るものであり，それらを使用していることが強く示唆された．また，それら製品のほとんどは複数種の植物の混合という製品形態であった．さらに，赤，青，黄色などの花弁を混入させる製品も多数見られた．大麻，ケシなどの国内で規制されている植物は検出されなかった．一方で，外部から分析依頼を受けた植物種は，現在，規制を受けないが幻覚性成分を有する植物種であり今後の市場への出現などが懸念された．これら製品から法規制植物を検出する簡易分析法としてLAMPを用いたスクリーニング法の検討を行い，目視による全工程3時間ほどでPCR装置，検出器を使用しない手法を確立した．

平成25年3月に合成カンナビノイドに，平成26年1月にカチノン系化合物に対し，包括規制が施行された．合成カンナビノイド包括規制後，包括範囲化合物は危険ドラッグ市場から速やかに消え，インドールの替わりにインダゾール，カルボニルの替わりにカルボキサミドもしくはカルボン酸エステル等，様々な構造を有する合成カンナビノイド類が次々と市場に出現した．特に，平成26年後半に出現した，インドールもしくはインダゾールの3位に2-アセトアミド-3-メチルブタン酸メチルエステル構造もしくは2-アセトアミド-3,3-ジメチルブタン酸メチルエステル構造を有する合成カンナビノイド類（例えば5F-AMBと5F-ADB，MDMB-CHMICAとMDMB-CHMINACA等）はカンナビノイド$CB_1$受容体に対する結合性が極めて強く，摂取により健康被害が憂慮される化合物群となっている．カチノン系化合物についても，ベンゼン環上にハロゲンやメチル基，メトキシ基等が導入された構造，フェニルプロパン骨格のアルキル鎖がC3からC9まで延長した構造，*N*-アルキル鎖が延長した構造，ピロリジニル基が導入された構造等，様々に修飾された化合物が次々と出現している．特に，ピロリジニル基が導入された化合物は，モノアミントランスポーターに結合し，モノアミンの取り込みを阻害する作用が強く，世界的にも被害事例報告が多い．なお，合成カンナビノイド類やカチノン系化合物の他にも，平成24年度以降，フェネチルアミン系幻覚薬（セロトニン受容体アゴニスト）であるNBOMe，NBFシリーズの化合物，オピオイド受容体アゴニストであるAH-7921，MT-45及びacetylfentanyl，NMDA受容体アンタゴニストであるdiphenidine等の危険ドラッグが次々と出現している．これらはいずれも，少量で極めて強い薬理作用を示す化合物である．

危険ドラッグにおいては，医薬品とは異なり，製品中に実際にどのような化合物が含まれているか不明であることが多く，同じ名称，同じ包装形態の製品でも，含有化合物の種類や量が異なる場合が少なくない．従って，使用者が過去に使用した製品と同名製品を使用しても，同等の作用がでるとは限らない．また，製品によっては複数の化合物を含有しており，鎮静，興奮，幻覚など，薬理作用が異なる複数の薬物が混在している場合も多い．さらに，近年流通する合成カンナビノイドは，標的部位であるカンナビノイド$CB_1$受容体に対し，大麻の活性成分$\Delta^9$-THCよりも強い親和性を有することが多い．一方，規制を逃れるために化学構造を一部修飾しているうちに，予想外の薬理活性を有する化合物が登場する可能性も考えられる．また，合成原料，合成副生成物や反応生成物（複数の含有化合物が製品調製中に反応して生成した化合物など），分解物などが製品から検出される場合もあるが，それら「不純物」の薬理作用はまったく予測できない．さらに，流通化合物の移り変わりが早いため，危険ドラッグによる健康被害に対し，病院側で原因化合物を特定することが困難であり，治療法も存在していない．

次から次へと新規流通化合物が出現する危険ドラッグにおいては，国内外の研究機関間における情報の共有化が重要となる．国立医薬品食品衛生研究所では，平成26年4月に「違法ドラッグデータ閲覧システム」を公開した（http://npsdb.nihs.go.jp/Search/）．本システムでは，指定薬物やその構造類似体，また今後流通が予想される危険ドラッグ成分について，化合物名（一般名，通称，IUPAC名），CAS番号，分子式，分子量，

モノアイソトピック質量, GC-EI-MS の代表的なフラグメントイオン, LC-ESI-MS のプロトン付加分子イオン及び代表的なフラグメントイオン等から化合物情報が検索可能である. また, これら化合物を含有する危険ドラッグ製品情報も検索可能となっている. 本研究班の研究結果は, この違法ドラッグデータ閲覧システムに反映されている. 各分析データは, 国連薬物犯罪事務所(United Nations Office on Drugs and Crime, UNDOC)などの国際機関にも提供している. 平成 26 年 12 月末時点で, 633 化合物, 1980 製品の情報が収載されており, 国内 260 機関(部署), 海外 17 機関(部署)が登録をしている.

上記の研究結果の一部は, 平成 24 年度から 26 年度に開催された 18 回に及ぶ薬事・食品衛生審議会の指定薬物部会において(平成 24 年 4 月 18 日, 8 月 30 日, 10 月 16 日, 11 月 28 日, 平成 25 年 2 月 15 日, 5 月 14 日, 8 月 19 日, 9 月 17 日, 12 月 17 日, 平成 26 年 3 月 25 日, 7 月 31 日, 9 月 16 日, 10 月 23 日, 11 月 17 日, 12 月 25 日, 平成 27 年 1 月 29 日, 2 月 17 日及び 3 月 24 日), また 7 月 25 日緊急施行された特例指定において, 問題となる薬物を指定薬物に指定するための判断根拠となる科学的データとして提示された. さらに, その審議結果をうけ, 薬事法第 2 条第 14 項に規定する指定薬物及び同法第 76 条の 4 に規定する医療等の用途を定める省令の一部を改正する省令により(平成 25 年厚生労働省令第 128 号/平成 25 年 12 月 13 日公布/2-アミノ-1-フェニル-プロパン-1-オン(通称カチノン)を基本骨格とする物質群 495 物質(新規 474 物質)を含む), 合計 679 化合物が, 新たに指定薬物として規制された. また, 上記新規指定薬物について, 本研究において検討した分析データを参考とし, 標準分析法を国立衛研が取りまとめ, 厚生労働省より全国に通知した(平成 24 年 6 月 28 日厚生労働省監視指導・麻薬対策課長通知薬食監麻発 0628 第 1 号, 11 月 6 日薬食監麻発 1106 第 1 号, 平成 25 年 1 月 7 日薬食監麻発 0107 第 1 号, 5 月 15 日薬食監麻発 0515 第 2 号, 5 月 28 日薬食監麻発 0528 第 1 号, 7 月 26 日薬食監麻発 0726 第 1 号, 11 月 18 日薬食監麻発 1118 第 2 号, 平成 26 年 1 月 27 日薬食監麻発 0127 第 14 号, 3 月 6 日薬食監麻発 0306 第 10 号, 0707 第 1 号, 8 月 25 日薬食監麻発 0825 第 2 号, 10 月 20 日薬食監麻発 1020 第 3 号, 10 月 20 日薬食監麻発 1020 第 4 号, 11 月 27 日薬食監麻発 1127 第 1 号, 11 月 27 日薬食監麻発 1127 第 3 号, 平成 27 年 1 月 6 日薬食監麻発 0106 第 4 号, 2 月 9 日薬食監麻発 0209 第 3 号, 3 月 3 日薬食監麻発 0303 第 7 号「指定薬物の測定結果等について」). さらに, 研究成果の一部については, 平成 25 年 2 月 22 日に, 厚生労働省の依頼により国立衛研究で開催した「平成 24 年度指定薬物分析研修会議」(全国 46 都道府県の地方衛生研究所から 62 名が参加), 平成 25 年月 22 日に開催した「平成 25 年度指定薬物分析研修会議」(全国 44 都道府県の地方衛生研究所から 58 名が参加), 平成 27 年 1 月 16 日に開催した「平成 26 年度指定薬物分析研修会議」(全国 46 都道府県の地方衛生研究所から 60 名が参加)において説明を行った. 厚生労働省を通じて, 地方衛生研究所及び科学捜査研究所等各検査機関へ分析用標品を交付した. その他, 厚生労働省を通して正式な依頼を受け, 地方衛生研究所等の公的分析機関から送付された違法ドラッグ製品について含有成分分析を実施した.

また, 研究代表者は, シンポジウムもしくは特別講演において, 本研究成果を 国内 14 回, 国際 5 回, 講演した. 特に, 海外においては, 平成 25 年には, 国連 UNODC が主催する専門家会議で本研究成果に基づいた日本における危険ドラッグの流通実態を講演した. さらに, 平成 24 年には, 米国 NIDA (National Institute on Drug Abuse), 平成 25 年には EMCDDA (European Monitoring Centre for Drugs and Drug Addiction), 平成 26 年にはイタリア政府が主催する国際フォーラム等に招待され, 本研究成果を発表した. 平成 25 年度には日本薬学会第 134 年会シンポジウム「“脱法ドラッグ(脱法ハーブ)”による

健康被害を防ぐために」を企画し，分担研究者とともに研究成果を講演した．なお，平成 26 年度には，分担研究者が，本研究成果で，日本法中毒学会から吉村賞（学術奨励賞）を授与された．さらに，研究協力者が，第 37 回日本神経科学大会において学会大会前日の一般向けプレスリリースに選ばれた．

以上，本研究結果は，本研究成果で，厚生労働省の監視指導行政に直接貢献する研究であり，国の違法ドラック対策に即したものと考えられる．

E. 健康危機情報

1) MAM-2201 は，平成 24 年度に最も流通した違法ドラッグ成分のひとつであり，交通事故等を含む他害事故にも数多く係わっている可能性が指摘されている．今回の *in vitro* 評価において，カンナビノイド $CB_1$ 受容体に対する親和性が極めて強いことが示され，本化合物による健康被害が憂慮された（平成 25 年 5 月 26 日より麻薬として規制）．また，日本においては今のところ流通が認められていないが，欧州において死亡事例を含む健康被害が報告されている 5-IT については，極めて強いモノアミン再取り込み阻害作用（特にドパミン及びノルエピネフリン）が認められ，本化合物についても日本での流通が認められた場合，健康被害が憂慮された（平成 25 年 1 月 16 日より指定薬物として規制）．

2) 5F-QUPIC は，平成 24 年度後半から平成 25 年度全般に最も流通した違法ドラッグ成分であり，死亡事例や交通事故等を含む他害事故にも数多く係わっている可能性が指摘されている．今回の in vitro 評価において，カンナビノイド $CB_1$ 受容体に対する親和性が極めて強いことが示され，本化合物による健康被害が憂慮された（平成 26 年 8 月 1 日より麻薬として規制）．また，平成 25 年度後半に最も流通が認められた FUB-PB-22 についても極めて強い $CB_1$ 受容体親和性が認められ，健康被害が憂慮された（平成 26 年 1 月 11 日より指定薬物として規制）．

3) 平成 26 年度になり流通が広がった 5F-ADB（平成 26 年 11 月 8 日に指定薬物として規制）や MDMA-CHIMINACA（平成 27 年 1 月 15 日に指定薬物として規制）などに代表される idole/indazole-carboxamide-methyl 3-methylbutanoate/methyl 3,3-dimethylbutanoate もしくは 3-methylbutanamide/3,3-dimethyl butanamide 構造，5F-QUPIC などに代表される indole/indazol-carboxylate ester-naphthyl/ quinolinyl 構造を有する化合物群は，極めてカンナビノイド $CB_1$ 受容体に対する結合親和性が高い．また，5F-ADB や MDMA-CHIMINACA においては，薬物投与マウスの死亡が確認され，これら化合物群のヒトへの健康被害が懸念された．

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G. 知的所有権の取得状況

1. 特許取得

特になし

2. 実用新案登録

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3. その他

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研究成果の刊行に関する一覧表

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# L-glutamate released from activated microglia downregulates astrocytic L-glutamate transporter expression in neuroinflammation: the 'collusion' hypothesis for increased extracellular L-glutamate concentration in neuroinflammation

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## Abstract

**Background:** In the central nervous system, astrocytic L-glutamate (L-Glu) transporters maintain extracellular L-Glu below neurotoxic levels, but their function is impaired with neuroinflammation. Microglia become activated with inflammation; however, the correlation between activated microglia and the impairment of L-Glu transporters is unknown.

**Methods:** We used a mixed culture composed of astrocytes, microglia, and neurons. To quantify L-Glu transporter function, we measured the extracellular L-Glu that remained 30 min after an application of L-Glu to the medium (the starting concentration was 100 μM). We determined the optimal conditions of lipopolysaccharide (LPS) treatment to establish an inflammation model without cell death. We examined the predominant subtypes of L-Glu transporters and the changes in the expression levels of these transporters in this inflammation model. We then investigated the role of activated microglia in the changes in L-Glu transporter expression and the underlying mechanisms in this inflammation model.

**Results:** Because LPS (10 ng/mL, 72 h) caused a significant increase in the levels of L-Glu remaining but did not affect cell viability, we adopted this condition for our inflammation model without cell death. GLAST was the predominant L-Glu transporter subtype, and its expression decreased in this inflammation model. As a result of their release of L-Glu, activated microglia were shown to be essential for the significant decrease in L-Glu uptake. The serial application of L-Glu caused a significant decrease in L-Glu uptake and GLAST expression in the astrocyte culture. The hemichannel inhibitor carbenoxolone (CBX) inhibited L-Glu release from activated microglia and ameliorated the decrease in GLAST expression in the inflammation model. In addition, the elevation of the astrocytic intracellular L-Glu itself caused the downregulation of GLAST.

**Conclusions:** Our findings suggest that activated microglia trigger the elevation of extracellular L-Glu through their own release of L-Glu, and astrocyte L-Glu transporters are downregulated as a result of the elevation of astrocytic intracellular L-Glu levels, causing a further increase of extracellular L-Glu. Our data suggest the new hypothesis that activated microglia collude with astrocytes to cause the elevation of extracellular L-Glu in the early stages of neuroinflammation.

**Keywords:** L-glutamate, Microglia, Transporter, Astrocytes, Inflammation, Hemichannel

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## Background

L-glutamate (L-Glu) is one of the most important excitatory neurotransmitters in the mammalian central nervous system (CNS). However, high concentrations of L-Glu cause excessive stimulation of L-Glu receptors and lead to neurotoxicity [1,2]. In astrocytes, GLAST (EAAT1 in humans) and GLT-1 (EAAT2) are the major functional L-Glu transporters in the CNS, and they play an important role in maintaining extracellular L-Glu concentrations below neurotoxic levels [3]. Impairments of L-Glu transporter function have been reported in numerous neurological diseases associated with inflammation, for example, Alzheimer's disease [4], amyotrophic lateral sclerosis [5], major depressive disorder [6,7], and epilepsy [8-10]. Furthermore, elevated extracellular L-Glu content has been reported in *in vivo* and *in vitro* inflammation models [11,12]. Accordingly, the impairment of L-Glu transporters has been suggested to contribute to elevated extracellular L-Glu concentrations in inflammation; however, the specific role of such transporters remains unknown, as some inflammation models also cause cell death.

The CNS is composed of neurons and the following three types of glial cells: astrocytes, microglia, and oligodendrocytes [13]. Microglia are the primary cells that are activated in response to inflammatory stimulation [14,15] and are the resident innate immune cells in the CNS. Once activated, microglia exhibit a phenotypic switch from a resting ramified type to a motile amoeboid type [16,17] and release various soluble factors, including pro-inflammatory cytokines [18,19], reactive oxygen species [20], nitric oxide (NO) [16], L-Glu [21,22], and ATP [23,24]. Although the direct application of some of these factors has been reported to inhibit L-Glu transporters [25-28], few studies have examined the interaction between activated microglia and astrocyte L-Glu transporters in inflammation.

In this study, we aimed to clarify the interaction between activated microglia and astrocyte L-Glu transporters in inflammation. To quantify L-Glu transporter function, we measured the extracellular concentrations of L-Glu (that is, the concentration of L-Glu remaining) after a single exogenous application of L-Glu to the medium. To ensure that we measured the effects on live cells (and not L-Glu released from dying cells), we identified a condition of lipopolysaccharide (LPS) application that was suitable to induce inflammation without cell death. In this model, we found that activated microglia released L-Glu, the resultant elevation in extracellular L-Glu led to the elevation of intracellular L-Glu content in astrocytes through L-Glu transporters, and the increased level of intracellular L-Glu in astrocytes decreased GLAST expression. These reactions caused a further elevation of the extracellular concentration of L-Glu. Our data suggest a new hypothesis in which activated microglia collude with astrocytes to cause the elevation of extracellular L-Glu in the early stages of neuroinflammation.

## Methods

All procedures using live animals in this study were conducted in accordance with the guidelines of the National Institute of Health Sciences (NIHS), Japan, as developed under the Guide for the Care and Use of Laboratory Animals by the National Research Council. Also all experiments were approved by the ethics committee of the NIHS.

### Materials

L-Glu, LPS, CBX, anti-rabbit Iba-1 polyclonal antibody (019–19741), and paraformaldehyde (PFA) were purchased from Wako (Osaka, Japan). Dihydrokainic acid (DHK), adenosine 5′-triphosphate disodium salt hydrate (ATP), 2′(3′)-O-(4-benzoylbenzoyl)ATP triethylammonium salt (BzATP), 2′,3′-O-(2,4,6-trinitrophenyl)ATP salt hydrate (TNP-ATP), adenosine 5′-triphosphate, periodate oxidized sodium salt (OxATP), poly-L-lysine hydrobromide, polyethylenimine, β-nicotinamide adenine dinucleotide (β-NAD), 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 1-methoxy-5-methyl-phenazinium methyl sulfate (MPMS), Triton-X100, lactate lithium salt, anti-mouse β-actin monoclonal antibody (A5316), sodium deoxycholate, 2-mercaptoethanol, bromophenol blue sodium salt (BPB), and bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO, USA). DL-threo-β-benzyloxyaspartic acid (TBOA) was purchased from TOCRIS (Ellisville, MO, USA). An MTT Cell proliferation assay kit was purchased from Life Technologies (Grand Island, NY, USA). Rat glutamate transporter (GLAST/EAAT1) control peptide (GLAST11-P) and rat glutamate transporter (GLT1/EAAT2) control peptide (GLT11-P) were purchased from Alpha Diagnostic (San Antonio, TX, USA). Clodronate disodium salt and polyoxyethylene (9) octylphenyl ether (NP-40) were purchased from Calbiochem (Darmstadt, Germany). Dulbecco's modified eagle medium (DMEM), fetal bovine serum (FBS), and horse serum (HS) were purchased from GIBCO (Grand Island, NY, USA). Bovine liver glutamate dehydrogenase (GlDH) was purchased from Roche (Mannheim, Germany). RNeasy Mini Kits and an RNase-Free DNase set were purchased from Qiagen (Hilden, Germany). TaqMan one-step RT-PCR master mix reagents and TaqMan ribosomal RNA control reagents (VIC Prove) were purchased from Applied Biosystems (Foster City, CA, USA). 2-Amino-5,6,7,8-tetrahydro-4-(4-methoxyphenyl)-7-(naphthalen-1-yl)-5-oxo-4H-chromene-3-carbonitrile (UCPH-101), rabbit anti-GLAST polyclonal antibody (ab65978), and anti-chicken glial fibrillary acidic protein (GFAP) polyclonal antibody (ab4674)

were purchased from Abcam (Cambridge, UK). Goat anti-EAAT2 (GLT1) antibody (sc-7760) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-mouse Tuj1 (β3 tubulin) antibody (MAB5564) was purchased from Chemicon (Temecula, CA, USA). Donkey anti-rabbit IgG conjugated with horseradish peroxidase was purchased from Amersham Biosciences (Washington, DC, USA). The SuperSignal West Femto Trial Kit was purchased from Thermo Scientific (Rockford, IL, USA). The BCA protein assay kit was purchased from Pierce Chemical (Rockford, IL, USA). Tris (hydroxymethyl) aminomethane (Tris–HCl) was purchased from Bio-Rad (Hercules, CA, USA). Ethylenediaminetetraacetate (EDTA) and ethyleneglycoldiaminetetraacetate (EGTA) were purchased from Dojindo (Kumamoto, Japan). Goat serum, anti-mouse IgG-conjugated Alexa Fluor 488, anti-chicken IgG-conjugated Alexa Fluor 594, and anti-rabbit IgG-conjugated Alexa Fluor 647 were purchased from Vector Laboratories (Burlingame, CA, USA).

### Cell culture

#### *Astrocyte-microglia-neuron mixed culture*

The brains of 2-day-old Sprague–Dawley (SD) rats were aseptically removed, and the cerebral cortices were dissected. The tissues were dissociated by trituration and trypsinization. After centrifugation at 1,500 rpm for 5 min, the cells were suspended in DMEM supplemented with 10% FBS and 1% antibiotic-antimitotic agent, and the residual tissue aggregates were removed by filtration through a cell strainer with a pore size of 40 to 45 μm. The cells were seeded onto appropriately sized poly-L-lysine-coated plastic dishes or polyethyleneimine-coated cover glass, and grown for 8 days at 37°C in a humidified atmosphere containing 5% $CO_2$. The medium was changed every 2 days.

#### *Astrocyte culture*

The primary culture of rat astrocytes was prepared according to a method previously described [29,30]. The cortical cells were obtained by the methods described above and were seeded in uncoated 75-$cm^2$ flasks at a density of $5 \times 10^4$ cells/$cm^2$. The medium was changed 24 h after plating and then every 3 to 4 days. When the cells became confluent (10 to 14 days *in vitro* (DIV)), the non-astrocyte cells were detached from the flasks by shaking and removed by changing the medium. The remaining cells were dissociated by trypsinization and seeded onto appropriately sized poly-L-lysine-coated plastic dishes or polyethyleneimine-coated cover glass at a density of $3 \times 10^5$ cells/$cm^2$. The cells became confluent again 2 to 3 days after plating.

#### *Microglia culture*

The primary culture of the rat microglia was prepared according to a method previously described [31]. In brief, the cells were obtained from the cerebral cortices of 1-day-old SD rats and seeded in poly-L-lysine-coated 75-$cm^2$ flasks at a density of $2.5 \times 10^5$ cells/$cm^2$. The cells were then grown in DMEM containing 10% FBS, 10 U/mL penicillin, and 10 mg/mL streptomycin for 10 to 14 days at 37°C. The medium was changed every 2 to 3 days. The microglia were removed from the flask bottoms by gentle shaking (60 rpm, 2 min) and collected by centrifugation at 1,000 × *g* for 5 min. The microglia were resuspended and seeded at a density of $6 \times 10^4$ cells/$cm^2$ onto appropriately sized poly-L-lysine-coated plastic dishes or polyethyleneimine-coated cover glass.

#### *Astrocyte-microglia co-culture*

The microglia prepared by the methods described above were seeded at a density of $6 \times 10^4$ cells/$cm^2$ onto confluent astrocytes and cultured for 1 to 2 days.

#### *Astrocyte-neuron co-culture*

The astrocyte-microglia-neuron co-culture was treated with clodronate [32] at a concentration of 10 μg/mL for 4 days from 5 DIV to reduce the density of microglia to below $1.2 \times 10^3$ cells/$cm^2$.

### Drug treatment

Stock solutions of 100 mM L-Glu, 10 μg/mL LPS, 100 μg/mL clodronate, 10 mM TBOA (non-selective L-Glu transporter inhibitor, IC50: 48 μM for GLAST, 7 μM for GLT-1), 10 mM UCPH-101 (a GLAST specific inhibitor, IC50: 0.66 μm for GLAST, >400-fold selectivity over EAAT2 and EAAT3), 10 mM DHK (a GLT-1-specific inhibitor, IC50: >3,000 μM for GLAST, 23 μM for GLT-1), 100 mM ATP, 10 mM BzATP, 10 mM, and 100 mM CBX in phosphate-buffered saline (PBS) were dissolved into the culture medium at the time of application. At 8 DIV, the astrocyte-microglia-neuron culture was treated with LPS at concentrations of 1 to 100 ng/mL for 6 to 72 h. TBOA, UCPH-101, or DHK was applied to the astrocyte-microglia-neuron co-culture for 24 h at 10 DIV. ATP or BzATP was applied to the astrocyte culture at concentrations of 100 to 3,000 μM or 10 to 300 μM, respectively, for 72 h. At 8 DIV, CBX was applied to the astrocyte-microglia-neuron culture at concentrations of 10 to 100 μM from 1 h before to the end of the LPS treatment.

### The measurement of the extracellular L-Glu concentration in the medium

The measurement of L-Glu concentration in the medium was performed according to a previously described method [33]. The culture medium in the 96-well plates was replaced with fresh medium containing 100 μM L-Glu. After 30 min, 50 μL of the culture medium in each well was collected. The L-Glu concentration was

measured by mixing the medium with 50 μL of substrate mixture (20 U/mL GlDH, 2.5 mg/mL β-NAD, 0.25 mg/mL MTT, 100 μM MPMS, and 0.1% (vol/vol) Triton X-100 in 0.2 M Tris–HCl buffer (pH 8.2)) and incubating the mixture at 37°C for 30 min. The reaction was stopped by adding 100 μL of stop solution (50% (vol/vol) dimethylformamide and 20% (wt/vol) sodium dodecyl sulfate (SDS) in water (pH 4.7)). The amount of the reaction product (MTT formazan) was determined by measuring the absorbance at 570 nm (test wavelength) and 655 nm (reference wavelength) with a microplate reader. The extracellular L-Glu concentration was estimated from a standard curve, which was constructed for each assay using cell-free medium containing known concentrations of L-Glu. When the L-Glu was not applied after washing with fresh medium, no changes in extracellular L-Glu concentrations were observed in the 30 min incubation period in any experiments in this study. To measure the L-Glu released from microglia, the extracellular concentration of L-Glu was measured after 24 h of LPS treatment. The control values were almost same in the same culture batch but variable among different batches (40 to 60 μM). We therefore confirmed the reproducibility of the results in three independent experiments using different culture batches.

### LDH and MTT assays

The lactate dehydrogenase (LDH) activity in the medium was evaluated according to a previously described method [33]. Briefly, 50 μL of culture medium from each well of a 96-well plate was mixed with 50 μL of substrate mixture (2.5 mg/mL lactate lithium salt, 2.5 mg/mL β-NAD, 0.25 mg/mL MTT, 100 μM MPMS, and 0.1% (vol/vol) Triton X-100 in 0.2 M Tris–HCl buffer (pH 8.2)). After a 10 min incubation at 37°C, the reaction was stopped by adding 100 μL of stop solution as described above. The amount of MTT formazan was determined using a microplate reader. The data were normalized to the averaged value of the group treated with 0.1% (vol/vol) Triton-X 100 for 1 h. MTT reductions were evaluated according to the manufacturer's instructions.

### Real-time quantitative polymerase chain reaction (TaqMan RT-PCR)

The total cellular ribonucleic acid (RNA) was extracted from cells with an RNeasy Mini Kit and treated with RNase-free DNase to eliminate genomic deoxyribonucleic acid (DNA) contamination. The amount of total RNA was quantified by measuring the OD260 using a Nanodrop spectrophotometer (Nanodrop, Wilmington, DE). The reactions (25 μL) contained 1 ng of total RNA, 900 nM forward and reverse primers, 250 nM TaqMan probe, and RNase inhibitor mix in the master mix solution. The RT-PCR was performed using the TaqMan One-Step RT-PCR master mix reagent kit according to the manufacturer's protocol. The data were analyzed with 7,900 System SDS Software 2.2.2 (Applied Biosystems, Foster City, CA, USA) using the standard curve method. The GLAST and GLT-1 mRNA expression levels were normalized to the ribosomal RNA control (18S) expression levels. The primer sequences were as follows: 5'-GATCGG CATAATCATTGTCATCA-3', 5'-CGATTTTACCTTCTC TGTACATGTTTC-3' (GLAST), and 5'-CCGAGCTGGA CACCATTGA-3' 5'-AATGGACTGCGTCTTGGTCAT-3' (GLT-1). Specific probes for GLAST (TCCACCCCGG AAAGGGCACG) and GLT-1 (CAACACCGAATGAATG CACGAAGACATCGA) were used.

### Western blotting

The cells were washed twice with PBS and once with lysate buffer (150 mM (wt/vol) NaCl, 10 mM (wt/vol) EDTA, 5 mM (wt/vol) EGTA, 0.5% mM (vol/vol) NP-40, and 0.5% (wt/vol) sodium deoxycholate in 10 mM Tris–HCl buffer (pH 7.4)). The protein concentration was measured using the BCA protein assay. The proteins (20 μg/lane) were mixed with SDS sample buffer (2% (wt/vol) SDS, 10% (vol/vol) glycerol, 0.25% (wt/vol) BPB, 5% (vol/vol) 2-mercaptoethanol in 125 mM Tris–HCl buffer (pH 6.8)), loaded onto a 10% polyacrylamide gel, electrophoresed, and transferred onto a PVDF membrane. The membrane was blocked with 5% (wt/vol) non-fat dry milk in Tris-buffered saline containing 0.1% (vol/vol) Tween 20. The membrane was incubated with rabbit anti-GLAST polyclonal antibody (1:4,000; ab65978, Abcam), mouse anti GLT-1 (1:2,000; sc-7760, Santa Cruz) or anti β-actin monoclonal antibody (1:5,000; A5316, Sigma-Aldrich) overnight at 4°C followed by incubation with the horseradish peroxidase-conjugated anti-rabbit antibody (1:20,000; Amersham Biosciences) or the anti-mouse or anti-goat antibody (1:20,000 Amersham Biosciences). The signals were scanned with an LAS3000 (Fuji Photo Film Co., Ltd., Tokyo, Japan) using an ECL western blot detection system (SuperSignal West Femto Trial Kit). For relative quantification of the expression levels of GLAST and GLT-1, we first compared the densities of the bands of the same amount of GLAST and GLT1 control proteins (full length) (1, 10 μg). In the LPS-exposure experiment, we normalized the band density of each subtype to the density of the 10 μg control band of the corresponding subtype. The bands of GLAST and GLT1 standard proteins were obtained at the same appropriate exposure time. The bands of GLAST and GLT1 in the LPS-exposure experiment were obtained at the same appropriate exposure time.

### Immunocytochemistry

The cells were washed with PBS three times and fixed with 4% PFA for 60 min at room temperature. After

more washes with PBS, the cells were permeabilized and blocked for 60 min with 0.1% (vol/vol) Triton X-100, 5% (vol/vol) goat serum, and 1% (wt/vol) BSA in PBS. After washes with PBS, the cells were incubated with primary antibodies overnight at 4°C. Mouse monoclonal anti-Tuj1 antibody (1:500, MAB5564, Chemicon), chicken polyclonal anti-GFAP antibody (1:400, ab4674, Abcam), and rabbit polyclonal anti-Iba1 antibody (1:1,000, 019–19741, Wako) were used to stain neurons, astrocytes, and microglia, respectively. After washes with PBS, the cells were incubated with secondary antibodies (1:500, Invitrogen) conjugated to fluorochromes for 2 h at room temperature in the dark. After washes with PBS, fluorescent images of the cells were obtained by confocal microscopy (LSM5 Pascal, Zeiss).

### Characterization of microglial releasing factors that downregulate L-Glu transporters

For the conditioned medium study, the astrocyte-microglia-neuron mixed culture was treated with LPS (10 ng/mL, 72 h), and the conditioned medium was transferred to the astrocyte culture. After 72 h, the L-Glu clearance assay was performed on the astrocyte culture. In a separate experiment, the astrocyte culture was incubated for 72 h with a transwell carrying microglia that had been treated with LPS, and the L-Glu clearance assay was performed in the astrocyte culture. For the serial applications of L-Glu, the medium of the astrocyte-microglia-neuron mixed cultures was replaced with fresh medium containing L-Glu (100 μM) every 2 h for 24 h. The L-Glu clearance assay, TaqMan RT-PCR, and western blotting were performed after the serial application of L-Glu.

### The measurement of the astrocytic intracellular L-Glu concentration

The astrocyte-microglia co-culture was treated with LPS (10 ng/mL, 72 h) and washed twice with gentle shaking to remove microglia. After confirmation of the microglial removal under a microscope, 0.1% TritonX-100 was applied and incubated for 1 h. The L-Glu concentration in the supernatant was measured as described above. TBOA was applied from 1 h before the start of LPS treatment.

### Statistical analysis

All data are expressed as the mean ± the S.E.M. Statistical analyses were performed with Student's *t* test or a one-way repeated-measures analysis of variance (ANOVA) followed by Tukey's post-hoc test for multiple pairwise comparisons, as shown in the figure legends. In all of the comparisons, the differences were considered statistically significant when $P < 0.05$. All of the experiments were repeated in triplicate, and the same results were obtained in all of the sessions.

## Results

### L-Glu uptake was decreased during inflammation without cell death through the downregulation of GLAST expression

To definitively investigate the interactions between activated microglia and astrocytes, we used a mixed culture composed of astrocytes, microglia, and neurons. To quantify L-Glu transporter function, we measured the extracellular concentrations of L-Glu (that is, the concentration of L-Glu remaining) 30 min after a single exogenous application of L-Glu to the medium (the starting concentration was 100 μM). In this manner, we first determined the optimal conditions for inflammation without cell death. The cultures were treated with LPS for 72 h. The L-Glu remaining was significantly increased after incubation with 10 and 100 ng/mL LPS (Figure 1A). Significant LDH leakage and decreases in MTT reduction were induced by 100 ng/mL LPS but not by LPS concentrations less than 10 ng/mL (Figure 1B). In pure astrocyte cultures, significant decreases in MTT reduction were induced by 100 ng/mL LPS, but LPS concentrations less than 10 ng/mL did not affect either the LDH leakage or MTT reduction (Figure 1C). Whereas increases in MTT reduction were induced by 1 to 100 ng/mL LPS in pure microglial culture, significant LDH leakage was induced by 100 ng/mL but not by LPS concentrations <10 ng/mL (Figure 1D). When the treatment duration was changed, 10 ng/mL LPS was found to inhibit L-Glu uptake in a time-dependent manner, and a significant decrease was observed at 72 h (Figure 1E). Therefore, we adopted a 72-h treatment with 10 ng/mL LPS for inflammation without cell death. We also confirmed the morphology and cell density of each cell type in this inflammation model. LPS dramatically changed the shape of Iba-1 (+) microglia from a ramified shape to an amoeboid shape, which is consistent with the typical morphological changes observed after activation of microglia in previous reports [16,17] (Figure 1G). No changes were observed in astrocytes or neurons. The cell densities of all cell types did not change (Figure 1F).

To determine which L-Glu transporter subtypes were responsible for the increase in the concentration of L-Glu remaining in the mixed cultures, we first examined the effects of various subtype-specific inhibitors on L-Glu uptake (Figure 2A). The cultures were treated with a non-selective L-Glu transporter inhibitor (TBOA) (10 to 1,000 μM), a GLAST-specific inhibitor (UCPH-101) (0.1 to 10 μM), or a GLT-1-specific inhibitor (DHK) (30 to 300 μM) for 24 h, and the concentration of L-Glu remaining in the medium 30 min after the application of L-Glu (the starting concentration was 100 μM) in the presence of the inhibitors was measured. TBOA and UCPH-101 increased the L-Glu remaining in a concentration-dependent manner to similar extents, whereas DHK did not. These results indicate that GLAST is the predominant functional transporter in

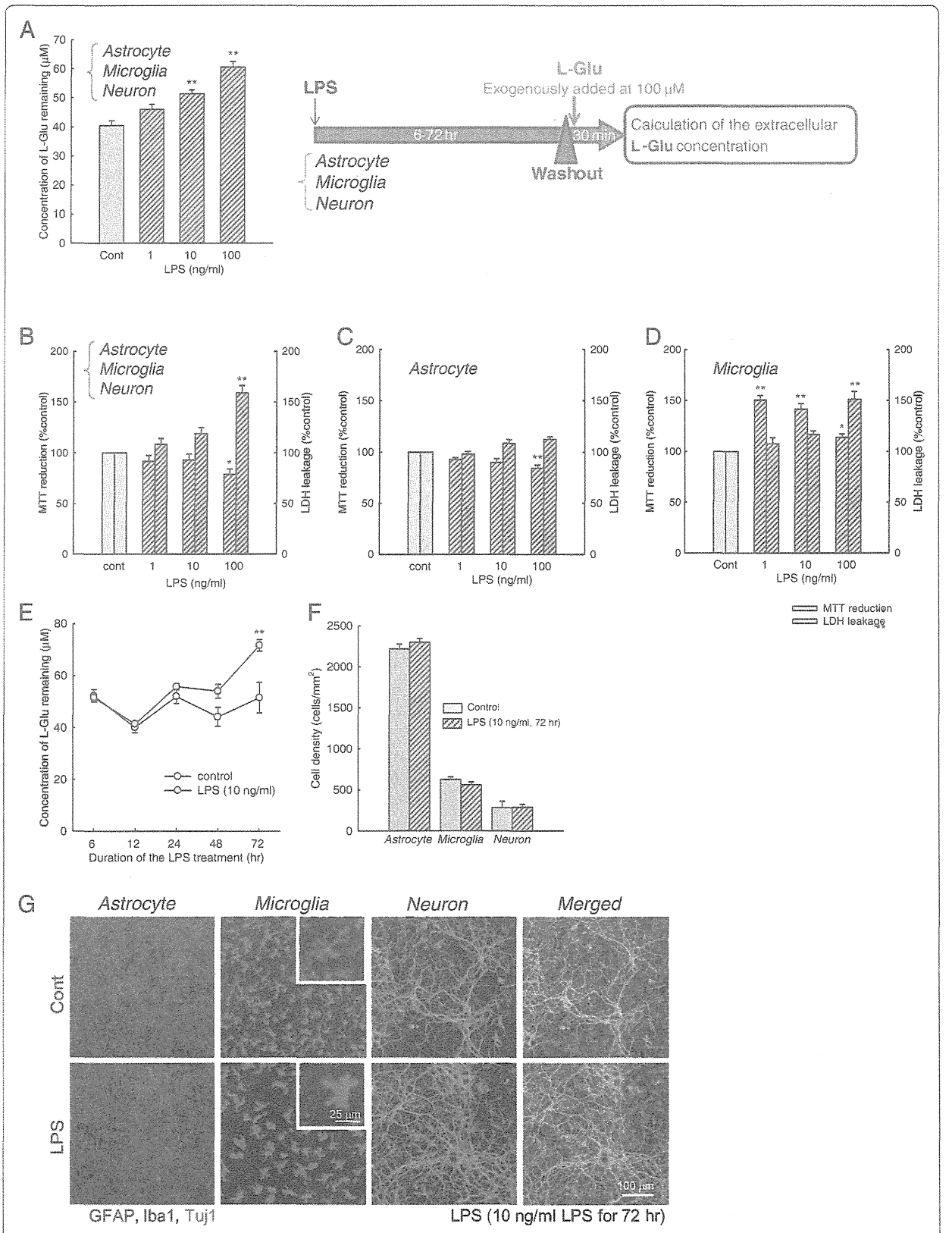


**Figure 1** (See legend on next page.)

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**Figure 1 The concentration of L-Glu remaining was increased during inflammation without cell death.** (**A**) Effects of LPS (1 to 100 ng/mL, 72 h) on the concentration of L-Glu remaining. The L-Glu remaining was significantly increased after incubation with 10 and 100 ng/mL LPS. $^{**}P < 0.01$ *vs.* control group ($n = 6$), Tukey's test following an ANOVA. (**B**, **C**, **D**) Effects of LPS on LDH leakage and MTT reduction in the astrocyte-microglia-neuron mixed cultures, astrocyte cultures, and microglia cultures. Significant LDH leakage and decreases in MTT reduction were induced by 100 ng/mL LPS but not by LPS concentrations <10 ng/mL in the mixed cultures (B). In the pure astrocyte culture, LPS concentrations <10 ng/mL affected neither LDH leakage nor MTT reduction (C). In the pure microglial culture, significant LDH leakage was induced by 100 ng/mL but not by LPS concentrations <10 ng/mL (**D**). (**E**) Time-dependent effects of LPS. The concentration of L-Glu remaining was measured after 6 to 72-h treatments with LPS (10 ng/mL). $^{**}P < 0.01$ *vs.* control group ($n = 6$), paired *t*-test. (**F**) The effects of LPS (10 ng/mL, 72 h) on the number of neurons, astrocytes, and microglia. LPS treatment had no effect on the cell numbers ($n = 5$). (**G**) Immunostaining of astrocytes, microglia, and neurons in the mixed culture with antibodies against GFAP (red), Iba-1 (blue), and Tuj1 (green) after treatment with 10 ng/mL LPS for 72 h. LPS dramatically changed the shape of the microglia from a ramified shape to an amoeboid shape.

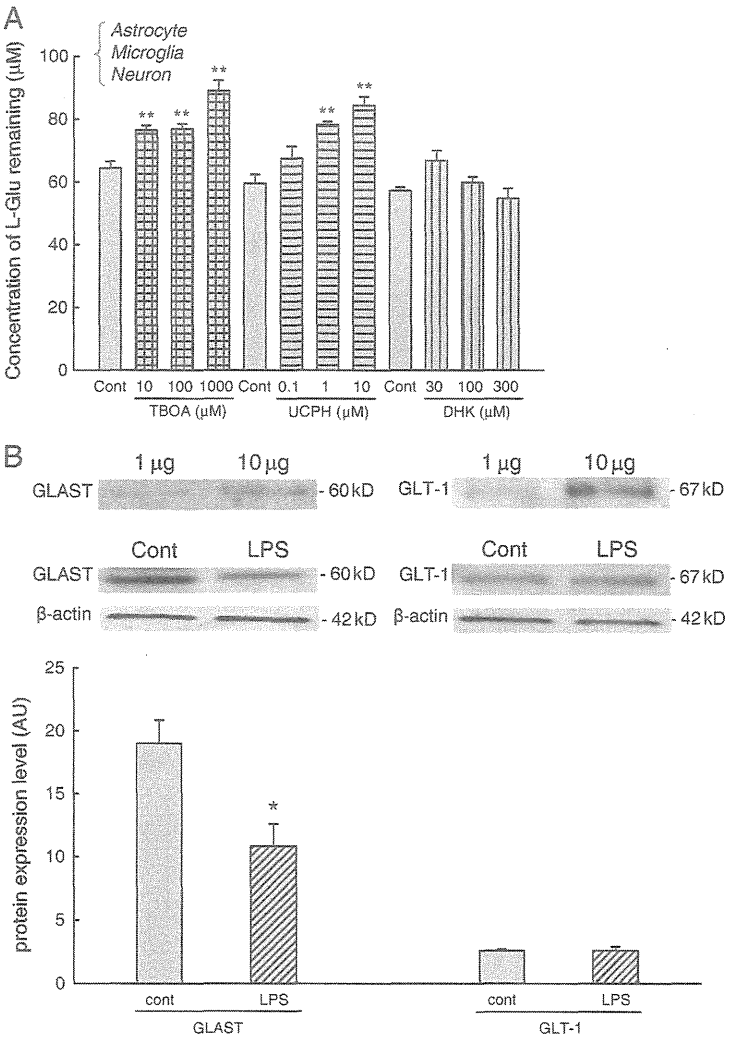


**Figure 2 The increase in the concentration of L-Glu remaining was mainly caused by the downregulation of GLAST.** (**A**) The effects of TBOA (30 to 300 μM), UCPH-101 (0.1 to 10 μM), and DHK (30 to 300 μM) on L-Glu clearance. Concentration-dependent inhibition was obtained by treatment with TBOA and UCPH-101. $^{**}P < 0.01$ *vs.* the control group ($n = 6$), Tukey's test following ANOVA. (**B**) The basal expression levels of astrocyte L-Glu transporters and the effect of LPS on their expression levels. Basally, the GLAST protein level is much higher than that of GLT1. The GLAST protein levels significantly decreased after the LPS treatment (10 ng/mL, 72 h), but GLT-1 protein levels did not change. $^{*}P < 0.05$ *vs.* the control group ($n = 4$), Student's *t*-test.