype that could explain the gender-dependent vulnerability of the brain. Female spinal astrocytes cleared more glutamate by GLAST than male ones. In addition, motoneurons seeded on female spinal astrocytes were less vulnerable to glutamate than those seeded on male ones. It is suggested that female astrocytes uptake more glutamate and reveal a stronger neuroprotective effect against glutamate than male ones. It should be noted that such an effect was independent of gonadal female hormones, suggesting that astrocytes have cell-autonomous regulatory mechanisms by which they transform themselves into appropriate phenotypes.

Keywords Astrocytes · Sex difference · GLAST · Glutamate · Neurotoxicity

Introduction

Astrocytes control synaptic transmission by releasing gliotransmitters such as ATP and glutamate or by uptaking excess neurotransmitters (Haydon 2001; Koizumi et al. 2003). As for the clearance of neurotransmitters, astrocytes express excitatory amino acid transporter 1 (EAAT1; GLAST) or EAAT2 (GLT-1), by which they control the extracellular glutamate concentrations and excitatory neurotransmission. Thus, the functions of these transporters are highly involved in glutamate-dependent excitotoxicity or various neuronal diseases.

It is well known clinically and experimentally that female brain is more resistant to various brain insults or neurodegenerative diseases than male brain, and such sex differences have been historically attributed to the protective effect of gonadal female hormones such as estrogen. Studies by Sato et al. (2003) and Pawlak et al. (2005) have already shown that, exogenously applied 17β-estradiol (E2), the most potent mammalian estrogen, affects the activity of glutamate uptake in astrocytes, which may in part explain the gender difference in brain vulnerability. However, sexual dimorphism generally persists well beyond menopause (Sacco et al. 1998), suggesting that sex differences in brain injury may not be entirely related to the

Y. Morizawa · K. Shibata · S. Koizumi (✉)
Department of Neuropharmacology, Graduate School of Medicine and Engineering, University of Yamanashi, Yamanashi, Japan
e-mail: skoizumi@yamanashi.ac.jp

Y. Morizawa · S. Ohta
Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

K. Sato · J. Takaki
Division of Pharmacology, National Institute of Health Sciences, Tokyo, Japan

J. Takaki · T. Suzuki
Division of Basic Biological Sciences, Keio University, Tokyo, Japan

A. Kawasaki
Center for Trans-Disciplinary Research, Niigata University, Niigata, Japan

Published online: 27 March 2012

influence of gonadal female hormones. In this study, we demonstrate that female astrocytes in vitro are a high glutamate-uptake phenotype that removes more glutamate and protects motoneurons more against glutamate than male ones. We also demonstrate that such a difference in astrocytic function does not depend on gonadal female hormones but depends rather on a local cell-autonomous mechanism (s).

Materials and Methods

All of the animals used in this study were obtained, housed, cared for, and used in accordance with the guidelines of the Universities of Yamanashi and Hiroshima.

Cell Culture

The culture of spinal astrocytes was prepared as described previously (Shibata et al. 2011) with minor modifications. The spinal cord was removed from neonatal male or female Wistar rats. Male and female rat pups were distinguished by the larger genital papilla and longer ano-genital distance in male versus female pups. To remove serum-derived hormones, charcoal-stripped fetal bovine serum was used. The culture of rat motoneurons was prepared as described previously (Nishijima et al. 2001).

Measurement of Glutamate Uptake

Glutamate clearance was measured as previously described (Sato et al. 2003).

Ca^{2+} Imaging in Single Motor Neurons

Changes in the intracellular Ca^{2+} concentration ($[Ca^{2+}]i$) were measured by the fura-2 method as previously described (Koizumi et al. 2003). The amplitude of the high K^{+}-evoked $[Ca^{2+}]i$ elevation in motoneurons seeded on either male or female astrocytes was used as an index of neuronal function (Koizumi et al. 1994).

Chemicals

DL-*threo*-β-Benzyloxyaspartic acid (TBOA) and Dihydrokainate (DHK) were purchased from TOCRIS Bioscience (Bristol, UK). Anti-neurofilament H non-phosphorylated (SMI-32) antibody was from COVANCE Japan Co. Ltd (Tokyo, Japan). All other reagents were from Sigma-Aldrich Japan (Tokyo, Japan).

Statistical Analysis

Experimental results are expressed as means ± S.E.M. Statistical analysis was performed using Student's *t* test. One way analyses of variance (ANOVA) followed by Tukey test were applied for multiple comparisons. The differences between means were considered to be significant when the *p* values were less than 5 %.

Results

Female astrocytes (grey columns) cleared significantly larger amounts of glutamate than male ones (open columns) both at 30 and 60 min (Fig. 1). At 30 min, female astrocytes showed two times higher glutamate uptake activity. When extracellular Na^{+} was removed, the glutamate clearance disappeared almost completely (data not shown), indicating that the extracellular glutamate was uptaken by Na^{+}-dependent glutamate transporter(s). To identify the predominant glutamate transporter(s) of cultured astrocytes, we co-applied 0.3 mM TBOA, an inhibitor of both GLAST and GLT-1 (Shimamoto et al. 2004) or 1 mM DHK, a selective inhibitor of GLT-1 (Johnston et al. 1974) with glutamate. TBOA dramatically inhibited the glutamate uptake in both male and female astrocytes, whereas DHK showed only slight inhibition or no effect, suggesting that GLAST was dominant in both cultures.

We then investigated whether such sex-dependent differences in glutamate uptake might affect neuronal damage/death induced by exogenously applied glutamate. Since the glutamate clearance by spinal astrocytes greatly affects the survival of motoneurons (Jimonet et al. 1999),

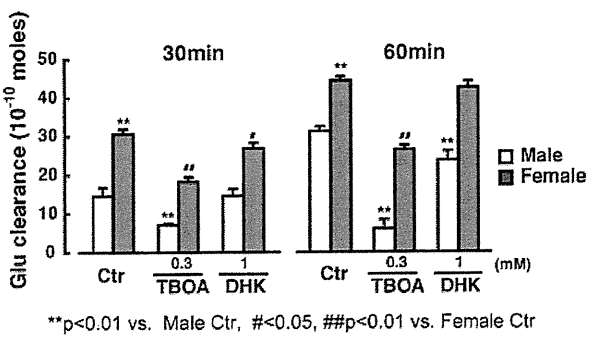


Fig. 1 Differences in glutamate (Glu) clearance in female and male astrocytes. The uptake activity of female (*grey columns*) and male (*open columns*) astrocytes, 30 and 60 min after incubation with glutamate in the absence (Ctr) and the presence (TBOA or DHK) of inhibitors of glutamate transporters. Female astrocytes uptook higher amounts of glutamate than male ones at both time periods (Ctr). DHK had no or only a slight effect on the glutamate uptake, but TBOA significantly decreased the glutamate clearance both in male and female astrocytes

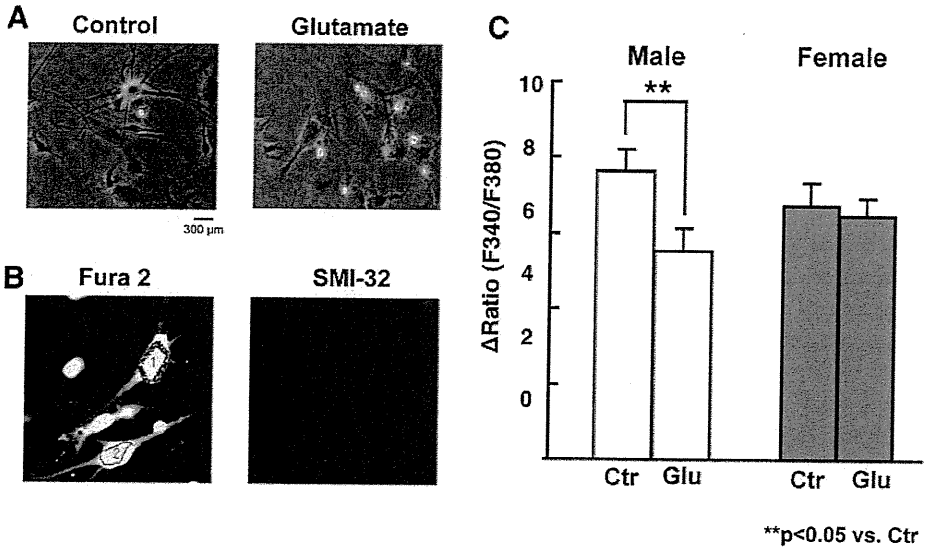


Fig. 2 Potent protection of motoneurons by female astrocytes. **a** Phase-contrast images of motoneurons seeded on male astrocytes, showing the effects of glutamate-treatment. *Left panel*, healthy motoneurons show phase bright morphology; *right panel*, motoneurons damaged by glutamate seeded on male astrocytes, show dark, flattened shape. **b** Fura-2 fluorescent images and immunostaining by SMI-32 antibody. *Left panel*, fura-2 fluorescence; *right panel*, immunocytochemical images of anti-SMI32 antibody of the motoneurons after Ca^{2+} imaging experiments. **c** The high K^+-evoked increase in $[Ca^{2+}]i$ in motoneurons after treatment of cells with glutamate (100 μM, 30 min, and then 24 h washout). Changes in $[Ca^{2+}]i$ in cells were expressed as Δ ratio of F340/F380. The high K^+-evoked increase in $[Ca^{2+}]i$ in motoneurons seeded on male astrocytes was decreased by glutamate but not in those seeded on female astrocytes

we used motoneurons cultured on either male or female spinal astrocytes. Figure 2a shows phase-contrast images of motoneurons, showing the effects of glutamate-treatment. Healthy motoneurons show phase bright morphology (left panel), but when damaged, they show a dark, flattened shape. Motoneurons were stimulated with glutamate (100 μM) for 30 min, and then washed-out and further incubated with glutamate-free medium for 24 h. The fraction of motoneurons with phase bright morphology was dramatically decreased by the glutamate-treatment. For quantitative analysis, we employed a high K^+-evoked increase in $[Ca^{2+}]i$ in neurons (Koizumi et al. 1994). The treatment with glutamate (100 μM, 30 min, and then 24 h washout) significantly decreased the high K^+-evoked responses in motoneurons on male astrocytes, whereas it had almost no effect on the $[Ca^{2+}]i$ responses in motoneurons on female astrocytes (Fig. 2c). After the Ca^{2+} imaging experiments, cells were stained with anti-SMI-32 antibody to confirm that the cells of interest were motoneurons (Fig. 2b, right).

Discussion

In this study, we demonstrated that (1) female astrocytes cleared more glutamate by GLAST than male ones; (2) spinal female astrocytes showed stronger protective action against glutamate-evoked neuronal damage in motoneurons than male ones; and most importantly, (3) these characteristic features of female astrocytes were not necessarily dependent on gonadal female hormones, since astrocytes were obtained separately from either female or male pups and cultured in the absence of sex hormones. Although differences in the vulnerability to several types of brain insults between female and male brains are often explained by the fact that gonadal female hormones act on and protect neurons, it is unlikely that such differences are entirely related to the hormonal effects on neurons. Thus, our present results could be novel and important as that we have shown that such sex differences could be explained by (i) functional differences in astrocytes but not neurons, and that (ii) these differences in astrocytic functions do not totally depend on gonadal sex hormones but, presumably, depend on the property of XX versus XY chromosomes, by which each astrocyte is transformed into a distinct phenotype in a cell-autonomous mechanism, although we must await further studies to clarify the detail molecular mechanisms.

As for the peripheral sex hormone-independent mechanisms, extragonadal production of E2 may be involved. E2 can be synthesized locally from testosterone by the aromatase cytochrome P450 in the CNS. In an experimental stroke model, mice with targeted deletion of *cyp*19, which codes for aromatase P450, showed more severe brain injury

than wild-type litter mates (McCullough et al. 2003), suggesting that aromatase and extragonadal E2 play an important role in protection of the brain. It should be noted that astrocytes express aromatase P450, and more importantly, that the expression of P450 is higher in female astrocytes than that in male astrocytes (Liu et al. 2007). These findings suggest that female astrocytes locally produce more estrogen than male, thereby leading to higher expression of glutamate-transporters. The higher capacity to clear glutamate causes the female astrocytes to have higher neuroprotection against glutamate. However, further study is required to clarify this issue.

Spinal astrocytes in vivo express more GLT-1 than GLAST. It is well known that, similar to cultured astrocytes obtained from the hippocampus or cortex, GLT-1 expression becomes less dominant by cultivation. If cultured with neurons, or in the presence of several factors such as cAMP-forming reagents or β-lactam antibiotics, GLT-1 expression is increased (Rothstein et al. 2005). When spinal astrocytes were co-cultured with motoneurons, it is possible that GLT-1 was upregulated contributing to the clearance of glutamate in female astrocytes. Thus, we do not exclude the involvement of GLT-1 in the higher uptake of glutamate in female astrocytes.

Taken together, we demonstrated that spinal astrocytes obtained from female pups showed higher glutamate uptake activity and more intensive neuroprotection against glutamate than those obtained from males. The effect was independent of gonadal female hormones, suggesting that astrocytes have cell-autonomous regulatory mechanisms by which they transform themselves into less vulnerable phenotypes.

Acknowledgments This study was partly supported by a Grand-in Aid for Scientific Research [(to SK)(A) 21240034], Grants-in-Aid for challenging Exploratory Research [(to SK) 23659810], by a Grant-in-Aid [(to SK and KS) 1003] from Food Safety Commission Japan, and by Diichi-Sankyo Foundation of Life Science.

References

Haydon PG (2001) GLIA: listening and talking to the synapse. Nat Rev Neurosci 2:185–193

Jimonet P, Audiau F, Barreau M, Blanchard JC, Boireau A, Bour Y, Coleno MA, Doble A, Doerflinger G, Huu CD, Donat MH, Duchesne JM, Ganil P, Gueremy C, Honor E, Just B, Kerphirique R, Gontier S, Hubert P, Laduron PM, Le Blevec J, Meunier M, Miquet JM, Nemecek C, Mignani S et al (1999) Riluzole series. Synthesis and in vivo "antiglutamate" activity of 6-substituted-2-benzothiazolamines and 3-substituted-2-imino-benzothiazolines. J Med Chem 42:2828–2843

Johnston GA, Curtis DR, Davies J, McCulloch RM (1974) Spinal interneurone excitation by conformationally restricted analogues of L-glutamic acid. Nature 248:804–805

Koizumi S, Kataoka Y, Niwa M, Yamashita K, Taniyama K, Kudo Y (1994) Endothelin increased [$Ca2^{+}$]i in cultured neurones and slices of rat hippocampus. Neuroreport 5:1077–1080

Koizumi S, Fujishita K, Tsuda M, Shigemoto-Mogami Y, Inoue K (2003) Dynamic inhibition of excitatory synaptic transmission by astrocyte-derived ATP in hippocampal cultures. Proc Natl Acad Sci USA 100:11023–11028

Liu M, Hurn PD, Roselli CE, Alkayed NJ (2007) Role of P450 aromatase in sex-specific astrocytic cell death. J Cereb Blood Flow Metab 27:135–141

McCullough LD, Blizzard K, Simpson ER, Oz OK, Hurn PD (2003) Aromatase cytochrome P450 and extragonadal estrogen play a role in ischemic neuroprotection. J Neurosci 23:8701–8705

Nishijima C, Kimoto K, Arakawa Y (2001) Survival activity of troglitazone in rat motoneurones. J Neurochem 76:383–390

Pawlak J, Brito V, Kuppers E, Beyer C (2005) Regulation of glutamate transporter GLAST and GLT-1 expression in astrocytes by estrogen. Brain Res Mol Brain Res 138:1–7

Rothstein JD, Patel S, Regan MR, Haenggeli C, Huang YH, Bergles DE, Jin L, Dykes Hoberg M, Vidensky S, Chung DS, Toan SV, Bruijn LI, Su ZZ, Gupta P, Fisher PB (2005) Beta-lactam antibiotics offer neuroprotection by increasing glutamate transporter expression. Nature 433:73–77

Sacco RL, Boden-Albala B, Gan R, Chen X, Kargman DE, Shea S, Paik MC, Hauser WA (1998) Stroke incidence among white, black, and hispanic residents of an urban community: the northern manhattan stroke study. Am J Epidemiol 147:259–268

Sato K, Matsuki N, Ohno Y, Nakazawa K (2003) Estrogens inhibit l-glutamate uptake activity of astrocytes via membrane estrogen receptor alpha. J Neurochem 86:1498–1505

Shibata K, Sugawara T, Fujishita K, Shinozaki Y, Matsukawa T, Suzuki T, Koizumi S (2011) The astrocyte-targeted therapy by Bushi for the neuropathic pain in mice. PLoS ONE 6:e23510

Shimamoto K, Sakai R, Takaoka K, Yumoto N, Nakajima T, Amara SG, Shigeri Y (2004) Characterization of novel L-threo-beta-benzyloxyaspartate derivatives, potent blockers of the glutamate transporters. Mol Pharmacol 65:1008–1015