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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.

Key Words: AMPA receptor; GluR2, RNA editing; adenosine deaminase acting on RNA type 2 (ADAR2); antidepressant; amyotrophic lateral sclerosis (ALS)

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a progressive neurodegenerative disease that affects approximately 1 in 2,000 people over their lifetime (Cleveland el al., 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 et al., 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 et al., 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; Verdoom et al., 1991; Burnashev et al., 1992). These properties of GluR2 are generated posttranscriptionally by RNA editing at the Q/R site in the putative second membrane domain, during which the glutamine (Q) codon is substituted by an arginine (R) codon (Verdoom et al., 1991; Burnashev etl 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 etl 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 et al., 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 et al., 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 (Tet-HeLaG2m 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 μg 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^{TM} 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 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^{TM} 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 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 mini-gene (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.

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 mini-gene, 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 μM and 10 μM amitriptyline (Fig. 3F) and 1μM imipramine had some effect (about 10%).

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 μM and 10 μM of fluvoxamine and 0.1 μM 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 μM and 10 μM) (Fig. 4D). The other drugs did not alter the amount of ADAR2 mRNA significantly (Fig. 4B, C, E, and F).

The expression of GluR2 mRNA was higher after treatment with 1 μM and 10 μM of fluoxetine and 0.1 μM, 1 μM, 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 μM and 10 μM) (Fig. 4C), and by 20% after treatment with amitriptyline (0.1

μM and 10 μM) (Fig. 4E). Similarly, after treatment with 0.1 μM of fluvoxamine, 0.1 μM of fluoxetine, 1 μM and 10 μM of milnacipran, and 0.1 μM 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 Figure 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-hour-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 & Kawahara et al., 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 Tet-HeLaG2m 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 (Fig. 3, 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-HT_{2c} receptor (5-HT_{2c}R) expressed in brains of both depressed suicide victims (Niswender et al., 2001; Iwamoto et al., 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-HT_{2c}R E site-editing in the brain (Gurevich et al., 2002). 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-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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Legends for Table and Figures

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

Figure 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.

Figure 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

Figure 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 to 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

Figure 4 Expression levels of ADAR2 mRNA, GluR2 mRNA and GluR2 pre-mRNA

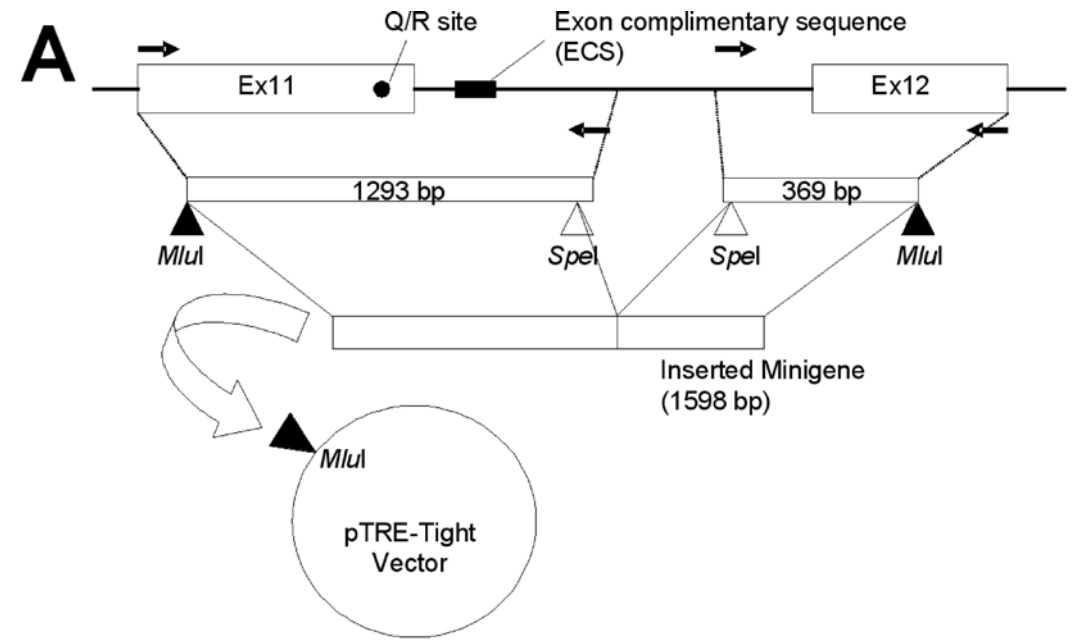
Amounts of RNA that were upregulated by seven out of eight antidepressants that upregulated by GluR2 Q/R site-editing (A, B, C, D, E, F, and G) were quantitatively analyzed. The amounts of ADAR2 mRNA, GluR2 mRNA, and GluR2 pre-mRNA are expressed as values relative to that of β-actin mRNA. The amount 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, ***P<0.001).

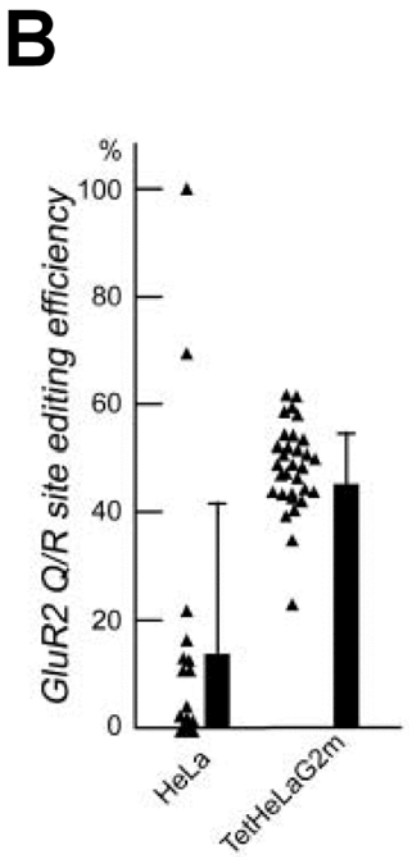
GluR, glutamate receptor; ADAR, adenosine deaminase acting on RNA; Q, glutamine; R, arginine

Table 1

	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’

Figure 1


A
Q/R site
Exon complimentary sequence
(ECS)
Ex11
Ex12
1293 bp
369 bp
MluI
SpeI
SpeI
MluI
Inserted Minigene
(1598 bp)
MluI
pTRE-Tight
Vector


B
%
GluR2 Q/R site editing efficiency
100
80
60
40
20
0
HeLa
TetHeLaG2m

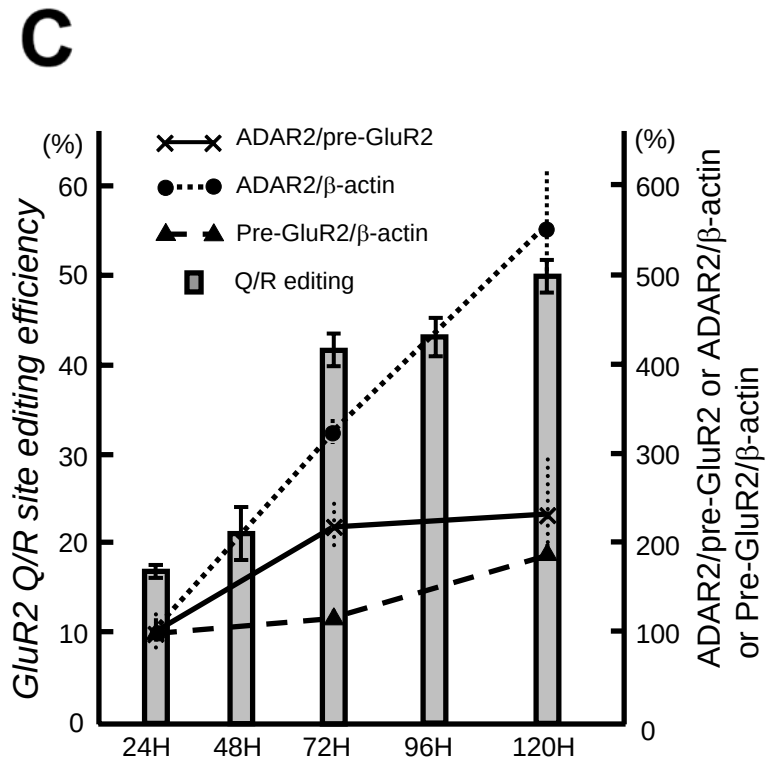
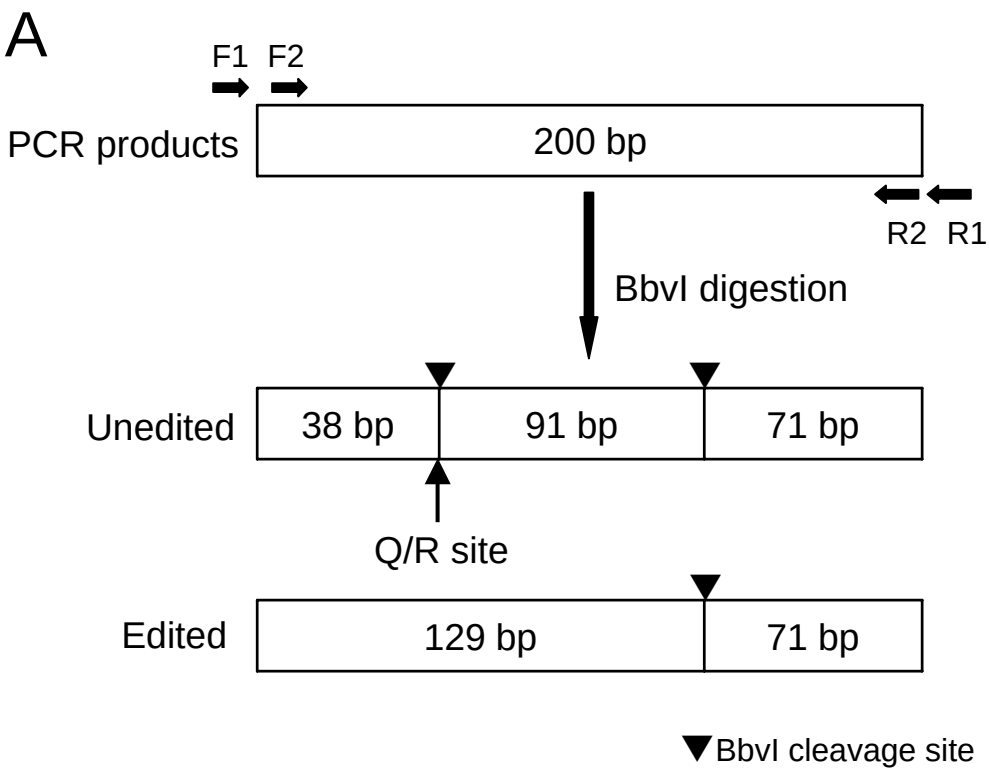

C
(%)
ADAR2/pre-GluR2
ADAR2/β-actin
Pre-GluR2/β-actin
Q/R editing
GluR2 Q/R site editing efficiency
60
50
40
30
20
10
0
(%)
600
500
400
300
200
100
0
ADAR2/pre-GluR2 or ADAR2/β-actin
or Pre-GluR2/β-actin
24H
48H
72H
96H
120H

Figure 2


A
F1
F2
PCR products
200 bp
R2
R1
BbvI digestion
Unedited
38 bp
91 bp
71 bp
Q/R site
Edited
129 bp
71 bp
BbvI cleavage site

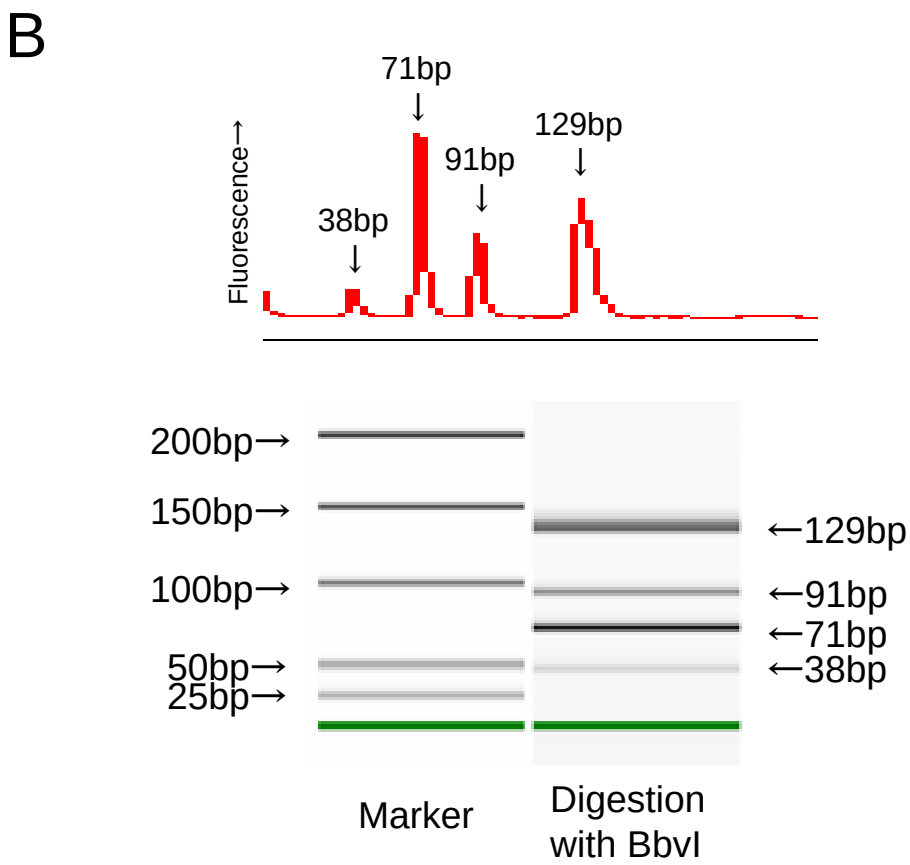

B
71bp
129bp
91bp
38bp
Fluorescence
200bp
150bp
100bp
50bp
25bp
129bp
91bp
71bp
38bp
Marker
Digestion with BbvI

Figure 3

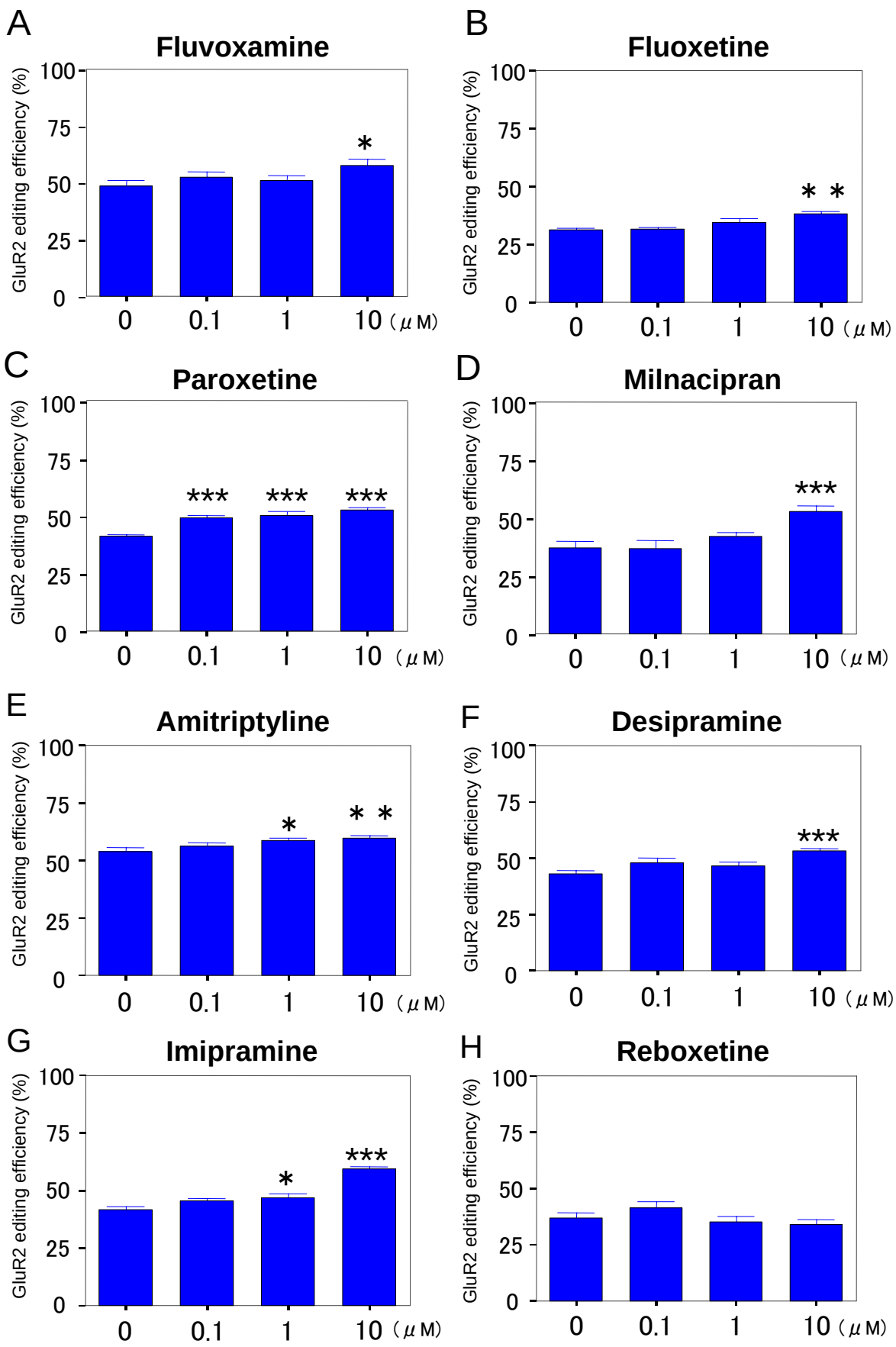
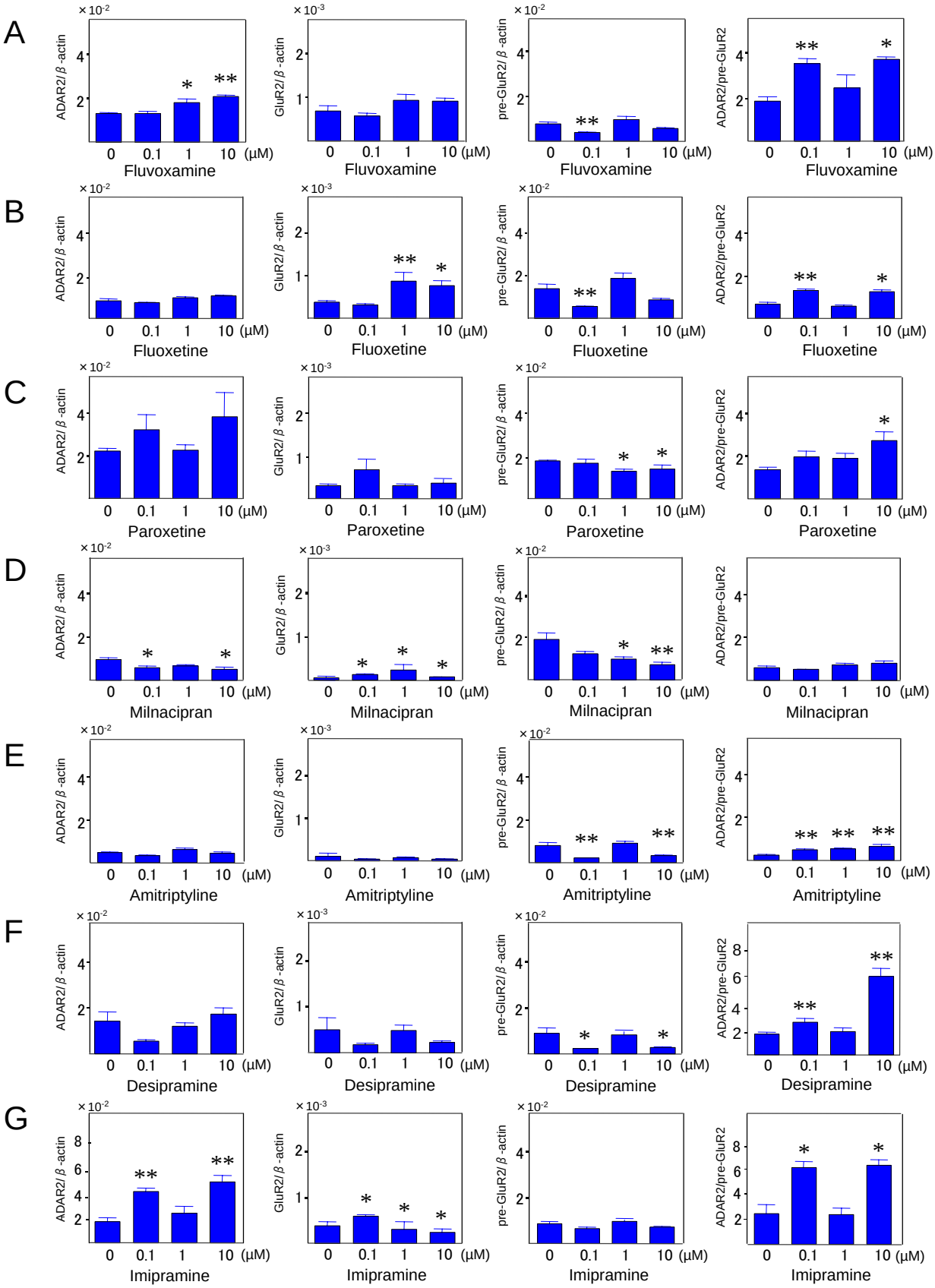

A
Fluvoxamine
B
Fluoxetine
C
Paroxetine
D
Milnacipran
E
Amitriptyline
F
Desipramine
G
Imipramine
H
Reboxetine
GluR2 editing efficiency (%)
100
75
50
25
0
0
0.1
1
10 (μM)
*
* *
***

Figure 4


A
B
C
D
E
F
G
ADAR2/β-actin
GluR2/β-actin
pre-GluR2/β-actin
ADAR2/pre-GluR2
0 0.1 1 10 (μM)
Fluvoxamine
Fluoxetine
Paroxetine
Milnacipran
Amitriptyline
Desipramine
Imipramine

Supplementary Materials and Methods, Results, and Tables

Supplementary Materials and Methods

Cell cultures of N1E-115 and NSC34.

N1E-115 cells (CRL-2263; American Type Culture Collection) and NSC34 cells (Cellutions Biosystems, Inc.) were seeded at 1×10^7 cells/well in 10 cm plastic wells. The N1E-115 cells and NSC34 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin-streptomycin, and 2 mM L-glutamine, and Dulbecco's modified Eagle's medium (DMEM) plus 10% fetal bovine serum, respectively, and incubated in a 5% CO_2 atmosphere. After growing to confluence, cells were plated at 2×10^6 cells/well in 6-well plates.

RNA extraction, reverse transcription, and following nested PCR and restriction digestion from these cell lines were performed as described in the Materials and Methods.

Supplementary Results

GluR2 Q/R site-editing of N1E-115 and NSC34 cells

The editing of GluR2 Q/R site of N1E-115 cells was 100% (n = 6) and that of NSC34 cells was 72 ± 5.3 % (n = 5). The editing levels of GluR2 Q/R site of these cells are too high to evaluate the drugs which promote editing of this site.