Table 1
Demographic and medical backgrounds of the patients

	Cancer patients		Statistic value	p
	With depressive episode $n=6$	Without depressive episode $n=15$		
Age (years old)[a]	69.5±3.3	62.5±1.7	$t=2.05$	$p=0.06$
Gender[b]: male	3 (50%)	9 (67%)	$\chi^2=0.18$	$p=0.52$
Height (cm)[a]	158.2±3.7	149.7±9.8	$t=0.54$	$p=0.60$
Weight (kg)[a]	55.5±3.4	53.8±1.9	$t=0.48$	$p=0.64$
Handedness (no. of patients)				
right	6 (100%)	15 (100%)	–	–
Education (years)[a]	12.8±1.1	12.9±0.7	$t=0.03$	$p=0.98$
PS (no. of patients)[b]				
0	6 (100%)	12 (80%)	$\chi^2=1.40$	$p=0.34$
I	0 (0%)	3 (20%)	–	–
Clinical stage[b]				
II	1 (17%)	1 (7%)	$\chi^2=0.50$	$p=0.50$
III–IV	5 (83%)	14 (93%)	–	–
Pain[c]	3 (0–7)	1 (0–6)	$U=29.0$	$p=0.24$
Fatigue[c]	2 (1–6)	1 (0–8)	$U=26.0$	$p=0.15$
Shortness of breath[c]	0.5 (0–2)	0 (0–1)	$U=30.5$	$p=0.27$
Appetite loss[c]	1.5 (0–2)	1 (0–10)	$U=43.5$	$p=0.91$
Nausea[c]	0 (0–0)	0 (0–8)	$U=39.0$	$p=0.68$
Opioids use (no. of patients)[b]	2 (33%)	3 (20%)	$\chi^2=0.42$	$p=0.45$
Psychotropic medication	1 (17%)[d]	1 (7%)[d]	$\chi^2=0.50$	$p=0.50$
Anticonvulsant medication	0 (0)	0 (0)	–	–
BS before PET (mg/dl)[a]	102±5.4	103±2.9	$t=0.23$	$p=0.82$
FDG injection (MBq)[a]	258.7±9.5	267.6±4.1	$t=1.03$	$p=0.32$
HDRS score[e]	10.8 (6–15)	5.7 (0–13)	$U=11.5$	$p<0.01$
STAI score[e]	88.3 (84–98)	75.1 (59–111)	$U=16.0$	$p=0.02$
IES-R score[e, f]	25.0 (14–44)	8.2 (0–28)	$U=11.5$	$p=0.01$

No.: number; PS: performance status; BS: blood sugar; PET: positron emission tomography; FDG: F18-fluorodeoxyglucose; HDRS: the Hamilton Depression Rating Scale 17 item version; STAI; the State and Trait Anxiety Inventory; IES-R; the Impact of Event Scale-Revised.

[a] Mean±standard deviation and t and p values of the Student's t-test.

[b] Number of patients (percentage) and χ^2 and p values of the χ^2 test.

[c] Pain, fatigue, shortness of breath, and nausea were measured by using the MDASI and indicated median (minimum−maximum) score, and U and p value of the Mann-Whitney U test.

[d] One subject in the depressed group used alprazolam and one subject in the nondepressed group used lormetazepam.

[e] Median (minimum−maximum) score, and U and p value of the Mann-Whitney U test.

[f] Number of cancer patients without depressive episode was 13.

The reliability of the diagnostic interview was tested by having another interviewer as a second rater (kappa=1.0, $n=21$). Medical data were collected by chart review, and demographic data were collected by a structured interview. The Hamilton Depression Rating Scale 17 item version (HDRS) was used to measure depression severity (Hamilton, 1960). To evaluate physical symptoms, the M.D. Anderson Symptom Inventory-Japanese version (MDASI) (Okuyama et al., 2003) was used to measure pain, fatigue, shortness of breath, appetite loss, nausea, and vomiting, rated using a 0-to-10 numerical system. The Impact of Event Scale-Revised, a 22-item self-rating questionnaire, was used (Asukai et al., 2002) to assess the level of symptomatic reaction to a traumatic experience i.e., disclosure of cancer diagnosis. The score ranges from 0 to 88. The State and Trait Anxiety Inventory (Spielberger et al., 1970), a 40-item self-rating questionnaire designed to evaluate state- and trait-anxiety, was used to measure the anxiety level. The score ranges from 40 to 160.

2.3. Measurement of regional glucose metabolism by PET

Under resting conditions in a dark room for thirty minutes after an injection of F18-fluorodeoxyglucose (FDG) (5 MBq/kg), 10-min transmission with ^{68}Ge and 20 min emission scans of the brain were performed using a GE ADVANCE NXi (GE Medical Systems, Milwaukee). The spatial resolution of this scanner is 4.8 mm full width at half maximum. Whole body PET scans to evaluate the clinical stage of pancreatic cancer were performed after the brain scans. Summed images were used for data analysis. Preprocessing of scanned images was done using SPM2 software (Wellcome Department of Cognitive Neurology, London). Images were normalized

to a template, globally normalized, and smoothed using a 12-mm Gaussian kernel.

2.4. Statistical analyses

Comparisons of the background and medical factors were performed by the Student's *t*-test, the Mann-Whitney *U* test, or the χ^2 test between patients with a current depressive episode and those without an episode. rCMRgluc was compared between the two groups on the SPM2 software using an ANCOVA model with age and gender as nuisance variables. A statistical *p* value of the comparison was uncorrected $p<0.005$ in the regions of interest (ROIs), including the prefrontal and limbic regions. Other brain regions were compared using $p<0.001$ as a reference, with no discussion of the results.

3. Results

Six of the 21 pancreatic cancer patients were diagnosed as having a depressive episode after pancreatic cancer diagnosis. Two had major depressive episodes and 4 had minor depressive episodes. In the 6 patients with a current depressive episode, none had any history of a major or minor depressive episode before their cancer diagnosis. In the remaining 15 cancer patients without a current depressive episode, one patient had a history of minor depression before the cancer diagnosis. The duration of the episode up to the time of the PET was less than 6 weeks (mean duration was 3 weeks). All of the episodes developed after the disclosure of the pancreatic cancer diagnosis by the patients' physicians. None of the patients had been diagnosed as having a current/past history of anxiety disorders, including post-traumatic stress disorder (PTSD), substance-use disorders, or bipolar disorder. Table 1 shows no significant difference in demographic and medical factors between the two groups. In the psychological factors, the scores of the HDRS, the STAI, and the IES-R were significantly different between the groups. Amongst the ROIs, the subgenual anterior cingulate cortex (sACC) had a significantly higher rCMRgluc (uncorrected $p=0.002$, $t=2.89$) (Fig. 1). The *xyz* coordinate of the MNI space was [−8 32 −10]. Sub-analyses showed no significant correlations between the rCMRgluc values at the coordinate and the HDRS scores ($r=0.259$, $p=0.285$) or the STAI scores ($r=0.116$, $p=0.635$) ($n=21$). Among the patients with depression, however, the partial correlation coefficients of rCMRgluc at the coordinate were $r=-0.992$ ($p=0.008$) for the HRSD scores and $r=-0.872$ ($p=0.128$) for the STAI scores ($n=6$). There was no region amongst the other ROIs which had a significantly lower rCMRgluc. Out

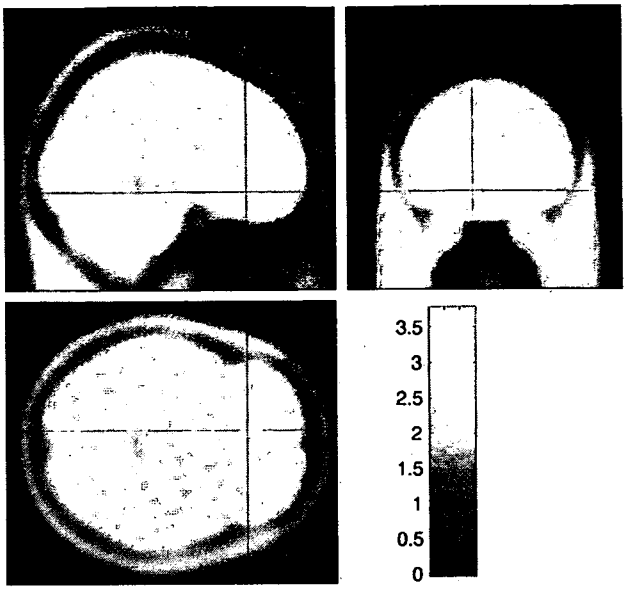

Fig. 1. Statistical parametric map illustrating a higher regional cerebral metabolic rate of glucose (uncorrected $p<0.005$) in the left subgenual anterior cingulate under resting condition in pancreatic cancer patients with the first depressive episode after cancer diagnosis compared with those without a depressive episode. The color bar indicates the *t* value.

with the ROIs, the region which did have a significantly lower rCMRgluc in the depressed patients was the cerebellum (uncorrected $p<0.001$, $t=3.23$, and size=30 voxels), of which the *xyz* coordinates of the MNI template were [20 −86 −32]. No region outside the ROIs had a significantly higher rCMRgluc.

4. Discussion

The present study showed a significantly higher rCMRgluc in the sACC in antidepressant-naive pancreatic cancer patients with a depressive episode after their diagnosis of cancer, before their cancer treatment.

To the best of our knowledge, no study has yet demonstrated rCMRgluc in depressed pancreatic cancer patients. A previous study demonstrated an inverse correlation between depressive mood measured by a self-rating scale and rCMRgluc of only the right caudate under resting condition in 8 cancer patients with lung, breast, colon, or prostate cancer before or after cancer treatment (Tashiro et al., 2001), which was inconsistent with the result of the present study. This inconsistency may be attributed to the differences in timing of the assessment, the use of an anti-cancer chemotherapeutic agent, and/or the characteristics of depression including some difference in the definition of "depression" between the two studies. On the other hand, in the previous study, a self-reported anxiety scale was positively correlated with

the metabolism of the anterior cingulate cortex, which is near the principal ROI in the present study. The STAI score to measure the anxiety was significantly different between the two groups in the present study. Anxiety could have contributed significantly to the findings in this group of generally mildly depressed patients.

Giving the reciprocal connections of the sACC with the orbital cortex, hypothalamus, amygdala, and accumbens, which are implicated in emotional behavior (Drevets, 2001), the result of the present study indicating the association of the sACC with a depressive episode in pancreatic cancer patients seems plausible. Many of the previous neuroimaging studies in major depression demonstrated lower glucose metabolism in the subgenual prefrontal cortex (Drevets, 2001). Although the definition of depression in the present study using a combination of major and minor depression was different from that of previous studies, the indication in the present study of a higher metabolism at the sACC was a possible inconsistency. Several reasons can be considered. Drevets et al. reported lower glucose metabolism in the region, which actually turned to be a higher glucose metabolism after adjustment for the smaller volume of the region in subjects with familial major depression (Drevets, 2001). Because the present study did not measure the volume of the regions, further study is needed to confirm the real activity after adjusting for the volume.

The other explanation for the inconsistent higher metabolism may be based on the findings in patients with PTSD indicating a higher metabolism in the regions (Shin et al., 1997) and in healthy non-depressed subjects during experimentally induced sadness in which blood flow increases in the regions were indicated (Damasio et al., 2000; Mayberg et al., 1999). Given the high comorbidity rate of depression in PTSD (Breslau et al., 2000; Kessler et al., 1995), a PTSD-like pathophysiology may be associated with depression after pancreatic cancer diagnosis. In fact, the total score of the IES-R was significantly higher in the depressed group compared with the non-depressed group. In addition, the PET scans for the investigation of pancreatic cancer may cause distressing recollections about the cancer-related traumatic event. Rumination may be related to the higher activity of sACC observed in the present study. This may be a specific finding in depression in pancreatic cancer patients.

Although the primary result of the present study indicated an increased rCMRgluc in the sACC of depressed subjects, the sub-analysis showed a paradoxical association between an increased rGMRgluc and a lower HDRS score. rCMRgluc in the sACC may not be linearly correlated with the severity of depression. A second study with a larger sample size is needed to clarify this point.

Taking the substrates secreted by pancreatic cancer into account, a different pathophysiology of depression in pancreatic cancer patients may exist, in addition to the psychological response. The fact that all of the depressive episodes had developed serially after the distressing event of disclosure of the cancer diagnosis indicates the possibility that psychological distress plays a role in developing the depressive episode. However, the possible effect of substances secreted by pancreatic cancer on the development of the depressive episode and increased metabolism in the sACC could not be excluded.

The present study has the following limitations: (1) the small number of depressed subjects; (2) the cross-sectional study design and the unclear causality of rCMRgluc; (3) patient use of opiates as a potential confounding factor; (4) the lack of a healthy control group; (5) the use of a clinical, rather than a pathological, diagnosis of pancreatic cancer; (6) the trend towards depressed patients being older than nondepressed patients; (7) the generally mild degree of depression in the depressed patients; and (8) the use of normalized rather than absolute rCMRgluc values.

The present study indicated a higher glucose metabolism in the sACC in pancreatic cancer patients with a depressive episode after their cancer diagnosis. However, a large sample size study with a longitudinal design is needed.

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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]-1H-pyrrole-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-aminophenoxy)ethane-N,N,N′,N′-tetraacetic acid-acetoxymethyl ester; LY294002, 2-(4-morpholinyl)-8-phenyl-1(4H)-benzopyran-4-one hydrochloride; WAY100635, [O-methyl-3H]-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(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) (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-

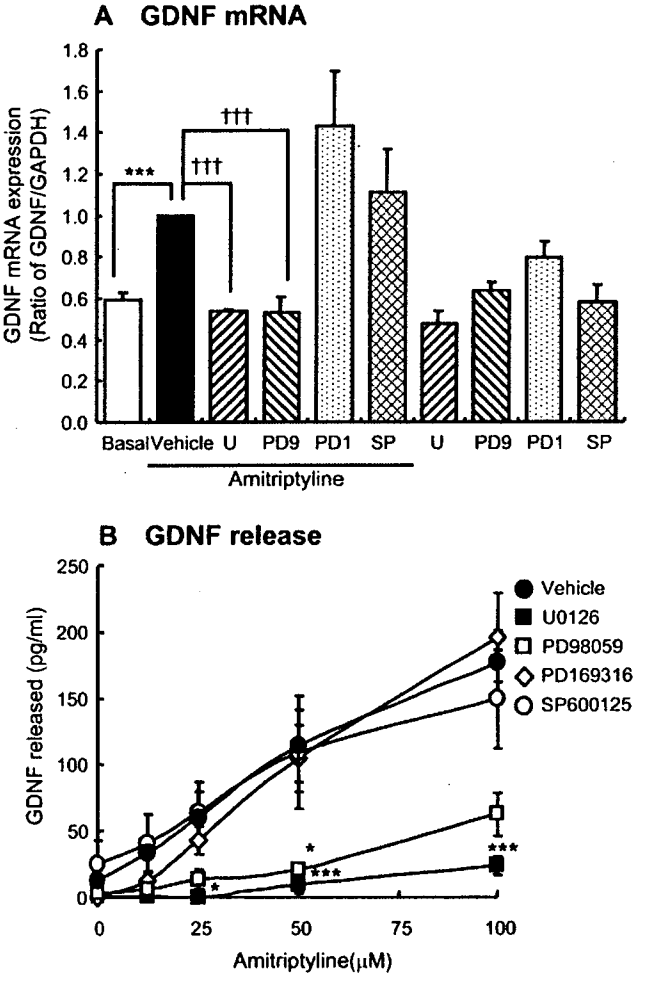

Fig. 1. Effects of U0126, PD98059, PD169316, and SP600125 on the amitriptyline-induced GDNF mRNA expression and GDNF release in C6 cells. A, effects of U0126, PD98059, PD169316, and SP600125 on the amitriptyline-induced GDNF mRNA expression. C6 cells were pretreated with 10 μM U0126 (U), 30 μM PD98059 (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 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).

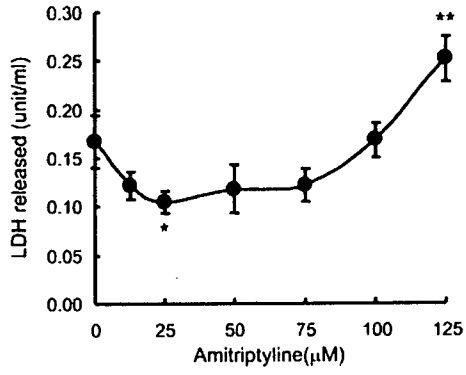

Fig. 2. Chemotoxicity of amitriptyline in C6 cells. C6 cells were treated with the indicated concentration of amitriptyline for 48 h. For the determination of the cytotoxicity of amitriptyline, we measured LDH in the conditioned medium. Values are expressed as mean ± S.E.M. of released LDH (units per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

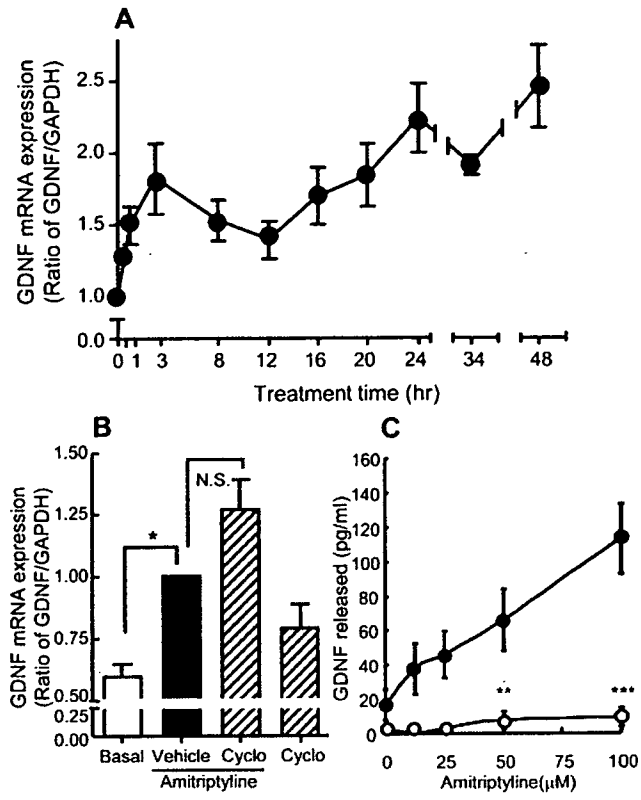

Fig. 3. Amitriptyline increased GDNF release through GDNF mRNA expression. A, C6 cells were treated with 25 μM amitriptyline for the indicated period. Values are shown as the ratio of GDNF mRNA versus GAPDH mRNA. Data are expressed as mean ± S.E.M. from three independent experiments. B, effects of cycloheximide 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; $n = 4$).

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

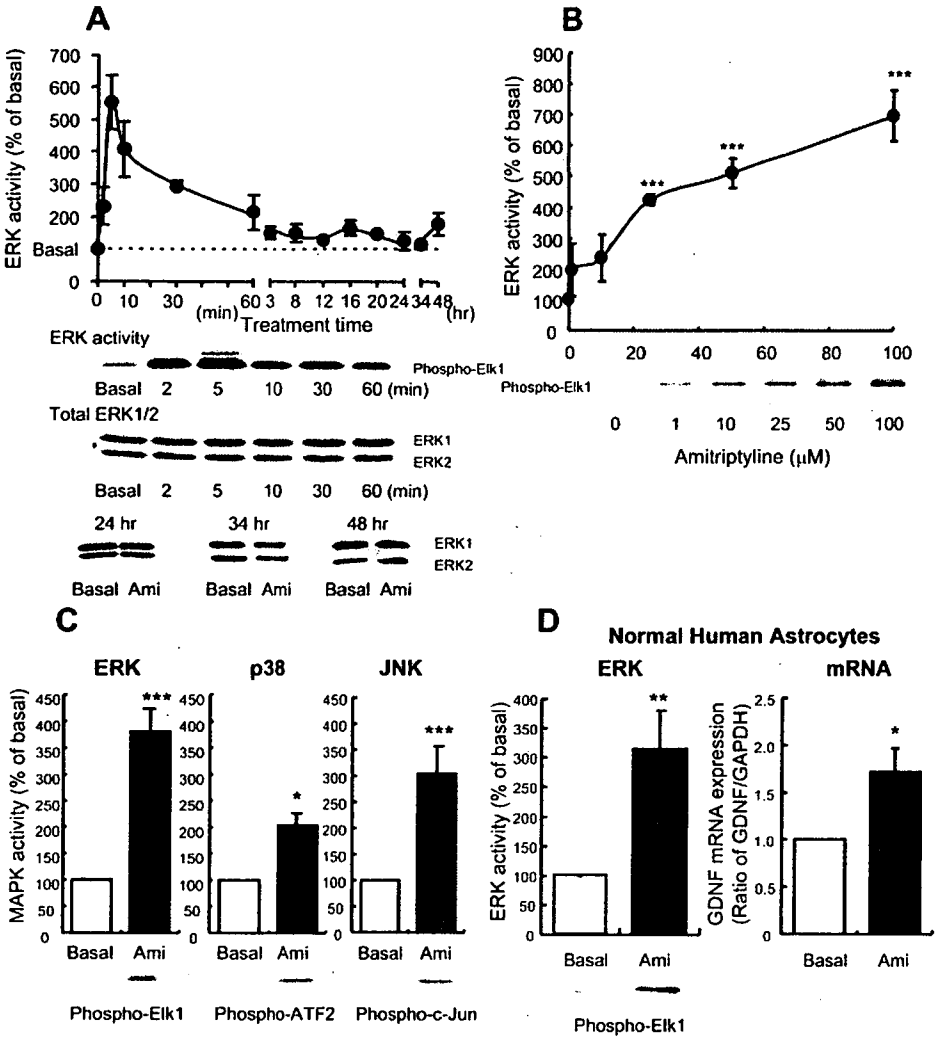

Fig. 4. Effects of amitriptyline on MAP kinase activation in C6 cells and normal human astrocytes. A, time course of the amitriptyline-induced ERK activation. C6 cells were treated with 25 μM amitriptyline for the indicated period, and ERK activity was measured. Data are expressed as mean ± S.E.M. from 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).

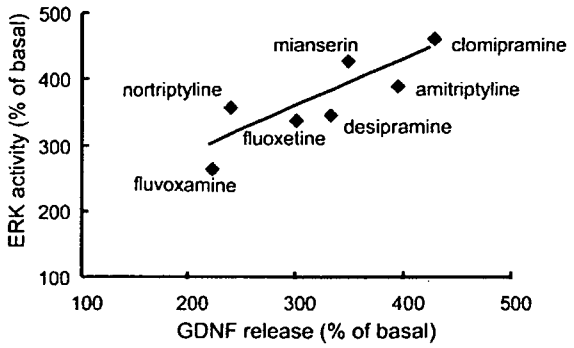

Fig. 5. Correlation between acute ERK activity and GDNF release induced by antidepressants. C6 cells were treated with 25 μM antidepressants for 5 min (ERK activity) or 48 h (GDNF release). The x-axis of the graph represents GDNF release, and the y-axis represents ERK activity. GDNF release and ERK activity 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).

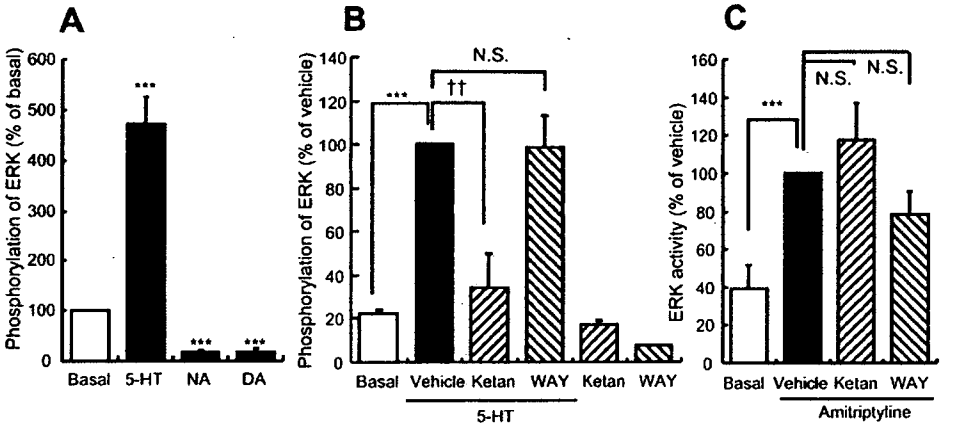

Fig. 6. 5-HT, but not NA or DA, increased ERK activation via 5-HT_{2A} receptors, but 5-HT receptor antagonist did not affect the amitriptyline-induced ERK activation in C6 cells. A, effects of 5-HT, NA, and DA on ERK activity. C6 cells were treated with 10 μM 5-HT, NA, or DA for 2 min, and phosphorylation 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-

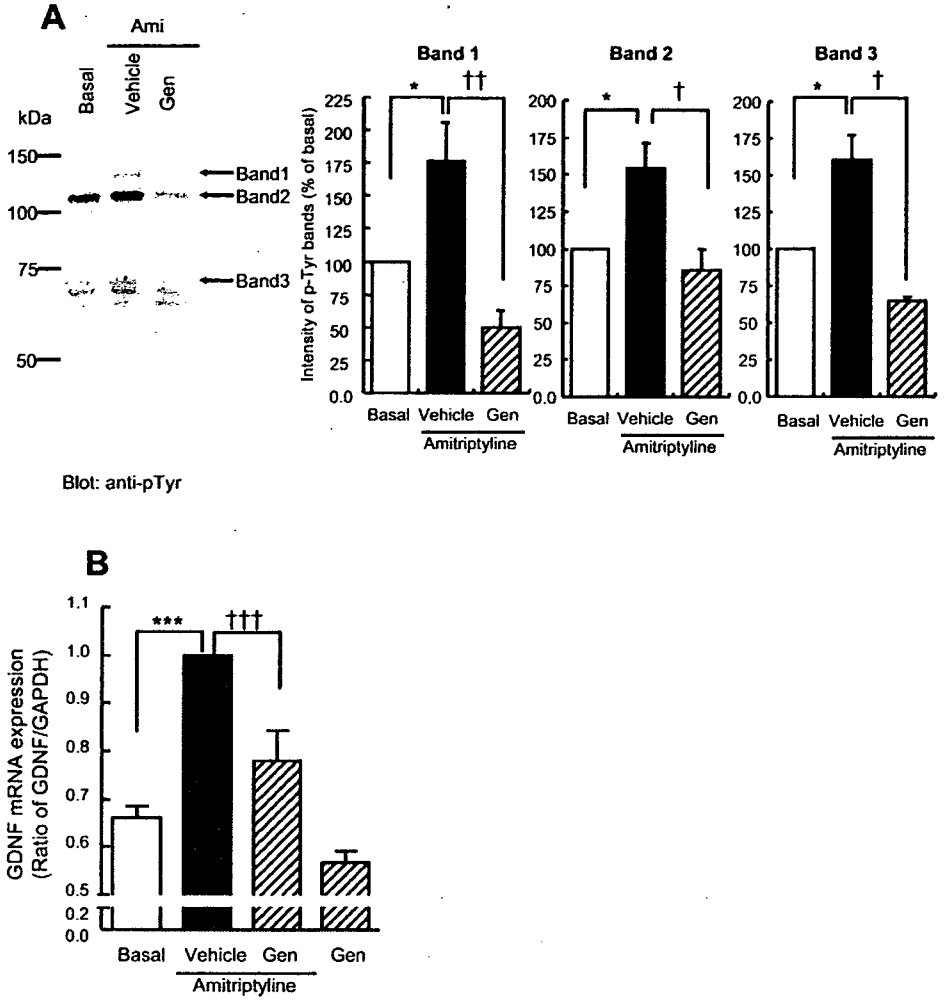

Fig. 7. Effects of genistein on the amitriptyline-induced phosphorylation level of tyrosine-containing proteins and GDNF mRNA expression in C6 cells. A, effect of genistein on the amitriptyline-induced phosphorylation level of tyrosine-containing proteins. C6 cells were treated with 100 μM genistein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

Antidepressants induce acute CREB phosphorylation and CRE-mediated gene expression in glial cells: a possible contribution to GDNF production

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ABSTRACT

Recently, the changes of neuronal and glial plasticity related gene expression following the increase of monoamine are suggested to be important for the therapeutic effect of antidepressants. We previously showed that antidepressants increased glial cell line-derived neurotrophic factor (GDNF) expression, which was dependent on acute activation of protein tyrosine kinase (PTK) and extracellular signal-regulated kinase (ERK) in rat C6 glioma cells (C6 cells) and normal human astrocytes (NHA). Transcription of many genes including GDNF is directed by the cAMP responsive element (CRE) and its cognate transcription factor CRE binding protein (CREB). In this study, we showed that amitriptyline, a tricyclic antidepressant, acutely increased phosphorylation of CREB, without altering the level of total CREB in C6 cells as well as in NHA. In contrast, acute amitriptyline treatment did not affect phosphorylation of CREB in SH-SY5Y cells, a human neuroblastoma cell line. Different classes of antidepressants as well as amitriptyline acutely increased phosphorylation of CREB, but haloperidol and diazepam did not. The amitriptyline-induced phosphorylation of CREB was completely blocked by U0126 [a mitogen-activated protein (MAP) kinase kinase 1 inhibitor] and genistein (a PTK inhibitor), but not by inhibitors of protein kinase A, p38 MAP kinase, or Ca^{2+}/calmodulin-dependent kinase. Amitriptyline treatment also increased the expression of luciferase reporter gene regulated by CRE elements. The amitriptyline-induced luciferase activity was completely inhibited by U0126 in the same as phosphorylation of CREB. These results suggest that antidepressants acutely increase CREB activity in PTK and ERK-dependent manners, which might contribute to gene expression including GDNF in glial cells.

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Abbreviations: BDNF, brain-derived neurotrophic factor; CaMK, Ca^{2+}/calmodulin-dependent kinase; CREB, cyclic AMP response element binding protein; C6 cells, rat C6 glioma cells; ERK, extracellular signal-regulated kinase; GDNF, glial cell line-derived neurotrophic factor; MAP, mitogen-activated protein; MEK, mitogen-activated protein kinase kinase; NA, noradrenaline; NHA, normal human astrocytes; PBS, phosphate-buffered saline; PKA, protein kinase A; PKC, protein kinase C; PTK, protein tyrosine kinase; Ser^{133}, Serine 133; 5-HT, serotonin; Tukey HSD, Tukey honestly significant differences

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1. Introduction

Antidepressants facilitate signaling of serotonin (5-HT) or noradrenaline (NA), either by inhibiting reuptake to presynaptic terminals, inhibiting catabolism, binding to 5-HT or NA receptors, or a combination of effects. All these events occur soon after drug administration; however, clinical antidepressant effects develop slowly in the weeks after continuous drug treatment (Nestler et al., 2002). Recent evidence indicates that changes in gene expression following an increase of monoamines are important for the therapeutic effects of antidepressant treatment. These changes of gene expression might be directed by different transcription factors, which after activation/phosphorylation by some intracellular signals, bind to specific DNA sequences located in the regulatory regions of target genes.

The best characterized among families of transcription factors is that including cyclic AMP response element (CRE)-binding protein (CREB). CREB is a 43 kDa protein and a member of the leucine zipper family of transcription factors, which regulates gene transcription by binding to CRE, a *cis*-acting enhancer element in the regulatory region of various genes. The function of CREB is regulated largely by its state of phosphorylation at Serine 133 (Ser^{133}), which results in CRE-dependent gene transcription (Lonze and Ginty, 2002). CREB is attractive to many researchers because it appears to be required for long-term memory (Silva et al., 1998). The list of putative CREB target genes now exceeds 100, and includes genes that control development, function, and plasticity of the nervous system (Mayr and Montminy, 2001). Among them, neurotrophic factors, such as brain derived neurotrophic factor (BDNF), is known to be regulated by antidepressants (Tardito et al., 2006).

We recently showed that several different classes of antidepressants increased GDNF production through acute activation of protein tyrosine kinase (PTK) and extracellular signal-regulated kinase (ERK) in rat C6 glioma cells (C6 cells) and normal human astrocytes (NHA) (Hisaoka et al., 2001, 2007). Consensus-binding sequences for CREB are identified in the promoter sequence of the GDNF gene (Baecker et al., 1999). Previous studies showed that CREB is associated with GDNF expression in rat cultured astrocytes and neurons (Koyama et al., 2004; Cen et al., 2006). Thus, we examined whether antidepressants activate CREB, and, if so, whether the effect of antidepressants on CREB is related to ERK and PTK in glial cells (C6 cells and NHA), compared to SH-SY5Y as a model of human neuron in this study.

2. Results

2.1. *Amitriptyline treatment acutely increased phosphorylation of CREB in C6 cells and NHA, but not in SH-SY5Y cells*

First, we examined the time course of the amitriptyline-induced phosphorylation of CREB. Because the amitriptyline-induced GDNF mRNA expression occurred as early as 1 h in C6 cells (Hisaoka et al., 2007), we examined not only the chronic but also the acute effects of amitriptyline on CREB phosphorylation. The phosphorylation of CREB induced by amitriptyline significantly increased after 5 min of treatment and reached a maximum at 10 min. This phosphorylation decreased to levels of around 1.5-fold after 1 h treatment and a little phosphorylation continued after 48 h (Fig. 1A). The amount of total CREB was not changed until after 48 h of amitriptyline treatment in C6 cells (Fig. 1B). Next, we examined the concentration-dependency of amitriptyline on CREB phosphorylation in C6 cells. The phosphorylation level of CREB by amitriptyline treatment (10 min) depended on the concentration of amitriptyline (Fig. 1C). Acute treatment (10 min) of amitriptyline also increased phosphorylation of CREB in NHA (Fig. 1D). Acute amitriptyline treatment did not affect the phosphorylation level of CREB in SH-SY5Y; however long-term of amitriptyline treatment (24 and 48 h) significantly decreased phosphorylation level of CREB (Fig. 1E). The amount of total CREB was not changed in SH-SY5Y cells until after 48 h (Fig. 1F).

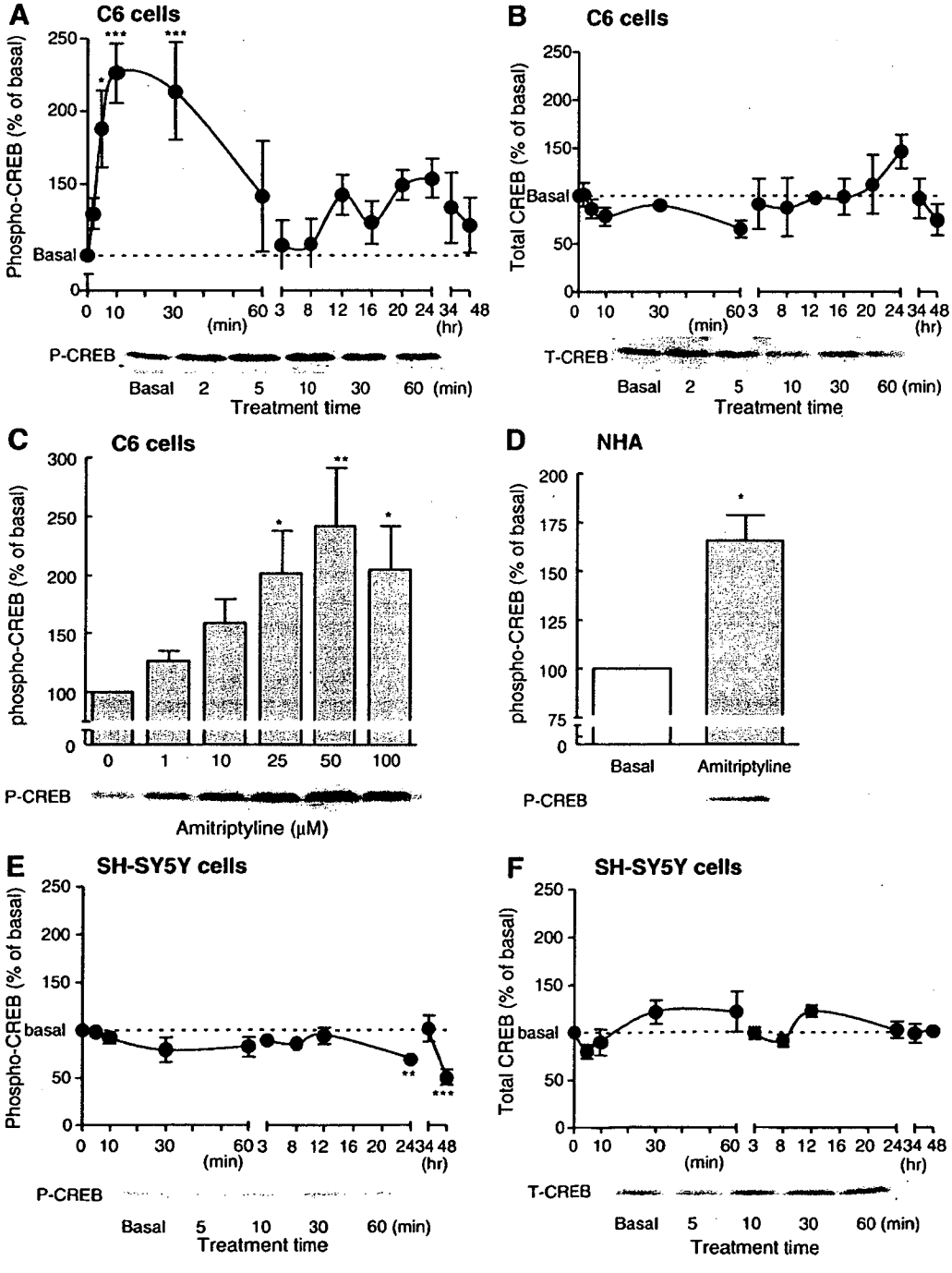
Fig. 1 – Effects of amitriptyline on phosphorylation of CREB in C6 cells, NHA and SH-SY5Y cells. A, Time course of phosphorylation of CREB in the amitriptyline-treated C6 cells. C6 cells were treated with 25 μM of amitriptyline, and phosphorylation of CREB was measured after the indicated period of treatment. Data are expressed as mean ± SEM [$F(13.36) = 4.933$, $p<0.001$]. *, $p<0.05$; ***, $p<0.001$ significantly different from the basal group (Tukey's HSD test). The phosphorylated CREB was detected by Western blotting. A representative result is shown. B, Time course of total CREB expression in the amitriptyline-treated C6 cells. C6 cells were treated with 25 μM of amitriptyline, and total CREB was measured after the indicated period of treatment. Data are expressed as mean ± SEM [$F(13.36) = 1.511$, N.S.]. The total CREB was detected by Western blotting. A representative result is shown. C, Concentration dependence of the amitriptyline-induced phosphorylation of CREB. C6 cells were treated with the indicated concentrations of amitriptyline for 10 min, and phosphorylation of CREB was detected. Data are expressed as mean ± SEM [$F(5.24) = 3.042$, $p<0.05$]. *, $p<0.05$; **, $p<0.01$ significantly different from the basal group (Dunnett's test). A representative result is shown. D, Effect of amitriptyline on phosphorylation of CREB in NHA. NHA were treated with 25 μM of amitriptyline for 10 min. Data are expressed as mean ± SEM from four independent experiments. *, $p<0.05$, significantly different from the basal group (Student's t-test). A representative result is shown. E, Time course of phosphorylation of CREB in the amitriptyline-treated SH-SY5Y cells. SH-SY5Y cells were treated with 25 μM of amitriptyline, and phosphorylation of CREB was measured after the indicated period of treatment. Data are expressed as mean ± SEM [$F(10.37) = 7.009$, $p<0.001$]. **, $p<0.01$; ***, $p<0.001$ significantly different from the basal group (Tukey's HSD test). A representative result is shown. F, Time course of total CREB expression in the amitriptyline-treated SH-SY5Y cells. SH-SY5Y cells were treated with 25 μM of amitriptyline, and total CREB was measured after the indicated period of treatment. Data are expressed as mean ± SEM [$F(10.38) = 2.409$, $p<0.05$]. There was no significant difference between basal group and amitriptyline treatment groups (Tukey's HSD test). The total CREB was detected by Western blotting. A representative result is shown.

2.2. *Antidepressants increased phosphorylation of CREB acutely, but haloperidol and diazepam did not*

To investigate whether the ability to increase phosphorylation of CREB is a common feature of antidepressants, we examined the effects of different classes of antidepressants and non-antidepressant psychotropic drugs, including amitriptyline (a tricyclic antidepressant), mianserin (a tetracyclic antidepressant), paroxetine (a selective 5-HT reuptake inhibitor), haloperidol (an antipsychotic-D2-dopamine receptor antagonist), and diazepam (a benzodiazepine), most of which are clinically used in Japan, on phosphorylation of CREB. Mianserin and paroxetine as well as amitriptyline significantly increased phosphorylation of CREB in C6 cells, but haloperidol and diazepam did not affect the phosphorylation level of CREB (Fig. 2).

2.3. *The amitriptyline-induced acute CREB phosphorylation was dependent on PTK and MEK/ERK pathway in C6 cells*

To clarify the intracellular mechanism by which amitriptyline induces acute CREB phosphorylation, we used various types of inhibitors of intracellular signal transduction. We examined the effects of U0126 [a mitogen-activated protein kinase kinase (MEK) 1 inhibitor], H-89 [a protein kinase A (PKA) inhibitor], PD169316 [a p38 mitogen-activated protein (MAP)



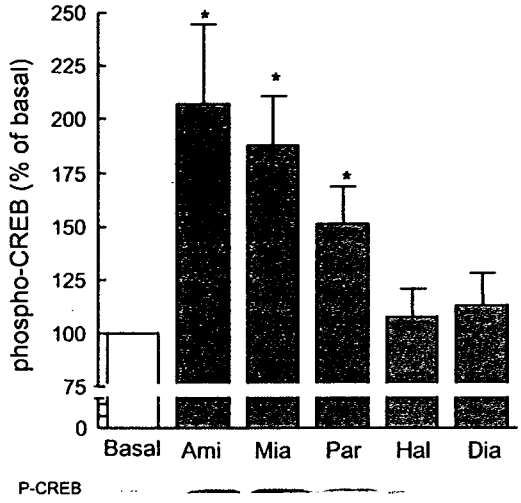


Fig. 2 – Antidepressants increased phosphorylation of CREB in C6 cells, but haloperidol and diazepam did not. C6 cells were treated with 25 μM of amitriptyline, mianserin, paroxetine, haloperidol, or diazepam for 10 min, and phosphorylation of CREB was detected by Western blotting. Data are expressed as mean ± SEM from six independent experiments. *, $p<0.05$, significantly different from the basal group (Student's t-test). A representative result is shown.

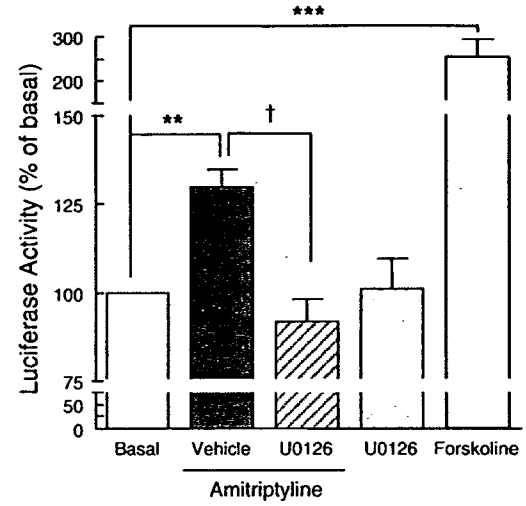


Fig. 3 – Effects of amitriptyline or forskoline on CRE-directed gene transcription in C6 cells. C6 cells were transiently transfected with pCRE-Luc plasmid. The cells were pretreated with 10 μM of U0126 for 30 min, and subsequently treated with 25 μM of amitriptyline or 1 μM of forskoline for 3 h. After drug treatment, the cells were collected, and luciferase activity was measured. Values are expressed as mean ± SEM of luciferase activity (% of basal) [$F(4.25)=60.09$, $p<0.001$]. **, $p<0.01$; ***, $p<0.001$, compared with the basal group, and †, $p<0.05$ compared with vehicle (amitriptyline only) group (Tukey's HSD test).

kinase inhibitor], KN93 [Ca^{2+}/calmodulin-dependent kinase (CaMK) inhibitor], genistein (a PTK inhibitor), and genistin (a negative analog of genistein). U0126 and genistein significantly inhibited the amitriptyline-induced phosphorylation of CREB, but H89, PD169316, KN93, and genistin did not have any effects (Table 1). U0126 and genistein alone did not have any significant effect on the basal level of CREB phosphorylation (94.5±10.5 and 129.8±22.2% of basal, respectively). Thus, it is apparent that PTK and ERK activations play an important role in phosphorylation of CREB after acute amitriptyline treatment. The amitriptyline-induced phosphorylation of CREB seems to be regulated by the same pathway as the antidepressant-induced GDNF production (Hisaoka et al., 2007).

Table 1 – Effects of inhibitors for intracellular signal transduction on the amitriptyline-induced acute CREB phosphorylation in C6 cells

Drugs	CREB phosphorylation (% of control)
Basal	56.7±6.5
Vehicle (Amitriptyline only)	100.0±0.0***
+H89 (PKA inhibitor)	102.2±10.8
+U0126 (MEK1 inhibitor)	53.4±11.4††
+PD169316 (p38 MAP kinase inhibitor)	111.1±17.4
+KN93 (CaMK inhibitor)	112.4±7.9
+Genistein (PTK inhibitor)	63.5±4.8†
+Genistin (negative analog of genistein)	111.8±7.8

C6 cells were pretreated with 10 μM of U0126 or 10 μM of PD169316 for 30 min; and 1 μM of H89, 1 μM of KN93, 100 μM of genistein or 100 μM of genistin for 1 h, and subsequently treated with 25 μM of amitriptyline for 10 min, and phosphorylation of CREB was detected by Western blotting. Data are expressed as mean ± SEM [$F(7.35)=12.09$ $p<0.001$]. ***, $p<0.001$ compared with the basal group, and †, $p<0.05$; ††, $p<0.01$ compared with the vehicle (amitriptyline only) group (Tukey's HSD test).

2.4. *Amitriptyline treatment increased CRE-dependent gene expression through MEK/ERK pathway in C6 cells*

Phosphorylation of CREB is an indicator of CREB activation, but the ultimate measure of CREB function is gene transcription. To investigate the effects of amitriptyline on CRE-directed gene transcription, we used the PathDetect *cis*-reporting system, in which the luciferase reporter gene activity reflects the CRE-mediated gene expression. C6 cells were transiently transfected with a pCRE-Luc plasmid, which contains CRE binding sites in front of the luciferase reporter gene, and then the transfected C6 cells were treated with amitriptyline or forskoline for 3 h. Amitriptyline or forskoline treatment significantly increased luciferase activity (Fig. 3). Furthermore, the amitriptyline-induced luciferase activity as well as the phosphorylation of CREB was completely inhibited by U0126 (Fig. 3). U0126 alone did not have any significant effect on the basal level of luciferase activity (Fig. 3).

3. Discussion

In the present study, we have shown that amitriptyline treatment acutely increased phosphorylation of CREB in C6 cells and NHA, but not in SH-SY5Y cells. The amitriptyline-

induced phosphorylation of CREB was specifically inhibited by inhibitors of PTK and MEK. Amitriptyline treatment also increased CRE-mediated gene expression, and it was completely inhibited by a MEK inhibitor. These results suggest that PTK- and ERK-dependent CREB activation by antidepressants might contribute to gene expression in glial cells.

Amitriptyline treatment acutely increased phosphorylation of CREB and a little increase of phosphorylation level continued after 48 h without altering the total amount of CREB until after 48 h amitriptyline treatment in C6 cells. These results suggest that sustained phosphorylation of CREB by prolonged amitriptyline treatment did not result from an increase of total amount of CREB. Different classes of antidepressants (paroxetine and mianserin) as well as amitriptyline acutely increased phosphorylation of CREB, but haloperidol or diazepam did not. Fluoxetine and imipramine acutely increased phosphorylation of CREB without altering CREB expression in jurket cells, which are human T cell lymphoblast-like cell lines (Koch et al., 2003). In this study, amitriptyline treatment increased CRE-mediated transcription in C6 cells, which were transiently transfected with reporter plasmid. The other group also demonstrated that desipramine treatment of HIT-T15 cells derived from a hamster insulinoma, transiently transfected with reporter plasmid, led to a slight increase (165.9±7.9% of basal) in CRE-mediated transcription (Schwaninger et al., 1995). These results suggest that several different types of antidepressants, but non-antidepressant drugs, may commonly increase phosphorylation of CREB and CREB activity. However, the acute amitriptyline treatment did not affect phosphorylation of CREB in SH-SY5Y cells, while long-term amitriptyline treatment decreased phosphorylation of CREB without altering the total level of CREB. The effect of antidepressants on CREB in SH-SY5Y cells appears opposite in direction to that seen in the glial cell lines. Thus, the effect of antidepressants on CREB seems to be dependent on different types of cells [glial cells (C6, NHA) or neuron-like cells (SH-SY5Y)], and different period of treatment time (acute or chronic). There is general agreement that antidepressants stimulate CREB function and affect neuroplasticity; however, some authors reported a decrease in CREB expression and/or function following antidepressant treatments (Tardito et al., 2006; Blendy, 2006; Carlezon et al., 2005). Although, it is not clear whether the regulation of CREB in the brain by antidepressants occurred in the glia or neurons, antidepressants might have differential effects on CREB, depending on the brain areas examined, cell types, and experimental paradigms.

CREB can be activated by PKA, CaMK, ERK, and p38 MAP kinase in the central nervous system (Lonze and Ginty, 2002), and it is conceivable that the antidepressants could interact with one or more of these enzymes to increase CREB phosphorylation. In this study, the amitriptyline-induced acute phosphorylation of CREB in C6 cells was completely inhibited by U0126 and genistein, but not by H89, KN93, or PD169316. Thus, the amitriptyline-induced acute phosphorylation of CREB seems to have occurred downstream of PTK and ERK activation. We recently showed that under the same experimental paradigm, GDNF production by antidepressants was dependent on monoamine-independent activation of PTK and ERK (Hisaoka et al., 2004, 2007) as well as phosphorylation of CREB. The amitriptyline-induced CRE-dependent transcription was inhibited by U0126 (Fig. 3) in agreement with phosphorylation of CREB. Consensus-binding sequences for the transcription factors, such as CREB and nuclear factor κB, among others, are identified in the promoter sequence of the GDNF gene (Baecker et al., 1999; Caumont et al., 2006). Several groups showed that CREB is associated with GDNF expression in rat cultured astrocytes and neurons (Koyama et al., 2004; Cen et al., 2006). These results suggest that although the precise mode of action of CREB in GDNF production is not yet known, the monoamine-independent PTK/ERK/CREB activation by antidepressants seem to play an important role on GDNF production in glial cells. The modulation of gene expression such as GDNF by antidepressant in glial cells might contribute to neuronal and glial plasticity, which seems to play an important role for therapeutic effects of antidepressants.

Although it has been proposed that the effects of antidepressant on CREB are mainly due to up-regulation of the cAMP-PKA cascade through monoamine receptors in neurons, little is known about the mechanism whereby antidepressants affect CREB in glial cells. Glia may be unique targets for novel strategies in the treatment of major depression, because glia synthesize and release many types neurotrophic/growth factors, such as GDNF, BDNF, nerve growth factor and basic fibroblast growth factor (Darlington, 2005). The expression of BDNF and GDNF, which supposed to be involved in the etiology of mood disorders (Nestler et al., 2002; Takebayashi et al, 2006), might be regulated by CREB in glial cells. Thus, clarifying the mechanism of PTK/ERK/CREB activation in glial cells might contribute to the elucidation of novel targets for antidepressants.

4. Experimental procedures

4.1. *Reagents*

Reagents were obtained from the following sources: amitriptyline, diazepam and haloperidol (Wako Pure Chemicals, Osaka, Japan); KN93, mianserin and paroxetine (Sigma-Aldrich, St. Louis, MO); H-89 (BIOMOL Research Laboratories, Plymouth Meeting, PA); genistein, genistin, PD169316 and U0126 (Calbiochem, San Diego, CA).

4.2. *Cell culture*

Cultures of C6 cells and NHA were described previously (Hisaoka et al., 2007). SH-SY5Y cells were grown in Dulbecco's modified Eagle's medium (Cambrex, Walkersville, MD) supplemented with 2 mM L-glutamine and 5% fetal bovine serum (JRH Biosciences, Lenexa, KS) in a 5% CO_2 humidified atmosphere. For drug treatment, 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 the drugs of interest.

4.3. *Western blotting*

Western blots were performed for the detection of phospho-CREB and total CREB using their respective antibodies (Cell Signaling, Beverly, MA). C6 cells, NHA, and SH-SY5Y cells were cultured at a density of $1.6\times10^5/cm^2$ on a 6-well plate with

3 mL of growth medium. After drug treatment, the cells were collected by using ice-cold phosphate-buffered saline (PBS) and solubilized in the sample buffer [100 mM Tris–HCl (pH 6.8), 20% glycerol, 4% SDS]. Total amounts of proteins in each sample were measured by BCA kit, and 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. The membranes were blocked with 5% (w/v) nonfat milk for 6 h at 4 °C and incubated with their 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).

4.4. *Transient transfection and luciferase assay*

PathDetect® CRE Cis-Reporting System (Stratagene, La Jolla, CA) was used to detect CREB mediated gene expression in C6 cells. C6 cells were cultured at a density of $0.4\times10^5/cm^2$ on a 6-well plate with 2 mL of growth medium. After 24 h incubation, transfection was performed using 0.5 μg of pCRE-Luc plasmid (encoding a luciferase gene driven by a promoter containing CRE binding sites) and 2.5 μL of Lipofectamine™2000 (Invitrogen, Carlsbad, CA) in 2 mL of Opti-MEM™. On transiently transfect cells, luciferase assays were performed 48 h following transfection using luciferase assay kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). Amitriptyline or forskolin was added 3 h before harvest, and U0126 was added 30 min before amitriptyline treatment. The cells were rinsed with PBS and incubated in 200 μL of cell lysis buffer for 15 min at room temperature. Aliquots containing equal amounts of protein were transferred to a white 96-well micro-plate, 100 μL of luciferase substrate-assay buffer was added, and the luminescence was measured in an ARVO MX/Light (PerkinElmer, Wellesley, MA).

4.5. *Data analysis*

We analyzed statistical tendencies by using SPSS software (SPSS, Chicago, IL). Results are expressed as mean±SEM. One-way ANOVA was used in most cases. Differences between the groups were analyzed by Tukey honest significant difference (HSD) or Dunnett's test. Differences between two groups were analyzed by Student's *t*-test. The significance level was set at $p<0.05$.

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