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孤発性ALSの分子異常を標的とした治療技術の確立

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（総括）研究報告書

孤発性ALSの分子異常を標的とした治療技術の確立

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［研究趣旨］

孤発性ALSの運動ニューロンで疾患特異的、部位選択的分子変化として我々が見出したGluR2 Q/R部位のRNA編集異常は、神経細胞死を引き起こす一次原因であり、疾患特異的な分子異常である．GluR2 Q/R部位のRNA編集は，RNA編集酵素adenosine deaminase acting on RNA 2（ADAR2）により触媒されることより，孤発性ALS運動ニューロンではADAR2活性が低下していると考えられる．したがって，ADAR2活性の賦活によりGluR2 Q/R部位のRNA編集が正常化し、孤発性ALSの神経細胞死を抑制する効果が期待される．本年度の研究において、1）ADAR2活性賦活物質のin vitroスクリーニング用に開発したTet-HeLaG2m細胞系を用いて10種類以上の候補薬剤を得，2）遺伝子治療に向けたADAR2遺伝子導入ベクターの作製，および血管内投与による脊髄運動ニューロンへの遺伝子導入に有用な改変ベクターの開発を行った．

分担研究者氏名・所属機関名及び所属機関における職名

相澤仁志　旭川医科大学　講師

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A. 研究目的

我々は孤発性ALS脊髄運動ニューロンに発現するグルタミン酸受容体であるAMPA受容体のGluR2サブユニットに、本来生ずべきQ/R部位 におけるRNA編集が行われないmRNAが含まれていること，この分子異常はALS運動ニューロンに疾患特異的かつ細胞選択的な変化であることを報告した[1,2]．AMPA受容体の大多数はサブユニットに編集型GluR2（Q/R部位がアルギニンR）を含むため、Ca^{2+}透過性が低い．他方，未編集型GluR2（Q/R部位がグルタミンQ）を含むAMPA受容体はGluR2を含まないAMPA受容体同様Ca^{2+}透過性が高い．正常のニューロンは編集型GluR2のみを発現し，未編集型GluR2を発現しないので，AMPA受容体の大多数はCa^{2+}非透過性である．孤発性ALSでは未編集型GluR2の発現によりCa^{2+}透過性AMPA受容体が増加している．GluR2 mRNAのQ/R部位は、RNA編集酵素ADAR2（adenosine deaminase acting on RNA 2）により特異的に編集され、ADAR2のノックアウト動物は幼弱期に死亡する[3]．したがって，ADAR2活性が低下すると未編集型GluR2が増加し、Ca^{2+}透過性AMPA受容体が増え，細胞内Ca^{2+}濃度上昇を通じて神経細胞死を引き起こすと考えられる．実際，孤発性ALSの脊髄前角組織では，ADAR2 mRNA発現レベルが低下しており、ADAR2活性が低下していることが未編集型GluR2の増加を引き起こしていると考えられる．また、ADAR2を欠損した脊髄運動ニューロンは緩徐な神経細胞死に陥ることがコンディショナルノックアウトマウスの解析から明らかになっている[4]。この仮説が正しければ，ADAR2活性を上げることにより運動ニューロン死を阻止し、孤発性ALSの進行を抑止する治療法の開発が可能であると考えられる．さらに、孤発性ALS運動ニューロンに特異的な病理変化として近年明らかにされたTDP-43病理がADAR2活性低下と極めて密接に関連していることが明らかになった[5]。これは、ADAR2活性低下が孤発性ALSの病因と密接に関連することをさらに示唆する結果であり、我々の仮説の妥当性を支持する。

本研究では、ADAR2活性賦活による孤発性ALSの特異治療法の開発のため、ADAR2活性賦活物質の探索、ADAR2遺伝子導入による治療技術基盤の確立を目的とする．

B. 研究方法

1. 培養細胞を用いたADAR2活性賦活物質のスクリーニング

我々が開発したTetHeLaG2m細胞を用いて，0.1ー10μM濃度の薬剤に24時間暴露後の

GluR2 Q/R 部位編集率の変化から，ADAR2 活性賦活作用のスクリーニングを行った．スクリーニング対象は US Drug Collection1040 種類とした．

２．アデノ随伴ウイルス（AAV）ベクターによる神経細胞への ADAR2 遺伝子導入技術の確立のため、ヒト ADAR2 cDNA を組み込んだ AAV ベクターを作成した．AAV ベクターを HeLa 細胞および Neuro2a 細胞に遺伝子導入し、その遺伝子発現，および ADAR2 活性を，ADAR2 遺伝子の発現 ADAR2 基質である GluR2 Q/R 部位および CYFIP2 K/E 部位の RNA 編集率の変化により解析した．対照としては ADAR2 遺伝子を組み込まない AAV ベクター、および RNA 編集活性を持たない変異型 ADAR2^{E396A} 遺伝子を導入した AAV ベクターを用いた．さらに、マウス脳組織へ AAV ベクターを注入し、ADAR2 遺伝子導入が機能するかどうかを検討した．

臨床応用のためには、血管内投与による遺伝子導入法の確立が必要である．脳内へ移行しやすい AAV ベクターを見出すため、各種 AAV カプシド蛋白を持ち、GFP を発現する psudotype AAV ベクターを作製し、マウス血管内投与による中枢移行を解析した．

（倫理面への配慮）

遺伝子操作に関しては、第二種使用等拡散防止措置における承認を得、全ての遺伝子操作は本学 DNA 組換え実験指診に従い行った．また動物実験については、東京大学医学部動物委員会の承認を得、実験方法については同動物実験指針に従い動物愛護面に十分配慮した．

C. 研究結果及び考察

1． Tet-HeLaG2m 細胞系を用い中枢移行性の高い市販薬を中心に 104 種類の化合物のスクリーニングを行い、ADAR2 活性賦活作用のある薬剤を 20 種類得た．これらの作用メカニズムは、ADAR2 mRNA 発現量の増加によるもの、ADAR2 mRNA/GluR2 pre-mRNA 比の増加によるものの他、これらの ADAR2 活性に関連しうる分子には影響を与えないものがあり、異なる分子メカニズムに依ることが明らかになった(5)．

２．ヒト ADAR2a、ADAR2^{E396A} cDNA を AAV ベクターに組み込んだコンストラクトを作製し、HeLa 細胞および Neuro2a 細胞に遺伝子導入した．ADAR2a の導入により、ADAR2 mRNA の増加、GluR2 Q/R 部位の RNA 編集率上昇が見られ、ADAR2^{E396A} の導入では ADAR2 mRNA 増加はみられたものの、GluR2 Q/R 部位の RNA 編集率はむしろ低下した．AAV のみの 導入では両者共に変化がなく、ADAR2 遺伝子導入により GluR2 Q/R 部位の RNA 編集活性が変化することを確認した．さらに、野生型マウス脳に ADAR2^{E396A} を導入することにより、GluR2 Q/R 部位の RNA 編集率が有意に低下することが確認でき、in vivo においても有効に遺伝子導入できることが明らかになった．

血管内投与による遺伝子導入のためのベクター開発研究では、野生型カプシドを持つ AAV8, AAV9 では、脳内少数の神経細胞・グリア細胞に発現がみられたが、脊髄神経細胞には発現がみられなかった．一方、神経細胞特異的プロモーターを持つ AAV ベクターでは、脊髄 ChAT 陽性細胞の 20%程度に発現がみられた．

D. 結論

ADAR2 活性賦活作用を持つ治療薬候補物質のスクリーニングを進めた．ADAR2 の遺伝子治療に必要なコンストラクトの開発、血管経由による神経細胞への遺伝子導入ためのベクターの開発を進めた．

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E. 健康危険情報

なし

F. 研究発表

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G. 知的財産権の出願・登録状況

1. 特許：

「筋萎縮性側索硬化症の予防及び治療のための医薬」出願日 2009 年 07 月 28 日　出願人 国立大学法人　東京大学　出願番号：特願 2009-175128　出願国 日本

2. 実用新案登録：なし

3. その他：なし

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（分担）研究報告書

孤発性 ALS の分子異常を標的とした治療技術の確立
———運動ニューロンの ADAR2 活性を賦活する薬物の開発———

研究分担者　相澤　仁志　旭川医科大学内科学講座講師

研究要旨：Tet-HeLaG2m細胞系培養細胞によりADAR2活性を賦活する薬剤を一次スクリーニンし、現在まで15種類の薬剤がADAR2活性を有する事を確認した。

A.研究目的
ALSの運動ニューロン死に特異的変化であるRNA編集酵素ADAR2の活性低下を正常化する薬剤のスクリーニングをする。

B.研究方法
Tet-HeLaG2m細胞を培養24時間後に薬剤を10 nM-10 μ Mの間で暴露し、24時間後に総RNAを抽出してGluR2 Q/R部位のRNA編集率を測定する．有意な編集率の変化を引き起こした物質については、ADAR2 mRNA、GluR2 pre-mRNAを定量しADAR2 活性賦活作用のメカニズムを検討する。対象は市販薬、活性のある市販薬に類似の構造を持つ化合物、転写活性物質とする。

（倫理面への配慮）
培養細胞を使用した薬剤スクリーニングは倫理面の問題はない。

C.研究結果
現在まで54種の薬剤のスクリーニングを行い、15種でADAR2 活性賦活作用を確認した。

D.考察
ADAR2 活性賦活作用を有する薬剤はADAR2活性をノックアウトさせた孤発性ALSのモデルマウスを用いたin vivo二次スクリーニングに使用可能と考えられる

E.結論
新たにADAR2 活性賦活作用を有する薬剤を複数見いだした。

F.健康危険情報
なし

G.研究発表
1. 論文発表
○Aizawa H, Sawada J, Hideyama T, Yamashita Y, Katayama T, Hasebe N, Kimura T, Yahara O, Kwak S. TDP-43 pathology in sporadic ALS occurs in motor neurons lacking the RNA editing enzyme ADAR2. Acta Neuropath, Epub Apr 7, 2010.

○Sawada J, Yamashita T, Aizawa H, Aburakawa Y, Hasebe N, Kwak S. Effects of antidepressants on GluR2 Q/R site-RNA editing in modified HeLa cell line. Neurosci Res. 64(3):251-258, 2009

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H．知的財産権の出願・登録状況
1. 特許取得：有効性を確認次第提出。
2. 実用新案登録：該当なし
3. その他：該当なし

厚生労働科学研究費補助金（こころの健康科学研究事業）
（分担）研究報告書

孤発性ALSの分子異常を標的とした治療技術の確立
———ADAR2遺伝子の運動ニューロン導入技術の確立———

研究分担者　村松慎一　自治医科大学大学内科学講座神経内科学部門・特命教授

研究要旨：アデノ随伴ウイルス(AAV)ベクターを応用し神経細胞へADAR2およびGluR2遺伝子を導入することにより運動神経変性を抑制する遺伝子治療法の基盤技術を開発することを目標に研究を行った．各種の型のAAVのカプシド蛋白およびゲノム配列を改変し，血管内投与により脳と脊髄の神経細胞に治療用遺伝子を効率よく送達可能なAAVベクターを開発した．

A.研究目的

筋萎縮性側索硬化症(ALS)に対する遺伝子治療として，アデノ随伴ウイルス(AAV)ベクターを応用して血管内投与により治療用の遺伝子を脳および脊髄の運動神経細胞へ送達する方法を開発する．

B.研究方法

血管内投与後に脳内へ移行しやすいAAVベクターを見出すため，AAV1, 2, 3, 5, 8, 9,10など各種の型のカプシド蛋白を持ち，GFPを発現するpseudotype AAVベクターを作製した．一部の型では，カプシド蛋白の数か所のアミノ酸配列を置換し，発現カセットの塩基配列も改変したベクターを作製した．また，神経細胞への親和性を持つRabies virusの糖タンパク質由来のRVGペプチド(29アミノ酸)の誘導体を化学合成しカプシド蛋白を修飾した．これらのベクターをマウスの血管内に投与し4週間後に組織を解析した．

C.研究結果

野生型カプシドを持つAAV8, AAV9ベクターでは，脳内の少数の神経細胞およびグリア細胞にGFPの発現が認められたが，脊髄の神経細胞では発現が見られなかった．神経細胞特異的プロモーターを搭載した改良型AAVベクターでは，脳の広範な領域と脊髄の神経細胞でGFPの発現が認められた．脊髄のChAT陽性細胞では20%程度にGFPの発現が得られた．

D.考察

従来，AAVベクターは，脳内での逆行性輸送および筋肉内投与による末梢神経終末から脊髄への輸送についての報告があるがその効率は高くない．カプシド・ゲノムの改変によりこの点を改善できればALSの遺伝子治療が可能になる．今回，開発した改良型ベクターでは，血管内投与により脊髄の運動神経細胞へ遺伝子導入が可能であり，臨床応用への期待が持てる．血液脳関門を通過する詳細な機序は不明であるが，炎症反応や組織破壊は認めていない．今後，ADAR2遺伝子を搭載したベクターを使用してさらに前臨床研究を実施する．

E.結論

カプシド蛋白およびゲノム配列を改変し，血管内投与により効率よく脳と脊髄の神経細胞へ遺伝子導入可能なAAVベクターを開発した．

F.健康危険情報

なし

G.研究発表

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H.知的所有権の取得状況（予定を含む）

1.特許取得
7. 実用新案登録
8. その他

なし

【研究成果の刊行に関する一覧表】

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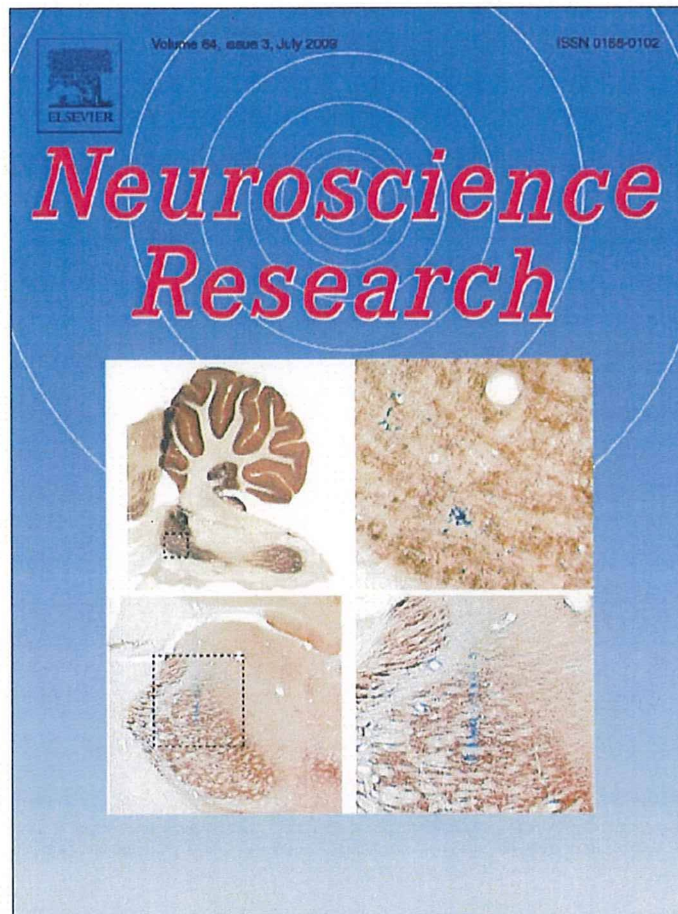

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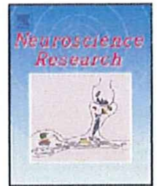

Effects of antidepressants on GluR2 Q/R site-RNA editing in modified HeLa cell line

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ABSTRACT

Marked reduction of RNA editing at the glutamine (Q)/arginine (R) site of the glutamate receptor subunit type 2 (GluR2) in motor neurons may be a contributory cause of neuronal death specifically in sporadic ALS. It has been shown that deregulation of RNA editing of several mRNAs plays a causative role in diseases of the central nervous system such as depression. We analyzed the effects of eight antidepressants on GluR2 Q/R site-RNA editing in a modified HeLa cell line that stably expresses half-edited GluR2 pre-mRNA. We also measured changes in RNA expression levels of adenosine deaminase acting on RNA type 2 (ADAR2), the specific RNA editing enzyme of the GluR2 Q/R site, and GluR2, in order to assess the molecular mechanism causing alteration of this site-editing. The editing efficiency at the GluR2 Q/R site was significantly increased after treatment with seven out of eight antidepressants at a concentration of no more than 10 μM for 24 h. The relative abundance of ADAR2 mRNA to GluR2 pre-mRNA or to β-actin mRNA was increased after treatment with six of the effective antidepressants, whereas it was unchanged after treatment with milnacipran. Our results suggest that antidepressants have the potency to enhance GluR2 Q/R site-editing by either upregulating the ADAR2 mRNA expression level or other unidentified mechanisms. It may be worth investigating the in vivo efficacy of antidepressants with a specific therapeutic strategy for sporadic ALS in view.

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that affects approximately 1 in 2000 people over their lifetime (Cleveland and Rothstein, 2001). ALS is characterized by a selective loss of upper and lower motor neurons that initiates a progressive paralysis with muscle wasting in mid-life, and is usually fatal within 1–5 years after onset. Approximately 5–10% of all ALS cases are familial, and at least five causal genes have been so far identified in individuals affected with familial ALS (SOD1, ALS2, senataxin, vesicle-trafficking protein/synaptobrevin-associated membrane protein, and TDP-43), although the mechanism underlying motor neuron death of familial ALS pathology has not been elucidated (Rosen et al., 1993; Hadano et al., 2001; Yang et al., 2001; Chen et al., 2004; Nishimura et al., 2004; Yokoseki et al., 2008; Gitcho et al., 2008; Kabashi et al., 2008; Sreedharan et al., 2008; Van Deerlin et al., 2008). However, sporadic ALS accounts for the majority of all ALS cases, and one clue to the pathomechanism of sporadic ALS, low editing efficiency of GluR2 mRNA, has been elucidated (Takuma et al., 1999; Kawahara et al., 2004).

One of the most plausible hypotheses for selective neuronal death in sporadic ALS is excitotoxicity mediated by abnormally Ca^{2+}-permeable α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) receptors, a subtype of ionotropic glutamate receptor (GluR) (Kwak and Kawahara, 2005; Carriedo et al., 1996; Lu et al., 1996). The contribution of excessive Ca^{2+} influx through glutamate receptors to the death of motor neurons is the basis for the recent suggestion that deficient GluR2 Q/R site-RNA editing might play a role in ALS (Kawahara et al., 2004; Kwak and Kawahara, 2005). A decrease or loss of RNA editing function occurring specifically in motor neurons could lead to AMPA-channels highly permeable to Ca^{2+}, mimicking or exacerbating the overexcitation of glutamate receptors due to excitatory amino acid transporter loss. The Ca^{2+} conductance of AMPA receptors differs markedly depending on whether the GluR2 subunit is a component of the receptor. AMPA receptors that contain at least one GluR2 subunit have low Ca^{2+} conductance, whereas those lacking a GluR2 subunit are Ca^{2+} permeable (Hollmann et al., 1991; Verdoorn et al., 1991; Burnashev et al., 1992). These properties of GluR2 are generated post-transcriptionally by RNA editing at the glutamine/arginine (Q/R) site in the putative second membrane domain, during which the glutamine codon is substituted by an arginine

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codon (Verdoorn et al., 1991; Burnashev et al., 1992). AMPA receptors containing the unedited form of GluR2Q have high Ca^{2+} permeability in contrast to the low Ca^{2+} conductance of those containing the edited form of GluR2R (Burnashev et al., 1995; Swanson et al., 1997). Editing of the GluR2 Q/R position was inefficient in a subset of motor neurons in sporadic ALS, whereas it was completely efficient in all the motor neurons of control cases (Takuma et al., 1999; Kawahara et al., 2004). This finding indicates that abnormal editing may be a contributory cause of neuronal death specifically in sporadic ALS.

A-to-I RNA editing is catalyzed by adenosine deaminase acting on RNA (ADAR) (Bass, 2002; Keegan et al., 2001; Gott and Emeson, 2000; Maas et al., 2003). An association between the level of ADAR type 2 (ADAR2) mRNA expression and editing efficiency at the GluR2 Q/R site has been demonstrated in human brain white matter (Kawahara et al., 2003). Hence, the expression level of ADAR2 mRNA is one factor determining the efficiency of GluR2 Q/R site-editing, although the nonlinear correlation suggests that another factor or factors may be also involved in the regulation of editing activity (Kawahara et al., 2004).

Several lines of evidence suggest an association between major psychiatric disorders and the pattern of RNA editing at several known A-to-I positions in the serotonin $(5\text{-HT})_{2c}$ receptor mRNA (Dracheva et al., 2003; Niswender et al., 2001; Iwamoto and Kato, 2003; Sodhi et al., 2001; Gurevich et al., 2002a). Furthermore, one research group reported that the extent of editing was altered at some of the A-to-I positions in glutamate receptors mRNAs in the pre-frontal/frontal cortex and hippocampus of rats after a continuous 2-week-treatment with antidepressants (Barbon et al., 2006).

Based on the evidence showing that antidepressant drugs affect the function of AMPA/kainite (KA) receptors (Barbon et al., 2006), we postulated that antidepressants had the potency to modulate GluR2 Q/R site-editing, thereby becoming a potential therapy for ALS. We established methods to analyze editing levels at the GluR2 Q/R site using a modified HeLa cell line, which stably expresses the half-edited GluR2 pre-mRNA (TetHeLaG2m cell). We investigated the abilities of three kinds of antidepressants, i.e., a selective serotonin reuptake inhibitor (SSRI), serotonin noradrenaline reuptake inhibitor (SNRI), and tricyclic antidepressant, to upregulate the GluR2 Q/R site-editing using a newly developed modified HeLa cell line (TetHeLaG2m).

2. Materials and methods

2.1. Generation of HeLa cell line stably expressing GluR2 mini-gene using Tet-on gene expression system (TetHeLaG2m cell line)

The GluR2 mini-gene was designed to include the Q/R site in exon 11 and its exon complementary sequence in the adjacent intron 11 of human GluR2 pre-mRNA in order to evaluate the efficacy of A-to-I editing by ADAR2. The regions of GluR2 pre-mRNA including the sequence between exon 11 and intron 11 (5′-PCR) and the sequence between intron 11 and exon 12 (3′-PCR) were separately amplified by PCR (Fig. 1A). For each PCR, the following primer pairs were used: for the 5′-PCR, 5′-AAAAACGCGTATGAAAGCTGATATTGCAATTGCTCCAT-3′ and 5′-TGTATCATGAAAGGCACCCGCTCCACTAGT-3′; for the 3′-PCR, 5′-TGTTTAATGATTTCCAGTTTCATTAACTAG-3′ and 5′-ATATTACGCGTCTACCTGAAAAACTCTTTAGTGGAGCCA-3′. Each PCR amplification began with a 10-min denaturation at 95 °C, followed by 30 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 68 °C for 90 s. The resulting PCR products were digested by MluI, which recognizes the exon 11-intron 11 junction of the 5′-PCR product and the intron 11-exon 12 junction of the 3′-PCR product. Then, both PCR-amplified fragments were ligated at the SpeI restriction sites in intron 11. The ligated products were inserted into the Tet-on pTRE-Tight Vector (Clontech, Palo Alto, CA, USA), and then transfected into Tet-on HeLa cells (Clontech). Tet-on HeLa cells were transfected with the GluR2-mini gene pTRE-Tight Vector and a linear puromycin marker (Clontech). Then, TetHeLaG2m cells were isolated from the puromycin-resistant clones.

2.2. Cell culture and drug treatment

TetHeLaG2m cells were seeded at 1×10^7 cells/well in 10 cm plastic wells, cultured in MEM-α medium (Wako, Tokyo, Japan) supplemented with 10% Tet System-approved fetal bovine serum, 0.75 μg/mL puromycin (both Clontech), 100 IU/mL penicillin, and 100 μg/mL streptomycin (Invitrogen) and incubated in a 5% CO_2 atmosphere. After growing to confluence, TetHeLaG2m cells were plated at 2×10^6 cells/well in 6-well plates.

Culture cells were incubated with 0–10 μM of antidepressant for 24 h and then harvested for RNA extraction. The antidepressants used in this study were SSRIs (fluvoxamine, fluoxetine, paroxetine), SNRIs (milnacipran, reboxetine), and tricyclic antidepressants (amitriptyline, desipramine, imipramine); these drugs were purchased from Sigma (St Louis, MO, USA). Fluvoxamine and paroxetine were dissolved in dimethyl sulfoxide, while the other drugs were dissolved in distilled water.

2.3. RNA extraction and reverse transcription

RNA was extracted from the cells in each well using an RNeasy mini kit according to the manufacturer's instructions (Qiagen, Hilden, Germany). One microgram of total RNA was incubated at 70 °C for 10 min with 500 ng of Oligo(dT). First-strand cDNA was synthesized from the total RNA with 4 μL of 5× first-strand buffer, 2 μL of 0.1 M DTT, 4 μL of 2.5 mM dNTPs, 1 μL of RNase inhibitor (Toyobo, Tokyo, Japan), and 1 μL of SuperScript™ II Reverse Transcriptase (Invitrogen) in a final volume of 20 μL. The reverse transcription started with incubation at 42 °C for 60 min, followed by incubation at 51 °C for 15 min, and was stopped by heating to 72 °C for 15 min.

2.4. Nested polymerase chain reaction and restriction digestion

To determine the editing efficiency at the Q/R site of GluR2 in TetHeLaG2m cells, nested PCR products including the Q/R site were digested with restriction enzyme BbvI (New England BioLabs, Beverly, MA, USA) as previously described (Kawahara et al., 2003). In brief, 2 μL of cDNA were subjected first to PCR in duplicate in a reaction mixture of 50 μL containing 10 μM each primer, 4 μL of 2.5 mM dNTPs, 5 μL of 10× PCR buffer, and 0.5 μL of Gene Taq (Nippon Gene, Tokyo, Japan). The PCR amplification began with a 2-min denaturation step at 95 °C, followed by 20 cycles of denaturation at 95 °C for 10 s, annealing at 66 °C for 30 s and extension at 68 °C for 60 s. Nested PCR was conducted on 2 μL of the first PCR product under the same conditions with the exception of the number of PCR cycles (30 cycles). For each PCR, the following primers were used (amplified product lengths are also indicated): for the first PCR (352 bp), F1 (5′-TTCCTGGTCAGCAGATTTAGCC-3′) and R1 (5′-GCAACATTCAAAGAACATTGTTC-3′), and for the nested PCR (200 bp), F2 (5′- TCTGGTTTTCCTTGGGTGCC-3′) and R2 (5′-CCGAAGCTAAGAGGATGTCCTTC-3′).

After gel purification using the Zymoclean Gel DNA Recovery Kit according to the manufacturer's protocol (Zymo Research, Orange, CA, USA), PCR products were quantified using a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA). An aliquot (0.5 μg) was then incubated at 37 °C for 12 h with 10× restriction buffer and 2 U of BbvI in a total volume of 20 μL and inactivated at 65 °C for 30 min.

The PCR products had one intrinsic BbvI recognition site, whereas the products originating from unedited GluR mRNA had

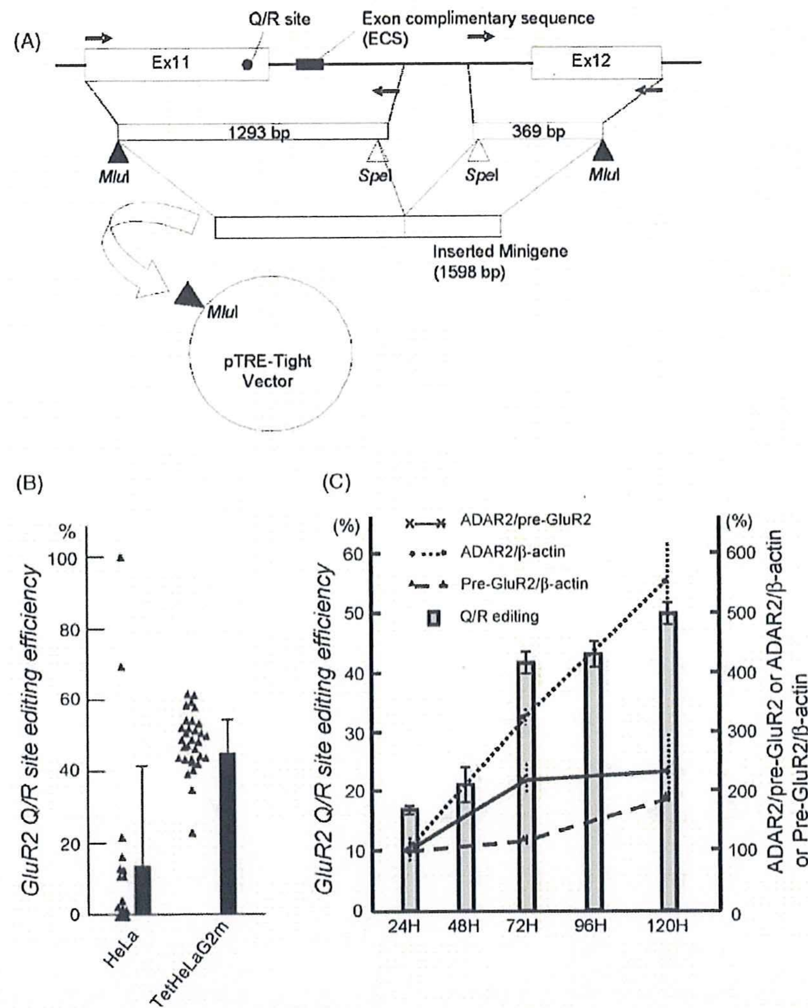

Fig. 1. TetHeLaG2m cell line. (A) Schematic figure of the GluR2 minigene with pTRE-tight vector. (B) Editing efficiencies at the Q/R site of GluR2 minigene pre-mRNA in conventional HeLa and TetHeLaG2m cell lines cultured in a dish for 48 h. Each symbol represents the extent of Q/R site-editing of GluR2 minigene pre-mRNA isolated from a single culture dish. Each large symbol represents the results of five culture dishes. For each cell line, the mean ± SEM (n = 15–30) is also indicated. (C) Culture time-dependent changes of editing efficiency at the Q/R site of GluR2 minigene pre-mRNA and expression levels of ADAR2 mRNA and GluR2 pre-mRNA, and relative abundance of ADAR2 mRNA to GluR2 pre-mRNA in the TetHeLaG2m cell line. TetHeLaG2m cells were plated at a low concentration (5×10^5 cells/well in 6-well plate). The relative abundance of ADAR2 mRNA to GluR2 pre-mRNA increases in parallel with the editing efficiency at the GluR2 Q/R site in a time-dependent manner. For each culture time, the mean ± SEM (n = 6) is also indicated.

an additional recognition site (Fig. 2). Thus, in TetHeLaG2m cells, restriction digestion of the PCR products originating from edited GluR2 mRNA should produce two bands at 129 and 71 bp, whereas those originating from unedited GluR2 mRNA should produce three bands at 91, 38, and 71 bp. As the 71-bp band would originate from both edited and unedited mRNA but the 129-bp band would originate from only edited mRNA, we quantified the molarity of the 129- and 71-bp bands using the 2100 Bioanalyser and calculated the editing efficiency as the ratio of the former to the latter for each sample.

2.5. Standard preparation for quantitative polymerase chain reaction

To prepare an internal standard for quantitative PCR of TetHeLaG2m cells, we inserted the genes we aimed to estimate into the plasmid vector. Total RNA was extracted from conventional HeLa cells by using an RNAspin Mini kit (GE Healthcare) and the cDNA was synthesized from the total RNA with Ready-To-Go You-Prime First-Strand Beads (Amersham Bioscineces). Gene-specific PCR products of GluR2, ADAR2, and β-actin were amplified from the cDNA with the primers noted in Table 1. Each PCR was done using the following program: the PCR amplification began with a 10-min denaturation step at 95 °C, followed by 30 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s, and extension at 68 °C for 40 s. Using the primers (Table 1) shown in the previous report (Nishimoto et al., 2008), 2 μL of cDNA extracted from HeLa cells (human control) was subjected to PCR with 1 μL of Advantage 2 Polymerase mix (BD Biosciences Clontech, Palo Alto, CA, USA). After gel purification, PCR products were subcloned using the TOPO TA cloning kit (Invitrogen), and clones containing inserts were sequenced with an ABI PRISM 3100 sequencer (Applied Biosystems, Foster City, CA). The concentration of each standard plasmid was measured spectrophotometrically at 260 nm (Nano Drop™ ND-1000; Nano Drop Technologies, Wilmington, DE). We prepared standard solutions by serial dilutions of the sample ranging from 10^{-11} to 10^{-10}, 10^{-9}, 10^{-8} and 10^{-6} μg per 1 μL.

2.6. Real-time quantitative polymerase chain reaction

PCR was performed on TetHeLaG2m cells using a LightCycler System (Roche Diagnostics, Mannheim, Germany). The PCR primers and probes were designed from the cDNA sequences of

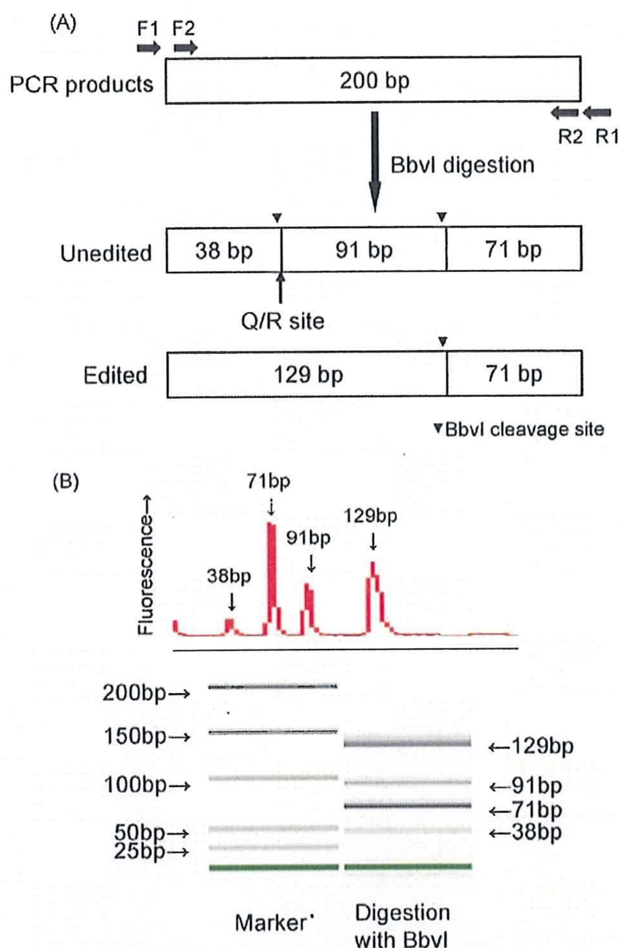

Fig. 2. Method of detecting editing efficiency at the Q/R site of GluR2. (A) Scheme for detecting editing efficiency at the Q/R site of GluR2. Open bars represent nested PCR products. Intrinsic BbvI recognition sites are indicated by vertical solid arrowheads. The sizes of the DNA fragments generated by restriction digestion are indicated. (B) Example of the quantification of editing efficiency using a 2100 Bioanalyzer. The molarity of the 129-bp (derived from edited GluR2 pre-mRNA) and 71-bp (derived from both edited and unedited GluR2 pre-mRNA) bands was quantified after restriction digestion with BbvI for 12 h, and the editing efficiency was calculated as the ratio of the former to the latter. The lower figure indicates gel-like image produced by the 2100 Bioanalyzer. GluR, glutamate receptor; Q, glutamine; R, arginine; PCR, polymerase chain reaction.

GluR2, ADAR2, and β-actin (as an internal control), which were obtained from GenBank (Table 1). The PCR primers and probes were designed from the cDNA sequences of GluR2, GluR2 minigene (pre-GluR2), ADAR2, and β-actin (as an internal control), which were obtained from GenBank (Table 1). A set of standard and cDNA samples was amplified in duplicate in a master mixture (20 μL total volume) comprising 2 μL of 5× TaqMan DNA polymerase (Roche Diagnostics) containing the reaction mix, 0.5 μM each primer, 0.1 μM Universal probes (Roche Diagnostics). Herring sperm DNA solution was coamplified as a negative control in each series of reactions. The reactions started with incubation for 10 min at 95 °C to activate TaqMan DNA polymerase. Templates were amplified by 60 cycles of denaturation at 95 °C for 10 s and primer annealing at 60 °C for 30 s. This was followed by fluorescence acquisition and extension at 72 °C for 1 s.

2.7. Statistical analysis

For the value of GluR2 Q/R site-editing efficiency, one-way analysis of variance (ANOVA), followed by the Dunnett's multiple comparison test, was used to compare the control group with antidepressant-treated groups, and Steel's test was used for multiple comparison to compare the mRNA expression levels of the treated group with the control group in the statistical analysis. Results are given as the mean value ± standard error.

Table 1
Sequences of primers and fluorescent hybridization probes used for quantitative polymerase chain reaction.

	Oligonucleotide sequence
GluR2	
Forward primer	5′-ATGCGATATTTCGCCAAGA-3′
Reverse primer	5′-CAGTCAGGAAGGCAGCTAAGTT-3′
Universal probe library probe #63	5′-CTCCTCCT-3′
pre-GluR2	
Forward primer	5′-GATGGTGTCTCCCATCGAAA-3′
Reverse primer	5′-TCCATAAGCAATTTCTGTTTGCT-3′
Universal probe library probe #73	5′-GCTGAGGA-3′
ADAR2	
Forward primer	5′-GTGTAAGCACGCGTTGTACTG-3′
Reverse primer	5′-CGTAGTAAGTGGGAGGGAACC-3′
Universal Probe Library probe #42	5′-GCTGGATG-3′
β-Actin	
Forward primer	5′-CCAACCGCGAGAAGATGA-3′
Reverse primer	5′-CCAGAGGCGTACAGGGATAG-3′
Universal Probe Library probe #64	5′-CCAGGCTG-3′

3. Results

3.1. Establishment of HeLa cell line (TetHeLaG2m) suitable for measurement of editing activity at GluR2 Q/R site

To measure RNA editing activity at the GluR2 Q/R site, we developed a double-stable HeLa cell line carrying a GluR2 minigene, which included human GluR2 exon 11, a part of intron 11, and exon 12, using Tet-on system (Fig. 1A). In contrast to HeLa cell line in which the editing efficiency at the GluR2 Q/R site varied widely from 0% to 100%, this cell line (TetHeLaG2m) stably expressed the pre-mRNA of GluR2-mini gene with both edited and unedited Q/R sites in nearly the same amounts after culture in vitro for 48 h (Fig. 1B). The extent of RNA editing at this site increased linearly with the length of culture until the cells were confluent in 6-well plates (Fig. 1C).Because the expression level of pre-GluR2 mRNA relative to β-actin mRNA in TetHeLaG2m cells was 100-fold high than that in HeLa cells (2.0E−05 ± 2.9E−06 (n = 18) and 2.6E−03 ± 2.4E−04 (n = 12) in HeLa cells and TetHeLaG2m cells, respectively), it is likely that the majority of pre-GluR2 mRNA in Fig. 4 were derived from the GluR2 mini gene in TetHeLaG2m cells.

3.2. Effect of antidepressant treatment on GluR2 Q/R site-editing

We investigated the editing efficiency at the GluR2 Q/R site in TetHeLaG2m cells after antidepressant treatment. The RNA editing level depends on culture time, but the variation of the editing level at given culture time is small among cells in sister culture (Fig. 1C). GluR2 Q/R site-editing was increased after incubation with each antidepressant except reboxentine. The effects of these drugs appeared to be dose-dependent, and the extents to which they increased editing were most marked after treatment with 10 μM milnacipran (Fig. 3D) and imipramine (Fig. 3G) (each about 40%), followed by 10 μM fluvoxamine (Fig. 3A), fluoxetine (Fig. 3B), paroxetine (Fig. 3C), and desipramine (Fig. 3G) (about 20%), and 1 and 10 μM amitriptyline (Fig. 3F) and 1 μM imipramine had some effect (about 10%).

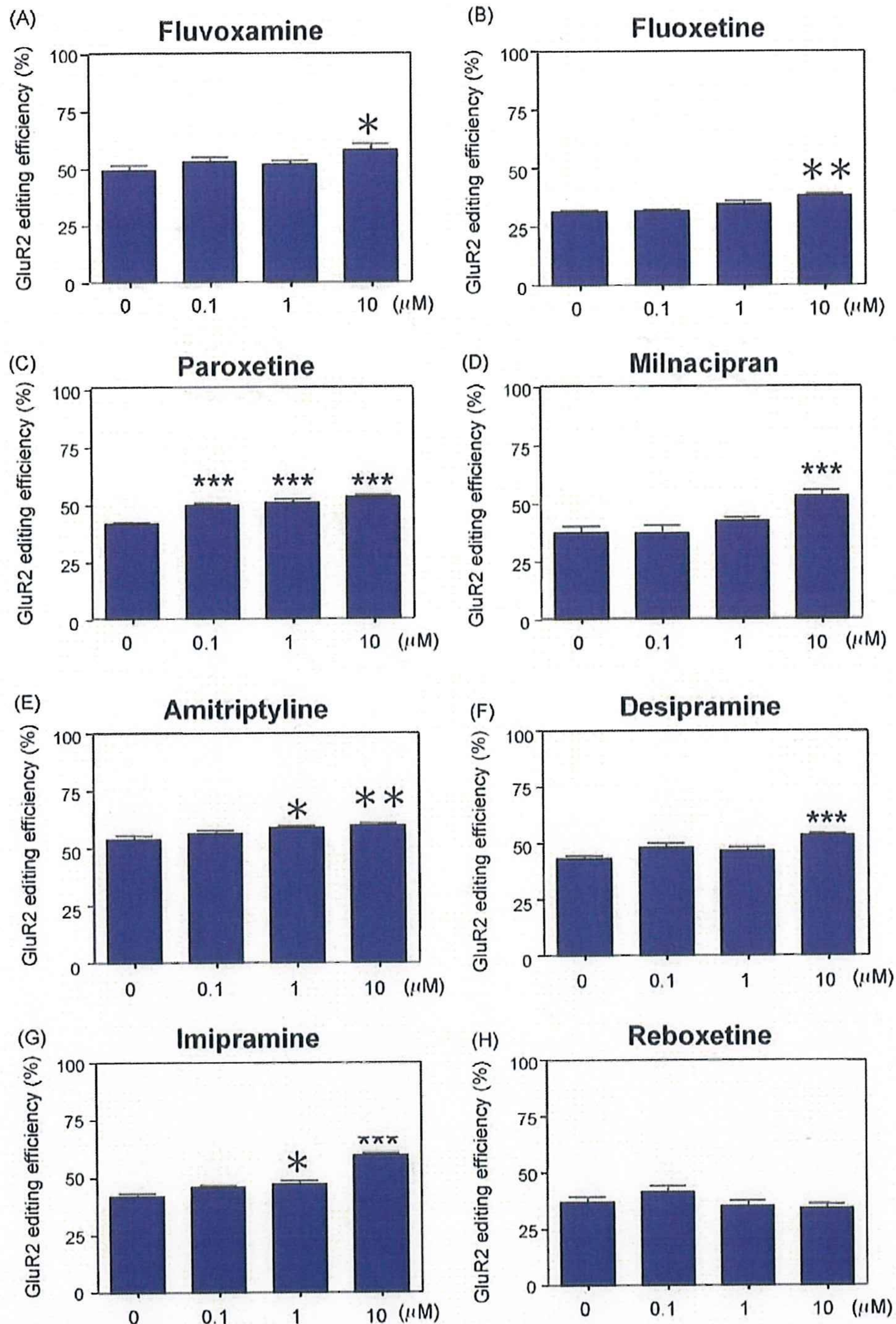

Fig. 3. Editing extent of GluR2 Q/R site. Editing efficiency in TetHeLaG2m cells after treatment with antidepressants (0–10 μM) for 24 h is expressed as mean ± SEM ($n = 5–8$). Statistical analysis was performed by the one-way ANOVA test followed by Dunnett's multiple comparison test (*$P < 0.05$, **$P < 0.01$, and ***$P < 0.001$). GluR, glutamate receptor; Q, glutamine; R, arginine; ANOVA, analysis of variance.

3.3. Changes in expression levels of ADAR2 mRNA, GluR2 mRNA, and GluR2 pre-mRNA

Because seven out of the eight antidepressants we examined (fluvoxamine, fluoxetine, paroxetine, milnacipran, amitriptyline, desipramine, and imipramine) significantly increased the GluR2 editing efficiency, we next investigated the relative changes in the expression levels of ADAR2, GluR2 mRNAs, and GluR2 pre-mRNA normalized to the expression level of β-actin mRNA before and after treatment with the above drugs. We also calculated the ratios of the amount of ADAR2 mRNA to that of GluR2 pre-mRNA in order to assess changes in the enzyme-substrate ratio after treatment with these antidepressants (Fig. 4).

The expression of ADAR2 mRNA was higher than that of the control group after treatment with 1 and 10 μM of fluvoxamine and 0.1 and 10 μM of imipramine (Fig. 4A and G), whereas it was lower than that of the control group after treatment with milnacipran (0.1 and 10 μM) (Fig. 4D). The other drugs did not alter the amount of ADAR2 mRNA significantly (Fig. 4B, C, E, and F).

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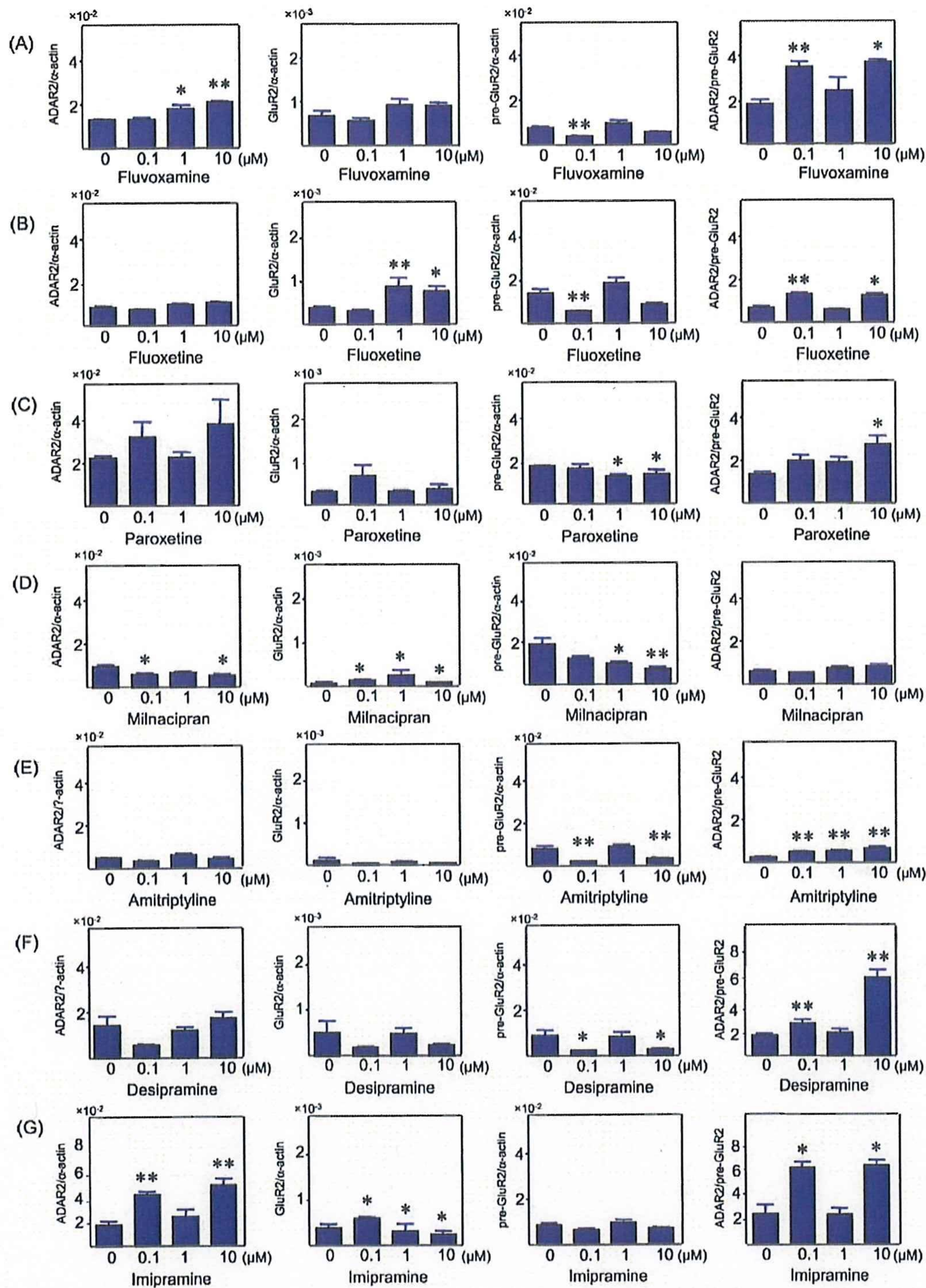

Fig. 4. Expression levels of ADAR2 mRNA, GluR2 mRNA and GluR2 pre-mRNA. Amounts of RNA that were upregulated by seven out of eight antidepressants that upregulated by GluR2 Q/R site-editing (A–G) were quantitatively analyzed. The amounts of ADAR2 mRNA, GluR2 mRNA, and GluR2 pre-mRNA are expressed as values relative to that of β-actin mRNA. The amount of ADAR2 mRNA is also expressed as a value relative to that of GluR2 mRNA. Mean ± SEM of at least 5–8 wells are displayed. Statistical analysis was performed with Steel's multiple comparison test (* $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$). GluR, glutamate receptor; ADAR, adenosine deaminase acting on RNA; Q, glutamine; R, arginine.

The expression of GluR2 mRNA was higher after treatment with 1 and 10 μM of fluoxetine and 0.1, 1, and 10 μM of milnacipran compared with that of the control group (Fig. 4B and D). In the imipramine-treated group, the expression of GluR2 mRNA with 0.1 μM of imipramine was higher than that of the control group, but at concentrations of 1 μM and 10 μM it was lower than that of the control group (Fig. 4G). The treatment with fluvoxamine, paroxetine, amitriptyline, and desipramine did not alter the amount of GluR2 mRNA significantly (Fig. 4A, C, E, and F).

The expression of GluR2 pre-mRNA was decreased by 70% after treatment with paroxetine (1 and 10 μM) (Fig. 4C), and by 20% after treatment with amitriptyline (0.1 and 10 μM) (Fig. 4E).

Similarly, after treatment with 0.1 μM of fluvoxamine, 0.1 μM of fluoxetine, 1 and 10 μM of milnacipran, and 0.1 and 10 μM of desipramine, the expression of GluR2 pre-mRNA was significantly lower than that of the control group (Fig. 4A, B, D, and F). On the other hand, imipramine did not significantly alter the expression of GluR2 pre-mRNA at each concentration compared with that of the control group (Fig. 4G).

The relative abundance of ADAR2 mRNA to GluR2 pre-mRNA was significantly higher than that of control groups after treatment with the antidepressants at the concentration of 10 μM or lower, except milnacipran (Fig. 4).

4. Discussion

We investigated the effects of three kinds of antidepressants including SSRI, SNRI, and tricyclic antidepressants on GluR2 Q/R site-editing using the newly developed cell line TetHeLaG2m. As shown in Fig. 1, TetHeLaG2m cells stably expressed the mini-GluR2 pre-mRNA with their Q/R sites both edited and unedited in nearly equal amounts. The GluR2 mini-gene pre-mRNA included a sequence identical to that found in the naturally occurring GluR2 pre-mRNA; hence, RNA editing at the Q/R site of this gene pre-mRNA was likely mediated by ADAR2. Thus, this cell line may be suitable for measurement of RNA editing activity, or in other words, ADAR2 activity at the GluR2 Q/R site.

In order to examine the effect of drugs on RNA editing of the GluR2 Q/R site in cell lines, basic editing level of the cells is required to be about 50% and these cells express GluR2 mRNA at a level abundant enough to be easily amplified by PCR. We tested various cell lines including N1E-115 (a mouse neuroblastoma cell line) and NSC34 (a hybrid cell line produced by fusion of motor neuron enriched, embryonic mouse spinal cord cells with mouse neuroblastoma), which, however, were not suitable for our purpose due to that the RNA editing at the Q/R site of GluR2 was either too high or too variable, or the expression of GluR2 mRNA was too low (data not shown). For these reasons, we created the modified HeLa cell line (TetHeLaG2m) which was suitable for our purpose to examine the effect of drugs on RNA editing of the GluR2 Q/R site.

We showed that seven of the eight antidepressants we examined significantly upregulated the editing efficiency at the Q/R site of the GluR2-mini pre-mRNA in TetHeLaG2m cells after 24-h-exposure, although the absolute increase in GluR2 Q/R site-RNA editing efficiency was rather small. This is the first report on the effects of antidepressants on GluR2 Q/R site-RNA editing in human cell lines. A moderate but persistent increase of Ca^{2+} permeability of AMPA channel causes degeneration of spinal motor neurons in the mouse (Kuner et al., 2005), suggesting that chronic moderate amending of the inactive GluR2 Q/R site-RNA editing observed in ALS would rescue the spinal motor neurons from death in ALS (Kwak and Kawahara, 2005). Treatment with reboxetine, an SNRI drug, did not increase the editing level at any concentration examined, suggesting that antidepressants upregulated GluR2 Q/R site-editing through a mechanism other than that exerting their anti-depressive effects.

Because the expression level of ADAR2 mRNA is one of the factors that determine the editing efficiency of GluR2 mRNA at the Q/R site (Kawahara et al., 2003), we next investigated the changes in the relative expression levels of ADAR2 mRNAs to GluR2 mRNA and GluR2 pre-mRNA (the majority were derived from the pre-mRNA of the GluR2-mini gene) in TetHeLaG2m cells after treatment with antidepressants that had significantly increased Q/R site-editing at concentrations ranging from a sub-μM order (paroxetine) to a 10 μM order (fluvoxamine, fluoxetine, milnacipran, and desipramine). The amount of GluR2 mRNA in TetHeLaG2m cells was less than 10% of GluR2 pre-mRNA, indicating that the majority of Q/R site-editing occurred in GluR2 pre-mRNA. Indeed, upregulation of editing efficiency at the GluR2 Q/R site after incubation with antidepressants seemed to be markedly influenced by the changes in the ratio of ADAR2 mRNA to GluR2 pre-mRNA, but not to GluR2 mRNA, except milnacipran (Figs. 3 and 4).

Treatment with fluvoxamine or imipramine increased the ADAR2 mRNA expression level (Fig. 4). On the other hand, treatment with fluoxetine, paroxetine, amitriptyline, or decipramine decreased the expression level of GluR2 pre-mRNA more than that of ADAR2 mRNA at a certain concentration. Thus, it seems likely that the increase in the ratio of ADAR2 mRNA to GluR2 pre-mRNA was mainly due to an increase in the expression level of ADAR2 mRNA after treatment with fluvoxamine or imipramine, whereas it was due to a decrease in the expression level of GluR2 pre-mRNA after treatment with the other antidepressants. The effects of antidepressants on GluR2 mRNA, GluR2 pre-mRNA, and ADAR2 mRNA expression levels may differ, even though they have the same antidepressant effects pharmacologically.

Several research groups reported alterations in RNA editing efficiency at A-to-I positions in the $5\text{-}HT_{2c}$ receptor ($5\text{-}HT_{2c}R$) expressed in brains of both depressed suicide victims (Niswender et al., 2001; Iwamoto and Kato, 2003) and individuals with major depression (Gurevich et al., 2002b). Similar changes were also observed in a rat model of depression, which was reversed after treatment with fluoxetine (Iwamoto et al., 2005). In addition, mice chronically treated with fluoxetine also exhibited decreased $5\text{-}HT_{2c}R$ E site-editing in the brain (Gurevich et al., 2002a,b). Although the expression level of ADAR2 mRNA is one determinant of the efficiency of GluR2 Q/R site-editing, it has been reported that editing extents of the various A-to-I editing sites in $5\text{-}HT_{2c}R$ mRNA correlated with the mRNA expression level of none of the members of ADAR families in cells from the rat hypothalamic tuberomamillary nucleus (Sergeeva et al., 2007). Taking our data and these reports together, antidepressants might have modulatory effects on A-to-I RNA editing sites in various mRNAs by direct upregulation of ADAR2 mRNA or other mechanisms. The molecular mechanism underlying the modulatory effects of antidepressants on A-to-I RNA editing remains to be elucidated, hence, further analysis of the activity and cellular localization of the ADAR enzymes (Sansam et al., 2003) and possible co-factors, such as nuclear RNA (Cavaille et al., 2000), that might be affected by antidepressants is necessary.

In conclusion, our results showed that antidepressants, although at rather high concentrations, increased the RNA editing efficiency at the GluR2 Q/R site in a human cell line. It is worth noting that this is the first report that the drugs could increase the RNA editing efficiency at the GluR2 Q/R site. Because a marked reduction of RNA editing at the GluR2 Q/R site in motor neurons may be a contributory cause of neuronal death specifically in sporadic ALS (Takuma et al., 1999; Kawahara et al., 2004), the drugs that upregulate GluR2 Q/R site-editing may be potential therapeutic tools for sporadic ALS. It is important to investigate whether these antidepressants could enhance GluR2 Q/R site-editing in vivo, and also to elucidate the mechanism underlying the upregulation of GluR2 Q/R site-editing by antidepressants.

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