Tricyclic Antidepressant Amitriptyline Activates Fibroblast Growth Factor Receptor Signaling in Glial Cells

IN INVOLVEMENT IN GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR PRODUCTION*

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Recently, both clinical and animal studies demonstrated neuronal and glial plasticity to be important for the therapeutic action of antidepressants. Antidepressants increase glial cell line-derived neurotrophic factor (GDNF) production through monoamine-independent protein-tyrosine kinase, extracellular signal-regulated kinase (ERK), and cAMP responsive element-binding protein (CREB) activation in glial cells (Hisaoka, K., Takebayashi, M., Tsuchioka, M., Maeda, N., Nakata, Y., and Yamawaki, S. (2007) J. Pharmacol. Exp. Ther. 321, 148–157; Hisaoka, K., Maeda, N., Tsuchioka, M., and Takebayashi, M. (2008) Brain Res. 1196, 53–58). This study clarifies the type of tyrosine kinase and mechanism of antidepressant-induced GDNF production in C6 glioma cells and normal human astrocytes. The amitriptyline (a tricyclic antidepressant)-induced ERK activation was specifically and completely inhibited by fibroblast growth factor receptor (FGFR) tyrosine kinase inhibitors and siRNA for FGFR1 and -2. Treatment with amitriptyline or several different classes of antidepressants, but not non-antidepressants, acutely increased the phosphorylation of FGFRs and FGFR substrate 2α (FRS2α). Amitriptyline-induced CREB phosphorylation and GDNF production were blocked by FGFR-tyrosine kinase inhibitors. Therefore, antidepressants activate the FGFR/FRS2α/ERK/CREB signaling cascade, thus resulting in GDNF production. Furthermore, we attempted to elucidate how antidepressants activate FGFR signaling. The effect of amitriptyline was inhibited by heparin, non-permeant FGF-2 neutralizing antibodies, and matrix metalloproteinase (MMP) inhibitors. Serotonin (5-HT) also increased GDNF production through FGFR2 (Tsuchioka, M., Takebayashi, M., Hisaoka, K., Maeda, N., and Nakata, Y. (2008) J. Neurochem. 106, 244–257); however, the effect of 5-HT was not inhibited by heparin and MMP inhibitors. These results suggest that amitriptyline-induced FGFR activation might occur through an extracellular pathway, in contrast to that of 5-HT. The current data show that amitriptyline-induced FGFR activation might occur by the MMP-dependent shedding of FGFR ligands, such as FGF-2, thus resulting in GDNF production.

Brain imaging studies have revealed that both the hippocampus and prefrontal cortex undergo selective volume reduction in major depressive disorder (MDD). One of the most consistent findings, associated with the volume reduction, in post-mortem studies of MDD is a decrease in the density and number of glia in several cortical areas (1). The decreases in glial density are accompanied by a reduction of astrocytic markers, such as glial fibrillary acidic protein and glutamine synthetase (1, 2), thus suggesting that glia, especially astrocytes might be involved in the pathophysiology of MDD.

One of the major role of astrocytes is the production of neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor, fibroblast growth factor (FGF), and glial cell line-derived neurotrophic factor (GDNF), which support neurogenesis, gliogenesis, development, plasticity, and survival (3).

GDNF, a member of the transforming growth factor-beta superfamily, was originally purified from a rat glial cell line supernatant as a trophic factor for midbrain dopamine neurons and was later found to have pronounced effects on other neuronal populations and glia (4). GDNF improves cognitive function (5, 6) while also inhibiting drug-induced dependence (7). These results suggest that GDNF plays a crucial role in not only neuronal development but also neuronal and glial plasticity in higher-ordered brain function.

A growing body of evidence suggests that GDNF as well as brain-derived neurotrophic factor is involved in the pathophysiology of MDD (8–12). GDNF has been shown to decrease in the peripheral blood of patients with MDD (8). In addition, the decreased blood level of GDNF in MDD has been reported to increase after antidepressant treatment (13). We have also previously shown that antidepressants increase the GDNF production in C6 glioma cells (C6 cells), rat astrocytes, and normal human astrocytes (NHA) (14, 15). Treatment with antidepressants alters the GDNF levels in rodents in vivo and glial cell culture in vitro (16–18). These findings suggest that an increase of GDNF production may be involved in the therapeutic effect

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2 The abbreviations used are: MDD, major depressive disorder; GDNF, glial cell line-derived neurotrophic factor; NHA, normal human astrocytes; CREB, cAMP-responsive element-binding protein; HSD, honest significant difference; Trk, tropomyosin-related kinase; FGFR, FGF receptor; FRS2α, FGFR substrate 2α; MMP, matrix metalloproteinase; nAb, neutralizing antibody; F, F-ratio.
for MDD. Therefore, understanding of the mechanism of GDNF production in response to antidepressants in glial cells might thus provide some novel insights into the treatment of MDD (19).

The monoamine-independent acute activation of protein-tyrosine kinase, extracellular signal-regulated kinase (ERK), and cAMP-responsive element-binding protein (CREB) signaling cascade by antidepressants plays a crucial role in GDNF production in glial cells. In fact, amitriptyline treatment increases the phosphorylation of several phosphotyrosine-containing proteins (15). Therefore, protein-tyrosine kinase seems to play an important role in GDNF production by antidepressants. However, the specific type of protein-tyrosine kinase involved the effect of antidepressants and the mechanism of protein-tyrosine kinase activation by antidepressants remain unknown (15, 20). This study attempts to clarify the type of protein-tyrosine kinase and elucidate its precise mechanism of GDNF production by antidepressants.

EXPERIMENTAL PROCEDURES

Reagents—Reagents were obtained from the following sources: amitriptyline, desipramine, diazepam, and haloperidol (Wako Pure Chemical Industries, Ltd., Osaka, Japan); AG1478, GM6001, and GM6001 negative control (Merck KGaA, Darmstadt, Germany); K252a, heparin, o-phenanthroline, ouabaine, fluoxetine, and anti-fibroblast growth factor-1 (FGF-1 nAb, developed in rabbit; Sigma); clopinarin, GM6001, GM6001 negative control, PD173074, SU5402, and (Wako Pure Chemical Industries, Ltd., Osaka, Japan); AG1478, Technology, Beverly, MA).

Western Blotting—Western blotting has been described previously (15, 21). Western blotting was performed with antibodies as follows: phospho-FGFR receptor (Tyr-653/654) antibody, phospho-FRS2α (Tyr-196) antibody, phospho-p44/42 MAPK (Erk1/2) (Thr-202/Tyr-204) antibody (Cell Signaling Technology), Flg (C-15) antibody, Bek (C-17) antibody, actin (C-2) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA), FRS2α (SNT-1) antibody (Sigma). Cells were collected by using ice-cold phosphate-buffered saline and solubilized in the sample buffer (100 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS). The total amounts of proteins in each sample were adjusted to the same amount. After the addition of 1,4-dithiothreitol, the samples were boiled for 5 min. The proteins were separated by SDS-polyacrylamide gel electrophoresis and transblotted to polyvinylidine difluoride membranes. The membranes were blocked with 5% (w/v) BSA or skim milk for 6 h at 4 °C and incubated with respective antibodies overnight at 4 °C. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody for 1 h at room temperature. Chemiluminescent detection was performed using Immun-Star WesternCTM Kit (Bio-Rad), and the net intensities of each band were quantified using ChemiDocTM XRS+ (Bio-Rad).

RNA Interference—The transfection of small interfering RNA (siRNA) has been described previously (21). Duplexed RNA oligonucleotides for FGFR1 (FGFR1 siRNA: Fgfr1 Stealth select RNAi (RSS330462)) and FGFR2 (FGFR2 siRNA: Fgfr2 Stealth select RNAi (RSS302936)) were designed and synthesized by Invitrogen. Control siRNA (Non-Targeting siRNA #5; Thermo Fisher Scientific, Lafayette, CO) was used as a negative control. In brief, the C6 cells plated on 6-well plates were transfected with the corresponding siRNA using Lipofectamine RNAi MAX (Invitrogen) according to the manufacturer’s instructions. In these experiments, cells were used 48 h after transfection. The efficiency of FGFR knockdown was determined by Western blotting and real-time RT-PCR assay.

RNA Isolation—For the collection of total RNA, the cells were cultured at a density of 1.6 × 10⁵/cm² on a 6-well plate with 3 ml of growth medium. After drug treatment, total RNA was isolated using an RNaseasy Mini kit (Qiagen, Valencia, CA) following the manufacturer’s protocols. RNA quantity and purity were determined with the Multi-Spectrophotometer (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan).

Real-time RT-PCR Assay—The real-time RT-PCR assay has been described previously (15). In brief, the first-strand cDNA was synthesized from 500 ng of total RNA by using a RNA PCR kit (AMV) Version 3.0 (Takara Bio Inc., Otsu, Japan). Real-time quantitative PCR was performed using the SmartCycler® system (Cepheid, Sunnyvale, CA) with TaqMan probes and primers for rat GDNF, FGFR1, FGFR2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Applied Biosystems, Foster City, CA). The mRNA levels were normalized for GAPDH mRNA in the same samples by the 2(-ΔΔC(T)) method, which is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments (22).

ELISA—For the assay of GDNF release or FGFR-2 shedding, C6 cells were cultured at a density of 1.3 × 10⁵/cm² on a 12-well plate with 0.5 ml of growth medium. After drug treatment,
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GDNF or FGF-2 protein levels in cell-conditioned media were determined using a GDNF Emax® ImmunoAssay System (Promega, Madison, WI) or a human FGF basic Quantikine HS ELISA kit (R&D Systems) according to the manufacturer's instructions.

Data Analysis—Statistical tendencies were analyzed using the SPSS software package (SPSS, Chicago, IL). The results are expressed as the means ± S.E. One-way analysis of variance was used in most cases to check statistical tendencies. Differences between the groups were analyzed by Tukey’s honest significant difference (HSD), Dunnett’s, or Games-Howell test. Differences between the two groups were analyzed by Student’s t test. The significance level was set at \( p < 0.05 \).

RESULTS

Effects of Tyrosine Kinase Inhibitors on the Amitriptyline-induced ERK Activation—Genistein, a general tyrosine kinase inhibitor, inhibited the amitriptyline-induced ERK activation and the following GDNF production (15). Actually, treatment with amitriptyline increased the phosphorylation levels of a number of phosphotyrosine-containing proteins in C6 cells (15). Selective inhibitors of tyrosine kinase were used to identify which types of protein-tyrosine kinase are involved in the effect of amitriptyline. SU5402 and PD173074 (FGFR inhibitors) completely inhibit the ERK activation induced by amitriptyline treatment in C6 cells. However, K252a (tropomysosin-related kinase (Trk) inhibitor) or AG1478 (epidermal growth factor (EGF) receptor inhibitor) had no effect (Fig. 1A). FGFR inhibitors alone have no effect on basal levels of ERK activity (21). FGFR inhibitors, but not Trk or EGF receptor inhibitors, also selectively and completely block the amitriptyline-induced ERK activation in NHA (Fig. 1B).

Effects of FGFR1 or FGFR2 Knockdown on the Amitriptyline-induced ERK Activation—The mammalian FGFR family consists of a group of four transmembrane proteins with intrinsic tyrosine kinase activity (FGFR1-FGFR4) (23). C6 cells express FGFR1 (145 kDa (glycosylated mature type) and 120 kDa (unglycosylated immature type)) and FGFR2 (100 kDa) (21). Therefore, the specific siRNAs were used for FGFR1 and FGFR2 knockdowns. The transfection of FGFR1 siRNA largely reduced the protein level of both 145- and 120-kDa forms of FGFR1 (20.2 ± 7.6 and 21.6 ± 7.4% of basal, respectively, \( p < 0.001 \)). The transfection of FGFR2 siRNA significantly reduced the protein level of FGFR2 (61.5 ± 2.4% of basal; \( p < 0.05 \)). Control siRNA did not affect the expression levels of FGFR1 and FGFR2 for up to 100 nm at 48 h after transfection (FGFR1 145 kDa, 114.7 ± 4.5% of basal; FGFR1 120 kDa, 112.6 ± 2.4% of basal; FGFR2 100.9 ± 13.2% of basal) (Fig. 1C). We also confirmed that siRNA for individual FGFR caused similar reductions at the mRNA level (FGFR1 mRNA, 59.0 ± 10.0% (\( p < 0.01 \)) of basal; FGFR2 mRNA, 77.6 ± 8.2% (\( p < 0.05 \)) of basal). The amitriptyline-induced ERK activation was significantly blocked by either FGFR1 siRNA or FGFR2 siRNA transfection, whereas control siRNA had no affect up to 100 nm (Fig. 1D).

The knockdown of FGFR1 is much more effective but only partially decreases the ERK activation by amitriptyline, whereas the knockdown of FGFR2 completely blocks the activation of ERK even though the protein is only reduced to 60% of the basal level. The transfection of FGFR1 siRNA increased FGFR2 mRNA expression (124.8 ± 8.1% (\( p < 0.05 \)) of basal), whereas transfection of FGFR2 siRNA had no effect on FGFR1 mRNA expression (98.2 ± 12.9% of basal). The adverse effect of FGFR1 siRNA on FGFR2 expression may attenuate the effect of FGFR1 siRNA transfection on the ERK activation by amitriptyline.

Amitriptyline Treatment Increased Phosphorylation of FGFRs and FRS2α—The amitriptyline-induced ERK activation was inhibited by specific inhibitors or siRNA for FGFRs. These results suggest the possibility that amitriptyline treatment induces FGFRs activation. Therefore, the effect of amitriptyline on FGFRs phosphorylation was examined by using phospho-FGFR (Tyr-653/654) antibody, which recognizes phosphorylated FGFR1 (145 and 120 kDa), and FGFR2 (100 kDa) (21). The phosphorylation level of all FGFR subtypes significantly increased after 5 min of amitriptyline treatment. The levels of total FGFRs did not change after 60 min of amitriptyline treatment (Fig. 2A).

Experiments confirmed that amitriptyline treatment increased FGFR phosphorylation. However, the level of FGFR phosphorylation by amitriptyline (25 μM) treatment was relatively low (about 1.5-fold increase) in comparison to the level induced by FGF-2 (10 ng/ml) treatment (about 4-fold increase) (21), and the standard deviation was high. Therefore, because of the sensitivity of the antibody, it was difficult to characterize the amitriptyline-induced FGFR phosphorylation in detail. Therefore, the effect of amitriptyline on FRS2α phosphorylation was examined by using phospho-FRS2α antibody, which is a member of FRS family of lipid-anchored docking protein, was examined as an alternative index of FGFR activation. FRS2α is the primary substrate for the FGFR kinase and links the FGFR and their Ras/MAP kinase signaling cascades (24, 25). A valine-threonine motif encoded by alternatively spliced sequences in the intracellular juxtamembrane domain of FGFR1 and FGFR2 is important for association with FRS2α (26, 27). The phosphorylation of FRS2α induced by amitriptyline treatment significantly increased after 5 min of treatment as well as FGFRs phosphorylation and sustained an increase at 60 min in C6 cells. The amount of total FRS2α did not change after 60 min of amitriptyline treatment (Fig. 2B).

FRS2α also plays a role in mediating the intracellular signals that are generated at the cell surface by activation of the NGF or EGF receptors (27, 28). The effects of inhibitors for FGFR, Trk, or EGF receptor were examined to confirm whether the amitriptyline-induced FRS2α phosphorylation is selectively mediated by FGFRs. As expected, the amitriptyline or FGF-2-induced FRS2α phosphorylation was selectively inhibited by SU5402 and PD173074 (FGFR inhibitors) in C6 cells. However, K252a (Trk inhibitor) or AG1478 (EGF receptor inhibitor) did not have any effect (data not shown). These results confirmed that FRS2α phosphorylation selectively indicated the activation of FGFR.

NHA express FGF-2 and FGF-2 (29). Acute treatment (5 min) with amitriptyline (25 μM) or FGF-2 (10 ng/ml) also increased the phosphorylation of FRS2α in NHA (amitriptyline, 142.5 ± 10.4% (\( p < 0.01 \)); FGF-2, 200.6 ± 30.7% (\( p < 0.05 \)) of basal, respectively).
Different Classes of Antidepressants Increased FRS2α Phosphorylation—The effects of several different classes of antidepressants and non-antidepressant drugs, including amitriptyline, clomipramine, nortriptyline, and desipramine (tricyclic antidepressants), mianserin (a tetracyclic antidepressant), fluvoxamine, and fluoxetine (selective 5-HT reuptake inhibitors (SSRI)), haloperidol (an antipsychotic-D2-dopamine receptor antagonist), and diazepam (a benzodiazepine) were examined to determine the pharmacological specificity of antidepressants on FRS2α phosphorylation. All antidepressants (25 μM, 5 min of treatment) significantly increased FRS2α phosphorylation in C6 cells, but haloperidol and diazepam did not have any effect (Table 1). The multiplication of NHA is very limited because of normal astrocytes; therefore, it was not pos-
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Effects of antidepressants and non-antidepressant drugs on the FRS2α phosphorylation

C6 cells and NHA were treated with 25 μM of drugs for 5 min, and the phosphorylation level of FRS2α was measured. Data are expressed as the mean ± S.E. from 3–13 independent experiments.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>C6 cells (0.01)</th>
<th>NHA (0.001)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tricyclic antidepressant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>176.2 ± 13.1</td>
<td>196.1 ± 29.9</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>228.1 ± 7.86</td>
<td>149.5 ± 13.9</td>
</tr>
<tr>
<td>Desipramine</td>
<td>216.3 ± 32.4</td>
<td></td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>231.1 ± 24.7</td>
<td></td>
</tr>
<tr>
<td>Tetracyclic antidepressant</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mianserin</td>
<td>197.1 ± 29.0</td>
<td>180.3 ± 15.4</td>
</tr>
<tr>
<td>Selective serotonin reuptake inhibitor</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>216.5 ± 33.7</td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>210.3 ± 37.1</td>
<td></td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haloperidol</td>
<td>115.9 ± 24.4</td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>118.8 ± 24.8</td>
<td></td>
</tr>
</tbody>
</table>

*p < 0.01 (**), significantly different from the basal (Student’s t-test). *p < 0.05 (*), significantly different from the basal (Student’s t-test).

Possible to examine the same kinds of drugs that had been examined in C6 cells. However, several different classes of antidepressant increased FRS2α phosphorylation in NHA (Table 1).

Effects of FGFR Inhibitors on CREB Phosphorylation and GDNF Production Induced by Amitriptyline Treatment—Antidepressants increase CREB phosphorylation and cAMP-responsive element-mediated GDNF mRNA expression in glial cells (20). The effects of FGFR inhibitors on the amitriptyline-induced CREB phosphorylation and GDNF production were examined. SU5402 and PD173074 significantly inhibited CREB phosphorylation, GDNF mRNA expression and GDNF release induced by amitriptyline treatment (Fig. 3). There was no effect on the basal level of CREB phosphorylation (SU5402 only, 42.6 ± 7.89%; PD173074 only, 49.0 ± 5.97% of control) and GDNF release (SU5402 only, 28.6 ± 8.9 pg/ml; PD173074 only, 3.7 ± 3.2 pg/ml) by inhibitors alone. A previous study confirmed that there was no effect on basal levels of GDNF mRNA by FGFR inhibitors alone (21).

Effects of Heparin on the Amitriptyline or 5-HT-induced ERK Activation—The results suggest that antidepressants acutely activate FGFRs and the subsequent intracellular signaling molecules (FRS2α, ERK, and CREB) and increase GDNF production. 5-HT increases GDNF production through 5-HT_{2} receptor-mediated FGFR2 transactivation, which does not involve FGFR ligands (21). We next attempted to elucidate whether the activation of FGFR signaling by amitriptyline is associated with FGFR ligands or not. The effect of heparin, a chelator of FGFR ligands that blocks binding of FGFR ligands to cell surface

Table 1: Effects of antidepressants and non-antidepressant drugs on the FRS2α phosphorylation
receptors, was examined (30, 31). We used the ERK activity as an index of FGFR activation to characterize the mechanism, because ERK is most sensitive among FGFR-related intracellular signaling molecules. The amitriptyline-induced ERK activation was dose-dependently and completely inhibited by heparin in C6 cells, whereas the 5-HT-induced ERK activation was not affected by heparin (Table 2). There was no effect on the basal levels of ERK activation by heparin (100 μg/ml) alone (19.2 ± 7.89% of control). Heparin also blocked the amitriptyline-induced ERK activation in NHA (Table 2). These results suggest that the heparin binding FGFR ligands are involved in the effect of amitriptyline. The mechanism of amitriptyline-induced FGFR activation thus seems to differ from that of 5-HT due to the involvement of the FGFR ligands.

### Effects of Neutralize Antibodies for FGFR Ligands on the Ami-triptylene-induced ERK Activation

The effect of specific non-inhibitory neutralizing antibodies for FGFR ligands was examined to clarify which kinds of FGFR ligands contribute to the effect of amitriptyline. C6 cells were pretreated with FGF-1 neutralizing antibody (FGF-1 nAb), two different types (monoclonal or polyclonal) of FGF-2 neutralizing antibodies (FGF-2 nAb (monoclonal) and FGF-2 nAb (polyclonal)), or negative control IgG (mouse IgG and goat IgG). Both FGF-2 nAbs significantly inhibited ERK activation by amitriptyline, whereas FGF-1 nAb and negative control IgG had no effect (Table 3). In NHA, FGF-2 nAb (polyclonal) also significantly inhibited ERK activation by the amitriptyline treatment (Table 3).

### Amitriptyline, but Not 5-HT Treatment, Increases FGF-2 Shedding in C6 Cells

We next measured the levels of FGF-2 in the media using a very sensitive FGF-2 ELISA kit. Acute amitriptyline treatment (2 min) dose-dependently and significantly increased the level of FGF-2 in the media, although the increase in the FGF-2 level was small. In contrast, 5-HT treatment did not affect the level of FGF-2 in the media at all (Table 4). We tried to increase the sensitivity of FGF-2 detection in the media by combining a FGFR1 antibody and FGFR inhibitors that block the receptor-mediated internalization of FGF-2; however, no improvement was observed. The effect of heparin and the
Amitriptyline Increases GDNF Production through FGFR

TABLE 3

Effects of neutralize antibodies on the amitriptyline-induced ERK activation

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>C6 cells</th>
<th>NHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>38.4 ± 6.33</td>
<td>61.6 ± 4.89</td>
</tr>
<tr>
<td>Control (Amitriptyline only)</td>
<td>100.0 ± 1.86</td>
<td>100.0 ± 3.54</td>
</tr>
<tr>
<td>+FGF-1 nAb</td>
<td>96.8 ± 18.0</td>
<td>97.7 ± 12.6</td>
</tr>
<tr>
<td>+FGF-2 nAb (monoclonal)</td>
<td>77.2 ± 7.42</td>
<td>71.9 ± 5.44</td>
</tr>
<tr>
<td>+FGF-2 nAb (polyclonal)</td>
<td>48.6 ± 4.74</td>
<td>71.9 ± 5.44</td>
</tr>
<tr>
<td>+Mouse IgG</td>
<td>91.3 ± 18.3</td>
<td>93.0 ± 7.82</td>
</tr>
</tbody>
</table>

% of control

* p < 0.001 in comparison to the basal group (Tukey's HSD test).

** p < 0.05 in comparison to the control group (Tukey's HSD test).

*** p < 0.01 in comparison to the control group (Tukey's HSD test).

TABLE 4

Effects of MMP Inhibitors or 5-HT on FGF-2 shedding in C6 cells

<table>
<thead>
<tr>
<th>Treatments</th>
<th>PG/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>2.51 ± 0.577</td>
</tr>
<tr>
<td>Amritriptyline (25 μM)</td>
<td>4.21 ± 1.01</td>
</tr>
<tr>
<td>Amritriptyline (50 μM)</td>
<td>9.92 ± 5.80</td>
</tr>
<tr>
<td>Basal</td>
<td>1.96 ± 0.422</td>
</tr>
<tr>
<td>5-HT (10 μM)</td>
<td>2.23 ± 0.694</td>
</tr>
<tr>
<td>5-HT (100 μM)</td>
<td>1.07 ± 0.540</td>
</tr>
</tbody>
</table>

p < 0.05 in comparison to the basal group (Dunnnett's test).

neutralizing antibodies demonstrate that the shedding of FGF-2, at least in part, might be involved in the effect of amitriptyline.

Effects of MMP Inhibitors or Na/K-ATPase Inhibitor on the Amitriptyline-induced ERK Activation—Unlike many other growth factors, FGF-2 lacks a classic peptide sequence for its secretion, and the mechanisms for its release remain controversial. Therefore, different mechanisms for FGF-2 release have been proposed, including mechanical strain, membrane disruption, Na/K-ATPase, and protease (32, 33). As a result of a lactate dehydrogenase assay, amitriptyline treatment does not induce nonspecific protein release due to cell damage (15). MMPs are involved in the shedding of plasma membrane-bound FGF-2 from its membrane anchor (34, 35). Therefore, cells were pretreated with Na/K-ATPase inhibitor (ouabine) or broad-spectrum inhibitors of MMP (o-phenanthroline, phosphoramidon, and GM6001) before treatment with amitriptyline. The amitriptyline-induced ERK activation was significantly reduced by MMP inhibitors, whereas Na/K-ATPase inhibitor or GM6001 negative control did not have effect in C6 cells (Fig. 4A). There was no effect on basal levels of ERK by inhibitors alone (o-phenanthroline, 21.3 ± 5.54%; phosphoramidon, 67.7 ± 9.38%; GM6001, 20.4 ± 7.47% of control, respectively). The amitriptyline-induced ERK activation in NHA was also inhibited by MMP inhibitor (basal, 36.7 ± 7.5%; amitriptyline only (25 μM for 5 min), 100.0 ± 1.3% (p < 0.001 in comparison to basal); amitriptyline + o-phenanthroline (200 μM), 41.5 ± 10.9% (p < 0.001 in comparison to amitriptyline only); F (2.17) = 25.02, p < 0.001). These results suggest the shedding of FGF-2 by MMP, thus leading to FGFR activation, may be involved in the effect of amitriptyline.

Effects of MMP Inhibitors on the 5-HT- or FGF-2-induced ERK Activation—Treatment with amitriptyline seems to activate MMPs in glial cells. The effect of MMP inhibitors (GM6001 and o-phenanthroline) on the 5-HT- or FGF-2-induced ERK activation was examined to determine whether MMP activation by amitriptyline occurred before or after FGFR activation. The 5-HT- or FGF-2-induced ERK activation was not inhibited by MMP inhibitors (Fig. 4, B and C). In addition, the amitriptyline-induced FRS2α phosphorylation was inhibited by MMP inhibitors, whereas the FGF-2-induced FRS2α phosphorylation was not affected by MMP inhibitors (data not shown). These results suggest that the involvement of MMP activation by amitriptyline occur upstream of FGFR activation (Fig. 6).

Effects of MMP Inhibitor on GDNF Production Induced by Amitriptyline Treatment—The effects of GM6001 or GM6001 negative control on GDNF release by amitriptyline treatment were investigated. GM6001, but not GM6001 negative control, significantly inhibited the GDNF release induced by amitriptyline treatment (Fig. 5). There was no effect on the basal level of GDNF release by inhibitor alone (GM6001, 33.0 ± 9.46 pg/ml).

DISCUSSION

The present study demonstrated that antidepressants used in this study acutely activate the FGFR/FRS2α/ERK/CREB signaling cascade through a monoamine-independent mechanism, finally resulting in GDNF production. The most significant finding of this study concerns the key role of FGFR on GDNF production by antidepressants. The amitriptyline-induced FGFR activation might be induced by MMP-dependent mobilization of FGFR ligands, such as FGF-2 from membrane store. The same signaling components (MMP, FGF-2, FGFR, and FR52a) in C6 cells were identified to mediate the effect of amitriptyline on ERK in NHA. In addition, different types of antidepressants, but not non-antidepressants, commonly increased FR52a phosphorylation as well as GDNF release. These results provide the first evidence for FGFR activation by antidepressants in glial cells.

The experiment with siRNAs for FGFR suggests that the effect of amitriptyline occurred through both of FGFR1 and FGFR2. In accordance with the data of siRNA (Fig. 1D), amitriptyline increased both of FGFR1 and FGFR2 phosphorylation. However, the current data suggest that FGFR1 and FGFR2 participate differently in the effect of amitriptyline. There are two mechanisms for the difference in the contribution of FGFR1 and FGFR2 on the effect of amitriptyline. 1) The increase in the FGFR2 expression after FGFR1 siRNA transfection may attenuate the effect of FGFR1 siRNA. This result suggests that FGFR2 might play a role that is complementary to FGFR1. FGFR heterodimerization as well as homodimerization is required for signal transduction (32). Therefore, the interaction between FGFR1 and FGFR2 seems to be important in the effect of amitriptyline. 2) The other FGFR ligands, which have high affinity for FGFR2, might be involved in the effect of amitriptyline. For example, FGF-9 has a high affinity for FGFR2.
rather than FGFR1 (32). Taken together, the interaction between FGFR1 and FGFR2 and the involvement of several FGFR ligands contribute to the complexity in FGF signaling activated by amitriptyline treatment.

The current data showed that the amitriptyline-induced FGFR activation occurs by FGFR ligands, such as FGF-2. The effect of amitriptyline was completely inhibited by heparin, whereas it was partially inhibited by FGF-2 nAbs. The mammalian FGF family consists of 22 ligands that are distributed throughout the central nervous system. FGF-1 and FGF-2 are expressed in C6 cells and NHA (29, 36, 37). FGF-9 has been detected in glial cells (38) and increases the GDNF expression.
Amitriptyline Increases GDNF Production through FGFR

FIGURE 5. Effect of MMP inhibitor on GDNF release induced by amitriptyline treatment. Effects of GM6001 and GM6001 negative control on the amitriptyline-induced GDNF release. C6 cells were pretreated with 25 μM GM6001 or GM6001 negative control (GM Nega) for 30 min and then were subsequently treated with 25 μM amitriptyline for 48 h. Values are expressed as the mean ± S.E. of released GDNF (pg/ml) (F (3.63) = 8.44, p < 0.001). ***, p < 0.001 in comparison to the basal group; +, p < 0.05 in comparison to the control group (Tukey’s HSD test).

in C6 cells (39). These results suggest that not only FGF-2, but also other heparin-sensitive ligands, such as FGF-9, might thus be involved in the effects of amitriptyline. The FGF ligands accumulated on cell surface heparin sulfate proteoglycans seem to play an important role in the effect of amitriptyline, because heparin and neutralizing antibodies trap FGF ligands on the outside of cell membrane (40). The level of FGF-2 in the media was barely detected even by the highly sensitive ELISA and slightly but significantly increased after amitriptyline treatment. Although the mechanisms for FGF-2 shedding remain controversial, MMP is important for the effect of amitriptyline. We examined whether MMP inhibitors block FGF-2 shedding by amitriptyline treatment, but the effects were unclear, because the amount of FGF-2 shedding by amitriptyline was relatively small. However, the differential effect of MMP inhibitors on amitriptyline or FGF-2-induced ERK activation suggests that amitriptyline seems to activate FGFR through MMP activation (Figs. 4, A and C, and 6). Furthermore, amitriptyline does not seem to bind directly to FGFR. Further studies investigating the overexpression of GFP-tagged FGF-2 (41) may elucidate how antidepressant induces FGF-2 shedding clearly.

Although the data showed the involvement of MMP in the effect of amitriptyline, the specific type of MMP and the precise mechanism of MMP activation by amitriptyline remains unknown. MMPs are a family of zinc-dependent endopeptidases with 24 identified members (42). There are reports showing that C6 cells express MMP-2, -9, -13, and membrane-type1 MMP (43, 44). GM6001 inhibits MMP-1, -2, -3, -8, and -9 (45). Therefore, MMP-2 or -9 might be involved in the effect of amitriptyline. There are two reports of MMPs-dependent FGFR activation (34, 35). The activation of μ-opioid receptor in C6 cells induces FGFR transactivation through α-phanenanthrolione and phosphoramide-sensitive MMP (34). The activation of EP3 subtype receptor by prostaglandin E2 in microvascular endothelial cells induces the intracellular activation of c-Src, the activation of MMP (predominantly MMP2), which in turn causes the mobilization of membrane-anchored FGF-2 and results in FGFR activation (35). The activity of MMPs is regulated by Ca2+, PKC, Src, Pyk2, and GTP-binding protein, depending on the cell type (46). Ca2+, PKC, and opioid receptors were not found to be involved in the mechanism of amitriptyline-induced ERK activation (15). Therefore, the precise mechanism of MMPs activation by antidepressants is currently being investigated in detail.

Both amitriptyline- and 5-HT-induced GDNF production were inhibited by FGFR and MEK inhibitors. These results suggest that the amitriptyline and 5-HT signaling to ERK share certain features, but they seem to differ in the mechanism of FGFR activation. The current results showed that amitriptyline activates both of FGFR1 and FGFR2 through an extracellular pathway, with the mobilization of FGFR ligands from a membrane store by MMP. In contrast, 5-HT selectively activates FGFR2 through an intracellular pathway that is regulated by Src family-tyrosine kinase and stabilized microtubules (21). FGFR is at the point of convergence of these two pathways and crucial for GDNF production. These differences in the mechanism of FGFR activation between amitriptyline and 5-HT confirmed that the effect of amitriptyline on ERK activation, after GDNF production, thus occurred through a monoamine-independent pathway (Fig. 6). It is noteworthy that the amitriptyline-induced actions occur independently of the actions of amitriptyline on serotonin reuptake transporter (SERT) because C6 cells lack SERT (47). Perhaps more importantly, these data suggest multiple sites of action for amitriptyline (and perhaps other antidepressants). Amitriptyline, but no other antidepressants, interacts directly with both TrkA and TrkB receptors (48). C6 cells express both TrkA and TrkB receptors (49); however, Trk inhibitor (K252a) had no effect on the amidriptyline-induced GDNF release.

**FIGURE 6. Proposed mechanism of antidepressant on FGFR activation and after GDNF production in glia.** HSPG, heparan sulfate proteoglycans; α-Phe, α-phanenanthrolione; Phospo, phosphoramidon.

tyline-induced ERK activation in C6 cells and NHA (Fig. 1, A and B). These results exclude the possibility of the involvement of Trk receptors in the effect of amitriptyline. Several different classes of antidepressants also increased GDNF production after the activation of FRS2α/ERK/CREB signaling cascade, although these antidepressants differ in structure and have no information about a different common target other than monoamine transporters. Therefore, it is likely that several presynaptic and postsynaptic molecular cascades contribute to the antidepressant effect (50) and that the GDNF production after FGFR activation results from one of these pathways.

The involvement of the FGF system is beginning to emerge from clinical and basic research studies for MDD. The FGF family has been shown to be down-regulated in the cortex and hippocampus in post-mortem studies of individuals with MDD (51, 52). On the other hand, both acute and chronic administrations of FGFR ligands have antidepressant treatment in glial cells (39, 54, 55), which is consistent with the current findings. Therefore, antidepressants activate both the FGF and GDNF systems in glial cells, and multiple neurotrophic/growth factor systems seem to be cooperatively involved in the therapeutic effect of antidepressants. Clarifying the monoamine-independent novel target of antidepressants in glial cells might, therefore, contribute to the development of more efficient treatment for MDD.

REFERENCES
39. Lázár, I., Szabó, T., Marincsák, R., Kovács, L., Blumberg, P. M., and Biró, T.
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Tricyclic Antidepressant Amitriptyline Activates Fibroblast Growth Factor Receptor Signaling in Glial Cells: INVOLVEMENT IN GLIAL CELL LINE-DERIVED NEUROTROPHIC FACTOR PRODUCTION
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