Antidepressants Increase Glial Cell Line-Derived Neurotrophic Factor Production through Monoamine-Independent Activation of Protein Tyrosine Kinase and Extracellular Signal-Regulated Kinase in Glial Cells

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ABSTRACT

Recent studies show that neuronal and glial plasticity are important for therapeutic action of antidepressants. We previously reported that antidepressants increase glial cell line-derived neurotrophic factor (GDNF) production in rat C6 glioma cells (C6 cells). Here, we found that amitriptyline, a tricyclic antidepressant, increased both GDNF mRNA expression and release, which were selectively and completely inhibited by mitogen-activated protein kinase kinase (MEK) inhibitors. Moreover, treatment of amitriptyline rapidly increased extracellular signal-regulated kinase (ERK) activity, as well as phospho-specific mitogen-activated protein kinase and c-Jun NH2-terminal kinase activities. Furthermore, different classes of antidepressants also rapidly increased ERK activity. The extent of acute ERK activation and GDNF release were significantly correlated to each other in individual antidepressants, suggesting an important role of acute ERK activation in GDNF production. Furthermore, antidepressants increased the acute ERK activation and GDNF mRNA expression in normal human astrocytes as well as C6 cells. Although 5-hydroxytryptamine (serotonin) (5-HT), but not noradrenaline or dopamine, increased ERK activation and GDNF release via 5-HT2A receptors, ketanserin, a 5-HT2A receptor antagonist, did not have any effect on the amitriptyline-induced ERK activation. Thus, GDNF production by amitriptyline was independent of monoamine. Both of the amitriptyline-induced ERK activation and GDNF mRNA expression were blocked by genistein, a general protein tyrosine kinase (PTK) inhibitor. Actually, we found that amitriptyline acutely increased phosphorylation levels of several phosphotyrosine-containing proteins. Taken together, these findings indicate that ERK activation through PTK regulates antidepressant-induced GDNF production and that the GDNF production in glial cells may be a novel action of the antidepressant, which is independent of monoamine.

Major depression is a common and severe illness affecting a large number of individuals during their lifetime, and it is primarily treated with antidepressants. Most of the antidepressants are known to inhibit 5-hydroxytryptamine (serotonin, 5-HT) and/or noradrenaline (NA) reuptake; however, the efficacy of these antidepressants cannot be solely explained by their actions on the monoaminergic system. The molecular and cellular adaptations that underlie the therapeutic action of antidepressants have remained obscure.

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); NA, noradrenaline; BDNF, brain-derived neurotrophic factor; FGF, fibroblast growth factor; GDNF, glial cell line-derived neurotrophic factor; N2[(4-bromocinnamylamino)ethyl]-5-isoquinoline; U73122, 1-[6-[[17-me-thoxyestra-1,3,5(10)-trien-17-yl]aminio][hexyl]-1H-pyrole-2,5-dione; PD169316, 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)1H-imidazole; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)-butadiene; BAPTA-AM, 1,2-bis-(2-aminophenoxylethane-N,N,N’,N’-tetraacetic acid-acetoxyethyl ester; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; WAY106655, [O-methyl-3H]-N-(2-(4-(2-methoxyphenyl)-1-piperazineyl)ethyl]-N-(2-pyridinyl)cyclohexancarboxamidine trihydrochloride; NHA, normal human astrocyte(s); RT-PCR, reverse transcription-polymerase chain reaction; GDPN, glyceraldehyde-3-phosphate dehydrogenase; MAP, mitogen-activated protein; IP, immunoprecipitation; ERK, extracellular signal-regulated kinase; JNK, c-Jun NH2-terminal protein kinase; LDH, lactate dehydrogenase; ANOVA, analysis of variance; PLSD, protected least significant difference; HSD, honestly significant difference; MEK, mitogen-activated protein kinase kinase; PKA, protein kinase A; PKC, protein kinase C; DA, dopamine; P3K, phosphoinositide-3 kinase; SP600125, anthra[1,9-cd]pyrazol-6(2H)-one.
Recently, it was demonstrated that adult neurogenesis induced by antidepressant is critical to antidepressant effects (Santarelli et al., 2003). Adult neurogenesis is regulated by several trophic factors and growth factors, such as brain-derived neurotrophic factor (BDNF), fibroblast growth factor (FGF), insulin-like growth factor, vascular endothelial growth factor, and glial cell line-derived neurotrophic factor (GDNF) (Aberg et al., 2000; Jin et al., 2003; Newton and Duman, 2004; Chen et al., 2005; Scharfman et al., 2005). Accumulating evidence from animal studies indicates that the changes of gene expression and signal transduction related to neuronal and glial plasticity and adaptations after chronic antidepressant treatment are important for the therapeutic effect of antidepressants (Duman, 2004). Furthermore, a number of imaging and post-mortem studies in patients with mood disorders have revealed a reduction of particular areas such as the prefrontal cortex, hippocampus, and amygdala in total volume and cell density/size, especially glial cells (Öngür et al., 1998; Manji et al., 2001; Rajkowska, 2002). Thus, a leading hypothesis is that depression is associated with a loss of neural and glial plasticity and neurotrophic support and that antidepressant treatments increase neurogenesis through neurotrophic factor production, which reverse adverse effects of depression (Newton and Duman, 2004; Malberg and Schechter, 2005).

GDNF, a member of the transforming growth factor-β superfamily, was originally purified from a rat glial cell line supernatant as a trophic factor for midbrain dopamine neurons, and it was later found to have pronounced effects on other neuronal populations (Airaksinen and Saarma, 2002). The infusion of GDNF increased neurogenesis in the hippocampus of adult rat (Chen et al., 2005). GDNF has been reported to play important roles in higher order brain function such as cognitive abilities and drug addiction (Messer et al., 2000; Gerlai et al., 2001). These results suggest that GDNF is potentially important in neuronal and glial plasticity.

We previously demonstrated that several different classes of antidepressants increase GDNF production in rat astrocytes and rat C6 glioma cells (C6 cells) (Hisaoa et al., 2001). Furthermore, we recently reported that total GDNF levels in whole blood in patients with mood disorders were significantly lower GDNF levels in C6 cells grown in Dulbecco’s modified Eagle’s medium (Cambrex Bio ScienceWalkersville, Inc.,Walkersville, MD) supplemented with 2 mM l-glutamine and 5% fetal bovine serum (SRH Biosciences, Lenexa, KS) in a 5% CO2-humidified atmosphere. Normal human astrocytes (NHA), derived from fetal tissue (male; 18 weeks), were purchased from Cambrex Bio Science and grown in ABM (Cambrex Bio Science) in a 5% CO2-humidified atmosphere. More than 80% NHA expressed glial fibrillary acidic protein. For drug treatment, the medium was replaced with serum-free Opti-MEM (Invitrogen, Carlsbad, CA) containing 0.5% bovine serum albumin (Sigma-Aldrich), and the cells were incubated in a 5% CO2 environment for 24 h, and then the cells were treated with drugs of interest.

**RNA Isolation.** For collection of total RNA, cells were cultured at a density of 8 to 16 × 10^6/cm² on a six-well plate with 3 ml of growth medium. After drug treatment, total RNA was isolated using an RNasy mini kit (QIAGEN, Valencia, CA) following the manufacturer’s protocols. RNA quantity and purity were determined with the Multi-Spectrophotometer (Dainippon, Osaka, Japan).

**Real-Time Reverse Transcription-Polymerase Chain Reaction Assay.** GDNF mRNA was measured by real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR). The first-strand cDNA was synthesized from 500 ng of total RNA by using a RNA PCR kit (avian myeloblastosis virus), version 3.0 (Takara Biochemicals, Tokyo, Japan). Real-time quantitative PCR was performed by the SmartCycler system (Cepheid, Sunnyvale, CA), using TaqMan probes and primers for rat or human GDNF and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Applied Biosystems, Foster City, CA). The cycling conditions for all primers were as follows: hold for 10 min at 95°C, followed by 40 cycles consisting of two steps, 15 s at 95°C (denaturing), and 1 min at 60°C (annealing-extension). The threshold cycle, which correlates inversely with the mRNA levels of target, was measured as the cycle number at which the reporter fluorescent emission increases above a threshold level. The GDNF mRNA levels were normalized for GAPDH mRNA in the same samples by the 2^(-ΔΔCT) method, which is a convenient way to analyze the relative changes in gene expression from real-time quantitative PCR experiments (Livak and Schmittgen, 2001).

**GDNF Enzyme-Linked Immunosorbent Assay.** For the assay of GDNF release, C6 cells were cultured at a density of 13 × 10^3/cm² on a 12-well plate with 0.5 ml of growth medium. After drug treatment, conditioned medium was collected and stored at −80°C until assayed. GDNF protein levels in cell-conditioned media were determined using a GDNF enzyme-linked immunosorbent assay according to the manufacturer’s instructions (Promega, Madison, WI).

**Mitogen-Activated Protein Kinase Activity Assay.** We used two different methods for measuring mitogen-activated protein (MAP) kinase activities. The nonradioactive conventional immunoprecipitation (IP)/kinase assay is more sensitive and specific method compared with the detection of phosphorylation of MAP kinase by Western blotting method. Therefore, we used the IP/kinase assay to measure MAP kinase activation by antidepressant, because the antidepressant-induced MAP kinase activation is relatively smaller than the 5-HT-induced MAP kinase activation, which could be detected by Western blotting method. For the nonradioactive IP/kinase assay of MAP kinase activities [extracellular signal-regulated kinase (ERK), p38 MAP kinase, and c-Jun N-terminal protein kinase (JNK)], cells were cultured at a density of 8 to 16 × 10^4/cm² on a six-well plate with 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 Chemical, Rockford, IL), and it was adjusted to the same amount for all samples. MAP kinase activities were determined using an assay kit according to the manufacturer’s
instructions (Cell Signaling Technology Inc., Beverly, MA). In brief, cell lysate were immunoprecipitated by adding immobilized antibodies, after immunoprecipitation, pellets were washed twice with 50 μl of cell lysis buffer and twice with 500 μl of kinase buffer. The pellets were suspended in 50 μl of kinase buffer supplemented with ATP and individual substrate proteins and incubated for 30 min at 30°C. The reaction was terminated with 25 μl of 3× SDS sample buffer. After heating to 95°C for 5 min, the protein samples were separated by SDS-polyacrylamide gel electrophoresis and transblotted to polyvinylidene difluoride membranes. The membranes were blocked with 5% (w/v) nonfat milk for 1 h at room temperature with horseradish peroxidase-conjugated secondary antibody. Protein bands were detected by exposure to X-ray film, scanned digitally, and densitometrically analyzed by a computer-based analysis system with Kodak 1D Image Analysis software (Eastman Kodak, Rochester, NY). The amounts of phospho-Elk-1, phosphoactivating transcription factor-2, or phospho-c-jun indirectly show ERK activity, p38 activity, or JNK activity.

Western Blotting. Western blots were performed using respective antibodies for the detection of total ERK, phosphorylated ERK (Cell Signaling Technology Inc.), and phosphorylated tyrosine-containing proteins (4G10; Upstate Biotechnology, Lake Placid, NY). C6 cells were collected by using ice-cold phosphate-buffered saline and solubilized in the sample buffer (100 mM Tris-HCl, pH 6.8, 20% glycerol, and 4% SDS). Total amounts of proteins in each sample were adjusted to the same amount for all samples. After addition of 1,4-dithiobis(benzil), samples were boiled for 5 min. Proteins were separated by SDS-polyacrylamide gel electrophoresis and transblotted to polyvinylidene difluoride membranes. Further procedures were as same as for the MAP kinase activity assay.

Lactate Dehydrogenase Release Assay. To determine the cytoxicity of amitriptyline, we measured lactate dehydrogenase (LDH) release by using a cytotoxicity colorimetric assay kit (Oxford Biomedical Research, Oxford, MI) according to the manufacturer’s instructions. For the assay of LDH release, C6 cells were cultured at a density of 13 × 10⁴/cm² on a 12-well plate with 0.5 ml of growth medium. After drug treatment, conditioned medium was collected and stored at −80°C until assayed.

Measurement of 5-HT. The measurement of 5-HT was outsourced to SRL (Tokyo, Japan), and its concentration in the cultured cells and the medium was measured with high-performance liquid chromatography.

Data Analysis. Results are expressed as means ± S.E.M. One-way analysis of variance was used in most cases to check statistical tendencies. Differences between groups were analyzed by Fisher’s protected least significant difference (PLSD) if the group sizes were equal, or Tukey honest significant difference (HSD) if the group sizes were not equal. Differences between two groups were analyzed by Student’s t test. Two-way analysis of variance was used in Figs. 1B and 3C, and differences between groups were analyzed by Bonferroni post-tests. The significance level was set at p < 0.05. The relationship between two variables was examined using Pearson’s correlation coefficient; results with p < 0.05 were considered significant.

Results
Effects of MAP Kinase Inhibitors on GDNF mRNA Expression and GDNF Release Induced by Amitriptyline Treatment. We previously demonstrated that mitogen-activated protein kinase kinase (MEK) inhibitors, but not p38 inhibitors, a protein kinase A (PKA) inhibitor or a protein kinase C (PKC) inhibitor, inhibited the amitriptyline-induced GDNF release (Hisaoka et al., 2001). To clarify the role of MAP kinase on the amitriptyline-induced GDNF mRNA expression and GDNF release, we used inhibitors of MAP kinase cascades: U0126 (an MEK1 inhibitor), PD98059 (an MEK inhibitor), PD169316 (a p38 inhibitor), and SP600125 (a JNK inhibitor). U0126 and PD98059 significantly inhibited both GDNF mRNA expression and GDNF release by amitriptyline treatment (Fig. 1, A and B). There were no effect on basal levels of GDNF mRNA and GDNF release by inhibitors alone (Fig. 1, A and B).

To ensure that GDNF mRNA expression and GDNF release by amitriptyline treatment were not confounded by nonspecific protein release due to cell damage, chemotoxicity of amitriptyline Treatment.

![Fig. 1. Effects of U0126, PD98059, PD169316, and SP600125 on the amitriptyline-induced GDNF mRNA expression and GDNF release in C6 cells. A, effects of U0126, PD98059, PD169316, and SP600125 on the amitriptyline-induced GDNF mRNA expression. C6 cells were pretreated with 10 μM U0126 (U), 30 μM PD98059 (PD9), 10 μM PD169316 (PD1), or 10 μM SP600125 (SP) for 30 min and subsequently treated with 25 μM amitriptyline for 8 h with inhibitors or inhibitors alone. Values are shown as the ratio of GDNF mRNA versus GAPDH mRNA. Data are expressed as mean ± S.E.M., **p < 0.01 compared with the basal group, and †††, p < 0.001 compared with the vehicle (amitriptyline only) group (Tukey’s HSD test; n = 3–5). B, effects of U0126, PD98059, PD169316, and SP600125 on the amitriptyline-induced GDNF release. C6 cells were treated with 10 μM U0126 ( ), 30 μM PD98059 ( ), 10 μM PD169316 ( ), 10 μM SP600125 ( ), or vehicle ( ) for 30 min and treated with the indicated concentration of amitriptyline for 48 h. Values are expressed as mean ± S.E.M. of released GDNF (picograms per milliliter) from three independent experiments. *, p < 0.05; **, p < 0.01, significantly different from the vehicle (amitriptyline only) group (Bonferroni post-tests; n = 3).
line was quantified by a standard measurement of LDH. The amount of released LDH significantly decreased after treatment with 25 μM amitriptyline, and it increased after treatment with 125 μM amitriptyline (Fig. 2). These results showed that treatment with amitriptyline up to 100 μM for 48 h was not toxic to C6 cells. Furthermore, treatment with 25 μM amitriptyline was significantly protective rather than toxic.

**Amitriptyline Treatment Increased GDNF Production through GDNF mRNA Expression.** We previously measured the expression of GDNF mRNA by semiquantitative RT-PCR and showed that amitriptyline-induced GDNF mRNA expression was detectable after 12-h treatment and continued up to 48 h (Hisaoa et al., 2001). To elucidate the precise process of GDNF synthesis by amitriptyline treatment, we examined the time course of GDNF mRNA expression using real-time quantitative RT-PCR, which is more sensitive and precise than semiquantitative RT-PCR. GDNF mRNA expression was enhanced as early as 1 h after the addition of 25 μM amitriptyline, and the increased level remained constant at approximately 2-fold for at least 48 h (Fig. 3A). As demonstrated previously (Hisaoa et al., 2001), the amitriptyline-induced GDNF mRNA expression at 24-h treatment was increased in concentration-dependent manner (data not shown).

We next examined the effect of cycloheximide (a protein synthesis inhibitor) on the amitriptyline-induced GDNF mRNA expression and GDNF release. Cycloheximide significantly inhibited GDNF release (Fig. 3C), but it did not have any significant effect on GDNF mRNA expression by amitriptyline treatment (Fig. 3B).

**Amitriptyline Treatment Increased MAP Kinase Activites.** Our results showed that both the amitriptyline-induced GDNF mRNA expression and GDNF release were inhibited by U0126 and PD98059 (Fig. 1, A and B). These results suggest the possibility that amitriptyline treatment induces MEK-ERK activation. In this study, we examined not only chronic but also acute effect of amitriptyline on ERK activity, because the amitriptyline-induced GDNF mRNA expression occurred as early as 1 h in C6 cells (Fig. 3A). First, we examined the time course of the amitriptyline-induced ERK activation. The ERK activation by amitriptyline occurred after 2 min of treatment and reached a maximum at 5 min. This activation decreased to levels of around 2-fold after 1 h of treatment, and a little activation continued after 48 h (Fig. 4A). The amount of total ERK was not changed until after 48 h of amitriptyline treatment (Fig. 4A).

We next examined the concentration dependence of amitriptyline on ERK activation in C6 cells. The level of ERK activation (5 min) depended on the concentration of the amitriptyline-induced GDNF mRNA expression (Fig. 4B). To specify the effect of amitriptyline on ERK activation, we examined the effects of amitriptyline on p38 and JNK. Interestingly, acute treatment with amitriptyline also increased p38 and JNK activities (Fig. 4C).

**Amitriptyline Treatment Increased ERK Activity and GDNF mRNA Expression in Normal Human Astrocytes.** We next examined whether amitriptyline shows the same effects as C6 cells on NHA. Acute treatment (5 min) of amitriptyline increased ERK activity in NHA (Fig. 4D). Amitriptyline treatment (24 h) also increased GDNF mRNA expression in NHA (Fig. 4D).

**Antidepressant Treatments Increased Acute ERK Activation, but Nonantidepressant Drugs Did Not.** To
determine a pharmacological specificity of antidepressants on ERK activation, we examined the effects of several different classes of antidepressants and nonantidepressant drugs, including amitriptyline, clomipramine, nortriptyline, and desipramine (tricyclic antidepressants), mianserin (a tetracyclic antidepressant), fluvoxamine, and fluoxetine (selective 5-HT reuptake inhibitors), haloperidol (an antipsychotic-D2-dopamine receptor antagonist), diazepam (a benzodiazepine), lithium (a mood stabilizer), diphenhydramine (an antihistaminergic drug), and trihexyphenidyl (an anticholinergic drug), most of which are clinically used in Japan, on ERK activity. All antidepressants significantly increased ERK activity in C6 cells, but haloperidol, diazepam, lithium, diphenhydramine, and trihexyphenidyl did not influence the ERK activity (Table 1). The multiplication of NHA is very limited because of normal astrocytes; therefore, we could not repeat examination on C6 cells. However, we also confirmed that several different types of antidepressants increased ERK activity in NHA (Table 1).

We next examined the effects of antidepressants (Table 1) on GDNF release in C6 cells. We plotted the amounts of ERK activity and GDNF release by individual antidepressants. There is a positive correlation between acute ERK activation and GDNF release in C6 cells (Fig. 5). Pearson's correlation...
TABLE 1

<table>
<thead>
<tr>
<th>Drug</th>
<th>C6 Cells % basal</th>
<th>NHA % basal</th>
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<tr>
<td>Amitriptyline</td>
<td>394.8 ± 85.6***</td>
<td>313.4 ± 66.5***</td>
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<tr>
<td>Clomipramine</td>
<td>466.4 ± 34.7***</td>
<td>373.0 ± 90.9***</td>
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<tr>
<td>Desipramine</td>
<td>350.3 ± 55.1*</td>
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</tr>
<tr>
<td>Nortriptyline</td>
<td>362.8 ± 100.4*</td>
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<tr>
<td>Mianserin</td>
<td>431.6 ± 41.5*</td>
<td>279.3 ± 55.6*</td>
</tr>
<tr>
<td>Selective 5-HT uptake inhibitor</td>
<td></td>
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<tr>
<td>Fluvoxamine</td>
<td>268.8 ± 69.1*</td>
<td>252.8 ± 25.6**</td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>342.2 ± 31.9*</td>
<td></td>
</tr>
<tr>
<td>Other</td>
<td></td>
<td></td>
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<tr>
<td>Haloperidol</td>
<td>72.9 ± 36.5</td>
<td></td>
</tr>
<tr>
<td>Diazepam</td>
<td>70.5 ± 15.1</td>
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<tr>
<td>Lithium</td>
<td>118.3 ± 46.2</td>
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<tr>
<td>Diphenhydramine</td>
<td>105.5 ± 22.4</td>
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<tr>
<td>Trihexyphenidyl</td>
<td>74.5 ± 14.7</td>
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*p < 0.05, significantly different from the basal (Student’s t test).
**p < 0.01, significantly different from the basal (Student’s t test).
***p < 0.001, significantly different from the basal (Student’s t test).

Amitriptyline Increased GDNF mRNA Expression through PTK Activation. We examined the effect of amitriptyline on phosphorylation of tyrosine residues. The phosphorylated tyrosine-containing proteins were immunodetected with monoclonal antiphosphotyrosine antibody (4G10). Treatment of amitriptyline acutely increased phosphorylation levels of a number of phosphotyrosine containing proteins in C6 cells. The majority of the tyrosine-phosphorylated proteins were located within the molecular size range of 50 to 150 kDa, three of which are indicated by arrows (bands 1, 2, and 3) (Fig. 7A). The phosphorylation levels of these proteins were significantly increased by amitriptyline and reversed to basal levels by genistein (Fig. 7A). Finally, we examined the effect of genistein on the amitriptyline-induced GDNF mRNA expression (Fig. 7B). Genistein alone did not have any significant effect on basal level of GDNF mRNA expression (p = 0.58; Fig. 7B).
Effects of ketanserin and WAY100635 on the amitriptyline-induced ERK activation in C6 cells. C6 cells were pretreated with 100 nM ketanserin (Ketan) or 100 nM WAY100635 (WAY) for 10 min and treated with or without 100 nM 5-HT for 2 min. Phosphorylation levels of ERK1/2 were detected by Western blotting. Data are expressed as mean ± S.E.M. B, effects of ketanserin and WAY100635 on the 5-HT-induced ERK phosphorylation. C6 cells were pretreated with 100 nM ketanserin (Ketan) or 100 nM WAY100635 (WAY) for 10 min and treated with 25 µM amitriptyline for 5 min. ERK activity was measured. Data are expressed as mean ± S.E.M.

Discussion

In the present study, we have shown that PTK-dependent ERK activation plays an important role in GDNF production by amitriptyline, and this effect of amitriptyline seems to be independent of the monoamine system. We also showed that amitriptyline activates not only ERK but also p38 and JNK. Although the activation of p38 and JNK did not contribute to GDNF production by amitriptyline, these activations probably affect gene expression or cellular function. In addition, different types of antidepressants, but nonantidepressants, commonly increased ERK activity and GDNF release. These results suggest a possible specificity of these effects for antidepressants. Furthermore, we showed that amitriptyline increased ERK activity and GDNF mRNA expression in NHA. These results suggest that treatment of antidepressants seems to commonly increase ERK activity and GDNF mRNA expression not only in rat glial cell line but also in normal human astrocytes.

Although the precise mode of action of ERK in GDNF production is not fully investigated, we demonstrated that the MEK-ERK pathway regulates both GDNF mRNA expression and GDNF release. The time course showed that the increase of GDNF mRNA expression occurred before the increase of GDNF release (Fig. 3A). In addition, a protein synthesis inhibitor significantly inhibited GDNF release by amitriptyline, but it did not have any effect on the amitriptyline-induced GDNF mRNA expression (Fig. 3B and C). These results suggest that amitriptyline seems to increase GDNF release resulting from an induction of mRNA expression and de novo protein synthesis. The acute ERK activation (5 min) is important for GDNF release (48 h), because the extent of acute ERK activation and GDNF release was significantly correlated to each other in individual antidepressants. We previously showed the time course, and the amitriptyline-induced GDNF release was significantly increased at 48 h after treatment (Hisaoka et al., 2001). Thus, the production of GDNF seems to be triggered by ERK activation at 5 min, followed by mRNA expression as early as 1 h, resulting in an increase of GDNF release at 48 h. However, we cannot rule out a possibility that ERK activation is required, but not sufficient, to stimulate GDNF production in this study. Further investigations (for example, to study whether transfection of constitutively activated forms of MEK stimulate GDNF production) are needed to clarify the possibility.

The induction of GDNF mRNA by amitriptyline remained after 48 h (Fig. 3A), whereas the activation of ERK reached maximum at 5 min (Fig. 4A). These data suggest that not only acute ERK activation but also a little lasting activation of ERK might be important for the antidepressant-induced GDNF mRNA expression. This sustained ERK activation by prolonged amitriptyline treatment did not result from an increase of total amount of ERK, because total levels of ERK1/2 were not changed over a 48-h period by amitriptyline. ERK plays an important role not only at the transcriptional level but also at the translational level. However, the precise mode of action of ERK in GDNF production is not fully investigated, we demonstrated that the MEK-ERK pathway regulates both GDNF mRNA expression and GDNF release. The time course showed that the increase of GDNF mRNA expression occurred before the increase of GDNF release (Fig. 3A). In addition, a protein synthesis inhibitor significantly inhibited GDNF release by amitriptyline, but it did not have any effect on the amitriptyline-induced GDNF mRNA expression (Fig. 3B and C). These results suggest that amitriptyline seems to increase GDNF release resulting from an induction of mRNA expression and de novo protein synthesis. The acute ERK activation (5 min) is important for GDNF release (48 h), because the extent of acute ERK activation and GDNF release was significantly correlated to each other in individual antidepressants. We previously showed the time course, and the amitriptyline-induced GDNF release was significantly increased at 48 h after treatment (Hisaoka et al., 2001). Thus, the production of GDNF seems to be triggered by ERK activation at 5 min, followed by mRNA expression as early as 1 h, resulting in an increase of GDNF release at 48 h. However, we cannot rule out a possibility that ERK activation is required, but not sufficient, to stimulate GDNF production in this study. Further investigations (for example, to study whether transfection of constitutively activated forms of MEK stimulate GDNF production) are needed to clarify the possibility.

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tional level but also at the translational level (Kelleher et al., 2004). Thus, a little lasting activation of ERK may regulate translation and secretion of GDNF. However, pathways of processing and secretion of GDNF are unknown at present, although NGF and BDNF are known to cleave extracellularly by the serine protease plasmin and selective matrix metalloproteinases (Lee et al., 2001). Because a signal sequence for cleavage is found in a precursor of GDNF (Airaksinen and Saarma, 2002), secretion of GDNF is assumed to occur by proteolytic cleavage. Thus, the role of a little lasting activation of ERK by amitriptyline should be further considered.

In this study, we treated C6 cells with antidepressants at higher concentration than in the human plasma level, but LDH release assay showed that the micromolar range of amitriptyline was not toxic. Furthermore, it has been reported that most antidepressants accumulate in the brain because they have highly lipophilic properties (Prouty and Anderson, 1990). For example, the brain concentration of amitriptyline is approximately 10 to 35 times higher than the corresponding blood levels (Prouty and Anderson, 1990). Therefore, these finding suggest that the accumulation of antidepressant may occur under therapeutic conditions, and antidepressant concentrations in the brain might be in the concentration range in which promotion of MAP kinase activation and gene induction in C6 cells take place.

The mechanisms by which antidepressants lead to the activation of MAP kinases still need proper characterization. We showed that 5-HT2A antagonist and MEK inhibitor significantly inhibited the 5-HT-induced GDNF production (Hisaoka et al., 2004) and that 5-HT2A antagonist completely inhibited the 5-HT-induced ERK activation in this study. These results suggest that 5-HT increased GDNF production through 5-HT2A receptor-dependent ERK activation. We are now investigating the precise mechanism by which 5-HT induces ERK activation and GDNF production in C6 cells (Tsuchioka et al., 2005). However, the 5-HT2A antagonist did not inhibit the amitriptyline-induced ERK activation and GDNF release. Furthermore, there was no detectable amount of 5-HT in both of C6 cells lysate and the conditioned medium after amitriptyline treatment. Our data are supported by another report that no detectable amount of 5-HT

![Figure 7](https://example.com/fig7.jpg)

**Fig. 7.** Effects of genistein on the amitriptyline-induced phosphorylation level of tyrosine-containing proteins and GDNF mRNA expression in C6 cells. A, effect of genistein on the amitriptyline-induced phosphorylation level of tyrosine-containing proteins. C6 cells were treated with 100 μM genistein (Gen) for 1 h and treated with 25 μM amitriptyline for 2 min. C6 cell lysates were prepared, and protein tyrosine phosphorylation was immunodetected by 4G10 antibody. A representative result is shown. Arrows indicate three bands (bands 1, 2, and 3), which were increased by amitriptyline and reversed by genistein. Data are expressed as mean ± S.E.M., *p < 0.05 compared with the basal group, and †, p < 0.05; ††, p < 0.01 compared with the vehicle (amitriptyline only) group (Tukey's HSD test; n = 3–5). B, effect of genistein on the amitriptyline-induced GDNF mRNA expression. C6 cells were treated with 100 μM genistein (Gen) for 1 h and treated with 25 μM amitriptyline or genistein alone for 3 h. Values are shown as the ratio of GDNF mRNA versus GAPDH mRNA. Data are expressed as mean ± S.E.M. ***, *p < 0.001 compared with the basal group, and †††, p < 0.001 compared with the vehicle (amitriptyline only) group (Tukey's HSD test; n = 10–11).
was observed in either C6 cells or their conditioned medium in the presence or absence of antidepressants (Muraoa et al., 1998). In addition, we showed that NA and DA did not have any effect on GDNF production (Hisaoka et al., 2004) and that NA and DA decreased ERK activation in C6 cells (Fig. 6A). Because NA and DA increase cAMP in C6 cells (Zumwalt et al., 1999), the increases of cAMP level might have an effect on phosphorylation levels of ERK. These results suggest that the effect of amitriptyline on ERK activation and GDNF production in C6 cells might not involve monoamine system.

Furthermore, we examined the effects of an anticholinergic or an antihistaminergic drug on GDNF production, because tricyclic antidepressants, such as amitriptyline, produce adverse side effects by histamine or muscarinic receptor blockade (Burke and Preskorn, 1995). We showed that diphenhydramine and trihexyphenidyl did not have any effect on ERK activity in this study. These results suggest that the effect of amitriptyline on ERK activation and GDNF production in C6 cells might not involve anticholinergic or antihistaminergic action as well as monoamine system.

Because the monoamine-independent site of action by antidepressant is unknown, we attempted to clarify the 5-HT-independent intracellular mechanism that resulted in activation of ERK, following amitriptyline treatment, by using various inhibitors of intracellular signal transduction. Our data suggest that only PTK but not PKA, pan-PKC, PKCδ, calcium, Pi3K, or phospholipase C might be involved in ERK activation by amitriptyline. Furthermore, we found that amitriptyline increased phosphorylation levels of several phospho-tyrosine-containing proteins, which were reversed by genistein. From both of the molecular mass of these bands and the information about the expression of tyrosine kinases in C6 cells, we suggest that the Src family (60 kDa), proline-rich tyrosine kinase 2 (116 kDa), FGF receptor (120 or 145 kDa), focal adhesion kinase (125 kDa), or trk (A, B, and C; 140 kDa) might be involved in the effect of amitriptyline (Tsuda et al., 1997; Belcheva et al., 2002; Kawanabe et al., 2003; Lazar et al., 2004). The further characterization of the nature of PTKs is now under investigation.

Recent findings showed that several neurotrophic factors such as BDNF, neurotrophin-3, FGF, and GDNF are altered in post-mortem brain, cerebrospinal fluid, or blood from patients with mood disorders (Hock et al., 2000; Shimizu et al., 2003; Evans et al., 2004; Takebayashi et al., 2006). These observations suggest that the dysregulation of multiple neurotrophic/growth factor systems might be involved in the etiology of mood disorders. Thus, regulation of neurotrophic/growth factor production following antidepressant treatment may contribute to therapeutic effects. As shown in this work, rapid activation of signaling pathways would induce the expression of genes coding for neurotrophins and other proteins in hours or days. These factors might increase neurogenesis and improve the survival, plasticity, and activity of various glia and neighboring neurons in the brain, including monoaminergic systems, and finally reverse adverse effects of depression.

Here, we provided novel information about the monoamine-independent mechanisms that underlie the antidepressant-induced production of GDNF via activation of PTK and ERK in glial cells. Because a monoamine, such as 5-HT, induces GDNF production, antidepressants might increase GDNF production additively through two pathways in the brain. One pathway is the monoamine-dependent pathway, which increases monoamine by inhibiting reuptake of monoamine; the other pathway is the monoamine-independent pathway, which involves PTK-ERK activation. The antidepressant-induced GDNF might increase neurogenesis and gliogenesis and probably mediates the therapeutic effect of antidepressants. We suggest that clarifying the monoamine-independent novel target of antidepressants might contribute to the development of more efficient treatment for depression.

References


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