Introduction

Imipramine, a classic tricyclic antidepressant drug, had been known to inhibit reuptake of monoamines (1, 2). The generally accepted mechanism of action for antidepressants is so-called “monoamine hypothesis”, in which antidepressants restore the compromised monoaminergic neurotransmission. However, therapeutic responses with antidepressants could only be achieved after at least 2 – 3 weeks of treatment in spite of the fact that antidepressants alter synaptic monoamine levels within hours. Since the monoaminergic hypothesis cannot fully explain this delay of antidepressant effect, it has been suggested that the delay is required in order to produce neuroadaptive mechanisms that may enhance neuronal plasticity and resilience (3, 4). Besides, antidepressants are effective in less than 50% of patients (2), suggesting that additional biological aspects could provide potential therapeutic targets.

Based on clinical and animal studies it has been suggested that depression is associated with neuronal atrophy and neuronal loss, especially in the hippocampus and cerebral cortex (5). Therefore, neurotrophic agents such as neurotrophins are recognized as important new leads in the quest for a deeper understanding of depression and for the mechanisms of the antidepressants effect (6). In the neurotrophic hypothesis of depression, a deficiency in neurotrophic support may contribute to hippocampal pathology during the development of depression, and the reversal of this deficiency by antidepressant treatments may contribute to the resolution of depressive symptoms. Among various neurotrophins, most studies have focused on brain-derived neurotrophic factor (BDNF), one of the most prevalent neurotrophins in adult brain (7).

BDNF has been implicated in various stages in the CNS, including development, neural regeneration, synaptic transmission, synaptic plasticity, and neurogenesis (2, 8 – 14). Studies in humans have shown decreased

Full Paper

Imipramine Induces Brain-Derived Neurotrophic Factor mRNA Expression in Cultured Astrocytes

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Abstract. Depression is one of the most prevalent and livelihood-threatening forms of mental illnesses and the neural circuitry underlying depression remains incompletely understood. Recent studies suggest that the neuronal plasticity involved with brain-derived neurotrophic factor (BDNF) plays an important role in the recovery from depression. Some antidepressants are reported to induce BDNF expression in vivo; however, the mechanisms have been considered solely in neurons and not fully elucidated. In the present study, we evaluated the effects of imipramine, a classic tricyclic antidepressant drug, on BDNF expression in cultured rat brain astrocytes. Imipramine dose-dependently increased BDNF mRNA expression in astrocytes. The imipramine-induced BDNF increase was suppressed with inhibitors for protein kinase A (PKA) or MEK/ERK. Moreover, imipramine exposure activated transcription factor cAMP response element binding protein (CREB) in a dose-dependent manner. These results suggested that imipramine induced BDNF expression through CREB activation via PKA and/or ERK pathways. Imipramine treatment in depression might exert antidepressant action through BDNF production from astrocytes, and glial BDNF expression might be a target of developing novel antidepressants.

Keywords: astrocyte, antidepressant drug, brain-derived neurotrophic factor (BDNF), protein kinase A (PKA), cAMP response element binding protein (CREB)
plasma levels of BDNF in bipolar disorder, manic, and depressive patients (15, 16). Many preclinical and clinical studies provide direct evidence suggesting that modulation in expression of BDNF could be involved in behavioral phenomena associated with depression. The role of BDNF in depression has also been supported by the hypothesis that BDNF mediates the action of antidepressants (17, 18).

Along this line of reasoning, several studies have shown that BDNF may mediate the therapeutic action of antidepressants (19–22). There is plenty of evidence documenting that antidepressant treatments, including SSRIs and electroconvulsive shock, increase the expression of BDNF and its receptor tropomyosin-related kinase B (TrkB) in the hippocampus in animal models. These effects are dependent on chronic administration of antidepressant therapy, consistent with the time course of antidepressant treatments (23, 24). This suggests that the regulations of BDNF and TrkB are involved in development of depression and also in antidepressant treatments.

Antidepressants-induced BDNF expression has been investigated so far mostly in neurons. There are many studies about the mechanisms suggesting the involvement of extracellular signal-regulated protein kinase (ERK) activation, protein kinase A (PKA) activation through cAMP elevation, or calmodulin-dependent protein kinase activation by intracellular Ca2+ elevation, followed by phosphorylation of cAMP response element binding protein (CREB) (25). Recently, an antidepressant agent, fluoxetine, was reported to produce BDNF in astrocytes other than neurons (26). The mechanisms of antidepressant-induced BDNF expression in glial cells must be dissected in more detail. Although the relationship between depression and BDNF has been reported in recent years, the reports indicating effects of imipramine on BDNF appear to be fewer than other antidepressants.

In the present study, we examined whether BDNF expression increases in cultured rat astrocytes treated with imipramine. We found that the exposure to imipramine could increase BDNF mRNA expression and that CREB activation through the activation of PKA and/or ERK might be involved in this induction pathway.

Materials and Methods

Materials

Polyethyleneimine, DNase I (DN-25), trypsin, protease inhibitor cocktail, mouse anti-β-actin antibody, 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA), horseradish peroxidase–conjugated goat anti-rabbit IgG (whole molecule) antibody, and U0126 monoethanolate (MEK inhibitor) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Dulbecco’s modified Eagle medium (DMEM) and horse serum was obtained from Gibco BRL (Grand Island, NY, USA). Fetal bovine serum was obtained from Nichirei Biosciences Inc. (Tokyo). Imipramine hydrochloride, dl-propranolol hydrochloride, noradrenaline hydrogen tartrate monohydrate, dopamine hydrochloride, 5-hydroxytryptamine hydrochloride, and N′,2′-O-dibutryl adenosine 3′,5′-(cyclic)monophosphate (dbcAMP) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka). Phenoxybenzamine hydrochloride was obtained from Funakoshi (Tokyo). PKA inhibitor 14-22 amide, cell-permeable, myristoylated was purchased from Calbiochem (Tokyo). Horseradish peroxidase–conjugated goat anti-mouse IgG (H+L) antibody was purchased from Bio-Rad Laboratories, Inc. (Hercules, CA, USA). Anti-dual phosphor ERK1/2 antibody and anti-ERK1/2 antibody were purchased from Enzo Life Sciences, Inc. (Farmingdale, NY, USA). Anti-phospho-CREB (Ser133) antibody and anti-CREB antibody were purchased from Cell Signaling Technology Inc. (Danvers, MA, USA). Anti-BDNF antibody was purchased from Millipore (Billerica, MA, USA). BDNF Emax® ImmunoAssay System, an ELISA kit for BDNF, was purchased from Promega (Madison, WI, USA). [γ-32P]Adenosine 5′-triphosphate ([γ-32P]ATP), Isoblu® tetra-triethylammonium salt, was obtained from MP Biomedicals, Inc. (Santa Ana, CA, USA).

Preparation of astrocytes culture

This study was carried out in compliance with the Guideline for Animal Experimentation at Osaka Prefecture University, with an effort to minimize the number of animals used and their suffering. Astrocytes were prepared as described previously (27). In brief, hippocampi from 19- or 20-day-old embryos, which were taken out from deeply anesthetized pregnant Wistar rats, were cleared of meninges, cut into about 1-mm3 blocks, and treated with 0.25% trypsin in Ca2+, Mg2+-free phosphate-buffered saline containing 5.5 mM glucose for 20 min at 37°C with gentle shaking. An equal volume of horse serum supplemented with 0.1 mg/ml of DNase I was added to the medium to inactivate the trypsin. Then, the tissues were centrifuged at 350 × g for 5 min. The tissue sediments were triturated through a pipette with DMEM containing 10% fetal bovine serum, 100 μg/ml streptomycin, and 50 unit/ml penicillin. After filtering cell suspensions through a lens-cleaning paper (Fujifilm Co., Tokyo), the cells were plated on polyethyleneimine-coated 100-mm-diameter plastic dishes (Iwaki, Asahi Glass Co., Tokyo) at a density of 0.8 – 1.3 × 105 cells/cm². Cultures were maintained in a humidified atmosphere of 5% CO2 and 95% air at 37°C, with the medium being changed every 3 days. After one week, astrocytes
were replated to remove neurons. On days 12 – 14, they were replated onto 96-well plates (Sumitomo, Tokyo), 12-well plates (Iwaki), 24-well plates (Iwaki), or 60-mm-diameter plastic dishes (Iwaki) using an ordinary trypsin-treatment technique at a density of $4 \times 10^5$ cells/ml and stabilized for 1 day, and then we used them for the experiments.

More than 90% of the cells were immunoreactively positive to an astrocyte marker, glial fibrillary acidic protein; using the antibody (Sigma) and FITC-conjugated anti-rabbit IgG antibody. Less than 10% of the cells were positive to a microglial marker, lectin from Bandeiraea simplicifolia bs-1 isolecitin b4 (peroxidase-labeled, Sigma).

Reverse transcription–polymerase chain reaction (RT-PCR)

Cultured astrocytes were washed with PBS, followed by extraction of mRNA using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and subsequent synthesis of complementary DNA (cDNA) with oligo dT primers, mixture of dNTP (deoxyribonucleotide triphosphate), RNase inhibitor, Buffer RT, and Omniscript Reverse Transcriptase (Omniscript Reverse Transcription Kit; Qiagen). The reverse transcriptase reaction was run at 37°C for 60 min, followed by inactivation of the enzyme at 94°C for 5 min, and an aliquot of synthesized cDNA was directly used for PCR.

Realtime PCR was performed in buffer containing SYBR® Green Realtime PCR Master Mix (Toyobo) was directly used for PCR. Extension at 60°C for 30 s. The results were analyzed at 95°C for 15 s, annealing at 60°C for 15 s, and extension at 60°C for 30 s. The results were analyzed using Realtime PCR System (StepOne™; Applied Biosystems, Tokyo).

Specific primers used were as follows: BDNF, sense 5′-CAGGGGCATAGACAAAG-3′; antisense 5′-CT TCCCTTTTTACTGTC-3′; GAPDH, sense 5′-TTGT CAGCAATGCATCCTGC-3′; antisense 5′-GGATGCA GGGATGATTCT-3′.

It is reported that rat BDNF mRNA has multiple different transcripts, which differ in the 5′-promoter regions and that astrocytic BDNF is translated by exon-III– and exon-IV–containing transcripts (28, 29). The primers for BDNF mRNA used in the present study could not distinguish these transcripts.

Cell viability

To evaluate cell viability, we measured total mitochondrial activity with the so-called MTT assay [MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium bromide; Dojindo, Kumamoto]. In brief, after the cells were stimulated, the medium was changed with a fresh one and one-tenth volume of 5 mg/ml MTT solution was added. The cells were incubated for 1 h at 37°C and the formazan generated by total mitochondrial activity was dissolved in DMSO, and then the color development was measured at 585 nm with a microplate reader (ARVO 1420 Multilabel counter; Wallac, Turuk, Finland). When we observed cell morphology under a phase-contrast microscope, the remaining cell number is almost consistent with the results of the MTT assay.

Measurement of reactive oxygen species (ROS)

Intracellular ROS levels were evaluated using a cell-permeable fluorescent dye, H$_2$DCF-DA. The cells were incubated with 5 μM H$_2$DCF-DA in serum-free medium for 30 min at 37°C, and then the medium was changed to Heps-Krebs Ringer buffer containing Ca$^{2+}$ and glucose with or without imipramine. The level of intracellular production of ROS was evaluated by fluorescent intensity of dichlorofluorescein (DCF) by measurement with a microplate reader (ARVO 1420 Multilabel counter).

Western blotting

Cultured astrocytes were homogenized in 20 mM Tris- HCl (pH 7.5) buffer containing 1 mM EDTA and protease inhibitor cocktail (Sigma P8340). In the case of detection of phosphorylated protein, the cells were homogenized in 20 mM Tris-HCl (pH 7.5) buffer containing 1 mM EDTA, 1 mM EGTA, 10 mM sodium fluoride, 10 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, and protease inhibitor cocktail. Each homogenate was added at a volume ratio of 4:1 to 50 mM Tris-HCl buffer (pH 6.8) containing 50% glycerol, 10% sodium dodecyl sulfate, 0.05% bromophenol blue, and 25% 2-mercaptoethanol, followed by mixing and boiling at 100°C for 5 min. Each aliquot of 20 μg proteins was loaded on a 10% polyacrylamide gel for electrophoresis at a constant voltage of 120 V for 2 h at room temperature and subsequent blotting to a polyvinylidene fluoride membrane previously treated with 100% methanol. After blocking by 5% skimmed milk dissolved in 20 mM Tris-HCl buffer (pH 7.5) containing 137 mM NaCl and 0.05% Tween 20, the membrane was reacted with antibodies against phosphorylated ERK1/2 (p-ERK1/2), ERK1/2, phosphorylated CREB (p-CREB), CREB, BDNF, or β-actin followed by a reaction with anti-rabbit or anti-mouse IgG antibodies conjugated with peroxidase. Proteins reactive with those antibodies were detected with the aid of ECL detection reagents and analyzed with lumino-image-analyzer.
(LAS-4000; Fujifilm). The graphs showed the objective proteins/β-actin ratio of the density of detection bands. Protein concentrations were determined by the method of Bradford using CBB color solution (Nacalai Tesque, Kyoto), according to the manufacturer’s protocol, with bovine serum albumin (BSA) as the standard.

**Nuclear extracts**

Nuclear extracts were prepared according to the procedures described by Schreiber et al. (30) with minor modifications (31). In brief, cultured astrocytes were homogenized in 10 mM HEPES-NaOH buffer (pH 7.9) containing 10 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM dithiothreitol (DTT), 10 mM each of phosphatase inhibitors (sodium fluoride and sodium β-glycerophosphate), and protease inhibitor cocktail, using a Dounce homogenizer with a tight-type pestle. Following the addition of 10% Nonidet P-40 to make a final concentration of 0.6% and incubation for 5 min on ice, the homogenates were centrifuged at 20,000 × g for 5 min. Pellets were suspended in 50 mM Tris-HCl buffer (pH 7.5) containing 10% glycerol, 400 mM KCl, 1 mM EDTA, 1 mM EGTA, 5 mM DTT, and the aforementioned phosphatase inhibitors and protease inhibitor cocktail. Protein concentrations were determined by the method of Bradford using CBB color solution (Nacalai Tesque), according to the manufacturer’s protocol, with BSA as the standard. Nuclear extracts were stored at −80°C until use. Buffers and any solutions used in this study were all sterilized by autoclave or filtration before each use.

**Gel retardation electrophoresis**

Assays were carried out using one of the double stranded oligonucleotides with a base length of 27-mer containing consensus core sequences for CREB as probe for detection of the corresponding DNA binding activity. Nucleotide sequences of the probe were designed with a manufacturer’s kit: Gel Shift Assay Systems (Promega) and shown below with the respective consensus elements in bold letters: CREB, 5′-AGA GAT TGC ACG TCA GAG AGC TAG-3′; 3′-TCT CTA ACG GAC TGC AGT CTC TCG ATC-5′.

These oligonucleotides were labeled with [γ-32P]ATP. The labeling reaction was carried out using T4 polynucleotide kinase in 70 mM Tris-HCl buffer (pH 7.6) containing 10 mM MgCl2 and 5 mM DTT at 37°C for 10 min, followed by purification with gel filtration chromatography on a spin column.

An aliquot of nuclear extracts was incubated at a fixed amount of 5 μg protein in 10 μl 10 mM Tris-HCl buffer (pH 7.5) containing 4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT, 50 mM NaCl, 0.5 μg poly(dI-dC)-poly(dI-dC), and 1 μl labeled probe for 30 min at room temperature (20°C – 25°C). Bound and free probes were separated by electrophoresis on a 4% polyacrylamide gel in TBE buffer (pH 8.3) at a constant voltage of 350 V for 15 min at room temperature. Gels were fixed, followed by exposure to an imaging plate (Fujifilm) for different periods to obtain autoradiograms most adequate for quantitative densitometry. Densitometric analysis for quantification of these autoradiograms was carried out with the aid of the fluoro-image analyzer FLA-7000 (Fujifilm). In the absence of nuclear extracts, radioactivity was only found at the bottom on the gel. Radioactive bands were additionally detected with slow mobility on the gel in the sample of CREB specific DNA binding activity. Competition experiments with non-labeled probes clearly demonstrated the validity of determination of CREB-specific DNA-binding activity under the conditions employed (data not shown).

**Data analyses**

For statistical analysis of the data, one-way ANOVA followed by Tukey-Kramer multiple comparison procedure or Student’s t-test was used. Differences between treatments were considered statistically significant when P < 0.05.

**Results**

**Expression of BDNF mRNA increased in cultured astrocytes after imipramine exposure**

To examine the expression of BDNF in astrocytes, we used cultured astrocytes prepared from the cortex of rat embryo brains. Cultured astrocytes were stimulated with various concentrations of imipramine for 6 h, and then the expression level of BDNF mRNA was analyzed by realtime RT-PCR. The level of BDNF was increased by imipramine stimulation in a dose-dependent manner, becoming 3-fold of the control at 30 μM (Fig. 1A). Cell viability assessed by MTT assay after 24 h imipramine exposure was not affected at all up to 30 μM, but 100 μM imipramine decreased the cell viability to about a half (Fig. 1B). We examined whether imipramine induced BDNF protein by western blotting. However, immunopositive band for BDNF could not be detected. We also examined BDNF protein levels in the medium by ELISA. We could detect the tendency of released BDNF levels to increase when the cells were incubated with imipramine for 24 h (control: 0.73 ± 0.22, 30 μM imipramine: 1.08 ± 0.15 pg/ml), but not significantly.

It has been reported that oxidative stress increases BDNF expression in the brain (32). We assessed the effect of imipramine on intracellular ROS production in...
cultured astrocytes. The cells loaded with H₂DCF were treated with 30 μM imipramine; however, DCF-derived fluorescence did not change significantly until 60 min after imipramine treatment (data not shown).

Involvement of PKA in imipramine-induced BDNF expression

Activation of PKA via increase of intracellular cAMP is known to be involved in BDNF production in various cells (25). We examined the effect of dbcAMP, a cell-permeable cAMP analog, on BDNF expression in cultured astrocytes. The cells were stimulated with various concentrations of dbcAMP for 6 h, and then the expression level of BDNF mRNA was analyzed. The BDNF expression increased dose-dependently; significant increases were observed at more than 100 μM dbcAMP (Fig. 2A).

After the cells were preincubated with 5 μM of the PKA inhibitor 14-22 amide for 20 min, they were then stimulated with 100 μM dbcAMP or 30 μM imipramine for 6 h and the expression of BDNF was analyzed. Enhanced expression of BDNF mRNA induced by dbcAMP stimulation was completely blocked by the PKA inhibitor to the control level (Fig. 2B). Furthermore, imipramine-enhanced expression of BDNF mRNA was significantly suppressed by the PKA inhibitor (Fig. 2C).
Increased BDNF Expression by Imipramine

Involvement of ERK in imipramine-induced BDNF expression

To assess whether ERK activation is involved in the pathway of imipramine-induced BDNF expression, we examined the expression of phosphorylated ERK in imipramine-treated cells. The cells were stimulated with various concentrations of imipramine for 1 h, and then the expression of phosphorylated ERK protein was detected by western blotting. Imipramine increased the expression of both p-ERK1 and p-ERK2 in a dose-dependent manner (Fig. 3A, graphs). We also examined the expression of total ERK1/2 in imipramine-treated cells by western blotting. Imipramine stimulation for 1 h did not significantly affect the expression levels of total ERK1/2 (data not shown). We examined the effect of U0126, a MEK inhibitor, on BDNF expression in cultured astrocytes. After the preincubation with 5 μM U0126 for 20 min, the cells were stimulated by 30 μM imipramine for 6 h, and then the expression of BDNF was analyzed. Enhanced expression of BDNF induced by imipramine stimulation was significantly suppressed by U0126 preincubation (Fig. 3B).

The effect of imipramine on CREB activation

To dissect in more detail the mechanisms of imipramine-induced BDNF expression, we assessed imipramine-induced CREB activation. We quantified the activation of CREB by imipramine stimulation using a gel shift assay. Cultured astrocytes were stimulated with various concentrations of imipramine for 1 h and the nuclear extracts were prepared from each cell. Samples were incubated with the radiolabeled probe for CREB, followed by gel retardation electrophoresis. Imipramine markedly increased the CREB-specific DNA–binding activity in a dose-dependent manner, becoming about 2-fold at 30 μM (Fig. 4: A, C). In order to confirm CREB specificity further, we also conducted supershift assay using an anti-CREB antibody. Nuclear extracts prepared from the cells without imipramine were preincubated with anti-CREB antibody for 1 h at 4°C, and then incubated in buffer with the radiolabeled probe followed by gel retardation electrophoresis. Radioactive bands detected with slow mobility on the gel were shifted upward by preincubation with anti-CREB antibody (Fig. 4B).

We examined the expression of total and p-CREB in imipramine-treated cells. The cells were stimulated with or without 30 μM imipramine for 1 h, and then the expression of phosphorylated CREB protein was detected by western blotting. Imipramine increased the expression of p-CREB (Fig. 5: A, B). We also examined the expression of total CREB in imipramine-treated cells by western blotting. The expression levels of total CREB showed an upward tendency in response to imipramine stimulation.

Fig. 3. Inhibition of imipramine-induced BDNF expression by MEK inhibitor. A) Cultured astrocytes were stimulated with various concentrations of imipramine for 1 h, and then the expression of phosphorylated ERK1/2 proteins was detected by western blotting. The results are shown in the photograph and the graphs. Data are reported as the mean ± S.D. of five samples from different cell preparations. *P < 0.05, **P < 0.01, significantly different from the control. B) Cultured astrocytes were pretreated with 5 μM U0126 for 20 min and then stimulated by 30 μM imipramine for 6 h. The expression of BDNF mRNA was detected by realtime RT-PCR. Data are reported as the mean ± S.D. of four samples from different cell preparations. *P < 0.05, significantly different from imipramine.
for 1 h, but not significantly (Fig. 5: A, C). We examined the effects of PKA inhibitor and/or U0126, a MEK inhibitor, on total and p-CREB expressions in cultured astrocytes. After the preincubation with 5 μM PKA inhibitor and/or U0126 for 20 min, the cells were stimulated by 30 μM imipramine for 1 h, and then the expressions of total and p-CREB were analyzed. Both inhibitors did not significantly suppress the increased expression of p-CREB by imipramine, although U0126 tended to reduce the increase (data not shown).

Expression of BDNF mRNA after monoamines exposure

In the CNS, imipramine is pharmacologically known to inhibit monoamines reuptake. We examined the effect
of monoamines; serotonin, dopamine, and noradrenalin, on the expression of BDNF in cultured astrocytes. The cells were stimulated with various concentrations of monoamines for 6 h, and then the expression of BDNF was analyzed. Although serotonin and dopamine (up to 10 μM) did not affect BDNF mRNA expression, dopamine (100 μM) and noradrenalin (> 10 μM) increased the expression of BDNF (Fig. 6: A – C).

The cells were stimulated by 10 μM noradrenalin or 30 μM imipramine for 6 h together with or without 10 μM phenoxybenzamine (α-receptor antagonist) plus 10 μM propranolol (β-receptor antagonist), and then the expression of BDNF was analyzed. Enhanced expression of BDNF mRNA induced by noradrenalin stimulation was completely blocked by α- plus β-antagonists to the control level (Fig. 6D). On the other hand, enhanced expression of BDNF mRNA induced by imipramine stimulation was not affected by α- plus β-antagonists (Fig. 6E).

Discussion

In this study, we demonstrated that BDNF mRNA expression was enhanced 3-fold by imipramine treatment in cultured rat cortex astrocytes. The imipramine-enhanced BDNF expression was inhibited by a PKA inhibitor and a MEK inhibitor. Furthermore, imipramine treatment increased CREB activation to about 2-fold and it also increased ERK phosphorylation. It is suggested that imipramine increased BDNF expression by CREB activation and that PKA and/or ERK activation are also involved in the induction.

The first antidepressant drugs were discovered almost half a century ago and were soon found to inhibit metabolism or reuptake of monoamines, particularly serotonin and noradrenalin (2). These findings laid a foundation for the monoamine hypothesis of depression, and depression was typically considered equivalent to reduced serotonin levels in the brain. Several findings have, however, been difficult to explain solely based on the monoamine hypothesis. Thereafter, the focus of research about antidepressant action has been changed from the immediate effects to the slowly developing process of antidepressants during the past decade (2). The long-term antidepressant treatment produces molecular effects that resemble those induced by neuronal plasticity and learning. In particular, neurotrophins and CREB have received

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**Fig. 6.** Expression of BDNF mRNA in monoamines-treated cultured astrocytes. Cultured astrocytes were stimulated by various concentrations of serotonin (A), dopamine (B), or noradrenalin (C) for 6 h. The expressions of mRNAs of BDNF were detected by the realtime RT-PCR procedure. Cultured astrocytes were stimulated by 10 μM noradrenalin (D) or 30 μM imipramine (E) for 6 h in the presence of 10 μM phenoxybenzamine plus 10 μM propranolol. The expression of BDNF mRNA was detected by realtime RT-PCR. Data are reported as the mean ± S.D. of three or four samples from different cell preparations. **P < 0.01, significantly different from the control (B, C) or noradrenalin (D). n.s., not significant.
In our present study, dbcAMP increased the expression of CREB (42), induce BDNF expression via PKA activation. forskolin (40), isoproterenol (41), vasoactive intestinal peptide, and pituitary adenylate cyclase–activating peptide, might be a novel mechanism of imipramine-mediated neuroprotection. In contrast to the present study, Allaman et al. (26) reported that imipramine did not increase the expression of BDNF mRNA under the serum-free conditions in mouse astrocytes. The reason for this discrepancy is unknown; however, it might be due to the difference of animal and stimulation conditions.

About the mechanisms of imipramine-induced BDNF expression, several lines of evidence suggest that the cAMP signaling system is involved in the action of antidepressants (2, 25, 33). Increased cAMP signaling phosphorylates and activates the transcription factor CREB, which then stimulates the transcription of cAMP-responsive genes; including BDNF (33). It is reported that imipramine treatment increased CREB protein levels in the brain (39). In the present study, we demonstrated that imipramine increased the binding activity of CREB in the nuclear fraction from cultured astrocytes using the gel retardation assay (Fig. 4). These results strongly suggest that CREB activation is involved in imipramine-induced BDNF mRNA expression in astrocytes. The result of the supershift assay using an anti-CREB antibody in the gel retardation assay showed that imipramine-induced the CREB-CRE DNA complex contained CREB protein (Fig. 4B). CREB activity is regulated by induction of CREB proteins and by their phosphorylation (33). The expression of p-CREB increased by imipramine stimulation for 1 h, and the expression of total CREB showed an upward tendency but not significantly (Fig. 5). It is likely that imipramine might activate CREB both by phosphorylation and by induction of CREB proteins.

Moreover, diverse stimuli activate CREB by triggering different signal transduction pathways: the cAMP–PKA pathway, the Ras–MAPK pathway, and the Ca^{2+}/calmodulin-dependent protein kinase pathway, depending on the strength and type of stimuli. The involvements of PKA and ERK activation are well known (25). It is reported also in astrocytes that agents increasing cAMP, such as forskolin (40), isoproterenol (41), vasoactive intestinal peptide, and pituitary adenylate cyclase–activating peptide (42), induce BDNF expression via PKA activation. In our present study, dbcAMP increased the expression of BDNF mRNA and pretreatment with an inhibitor against PKA or MEK/ERK inhibited the imipramine-enhanced BDNF mRNA expression (Figs. 2, 3). Therefore, both PKA and ERK activation are likely to be involved in imipramine-induced BDNF expression in cultured astrocytes. On the other hand, imipramine stimulation for 1 h increased the expression of p-CREB protein and pretreatment of PKA inhibitor and/or ERK inhibitor did not significantly suppress the increase. Therefore, it is possible that a transcription factor other than CREB is also involved in imipramine-induced BDNF expression through PKA and/or ERK activation.

Concerning the involvement and relationship of PKA and ERK pathways in imipramine-induced BDNF mRNA expression, it is known that imipramine treatment increases PKA activation in rat brain (39). It is also reported that BDNF binds to its receptor TrkB and activates the downstream signal to ERK (25). Therefore, it is likely that imipramine might produce BDNF through PKA activation and then the produced BDNF might augment further BDNF production through ERK activation. Further investigation is needed to elucidate the mechanisms in more detail.

Pharmacological effect of imipramine is considered as a reuptake inhibitor of monoamines. Thus, we assessed the direct effects of monoamines on BDNF expression in cultured astrocytes. Consistent with previous research in astrocytes (43), noradrenaline increased BDNF mRNA expression (Fig. 6C). Dopamine also increased BDNF expression at the higher concentration of 100 μM (Fig. 6B); in this case, a higher concentration of dopamine might affect α- and/or β-receptors. It is also reported that BDNF expression is related to oxidative stress and inflammation (44, 45) and that high dose of dopamine produces ROS and affects adrenergic receptors (46, 47). Therefore, dopamine might contribute to the expression of BDNF irrespective of specific dopamine receptors. On the other hand, addition of α- and β-adrenergic receptor antagonists did not suppress enhanced expression of BDNF by imipramine treatment (Fig. 6E). These results suggest that imipramine-induced BDNF expression is not mediated via specific adrenergic receptors.

We could not exclude the possibilities that the mechanisms other than cAMP–PKA and ERK are involved in imipramine-induced BDNF expression via CREB activation. It has been reported that intracellular Ca^{2+} elevation increases BDNF mRNA expression in astrocytes (28). Long-term treatment of imipramine enhances serotonin-mediated intracellular Ca^{2+} elevation (48). It is also reported that imipramine have affinity to sigma-1 receptor and might enhance Ca^{2+} release from endoplasmic reticulum via IP_1 receptor (49). Further investigations are needed to elucidate the target molecule of imipramine.
In summary, imipramine increased BDNF expression through PKA and/or ERK activation in cultured astrocytes. CREB activation could be involved in the increase. BDNF production from astrocytes might be an important target in the development of antidepressants.

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