

# Tricyclic Antidepressant Amitriptyline-induced Glial Cell Line-derived Neurotrophic Factor Production Involves Pertussis Toxin-sensitive $G\alpha_{i/o}$ Activation in Astroglial Cells\*

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**Background:** A significant non-neural, monoamine-independent mechanism underlies the antidepressant effect of amitriptyline.

**Results:** Amitriptyline-evoked GDNF production is mediated by pertussis toxin (PTX)-sensitive  $G\alpha_{i/o}$ .

**Conclusion:** PTX-sensitive  $G\alpha_{i/o}$  activation is critical for the cascade that underpins the biological effect of amitriptyline.

**Significance:** Further elaboration of the intracellular mechanism of amitriptyline could lead to greater understanding of depression and novel antidepressant treatments.

Further elaborating the mechanism of antidepressants, beyond modulation of monoaminergic neurotransmission, this study sought to elucidate the mechanism of amitriptyline-induced production of glial cell line-derived neurotrophic factor (GDNF) in astroglial cells. Previous studies demonstrated that an amitriptyline-evoked matrix metalloproteinase (MMP)/FGF receptor (FGFR)/FGFR substrate 2 $\alpha$  (FRS2 $\alpha$ )/ERK cascade is crucial for GDNF production, but how amitriptyline triggers this cascade remains unknown. MMP is activated by intracellular mediators such as G proteins, and this study sought to clarify the involvement of G protein signaling in amitriptyline-evoked GDNF production in rat C6 astroglial cells (C6 cells), primary cultured rat astrocytes, and normal human astrocytes. Amitriptyline-evoked GDNF mRNA expression and release were inhibited by pertussis toxin (PTX), a  $G\alpha_{i/o}$  inhibitor, but not by NF449, a  $G\alpha_s$  inhibitor, or YM-254890, a  $G\alpha_q$  inhibitor. The activation of the GDNF production cascade (FGFR/FRS2 $\alpha$ /ERK) was also inhibited by PTX. Deletion of  $G\alpha_{o1}$  and  $G\alpha_{i3}$  by RNAi demonstrated that these G proteins play important roles in amitriptyline signaling. G protein activation was directly analyzed by electrical impedance-based biosensors (CellKey™ assay), using a label-free (without use of fluorescent proteins/probes or radioisotopes) and real time approach. Amitriptyline increased impedance, indicating  $G\alpha_{i/o}$  activation that was sup-

pressed by PTX treatment. The impedance evoked by amitriptyline was not affected by inhibitors of the GDNF production cascade. Furthermore, FGF2 treatment did not elicit any effect on impedance, indicating that amitriptyline targets PTX-sensitive  $G\alpha_{i/o}$  upstream of the MMP/FGFR/FRS2 $\alpha$ /ERK cascade. These results suggest novel targeting for the development of antidepressants.

Major depressive disorder (MDD)<sup>4</sup> is a severe, chronic, and often life-threatening illness with a lifetime prevalence of more than 10% (1). Since the late 1950s, a wide range of antidepressants targeting the monoaminergic neurotransmitter system have been available to alleviate the symptoms of MDD. However, the efficacy of these antidepressants cannot be solely explained by their modulatory effects on brain monoamines. The limited success in understanding the pathophysiology of MDD and the equivocal therapeutic effects of antidepressants could be the result of excessive focus on dysfunctional neurons, with scant studies performed in other types of cells of the brain such as glia.

In the past decade, glial degeneration or dysfunction, especially of astrocytes, has been postulated to play a critical role in the pathogenesis of MDD (2). One of the major roles of astrocytes is the production of neurotrophic/growth factors, which support neurogenesis, gliogenesis, brain development, neural plasticity, and survival (3). Recently, both clinical and preclinical animal studies have demonstrated that multiple neurotrophic/growth factors, such as glial cell line-derived neu-

<sup>4</sup> The abbreviations used are: MDD, major depressive disorder; GDNF, glial cell line-derived neurotrophic factor; MMP, matrix metalloproteinase; FGFR, fibroblast growth factor receptor; PTX, pertussis toxin; GPCR, G protein-coupled receptor; 5-HT, serotonin; NHA, normal human astrocyte; nor-BNI, nor-binaltorphimine dihydrochloride; DAMGO, [D-Ala<sup>2</sup>,N-MePhe<sup>4</sup>,Gly<sup>o</sup>]-enkephalin; GTP $\gamma$ S, 5'-3-O-(thio)triphosphate.

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retrophic factor (GDNF), a member of the transforming growth factor  $\beta$  superfamily, have been proposed to play an important role in the therapeutic effect of antidepressants (4, 5). Glial cell line-derived neurotrophic factor has been shown to be decreased in the peripheral blood of patients with MDD (6), whereas blood levels of GDNF in MDD are increased after antidepressant treatment (7). These findings suggest that modulating GDNF production may be a key therapeutic effect of antidepressants. Therefore, understanding the mechanism of GDNF production in response to antidepressant treatment in astroglial cells could provide novel insights into the pathogenesis of MDD, the pharmacological effect of antidepressants and potentially novel treatments for MDD.

Previously, reports demonstrated that treatment with several different classes of antidepressants, such as tricyclic antidepressants, tetracyclic antidepressants, selective serotonin (5-HT) reuptake inhibitors, and 5-HT and noradrenaline reuptake inhibitors, increased GDNF production in normal human astrocytes (NHA), rat primary cultured astrocytes, and rat C6 astroglial cells (C6 cells, an astrocytic cell line) (8–10). The tricyclic antidepressant amitriptyline increased GDNF production by astroglial cells through a monoamine-independent mechanism (9). The FGF receptor (FGFR)/FGFR substrate 2 $\alpha$  (FRS2 $\alpha$ )/ERK signaling cascade plays a crucial role in amitriptyline-induced GDNF production, independent of monoamine activation (11). Fibroblast growth factor receptor activation evoked by amitriptyline is derived from a matrix metalloproteinase (MMP)-dependent shedding of FGFR ligands in astroglial cells (11). However, how amitriptyline triggers MMP and the subsequent signaling cascade remain unknown. Matrix metalloproteinase is known to be activated by intracellular signaling mediators, such as G proteins (12). Thus, this study attempts to clarify the involvement of G protein in the amitriptyline-evoked production of GDNF. The effect of amitriptyline on G protein activation in living C6 cells and rat astrocytes was analyzed using a novel biosensor technology, the CellKey<sup>TM</sup> assay (MDS Sciex, Ontario, Canada). The CellKey<sup>TM</sup> assay utilizes cellular dielectric spectroscopy, which is specifically tailored to G protein-coupled receptor (GPCR) detection and can distinguish signals between the  $G\alpha_s$  ( $G_s$ ),  $G\alpha_{i/o}$  ( $G_{i/o}$ ), and  $G\alpha_q$  ( $G_q$ ) subfamilies (13, 14).

## Experimental Procedures

**Reagents**—Reagents were obtained from the following sources: amitriptyline (Wako Pure Chemical Industries, Ltd., Osaka, Japan); serotonin creatinine sulfate monohydrate, norbinaltorphimine dihydrochloride (nor-BNI), and [ $D$ -Ala<sup>2</sup>, $N$ -MePhe<sup>4</sup>,Gly-ol]enkephalin (DAMGO) (Sigma); FGF2 human recombinant (Roche Diagnostics); pertussis toxin, NF449, and U0126 (Calbiochem); GM6001, SU5402, and PD173074 (Merck KGaA, Darmstadt, Germany); acetylcholine and isoproterenol (Nacalai Tesque, Kyoto, Japan). YM-254890 was a gift from Taiho Pharmaceutical Co., Ltd. (Tokyo, Japan).

**Cell Culture**—Culturing of C6 cells, and NHA has been described previously (8, 11). In brief, C6 cells were grown in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 2 mM L-glutamine and 5% fetal bovine serum (Sigma) in a 5% CO<sub>2</sub> humidified atmosphere. Normal human

astrocytes, male fetuses 18 weeks of age, were purchased from Cambrex (Walkersville, MD) and grown in ABM<sup>TM</sup> (Cambrex) in a 5% CO<sub>2</sub> humidified atmosphere. More than 80% of NHA expressed glial fibrillary acidic protein. For drug treatment, the medium was replaced with serum-free Opti-MEM (Invitrogen) containing 0.5% bovine serum albumin (Sigma), and the cells were incubated for 24 h. The cells were then treated with various drugs of interest.

All experimental procedures and animal handling were performed according to both the Guiding Principles for the Care and Use of Laboratory Animals, as approved by the Japanese Pharmacological Society, and guidelines for the care and use of laboratory animals of Hiroshima University (Hiroshima, Japan). Primary cultured astrocytes were prepared from 1-day-old neonatal Wistar rats, as described previously (10, 15). Briefly, isolated cerebral cortices were minced and then incubated with trypsin and DNase I. Dissociated cells were suspended in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin (100 units/ml and 100  $\mu$ g/ml, respectively). Thereafter, cell suspensions were plated in 75-cm<sup>2</sup> tissue culture flasks (10  $\times$  10<sup>6</sup> cells/flask) precoated with poly-L-lysine (10  $\mu$ g/ml). The cells were maintained in a 10% CO<sub>2</sub> incubator at 37 °C. After 8–12 days, the growth flasks containing mixed glial cells were shaken by a rotary shaker at 100 rpm for 15 h and washed with PBS to remove neuronal and microglial cells. Adherent cells were trypsinized (0.25%) and plated into 75-cm<sup>2</sup> flasks. After the cells reached confluence (about 10 days), the confluent cells were shaken by hand for 10 min. Adherent cells were trypsinized (0.25%) and plated onto 35-mm diameter dishes (1  $\times$  10<sup>6</sup> cells/dish). After 2 days, the medium was replaced with serum-free DMEM. The cells were used for experiments on the following day. For the CellKey<sup>TM</sup> assay, rat astrocytes were prepared from the passaged cell cultures of 1-day-old rat cortex, as described previously (8, 16). Most of the cells (>95%) in culture are astrocytes, as they express the astrocytic marker glial fibrillary acidic protein.

**Electrical Impedance-based Biosensors (CellKey<sup>TM</sup> Assay)**—The CellKey<sup>TM</sup> assay has been described previously (17, 18). Cellular dielectric spectroscopy is a novel technology that employs a label-free, real time, cell-based assay approach for the comprehensive pharmacological evaluation of cells that exogenously or endogenously express GPCRs, and nuclear receptors on the same platform without any modification of the cells. Among the biosensors that use cellular dielectric spectroscopy technology, the CellKey<sup>TM</sup> system not only detects the activation of GPCRs but also distinguishes between signals through different subtypes of the  $G\alpha$  protein ( $G_s$ ,  $G_{i/o}$ , and  $G_q$ ). Many reports have indicated that cell signaling induced by GPCR activation can induce changes in morphology, and there are well established links between  $G_s$ ,  $G_i$ , and  $G_q$  signaling pathways (19–22). The activation of GPCR therefore changes the impedance ( $\Delta Z$ ), which can be interpreted as the differences of GPCR activation. In brief, the cells were cultured at a density of 2.0  $\times$  10<sup>4</sup> cells/well for C6 cells, 5.0  $\times$  10<sup>4</sup> cells/well for HEK293 cells, and 2.0  $\times$  10<sup>4</sup> cells/well for rat astrocytes on a standard CellKey<sup>TM</sup> 96-well microplate, which contained electrodes at the bottom of the wells, with 100  $\mu$ l of growth medium. All experiments were performed in CellKey<sup>TM</sup> assay buffer (Hanks'

## Amitriptyline Increases GDNF through $G\alpha_{i/o}$ Activation

balanced salt solution (1.3 mM  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ , 0.81 mM  $\text{MgSO}_4$ , 5.4 mM KCl, 0.44 mM  $\text{KH}_2\text{PO}_4$ , 4.2 mM  $\text{NaHCO}_3$ , 136.9 mM NaCl, 0.34 mM  $\text{Na}_2\text{HPO}_4$ , and 5.6 mM D-glucose) with 20 mM HEPES and 0.1% BSA) at 29 °C. Small voltages were applied to these electrodes every 10 s, and  $\Delta Z$  values were measured. Just before assay, the cells were washed with assay buffer and allowed to equilibrate in the assay buffer for 30 min before starting the assay. The CellKey™ instrument applied small voltages to these electrodes every 10 s and measured the  $\Delta Z$  of the cell layer. In this study, a 5-min baseline was recorded; drugs were added, and then  $\Delta Z$  was measured for 10 min. The extent of changes in  $\Delta Z$  was expressed in terms of  $\Delta Z$  maximum after drug injection.

**Cells Stably Expressing Opioid Receptors**—To visualize impedance changes from typical  $G_{i/o}$ -coupled receptors, as references, cells expressing the  $\mu$ -opioid receptor were prepared (see “Electrical Impedance-based Biosensors (CellKey™ Assay)” above). Human embryonic kidney 293 (HEK293) cells were plated in 35-mm dishes. After seeding for 24 h, the cells were transfected with  $\mu$ -opioid receptor tagged at the N terminus with FLAG using X-tremeGENE HP DNA transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the supplier’s instructions. After transfection for 24 h, the cells were re-plated in a 10-cm dish and selected with 700  $\mu\text{g}/\text{ml}$  G418 disulfate aqueous solution (Nacalai Tesque, Kyoto, Japan). These cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin mixed solution (Nacalai Tesque, Kyoto, Japan) in a 5%  $\text{CO}_2$  humidified atmosphere. Single clones expressing FL-MOR were then screened by both the conventional immunocytochemistry analysis using anti-FLAG (Wako Pure Chemical Industries, Ltd., Osaka, Japan) antibodies and a functional assay using CellKey™ assay.

**RNA Isolation**—For the collection of total RNA, the cells were cultured at a density of  $1.6 \times 10^5/\text{cm}^2$  for C6 cells and  $1.0 \times 10^5/\text{cm}^2$  for primary cultured rat astrocytes on a 6-well plate with 3 ml of growth medium. After drug treatment, total RNA was isolated using an RNeasy mini kit (Qiagen, Valencia, CA) following the manufacturer’s protocols. RNA quantity and purity were determined with a multi-spectrophotometer (Dainippon Sumitomo Pharma Co. Ltd., Osaka, Japan).

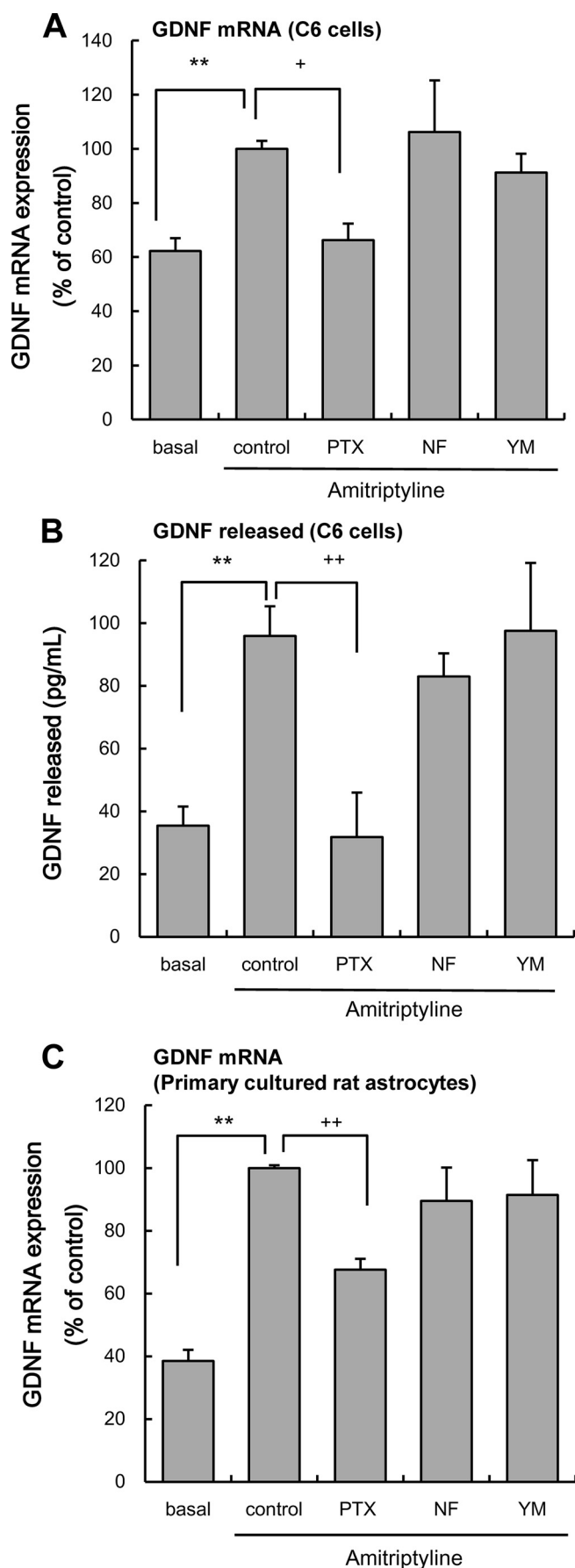
**Real Time RT-PCR Assay**—Real time RT-PCR assay has been described previously (10). In brief, the first strand cDNA was synthesized from 500 ng of total RNA by using an RNA PCR kit (AMV) Version 3.0 (Takara Bioscience, Shiga, Japan). Real time quantitative PCR was performed using the Thermal Cycler Dice® real time system II (Takara Bioscience), with TaqMan probes and primers for rat GDNF 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  $2(-\Delta\Delta C_T)$  method.

**ELISA**—For the GDNF release assay, C6 cells were cultured at a density of  $1.3 \times 10^5/\text{cm}^2$  on a 12-well plate with 0.5 ml of growth medium. After drug treatment, the concentrations of GDNF protein in cell-conditioned media were determined using a GDNF Emax® ImmunoAssay System (Promega, Madison, WI) according to the manufacturer’s instructions.

**ERK Activity Assay**—The ERK activity assay has been described previously (11). In brief, the cells were cultured at a density of  $1.6 \times 10^5/\text{cm}^2$  for C6 cells,  $0.8 \times 10^5/\text{cm}^2$  for normal human astrocytes and  $1.0 \times 10^5/\text{cm}^2$  for primary cultured rat astrocytes on a 6-well plate with 2 or 3 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 measured by a bicinchoninic acid kit (Pierce), and it was adjusted to the same amount for all samples. ERK activity was determined using an assay kit according to the manufacturer’s instructions (Cell Signaling Technology, Beverly, MA).

**Western Blotting**—Western blotting has been described previously (11). Western blotting was performed with the following antibodies: phospho-FGFR substrate 2 $\alpha$  (FRS2 $\alpha$ ) (Tyr-196) antibody (Cell Signaling Technology); actin (C-2) antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA); and FRS2 $\alpha$  (SNT-1) antibody (Sigma). Cells were collected in ice-cold phosphate-buffered saline (PBS) and solubilized in sample buffer (100 mM Tris-HCl (pH 6.8), 20% glycerol, 4% SDS). The total amount of protein between samples was adjusted to similar levels. After the addition of 1,4-dithiothreitol, the samples were boiled for 5 min. The proteins were separated by SDS-PAGE 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. After incubation, the membranes were washed, and the desired proteins were detected with Immun-Star WesternC™ kit (Bio-Rad), using GE Healthcare Image Quant LAS 4000 (Waukesha, WI). Band intensity was quantified with the software ImageJ (National Institutes of Health, Bethesda).

**RT-PCR Analysis**—Total RNA from C6 cells and control tissues (rat cortex and spleen) was prepared by the methods of RNA isolation and used to synthesize cDNA with murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA) and a random hexamer primer. Polymerase chain reactions were performed with the specific primers and AmpliTaqGold™ (Applied Biosystems) at 95 °C for 10 min followed by 35 (for  $G_{o1}$ ,  $G_{o2}$ , and  $G_z$ ) or 40 (for  $G_{i1}$ ,  $G_{i2}$ , and  $G_{i3}$ ) cycles of 95 °C for 30 s, 62 °C for 30 s, and 72 °C for 1 min with a final extension at 72 °C for 5 min. The sequences of the primers were as follows:  $G_{i1}$ , 5'-AAGGACAGCGGTGTGCAAGCCTGCT-TCAAC-3' (forward), and 5'-AATCTGTTCATCCGTACAA-GGTACTTAACA-3' (reverse);  $G_{i2}$ , 5'-AGTATGACGAGGC-AGCCAGCTACATCCAGAGCAA-3' (forward), and 5'-GTACTCCTCCAGACATAGGCCTTGGGAAACTCTGC-3' (reverse);  $G_{i3}$ , 5'-TGCTAGGAGAGCTCTAAGAGTATA-3' (forward), and 5'-GCTTGCTTCCCAAAGCAGTTCTGA-3' (reverse);  $G_{o1}$ , 5'-CATCCTCCGAACCAGGGTC-3' (forward), and 5'-CAAGCCACAGCCCCGGAG-3' (reverse);  $G_{o2}$ , 5'-CATCCTCCGAACCAGGGTC-3' (forward), and 5'-GGC-GATGATGACGTCCGT-3' (reverse); and  $G_z$ , 5'-CACCTGG-AGGACAACGCCGCT-3' (forward), and 5'-TTTCGGTTA-GGTCCTCGAACTGA-3' (reverse). The resulting PCR products were analyzed on a 1.5% agarose gel, and sizes were expected from known cDNA sequences.



**RNAi Experiments**—To suppress expression of  $G_{\alpha 1}$ ,  $G_{\alpha 12}$ , and  $G_{\alpha 13}$ , C6 cells were transfected with small interfering RNA (siRNA), which has been described previously (23). The cells were transfected with either siRNA, targeting rat  $G_{\alpha 1}$ ,  $G_{\alpha 12}$ ,  $G_{\alpha 13}$ , or nontargeting siRNA (siGENOME Nontargeting siRNA Pool 5, Thermo Fisher Scientific) by using Lipofectamine 2000® (Life Technologies, Inc.) according to the manufacturer's directions. In these experiments, cells were used 48 h after transfection. The efficiency of the knockdown of the  $\alpha$ -subunits of G proteins was determined by Western blotting.

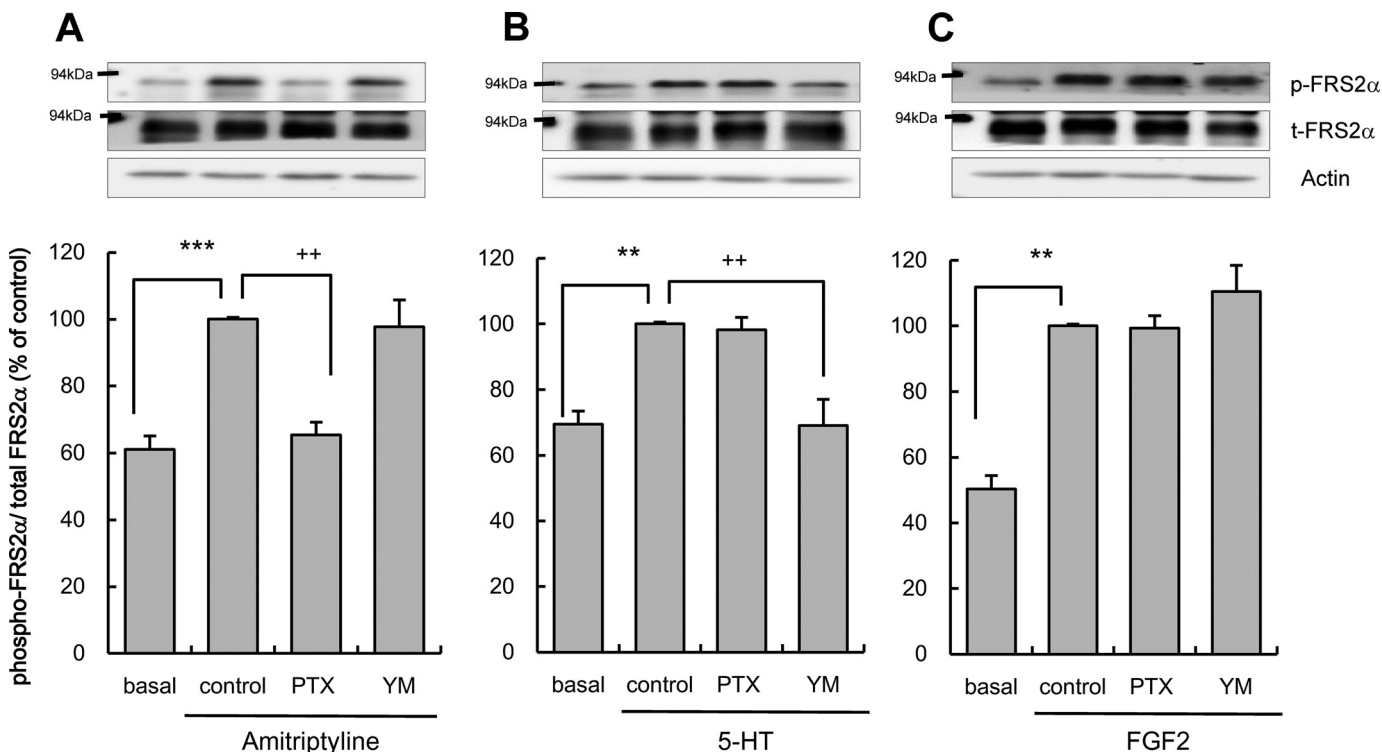
**Statistical Analysis**—Data were expressed as the mean  $\pm$  S.E. of at least three independent experiments. Statistically significant differences among the means were determined using one-way analysis of variance with pairwise comparison carried out by the Tukey's method.  $p$  values at less than 0.05 were taken as statistically significant.

## Results

**Effects of Pertussis Toxin, NF449, or YM-254890 on the GDNF Production Evoked by Amitriptyline in C6 Cells and Primary Cultured Rat Astrocytes**—To clarify the involvement of G proteins in the amitriptyline-evoked production of GDNF, the effects of the following inhibitors of the  $\alpha$ -subunits of G proteins were examined as described previously: pertussis toxin (PTX, 100 ng/ml;  $G_{i/o}$  inhibitor (24, 25)), NF449 (1  $\mu$ M;  $G_s$  inhibitor (26)), and YM-254890 (100 nM;  $G_q$  inhibitor (23)). A 3-h treatment with amitriptyline (25  $\mu$ M) significantly increased GDNF mRNA expression in C6 cells, and a 48-h amitriptyline treatment induced a significant release of GDNF from C6 cells. Both the amitriptyline-evoked expression of GDNF mRNA and release of GDNF were inhibited by PTX treatment. By contrast, neither NF449 nor YM-254890 had any effect on GDNF production (Fig. 1, A and B). Treatment with the inhibitors alone, without amitriptyline, had no significant effect on basal expression of GDNF mRNA (PTX only,  $74.1 \pm 8.3\%$ ; NF449 only,  $73.8 \pm 18.8\%$ ; YM-254890 only,  $69.2 \pm 7.6\%$  of control) and GDNF release (PTX only,  $19.1 \pm 9.0$  pg/ml; NF449 only,  $32.0 \pm 6.8$  pg/ml; YM-254890 only,  $47.9 \pm 11.7$  pg/ml).

**FIGURE 1. Effects of  $\alpha$ -subunit of G protein inhibitors on amitriptyline-evoked GDNF production.** A, effects of pertussis toxin (PTX), NF449 (NF), and YM-254890 (YM) on the amitriptyline-evoked GDNF mRNA expression. C6 cells were pretreated with 100 ng/ml PTX for 3 h and 1  $\mu$ M NF449 or 100 nM YM-254890 for 0.5 h and subsequently treated with 25  $\mu$ M amitriptyline for 3 h. Values are shown as the ratio of GDNF mRNA versus GAPDH mRNA (% of control). Data are expressed as the mean  $\pm$  S.E. for three to seven independent experiments. \*\*,  $p < 0.01$  in comparison with the basal group; +,  $p < 0.05$  in comparison with the control group (Tukey's test). B, effects of PTX, NF449, and YM-254890 on the amitriptyline-evoked GDNF release. C6 cells were pretreated with 100 ng/ml PTX for 3 h and 1  $\mu$ M NF449 or 100 nM YM-254890 for 0.5 h and subsequently treated with 25  $\mu$ M amitriptyline for 48 h. Values are expressed as the mean  $\pm$  S.E. of released GDNF (pg/ml) for 4–12 independent experiments. \*\*,  $p < 0.01$  in comparison with the basal group; ++,  $p < 0.01$  in comparison with the control group (Tukey's test). C, effects of pertussis toxin, NF449, and YM-254890 on the amitriptyline-evoked GDNF mRNA expression. Primary cultured rat astrocytes were pretreated with 100 ng/ml PTX for 3 h, 1  $\mu$ M NF449, or 100 nM YM-254890 for 0.5 h and subsequently treated with 25  $\mu$ M amitriptyline for 6 h. Values are shown as the ratio of GDNF mRNA versus GAPDH mRNA (% of control). Data are expressed as the mean  $\pm$  S.E. for 8–11 independent experiments. \*\*,  $p < 0.01$  in comparison with the basal group; ++,  $p < 0.01$  in comparison with the control group (Tukey's test).

## Amitriptyline Increases GDNF through $G_{\alpha_{i/o}}$ Activation

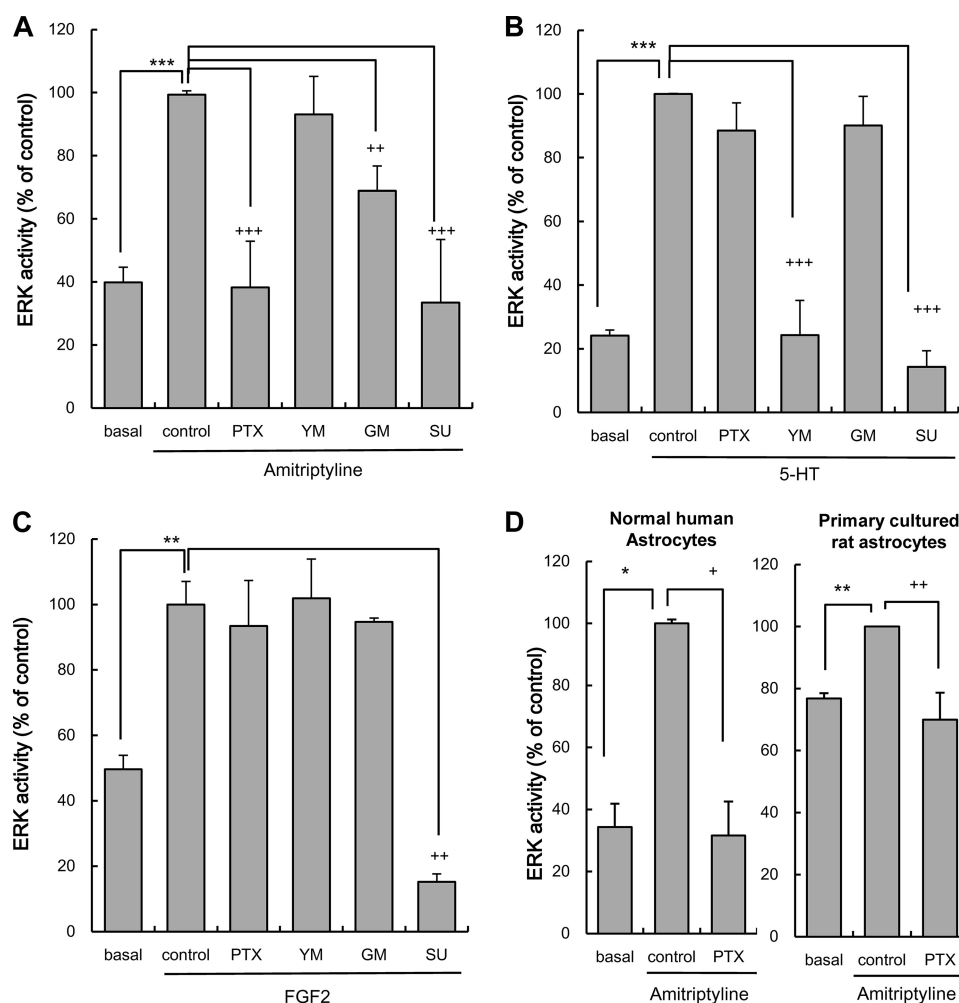


**FIGURE 2. Effects of pertussis toxin or YM-254890 on FRS2 $\alpha$  phosphorylation evoked by amitriptyline, 5-HT, or FGF2.** *A*, effects of pertussis toxin or YM-254890 (YM) on FRS2 $\alpha$  phosphorylation evoked by amitriptyline in C6 cells. C6 cells were pretreated with 100 ng/ml PTX for 3 h; 100 nM YM-254890 for 0.5 h and subsequently treated with 25  $\mu$ M amitriptyline for 5 min. Phosphorylated FRS2 $\alpha$ , total FRS2 $\alpha$ , and actin were quantified by Western blotting, and representative blots are shown. Values are shown as the ratio of phosphorylated FRS2 $\alpha$  to total FRS2 $\alpha$  (% of control). Data are expressed as the mean  $\pm$  S.E. for four to seven independent experiments. \*\*\*,  $p < 0.001$  in comparison with the basal group; ++,  $p < 0.01$  in comparison with the control group (Tukey's test). *B*, effects of PTX or YM-254890 on FRS2 $\alpha$  phosphorylation evoked by 5-HT in C6 cells. C6 cells were pretreated with 100 ng/ml PTX for 3 h, 100 nM YM-254890 for 0.5 h, and subsequently treated with 10  $\mu$ M 5-HT for 5 min. Phosphorylated FRS2 $\alpha$ , total FRS2 $\alpha$ , and actin were quantified by Western blotting, and representative blots are shown. Values are shown as the ratio of phosphorylated FRS2 $\alpha$  to total FRS2 $\alpha$  (% of control). Data are expressed as the mean  $\pm$  S.E. for six independent experiments. \*\*,  $p < 0.01$  in comparison with the basal group; ++,  $p < 0.01$  in comparison with the control group (Tukey's test). *C*, effects of PTX or YM-254890 on FRS2 $\alpha$  phosphorylation evoked by exogenous FGF2 in C6 cells. C6 cells were pretreated with 100 ng/ml PTX for 3 h, 100 nM YM-254890 for 0.5 h, and subsequently treated with 10 ng/ml FGF2 for 5 min. Phosphorylated FRS2 $\alpha$ , total FRS2 $\alpha$ , and actin were quantified by Western blotting, and representative blots are shown. Values are shown as the ratio of phosphorylated FRS2 $\alpha$  to total FRS2 $\alpha$  (% of control). Data are expressed as the mean  $\pm$  S.E. for five to eight independent experiments. \*\*,  $p < 0.01$  in comparison with the basal group (Tukey's test).

In primary cultured rat astrocytes, a 6-h treatment with amitriptyline (25  $\mu$ M) significantly increased GDNF mRNA expression (10). The amitriptyline-evoked expression of GDNF mRNA in primary cultured rat astrocyte was also inhibited by PTX, but not by NF449 or YM-254890 (Fig. 1C). Treatment with the inhibitors alone, without amitriptyline, had no significant effect on basal expression of GDNF mRNA (PTX only, 43.2  $\pm$  5.0%; NF449 only, 48.0  $\pm$  6.4%; YM-254890 only, 49.8  $\pm$  9.0% of control).

**Effects of PTX or YM-254890 on the FRS2 $\alpha$  Phosphorylation Evoked by Amitriptyline, 5-HT, or FGF2 in C6 Cells**—The effect of  $G_{i/o}$  inhibition by PTX and  $G_q$  inhibition by YM-254890 on the GDNF production cascade was evaluated. FGFR and the related signaling cascade were previously shown to be essential for amitriptyline-evoked GDNF production (11). Fibroblast growth factor receptor substrate 2 $\alpha$  is a member of the FRS family of lipid-anchored docking protein, and it is the primary substrate for the FGFR kinase and links the FGFR to Ras/MAPK signaling cascades (27, 28). Therefore, phosphorylation of FRS2 $\alpha$  was utilized as a surrogate index of FGFR activation. The phosphorylation of FRS2 $\alpha$  induced by amitriptyline treatment significantly increased and peaked 5 min after treatment in C6 cells (11). This increase was significantly reduced by PTX but

not by YM-254890 treatment of C6 cells (Fig. 2A). A previous study showed that 5-HT increased GDNF production through FGFR transactivation by the 5-HT $_2$   $G_q$ -coupled receptor in C6 cells (23). 5-HT treatment acutely increased FGFR phosphorylation in C6 cells (23). To determine whether  $G_q$  is involved in the 5-HT-evoked FRS2 $\alpha$  phosphorylation, unlike in the case of amitriptyline, the effects of PTX and YM-254890 on FRS2 $\alpha$  phosphorylation evoked by 5-HT were examined. A 5-min treatment with 10  $\mu$ M 5-HT significantly increased FRS2 $\alpha$  phosphorylation in C6 cells. The 5-HT-induced phosphorylation of FRS2 $\alpha$  was significantly reduced by YM-254890 but not by PTX (Fig. 2B). Next, to confirm the involvement of G proteins located upstream of FGFR, the effects of PTX and YM-254890 on exogenous FGF2 (a FGFR ligand)-evoked FRS2 $\alpha$  phosphorylation was examined. FGF2 treatment acutely increased FGFR phosphorylation in C6 cells (29). In this study, treatment with FGF2 (10 ng/ml, 5 min treatment) also significantly increased FRS2 $\alpha$  phosphorylation in C6 cells. The FGF2-evoked increase in FRS2 $\alpha$  phosphorylation was not affected by either PTX or YM-254890 (Fig. 2C). The inhibitors alone had no significant effect on basal levels of FRS2 $\alpha$  phosphorylation (PTX only, 99.0  $\pm$  14.5%; YM-254890 only, 122.9  $\pm$  26.3% of basal levels).

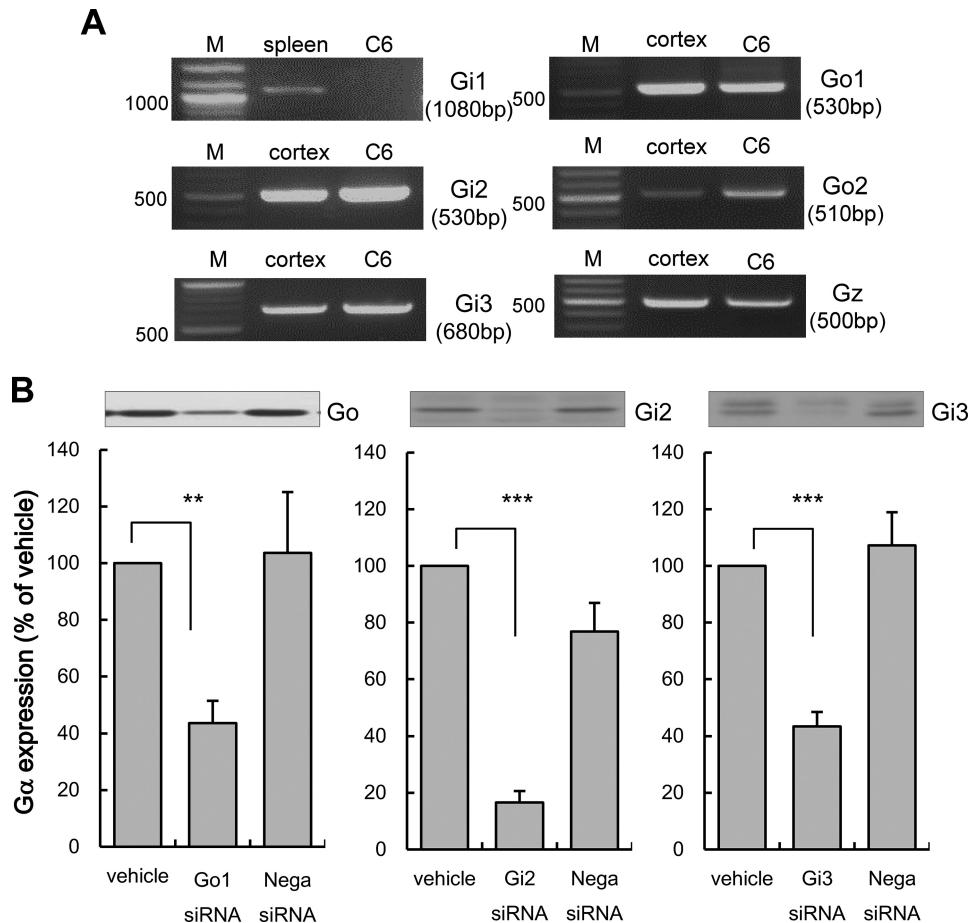


**FIGURE 3. Effects of pertussis toxin, YM-254890, GM6001, and SU5402 on ERK activation evoked by amitriptyline, 5-HT, and FGF2.** *A*, effects of pertussis toxin, YM-254890 (YM), GM6001 (GM), or SU5402 (SU) on ERK activation evoked by amitriptyline in C6 cells. C6 cells were pretreated with 100 ng/ml PTX for 3 h, 25  $\mu$ M SU for 1 h, and 100 nM YM-254890 or 25  $\mu$ M GM6001 for 0.5 h, and subsequently treated with 25  $\mu$ M amitriptyline for 5 min. Values are shown as ERK activity (% of control). Data are expressed as the mean  $\pm$  S.E. for four to five independent experiments. \*\*\*,  $p < 0.001$  in comparison with the basal group; ++,  $p < 0.01$ ; +,  $p < 0.05$  in comparison with the control group (Tukey's test). *B*, effects of PTX, YM-254890, GM6001, or SU5402 on ERK activation evoked by 5-HT in C6 cells. C6 cells were pretreated with 100 ng/ml PTX for 3 h, 25  $\mu$ M SU5402 for 1 h, 100 nM YM-254890 or 25  $\mu$ M GM6001 for 0.5 h, and subsequently treated with 10  $\mu$ M 5-HT for 2 min. ERK activity (phosphorylation levels of ERK1/2) was quantified by Western blotting. Values are shown as ERK activity (% of control). Data are expressed as the mean  $\pm$  S.E. for three independent experiments. \*\*\*,  $p < 0.001$  in comparison with the basal group; ++,  $p < 0.01$  in comparison with the control group (Tukey's test). *C*, effects of PTX, YM-254890, GM6001, and SU5402 on ERK activation evoked by FGF2 in C6 cells. C6 cells were pretreated with 100 ng/ml PTX for 3 h, 25  $\mu$ M SU5402 for 1 h, 100 nM YM-254890, or 25  $\mu$ M GM6001 for 0.5 h, and subsequently treated with 10 ng/ml FGF2 for 10 min. ERK activity (phosphorylation levels of ERK1/2) was quantified by Western blotting. Values are shown as ERK activity (% of control). Data are expressed as the mean  $\pm$  S.E. for three to five independent experiments. \*\*,  $p < 0.01$  in comparison with the basal group; ++,  $p < 0.01$  in comparison with the control group (Tukey's test). *D*, effects of PTX on ERK activation evoked by amitriptyline in NHA and primary cultured rat astrocytes. Normal human astrocytes were pretreated with 100 ng/ml PTX for 3 h and subsequently treated with 25  $\mu$ M amitriptyline for 5 min. Primary cultured rat astrocytes were pretreated with 100 ng/ml PTX for 3 h and subsequently treated with 25  $\mu$ M amitriptyline for 10 min. Values are shown as ERK activity (% of control). Data are expressed as the mean  $\pm$  S.E. for three to five independent experiments. \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  in comparison with the basal group; +,  $p < 0.05$ ; ++,  $p < 0.01$  in comparison with the control group (Tukey's test).

*Amitriptyline and 5-HT Increase ERK Activation through Different Intracellular Signaling Pathways in C6 Cells*—Amitriptyline-evoked ERK activation is elicited by FGFR activation through an MMP-dependent pathway that is coupled to GDNF production (11). To further determine whether PTX-sensitive  $G_{i/o}$  is specifically implicated in the cascade evoked by amitriptyline, the effect of PTX, YM-254890, MMP inhibitor (GM6001, 25  $\mu$ M), and FGFR inhibitor (SU5402, 25  $\mu$ M) on ERK activation evoked by either amitriptyline, 5-HT, or FGF2 was examined. The concentrations of inhibitors used were as described previously (23, 25, 30, 31). ERK activation by amitriptyline treatment significantly increased and peaked after 5 min of treatment in

C6 cells (9). The increase of ERK activity evoked by amitriptyline (25  $\mu$ M, 5 min treatment) was significantly inhibited by PTX, GM6001, and SU5402 but not by YM-254890 (Fig. 3A). The ERK activation by 5-HT treatment significantly increased and reached a maximum after 2 min of treatment in C6 cells (23). Serotonin-evoked ERK activation (10  $\mu$ M, 2 min treatment) was significantly inhibited by YM-254890 and SU5402 but not by either PTX or GM6001 (Fig. 3B). The ERK activation by FGF2 treatment significantly increased and reached a maximum after 10 min of treatment in rat astrocytes (32). FGF2-evoked ERK activation (10 ng/ml, 10 min treatment) was significantly inhibited by SU5402 but not by either PTX, YM-254890,

## Amitriptyline Increases GDNF through $G\alpha_{i/o}$ Activation



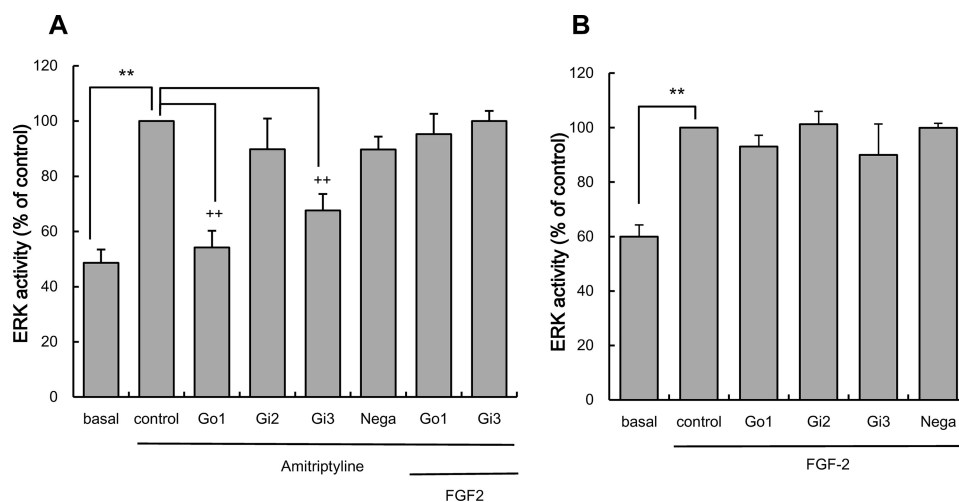
**FIGURE 4. Expressions of  $\alpha$ -subunits of G protein in C6 cells.** *A*, RT-PCR analysis of  $\alpha$ -subunits of G protein mRNA expression in C6 cells. Each lane represents the cDNA fragments of  $\alpha$ -subunits of G protein amplified from the RNA of either C6 cells, rat cortex, or rat spleen. Lane M indicates the molecular size marker. *B*, effects of  $G\alpha_{o1}$ ,  $G\alpha_{i2}$ ,  $G\alpha_{i3}$ , and negative control siRNA transfection on  $G\alpha_{o1}$ ,  $G\alpha_{i2}$ , and  $G\alpha_{i3}$  expressions. C6 cells were transfected with either  $G\alpha_{o1}$ ,  $G\alpha_{i2}$ ,  $G\alpha_{i3}$  siRNA (50 nM) or negative control siRNA (Nega siRNA; 50 nM) for 48 h. The  $\alpha$ -subunits of G protein were quantified by Western blotting. Representative blots are shown. Values shown are levels of  $\alpha$ -subunits of G protein (% of vehicle). Data are expressed as the mean  $\pm$  S.E. for 6–12 independent experiments. \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  in comparison with the vehicle group (Tukey's test).

or GM6001 (Fig. 3C). A previous study confirmed that there was no effect on basal levels of ERK activation by the inhibitors alone (23).

**Effect of PTX on ERK Activation Evoked by Amitriptyline in NHA and Primary Cultured Rat Astrocytes**—To confirm whether amitriptyline treatment evokes ERK activation through PTX sensitive G protein in both normal human and rat astrocytes, as it does in C6 cells, primary cultures of human and rat astrocytes were tested. In NHA, amitriptyline (25  $\mu$ M, 5 min treatment) significantly increased ERK activity. The ERK activation evoked by amitriptyline in NHA was almost completely suppressed by PTX (Fig. 3C). In primary cultured rat astrocytes, amitriptyline (25  $\mu$ M, 10 min treatment) significantly increased ERK activity. ERK activation evoked by amitriptyline in primary cultured rat astrocytes was also almost completely suppressed by PTX. The findings indicate that the intracellular mechanism observed in rat C6 cells is relevant to human astrocytes and primary cultured rat astrocytes.

**Effects of siRNA for  $G\alpha_{o1}$ ,  $G\alpha_{i2}$ , or  $G\alpha_{i3}$  on the ERK Activation Evoked by Amitriptyline in C6 Cells**—The effect of silencing PTX-sensitive  $\alpha$ -subunit of the G protein was examined to determine the role of each subtype on amitriptyline's effect. It has been reported that C6 cells express PTX-sensitive  $\alpha$ -sub-

units of the G protein, including  $G\alpha_{o1}$ ,  $G\alpha_{o2}$ ,  $G\alpha_{i2}$ , and  $G\alpha_{i3}$  (33, 34). The presence of these  $\alpha$ -subunits of G protein in C6 cells was confirmed by RT-PCR (Fig. 4A). Thus, the effect of siRNA specific for  $G\alpha_{o1}$ ,  $G\alpha_{i2}$ , and  $G\alpha_{i3}$  on amitriptyline-induced ERK activation was examined. The effect of siRNA on  $G\alpha_{o2}$  was not examined because an siRNA for  $G\alpha_{o2}$  is not commercially available. Because ERK is the most sensitive intracellular signaling molecule that responds to amitriptyline, ERK activity was used as an index of intracellular signaling cascade activation. Forty eight h of transfection of 50 nM siRNAs against  $\alpha$ -subunits of the G protein significantly reduced protein levels of individual subunits (Fig. 4B). An equivalent amount of negative control siRNA did not affect the expression levels of  $\alpha$ -subunits of G protein (Fig. 4B). Amitriptyline (25  $\mu$ M, 5 min treatment) significantly increased ERK activity in C6 cells. This activation was significantly blocked by  $G\alpha_{o1}$  siRNA and  $G\alpha_{i3}$  siRNA transfection, whereas  $G\alpha_{i2}$  siRNA transfection did not change ERK activity. Negative control siRNA had no effect on amitriptyline increased ERK activity (Fig. 5A). Thus, amitriptyline-evoked ERK activation appears to be more dependent on  $G\alpha_{o1}$  activity rather than  $G\alpha_{i3}$  activity and not at all on  $G\alpha_{i2}$  activity. Furthermore, addition of exogenous FGF2 increased ERK activity in cells that had  $G\alpha_{o1}$  and  $G\alpha_{i3}$  knocked down by siRNA transfection



**FIGURE 5. Effects of the  $\alpha$ -subunits of G protein knockdown on amitriptyline and FGF2-evoked ERK activation.** *A*, effect of either  $G_{\alpha_{o1}}$ ,  $G_{\alpha_{i2}}$ , or  $G_{\alpha_{i3}}$  knockdown on ERK activation evoked by amitriptyline. C6 cells were transfected with  $G_{\alpha_{o1}}$  siRNA,  $G_{\alpha_{i2}}$  siRNA, or  $G_{\alpha_{i3}}$  siRNA ( $G_{\alpha_{o1}}$ ,  $G_{\alpha_{i2}}$ , or  $G_{\alpha_{i3}}$ ; 50 nM) or negative control siRNA (*Nega*; 50 nM) for 48 h, and subsequently treated with 25  $\mu$ M amitriptyline alone or in conjunction with 10 ng/ml exogenous FGF2 for 5 min. Values are shown as ERK activity (% of control). Data are expressed as the mean  $\pm$  S.E. for 3–12 independent experiments. \*\*,  $p < 0.01$  in comparison with the basal group; ++,  $p < 0.01$  in comparison with the control group (Tukey's test). *B*, effect of either  $G_{\alpha_{o1}}$ ,  $G_{\alpha_{i2}}$ , or  $G_{\alpha_{i3}}$  knockdown on ERK activation evoked by FGF2. C6 cells were transfected with  $G_{\alpha_{o1}}$  siRNA,  $G_{\alpha_{i2}}$  siRNA, or  $G_{\alpha_{i3}}$  siRNA (*Go1*, *Gi2*, or *Gi3*; 50 nM) or negative control siRNA (*Nega*; 50 nM) for 48 h, and subsequently treated with 10 ng/ml FGF2 for 10 min. Values are shown as ERK activity (% of control). Data are expressed as the mean  $\pm$  S.E. for four to six independent experiments. \*\*,  $p < 0.01$  in comparison with the basal group (Tukey's test).

(Fig. 5A). Knock-out of  $G_{\alpha_{o1}}$  and  $G_{\alpha_{i3}}$  did not affect ERK activation by exogenous FGF2 (Fig. 5B), indicating that FGF2-induced ERK activation is independent of  $G_{\alpha_{o1}}$  and  $G_{\alpha_{i3}}$ .

**Effects of Amitriptyline on the Changes of Impedance ( $\Delta Z$ ) in C6 Cells and Rat Astrocytes Using Electrical Impedance-based Biosensors (CellKey™ Assay)**—A number of reports have demonstrated the CellKey™ assay's ability to distinguish the activation of  $G\alpha$  subfamily ( $G_{\alpha_{i/o}}$ ,  $G_s$ , and  $G_q$ ) by patterns of  $\Delta Z$  changes (19). To show typical different  $\Delta Z$  values caused by each ligand to specific types of GPCRs, HEK293 cells expressing  $\mu$ -opioid receptors, coupled to  $G_{\alpha_{i/o}}$ , and C6 cells endogenously expressing  $\beta$ -adrenergic receptors, coupled to  $G_s$ , were used (14, 35). In addition, HEK293 cells, which endogenously express the muscarinic ( $M_3$ ) acetylcholine receptor, were used to show the activation of  $G_q$ -coupled receptors (36, 37). In Fig. 6A, DAMGO, an agonist of the  $\mu$ -opioid receptor, induced an increase in  $\Delta Z$ ; the activation of  $G_{\alpha_{i/o}}$  increases  $\Delta Z$ . By contrast, isoproterenol, an agonist of  $\beta_1$ - and  $\beta_2$ -adrenergic receptors, induced a decrease in  $\Delta Z$ ; the activation of  $G_s$  decreases  $\Delta Z$  (Fig. 6B). Acetylcholine, an  $M_3$  acetylcholine receptor ligand, induced a rapid decrease in  $\Delta Z$  followed by a gradual increase in  $\Delta Z$ ; the activation of  $G_q$  leads to a unique  $\Delta Z$  pattern—rapid decrease and subsequent increase (Fig. 6C). As shown in Fig. 6, D and E, amitriptyline in C6 cells increased  $\Delta Z$  in a concentration-dependent manner. This increase peaked around 10 min after amitriptyline treatment and persisted for at least 30 min (data not shown). Amitriptyline treatment also increased  $\Delta Z$  in a concentration-dependent manner in rat astrocytes (Fig. 6, F and G). Comparing the pattern of  $\Delta Z$  induced by amitriptyline, the pattern suggests  $G_{\alpha_{i/o}}$  activation (Fig. 6, A, D, and F).

**Effects of PTX, NF449, or YM-254890 on the Increase in  $\Delta Z$  Evoked by Amitriptyline in C6 Cells and Rat Astrocytes**—To pharmacologically characterize the increase of  $\Delta Z$  evoked by amitriptyline, the effects of G protein  $\alpha$ -subunit inhibitors were examined. The increase in  $\Delta Z$  evoked by amitriptyline (25  $\mu$ M)

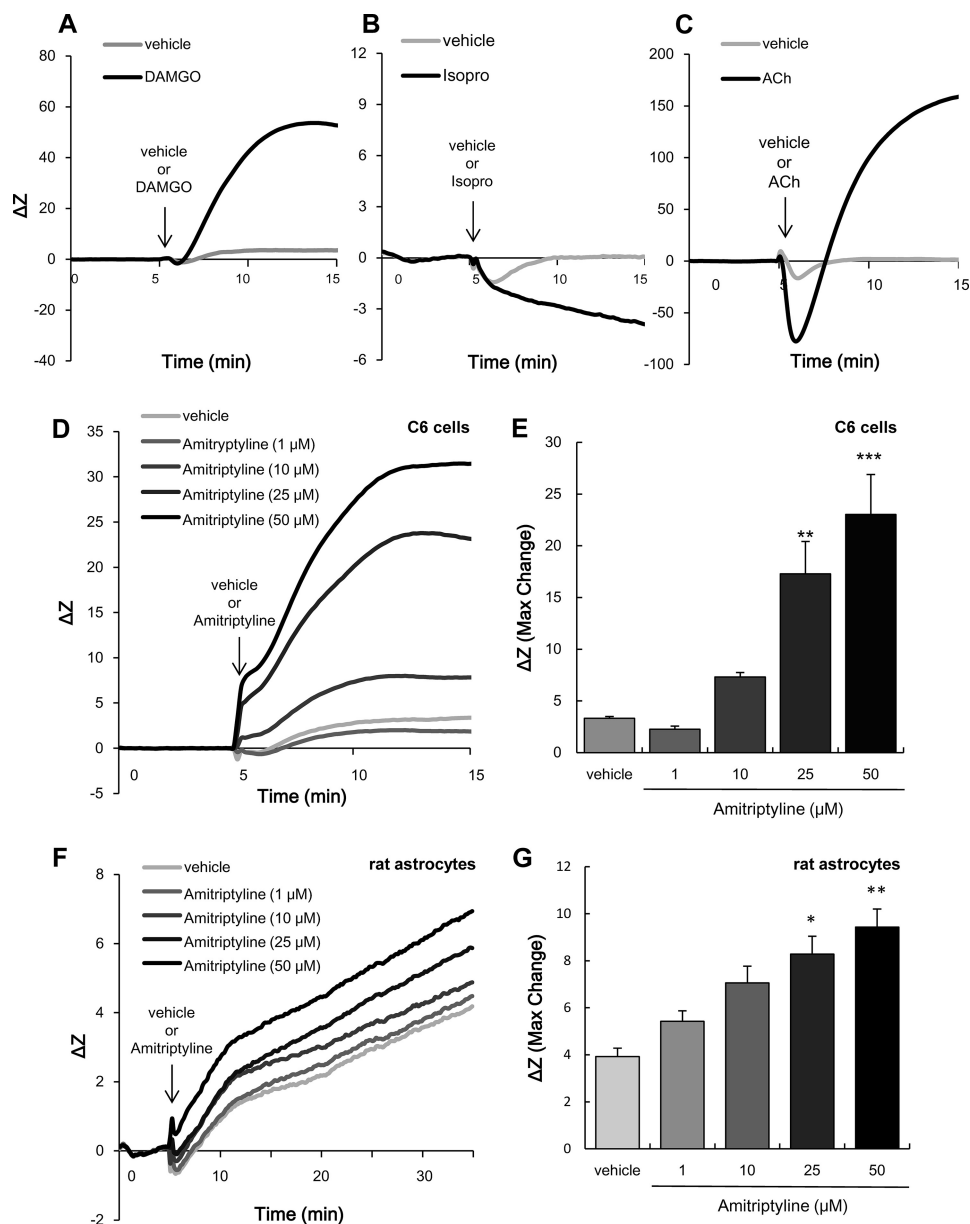
in C6 cells was significantly, but not completely, inhibited by PTX. However, neither NF449 nor YM-254890 inhibited the amitriptyline-induced increase in  $\Delta Z$  (Fig. 7A). Application of each inhibitor alone did not affect in  $\Delta Z$  (% of basal: PTX alone, 73.0  $\pm$  5.2%; NF449 alone, 111.3  $\pm$  24.7%; YM-254890 alone, 88.0  $\pm$  8.0%). The increase in  $\Delta Z$  evoked by amitriptyline (25  $\mu$ M) in rat astrocytes was also significantly, but not completely, inhibited by PTX (Fig. 7B). In  $\mu$ -opioid receptor-expressing HEK293 cells, the increase in  $\Delta Z$  evoked by DAMGO (1  $\mu$ M) was completely suppressed by a 3-h pretreatment of 100 ng/ml PTX (Fig. 7C). Application of PTX alone did not affect  $\Delta Z$  (% of basal: PTX alone, 155.2  $\pm$  47.8%).

**Effects of Inhibitors on the Cascade Related to GDNF Production on the Increase in  $\Delta Z$  Evoked by Amitriptyline in C6 Cells**—The effects of inhibitors on the cascade related to GDNF production (MMP/FGFR/FRS2 $\alpha$ /ERK) on the increase in  $\Delta Z$  evoked by amitriptyline were examined, to elucidate the point within the cascade that was responsible for the increase of  $\Delta Z$  ( $G_{\alpha_{i/o}}$  activation). The concentrations of inhibitors were used as described previously (30, 31, 38, 39). GM6001, SU5402, PD173074 (1  $\mu$ M; FGFR inhibitor), and U0126 (10  $\mu$ M; MEK inhibitor) had no effect on the amitriptyline-induced increase in  $\Delta Z$  (Fig. 8A). Application of each inhibitor alone did not affect  $\Delta Z$  (% of basal: GM6001 alone, 86.2  $\pm$  9.7%; SU5402 alone, 83.3  $\pm$  13.6%; PD173074 alone, 106.5  $\pm$  9.3%; U0126 alone, 75.9  $\pm$  6.9%). In addition, treatment with exogenous FGF2 (up to 500 ng/ml) did not change  $\Delta Z$  in C6 cells (Fig. 8, B and C).

**Increases of GDNF Production Evoked by Amitriptyline Are Independent of the  $\kappa$ -Opioid Receptor**—Recently, it has been reported that amitriptyline exerts direct agonist activity at  $\kappa$ -opioid receptors, which is coupled to  $G_{\alpha_{i/o}}$  (40). C6 cells are known to endogenously express  $\kappa$ -opioid receptors (41). To examine a potential involvement of  $\kappa$ -opioid receptors in GDNF production, we examined the effect of the  $\kappa$ -opioid



## Amitriptyline Increases GDNF through $G\alpha_{i/o}$ Activation



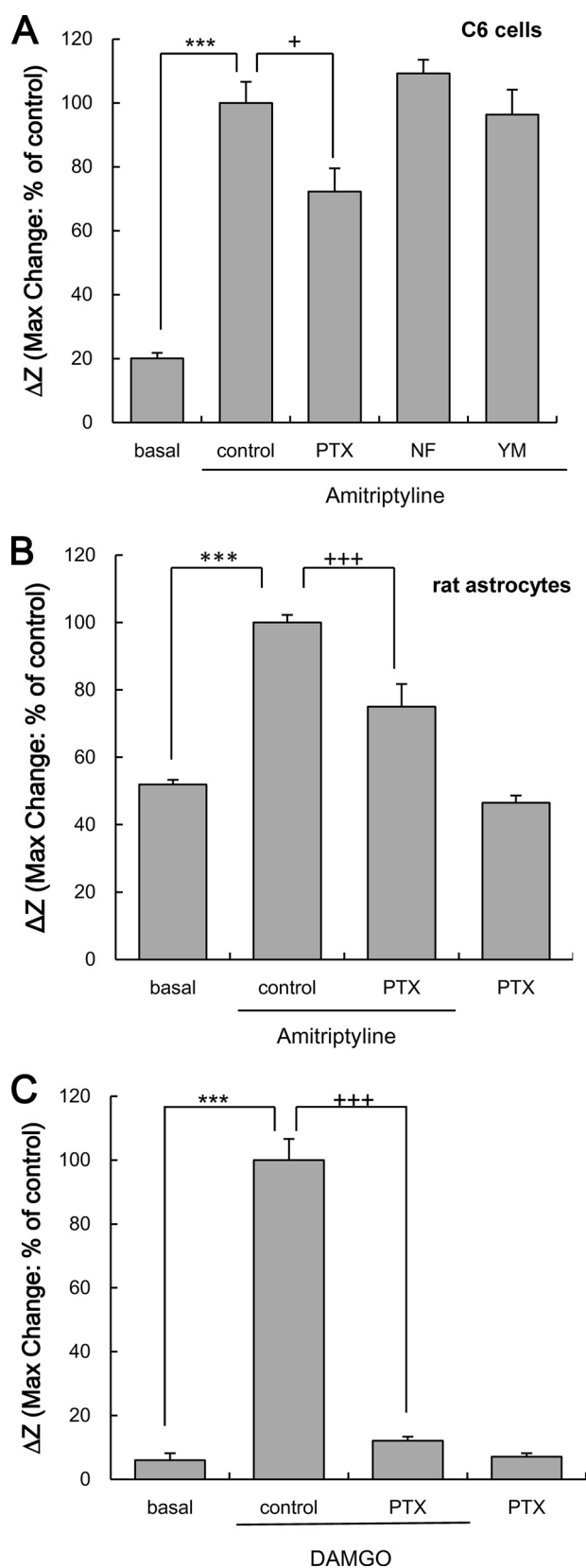
**FIGURE 6. Effects of amitriptyline on impedance ( $\Delta Z$ ) in C6 cells and rat astrocytes using electrical impedance-based biosensors (CellKey™ assay).** *A*, effects of DAMGO on  $\Delta Z$  in  $\mu$ -opioid receptor-expressing HEK293 cells. The cells were treated with vehicle or 1  $\mu M$  DAMGO for 10 min. The traces shown are representative of the mean increase in  $\Delta Z$  of cells from each well. Similar results were obtained in at least three independent experiments. *B*, effects of a  $\beta$ -adrenergic receptor agonist isoproterenol (*Isopro*) on  $\Delta Z$  in C6 cells endogenously expressing  $\beta$ -adrenergic receptors. The cells were treated with vehicle or 100 nM isoproterenol (*Isopro*) for 10 min. The traces shown are representative of the mean increase in  $\Delta Z$  of cells from each well. Similar results were obtained in at least three independent experiments. *C*, effects of acetylcholine on  $\Delta Z$  in HEK293 cells endogenously expressing muscarinic (M3) acetylcholine receptors. The cells were treated with vehicle or 1  $\mu M$  acetylcholine (*ACh*) for 10 min. The traces shown are representative of the mean increase in  $\Delta Z$  of cells from each well. Similar results were obtained in at least three independent experiments. *D*, effects of amitriptyline on  $\Delta Z$  in C6 cells. C6 cells were treated with amitriptyline (1, 10, 25, and 50  $\mu M$ ) for 10 min. The traces shown are representative of the mean increase in  $\Delta Z$  of cells from each well. Similar results were obtained in at least three independent experiments. *E*, extent of changes in  $\Delta Z$  was presented as the maximum  $\Delta Z$  after injection of vehicle or amitriptyline. Values are expressed as the mean  $\pm$  S.E. of  $\Delta Z$  (each group;  $n = 6$ ). \*\*,  $p < 0.01$ ; \*\*\*,  $p < 0.001$  in comparison with the vehicle group (Tukey's test). *F*, effects of amitriptyline on  $\Delta Z$  in rat astrocytes. Rat astrocytes were treated with amitriptyline (1, 10, 25, and 50  $\mu M$ ) for 30 min. The traces shown are representative of the mean increase in  $\Delta Z$  of cells from each well. Similar results were obtained in at least three independent experiments. *G*, extent of changes in  $\Delta Z$  was presented as the maximum  $\Delta Z$  after injection of vehicle or amitriptyline. Values are expressed as the mean  $\pm$  S.E. of  $\Delta Z$  (all each group;  $n = 12$ ). \*,  $p < 0.05$ ; \*\*,  $p < 0.01$  in comparison with the vehicle group (Tukey's test).

receptor antagonist, nor-BNI (1  $\mu M$ ), as described previously (40). The increase of GDNF release evoked by amitriptyline (25  $\mu M$ ) was not affected by pretreatment with nor-BNI (basal,  $15.9 \pm 3.87$  pg/ml; amitriptyline only,  $43.5 \pm 7.40$  pg/ml (\*,  $p < 0.05$  in comparison with basal); amitriptyline + nor-BNI,  $43.8 \pm 8.50$  pg/ml; nor-BNI only,  $15.8 \pm 4.83$  pg/ml). Thus,

$\kappa$ -opioid receptors are not involved in GDNF production in C6 cells.

### Discussion

The current data suggest that amitriptyline evokes GDNF production through a mechanism that is entirely independent



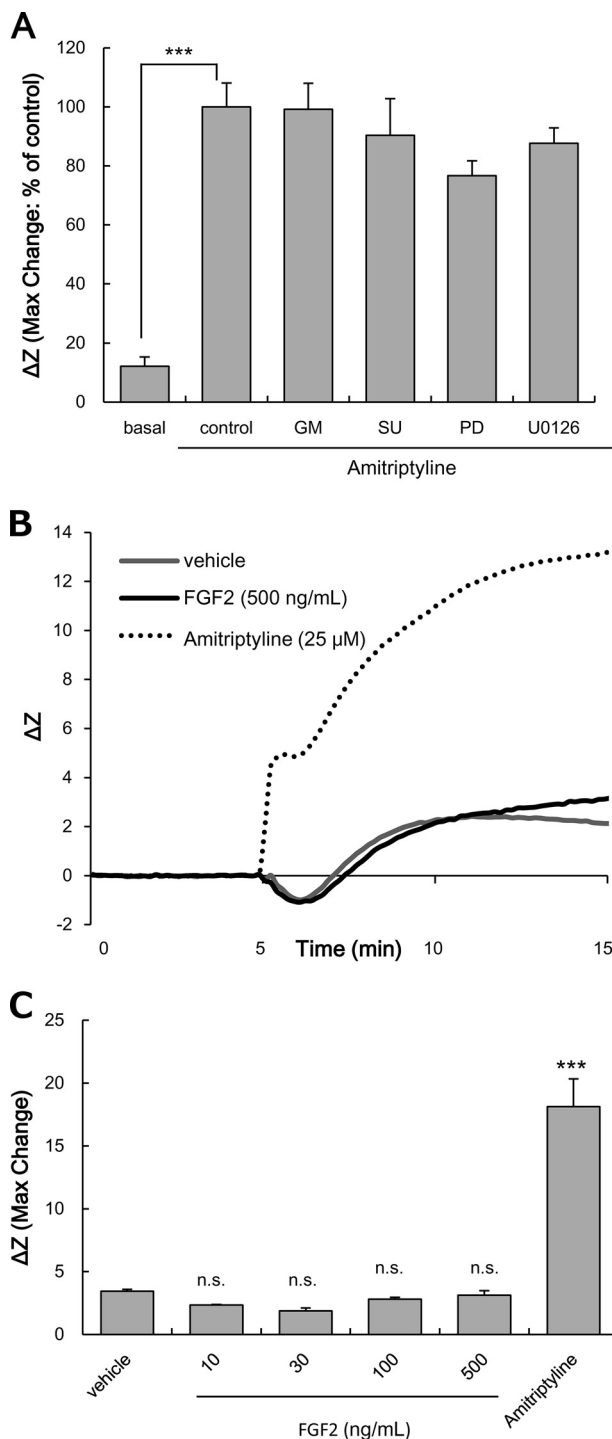
**FIGURE 7. Effects of G protein inhibitors on the increase in  $\Delta Z$  evoked by amitriptyline in C6 cells and rat astrocytes or  $\mu$ -opioid receptor agonist DAMGO in HEK293 cells expressing  $\mu$ -opioid receptors.** *A*, effects of PTX, NF449 (NF), and YM-254890 (YM) on amitriptyline-evoked increase in  $\Delta Z$  in C6 cells. C6 cells were pretreated with 100 ng/ml PTX for 3 h, 1  $\mu$ M NF449 or 100 nM YM-254890 for 0.5 h, and subsequently treated with 25  $\mu$ M of amitriptyline

of monoamines, via activation of a PTX-sensitive  $G_{i/o}$ /MMP/FGFR/FRS2 $\alpha$ /ERK cascade in astroglial cells (Fig. 9). This study demonstrated that the PTX-sensitive  $G_{i/o}$  subunits, especially  $G_o$  and  $G_{i3}$ , play important roles in the activation of ERK evoked by amitriptyline. The CellKey<sup>TM</sup> assay confirmed the pharmacological and siRNA findings, in that the pattern of the change in impedance induced by amitriptyline was similar to that of activation of  $G^{i/o}$ -coupled receptors, and not of  $G_s$ - or  $G_q$ -coupled receptors (13, 14, 17, 18). Although the C6 cells is an astrocyte model cell line derived from rat glioma, activation of the MMP/FGFR/FRS2 $\alpha$ /ERK cascade occurs in both normal human and rat astrocytes as well (9, 11), in a PTX-sensitive manner.

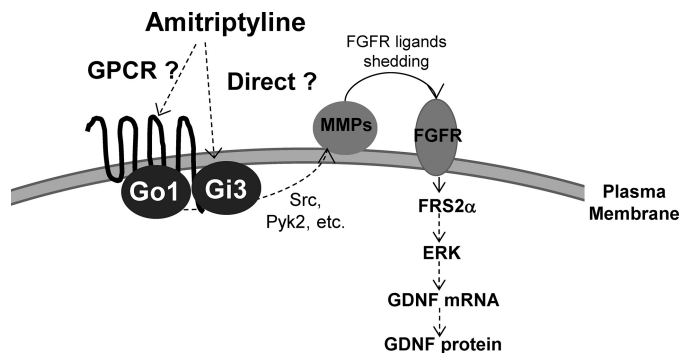
The  $G_\alpha$  family of G proteins is composed of the  $G_s$ ,  $G_{i/o}$ , and  $G_q$  subfamilies (14). Among the G protein inhibitors used in this study, only PTX inhibited amitriptyline-induced GDNF production and the amitriptyline-mediated response of the cascade (FRS2 $\alpha$  and ERK), indicating that PTX-sensitive  $G_{i/o}$  plays a critical role in the intracellular response to amitriptyline. The concentrations of the selective inhibitors for the various intracellular signal cascade molecules were based on previous reports for cell culture systems (23–26, 30, 31, 38, 39). Based on the  $IC_{50}$  value of these inhibitors, these inhibitors are in fact selective for their respective targets (30, 42–47). Although 25  $\mu$ M GM6001 was used, which is 1000-fold of the  $IC_{50}$ , a previous study used the negative analog of GM6001 to confirm selectivity of GM6001. The negative analog, at 25  $\mu$ M, showed no effect (11). To further confirm the involvement of PTX-sensitive  $G_{i/o}$  on the effects of amitriptyline, siRNAs specific for  $G_{o1}$ ,  $G_{i2}$ , and  $G_{i3}$  were used. Amitriptyline-evoked ERK activation was significantly blocked in C6 cells in which either  $G_{o1}$  or  $G_{i3}$  expression was reduced by siRNA. Although amitriptyline had no effect on ERK activation in these cells, treatment of the cells with exogenous FGF2 evoked ERK activation. PTX had no effect on the exogenous FGF2-mediated activation of FRS2 $\alpha$  and ERK. Furthermore, FGFR activation by exogenous FGF2 did not change  $\Delta Z$  ( $G_{i/o}$  activation). Conversely, FGFR inhibitor and ERK inhibitor had no effect on  $G_{i/o}$  activation by amitriptyline. These data indicate that activation of FGFR/FRS2 $\alpha$ /ERK is well downstream of  $G_{o1}$  and  $G_{i3}$  protein activation by amitriptyline, which is in line with our model system (Fig. 9). MMP is a key intermediary between  $G_{i/o}$  activation and FGFR/FRS2 $\alpha$ /ERK activation. Activation of FGFR/FRS2 $\alpha$ /ERK by amitriptyline was inhibited by a MMP inhibitor, whereas activation of FGFR/FRS2 $\alpha$ /ERK by exogenous FGF2 was not affected by a

for 10 min. Values are expressed as the mean  $\pm$  S.E. of  $\Delta Z$  (basal,  $n = 21$ ; control,  $n = 21$ ; PTX,  $n = 9$ ; NF449,  $n = 12$ ; YM-254890,  $n = 9$ ). \*\*\*,  $p < 0.001$  in comparison with the basal group; +,  $p < 0.05$  in comparison with the control group (Tukey's test). *B*, effects of PTX on amitriptyline-evoked increase in  $\Delta Z$  in rat astrocytes. Rat astrocytes were pretreated with 100 ng/ml PTX for 3 h and subsequently treated with 25  $\mu$ M amitriptyline for 30 min. Values are expressed as the mean  $\pm$  S.E. of  $\Delta Z$  (all each group;  $n = 6$ ). \*\*\*,  $p < 0.001$  in comparison with the basal group; +,  $p < 0.05$  in comparison with the control group (Tukey's test). *C*, effects of PTX on the increase in  $\Delta Z$  evoked by the  $\mu$ -opioid receptor agonist in HEK293 cells expressing  $\mu$ -opioid receptors. HEK293 cells expressing  $\mu$ -opioid receptors were pretreated with 100 ng/ml PTX for 3 h and subsequently treated with 1  $\mu$ M DAMGO for 10 min. Values are expressed as the mean  $\pm$  S.E. of  $\Delta Z$  (all each group;  $n = 12$ ). \*\*\*,  $p < 0.001$  in comparison with the basal group; +,  $p < 0.05$  in comparison with the control group (Tukey's test).

## Amitriptyline Increases GDNF through $G_{\alpha_{i/o}}$ Activation



**FIGURE 8. Effects of inhibitors related to the GDNF production cascade on the amitriptyline-evoked increase of  $\Delta Z$  in C6 cells.** *A*, effects of GM6001 (GM), SU5420 (SU), PD173074 (PD), and U0126 on the increase of  $\Delta Z$  evoked by amitriptyline in C6 cells. C6 cells were pretreated with 25  $\mu\text{M}$  GM6001, 25  $\mu\text{M}$  SU5402, 1  $\mu\text{M}$  PD173074, or 10  $\mu\text{M}$  U0126 for 0.5 h and subsequently treated with 25  $\mu\text{M}$  amitriptyline for 10 min. Values are expressed as the mean  $\pm$  S.E. of  $\Delta Z$  (basal,  $n = 23$ ; control,  $n = 23$ ; GM6001,  $n = 9$ ; SU5402,  $n = 5$ ; PD173074,  $n = 10$ ; U0126,  $n = 6$ ). \*\*\*,  $p < 0.001$  in comparison with the basal group (Tukey's test). *B*, changes in  $\Delta Z$  evoked by incubation in vehicle, FGF2 (10, 30, 100, and 500 ng/ml), or amitriptyline (25  $\mu\text{M}$ ) for 10 min in C6 cells. The traces shown are representative of the mean increase in  $\Delta Z$  of cells from each well. Similar results were obtained in at least three independent experiments. *C*, extent of changes in  $\Delta Z$  was presented as the maximum  $\Delta Z$  after injection of vehicle, FGF2, or amitriptyline. Values are expressed as the mean  $\pm$  S.E. of  $\Delta Z$  (vehicle,  $n = 9$ ; FGF2 (each dose),  $n = 3$ ; amitriptyline,  $n = 9$ ). \*\*\*,  $p < 0.001$  in comparison with the vehicle group (Tukey's test). n.s., no significant difference.



**FIGURE 9. Hypothesized monoamine-independent mode of action of amitriptyline-induced GDNF production in astroglial cells.** Amitriptyline either directly or indirectly activates PTX-sensitive  $G_{i/o}$  ( $G_{i3}$  or  $G_{o1}$ ) and MMP/FGFR/FRS2 $\alpha$ /ERK cascade, leading to GDNF production in astroglial cells.

MMP inhibitor (11). Furthermore, the increase of  $\Delta Z$  ( $G_{i/o}$  activation) by amitriptyline was not affected by an MMP inhibitor. These results indicate that MMP lies between  $G_{i/o}$  and FGFR/FRS2 $\alpha$ /ERK in the molecular signaling cascade evoked by amitriptyline. Thus, in our proposed pathway, although MMP and FGFR act as intermediaries,  $G_{o1}$  and  $G_{i3}$  are crucial as the initiating signaling molecules that leads to GDNF production by amitriptyline in astroglial cells (Fig. 9).

The intracellular signaling pathway mediating GDNF production by amitriptyline has not been completely elucidated. The MMPs are a family of zinc-dependent endopeptidases with 24 identified members (48). It has been reported that MMP is activated by intracellular mediators such as Src family kinases, calcium ( $\text{Ca}^{2+}$ ), proline-rich tyrosine kinase 2 (Pyk2), and protein kinase C (PKC), which are regulated by G proteins (12). We previously showed that ERK activation evoked by amitriptyline was not inhibited by calphostin C (pan-PKC inhibitor), rottlerin (PKC  $\delta$  inhibitor), EDTA ( $\text{Ca}^{2+}$  inhibitor), and 1,2-bis(2-aminophenoxy)ethane- $N,N,N',N'$ -tetraacetic acid tetrakis-(acetoxymethyl ester) (intracellular  $\text{Ca}^{2+}$  inhibitor) (9). Thus, either Src family kinases or Pyk2 could be involved in the  $G_{i/o}$ /MMP/FGFR/FRS2 $\alpha$ /ERK cascade activated by amitriptyline in this study. However, the molecular intermediaries between G protein and MMP in the cascade and the specific type of MMP involved have yet to be identified. Because of a potentially crucial role of MMP in mediating the physiological effect of amitriptyline, identification of the MMP in the cascade is currently under investigation.

In the CellKey<sup>TM</sup> assay, the pattern of  $\Delta Z$  evoked by amitriptyline indicated activation of  $G_{i/o}$  in C6 cells and rat astrocytes. Scott and Peters (19) reported that the CellKey<sup>TM</sup> assay is a highly sensitive assay compared with traditional assays such as GTP $\gamma$ S binding assay, cAMP assay, and  $\text{Ca}^{2+}$  imaging assay. However, there is currently a lack of data demonstrating changes in terms of the amount, activity, or distribution of  $G_{i/o}$  in C6 cells following chronic antidepressant treatment (49–51). The discrepancy between the current findings and previous findings could stem from differences in experimental protocols, such as treatment period (acute versus chronic), sample preparation (e.g. living cells, membrane preparation, or fixed tissues or cells), and assay readout (adenylyl cyclase activity or CellKey<sup>TM</sup>).

In this study, an acute treatment with amitriptyline activated  $G_{i/o}$  as indicated by increased  $\Delta Z$ . However, pretreatment with 100 ng/ml PTX for 3 h only partially inhibited the increase in  $\Delta Z$ . By contrast, the same treatment with PTX completely suppressed the DAMGO-induced increase of  $\Delta Z$ . Other experiments in this study showed a robust suppression of the GDNF production cascade using the current PTX treatment procedure. Thus, the partial inhibition by PTX treatment suggests involvement of a PTX-insensitive  $G_{i/o}$ . In the  $G_{i/o}$  subfamily,  $G_{11}$ ,  $G_{12}$ ,  $G_{13}$ ,  $G_{o1}$ ,  $G_{o2}$ , and  $G_z$  subunits have been identified and share similar properties in signal transduction (52). However, among these subunits,  $G_z$  lacks the C-terminal cysteine residue and is not inactivated by PTX treatment (53, 54). Wang *et al.* (55) reported that C6 cells express  $G_z$ . This study confirmed the expression of  $G_z$  in C6 cells by RT-PCR. Therefore, these data suggest that amitriptyline activates both PTX-sensitive  $G_{i/o}$  subunits,  $G_{o1}$  and  $G_{13}$ , and PTX-insensitive  $G_{i/o}$  subunit, such as  $G_z$ . Although the  $\Delta Z$  pattern following amitriptyline treatment was similar to that of DAMGO, indicating  $G_{i/o}$  activation, it was not the same. A rapid initial increase of  $\Delta Z$  was observed following amitriptyline treatment, whereas a gradual initial increase of  $\Delta Z$  was observed after DAMGO treatment. This differential response could be due to the type of  $G_{i/o}$  subunits activated by either ligand. Taken together, the predominant G protein subunit that mediates the effect of amitriptyline in this study is the PTX-sensitive  $G_{i/o}$  subunit, but it is possible, given the partial inhibition observed in this study, that amitriptyline also activates a PTX-insensitive  $G_{i/o}$  subunit such as  $G_z$ .

Although amitriptyline treatment acutely increased  $G_{i/o}$  activation in C6 cells and rat astrocytes, it is unknown whether amitriptyline directly or indirectly activates  $G_{i/o}$ . Amitriptyline may have two possible modes of action. One possibility is that amitriptyline directly acts on  $G_{i/o}$  associated with the plasma membrane, and another possibility is that amitriptyline activates  $G_{i/o}$  through a yet-to-be identified GPCR (Fig. 9). Yamamoto *et al.* (56) reported that tricyclic antidepressants increased GTPase activity of  $G_o$  in bovine brain membranes reconstituted into a phospholipid mixture without receptors. The effect of tricyclic antidepressants on the GTPase activity of  $G_o$  was abolished by pretreatment with PTX, indicating that tricyclic antidepressants, such as amitriptyline, could directly activate  $G_o$ , bypassing the need for receptor activation (56). However, the mechanism of GDNF production evoked by amitriptyline is reminiscent of receptor tyrosine kinase transactivation by GPCR (12). In this study, the data indicate the involvement of PTX-sensitive  $G_{i/o}$  subunits, especially  $G_{o1}$  and  $G_{13}$ . The histamine  $H_1$  and muscarinic  $M_3$  receptors, which are known to bind amitriptyline and are endogenously expressed in C6 cells, are functionally coupled to  $G_q$  (57–59). Like amitriptyline, 5-HT activated the FGFR/FRS2 $\alpha$ /ERK cascade, but as demonstrated in this study, the effect of 5-HT was elicited through  $G_q$ , but not  $G_{i/o}$ . Furthermore, it was previously confirmed that amitriptyline-evoked GDNF production in C6 cells occurred independently of monoamine and muscarinic and histamine receptor activation (9). Thus, these GPCR for which amitriptyline has high affinity are not likely involved in GDNF production in C6 cells. Although it has been reported that amitriptyline exerts direct agonist activity at the  $\kappa$ -opioid receptor

(40), this study demonstrated a lack of involvement of the  $\kappa$ -opioid receptor on GDNF production. Furthermore, it has been reported that in C6 cells, U69593, a  $\kappa$ -opioid receptor agonist, did not induce FGFR1 phosphorylation, which is a key step in the GDNF production cascade ( $G_{i/o}$ /MMP/FGFR/FRS2 $\alpha$ /ERK) (29); amitriptyline increased both of FGFR1 and FGFR2 phosphorylation in C6 cells (11). Thus, the  $\kappa$ -opioid receptor is not involved in the activation of the cascade that leads to the production of GDNF. A GPCR with high affinity for amitriptyline could, however, be involved in GDNF production, as numerous GPCRs have been identified in astrocytes. The specific GPCR mediating amitriptyline's effect has yet to be identified and requires further investigation. Identifying the GPCR could facilitate understanding the source of the adverse effects as well as the beneficial effects of antidepressants.

Because of their high lipophilic properties, antidepressants accumulate in the brain at concentrations severalfold higher than that in blood. In fact, it has been previously shown that the concentration of amitriptyline in the brain is  $\sim 10$ – $35$  times higher than the plasma concentration of amitriptyline (60, 61). The therapeutic plasma concentration of amitriptyline is commonly regarded to range from  $\sim 0.36$  to  $0.9 \mu\text{M}$  (62). At therapeutic plasma concentrations, the brain concentration of amitriptyline can therefore be expected to be between 3.6 and  $31.5 \mu\text{M}$ . Previous studies have shown that the minimum concentration of amitriptyline that induced GDNF release is  $10 \mu\text{M}$  and a trend toward increased ERK activity with amitriptyline concentrations greater than  $1 \mu\text{M}$  in astroglial cells was observed (9); these concentrations of amitriptyline are within the range of the estimated brain concentration that is achieved after treatment with clinically relevant doses. Furthermore, the concentrations of amitriptyline used in this study were not toxic to C6 cells and primary cultured rat astrocytes (9, 10). Amitriptyline inhibits the serotonin transporter and the norepinephrine transporter at a  $K_i$  value of 16 and  $8.6 \text{ nM}$ , respectively (63). Thus, the estimated brain concentration of amitriptyline is much higher than the  $K_i$  value for these transporters, indicating the likelihood that amitriptyline could interact with additional targets, such as  $G_{o1}$ - and/or  $G_{13}$ -coupled receptors or other membrane-binding sites.

G proteins are crucial mediators between extracellular signaling and the intracellular response, which, in turn, could lead to either neurological homeostasis or disorder. It remains unclear as to how amitriptyline-evoked  $G_{i/o}$  activation is related to the antidepressive effect of amitriptyline. An *in vivo* study showed that intracerebroventricular administration of PTX completely prevented the antidepressant-like effect of amitriptyline in the mouse forced swimming test (64). Furthermore, Galeotti *et al.* (64) showed that  $G_{11}$ ,  $G_{i2}$ ,  $G_{13}$ , and  $G_{o1}$ , but not  $G_{o2}$ , play important roles in the antidepressant-like effect of amitriptyline, administering antisense oligonucleotides against the  $\alpha$ -subunits of the G protein. This study confirms the importance of PTX-sensitive  $G_{i/o}$  in the effect of antidepressants such as amitriptyline and further elaborates a novel mechanism that could explain the efficacy of antidepressants, increased GDNF production by astrocytes.

In conclusion, this study showed that the PTX-sensitive  $\alpha$ -subunit of G proteins,  $G_{o1}$  and  $G_{13}$ , plays an important role in

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a monoamine-independent production of GDNF evoked by amitriptyline. Previous studies have shown that several classes of antidepressants (tricyclic antidepressant, tetracyclic antidepressant, and selective 5-HT reuptake inhibitor) increase FRS2 $\alpha$  phosphorylation, activate ERK, and stimulate GDNF production in both rat and human astrocytes (8, 9, 11). It is not yet clear whether these different antidepressants act similarly to amitriptyline. Further studies are needed to clarify whether the effect of amitriptyline on  $G_{i/o}$  is a feature shared among antidepressants. It is hoped that by elaborating the  $G_{i/o}$ -dependent mechanism that underlies amitriptyline's therapeutic effect, there will be greater understanding of the non-monoaminergic and non-neural mechanisms of antidepressant activity, thereby setting the stage for the development of antidepressants with truly novel mechanisms of action.

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