Friston 2000; Yamasue et al 2003). On the other hand, false-positive or false-negative VBM findings may arise from the changes in the shape or displacement of structures in the course of spatial normalization, especially for such small structures as the amygdala (Wright et al 1999). The aim of this study was to compare the gray matter density using voxel-based morphometry and supplement the analysis with a classical region of interest (ROI) analysis of the amygdala among breast cancer survivors (BCS) with their first minor depressive episode after cancer diagnosis, BCS with their first major depressive episode after cancer diagnosis, and BCS with no history of any depressive episode after cancer diagnosis.

Methods and Materials

Subjects

This study was approved by the Institutional Review Board and the Ethics Committee of the National Cancer Center, Tokyo, and was performed after obtaining written informed consent from the patients. The subjects were recruited between February 1998 and April 1999 during their follow-up visits to the Division of Breast Surgery, National Cancer Center Hospital East. The aim of this study was to investigate the associations between brain morphology and depression and distressing cancer-related recollections in breast cancer survivors. Previously, we reported the absence of any association between the first major depressive episode after cancer diagnosis and the hippocampal volume in breast cancer survivors (Inagaki et al 2004). The present study population was part of the previous study population. The inclusion criteria were 1) female gender, and 2) age between 18 and 55 years. The exclusion criteria were 1) double cancer or clear evidence of residual or recurrent cancer; 2) history of any neurological disorder or traumatic brain injury; 3) history of substance abuse or dependence; 4) family history of early dementia among first- or second-degree relatives; 5) physical symptoms that interfered with daily life; 6) ingestion of psychotropic medication within the previous month; 7) cognitive impairment; and 8) history of minor and/or major depressive episode before the cancer diagnosis and/or other psychiatric disorder. Of the 394 breast cancer patients who had survived for more than 3 years after surgery, 187 patients satisfied the aforementioned criteria. Among these, 137 could be contacted at the clinic. Fifty-three of the 137 patients refused to participate in the study, and the remaining 84 subjects were interviewed in a semistructured interview included in the *Structured Clinical Interview for DSM-IV Axis I Disorders (SCID-I), Clinician Version* (First et al 1997), by a trained psychiatrist (T.N.). Minor depressive episode is defined as research criteria for DSM-IV. The interrater reliability of the diagnostic interview was excellent (kappa = 1.0). After the interview, 26 subjects who had a history of major or minor depressive episode before the cancer diagnosis were excluded from the analysis. Seven other subjects were excluded because of an MRI acquisition error; finally, 51 BCS were included for this analysis. Eleven of the 51 patients suffered their first minor depressive episode after cancer diagnosis (mean duration ± SD of minor depression: 7.1 ± 5.6 weeks). Another 11 of the 51 patients experienced their first major depressive episode after cancer diagnosis (mean duration ± SD of major depression: 10.9 ± 14.7 weeks). The remaining 29 subjects did not experience any depressive episode or other neuropsychiatric disorder even after their cancer diagnosis. All of the subjects with history of first minor or major depressive episode after cancer diagnosis recovered from the depression without any psychiatric treatment or use of antidepressants. There were trend-level differences in the clinical stage of the cancer but no significant differences in the demographic or other medical background characteristics among the three groups (Table 1).

MRI Image Acquisition

The methods of MRI acquisition and data analysis are described in detail elsewhere (Inagaki et al 2004; Matsuoka et al 2003). Briefly, MRI was conducted on a 1.5-T MRI unit (Signa Scanner, GE Medical Systems, Milwaukee, Wisconsin), with three-dimensional spoiled gradient-recalled acquisition of 1.5-mm contiguous sections under the following conditions: field of view = 230 mm, matrix = 256 × 256 pixels, repetition time (TR) = 25 milliseconds, echo time (TE) = 5 milliseconds, and flip angle = 45°.

Table 1. Subject Characteristics and Clinical Measurements in Breast Cancer Survivors with No History of Any Depressive Episodes After Cancer Diagnosis, a History of First Minor Depressive Episode, and a History of First Major Depressive Episode

	History of First Depressive Episode After Cancer Diagnosis				
	None (n = 29)	Minor (n = 11)	Major (n = 11)		
	Mean (SD)	Mean (SD)	Mean (SD)	F^a	p
Age (years)	48.6 (5.1)	47.5 (5.1)	48.5 (4.7)	.211	.810
Education (years)	12.6 (1.7)	12.5 (1.8)	12.6 (2.0)	.029	.971
Alcohol Consumption (years g/week)	445.0 (1039)	660.1 (1647)	914.1 (1798)	.484	.619
	n (%)	n (%)	n (%)	chi-square[b]	p
Left-Handed	1 (3.4)	0 (0)	1 (9.1)	1.182	.554
Postmenopausal	12 (41.3)	6 (54.5)	5 (45.5)	.559	.756
Lymph Node Metastasis (positive)	9 (31.0)	5 (45.4)	4 (36.3)	.733	.693
Clinical Stage (0 or I)	18 (62.1)	9 (81.8)	5 (45.5)	5.181	.075
Surgical Procedure (Total Mastectomy)	20 (69.1)	10 (91.0)	7 (63.6)	.249	.288
Previous Adjuvant Chemotherapy (Received)	16 (55.2)	7 (63.6)	6 (54.5)	.264	.877
Tamoxifen (Received)	11 (37.9)	5 (45.5)	6 (54.5)	.928	.629

ANOVA, analysis of variance.
[a]Differences in continuous variables were analyzed by ANOVA.
[b]Differences in categorical variables were analyzed by the chi-square test.

Voxel-Based Morphometry

The theory and algorithm of voxel-based morphometry using the Statistical Parametric Mapping (SPM)2 software (Wellcome Department of Cognitive Neurology, London, United Kingdom) have been well documented (Ashburner and Friston 2000). Voxel-based morphometry was carried out using an optimized method (Good et al 2001). First, an optimized study-specific template set consisting of a T1 image and apriori gray, white, and cerebrospinal fluid (CSF) images was created for the VBM analysis. This template set was constructed from brain scans taken from all the study subjects. All the scans were then spatially normalized to the customized template, then smoothed with an 8-mm, full-width half maximum (FWHM) smoothing kernel, followed by averaging to create a customized template. The images were then segmented into gray matter (GM) and white matter (WM), using customized prior probability maps. These segments were then modulated to correct for volume changes occurring during the spatial normalization. Finally, the images were smoothed using a 12-mm FWHM Gaussian kernel. Smoothing was performed to convert the tissue classification images into images of local gray matter density. Gray matter density, in this context, means the proportion of the smoothing kernel that was classified as gray matter. This size of this kernel specifies the spatial scale of gray matter density changes.

For group comparisons, the main analysis was performed first using a two-tailed analysis for analysis of covariance (ANCOVA) in a basic model of the SPM2 among the three groups (BCS with a history of first minor depressive episode after cancer diagnosis, BCS with a history of first major depressive episode after cancer diagnosis, and BCS with no history of any depressive episode after cancer diagnosis), with age as the nuisance variable. In post hoc analysis, small volume corrections were used with the coordinates of the main analysis as the center and with 40-mm radius as the size of spherical searched volume. An additional analysis was performed between the two groups (BCS with a history of first minor or major depressive episode and BCS with no history of any depressive episode), with age as the nuisance variable, to investigate the presence of any common region associated with depression. Additionally, to investigate the brain region associated with the severity of depression, covariate models in SPM2 were used, with the severity of the depressive episode (0 = no history of depressive episode; 1 = history of the first minor depressive episode; 2 = history of the first major depressive episode) as the covariate and age as the nuisance variable. The possible confounding effects of age were controlled by entering these variables into the models.

Significance levels were set at a p value of $<.05$ (two-tailed) corrected for multiple comparisons using the false discovery rate (FDR) approach (Genovese et al 2002) in regions without hypothesis. Based on the findings in previous studies, we expected gray matter changes in the PFC (Bremner 2002; Drevets et al 1997; Kumar et al 1998). For these regions with hypothesis, significance was assumed at an uncorrected p value of $<.002$ (two-tailed) and an additional extent threshold of 400 voxels to suppress small clusters possibly arising by chance. We also examined associations between medical factors (clinical stage, history of adjuvant chemotherapy, and tamoxifen therapy) and regional brain volume by VBM, because all subjects were cancer survivors and no healthy control subjects were examined. Significance levels were set at a p value of $<.05$ (two tailed) corrected for multiple comparisons using the FDR approach (Genovese et al 2002).

Manual Tracing Method

We used the manual tracing method with ANALYZE-PC software (Biomedical Imaging Resource, Mayo Foundation, Rochester, Minnesota) to analyze the volume of the amygdala and hippocampus of either side and the intracranial volume. The volumetric procedure for measuring these volumes has been described previously (Inagaki et al 2004; Matsuoka et al 2003).

Table 2. Amygdala and Hippocampal Volume in Breast Cancer Survivors with No History of Any Depressive Episodes After Cancer Diagnosis, a History of First Minor Depressive Episode, and a History of First Major Depressive Episode

	History of First Depressive Episode After Cancer Diagnosis				
Normalized Volume × 10^{-3} (Absolute Volume/Intracranical Volume)	None Mean (SD)	Minor Mean (SD)	Major Mean (SD)	F^a	p
Amygdala					
Left	1.05 (.11)	.97 (.09)	.99 (.10)	3.12	.054
Right	1.09 (.14)	1.06 (.07)	1.06 (.12)	.37	.69
Hippocampus					
Left	2.32 (.31)	2.38 (.24)	2.23 (.21)	.84	.44
Right	2.45 (.28)	2.45 (.15)	2.31 (.22)	1.36	.27
	None	Minor and/or Major			
	Mean (SD)	Mean (SD)			
Amygdala					
Left	1.05 (.11)	.98 (.09)		6.17	.02
Right	1.09 (.14)	1.06 (.99)		.75	.39
Hippocampus					
Left	2.32 (.31)	2.30 (.23)		.03	.86
Right	2.45 (.28)	2.38 (.20)		.81	.37

ANOVA, analysis of variance.
[a]Differences were analyzed by ANOVA.

The intraclass correlation coefficients for intrarater variability based on the assessment in 30 subjects and the interrater reliability based on the assessment in 10 subjects were .94 and .82, respectively, for the amygdale; .97 and .91, respectively, for the hippocampus; and .99 and .99, respectively, for the intracranial volume.

The normalized volume values, defined as the absolute volume/intracranial volume, were analyzed by one-way analysis of variance (ANOVA) and ANCOVA adjusted for age as a covariate among three groups (BCS with a history of first minor depressive episode after cancer diagnosis, BCS with a history of first major depressive episode after cancer diagnosis, and BCS with no history of any depressive episode after cancer diagnosis) for the main analysis. Least significant difference (LSD) was used for post hoc analysis. An additional comparative analysis was performed between the two groups (BCS with a history of first minor or major depressive episode and BCS with no history of any depressive episode). Additionally, we also examined associations between the severity of depressive episode after cancer diagnosis (0 = no history of depressive episode; 1 = history of the first minor depressive episode; and 2 = history of the first major depressive episode) and the normalized amygdala and hippocampus volumes by the two-tailed Spearman's correlation test. We also examined associations between medical factors (clinical stage, history of adjuvant chemotherapy, and tamoxifen therapy) and the normalized amygdala and hippocampus volumes by ANOVA. Alpha levels were set at $p < .05$ (two-tailed).

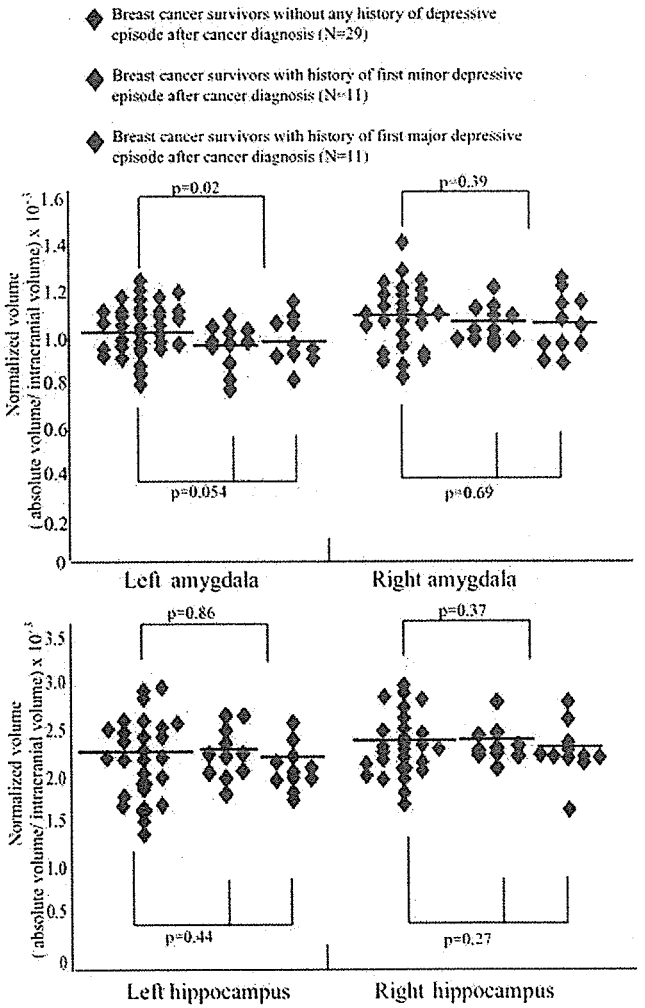

Figure 1. Amygdala and hippcampal volume in breast cancer survivors with no history of any depressive episodes, with a history of first minor depressive episode after cancer diagnosis, and with a history of first major depressive episode after cancer diagnosis. Horizontal bars indicate mean normalized volumes (absolute amygdala and hippocampal volume/intracranial volume) in left and right amygdala and hippocampal volume.

Results

VBM Analysis

The VBM analysis revealed no significant group effect in terms of either the GM voxels or WM densities in any brain region, including the PFC with hypothesis, among the three groups (BCS with a history of first minor depressive episode after cancer diagnosis, BCS with a history of first major depressive episode, and BCS with no history of any depressive episode). Also, no significant differences were observed between the two groups (BCS with a history of first minor or major depressive episode and BCS with no history of any depressive episode) in terms of either the GM voxels or WM densities in any of the brain regions, including the PFC with hypothesis. Furthermore, there were no brain regions significantly associated, in terms of either the GM voxels or WM densities, with the severity of the depressive episode. There were also no significant brain regions, including PFC, significantly associated, in terms of either the GM voxels or WM densities, with medical factors (clinical stage, history of adjuvant chemotherapy, and tamoxifen therapy).

Amygdala and Hippocampal Volume as Estimated by the Manual Tracing Method

The mean (SD) amygdala and hippocampus normalized volumes and their scatter plots are shown in Table 2 and Figure 1. An ANOVA among the three groups revealed a trend-level group effect ($F = 3.12$, $df = 2, 48$, $p = .054$) in the left normalized amygdala volume (absolute volume x 100 / intracranial content). Post hoc analysis revealed that the normalized left amygdala volume in the BCS with a history of first minor depressive episode was significantly smaller ($p = .03$) than that in the BCS with no history of any depressive episode, that normalized left amygdala volume in the BCS with a history of first major depressive episode was smaller with trend-level significance ($p = .09$) than that in the BCS with no history of any depressive episode, and that there was no significant difference between BCS with a history of first minor and major depressive episode ($p = .695$). Additional two-group analysis revealed that the normalized left amygdala volume was significantly smaller in the BCS with a history of first minor and/or major depressive episode than that in the BCS with no history of any depressive episode ($F = 6.17$, $df = 1, 49$, $p = .02$), and the differences were still significant after adjusting for age ($F = 5.88$, $df = 1, 48$, $p = .02$). The normalized left amygdala volumes showed a significant negative association with the severity of the depressive episodes (rho = −.32, $p = .02$). However, there was no significant difference in the normalized right amygdala volume either in the three groups ($F = .37$, $df = 2, 48$, $p = .69$) or in the two groups ($F = .77$, $df = 1, 49$, $p = .39$) in ANOVAs. The normalized right amygdala volume showed no significant association with the severity of the depressive episodes (rho = −.12, $p = .42$). There was no significant difference in the normalized hippocampal volume of either side in the three groups (left: $F = .84$, $df = 2, 48$,

$p = .44$; right: $F = 1.34$, $df = 2, 48$, $p = .27$) or in the two groups (left: $F = .03$, $df = 1, 49$, $p = .86$; right: $F = .81$, $df = 1, 49$, $p = .37$) in ANOVAs. The normalized hippocampal volume of either side showed no significant association with the severity of the depressive episodes (left: rho = −.07, $p = .67$; right: rho = −.17, $p = .24$). There were also no significant differences between the normalized amygdala or hippocampus volumes of either side and medical background factors (clinical stage of the cancer, history of adjuvant chemotherapy, and tamoxifen therapy).

Discussion

This is the first study to compare the volume of the PFC by voxel-based morphometry with hypothesis and the amygdala volume by the manual tracing method among BCS with a history of first minor depressive episode, a history of first major depressive episode, and with no history of depressive episodes after cancer diagnosis. Voxel-based morphometry demonstrated no brain region in the PFC whose volume was significantly different among the three groups. There were also no significant differences in the volume of the hippocampus among the three groups, consistent with the results of our previous study (Inagaki et al 2004). On the other hand, the left amygdala volumes, which were measured by manual tracing method, in the BCS group with a history of first minor or major depressive episode were significantly smaller than that in the group with no history of depressive episodes. However, VBM revealed no significant region that had a significant difference among the groups in amygdala. The present study, in addition, employed the small volume correction (SVC) method, in which we defined the center of amygdala as Montreal Neurological Institute (MNI) coordinates (24, 5, 17) in customized template and the searched volume as 16 mm sphere over the amygdala (alpha level: FDR corrected $p < .05$). These analyses also revealed no significant difference in amygdala. Differences of the results between the traditional manual tracing method and VBM analysis were demonstrated in several volumetric analyses (Good et al 2002; Testa et al 2004). Since it was reported that the manual tracing method appeared more sensitive than VBM to detect atrophy of the amygdala (Good et al 2002), the manual tracing method with sufficient reliability in this study may be more accurate than VBM. Therefore, the findings in this study might suggest that the volume of the amygdala, but not the volume of the PFC, was associated with the first minor and/or major depressive episode after cancer diagnosis.

Several volumetric studies in major depression have demonstrated a smaller PFC volume in these patients as compared with healthy control subjects (Botteron et al 2002; Bremner et al 2002; Drevets et al 1997; Lai et al 2000). On the other hand, there are few volumetric studies using quantitative MRI in minor depression as compared with those in cases of major depression. Kumar et al (1998) demonstrated that subjects with late-life–onset minor depression also had a smaller PFC, similar to the corresponding observations in those with late-life–onset major depression, as compared with healthy control subjects. In these studies, the magnitude of reduction of the PFC showed a negative association with the severity of the clinical depression, with the frontal lobe volume in the late-life–onset minor depression group being intermediate between that in the late-life–onset major depression and healthy control groups. These associations observed between the PFC volume and late-life–onset minor and major depression were not observed for first minor or major depressive episode after cancer diagnosis. The first minor and major depressive episode after cancer diagnosis, characterized by nonchronicity, existence of an obvious stressor such as cancer, improvement without psychiatric intervention, mild severity, and midlife onset in females, may not be related to the PFC volume.

In volumetric studies of the amygdala in major depression, this region has been demonstrated to show a larger volume in cases with a first major depressive episode (Frodl et al 2002), loss of normal asymmetry in cases with treatment-resistant major depression (Mervaala et al 2000), and a smaller core volume in cases with recurrent major depression (Sheline et al 1998). However, there are no reported results on the volume of the amygdala in cases of minor depression, especially in relation to major depression in cancer by consecutive sampling. In the present study, the volumetric difference in the amygdala not observed in the previous study (Inagaki et al 2004) might be due to the inclusion of cases of minor depression for the analysis. Because of the short duration of the first minor and major depressive episodes, the smaller volumes of the amygdala observed in this study could be considered as being not so much the result of the depression as a precedent to the development of a depressive episode, although the causal relationship remains unknown due to the study design. The amygdala is activated by fear or sadness in healthy participants, as demonstrated in emotion activation studies (Phan et al 2002). In major depression, elevated cerebral blood flow and glucose metabolism have been observed in the amygdala at rest (Drevets et al 2002), in relation to the severity of depression (Abercrombie et al 1998), and as an exaggerated response to emotional stimulation (Sheline et al 2001; Siegle et al 2002). Furthermore, a negative association has been suggested between the amygdala volume and the activity during emotional information processing tasks in depressed individuals (Siegle et al 2003). Smaller amygdalas, which might hyperrespond to emotional stimulations, might be associated with first minor and/or major depressive episode after cancer diagnosis. However, longitudinal studies with healthy control subjects would be needed before any definitive conclusions can be drawn.

The present study had the following limitations: 1) since the results were obtained from only one institution, the subjects are not representative of cancer survivors, in general; 2) all of the subjects were cancer survivors, and no healthy control subjects were examined; we could not exclude the possibility of the cancer experience itself having unknown effects on the brain, although there were no significant associations between the regional brain volumes and any of the medical factors in the present study; and 3) the presence of important depression-associated factors, such as a family history of major depressive episodes, was not examined in the study (Drevets et al 1997).

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BRIEF REPORT

Decreased levels of whole blood glial cell line-derived neurotrophic factor (GDNF) in remitted patients with mood disorders

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Abstract

Recent post-mortem and imaging studies provide evidence for a glial reduction in different brain areas in mood disorders. This study was aimed to test whether glial cell line-derived neurotrophic factor (GDNF), a member of transforming growth factor (TGF)-β superfamily, in blood levels was associated with mood disorders. We measured GDNF and TGF-β levels in whole blood in remitted patients with mood disorders [$n=56$; major depressive disorders (MDD) 39, bipolar disorders (BD) 17] and control subjects ($n=56$). GDNF and TGF-β were assayed with the sandwich ELISA method. Total GDNF levels were significantly lower in MDD and in BD than in control subjects (MDD, $p=0.0003$; BD, $p=0.018$), while no significant difference in total TGF-β1 or total TGF-β2 levels was found in these groups. Our study suggests that lower GDNF levels might be involved in the pathophysiology of mood disorders, although this preliminary study has several limitations.

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Key words: GDNF, mood disorders, transforming growth factor (TGF), whole blood.

Introduction

Mood disorders, including major depressive disorders (MDD) and bipolar disorders (BD), are common mental dysfunction with uncertain aetiology. 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 (Manji et al., 2001; Ongur et al., 1998; Rajkowska, 2002). In correspondence with these studies, recent findings showed that brain-derived neurotrophic factor (BDNF), neurotrophin-3 and fibroblast growth factor (FGF) systems, which are potent regulators for neuronal plasticity, survival and development, are altered in different samples such as post-mortem brain, cerebrospinal fluid, and blood from patients with mood disorders (Evans et al., 2004; Hock et al., 2000; Shimizu et al., 2003). It suggests that the dysregulation of multiple neurotrophic/growth factor systems might be involved in the aetiology of mood disorders.

Glial cell line-derived neurotrophic factor (GDNF), a member of transforming growth factor (TGF)-β superfamily, was originally purified from a rat glial cell line supernatant as a trophic factor for midbrain dopamine neurons, and was later found to have pronounced effects on other neuronal populations (Airaksinen and Saarma, 2002). GDNF has been reported to play important roles in higher-ordered brain function such as cognitive abilities and drug addiction (Gerlai et al., 2001; Messer et al., 2000). However, to the best of our knowledge, a relationship between GDNF and mood disorders has not been investigated.

Here, we examine whether blood levels of GDNF in patients with mood disorders would be altered from healthy control subjects.

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Method

Fifty-six Japanese patients with mood disorders (37 male, 19 female; mean age ± S.D. = 59.0 ± 12.4 yr; age range = 36–85 yr) were recruited from the Department of Psychiatry at NHO Kure Medical Center and the Department of Neuropsychiatry at Hiroshima University Hospital.

According to the DSM-IV (APA, 1994), diagnosis of mood disorders and evaluation of symptoms were determined after a clinical interview and a review of clinical records by two psychiatrists. The fifty-six patients consisted of 39 MDD, a single episode or recurrent, and 17 BD, including 9 bipolar I disorder and 8 bipolar II disorder. All patients were under partial or full remitted state when their blood was drawn, and were treated with psychotropic medication such as antidepressants and mood stabilizers. The following antidepressant drugs were administered: paroxetine ($n=15$), amoxapine ($n=13$), sulpiride ($n=10$), trazodone ($n=7$), fluvoxamine ($n=7$), mianserin ($n=6$), milnacipran ($n=3$), imipramine ($n=3$), clomipramine ($n=3$), maprotiline ($n=2$). We estimated 150 mg of imipramine-equivalent dose of each antidepressant as follows; paroxetine, 40 mg; mianserin, 60 mg; milnacipran, 150 mg; fluvoxamine, 150 mg; amitriptyline, 150 mg; clomipramine, 150 mg; amoxapine 150 mg; maprotiline, 150 mg; sulpiride, 300 mg; trazodone, 300 mg. Twenty-three of patients received combined antidepressant treatment with more than two types of antidepressants. Twenty-nine patients received mood stabilizers such as lithium ($n=27$, 600 ± 175 mg/d), valproate ($n=2$, 400–800 mg/d) and carbamazepine ($n=1$, 600 mg/d). Five patients received a monopharmacotherapy of mood stabilizer without any antidepressant.

Fifty-six race- and gender-matched healthy control subjects (17 male, 39 female; mean age ± S.D. = 47.5 ± 9.41 yr, age range = 27–60 yr) were recruited from volunteers in NHO Kure Medical Center and the Hiroshima University Hospital. Subjects with any other diagnosed mental or physical illness were excluded.

The study was approved by the NHO Kure Medical Center Ethics Committee and the Hiroshima University Medical Ethics Committee. Written informed consent was obtained after a full written and verbal explanation of the study.

The blood samples from the patients and the controls were drawn into tubes with ethylenediaminetetraacetic acid around noon and directly transferred to other tubes for storage at −80 °C until assayed. Total and free levels of GDNF, TGF-β1, and TGF-β2 levels were measured by using the enzyme linked immunosorbent assay (ELISA; Emax Immunoassay System kit, Promega, Madison, WI, USA), according to the manufacturer's instructions. GDNF is likely to interact with its ligands from receptors or binding proteins in blood and many types of tissues; thus, the acid treatment procedure is reported to cause dissociation of the ligands from GDNF and to increase the detectable amount of GDNF (total GDNF) (Okragly and Haak-Frendscho, 1997). Therefore, we measured total GDNF, TGF-β1 and TGF-β2 levels by the acid treatment procedure (the diluted samples were acidified to pH 2.6, followed by neutralization to pH 7.6 and centrifuged) according to the manufacturer's instructions, and measured free GDNF level without the procedure. All assays were performed in duplicate. For statistical analysis, the data were presented as the mean ± S.D. χ^2 analysis was performed on binomial data such as gender, and Student's t test was employed for the continuous variables (Table 1). One-way analysis of variance (ANOVA) was used to check statistical tendencies. When significant tendency was suggested, differences between groups were analysed by Fisher's protected least significant differences post-hoc test (Figures 1, 2, Table 2). The relationship between two variables was examined using Pearson's correlation coefficient. p values <0.05 were considered significant.

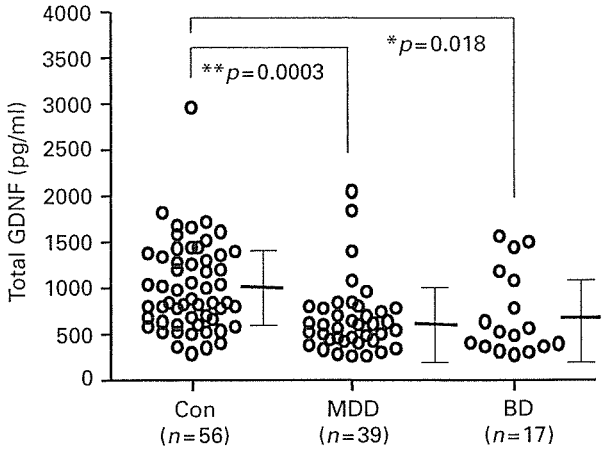

Figure 1. Lower total GDNF concentrations in whole blood in patients with major depressive disorders (MDD) and bipolar disorders (BD) compared with control subjects (Con). The horizontal lines indicate mean ± S.D. levels. * Significance level $p<0.05$, ** Significance level $p<0.01$.

Results

One-way ANOVA indicated a significant difference in whole blood levels of total GDNF between the patients with MDD, BD, and the control subjects ($F=7.8$,

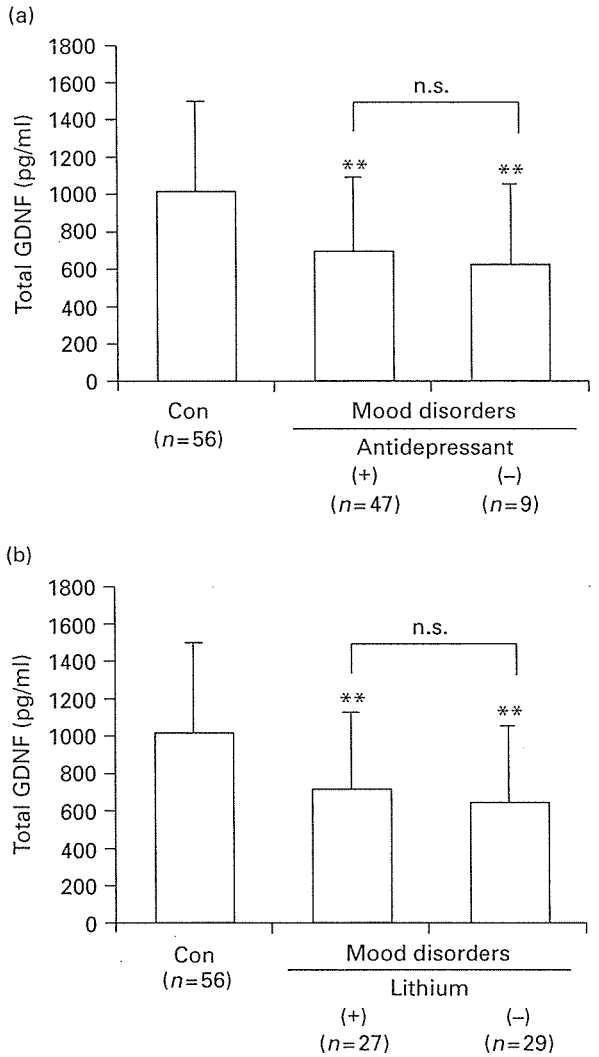

Figure 2. (a) Comparison in total GDNF concentrations between subgroups of control subjects (Con), mood disorders treated with and without antidepressants. (b) Comparison in total GDNF concentrations between subgroups of control subjects (Con), mood disorders treated with and without lithium. The horizontal lines indicate mean ± S.D. levels. ** Significance level $p<0.01$, n.s., not significant.

$p=0.0007$). The levels of total GDNF were significantly lower in the both patients with MDD (mean = 669 ± 383 pg/ml, $p=0.0003$) and BD (mean = 718 ± 457 pg/ml, $p=0.018$) compared with the control subjects (mean = 1016 ± 482 pg/ml), although there was no difference of total GDNF levels between patients with MDD and BD ($p=0.702$) (Figure 1). The levels of free GDNF were ~50 times lower than that of total GDNF (control subjects: 18.5 ± 21.7 pg/ml; MDD: 15.4 ± 46.6 pg/ml; BD: 17.6 ± 33.0 pg/ml). Because free GDNF values in 86 in total subjects ($n=112$) were below minimum sensitivity (16 pg/ml), we could not statistically estimate these values, although the values of these groups appeared similar. Further, one-way ANOVA revealed that there were no group differences in the levels of total TGF-β1 ($F=0.1$, $p=0.91$) and TGF-β2 ($F=2.7$, $p=0.07$) (Table 2). Table 1 shows the participants' demographics for which gender was matched, but age differed significantly between the groups, although no significant correlation was detected between levels of any TGF-β superfamily (including GDNF) and age in any of the subjects ($n=112$; total GDNF: $r=-0.126$, $p=0.186$; total TGF-β1: $r=0.22$, $p=0.819$; total TGF-β2: $r=0.009$, $p=0.924$). There was a tendency of gender difference in total GDNF levels in any subject ($n=112$; male = 34, 719 ± 462 pg/ml; female = 78, 907 ± 469 pg/ml, $p=0.052$). Neither age of onset ($n=56$, $r=0.045$, $p=0.744$), number of episodes ($n=56$, $r=0.007$, $p=0.9582$), dose of antidepressants (imipramine equivalents/d) ($n=56$, $r=0.09$, $p=0.5156$) or dose of lithium ($n=27$, $r=0.058$, $p=0.778$) correlated significantly with the levels of total GDNF in patients with mood disorders. In addition, there was no difference in total GDNF levels between subgroups of mood disorders treated with and without antidepressants, although the total GDNF levels in each group were significantly lower than that in control subjects (Figure 2a). In the same way, there was no difference in total GDNF levels between subgroups of mood disorders treated with and without lithium (Figure 2b). The total GDNF levels in patients with a family history of mood disorders ($n=10$, 656 ± 330 pg/ml) did not differ from those in patients without one ($n=46$, 706 ± 423 pg/ml, $p=0.73$). There was no difference in total GDNF levels between subgroups of partial and full remitted patients (partial: $n=47$, 717 ± 428 pg/ml; full: $n=9$, 511 ± 152 pg/ml, $p=0.16$), as well as between subgroups of bipolar I and bipolar II disorders (bipolar I: $n=9$, 707 ± 152 pg/ml; bipolar II: $n=8$; 731 ± 173 pg/ml, $p=0.92$).

Discussion

We found that total GDNF levels in whole blood in remitted patients with mood disorders were significantly lower than those in healthy control subjects, whereas total TGF-β1 and total TGF-β2 were not altered between the groups. This is the first report showing a possible association of GDNF to mood disorders.

In this study, we could not exclude the effects of antidepressants and mood stabilizers on the total GDNF levels in the patients. In the case of antidepressants, previous animal studies have reported

Table 1. Demographics of subjects and controls

	Mood disorders	Control subjects	t or χ^2	p value
No. of subjects	56	56		
Subtype (MDD/BD)	39/17	–		–
Gender (M/F)	17/39	17/39	$\chi^2=0.548$	0.76
MDD	13/26			
BD	4/13			
Mean age (yr)	59.0±12.4	47.5±9.41	$t=5.52$†	0.0001
MDD	60.0±13.0			
BD	56.9±11.2			
Onset (yr)	51.9±12.0	–	$t=1.59$‡	0.12
MDD	53.6±11.8			
BD	48.0±11.9			
No. of episodes	3.01±2.87	–	$t=-2.81$‡	0.007
MDD	2.42±1.72			
BD	4.79±4.41			
Imipramine-equivalents (mg/d)	118±93.3	–	$t=2.178$‡	0.034
MDD	135±84.0			
BD	76.5±104			

MDD, Major depressive disorder; BD, bipolar disorder.
Data are shown as mean±S.D.
† Comparisons between two groups of mood disorders and control subjects.
‡ Comparisons between MDD and BD.

Table 2. Total levels of whole blood transforming growth factor (TGF) in patients with mood disorders and control subjects

Variables (pg/ml)	Control subjects ($n=56$)	MDD ($n=39$)	BD ($n=17$)	p value
Total TGF-β1	4499±2475	4299±1615	4938±2407	$F=0.1$, $p=0.91$
Total TGF-β2	4565±2969	3606±2949	5556±3245	$F=2.7$, $p=0.07$

that chronic treatments with antidepressants did not affect on GDNF mRNA and protein levels in several areas of rat brain (Chen et al., 2001). In contrast, subchronic treatment with antidepressants increased GDNF mRNA and protein levels in glial cultured cells (Hisaoka et al., 2001; Mercier et al., 2004). In the case of mood stabilizers including lithium, chronic treatments with lithium and valproate did not affect on GDNF mRNA and protein levels in several areas of rat brain (Fukumoto et al., 2001). In the Finders Resistant Line rats, chronic treatment with lithium increased GDNF protein levels in the frontal cortex and occipital cortex, decreased the GDNF levels in the hippocampus, and did not alter the GDNF levels in the striatum (Angelucci et al., 2003). These previous reports suggest different effects of antidepressants and mood stabilizers on the GDNF expressions by strain, areas of brain, and types of experimental system. In this clinical study, there was no difference in total GDNF levels between subgroups of mood disorders treated with and without antidepressants, as well as between subgroups of mood disorders treated with and without lithium (Figure 2). In addition, no correlation between total GDNF levels in whole blood and dose of antidepressants/mood stabilizers among the patients was observed. Consequently, it is unlikely that medication may decrease the total GDNF levels in the patients. Another important concern is whether the GDNF effect in remitted patients with mood disorders is a state or trait marker. Our study indicates that degree of psychiatric symptom might not affect the GDNF levels because there was no difference in total GDNF

levels between subgroups of partial and full remitted patients, although the results are not conclusive. Therefore, additional studies by investigating drug responses of GDNF levels to drug-naive patients with psychiatric symptoms are needed to clarify whether a decrease of GDNF levels is secondary (i.e. effects of the drug medication or psychiatric symptoms) or primary (a genetic vulnerability) in patients with mood disorders.

The source of circulating GDNF in blood is totally unknown, although glia, neuron, kidney and ovary appear to be candidates (Golden et al., 1999). In the case of BDNF, the sources of circulating BDNF are considered to be platelets, brain neurons and vascular endothelial cells (Radka et al., 1996), and a positive correlation between blood and cortical BDNF levels was observed in rats (Karege et al., 2002). This suggests that blood cells could store and release neurotrophic factors. Actually, total GDNF levels in whole blood in healthy subjects were higher than those in plasma and serum were (data not shown), suggesting that an unknown source of blood cells might be involved in the total GDNF blood levels. Therefore, in this study, we measured concentrations of total GDNF in whole blood including the contents of all blood cell types. It is vital to confirm whether blood GDNF levels positively correlate with cerebral GDNF levels, as blood BDNF levels do.

This study is a preliminary report that has a number of limitations such as age difference and medication use. Recent imaging and biochemical studies in mood disorders have revealed a morphological reduction of several brain areas (Manji et al., 2001; Ongur et al., 1998; Rajkowska, 2002) and alternations of multiple neurotrophic/growth factor systems (Evans et al., 2004; Hock et al., 2000; Shimizu et al., 2003), although a direct association among both of them is still unclear. Taken together with our results that total GDNF levels in whole blood were reduced in patients with mood disorders, it is suggested that dysregulation of multiple neurotrophic/growth factor systems such as BDNF, FGF, and GDNF may be involved in the aetiology of the complex disorders. Further in-depth study will be necessary.

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Statement of Interest

None.

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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 inhibitors. Indeed, treatment of amitriptyline rapidly increased extracellular signal-regulated kinase (ERK) activity, as well as p38 mitogen-activated protein kinase and c-Jun NH_2-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-HT_{2A} receptors, ketanserin, a 5-HT_{2A} 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.

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); NA, noradrenaline; BDNF, brain-derived neurotrophic factor; FGF, fibroblast growth factor; GDNF, glial cell line-derived neurotrophic factor; H89, *N*-[2-(4-bromocinnamylamino)ethyl]-5-isoquinoline; U73122, 1-[6-[[17β-methoxyestra-1,3,5(10)-trien-17-yl]amino]hexyl]-1*H*-pyrrole-2,5-dione; PD169316, 4-(4-fluorophenyl)-2-(4-nitrophenyl)-5-(4-pyridyl)1*H*-imidazole; U0126, 1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene; BAPTA-AM, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid-acetoxymethyl ester; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4*H*)-benzopyran-4-one hydrochloride; WAY100635, [O-methyl-3*H*]-*N*-(2-(4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-*N*-(2-pyridinyl)cyclohexanecarboxamide trihydrochloride; NHA, normal human astrocyte(s); RT-PCR, reverse transcription-polymerase chain reaction; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; MAP, mitogen-activated protein; IP, immunoprecipitation; ERK, extracellular signal-regulated kinase; ; JNK, c-Jun NH_2-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; PI3K, phosphoinositide-3 kinase; SP600125, anthra(1,9-*cd*)pyrazol-6(2*H*)-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) (Hisaoka et al., 2001). Furthermore, we recently reported that total GDNF levels in whole blood in patients with mood disorders were significantly lower than those in healthy control subjects (Takebayashi et al., 2006). These results suggest that lower GDNF levels might be involved in the pathophysiology of mood disorders, and increase of GDNF by antidepressants might be involved in their therapeutic action. The identification of the mechanism of GDNF production by antidepressants may contribute to the search for novel targets, which might be related to the therapeutic action. Thus, we attempt to clarify the mechanism of antidepressant-induced GDNF production in this study.

Materials and Methods

Reagents. Reagents were obtained from the following sources: amitriptyline, cycloheximide, calphostin C, desipramine, diazepam, diphenhydramine, trihexyphenidyl, and haloperidol (Wako Pure Chemicals, Osaka, Japan); H89 and U73122 (BIOMOL Research Laboratories, Plymouth Meeting, PA); genistein, genistin, PD169316, PD98059, SP600125, and U0126 (Calbiochem, San Diego, CA); BAPTA-AM, LY294002, lithium chloride, fluoxetine, and rottlerin (Sigma-Aldrich, St. Louis, MO); ketanserin and WAY100635 (Tocris Cookson Inc., Ellisville, MO); clomipramine (Nihon Chiba-Geigy K.K., Hyogo, Japan); and EDTA (Amresco, Solon, OH).

Cell Culture. Cultures of C6 cells were described previously (Hisaoka et al., 2001). In brief, C6 cells were grown in Dulbecco's modified Eagle's medium (Cambrex Bio Science Walkersville, Inc., Walkersville, MD) supplemented with 2 mM L-glutamine and 5% fetal bovine serum (JRH Biosciences, Lenexa, KS) in a 5% CO_2-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% CO_2-humidified atmosphere. More than 80% NHA expressed glial fibrillary acid 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% CO_2 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 $\times$ $10^4/cm^2$ on a six-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 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 Biomedicals, Ohtsu, 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 glyceraldehydes-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 $2(-\Delta\Delta C(T))$ 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 $\times$ $10^4/cm^2$ 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 NH_2-terminal protein kinase (JNK)], cells were cultured at a density of 8 to 16 $\times$ $10^4/cm^2$ 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 500 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 6 h at 4°C and incubated with respective antibodies overnight at 4°C. After washing, the membranes were incubated 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-dithiothreitol, 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 cytotoxicity 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 $13 \times 10^4/cm^2$ 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 amitripty-

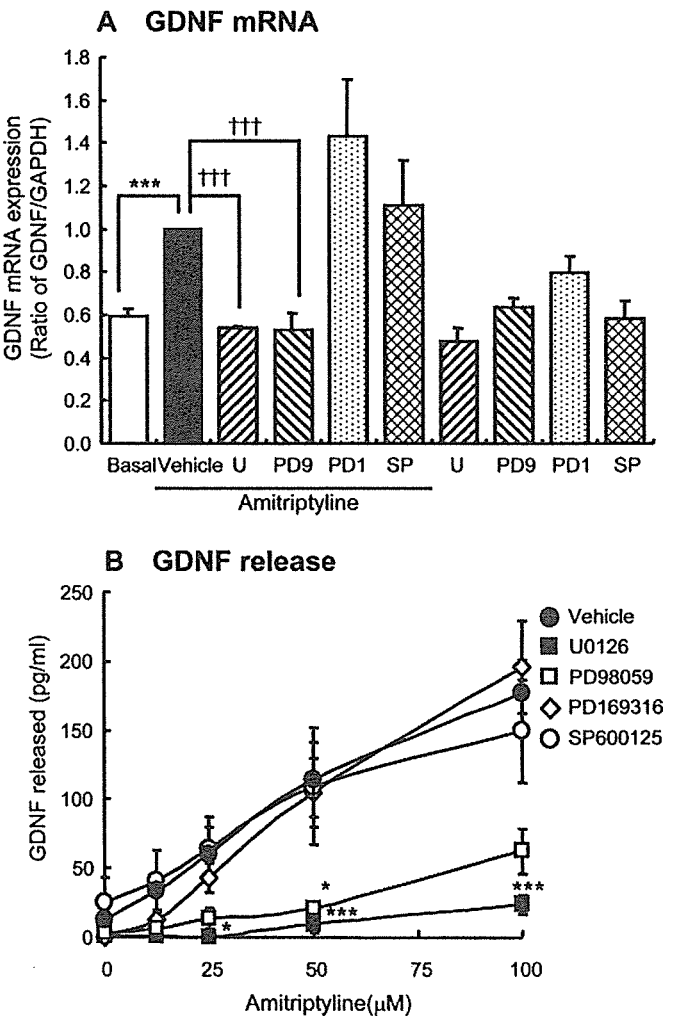

Fig. 1. Effects of U0126, PD98059, PD169316, and SP600125 on the amitriptyline-induced GDNF mRNA expression and GDNF release in C6 cells. A, effects of U0126, PD98059, PD169316, and SP600125 on the amitriptyline-induced GDNF mRNA expression. C6 cells were pretreated with 10 μM U0126 (U), 30 μM PD98059 (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. ***, $p < 0.001$ 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. *, $p < 0.05$; ***, $p < 0.001$, 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 (Hisaoka 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 (Hisaoka 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 Activities. 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

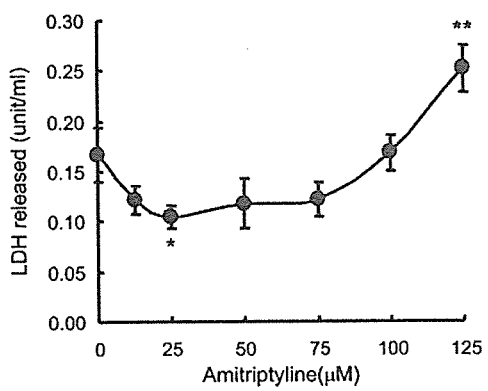

Fig. 2. Chemotoxicity of amitriptyline in C6 cells. C6 cells were treated with the indicated concentration of amitriptyline for 48 h. For the determination of the cytotoxicity of amitriptyline, we measured LDH in the conditioned medium. Values are expressed as mean ± S.E.M. of released LDH (units per milliliter) from four independent experiments. *, $p < 0.05$; **, $p < 0.01$, significantly different from the basal group (Fisher's PLSD test; $n = 4$).

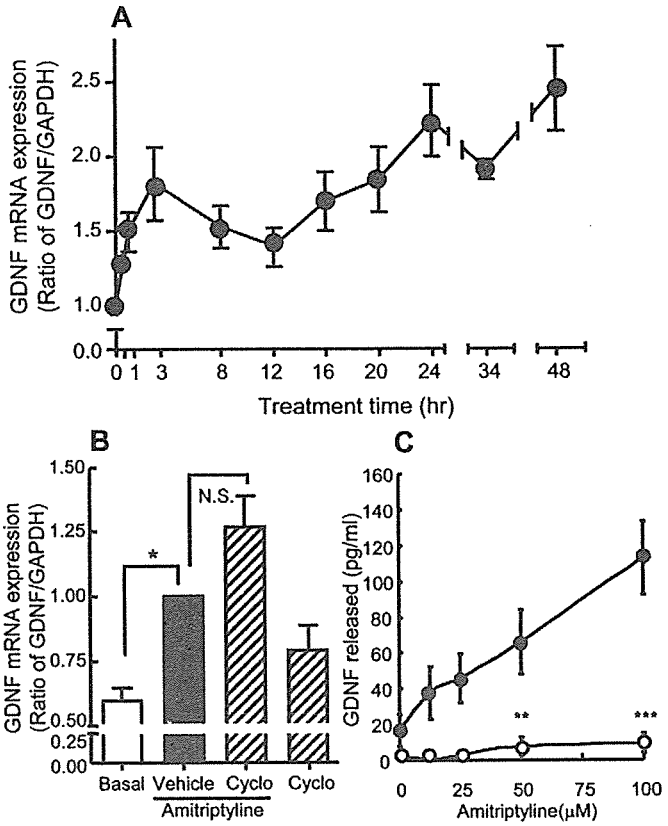

Fig. 3. Amitriptyline increased GDNF release through GDNF mRNA expression. A, C6 cells were treated with 25 μM amitriptyline for the indicated period. Values are shown as the ratio of GDNF mRNA versus GAPDH mRNA. Data are expressed as mean ± S.E.M. from three independent experiments. B, effects of cycloheximide on the amitriptyline-induced GDNF mRNA expression. C6 cells were pretreated with 1 μg/ml cycloheximide (Cyclo) for 30 min and subsequently treated with 25 μM amitriptyline for 3 h. Values are shown as the ratio of GDNF mRNA versus GAPDH mRNA. Data are expressed as mean ± S.E.M. *, $p < 0.05$ compared with the basal group (Tukey's HSD test; $n = 3$–5). C, effects of cycloheximide on the amitriptyline-induced GDNF release. C6 cells were treated with 1 μg/ml cycloheximide (○) 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 four independent experiments. **, $p < 0.01$; ***, $p < 0.001$, significantly different from the vehicle (amitriptyline only) group (Bonferroni post-tests; $n = 4$).

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 (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

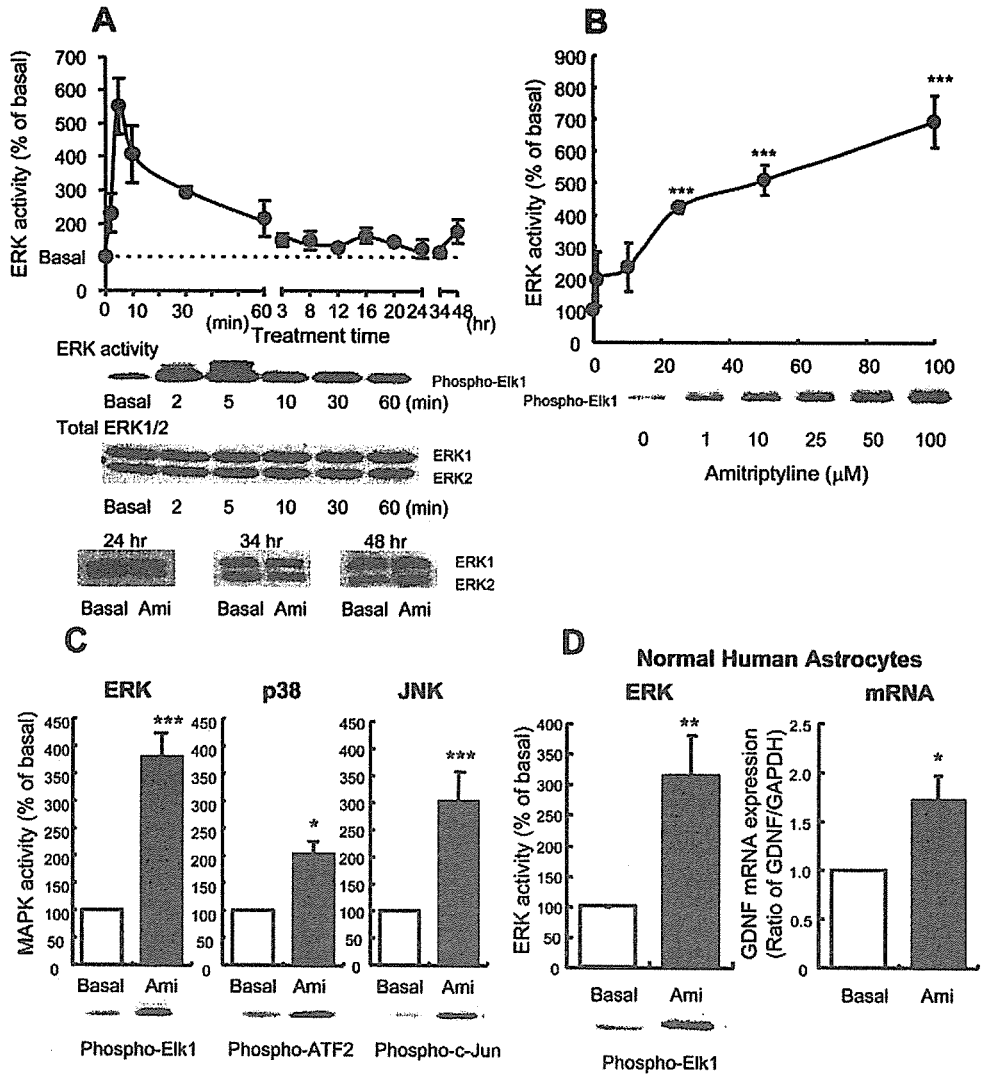

Fig. 4. Effects of amitriptyline on MAP kinase activation in C6 cells and normal human astrocytes. A, time course of the amitriptyline-induced ERK activation. C6 cells were treated with 25 μM amitriptyline for the indicated period, and ERK activity was measured. Data are expressed as mean ± S.E.M. from four independent experiments. Middle, phosphorylated Elk-1 was detected by Western blotting, and a representative result is shown. Bottom, C6 cells were treated with 25 μM amitriptyline (Ami) for the indicated periods. Total levels of ERK1/2 were detected by Western blotting, and representative results are shown. B, concentration dependence of the amitriptyline-induced ERK activation. C6 cells were treated with the indicated concentrations of amitriptyline for 5 min, and ERK activity was measured. Data are expressed as mean ± S.E.M. from four independent experiments. ***, $p < 0.001$, significantly different from the basal (Fisher's PLSD test). The phosphorylated Elk-1 was detected by Western blotting, and a representative result is shown. C, effects of amitriptyline on ERK, p38, and JNK activation. C6 cells were treated with 25 μM amitriptyline for 5 min, and ERK, p38, and JNK activity was measured. Data are expressed as mean ± S.E.M. from three independent experiments. *, $p < 0.05$; ***, $p < 0.001$, significantly different from the basal group (Student's t test). Phosphorylated Elk-1, activating transcription factor-2, and c-Jun were detected by Western blotting, and representative results are shown. D, effect of amitriptyline on ERK activation and GDNF mRNA expression in NHA. NHA were treated with 25 μM amitriptyline for 5 min (ERK activity) or 24 h (GDNF mRNA expression). Data are expressed as mean ± S.E.M. from three to five independent experiments. *, $p < 0.05$ significantly different from the basal group (Student's t test). The phosphorylated Elk-1 was detected by Western blotting. A representative result is shown.

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

Antidepressants increased acute ERK activation, but haloperidol, diazepam, and lithium did not in C6 cells and NHA

C6 cells were treated with 25 μM amitriptyline, nortriptyline, desipramine, mianserin, clomipramine, fluvoxamine, fluoxetine, haloperidol, diazepam, diphenhydramine, and trihexyphenidyl or 1 mM lithium for 5 min, and ERK activity was measured. NHA were treated with 25 μM amitriptyline, clomipramine, mianserin, or fluvoxamine for 5 min, and ERK activity was measured. Data are expressed as mean ± S.E.M. from three to five independent experiments.

Drug	ERK Activation	
	C6 Cells	NHA
	% basal	
Tricyclic antidepressant		
Amitriptyline	394.8 ± 85.6***	313.4 ± 66.5**
Clomipramine	466.4 ± 34.7**	373.0 ± 90.9*
Desipramine	350.3 ± 55.1*	
Nortriptyline	362.8 ± 100.4*	
Tetracyclic antidepressant		
Mianserin	431.6 ± 41.5*	279.3 ± 55.6*
Selective 5-HT reuptake inhibitor		
Fluvoxamine	268.8 ± 69.1*	225.8 ± 25.6**
Fluoxetine	342.2 ± 31.9*	
Other		
Haloperidol	72.9 ± 36.5	
Diazepam	70.5 ± 15.1	
Lithium	118.3 ± 46.2	
Diphenhydramine	105.5 ± 23.4	
Trihexyphenidyl	74.5 ± 19.7	

* $p < 0.05$, 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).

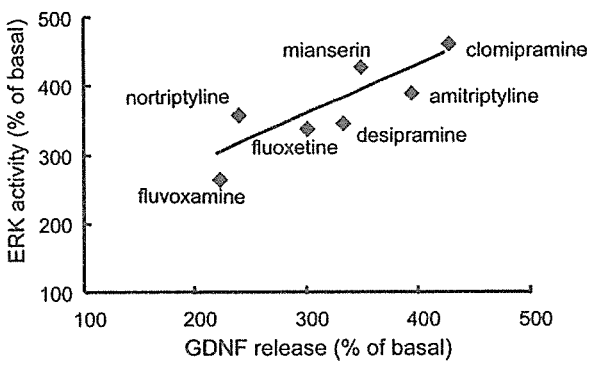

Fig. 5. Correlation between acute ERK activity and GDNF release induced by antidepressants. C6 cells were treated with 25 μM antidepressants for 5 min (ERK activity) or 48 h (GDNF release). The x-axis of the graph represents GDNF release, and the y-axis represents ERK activity. GDNF release and ERK activity by individual antidepressants are expressed as mean of the percentage compared with basal group from three to five independent experiments.

coefficient value was 0.839 ($p = 0.018$, $Y = 0.7147X + 144.57$).

Amitriptyline-Induced Acute ERK Activation Was Independent of Monoamine. Antidepressants are known to inhibit monoamine transporters or monoamine oxidase and to increase monoamine levels in the extracellular space. We previously demonstrated that 5-HT, but not NA or dopamine (DA), increased GDNF release and that the 5-HT-induced GDNF release was blocked by ketanserin, a 5-HT_{2A} receptor antagonist in C6 cells (Hisaoka et al., 2004). In this study, we examined the effect of monoamine on ERK activity in C6 cells. We showed that 5-HT increased ERK activity as well as GDNF release but that NA and DA decreased ERK activity (Fig. 6A). To clarify which 5-HT receptor subtypes were related to the effect of 5-HT, we examined the effects of antagonists for 5-HT_{2A} and 5-HT_{1A} receptors (ketanserin and WAY100635) on the 5-HT-induced ERK activation. Several reports demonstrated that C6 cells functionally express 5-HT_{2A} and 5-HT_{1A} receptors (Shinagawa, 1994; Elliott et al., 1995). Ketanserin, but not WAY100635, completely inhibited the 5-HT-induced ERK activation in C6 cells (Fig. 6B), whereas ketanserin and WAY100635 alone did not have any significant effect on basal ERK activity (Fig. 6B). Next, to clarify the role of 5-HT in the antidepressant-induced ERK activation in C6 cells, we examined the effects of ketanserin and WAY100635 on the amitriptyline-induced ERK activation. However, neither ketanserin nor WAY100635 affected the amitriptyline-induced ERK activation (Fig. 6C). Furthermore, ketanserin did not affect the amitriptyline-induced GDNF release (data not shown).

To eliminate the possibility that 5-HT is involved in the mechanism of the amitriptyline-induced ERK activation, we analyzed the 5-HT concentration in the cell lysate and the medium in C6 cells with or without 25 μM amitriptyline treatment for 48 h. No detectable amount of 5-HT in both of the cells lysate and the conditioned medium was observed. The detection limit for 5-HT by high-performance liquid chromatography was 4 nM.

In addition, histamine and acetylcholine (10 μM; 2-min treatment) did not have any effect on phosphorylation levels of ERK (83.1 ± 14.3 and 91.5 ± 2.4% basal level, respectively; $n = 4$) in C6 cells. Histamine and acetylcholine did not have any effect on GDNF production in C6 cells (data not shown).

Amitriptyline-Induced Acute ERK Activation Was Dependent on PTK. To clarify the intracellular mechanism by which amitriptyline induces acute ERK activation, we used various types of inhibitors of intracellular signal transduction. We examined effects of H89 (a PKA inhibitor), calphostin C (a pan-PKC inhibitor), rottlerin (a PKCδ inhibitor), EDTA (a calcium chelator), BAPTA-AM (an intracellular calcium chelator), LY294002 [a phosphoinositide-3 kinase (PI3K) inhibitor], U73122 (a phospholipase C inhibitor), and genistein (a PTK inhibitor) on the amitriptyline-induced ERK activation. Only genistein significantly inhibited the amitriptyline-induced ERK activation, whereas genistin, a negative analog of genistein, did not have any effect (Table 2). In addition, genistein alone did not have any significant effect on basal ERK activity (29.9 ± 9.14% vehicle versus basal ERK activity; $p = 0.89$).

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 (band 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. Genistein significantly inhibited 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).

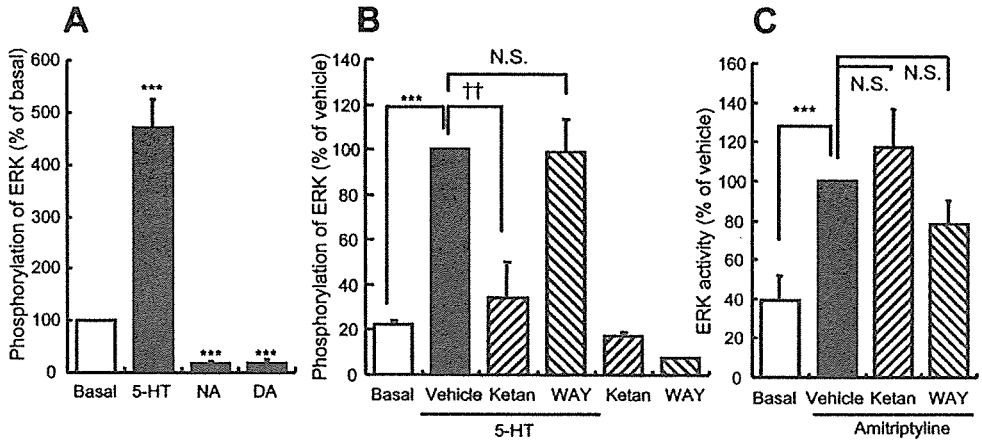

Fig. 6. 5-HT, but not NA or DA, increased ERK activation via 5-HT_{2A} receptors, but 5-HT receptor antagonist did not affect the amitriptyline-induced ERK activation in C6 cells. A, effects of 5-HT, NA, and DA on ERK activity. C6 cells were treated with 10 μM 5-HT, NA, or DA for 2 min, and phosphorylation 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 or without 10 μM 5-HT for 2 min. Phosphorylation levels of ERK1/2 were detected by Western blotting. Data are expressed as mean ± S.E.M. ***, $p < 0.001$ compared with the basal group, and ††, $p < 0.01$ compared with the vehicle (5-HT only) group (Tukey's HSD test; n = 3–5). C, effects of ketanserin and WAY100635 on the amitriptyline-induced ERK activation. 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. ***, $p < 0.001$ compared with the basal group (Tukey's HSD test; n = 3–5).

TABLE 2

Effects of inhibitors for intracellular transduction on the amitriptyline-induced ERK activation in C6 cells

C6 cells were pretreated with 1 mM EDTA for 10 min; 100 nM calphostin C, 5 μM rottlerin, 25 μM BAPTA-AM, or 1 μM U73122 for 30 min; and 1 μM H89, 25 μM LY294002, 100 μM genistein, or 100 μM genistin for 1 h and subsequently treated with 25 μM amitriptyline for 5 min. ERK activity was measured. Data are expressed as mean ± S.E.M. from three to four independent experiments.

Drug	ERK Activation
	% vehicle
Basal	31.6 ± 3.98
Vehicle (amitriptyline only)	100.0 ± 0.0***
+ H89 (PKA inhibitor)	89.8 ± 3.87
+ Calphostin C (pan-PKC inhibitor)	101.0 ± 4.16
+ Rottlerin (PKCδ inhibitor)	107.2 ± 11.8
+ EDTA (Ca^{2+} inhibitor)	103.5 ± 6.38
+ BAPTA-AM (intracellular Ca^{2+} inhibitor)	100.8 ± 10.3
+ LY294002 (PI3K inhibitor)	99.1 ± 27.4
+ U73122 (PLC inhibitor)	99.3 ± 11.2
+ Genistein (PTK inhibitor)	20.6 ± 6.21†††
+ Genistin (a negative analog of genistein)	100.7 ± 34.6

*** $p < 0.001$ compared with the basal group (Tukey's HSD test).
††† $p < 0.001$ compared with the amitriptyline treatment (vehicle) group (Tukey's HSD test).

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 not 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 antidepressant 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. 3, B 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 trigged 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 transcrip-

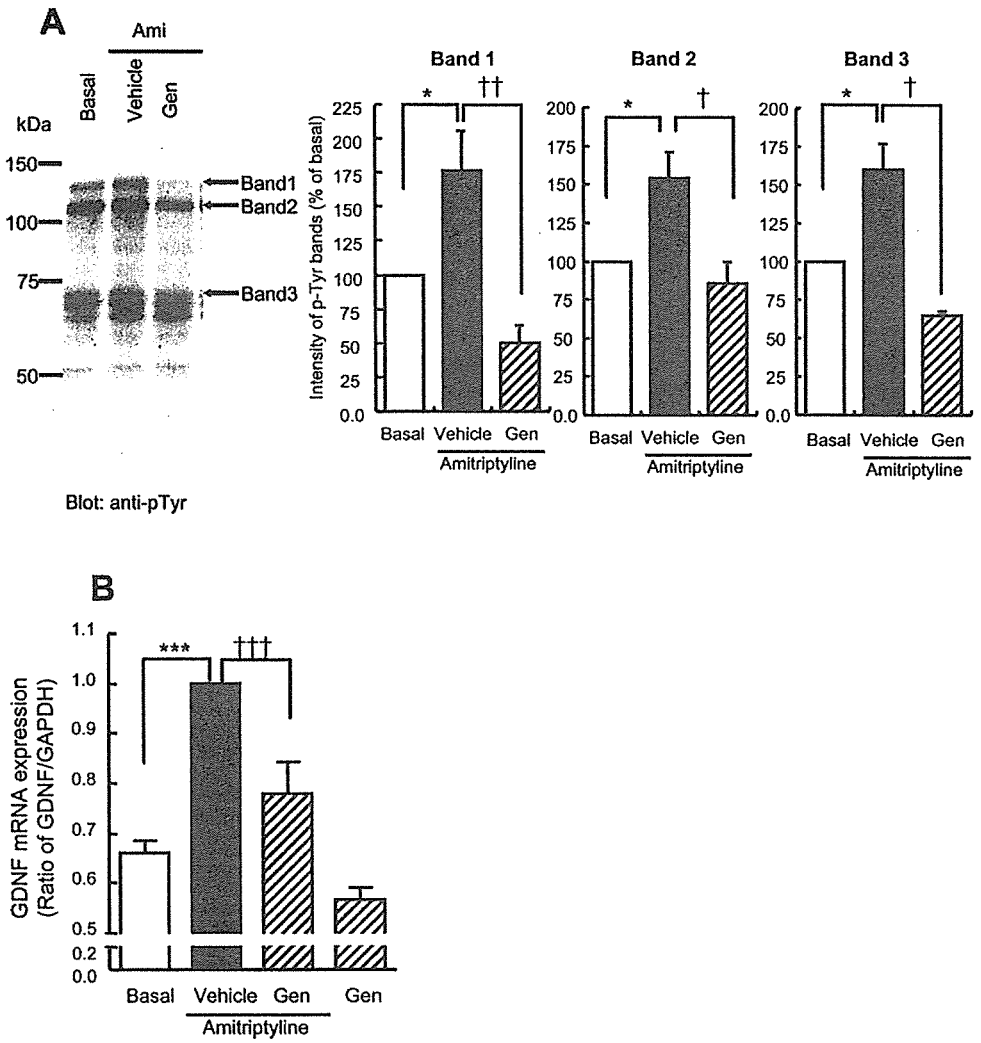

Fig. 7. Effects of genistein on the amitriptyline-induced phosphorylation level of tyrosine-containing proteins and GDNF mRNA expression in C6 cells. A, effect of genistein on the amitriptyline-induced phosphorylation level of tyrosine-containing proteins. C6 cells were treated with 100 μM genistein (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).

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 (Glotzbach and Preskorn, 1982; Baumann et al., 1984; Miyake et al., 1990), although the therapeutic plasma concentrations of amitriptyline range approximately from 0.36 to 0.9 μM (Baldessarini, 2001). In addition, the mean brain concentrations of the tricyclic antidepressants in post-mortem humans were approximately 20 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-HT_{2A} antagonist and MEK inhibitor significantly inhibited the 5-HT-induced GDNF production (Hisaoka et al., 2004) and that 5-HT_{2A} antagonist completely inhibited the 5-HT-induced ERK activation in this study. These results suggest that 5-HT increased GDNF production through 5-HT_{2A} 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-HT_{2A} 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

was observed in either C6 cells or their conditioned medium in the presence or absence of antidepressants (Muraoka 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 (Table 1) and that diphenhydramine did not induce GDNF release in C6 cells (Hisaoka et al., 2001). In addition, histamine or acetylcholine by themselves 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 antihistaminergic or anticholinergic 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 phosphotyrosine-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 involve 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.

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