Chronic Antidepressants Potentiate via Sigma-1 Receptors the Brain-derived Neurotrophic Factor-induced Signaling for Glutamate Release**

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Up-regulation of BDNF (brain-derived neurotrophic factor) has been suggested to contribute to the action of antidepressants. However, it is unclear whether chronic treatment with antidepressants may influence acute BDNF signaling in central nervous system neurons. Because BDNF has been shown by us to reinforce excitatory glutamatergic transmission in cultured cortical neurons via the PLC-γ/IP3/Ca2+ pathway (Numakawa, T., Yamagishi, S., Adachi, N., Matsumoto, T., Yokomaku, D., Yamada, M., and Hatanaka, H. (2002) J. Biol. Chem. 277, 6520–6529), we examined in this study the possible effects of pretreatment with antidepressants on the BDNF signaling through the PLC-γ/IP3/Ca2+ pathway. Furthermore, because the PLC-γ/IP3/Ca2+ pathway is regulated by sigma-1 receptors (Hayashi, T., and Su, T. P. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 491–496), we examined whether the BDNF signaling is modulated by sigma-1 receptors (Sig-1R). We found that the BDNF-stimulated PLC-γ activation and the ensuing increase in intracellular Ca2+ ([Ca2+]i) were potentiated by pretreatment with imipramine or fluvoxamine, so was the BDNF-induced glutamate release. Furthermore, enhancement of the interaction between PLC-γ and TrkB (receptor for BDNF) after imipramine pretreatment was observed. Interestingly, BD1047, a potent Sig-1R antagonist, blocked the imipramine-dependent potentiation on the BDNF-induced PLC-γ activation and glutamate release. In contrast, overexpression of Sig-1R per se, without antidepressant pretreatment, enhances BDNF-induced PLC-γ activation and glutamate release. These results suggest that antidepressant pretreatment selectively enhances the BDNF signaling on the PLC-γ/IP3/Ca2+ pathway via Sig-1R, and that Sig-1R plays an important role in BDNF signaling leading to glutamate release.

Antidepressant drugs increase the levels of serotonin and/or norepinephrine by blocking reuptake of these neurotransmitters to presynaptic terminals (3, 4). However, although increase in monoamine levels occurs soon after drug administration, the effects of antidepressants emerge gradually over several weeks of continuous application (5). Therefore, it is possible that in addition to an increase in monoamine levels, other more temporal neuronal events may be responsible for the action of antidepressants. Alterations in cell signaling are supposed to be essential for the clinical effects of antidepressants because signal abnormalities have been shown to underlie both the pathophysiology and the treatment of depressive disorders (3). Many studies show the involvement of the cAMP pathway in depressive disorders (6, 7). Electroconvulsive therapy as well as antidepressant treatments up-regulate components of the cAMP pathway including transcription factor cAMP response element-binding protein and its phosphorylation. These events have been proposed to play important roles in the clinical manifestation of antidepressants that are characterized by slow onsets in their effects (8–10).

Expression of BDNF2 is regulated through cAMP-response element-binding protein (11–13). BDNF may be one of the major targets in the clinical action of antidepressants. For example, chronic, but not acute, antidepressant (e.g. tranylcypromine, sertraline, desipramine, and mianserin) treatments or electroconvulsive seizure up-regulates BDNF mRNA in the rat brain (14, 15). The infusion of BDNF into the rat midbrain area or hippocampus mimics the effects of antidepressants in behavioral models of depression such as learned helplessness and the forced swim test (16, 17). Furthermore, the levels of BDNF were higher in the post-mortem hippocampal tissue obtained from antidepressant-treated subjects than those from untreated subjects (18). These results suggest that antidepressants might exert their effects through an up-regulation of BDNF. Consistently, a reduction of BDNF mRNA expression in rat hippocampus was observed after the immobilization of stress (19). A decrease in the serum BDNF level in subjects diagnosed with severe depression was also reported (20). Dwivedi et al. (21) reported that BDNF mRNA and protein were reduced in the postmortem hippocampus and frontal cortex of suicide victims, most of whom were diagnosed with major depression. These studies strongly suggest that BDNF is a key molecule involved in the pathophysiology of depressive disorder and perhaps in the pharmacological action of antidepressants as well.

BDNF/TrkB signaling pathways have been well studied. Ras/ERK, phosphoinositide 3-kinase/Akt, and PLC-γ pathways are activated after BDNF administration (22, 23). These pathways are required for cellular

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§ The abbreviations used are: BDNF, brain-derived neurotrophic factor; Mes, 2-morpholinoethanesulfonic acid; PI, phosphoinositide; ERK, extracellular signal-regulated kinase; PLC-γ, phospholipase-γ; IP3, inositol 1,4,5-trisphosphate; MAPK, mitogen-activated protein kinase; DIV, days in vitro; PtdIns3P, phosphatidylinositol bisphosphate; GFP, green fluorescent protein; MAP2, microtubule-associated protein 2; Sig-1R, sigma-1 receptors.
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differentiation, survival promotion, neurotransmission, and neuronal plasticity in the central nervous system (24–26). ERK (extracellular signal-regulated kinase) and phosphoinositide 3-kinase pathways have been implicated in BDNF-mediated survival and axon outgrowth. BDNF protects cortical neurons against campothecin and serum deprivation-induced apoptosis via ERK and phosphoinositide 3-kinase/Akt pathways (27). Primary dendrite formation is also promoted via the same pathways (28). BDNF-mediated PLC-γ signaling also plays an important role in the central nervous system neurons. We previously reported that BDNF induces the release of excitatory neurotransmitter, glutamate, through the TrkB/PLC-γ/IP3 pathway in cultured cortical and cerebellar neurons (1, 29–31). Interestingly, Saarelainen et al. (32) reported that TrkB-T1 (a kinase negative type of TrkB) transgenic mice show a reduced activation of TrkB in the brain, and are resistant to the antidepressants effect in the forced swim test, suggesting that TrkB signaling is involved in the action of antidepressants. However, the mechanism underlying the therapeutic effect of antidepressants that is characterized by slow onset, and the associated roles of BDNF/TrkB signaling, if any, are still unknown.

Sig-1R are endoplasmic reticular proteins that, when stimulated by ligands or when overexpressed, can translocate (33, 34). The translocation of Sig-1R has been shown to increase [Ca2+]i, by enhancing the IP3 receptor signaling at the endoplasmic reticulum via the removal of an inhibitory cytoskeletal adaptor protein ankyrin from the IP3 receptor (2). Interestingly, most antidepressants have high affinities to Sig-1R (35). Therefore, we hypothesize that Sig-1R may participate in modulation of the BDNF-stimulated PLC-γ/IP3/Ca2+ pathway.

In the present study, we focused on the influence of pretreatment with antidepressants on the BDNF-induced glutamate release through the PLC-γ/IP3/Ca2+ pathway and found that pretreatments with structurally different antidepressants that are Sig-1R agonists (i.e. imipramine and fluvoxamine) significantly potentiate the BDNF-triggered glutamate release in cultured cortical neurons. Such potentiation by antidepressants occurred via enhancing activation of the PLC-γ/IP3/Ca2+ pathway. Furthermore, we found that the potentiation of the PLC-γ/IP3/Ca2+ pathway may be through the Sig-1R-mediated action. Thus, it is possible that the potentiation of BDNF-induced glutamate release by antidepressant pretreatment may underlie the therapeutic effect of antidepressants and that antidepressants may exert this effect via Sig-1R.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Primary cultures were prepared from postnatal 2-day-old rat (SLC, Shizuoka, Japan) cortex as reported previously (1). Postnatal day 2 cortical cells were plated at a density of 5 × 10^4/cm² on polyethyleneimine-coated culture plates (Corning, Corning, NY) or cover glasses (Matsunami, Osaka, Japan) attached to flexiperm (VIVASCIENCE, Gottingen, Germany). The culture medium (5/5 DF) consisted of 5% fetal bovine serum, 5% heated-inactivated horse serum, 90% of a 1:1 mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium containing 15 mM HEPES buffer, pH 7.4, 30 mM Na₂HPO₄, and 1.9 mg/ml NaHCO₃.

**The Pretreatment with Antidepressants**—After maintenance of the cultured neurons for 5 days in vitro (DIV 5) with 5/5 DF media, antidepressants (imipramine hydrochloride and fluvoxamine maleate, Sigma) were added to cortical cultures by bath application. Then, the cultures were incubated for 48 h in the presence of antidepressants before performing collection of samples for amino acid measurement. BD1047 (1 µM, Tocris Cookson Ltd., Avonmouth, United Kingdom), an antagonist of Sig-1/2Rs (36), was applied 30 min before adding the antidepressants.

To examine the effect of BD1047, the antidepressant pretreatment was maintained in the presence of BD1047.

**Detection of Amino Acid Neurotransmitters**—The amount of amino acid released from cultured cortical neurons were measured as described previously (29). Briefly, amino acids released into the assay buffer (modified HEPES-buffered Krebs Ringer solution: KRH containing 130 mM NaCl, 5 mM KCl, 1.2 mM NaH₂PO₄, 1.8 mM CaCl₂, 10 mM glucose, 1% bovine serum albumin, and 25 mM HEPES, pH 7.4) were measured by high performance liquid chromatography (Shimadzu Co., Kyoto, Japan) with a fluorescence detector (Shimadzu Co., Kyoto, Japan). After cortical cultures were washed three times with KRH assay buffer, initially, fresh KRH buffer was added to cultures and collected without stimulation (1 min), that is, the amount of glutamate in the sample was considered as a basal release. Next, BDNF (final 100 ng/ml, 1 min) dissolved in phosphate-buffered saline containing bovine serum albumin (1 mg/ml) was added to cultures by bath application. The basal or BDNF-stimulated samples were treated with o-phthalaldehyde and 2-mercaptoethane acid for 5 min at 10 °C before being injected into the high performance liquid chromatography system and analyzed using a fluorescence monitor (excitation wavelength, 340 nm; emission wavelength, 445 nm). U73122 (Wako, Osaka, Japan), a PLC-γ inhibitor, was applied for 30 min at 2 µM before BDNF application. Xestospongic C (10 µM, Cayman Chemicals, Ann Arbor, MI), an IP3 receptor inhibitor, was added 1 h before the application of BDNF. Representative data from a sister culture are shown in the figures. N indicates the well number of each plate. The reproducibility was confirmed in separated cultures.

**Imaging of Intracellular Ca²⁺**—Ca²⁺ imaging was carried out as reported previously (1). Cultured cells were maintained on polyethyleneimine-coated cover glasses (Matsunami, Osaka, Japan) attached to flexiperm. The dye intensity was monitored using a fluorescent microscope (Axiovert 200 controlled by Slide Book TM 3.0, ZEISS, Tokyo, Japan). The emitted fluorescence was guided through a ×20 objective. Image data were obtained every 2 or 4 s. The data were stored and analyzed with the Slide Book™ 3.0 (Intelligent Imaging Innovations Inc., CO). All imaging experiments were performed at least 3 times with separate cultures. Representative data from neurons in a sister culture are shown in the figures.

**Immunoblotting**—Pretreatment with antidepressants was carried out at 0.1 µM and the cultures were incubated for 48 h. Afterward, BDNF was applied at 100 ng/ml for 0.5, 1, or 3 min. Cells were lysed in a SDS lysis buffer containing 1% SDS, 20 mM Tris-HCl, pH 7.4, 5 mM EDTA, pH 8.0, 10 mM NaF, 2 mM Na₃VO₄, 0.5 mM phenylarsine oxide, and 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged at 15,000 × g for 60 min at 4 °C, and the supernatants were collected for analysis. Primary antibodies for immunoblotting were used at the following dilutions: anti-Akt (1:1000, Cell Signaling, MA), anti-phospho-Akt (1:1000, Cell Signaling), anti-ERK (1:1000, Cell Signaling), anti-phospho-ERK (1:1000, Cell Signaling), affinity purified anti-Sig-1R (1:2000), anti-ankyrin (CALBIOCHEM, Darmstadt, Germany), anti-IP3 receptor1 (1:1000, Affinity BioReagents), anti-synapsin I (1:1000, CHEMICON, CA), anti-synaptotagmin I (1:3000, Transduction Laboratories, KY), anti-TUJ1 (1:5000, Berkeley Antibody Co., CA), and anti-GFP antibodies (1:1000, MBL, Nagoya, Japan). Anti-Sig-1R antisera was raised in rabbits by using a peptide antigen corresponding to amino acids 144–165 of guinea pig Sig-1R. Affinity purified anti-Sig-1R antibodies were raised in rabbits by using a peptide antigen corresponding to amino acids 52–69 of rat Sig-1R. To detect activation of TrkB or PLC-γ, immunoprecipitation was carried out as described previously (1). Anti-phospho-Tyr (1:1000, Upstate, VA), anti-PLC-γ (1:1000, Santa
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![Image of a fluorescence microscope showing cultured cortical neurons labeled with MAP2 and GFAP with merged images.](Image)

**FIGURE 1.** Imipramine enhanced BDNF-induced glutamate release and intracellular Ca²⁺ increase in cultured cortical neurons. A, cultured cortical cells at DIV 7 were immunostained with a, anti-MAP2 (red, microtubule-associated protein 2, a neuronal marker); and b, glial fibrillary acidic protein (green, a glial fibrillary acidic protein, an astroglial marker). c, overlay of a and b. Bar = 50 μm. B, dose-dependent effect of imipramine and fluvoxamine on BDNF-induced glutamate release. Pretreatment with imipramine (a) (0.01–10 μM) or fluvoxamine (b) (0.01–10 μM) was performed at DIV 7. BDNF was added to DIV 7 cortical neurons at a concentration of 100 ng/ml for 1 min. Basal release was collected 1 min before BDNF application. Data represent mean ± S.D. (n = 6). Statistical analysis was performed using Student’s t test. ***, p < 0.001**; **, p < 0.01 versus BDNF-induced release in 0 μM antidepressant pretreatment cultures. C, pretreatment with imipramine and fluvoxamine enhanced BDNF-increased intracellular Ca²⁺, a, the traces indicate the change in intensity of fluo-3 fluorescent from 6 neurons before or after BDNF (100 ng/ml) application. The bar indicates the exposure time of BDNF. B, images of fluo-3-filled cells before (a, c, and e) and 4 s after (b, d, and f) BDNF application are shown. Bar = 50 μm. c. plots summarize data from 50 selected cells from sister cultures at DIV 7. The ratio (F/F₀) of fluorescence was calculated from the intensities of fluorescence before and after BDNF stimulation. F/F₀, BDNF-induced level/basal level; ***, p < 0.001 versus BDNF-induced in none (t test).

Cruz Biotechnology Inc., Santa Cruz, CA), anti-TrkB (1:1000, BD Biosciences), and anti-Trk antibodies (1:1000, Santa Cruz Biotechnology Inc.) were used. To examine the effect of phosphatase inhibitor, Na₃VO₄ (CALBIOCHEM), on the PLC-γ activation, Na₃VO₄ was applied to cortical cultures 1 h before BDNF stimulation. The immunoblotting experiments were performed three or four times with independent cultures and they were quantitatively analyzed as described previously (37).

**Measurement of PLC-γ Activity**—PLC-γ activity was determined by hydrolysis of PtdInsP₂, by mixing 10 μl of the PLC solution with 20 μl of distilled water, 10 μl of reaction buffer, and 10 μl of phospholipid micelle solution (38, 39). The final concentration was 50 μM phosphatidylyethanolamine (Sigma), 40 μM PtdInsP₂ (Sigma), 0.5 μCi/ml [³H]PtdInsP₂ (PerkinElmer Life Sciences), 50 mM Mes, pH 7.0, 0.4 mM CaCl₂, and 0.5 mg/ml bovine serum albumin. The reaction mixture containing 30,000 dpm of [³H]PtdInsP₂ was incubated at 37 °C for 15 min, and the reaction was stopped by adding 1 ml of chloroform:methanol (2:1, v/v). Radioactive InsP₃ was extracted by adding 250 μl of 1 N HCl, and radioactivity in the upper aqueous phase was measured for 1 min in a liquid scintillation counter.

**Immunocytochemistry**—Cultured neurons were fixed with 4% paraformaldehyde for 20 min and then rinsed three times with phosphate-buffered saline. Cells were permeabilized with 0.1% Triton X-100 in phosphate-buffered saline for 5 min at room temperature. Anti-MAP2 (1:1000, Sigma) and anti-glial fibrillary acidic protein (1:1000, CHEMICON) antibodies were applied overnight at 4 °C. Anti-Sig-1R (Santa Cruz Biotechnology Inc.) and anti-Sig-1R antisera (1:100) were also used. Alexa Fluor 488- or Alexa Fluor 594-conjugated anti-mouse IgG (1:1000, Invitrogen) or anti-rabbit IgG (1:1000, Invitrogen) were used as secondary antibodies. Immunoreactivity was monitored with a fluorescence microscope (Axiovert 200, ZEISS). The images of GFP (green fluorescent protein) were obtained using the same system.

**Simdhis Virus**—A bicistronic vector plasmid (pSinEGdsp) was provided by Dr. Kawamura (Niigata University, Japan). The plasmid was derived from pSinRep5 (Invitrogen, Tokyo, Japan) and had two subgenomic promoters followed by a multiple cloning site for arbitrary gene insertion and an enhanced GFP open reading frame, thus the virus can produce arbitrary protein and enhanced GFP independently in the infected cell, as previously described (40). Rat Sig-1R cDNA amplified by reverse transcriptase-PCR with primers (forward 5′-GGAATTCACTAGTCGTCGCAGAGTTGTGGTA-3′ and reverse 5′-GCTCTA-GACGGCGTGCTCCTTCCCCCCACACAGACGAG3′) were cleaved with SpeI and MluI sites. Cleaved fragments were inserted at the XbaI-MluI sites of the plasmid. Each plasmid was cleaved with NotI, and used as a
template for mRNA transcription in vitro using a mMESSAGE mMACHINE kit (Ambion, TX). Pseudovirions were produced according to the experimental procedure of Invitrogen. Baby hamster kidney cells were transfected with each mRNA and 26 S helper mRNA (Invitrogen) by electroporation (500 V/cm, 25 μF, double pulse) using Gene Pulser (Bio-Rad). The cells were incubated with 5/5 DF media for 24 h at 37°C, the supernatants were collected as pseudovirion-containing solutions.

Materials—Regeneron Pharmaceutical Co. and Takeda Chemical Industries, LTD. generously donated the BDNF.

Statistical Analysis—Data shown in this study are expressed as mean ± S.D. Statistical significance was evaluated with Student’s t test, and probability values less than 5% were considered significant.

RESULTS

Pretreatment with Antidepressants Enhanced BDNF-evoked Release of Glutamate and Increase in Intracellular Ca²⁺ Concentration in Cultured Cortical Neurons—Immunocytochemical analysis, using anti-microtubule-associated protein 2 (MAP2, a neuronal marker) and anti-glia fibrillary acidic protein (an astroglial marker), revealed that the proportion of MAP2 positive cells in our cortical cultures (7 days in vitro, DIV 7) was ~80% (n = 4, the number of selected fields), suggesting that the majority of cultured cells were neurons (Fig. 1A). After cultured neurons were pretreated with 0.01–10 μM imipramine or fluvoxamine for 48 h, BDNF-induced glutamate release was measured. As shown in Fig. 1B, both antidepressants enhanced the BDNF-induced glutamate release. Both drugs were effective at 0.1–10 μM, although these antidepressants had no effect at the concentration of 0.01 μM (a and b in Fig. 1B). The basal release of glutamate was not influenced by either drug. In our previous study (1, 29), we showed that the BDNF-induced glutamate release depends on increases in [Ca²⁺], through IP₃-sensitive Ca²⁺ channels. Therefore, we monitored changes in the intracellular concentration of Ca²⁺ stimulated by BDNF. Pretreatment with imipramine or fluvoxamine potentiated the BDNF-elicted [Ca²⁺], (Fig. 1C). The time dependence of the [Ca²⁺], increase in cell bodies before and after BDNF application is shown (a in Fig. 1C). The changes in [Ca²⁺], were quantified by normalizing the Fₐ/F₀ (an increase in cell bodies before and after BDNF application) to a level of 0.5 min after BDNF stimulation without imipramine pretreatment. Data were normalized to a level of 0.5 min after BDNF stimulation without imipramine pretreatment. N.D., not detected. **, p < 0.01; *, p < 0.05 versus BDNF-induced in none (without imipramine pretreatment), respectively (t test). Pretreatment with imipramine or fluvoxamine increased the release of Ca²⁺ stimulated by BDNF, indicating a time- and concentration-dependent manner. The duration of the increase in [Ca²⁺], by BDNF was longer with imipramine (a and b in Fig. 1C). Pretreatment with imipramine (0.1 μM) or fluvoxamine (0.1 μM) potentiated the BDNF-elicited [Ca²⁺], up to 3 min after BDNF stimulation following pretreatment with imipramine. Pretreatment with imipramine or fluvoxamine at the concentration of 0.01–10 μM enhanced the BDNF-evoked increase in the level of intracellular Ca²⁺ (Fig. 1D), in a concentration-dependent manner (a and b in Fig. 1D).

Pretreatment with Imipramine Increased Activation of PLC-γ by BDNF—We previously reported that the PLC-γ/IP₃/Ca²⁺ (an increase in [Ca²⁺], from the IP₃-sensitive Ca²⁺ channel receptors) pathway, downstream of TrkB, is essential for the BDNF-induced glutamate release (1, 29). Therefore, the effect of pretreatment with imipramine on BDNF-stimulated PLC-γ activation (phosphorylation) was examined. To determine PLC-γ activity, the blotting with anti-phospho-Tyr antibody following immunoprecipitation with anti-PLC-γ antibody was performed. As shown in Fig. 2A, PLC-γ was activated within 0.5 min after the application of BDNF with or without imipramine pretreatment. However, the magnitude of PLC-γ activation was significantly greater in imipramine-treated cultures, compared with non-treated cultures. The enhancement of the PLC-γ activation was confirmed at 0.5, 1.0, or 3.0 min after the BDNF stimulation as compared with that obtained from non-treated cultures (a in Fig. 2A). Quantification of the levels of activated PLC-γ is shown (b in Fig. 2A). The enzymatic activity of PLC-γ stimulated by BDNF were also examined (supplementary Fig. S1). The enhancement of PLC-γ enzymatic activity was confirmed (supplementary Fig. S1). Activation of TrkB was also examined (Fig. 2B). TrkB was equally activated by BDNF with or without imipramine pretreatment (a in Fig. 2B). Quantification of the activated TrkB is shown (b in Fig. 2B). We then investigated other pathways activated by TrkB, i.e. phosphoinositide 3-kinase and MAPK/ERK pathways. However, imipramine pretreatment did not affect the activation of Akt (pAkt, phosphorylated Akt) or ERK1/2 (pERK1/2) caused by BDNF (a in Fig. 2C). Total expression of Akt or ERK1/2 was not influenced by imipramine. Quantifications of phosphorylated Akt and pERK1/2 levels are shown (b, c, and d in Fig. 2C). These results suggest that the chronic treatment with imipramine specifically affects the PLC-γ pathway. The duration of the magnification of PLC-γ activation was examined. As shown in supplementary Fig. S2, the magnification of BDNF-stimulated PLC-γ in imipramine-treated cultures was maintained until at least 24 h. In the present study, to investigate the effects of imipramine pretreatment on the acute action of BDNF, we focused on the neuronal responses within 3 min after BDNF stimulation following pretreatment with antidepressants.

To further consolidate the possibility that the PLC-γ pathway is involved in the imipramine-potentiated glutamate release and the increase of intracellular Ca²⁺, we examined the effect of U73122, a PLC-γ inhibitor, in our system. In the presence of U-73122 (2 μM), the

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FIGURE 2. Pretreatment with imipramine potentiated activation of PLC-γ stimulated by BDNF. A, α, activation of PLC-γ by BDNF increased in cultures pretreated with imipramine. Imipramine (0.1 μM) was applied to cultures at DIV 5. Forty-eight hours after this application, BDNF stimulation was carried out for 0, 0.5, 1, or 3 min. Collected lysates were immunoprecipitated with anti-PLC-γ antibody. Blotting was performed with anti-phospho-Tyr (top) and anti-PLC-γ antibodies (bottom), respectively. b, the amount of phospho-PLC-γ (pPLC-γ) was quantified by densitometry. Data represent mean ± S.D. (n = 4, the number of experiments in separated cultures). Data were normalized to a level of 0.5 min after BDNF stimulation without imipramine pretreatment. N.D., not detected. **, p < 0.01, *, p < 0.05 versus BDNF-induced in none (without imipramine pretreatment), respectively (t test). B, α, blotting with anti-phospho-Tyr (pTyr) (top) or anti-TrkB antibodies (bottom) after immunoprecipitation using anti-TrkB antibody. b, quantification of the levels of activated TrkB after exposure to BDNF. Data were normalized to a level of 0.5 min after BDNF stimulation without imipramine pretreatment. Data represent mean ± S.D. (n = 4). C, α, activation of Akt and ERK1/2 were determined. Quantification of the levels of activated Akt (pAkt, phosphorylated Akt) and activated ERK1/2 (pERK1/2) are shown. Normalization was level at 0 min. Data represent mean ± S.D. (n = 4). D, b, immunoblots.
BDNF-induced glutamate releases in both non- and imipramine-pretreated cortical cultures were completely blocked (a in Fig. 3A). The IP₃-sensitive Ca²⁺ channel inhibitor, xestospongin C, also blocked glutamate release in both non- and imipramine-pretreated cultures (b in Fig. 3A). The effects of U73122 or xestospongin C on the BDNF-induced Ca²⁺ increase were examined. The intracellular Ca²⁺ mobilization triggered by BDNF was abolished by U73122 with or without imipramine pretreatment (a in Fig. 3B). Fluvoxamine-potentiated Ca²⁺ increase was also U73122-sensitive (a in Fig. 3B). In the presence of xestospongin C, the potentiation in Ca²⁺ increase was slightly effective (Fig. 4). The influence of pretreatment with Na₃VO₄, a general phosphatase inhibitor, was tested. However, significant enhancement of PLC-γ activation by Na₃VO₄ was not observed at concentrations of 1 or 10 μM although a high concentration of Na₃VO₄ was slightly effective (Fig. 4C).

Sig-1R Was Involved in Antidepressant-Potentiated Glutamate Release—To further investigate the mechanisms underlying the antidepressant-potentiated glutamate release, the possible involvement of Sig-1R of 1 ng/ml, for 1 min) to cultured neurons at DIV 7. Pretreatment with imipramine (0.1 μM) was added 30 min before application of BDNF (100 ng/ml, for 1 min). Xestospongin C (10 μM) was added 1 h before application of BDNF (100 ng/ml, for 1 min). Xestospongin C (10 μM) was added 1 h before application of BDNF (100 ng/ml, for 1 min). Xestospongin C (XestC) (10 μM) was added 1 h prior to BDNF (100 ng/ml). The data were determined from 50 selected cells from sister cultures. ***: p < 0.001 (t test).

Binding of the PLC-γ to TrkB Receptor Was Reinforced by Imipramine Pretreatment—To identify the mechanism involved in the potentiation of PLC-γ/IP₃/Ca²⁺ signaling, we determined TrkB-PLC-γ interaction after imipramine pretreatment. First, we examined the binding of PLC-γ to TrkB after BDNF stimulation in imipramine-pretreated cortical cultures. As expected, imipramine pretreatment enhanced binding of PLC-γ to TrkB (a in Fig. 4A). Quantification of the binding of PLC-γ to TrkB is shown (b in Fig. 4A). We examined the endogenous levels of PLC-γ, TrkB, and BDNF after imipramine pretreatment. However, the levels of these proteins were not changed (a and b in Fig. 4B). TU1 expression is shown as a negative control (a and b in Fig. 4B). These results suggest that interaction between the PLC-γ and TrkB receptor was reinforced by pretreatment with imipramine. Next, the involvement of phosphatase in potentiation in PLC-γ activation was examined because it is possible that a loss of phosphatase activity results in up-regulation of PLC-γ activation. As shown in Fig. 4C, the influence of Na₃VO₄, a general phosphatase inhibitor, was tested. However, significant enhancement of PLC-γ activation by Na₃VO₄ was not observed at concentrations of 1 or 10 μM although a high concentration of Na₃VO₄ was slightly effective (Fig. 4C).

Sig-1R Was Involved in Antidepressant-Potentiated Glutamate Release—To further investigate the mechanisms underlying the antidepressant-potentiated glutamate release, the possible involvement of Sig-1R was examined because several reports have shown that Sig-1R is important for the biological effects of antidepressants (41, 42). Initially, the expression of endogenous Sig-1R was checked in our cultures. Immunocytochemical analysis showed that Sig-1R was present in the cell body and neurites of MAP2-positive neuronal cells (DIV 14, a–c in Fig. 5A). The expression of endogenous Sig-1R during in vitro maturation is shown (Fig. 5B). The Sig-1R expression was observed from DIV 4 to DIV 14 (Fig. 5B). To estimate synaptic maturation in our system, synaptic proteins (synapsin I and synaptotagmin) were also examined. Expressions in both synapsin I and synaptotagmin were significantly increased during culture (Fig. 5B), confirming synaptic maturation in vitro. TU1 (class III β-tubulin) is shown as a neuronal marker. Next, we tested the effect of BD1047 (an antagonist of Sig-1/2Rs) on the antidepressant-enhanced BDNF-induced glutamate release (Fig. 5C). The effect of BD1047 (an antagonist of Sig-1/2Rs) on the antidepressant-enhanced BDNF-induced glutamate release (Fig. 5C).
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In the present study, we found, for the first time, that pretreatment with antidepressants (imipramine and fluvoxamine) potentiated BDNF-regulated glutamatergic neuronal function in cultured cortical neurons. Chronic pretreatment with antidepressants enhanced release of glutamate induced by acute BDNF application via the potentiation of the PLC-γ/IP$_3$/Ca$^{2+}$ pathway. In our previous study, we confirmed that the BDNF-increased Ca$^{2+}$ occurred in neuronal cells. Furthermore, BDNF failed to elicit release of glutamate in glial pure cultures. Therefore, we concluded that the action of antidepressants in the present study was a neuronal (not glial) response to BDNF.

The Sig-1R antagonist, BD1047, blocked the effects of the antidepressant. Furthermore, overexpression of Sig-1R reinforced these neuronal responses to BDNF, suggesting that antidepressants have facilitating effects on the BDNF-dependent excitatory neurotransmission, and that its potentiation is, at least in part, through Sig-1R. Although further experiments are required, our results with Sig-1R potentiation the BDNF action are in support of our hypothesis that Sig-1R serve as amplifiers for intracellular signal transduction in the biological system.

In Fig. 5C, we show representative immunofluorescence images of cortical cultures infected with a Sindbis virus carrying a GFP construct. The infected cells were used as a control in the immunofluorescence analysis. Furthermore, we used a Sindbis virus carrying a GFP construct as a control in the immunofluorescence analysis.

In the present study, we found, for the first time, that pretreatment with antidepressants (imipramine and fluvoxamine) potentiated BDNF-regulated glutamatergic neuronal function in cultured cortical neurons. Chronic pretreatment with antidepressants enhanced release of glutamate induced by acute BDNF application via the potentiation of the PLC-γ/IP$_3$/Ca$^{2+}$ pathway. In our previous study, we confirmed that the BDNF-increased Ca$^{2+}$ occurred in neuronal cells. Furthermore, BDNF failed to elicit release of glutamate in glial pure cultures. Therefore, we concluded that the action of antidepressants in the present study was a neuronal (not glial) response to BDNF.

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Antidepressant Enhances BDNF-induced Glutamate Release

FIGURE 6. BDNF-induced glutamate release was reinforced in Sig-1R-infected cortical cultures. A: a and d, MAP2, and b and e, GFP-positive images. c, overlay of a and b. f, overlay of d and e. Upper a–c, images from GFP-infected control cultures. Lower d–f, images from overexpression of both GFP and Sig-1R cultures. g, Sig-1R, and h, GFP-positive cell in Sig-1R-infected cultures. i, overlay of g and h. Viral infection was performed at DIV 5, and fixed at DIV 7. Bar = 50 μm. B, increase in BDNF-induced glutamate release in Sig-1R-overexpressing cortical cultures. Cortical cultures were prepared without viral infection (None), with viral infection of GFP, or with viral infection of GFP/Sig-1R at DIV 5. Basal or BDNF (100 ng/ml)-induced release of glutamate was measured at DIV 7 (48 h after infection). Data represent mean ± S.D. (n = 4). ***, p < 0.001 (t test). C, a, BDNF-stimulated PLC-γ activation was enhanced in Sig-1R-overexpressing cultures, b, quantification of the levels of BDNF-activated PLC-γ after viral infection. Data represent mean ± S.D. (n = 4). Data were normalized to a level in BDNF stimulation in GFP-infected control cultures without imipramine pretreatment. *, p < 0.05 (t test). IB, immunoblot.

TABLE 1
The expression of Sig-1R, ankyrin B, or IP3 receptor (Type 1) in Sig-1R-overexpressing cultures

Cortical cultures were prepared with viral infection of GFP, or with viral infection of GFP/Sig-1R at DIV 5. Forty-eight hours later, cell lysates were collected for immunoblotting. The significant reduction of ankyrin B was observed in Sig-1R-overexpressing cortical cultures. In contrast, IP3 receptor or TUJ1 (as a negative control) were not changed. Data represent mean ± S.D. (n = 3). Data were normalized to a level in GFP-infected control cultures, respectively.

| Control (GFP only) | Sig-1R/GFP |
|-------------------|------------|
| Sig-1R            | 1.00       |
| Ankyrin B         | 1.00       |
| IP3R1             | 1.00       |
| TUJ1              | 1.00       |

* p < 0.01.
** p < 0.001 (t test).

subjects may have clinical relevance to the pathophysiology of suicidal behavior. In their report, although there was no significant difference in PI-PLC activity between suicidal subjects with a history of mental disorders and those without such a history, both groups had significantly lower PI-PLC activity than in normal subjects. In our system, activation or increase of PLC-γ did not occur in cultured cortical neurons merely by the exposure to antidepressants for 48 h. In contrast, marked potentiation in the PLC-γ activation or glutamate release after BDNF stimulation was observed. Therefore, it was possible that chronic treatment with antidepressants might strengthen the coupling between PLC-γ and signal acceptors such as TrkB. As expected, we found that imipramine pretreatment enhanced binding of PLC-γ to TrkB. Although the mechanism of enhancement in coupling between PLC-γ and TrkB was still unclear, it is possible that potentiation of PLC-γ activation was crucial for the effects of antidepressants.

We focused on the enhancement of BDNF-induced glutamate release by pretreatment with antidepressants, because we previously found that BDNF potentiates the glutamatergic transmission through the PLC-γ/IP3/Ca^2+ pathway (1). On the other hand, several studies have shown involvement of the MAPK/ERK pathway in the effects of antidepressants. Infusion of BDNF into the rat hippocampus, dentate gyrus granule cell layer, and CA3 pyramidal cell layer, significantly decreases escape failure in the learned helplessness paradigm, however, a selective MEK inhibitor, U0126, blocked these effects in a rat model of learned helplessness (17). A recent postmortem study has indicated that the level of ERK expression or activity is decreased in the hippocampus and cerebral cortex of depressed suicide victims (49), implying that activa-
tion of the MAPK pathway contributes to the antidepressant effect. In our system, antidepressant treatment for 48 h did not alter the activation of MAPK/ERK stimulated by BDNF. If antidepressant application were carried out for a longer time, an influence on activation of the MAPK pathway might have been observed. In this study, the acute effect of BDNF on MAPK activation (0.5–3.0 min) was monitored because the BDNF-induced glutamate release occurs within 1 min (50).

Some tricyclic antidepressants and selective serotonin reuptake inhibitors possess moderate to high affinities to Sig-1R (51, 52). Indeed, a relevant mechanism through Sig-1R in the actions of antidepressant has been reported (41, 42). Sig-1R agonists including igmesine, (+)-SKF-10,047, or steroid dehydroepiandrosterone sulfate showed some antidepressant-like activity by shortening the immobility time in Swiss mice in a forced swim test. These effects were blocked by the Sig-1R antagonist BD1047 (42). In our system, we found that BD1047 altered the potentiation of BDNF-induced effects by antidepressants and over-expression of Sig-1R mimicked the action of antidepressants (Figs. 5 and 6). Our results indicate that signal transduction via Sig-1R is important for the antidepressant-mediated glutamatergic function. How Sig-1R signaling interacts with the PLC-γ/IP₃/Ca²⁺ pathway after antidepressant exposure is an open question. Morin-Surum et al. (53) reported that Sig-1R modulates activity of PLC in the brainstem via an unknown mechanism. Furthermore, translocation of Sig-1R to the plasma membrane from the endoplasmic reticulum was reported (34). Therefore, it is possible that Sig-1R affects the activation of PLC-γ at the plasma membrane directly or indirectly, although further studies are required. Alternatively, Sig-1R may influence alternation in the ankyrin-IP₃ complex, because the translocation of Sig-1R has been shown to increase [Ca²⁺], by enhancing the IP₃ receptor signaling via the removal of ankyrin from the IP₃ receptor (2). That is, it is possible that the change in the level of ankyrin is essential for IP₃ receptor signaling. To clarify the possibility, the expression of ankyrin B was examined after Sig-1R overexpression in our cortical cultures (Table 1). Indeed, the significant reduction of ankyrin B was observed in Sig-1R-overexpressing cortical cultures, implying that reduction in ankyrin B results in the activation of IP₃ receptor signaling. Intracellular signaling through Sig-1R may be involved in not only TrkB-PLC-γ pathway but also ankyrin/IP₃ receptor interaction, downstream of TrkB receptor, after BDNF stimulation.

In this study, antidepressants significantly potentiated BDNF-induced excitatory glutamate release, suggesting the possibility that potentiation in BDNF-mediated neuronal transmission is involved in the underlying mechanism of action of antidepressants. Up-regulation of TrkB-mediated signaling, especially the PLC-γ pathway, may be involved in the therapeutic effects of antidepressants. Saarelainen et al. (32) showed that antidepressants acutely increase TrkB signaling in the cerebral cortex and that this signaling is required for the behavioral effects caused by antidepressants. They suggested that antidepressants exert their effects, at least in part, through endogenous BDNF. In our system, the PLC-γ pathway was up-regulated by antidepressants, although the activation of TrkB was not affected. In addition, endogenous levels of PLC-γ, TrkB, or BDNF expression were not changed after imipramine pretreatment for 48 h (Fig. 4B). The differences in experimental conditions such as concentration or exposure time of antidepressants and/or type of cells may influence neuronal responses to antidepressants, however, these findings highlight the importance of investigating alternations of TrkB-mediated intracellular signaling after BDNF stimulation in addition to monitoring trophic factor levels.

Neurotrophin expression is regulated in a activity-dependent manner, namely, glutamatergic activations up-regulate the expression of neurotrophins (26, 54). Thus, antidepressant-potentiated BDNF-mediated neurotransmission may influence not only plasticity of the glutamatergic network but also BDNF expression itself through these positive feed-forward loops, which may result in therapeutic effects of antidepressants. As Sig-1R are intimately related to neurotrophins and antidepressants, we believe that our current results provide the possibility that Sig-1R serves as an important clinical link between antidepressant effects and neurotrophin action such as that exerted by BDNF.
Antidepressant Enhances BDNF-induced Glutamate Release

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