Tricyclic antidepressant amitriptyline activates fibroblast growth factor receptor (FGFR) signaling in glial cells: Involvement in glial cell line-derived neurotrophic factor (GDNF) production

Kazue Hisaoka1,2, Mami Tsuchioka1, Ryoya Yano1,2, Natsuko Maeda1, Naoto Kajitani1,2, Norimitsu Morioka2, Yoshihiro Nakata2 and Minoru Takebayashi1,3

1Division of Psychiatry and Neuroscience, Institute for Clinical Research, 3Department of Psychiatry, National Hospital Organization (NHO) Kure Medical Center and Chugoku Cancer Center, 3-1 Aoyama, Kure 737-0023, Japan, 2Department of Pharmacology, Hiroshima University Graduate School of Biomedical Sciences, 1-2-3 Kasumi, Minami-ku, Hiroshima 734-8553, Japan

Address correspondence to: Minoru Takebayashi, MD, PhD. Department of Psychiatry, NHO Kure Medical Center and Chugoku Cancer Center, 3-1 Aoyama, Kure 737-0023, Japan. E-mail: mtakebayashi@kure-nh.go.jp, Phone: +81-823-22-3111, Fax: +81-823-21-0478

Running title: Amitriptyline increases GDNF production through FGFR

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 (PTK), extracellular signal-regulated kinase (ERK) and cAMP responsive element binding protein (CREB) activation in glial cells (Hisaoka et al., 2007, 2008). 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 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 et al., 2008); 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 postmortem 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 (GFAP) 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 (BDNF), 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 (TGF)-β super family, 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 the 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 BDNF 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 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 (PTK), 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, PTK seems to play an important role in GDNF production by antidepressants. However, the specific type of PTK involved the effect of antidepressants, and the mechanism of PTK activation by antidepressants remain unknown (15,20). This study attempts to clarify the type of PTK 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, GM6001 negative control, PD173074, SU5402 and genistein (Merck KGaA, Darmstadt, Germany); K252a, heparin, o-phenanthroline, ouabine, fluoxetine and anti-fibroblast growth factor-1 (FGF-1 nAb: developed in rabbit; Sigma-Aldrich Co., St. Louis, MO); clomipramine (Nihon Chiba-Geigy K.K. Tokyo, Japan); FGF-2 human recombinant (Roche Diagnostics, Indianapolis, IN); phosphoramidon (BIOMOL International L.P., Philadelphia, PA); anti-FGF-2 (neutralizing), clone bFM-1 (FGF-2 nAb, monoclonal antibody, developed in mouse; Up state, Temecula, CA); anti-FGF-2 antibody (FGF-2 nAb, polyclonal antibody, developed in...
goat), normal goat IgG, mouse IgG sub isotype control (R&D Systems, Minneapolis, MN).

**Cell culture**

The cultures of C6 cells have been described previously (15). In brief, C6 cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen, Carlsbad, CA) supplemented with 2 mM L-glutamine and 5% fetal bovine serum (Sigma-Aldrich Co.) in a 5% CO₂ humidified atmosphere. Normal human astrocytes (NHA), derived from fetal tissue (male, 18 weeks), were purchased from Cambrex (Walkersville, MD), and grown in ABM™ (Cambrex) in a 5% CO₂ humidified atmosphere. More than 80% of NHA expressed GFAP. For drug treatment, the medium was replaced with serum-free Opti-MEM (Invitrogen) containing 0.5% bovine serum albumin (Sigma-Aldrich Co.), and the cells were incubated for 24 h, and then the cells were treated with various drugs of interest.

**Extracellular signal-regulated kinase (ERK) activity assay**

The ERK activity assay has been described previously (15). In brief, C6 cells were cultured at a density of $1.6 \times 10^5$ cells/cm² on a 6-well plate with 3 mL of growth medium. NHA were then cultured at a density of $0.8 \times 10^5$ cells/cm² on a 6-well plate with 2 mL of growth medium. After drug treatment, the cells were collected in a cell lysis buffer. The total amount of protein in each sample was adjusted to the same amount. The ERK activities were determined using an assay kit according to the manufacturer’s instructions (Cell Signaling Technology, Beverly, MA).

**Western blotting**

Western blotting has been described previously (15,21). Western blotting was performed with antibodies as follows: phospho-FGF receptor (Tyr653/654) antibody, phospho-FRS2α (Tyr196) antibody, phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) 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-Aldrich Co.). Cells were collected by using ice-cold phosphate-buffered saline (PBS) 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 polyvinylidene 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 WesternC™ kit (Bio-Rad Laboratories, Inc., Hercules, CA), and the net intensities of each band were quantified using ChemiDoc™ XRS+ (Bio-Rad Laboratories, Inc.).

**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 knock down 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 x 10^5/cm^2 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) Ver.3.0 (Takara Bio Inc., Ohtsu, 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 glyceraldehydes-3-phosphate dehydrogenase (GAPDH; Applied Biosystems, Foster City, CA). The mRNA levels were normalized for GAPDH mRNA in the same samples by the 2(-delta delta C (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 FGF-2 shedding, C6 cells were cultured at a density of 1.3 x 10^5/cm^2 on a 12-well plate with 0.5 mL of growth medium. After drug treatment, 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 ± SEM. One-way ANOVA 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 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 PTK 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 [tropomyosin-related kinase (Trk) inhibitor] or AG1478 [epidermal growth factor receptor (EGFR) inhibitor] have no effect (Fig 1A). FGFR inhibitors alone have no effect on basal levels of ERK activity (21). FGFR inhibitors, but not Trk or EGFR inhibitors, also selectively and completely block the amitriptyline-induced ERK activation in NHA (Fig 1B).

**Effects of FGFR1 or FGFR2 knock down 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 2 knock downs. The transfection of FGFR1 siRNA largely reduced the protein level of both 145 and 120 kDa of FGFR1 (20.2 ± 7.6%***, 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%; FGFR1 120 kDa, 112.6 ± 2.4%; FGFR2 100.9 ± 13.2% of basal, respectively) (Fig 1C). We also confirmed that siRNA for individual FGFR caused similar reductions at the mRNA level (FGFR1 mRNA, 59.0 ± 10.0% **; FGFR2 mRNA, 77.6 ± 8.2% * of basal, respectively, *p<0.05, **p<0.01). The amitriptyline-induced ERK activation was significantly blocked by either FGFR1 siRNA or FGFR2 siRNA transfection, while 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, while 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% * of basal, *p<0.05), while 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 (Tyr653/654) 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 following 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, due to the sensitivity of the antibody, it was difficult to characterize the amitriptyline-induced FGFR phosphorylation in detail. Therefore, the effect of amitriptyline on FGFR substrate 2α (FRS2α), 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 following 60 min of amitriptyline treatment (Fig 2B).

FRS2α also plays 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 EGFR 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 (EGFR 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 FGFR (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%**; FGF-2, 200.6 ± 30.7%* of basal, respectively, *p<0.05, **p<0.01).

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, 5min 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 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 CRE-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-HT2 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 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, while 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 amitriptyline-induced ERK activation.

The effect of specific non-permeant 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 type (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 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 lactate dehydrogenase (LDH) assay, amitriptyline treatment does not induce nonspecific protein release due to cell damage (15). Matrix metalloproteinases (MMP) 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, while 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 5 min): 100.0 ± 1.3%***, Amitriptyline+ o-phenanthroline (200 μM): 41.5 ± 10.9%** of amitriptyline only, respectively, ***p<0.001 in comparison to basal, **p<0.001 in comparison to amitriptyline only, F (2.17) =25.02, p<0.001]. These results suggest the shedding of FGFR ligands, such as FGF-2 by MMP, thus leading to FGFR activation, may be involved in the effect of amitriptyline.

Effects of MMP inhibitors or Na/K-ATPase inhibitor 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 4BC). In addition, the amitriptyline-induced FRS2α phosphorylation was inhibited by MMP inhibitors; while 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 FRS2α) 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 FRS2α 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 differently participate 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 following 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 hetero-dimerization as well as homo-dimerization 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, with 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, while 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 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 FGFR ligands accumulated on cell surface heparin sulfate proteoglycans (HSPG) seem to play an important role in the effect of amitriptyline, because heparin and neutralizing antibodies trap FGFR 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 4A, 4C, 6). Furthermore, amitriptyline does not seem to bind directly to
FGFR. Further studies investigating the over-expression 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 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 o-phenanthroline and phosphoramidon-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, 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) (unpublished data). 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, following 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 amitriptyline-induced ERK activation in C6 cells and NHA (Figs 1A, 1B). 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 following 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 following 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 downregulated 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-like effects in animal models (53). The current study found that
antidepressants acutely activate FGFR signaling, and subsequently FGFR is involved in GDNF production by antidepressant treatment in glial cells. There are reports showing that FGF-2 treatment increases GDNF production 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

the role of GDNF. in *Recent Developments on Depression Research* (Shirayama, Y., and Chaki, S. eds.), Research Signpost, Kerala, India. pp 125-143


Figure 1. Effects of tyrosine kinase inhibitors and FGFR knock down on the amitriptyline-induced ERK activation.
A, Effects of Genistein, SU5402, PD173074, K252a and AG1478 on the amitriptyline-induced ERK activation in C6 cells. C6 cells were pretreated with 100 nM of AG1478 for 30 min; 100 μM of Genistein, 25 μM SU5402 or 200 nM K252a for 1 h; and 1 μM of PD173074 for 2 h and subsequently treated with 25 μM amitriptyline for 5 min. Values are shown as ERK activity (% of control). Data are expressed as the mean ± SEM [F (6.45) = 29.25, p<0.001]. ***p<0.001 in comparison to the basal group and †††p<0.001 in comparison to the control group (Tukey’s HSD test).

B, Effects of SU5402, PD173074, K252a and AG1478 on the amitriptyline-induced ERK activation in NHA. NHA were pretreated with 100 nM of AG1478 for 30 min; 25 μM SU5402 or 200 nM K252a for 1 h; and 1 μM of PD173074 for 2 h and subsequently treated with 25 μM amitriptyline for 5 min. Values are shown as the ERK activity (% of control). Data are expressed as the mean ± SEM [F (5.35) = 13.09, p<0.001]. ***p<0.001 in comparison to the basal group and †††p<0.001 in comparison to the control group (Tukey’s HSD test).

C, Effect of siRNA transfection on FGFR expressions. C6 cells were transfected with FGFR1 siRNA (50 nM), FGFR2 siRNA (100 nM), or control siRNA (50 or 100 nM) for 48 h. The FGFR1 or FGFR2 were detected by Western blotting. A representative result is shown.

D, Effect of FGFR knock down on the amitriptyline-induced ERK activation. C6 cells were transfected with FGFR1 siRNA (50 nM), FGFR2 siRNA (100 nM), or control siRNA (50 or 100 nM) for 48 h, and subsequently treated with 25 μM amitriptyline for 5 min. Values are shown as ERK activity (% of control). Data are expressed as the mean ± SEM [F (5.96) = 8.14, p<0.001]. ***p<0.001 in comparison to the basal group and †††p<0.001 in comparison to the control group (Tukey’s HSD test).

Figure 2. Amitriptyline treatment increased phosphorylation of FGFRs and FRS2α.
A, Time course of phosphorylation of FGFRs in the amitriptyline-treated C6 cells. C6 cells were
treated with 25 μM of amitriptyline, and phosphorylation of FGFRs was measured after the indicated period of treatment. The phosphorylated FGFRs (P-FGFR1 and 2), total FGFRs (T-FGFR1 and 2) and actin were detected by Western blotting. A representative result is shown. The values are shown as the ratio of phosphorylated FGFR to total FGFR (% of basal) (●: 100 kDa, FGFR2; ▲: 120 kDa, FGFR1; ■: 145 kDa, FGFR1). Data are expressed as the mean ± SEM [100 kDa FGFR2; F (5.42) =1.33, p=0.273, 120 kDa FGFR1; F (5.65) =2.98, p<0.05, 145 kDa FGFR1; F (5.65) =3.36, p<0.01]. *p<0.05; **p<0.01, significantly different from the basal group (Games-Howell test).

B. Time course of phosphorylation of FRS2α in the amitriptyline-treated C6 cells. C6 cells were treated with 25 μM of amitriptyline, and phosphorylation of FRS2α was measured after the indicated period of treatment. The phosphorylated FRS2α, total FRS2α and actin were detected by Western blotting, and a representative result is shown. The values are shown as the ratio of phosphorylated FRS2α to total FRS2α (% of basal). Data are expressed as the mean ± SEM [F (5.43) =4.71, p<0.01]. *p<0.05; **p<0.01 in comparison to the basal group (Tukey’s HSD test).

Figure 3. Effects of FGFR inhibitors on CREB phosphorylation and GDNF production induced by amitriptyline treatment.
A. Effect of SU5402 and PD173074 on the amitriptyline-induced CREB phosphorylation. C6 cells were pretreated with 25 μM of SU5402 for 1 h or 1 μM of PD173074 for 2 h and subsequently treated with 25 μM amitriptyline for 10 min. Values are shown as CREB phosphorylation (% of control). Data are expressed as the mean ± SEM [F (3.16) =19.64, p<0.001]. ***p<0.001 in comparison to basal, **p<0.01, ***p<0.001 in comparison to control group (Tukey’s HSD test).
B. Effect of SU5402 and PD173074 on the amitriptyline-induced GDNF mRNA expression. C6 cells were pretreated with 25 μM of SU5402 for 1 h or 1 μM of PD173074 for 2 h and subsequently treated with 25 μM amitriptyline for 3 h. Values are shown as the ratio of GDNF mRNA versus GAPDH mRNA. Data are expressed as the mean ± SEM [F (3.25) =45.10, p<0.001]. ***p<0.001 in comparison to the basal group and †††p<0.001 in comparison to the control group (Tukey’s HSD test).
C. Effects of SU5402 and PD173074 on the amitriptyline-induced GDNF release. C6 cells were pretreated with 25 μM of SU5402 for 1 h or 1 μM of PD173074 for 2 h and subsequently treated with 25 μM of amitriptyline for 48 h. Values are expressed as the mean ± SEM of released GDNF (pg/mL) [F (3.59)=11.22, p<0.001]. ***p<0.001 in comparison to the basal group and †††p<0.001 in comparison to the control group (Tukey’s HSD test).

Figure 4. Effects of MMP inhibitors or Na/K-ATPase inhibitor on the amitriptyline, 5-HT or FGF-2-induced ERK activation.
A. Effects of phosphoramidon, o-phenanthroline, GM6001, GM6001 negative control and ouabine on the amitriptyline-induced ERK activation in C6 cells. C6 cells were pretreated with 25 μM of GM6001 or GM6001 negative control (GM Nega) for 30 min; 300 μM of phosphoramidon (Phospho), 200 μM of o-phenanthroline (o-Phe) or 100 μM of ouabine for 1 h and subsequently treated with 25 μM amitriptyline for 5 min. Values are shown as ERK activity (% of control). Data are expressed as the mean ± SEM [F (5.37) =24.40, p<0.001]. ***p<0.001 in comparison to the basal group and †††p<0.001 in comparison to the control group (Tukey’s HSD test).
B. Effects of o-phenanthroline or GM6001 on the 5-HT-induced ERK activation in C6 cells. C6 cells were pretreated with 25 μM of GM6001 for 30 min or 200 μM of o-phenanthroline (o-Phe) and subsequently treated with 10 μM 5-HT for 2 min. ERK activity (phosphorylation levels of ERK1/2) were detected by Western blotting. Values are shown as ERK activity (% of control). Data are expressed as the mean ± SEM [F (3.24) =22.67, p<0.001]. ***p<0.001 in comparison to the basal
group (Tukey’s HSD test).

C. Effects of o-phenanthroline or GM6001 on the FGF-2-induced ERK activation in C6 cells. C6 cells were pretreated with 25 μM of GM6001 for 30 min or 200 μM of o-phenanthroline (o-Phe) and subsequently treated with 10 ng/mL FGF-2 for 10 min. ERK activity (phosphorylation levels of ERK1/2) were detected by western blotting. Values are shown as ERK activity (% of control). Data are expressed as the mean ± SEM [F (3.25) = 22.52, p<0.001]. ***p<0.001 in comparison to the basal group (Tukey’s HSD test).

**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 of GM6001 or GM6001 negative control (GM Nega) for 30 min and then were subsequently treated with 25 μM of amitriptyline for 48 h. Values are expressed as the mean ± SEM of released GDNF (pg/mL) [F (3.63) = 8.44, p<0.001]. ***p<0.001 in comparison to the basal group and †p<0.05 in comparison to the control group (Tukey’s HSD test).

**Figure 6. Proposed mechanism of antidepressant on FGFR activation and following GDNF production in glia.**
HSPG (Heparan-sulfate proteoglycans), o-Phe (o-phenanthroline), Phospho (Phosphoramidon)
### Table 1 Effects of antidepressants and non-antidepressant drugs on the FRS2α phosphorylation.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>FRS2α phosphorylation (% of Basal)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C6 cells</td>
</tr>
<tr>
<td><strong>Tricyclic antidepressant</strong></td>
<td></td>
</tr>
<tr>
<td>Amitriptyline</td>
<td>176.2 ± 13.1***</td>
</tr>
<tr>
<td>Clomipramine</td>
<td>228.1 ± 7.86**</td>
</tr>
<tr>
<td>Desipramine</td>
<td>216.3 ± 32.4*</td>
</tr>
<tr>
<td>Nortriptyline</td>
<td>231.1 ± 24.7*</td>
</tr>
<tr>
<td><strong>Tetracyclic antidepressant</strong></td>
<td></td>
</tr>
<tr>
<td>Mianserin</td>
<td>197.1 ± 29.0*</td>
</tr>
<tr>
<td><strong>Selective serotonin reuptake inhibitor</strong></td>
<td></td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>216.5 ± 33.7*</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>210.3 ± 37.1*</td>
</tr>
<tr>
<td><strong>Others</strong></td>
<td></td>
</tr>
<tr>
<td>Haloperidol</td>
<td>115.9 ± 24.4</td>
</tr>
<tr>
<td>Diazepam</td>
<td>118.8 ± 24.8</td>
</tr>
</tbody>
</table>

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 ± SEM from 3 to 13 independent experiments. *p<0.05; **p<0.01; ***p<0.001, significantly different from the basal (Student’s t-test).
Table 2 Effects of heparin on the amitriptyline or 5-HT-induced ERK activation.

<table>
<thead>
<tr>
<th></th>
<th>ERK activation (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C6 cells</td>
</tr>
<tr>
<td>Basal</td>
<td>38.0 ± 4.51</td>
</tr>
<tr>
<td>Control (Amitriptyline only)</td>
<td>100.0 ± 1.86***</td>
</tr>
<tr>
<td>+Heparin (10 µg/mL)</td>
<td>76.8 ± 5.99†</td>
</tr>
<tr>
<td>+Heparin (100 µg/mL)</td>
<td>21.3 ± 8.45+++</td>
</tr>
<tr>
<td>Basal</td>
<td>25.3 ± 2.93</td>
</tr>
<tr>
<td>Vehicle (5-HT only)</td>
<td>100.0 ± 2.68***</td>
</tr>
<tr>
<td>+Heparin (100 µg/mL)</td>
<td>103.4 ± 9.84</td>
</tr>
</tbody>
</table>

C6 cells were treated with 10 or 100 µg/mL of heparin for 30 min and subsequently treated with amitriptyline (25 µM) or 5-HT (10 µM) for 5 min. Values are shown as the ERK activity (% of control). Data are expressed as the mean ± SEM [Amitriptyline, F (4.41) =46.78, p<0.001; 5-HT, F (2.10) =65.23, p<0.001]. ***p<0.001 in comparison to the basal group and †p<0.05, †††p<0.001 in comparison to the control group (Tukey’s HSD test). NHA were treated with 100 µg/mL of heparin for 30 min and subsequently treated with 25 µM amitriptyline for 5 min. Values are shown as the ERK activity (% of control). Data are expressed as the mean ± SEM [F (2.8) =19.00, p<0.001]. **p<0.01 in comparison to the basal group and ††p<0.01 in comparison to the control group (Tukey’s HSD test).
Table 3 Effects of neutralize antibodies on the amitriptyline-induced ERK activation.

<table>
<thead>
<tr>
<th>Antibodies</th>
<th>ERK activation (% of Control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C6 cells</td>
<td>NHA</td>
</tr>
<tr>
<td>Basal</td>
<td>38.4 ± 6.33</td>
</tr>
<tr>
<td>Control (Amitriptyline only)</td>
<td>100.0 ± 1.86***</td>
</tr>
<tr>
<td>+FGF-1 nAb</td>
<td>96.8 ± 18.0</td>
</tr>
<tr>
<td>+FGF-2 nAb (monoclonal)</td>
<td>77.2 ± 7.42†</td>
</tr>
<tr>
<td>+FGF-2 nAb (polyclonal)</td>
<td>48.6 ± 4.74†</td>
</tr>
<tr>
<td>+Mouse IgG</td>
<td>91.3 ± 18.3</td>
</tr>
<tr>
<td>+Goat IgG</td>
<td>91.5 ± 16.8</td>
</tr>
</tbody>
</table>

C6 cells and NHA were pretreated with 5 μg/mL of antibodies for 30 min and subsequently treated with 25 μM amitriptyline for 5 min, and ERK activity was measured. Data are expressed as the mean ± SEM [C6, F (6.42) = 7.58, p < 0.001; NHA, F (4.49) = 7.47, p < 0.001]. ***p < 0.001 in comparison to the basal group, and †p < 0.05, ††p < 0.01 in comparison to the control group (Tukey’s HSD test).
Table 4 Effects of amitriptyline or 5-HT on FGF-2 shedding in C6 cells.

<table>
<thead>
<tr>
<th></th>
<th>FGF-2 (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>2.51 ± 0.577</td>
</tr>
<tr>
<td>Amitriptyline (25 μM)</td>
<td>4.21 ± 1.01</td>
</tr>
<tr>
<td>Amitriptyline (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>

C6 cells were treated with amitriptyline or 5-HT for 2 min. Data are expressed as the mean ± SEM of FGF-2 in the media (pg/mL) [Amitriptyline, F (2.41) =2.903, p=0.067; 5-HT, F (2.23) =1.164, p=0.331]. *p<0.05 in comparison to the basal group (Dunnett’s test).
**Fig 2**

**A**

Phosphorylated FGFR / total FGFR (% of Basal)

![Graph showing phosphorylation of FGFR over time](image)

**B**

Phosphorylated FRS2α / total FRS2α (% of Basal)

![Graph showing phosphorylation of FRS2α over time](image)
Amitriptyline

Fig 4

A

ERK activity (% of Control)

Basal  Control  Phospho  α-Phe  GM6001  GM Nega  Ouabine

Amitriptyline

B

ERK activity (% of Control)

5-HT

Basal  Control  α-Phe  GM6001

C

ERK activity (% of Control)

FGF-2

Basal  Control  α-Phe  GM6001
Fig 5

The graph shows the GDNF release (pg/mL) in response to different treatments. The x-axis represents the treatments: Basal, Control, GM6001, and GM Nega. The y-axis represents the GDNF release in pg/mL, ranging from 0 to 150.

Amitriptyline is indicated by the bars: Basal and Control show a significant increase in GDNF release compared to the Basal baseline, marked by ***. GM6001 and GM Nega also show an increase, marked by +. The error bars indicate the standard deviation.
Tricyclic antidepressant amitriptyline activates fibroblast growth factor receptor (FGFR) signaling in glial cells: Involvement in glial cell line-derived neurotrophic factor (GDNF) production
Kazue Hisaoka, Mami Tsuchioka, Ryoya Yano, Natsuko Maeda, Naoto Kajitani, Norimitsu Morioka, Yoshihiro Nakata and Minoru Takebayashi

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