4-Methylcatechol Increases Brain-Derived Neurotrophic Factor Content and mRNA Expression in Cultured Brain Cells and in Rat Brain In Vivo

ATSUMI NITTA, MEGUMI ITO, HIDEFUMI FUKUMITSU, MAKOTO OHMIYA, HISANORI ITO, AYAKO SOMETANI, HIROSHI NOMOTO, YOSHIKO FURUKAWA, and SHOEI FURUKAWA

Laboratory of Molecular Biology, Gifu Pharmaceutical University, Mitahora-higashi, Japan (A.N., M.I., H.F., M.O., H.I., A.S., H.N., S.F.); and Aichi Bunkyo Women’s College, Nishi-Machi, Inazawa, Japan (Y.F.)

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ABSTRACT

Practical use of brain-derived neurotrophic factor (BDNF) as therapy is limited by two serious problems, i.e., its inability to cross the blood-brain barrier and its instability in the bloodstream. In the present study, we investigated the effects of 4-methylcatechol (4-MC), which stimulates nerve growth factor synthesis and protects against peripheral neuropathies in rats, on BDNF content and mRNA expression in cultured brain cells and in vivo in the rat brain. 4-MC elevated BDNF content in culture media of both rat astrocytes and neurons with different dose-response relations. The increase in BDNF mRNA level was correlated with the increase in BDNF content, demonstrating that 4-MC can stimulate BDNF synthesis of both neurons and astrocytes. Then we examined the in vivo effects of 4-MC. First, we found that ventricularly administered 4-MC facilitated an increase in the BDNF content in the cerebral cortex and hippocampus in association with its diffusion into the brain parenchyma. Second, i.p. administration of 4-MC enhanced BDNF mRNA expression in the infant rat brain, in which the blood-brain has not yet fully been established. These results demonstrate that 4-MC, once delivered into the brain, can stimulate BDNF synthesis.

Brain-derived neurotrophic factor (BDNF) is one of the members of the neurotrophin family of proteins, which includes nerve growth factor (NGF), neurotrophin (NT)-3, NT-4/5, and NT-6 (Leibrock et al., 1989; Hohn et al., 1990; Halbrook et al., 1991). Widespread expression of BDNF mRNA in neurons of the central nervous system (CNS) suggests important roles for BDNF there (Phillips et al., 1990). BDNF affects the survival or differentiation of cultured motor neurons (Henderson et al., 1993), mesencephalic dopaminergic neurons (Knüsel et al., 1991), and septal cholinergic neurons (Knüsel et al., 1991). In adult rats, BDNF mRNA is more widely distributed in the whole brain than mRNAs of NGF and NT-3 (Phillips et al., 1990) and is regulated by glutamate or γ-aminobutyric acid neurotransmission (Zafra et al., 1991). And enhanced expression is following establishment of long-term potentiation (Rutherford et al., 1997). BDNF thus seems to participate in various activity-dependent events, including synapse plasticity.

BDNF mRNA expression is evoked in association with various brain insults such as traumatic injury (Yang et al., 1996) and infusion of kainic acid (Ballarin et al., 1992) or 6-hydroxy dopamine (Zhou et al., 1996) in limited brain regions. These observations suggest the involvement of BDNF in neuronal regeneration processes. In fact, intraventricular administration of BDNF prevented neuronal death of nigral dopaminergic neurons induced by infusion of neurotoxins (Tsukahara et al., 1995) and the nigrostriatal pathway (Yan et al., 1992) and suppressed the neuronal death that occurred in hippocampal pyramidal neurons following transient forebrain ischemia (Beck et al., 1994), demonstrating that BDNF is protective against particular neuronal degeneration. Therefore, BDNF is expected to be useful as a therapeutic tool for neurological disorders such as Parkinson’s disease, amyotrophic lateral sclerosis, and Alzheimer’s disease because of its potent actions on the neurons responsible for these disorders. However, there are two obstacles against the therapeutic application of BDNF to diseases of the CNS.

ABBREVIATIONS: BDNF, brain-derived neurotrophic factor; NGF, nerve growth factor; NT, neurotrophin; CNS, central nervous system; BBB, blood-brain barrier; 4-MC, 4-methylcatechol; DMEM, Dulbecco’s modified Eagle’s medium; FCS, fetal calf serum; RT-PCR, reverse transcription-polymerase chain reaction; CM, conditioned medium; EIA, enzyme immunoassay; GDNF, glial cell line-derived neurotrophic factor; PFA, paraformaldehyde.
First, BDNF is a macromolecule that cannot pass through the blood-brain barrier (BBB), making it difficult to deliver BDNF from the periphery to the CNS. Second, BDNF is rapidly incorporated into the liver due to its cationic charge (Partridge et al., 1994), resulting in a short-term circulation of BDNF in the bloodstream. These drawbacks may force consideration of intraventricular infusion of BDNF as therapy, although this approach involves severe technical and/or ethical problems. Transfection of cells in vivo with the BDNF gene delivered by viral vectors and the transplantation of cells engineered to contain the normal BDNF gene may be promising approaches because a few reports demonstrate their effective protection against dopaminergic neurotoxins (Levivier et al., 1998). However, the clinical safety of these applications has not yet been fully established. Another promising approach to use neurotrophic actions for therapeutic purposes is the stimulation of synthesis of neurotrophic factors. 4-Methylcathexyl (4-MC), a potent stimulator of NGF synthesis in vivo and in vitro (Furukawa et al., 1989; Kaechi et al., 1993; Hanaoka et al., 1994), stimulates regeneration of the sciatic nerve (Kaechi et al., 1995) and protects against or improves diabetes- and acrylamide-induced neuropathies (Hanaoka et al., 1994; Saita et al., 1996). These effects involve nerve regeneration and/or amelioration of the activity of motor neurons and/or sensory neurons with large-diameter myelinated axons, whose cells do not respond to NGF. Therefore, it is possible that 4-MC could stimulate synthesis of some neurotrophic factor(s) other than NGF. In this study, we investigated the effects of 4-MC on BDNF production in the CNS in vivo and in vitro, and obtained evidence suggesting that 4-MC may be a potential therapeutic tool for the treatment of certain neurological disorders.

**Experimental Procedures**

**Materials.** 4-MC was purchased from Tokyo Kasei (Tokyo, Japan). BDNF was generously donated by Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan), and anti-BDNF antibody was prepared as described previously (Nitta et al., 1999a). Antibodies for microtubule-associated protein-2 and glial fibrillary acidic protein were from Chemicon International, Inc. (Temecula, CA) and DAKO Japan (Osaka, Japan), respectively. All other materials used were reagent grade. Rats were purchased from Nippon SLC (Shizuoka, Japan) and were treated according to the Guideline of Experimental Animal Care issued from the Office of the Prime Minister of Japan.

**Cell Cultures.** Neurons were cultured from the hippocampi of 18-day-old rat embryos as described previously (Nitta et al., 1999b). Briefly, the hippocampi were incubated in PBS containing 0.25% trypsin (Life Technologies Laboratories, Grand Island, NY), 10 mM glucose, and DNase (6 μg/ml; Sigma Chemical Co., St. Louis, MO) for 20 min at 37°C and triturated with a plastic pipette to dissociate the tissue into single cells. Following centrifugation (900g; 3 min), the cell pellet was resuspended in medium composed of Dulbecco’s modified Eagle’s medium (DMEM) and nutrient mixture F-12 Ham’s (1:1) (Life Technologies Laboratories), which contained 5% horse serum and 5% newborn calf serum (Irvine Scientific, Santa Ana, CA). The cells were plated in 24-well plates or 10-cm plastic dishes (10^5 cells/cm²) precoated with poly d-ornithine (0.5 μg/ml; Sigma Chemical Co.). After a 24-h culture, the medium was changed to serum-free medium containing insulin (5 μg/ml; Sigma Chemical Co.), transferrin (5 μg/ml; Sigma Chemical Co.), progesterone (2 μg/ml; Sigma Chemical Co.), and 5% BSA. More than 97% of the cells thus obtained expressed microtubule-associated protein-2 when stained with antibody specific for it, thus demonstrating that most of the cells in our cultures were neurons.

Astrocytes cultured from the whole brain of newborn rats were maintained in DMEM containing 10% fetal calf serum (FCS; Irvine Scientific), as described in Furukawa et al. (1986). Immunohistochemical analysis revealed that >97% of the cells were positive for glial fibrillary acidic protein, a marker of astrocytes. Quiescent astrocytes were prepared as described in Furukawa et al. (1986). Namely, astrocytes were inoculated sparsely (10^4 cells/cm²) into 24-well plates and 10-cm dishes, and cultured in the FCS-containing DMEM. After having reached confluence, the astrocytes were cultured for about another 10 days in FCS-free DMEM containing 0.5% BSA, with a medium change every 2 days.

**Semiquantification of BDNF mRNA Expression.** Reverse transcription polymerase chain reaction (RT-PCR) was used to evaluate the BDNF and β-actin mRNA levels, as described earlier in Nitta et al. (1999b). Total RNA was prepared from the cells by use of Isogen (Nippon Gene, Tokyo, Japan), which is basically composed of guanidine isothiocyanate. Oligonucleotide primers for the respective genes of rat BDNF and β-actin were used. RT-PCR was performed with a GeneAmp Thermostable rTth reverse transcriptase RNA PCR kit (Perkin-Elmer, Oak Brook, IL) used according to the manufacturer’s instructions. In short, 500 ng of total RNA was reverse-transcribed with 0.75 mM downstream primer by rTth polymerase in the presence of Mg²⁺ for 15 min at 60°C. Synthesized cDNA was amplified by PCR in the presence of Mg²⁺ with both up- and downstream primers. The thermal cycle profile used for amplification was 28 cycles of 1) 94°C for 1 min, 2) 55°C for 1 min, and 3) 72°C for 2 min. A portion (10 μl) of the PCR products was resolved by polyacrylamide gel electrophoresis and visualized by ethidium bromide staining. The density of the BDNF PCR products was analyzed by image analysis software (Mac Bass 3000; Fuji Photo Co. Ltd, Tokyo, Japan) run in a Macintosh system and was expressed as the ratio of the sample density to the density of the β-actin PCR products amplified from an identical RNA sample.

**Measurement of BDNF Content.** BDNF content in the conditioned medium (CM) and brain tissues was determined with a newly developed two-site enzyme immunoassay (EIA) that was described recently (Nitta et al., 1999a). CM was directly applied for the EIA system. Each brain tissue was added to 19 ml/g wet weight homogenizing buffer (0.1 M Tris-HCl (pH 7.4) containing 1 M NaCl, 2% BSA, 2 mM EDTA, 0.2% Na₃N), pulse-sonicated for 30 s, and centrifuged at 100,000 g for 30 min. The supernatant was then mixed vigorously with 100 μl of chloroform and centrifuged at 20,000 g for 15 min, after which the aqueous phase was collected and used for the EIA measurement.

The EIA system for BDNF was based on the method originally developed for NGF (Kaechi et al., 1995). In short, multwell plates (Falcon 3910; Becton Dickinson and Co., Franklin Lakes, NJ) were incubated with 5 μl of anti-BDNF antibody in 0.1 M Tris-HCl buffer (pH 9.0) (10 μg/ml) per well for 12 h, washed with washing buffer (0.1 M Tris-HCl buffer, pH 7.4, containing 0.4 M NaCl, 0.02% Na₃N, 0.1% BSA, and 1 mM MgCl₂), and then blocked with washing buffer containing 1% (v/v) skim milk. Tissue extract or BDNF standard (30 μl) in washing buffer was then added to each antibody-coated well; and incubation was carried out for 5 h at 25°C. After three washes with washing buffer, 30 μl of biotinylated anti-BDNF-antibody (10 ng/ml) in washing buffer was added to each well; and the plate was incubated for 12 to 18 h at 4°C. The biotinylated secondary antibodies were reacted with avidin-conjugated β-galactosidase (Boehringer Mannheim GmbH, Mannheim, Germany) for 1 h. Then following thorough washing with washing buffer, enzyme activity retained in each well was measured by incubation with fluorogenic substrate; 4-methylumbelliferyl-β-D-galactoside (100 μM) in the washing buffer. The intensity of fluorescence was monitored with a 360-nm excitation and 448-nm emission. The detection limit of the EIA was as low as 5 pg/ml. The recovery of BDNF (61.8 pg/ml) exogenously added into the homogenizing buffer following disruption of the rat hippocampus was 80.5%. The value of BDNF content thus obtained was expressed without correction. In the sample from brain tissue of...
mutant mice lacking BDNF gene, any signal could not be detected by the EIA system (A.N. and S.F., unpublished data). NGF, NT-3, and glial cell line-derived neurotrophic factor (GDNF) concentration in the CMs of cultured neurons were measured by the same procedure as the EIA for BDNF.

**Estimation of i.c.v. Administration of 4-MC on BDNF Content in Rat Brain.** Male Std Wistar rats, weighing 180 to 200 g, were anesthetized with sodium pentobarbital (35 mg/kg i.p.) and fixed in a stereotaxic apparatus (Narishige Co. Ltd., Tokyo, Japan). 4-MC (10 nmol) dissolved in 2.5 µl of PBS, or PBS alone, was injected into the left ventricle (A, −0.8; L, 1.6; V, 3.6, according to the atlas of Paxinos and Watson, 1998). Twenty-four hours after the injection, the rats were decapitated while under light ether anesthesia. Brains, including injection site, were removed and cut coronally into three 2-mm-slices, and each slice was separated into four parts as shown in Fig. 1B. The whole hippocampus was dissected and separated into four parts from rostral to caudal as shown in Fig. 2A.

**Estimation of BDNF mRNA Expression.** 4-MC dissolved in PBS was i.p. administered to newborn rats i.p. 5 times at 12-h intervals at a dose of 10 or 150 µg/kg b.wt., which was shown earlier to induce effectively NGF synthesis in rat peripheral tissues (Kaechi et al., 1993, 1995). The rats were anesthetized 4 h after the final injection, and cardio-perfused with cold 4% paraformaldehyde (PFA). The brain was sliced into 5-mm pieces, postfixed with the same fixative for 1 day, and frozen in embedding compound (Tissue-Tek, Sakura Finetechnical Co., Ltd., Tokyo, Japan). Frozen sections of 10-µm thickness prepared with a cryostat (model 1800; Leica Inc., Deerfield, IL) were thawed on coverslips (MS 92130; Sumitomo Bakelite Co., Ltd., Osaka, Japan), and treated with 4% PFA to cross-link them to amino groups on the coverslips. Then the brain sections were used for in situ hybridization.

pGEM-7zf (+) plasmid (Promega Biotec, Madison, WI) with 0.93 kilobase of a full-length of mouse BDNF cDNA was linearized with AvaI or NcoI and used as a template for production of digoxigenin-labeled antisense cRNA (471 bases) or sense cRNA (445 bases) with T7 or SP6 RNA polymerase (Boehringer Mannheim). The brain sections were prehybridized for 1 h at 50°C in a solution containing 50% formamide, 1 mM EDTA, 0.6 M NaCl, 10 mM dithiothreitol, 1× Denhardt’s solution, 0.25% SDS, 10% dextran sulfate, and 200 µg/ml E. coli tRNA, and subsequently hybridized for 16 h at 50°C with 0.4 µg/ml of each cRNA probe dissolved in the same solution used for prehybridization. The sections were washed for 30 min at 37°C with 2× standard saline citrate (20× standard saline citrate: 0.3 M sodium citrate buffer, pH 7.0, containing 3 M NaCl) containing 50% formamide, and then treated with RNase (1 µg/ml) for 30 min at 37°C. Finally, the sections were reacted for 2 h at 25°C with alkaline phosphatase-conjugated antidigoxigenin antibody (Boehringer Mannheim) dissolved in 0.1 M Tris-HCl buffer (pH 7.6) containing 0.3% Triton X-100 and 4% Block Ace (Dainippon Pharm. Co. Ltd., Osaka, Japan). The sections were washed with 0.1 M Tris-HCl buffer (pH 9.5) containing 0.1 M NaCl and 5 mM MgCl₂ for 5 min, and the enzyme activity was visualized following incubation for 16 h at 25°C in the dark in the same buffer.

![Fig. 1. A, no cytotoxicity of i.c.v. injection of 4-MC in the ependymal cells around ventricular space. PBS-injected rat (a) and 4-MC-injected rat (b); scale bar, 10 µm. B and C, regional effects of i.c.v. injection of 4-MC on BDNF content around the injection site of the adult rat brain.](image-url)
Results

Effects of 4-MC on BDNF Content and BDNF mRNA Expression in Culture. In neuronal cultures, the BDNF content was 16.1 ± 2.2 pg/ml in the medium conditioned for 1 day without 4-MC, whereas it increased to 2260 ± 250 pg/ml with the addition of 0.5 mM 4-MC. The level was still significantly high, but lower with 1.0 mM 4-MC. NGF, NT-3, and GDNF contents also were measured in the CM of cultured neurons treated with 4-MC. Another factors contents were not changed except BDNF (Table 1). As shown in Fig. 3, the concentration of 1.0 mM was toxic for neurons cultured with it for 1 day, which may be the reason for the observed reduction in BDNF content. The ratio of RT-PCR product of BDNF mRNA to that of β-actin mRNA was monitored in each RNA preparation to evaluate 4-MC action on BDNF mRNA expression. The ratio significantly increased by the addition of 0.5 or 1.0 mM 4-MC. Toxicity of the 1 mM 4-MC was negligible in this case because the cells were used for RNA preparation following a short-term exposure (6 h) to the 4-MC.

In the cultures of astrocytes, as shown in Fig. 4, the BDNF level was determined with the EIA to be 163 ± 13.9 pg/ml in the medium conditioned for 1 day in the absence of 4-MC. The content rose in a dose-dependent manner from 0.01 to 0.1 mM 4-MC, attained its maximum at 0.1 mM, and then remained constant until 10 mM. RT-PCR analysis showed that the ratio of BDNF product/β-actin product was significantly elevated at the doses of 5 and 10 mM. Concentrations of 4-MC that caused significant increases were much higher in RT-PCR analysis than those in the EIA analysis, probably because of a low accuracy of quantification in the former analysis. 4-MC was not toxic for astrocytes even when the cells were exposed to 10 mM 4-MC.

Effects of Ventricular Injection of 4-MC on BDNF Content in Brain. 4-MC was injected into the left ventricle of the adult rat brain, and the animals were processed 24 h later. We also investigated the toxicity of 4-MC in the ependymal cells around ventricular space. As shown in Fig. 1A, any change in the morphology of ependymal cells could not be observed. The concentration of 4-MC in the injected solution was 4 mM, which was in the toxic range compared with the above-mentioned in vitro experiments. The solution probably will be lowered immediately after the injection because cerebral spinal fluid diluted it. Because the local BDNF level seemed to depend on the concentration of 4-MC that diffused from the ventricular space, we defined areas differently apart from the 4-MC-injected ventricle in two ways. The BDNF content as a function of 4-MC was expressed as a fold increase of that of the respective portions in animals treated with only PBS. First, coronal brain slices including the injection site were cut into four particular portions, and the pieces were alphabetically numbered as indicated in Fig. 1A. The BDNF content was highest in portion E, which included the 4-MC-injected ventricle. Portion B, including the contralateral ventricle, ranked next to portion E. Portion F, caudally adjacent to E, also showed a significantly elevated content. Second, the ipsilateral left hippocampus was cut into four portions from rostral to caudal, and numbered as indicated in Fig. 2A. The BDNF content was the highest in portion I, which faced the ventricle, and gradually decreased in portions II, III, and IV as a function of distance from the ventricle. These observations demonstrate that 4-MC facilitated an increase in BDNF content in the brain in association with diffusion of 4-MC into the brain parenchyma.

Effects of Peripheral Administration of 4-MC on BDNF mRNA Expression in Infant Rat Brain. In control rat brain, BDNF mRNA is predominantly expressed in the cerebral cortical neuronal layers II, III, V, and VI, and in the

<table>
<thead>
<tr>
<th>Neurotrophic Factors</th>
<th>4-MC Concentration (pg/ml)</th>
<th>4-MC Concentration (mM)</th>
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<tbody>
<tr>
<td></td>
<td>0</td>
<td>0.3</td>
</tr>
<tr>
<td>NGF</td>
<td>5.3 ± 2.1</td>
<td>4.7 ± 3.1</td>
</tr>
<tr>
<td>BDNF</td>
<td>21.1 ± 0.3</td>
<td>260 ± 83*</td>
</tr>
<tr>
<td>NT-3</td>
<td>55.5 ± 2.8</td>
<td>21.5 ± 6.5</td>
</tr>
<tr>
<td>GDNF</td>
<td>2.9 ± 0.1</td>
<td>2.1 ± 0.9</td>
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* p < .001 compared with control (Tukey’s test).
hippocampal neuronal layers (Phillips et al., 1990). The BBB has not been fully established in newborn and infant animals (Cornford and Cornford, 1986), which may permit 4-MC to cross the BBB. Therefore, we tested this possibility with infant rats. Repetitive i.p. administration of 4-MC facilitated BDNF mRNA expression in the cerebral cortex and the hippocampus in a dose-dependent manner, when judged by in situ hybridization with an antisense probe (Fig. 5). The cells that responded to the 4-MC were neurons, and astrocytes and oligodendrocytes were unresponsive. No signals could be detected in the brain sections exposed to the sense probe.

Discussion

BDNF protein and mRNA are predominantly expressed in neurons, and regulated by glutamate or γ-aminobutyric acid neurotransmission in vivo and in vitro (Zafra et al., 1991, 1992). Enhanced expression occurred following establishment of long-term potentiation (Rutherford et al., 1997). BDNF thus seems to participate in various activity-dependent events, including synapse plasticity. In this study we found BDNF mRNA expression in cultured neurons and astrocytes (Figs. 3 and 4). Furthermore, BDNF secretion into the culture medium was detected in both types of cultures by use of a sensitive EIA (Figs. 4 and 5). The basal level of secretion in astrocytes was comparable with that in neurons in spite of a failure of BDNF expression in astrocytes in the in vivo brain (Furukawa et al., 1998). This difference may reflect astrocytic properties that may be evoked by brain damage during preparation of the astrocytes for the in vitro study. Regardless, we confirmed that astrocytes have the ability to synthesize and secrete BDNF. The transport system of BDNF in the CNS is not completely revealed. Physiologically active 125I-labeled BDNF was transported retrogradely following injection into the rat sciatic nerve (DiStefano et al., 1992). Anterograde transport system of BDNF has recently been studied in the developing visual system (Bartheld et al., 1996). 4-MC markedly enhanced BDNF mRNA expression and BDNF secretion in cultured neurons and astrocyte (Figs. 3 and 4). As shown in Fig. 5, 4-MC increased BDNF mRNA expression in the neuronal cells in vivo. The basal level of secretion in astrocytes was comparable with that in neurons in spite of a failure of BDNF expression in astrocytes in the in vivo brain. Thus, 4-MC did not change the localization, but did change the contents of BDNF in brain. The present data suggest that 4-MC does not affect the transport system(s) of BDNF.

The stimulative nature of 4-MC was originally noticed during experiments designed with cultured astrocytes to examine regulatory mechanism of NGF synthesis (Furukawa et al., 1993). Presently, 4-MC also has stimulatory effects for BDNF synthesis and secretion in cultured neuronal cells. Together with these results, there is a high probability that 4-MC could stimulate many kinds of a growth factor. We measured other neurotrophic factors, such as NGF, NT-3, and GDNF, contents in the CMs of cultured neurons with 4-MC. 4-MC did not change any neurotrophic factor concentration except that of BDNF (Table 1). Thus, the stimulatory effects of 4-MC are within limit to synthesis of NGF and BDNF in cultured astrocyte, or BDNF in cultured neurons. It was found to stimulate the production of physiologically active NGF in the peripheral nervous system in rats (Kaechi et al., 1993), to facilitate nerve sprouting following sciatic nerve transection (Kaechi et al., 1995), and to ameliorate the neuropathy associated with diabetes (Hanaoka et al., 1994). The therapeutic availability of 4-MC in the peripheral nervous system suggests the possible use of drugs with neurotrophic factor synthesis-stimulating activity, and the involvement of

Fig. 3. Stimulatory effects of 4-MC on BDNF content in the culture medium and cellular mRNA content in cultured neurons. Neurons of the hippocampi of 18-day-old rat embryos were cultured in the serum-free defined medium containing various concentrations of 4-MC as described in Results. A, CMs were taken 24 h after the addition of 4-MC and their BDNF content was measured by the EIA. B, total cellular RNAs were prepared 6 h after the addition of 4-MC. An aliquot of the total RNA (500 ng) was subjected to the RT-PCR analysis as described in the text. BDNF and β-actin cDNAs were separately amplified with 28 thermal cycles. The PCR products were electrophoresed and visualized by ethidium bromide staining. The intensity of each band was digitized with an image analyzer and the ratio of the intensity of the BDNF band to that of the β-actin band was calculated for each culture dish. Values are expressed as means ± S.E. of six or seven dishes. *p < .05; **p < .01; and ***p < .005 compared with control (Tukey test).
neurotrophic factors in addition to NGF in the response to 4-MC. Doses for stimulation were relatively high, but comparable with those required for stimulation of NGF synthesis in cultured non-neuronal cells (Furukawa et al., 1989). This may be due to the instability of 4-MC in the culture medium, and/or weak activity on penetration through the plasma membrane; however, the dose of 4-MC optimal for in vivo stimulation is low enough for practical use in the case of NGF (10 μg/kg b. wt.) (Kaechi et al., 1995; Saita et al., 1996). Therefore, a substantial increment of BDNF content could be anticipated by i.v. administration of 4-MC in a small aliquot (10 nmol) (Figs. 1 and 2). A good correlation between the increase in BDNF content and expected 4-MC diffusion supports the direct effect of 4-MC on BDNF content. So far the stimulation of BDNF mRNA expression has been observed with agents that increase cAMP (Zafra et al., 1992) in astrocytes, and with glutamate receptor agonists in neurons (Zafra et al., 1990; Sano et al., 1996). These observations suggest cAMP-dependent and/or Ca2⁺-induced signalings. To obtain the evidence for the supposition, we did further experiments to investigate the mechanism(s) of BDNF synthesis. BDNF contents significantly increased in CM of cultured neurons treated with Ca2⁺ ionophore or dibutyryl cAMP (A.N. and S.F., unpublished data). These results suggest that Ca²⁺ and/or cAMP system regulate BDNF synthesis. In cultured cortical neurons, Ca²⁺ influx regulates BDNF transcription by a cAMP response element-binding protein family transcription factor (Tao et al., 1998). However, intracellular cAMP contents in cultured neurons was not changed by 4-MC (A.N. and S.F., unpublished data). These data suggest that the regulatory system of 4-MC is independent of a known pathway at the present. How does the 4-MC up-regulate BDNF synthesis? There is no plausible mechanism to explain the 4-MC action toward the BDNF gene at present. 4-MC is thought to be incorporated into the cells via a mechanism similar to that for “uptake 2” of catecholamines (Furukawa et al., 1986), which is not mediated by adrenergic receptors, and to regulate NGF gene expression via both protein kinase C- and cAMP-independent mechanisms in cultured astrocytes (Furukawa et al., 1993). A long-lasting enhancement of c-jun mRNA expression was a response to 4-MC (Omae et al., 1994). c-jun generates activator protein-1 that drives NGF...
gene expression (Hengerer et al., 1990). However, activator protein-1 is not required for the activation of BDNF gene (Sano et al., 1996).

The most serious problem of 4-MC for therapeutic use is its inability to cross the BBB of the mature brain. It is reported that the BBB is partially destroyed in some neurological disorders such as multiple sclerosis and Alzheimer’s disease (Elovaara et al., 1985; Cornford and Cornford, 1986). This may become conversely advantageous for site-specific delivery of the drug, if the BBB failure occurs at sites associated with the disease. In fact, in our present study repetitive peripheral administration of 4-MC enhanced BDNF mRNA expression in infant rats, in which the BBB has not yet fully been established (Fig. 5). Otherwise, chemical modifications that could permit delivery of 4-MC into the brain would be promising for patients with healthy BBB functions. Kouroumakis et al. (1997) succeeded in delivering a substantial amount of 4-MC esterized with dihydropyridine into the brain by peripheral administration, and observed a significant elevation of brain NGF content.

Recent investigations have added novel roles of BDNF action in the CNS, such as facilitation of neural transmission (Tongiorgi et al., 1997) and expression of genes acting on brain development (Ringstedt et al., 1998). Furthermore, it has been recently reported that a stimulating environment has positive effects on cerebral health, providing some resilience to cerebral insults by increasing BDNF expression (Young et al., 1999). These observations demonstrate the importance of BDNF for brain development, maintenance of functions, and protection of neurons from various insults, and suggest that medical enhancement of BDNF synthesis in the brain should prove profitable for prevention and amelioration of particular degenerative neurological disorders. From this point of view, the finding of present study, that 4-MC can elevate BDNF content and/or BDNF mRNA expression in vivo in the brain, is encouraging.

Our present results demonstrate that stimulators of BDNF in vitro, such as 4-MC, are promising candidates as therapeutic drugs for certain neurological diseases. Much more investigation is needed before 4-MC could be used as an effective and safe drug for clinical use.

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Send reprint requests to: Shoei Furukawa, Ph.D., Laboratory of Molecular Biology, 5-6-1 Mitahora-higashi, Gifu 502-8585, Japan. E-mail: furukawa@gifu-pu.ac.jp