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Cyclic AMP/Protein Kinase A Signal Attenuates Ca^{2+}-Induced Fibroblast Growth Factor-1 Synthesis in Rat Cortical Neurons

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Fibroblast growth factor (FGF)-1 is increased in particular brain regions after birth, suggesting an involvement of some regulatory neuronal circuits. To address the neuronal activity responsible for FGF-1 synthesis, effects of various neurotransmitter receptor activation on cellular FGF-1 content were examined using cultured rat cortical neurons. Histamine, glutamate, carbachol, serotonin or γ-aminobutyric acid (GABA) caused an increase of FGF-1 content. Because this effect was mimicked by (1) *N*-methyl-D-aspartate, a glutamatergic agonist; (2) Ca^{2+} ionophore; (3) depolarization with high concentration of KCl, but was abolished in Ca^{2+}-free medium, Ca^{2+} influx was thought to trigger FGF-1 synthesis. Such Ca^{2+}-mediated enhancement of FGF-1 synthesis, however, did not occur in the presence of norepinephrine (NE), but was restored by KT-5720, an inhibitor of protein kinase A (PKA), suggesting an interplay between Ca^{2+}-activated and cAMP/PKA signals for neuronal FGF-1 synthesis. This mechanism was proved to function in vivo by stimulation of FGF-1 expression in neurons of the cerebral cortex after intracerebral administration of propranolol, an antagonist of adrenergic β receptors. This demonstrates that FGF-1 synthesis is essentially upregulated by Ca^{2+} influx through excitatory neuronal activities, but such an effect is abolished by neurotransmission that evokes cAMP/PKA signals. FGF-1 produced is thought to act on establishment and maintenance of particular neuronal circuits in the brain, which may be one of the ways neurotransmitters regulate brain function.

Key words: fibroblast growth factor-1 (FGF-1); neurotrophic factor; synthesis; neurotransmitter; Ca^{2+}; cyclic AMP; protein kinase

Fibroblast growth factor (FGF)-1 is a prototype of the structurally related FGF family of proteins (Basilico and Moscatelli, 1992; Smallwood et al., 1996) that have 30–50% amino acid sequence homology and a similar exon/intron structure in the coding region of their gene (Wang et al., 1989; Goldfarb, 1990). FGF-1 promotes neuronal regeneration (Cordeiro et al., 1989) and survival (Unsicker et al., 1987) of cultured peripheral neurons, stimulates glial proliferation (Besnard et al., 1989; Davis and Stroobant, 1990), and exhibits neurotrophic effects on neuronal cultures and neuronal cell lines derived from the central nervous system (CNS) (Rydel and Greene, 1987; Unsicker et al., 1987; Lipton et al., 1988; Rifkin and Moscatelli, 1989; Mattson et al., 1989; Goldfarb, 1990). FGF-1 is expressed predominantly in neural tissues including brain, spinal cord, and retina, and exerts its effect through high-affinity receptors distributed throughout the nervous system (Heuer, 1990; Wanaka et al., 1990; Partanen et al., 1991). FGF-1 expression in brain is found exclusively in neuronal cells (Lipton et al., 1988; Heuer, 1990; Wanaka et al., 1990), but not in glial cells, and increases with brain development after birth (Ishikawa et al., 1991, Partanen et al., 1991). The role of FGF-1 in the CNS, however, is not yet well understood.

We have found that the FGF-1 level is increased within 3 weeks after birth, predominantly in the thalamus, pons/medulla, and cerebellum of rat brain, suggesting its involvement in the differentiation of particular neurons or neuronal circuits (Ishikawa et al., 1991). Such a development-dependent expression is observed in members of the neurotrophin family. Expression of nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) is enhanced postnatally in the brain regions in which FGF-1 expression is low, such as the hippocampus and cerebral cortex (Shelton and Reichardt, 1986; Das et al., 2001). NGF and BDNF have been shown expressed in an activity-dependent manner through excitatory and suppressive neuronal circuits (Zafra et al., 1990, 1991; Con-

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dorelli et al., 1994), demonstrating the involvement of neurotransmission in the neurotrophin synthesis (Thoenen et al., 1991). These findings suggest a possible activity- or transmission-dependent regulation of FGF-1 synthesis. Riva et al. (1996) have shown that cellular cAMP negatively regulates FGF-1 mRNA expression in cultured astrocytes; however, regulatory mechanisms of FGF-1 gene expression in neurons, the predominant source of FGF-1 in CNS, remain unknown.

We evaluated regulation of cellular FGF-1 in cultured rat cortical neurons using an enzyme immunoassay system (EIA), because of its high sensitivity, specificity for FGF-1, and superiority in quantification (Ishikawa et al., 1991). Our present results demonstrate that FGF-1 synthesis is enhanced by Ca^{2+}-activated signaling, but this enhancement is downregulated by cAMP/protein kinase A (PKA) signaling. Indeed, the blockade of cAMP/PKA signaling by propranolol, an antagonist of β-adrenergic receptors, administered into the adult rat cerebral cortex resulted in marked enhancement of FGF-1 mRNA and protein expressions. These in vitro and in vivo results suggest interplay between both signal transduction pathways to regulate FGF-1 synthesis in particular neurons of rat brain.

MATERIALS AND METHODS

Materials

FGF-1 and anti-FGF-1 antisera were prepared as described previously (Ishikawa et al., 1991). Anti-glial fibrillary acidic protein (anti-GFAP) antibody was purchased from DAKO Chemicals (Copenhagen, Denmark); and anti-microtubule-associated protein 2 (anti-MAP2) antibody, from Chemicon (Temecula, CA). γ-Aminobutyric acid (GABA), glutamate, carbachol, norepinephrine (NE), 5-hydroxytryptamine (5-HT; serotonin), histamine, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), quisqualic acid, A23187, H89, and KT-5720 were obtained from Research Biochemicals (Natick, MA). α-Bungarotoxin, ranitidine, kainic acid and cycloheximide came from Sigma (St. Louis, MO).

Cell Cultures

Neuronal cells were cultured from the cerebral cortices of 17-day-old rat embryos as described previously (Nakajima et al., 1993). Briefly, the cerebral cortices were incubated in phosphate-buffered saline (PBS) containing 0.25% trypsin, 10 mM glucose, and DNase (6 μg/ml; Sigma) for 20 min at 37°C, and triturated with a plastic pipette to dissociate the tissue into single cells. After centrifugation (900 × *g* for 3 min), the cell pellet was resuspended in medium composed with Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo, Japan) and nutrient mixture Ham's/F-12 (1:1; Gibco BRL), which contained 5% horse serum and 5% newborn calf serum. Suspended cells were plated in culture vessels (10^5 cells/cm^2) precoated with poly-DL-ornithine (0.5 μg/ ml; Sigma). After a 24-hr culture, the medium was changed to a serum-free one containing insulin (5 μg/ml; Sigma), transferrin (5 μg/ ml; Sigma), progesterone (2 pmol/ml; Sigma) and 5% bovine serum albumin (BSA). The cells were then cultured for 3 days, and provided for the experiments. Over 98% of the cells expressed MAP2 when stained with the specific antibody, demonstrating that most of cells in culture were neurons.

Determination of FGF-1 Content

Cells were cultured in 35-cm dishes for 24 hr in the presence or absence of various reagents. The culture media were collected, and the cells were washed twice with PBS, gently detached with a rubber scraper from the culture dishes into 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.6) containing 1 M NaCl, 2 mM EDTA, 2% BSA and 80 U aprotinin/ml, and disrupted by centrifugation at 100, 000 × *g* for 10 min. An aliquot of the culture media or the cell extracts were used for the EIA measurement. The cell number in five arbitrarily selected fields (total area, 2.4 mm^2) of each culture dish was counted under a phase-contrast microscope before scraping to calculate the total cell number. The values of FGF-1 content/10^6 cells were expressed as percent of the value obtained in the absence of the reagent.

The most critical event during extraction is the possible nonspecific binding of FGF-1 molecules to the surface of cell debris. To check this, we added exogenous FGF-1 to the extraction buffer before the second centrifugation. Recovery of exogenous FGF-1 (10–80 pg/ml) was about 95%, indicating that the loss of FGF-1 during extraction was negligible.

A two-site EIA for FGF-1 was carried out as described previously (Ishikawa et al., 1991). In short, immunoglobulin (Ig)G antibody (0.1 mg/ml) in 0.1 M Tris-HCl buffer (pH 7.6) was coated onto the well surface of 96-well U-bottom multiwell plates (20 μl/well) at room temperature for 1 hr. For evaluation of background signal, control wells were treated with normal rat IgG. The antibody IgG or control IgG solution was removed, and non-occupied space was blocked by incubation with 100 μl/well of 1% (wt/vol) skim milk for 1 hr. Each well was washed with 0.1 M Tris-HCl buffer (pH 7.6) containing 0.4 M NaCl, 0.1% BSA, 1 mM $MgCl_2$, and 0.02% NaN_3 (washing buffer) after every reaction. Each well then received 30 μl of test sample or serially diluted bovine FGF-1 as the standard. After incubation for 2 hr at room temperature, each well then received 30 μl of biotinylated affinity-purified antibody (0.2 μg/ml) in the washing buffer containing 1% normal rabbit serum. After overnight incubation at 4°C, 30 μl/well of β-D-galactosidase-conjugated streptavidin was added; 1 hr later, bound enzyme activity was measured by the addition of 30 μl of 30 μM 4-methylumbelliferyl-β-D-galactoside (Sigma). The amounts of 4-methylumbelliferone formed were analyzed fluorometrically (Model 850; Hitachi, Tokyo, Japan).

Analysis of FGF-1 mRNA Expression

The reverse transcription polymerase chain reaction (PT-PCR) was used to evaluate the FGF-1 and β-actin mRNA levels, as described previously (Miwa et al., 1997; Nitta et al., 1999). Total RNA of cultured cells or brain tissues was prepared by using Isogen (Nippon Gene). First-strand cDNA was synthesized with PowerScript Reverse Transcriptase (Clontech Laboratories) according to the manufacturer's instruction. The synthesized cDNA was amplified under the following conditions: denaturation, 94°C, 30 sec; annealing, 68°C (FGF-1) or

55°C (β-actin), 30 sec; extension, 68°C, 1 min. The cycle was repeated 38 times for FGF-1, and 26 times for β-actin. The amounts of all PCR products were within range of linear increase at indicated thermal cycles. The following primers were used: FGF-1 forward-primer, 5′-TTCAACCTGCCTCTAGGAAACT-3′, reverse-primer, 5′-CTTACAGCTCCCGTTCTTCTTG-3′; β-actin forward-primer, 5′-GTGGGCCGCTCTAGGCACCAA-3′, and reverse-primer, 5′-CTCTTTGATGTCACGCACGAT-3′. PCR products were electrophoresed in 2% agarose gels, and stained with ethidium bromide. DNA sequences of the PCR products were analyzed and their identity was confirmed.

Intracellular cAMP Level

Cells cultured in 35-mm dishes were washed with PBS, scraped from the culture vessels, and suspended in 0.05 M acetate buffer, pH 5.8, containing 0.2 mg/ml BSA. The cells were then disrupted by sonication and centrifuged at 100,000 × *g* for 10 min. The supernatant fluids were used for determination of cAMP level, which was measured with an EIA kit (Amersham) according to the manufacturer's instructions.

Monitoring of Ca^{2+} Influx

Cortical neurons were cultured in 3.5-cm dishes precoated with poly-DL-ornithine (10^5 cells/cm^2). The dishes were then washed with 10 mM HEPES buffer (pH 7.4) containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 0.55 M glucose, after which 1 μM fluo-3 AM (Molecular Probes) was added and incubation was carried out for 30 min at room temperature. The fluorescence of individual cells was recorded at 488 nm with a fluorescence microscope (Axiovert S 100; Carl Zeiss, Germany) after the addition of 100 μM glutamate, 100 μM NE, or both together.

Administration of Propranolol Into the Cerebral Cortex

All the experiments using animals were carried out according to the guidelines for animal experimentation of the NIH Guide for Care and Use of Laboratory Animals. Male Wistar rats (7 weeks old) purchased from Japan SLC (Shizuoka, Japan) were anesthetized with sodium pentobarbital (35 mg/kg, intraperitoneally), and fixed in a stereotaxic apparatus (Narishige, Tokyo). Five injections of 1 μl of propranolol dissolved in PBS (100 μg/μl) were made in the right side of the cerebral cortex. Stereotaxic coordinate of injection site a, b, c, d, or e (in mm anterior: −4.0, −3.0, −5.0, −5.0, or −3.0; lateral (lat.): 3.0, 2.0, 2.0, 4.0, or 4.0; height (ht.): 1.5, 1.3, 1.2, 1.4, or 1.8) was determined from the atlas of Paxinos and Watson (1998). PBS was then injected into the contralateral left side of the cerebral cortex. For RT-PCR analysis, rats were decapitated under the slight ether-anesthetized conditions at the indicated times after the injection. Brains were removed, and cut coronally into a 2-mm slice including all injection sites. The tissue containing all injection sites of propanolol or PBS was cut further into a $(2\ mm)^3$ cube, and used for RNA preparation. For immunohistochemical study, rats were anesthetized 24 hr after the injection, and cardio-perfused with 4% paraformaldehyde solution prepared with 0.1 M phosphate buffer, pH 7.3.

Immunohistochemical Study of Brain Sections

The brains were dissected out, cut coronally into 5-mm slices, and post-fixed for 2 hr in cold 4% paraformaldehyde solution (the fixative). Slices were then soaked in PBS containing 30% (wt/vol) sucrose for 1 day, and frozen in embedding compound (Miles). Coronal sections of 30-μm thickness were cut with a cryostat (Model CM 1800; Leica), and then thawed on slide glasses bearing covalently linked amino groups (Matsunami Glass, Tokyo, Japan). Tissue sections were dried and soaked in the fixative for 15 min to cross-link covalently the tissue sections to slide glasses. Sections were rinsed in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.3% (vol/vol) Triton X-100 for 15 min, boiled in Antigen unmasking solution (Vector) for 30 sec, and incubated for 1 day at 4°C with antibody against FGF-1 (Santa Cruz Biotechnology, Santa Cruz, CA), NeuN (Chemicon), or GFAP (Boehringer Mannheim Biochemicals) optimally diluted in PBS containing 10% FCS and 2% Block Ace (Dainippon Pharm., Osaka, Japan). Finally, the sections were reacted for 4 hr at 20°C with Alexa Fluor 488-labeled anti-rabbit IgG donkey antibody (Molecular Probes), or Alexa Fluor 546-labeled anti-mouse IgG goat antibody (Molecular Probes) diluted in PBS containing 10% FCS and 2% Block Ace, and observed for fluorescence with a confocal laser microscope (Model LSM510; Carl Zeiss).

RESULTS

Detection of FGF-1 Protein in Cultured Cells

For FGF-1 measurement, we used an EIA system having a sensitivity as low as 0.1 pg/ml of FGF-1 (Ishikawa et al., 1991). Even FGF-2, which has 55% amino acid sequence homology with FGF-1, did not give any significant signal. The intracellular FGF-1 content was 4.4 ± 0.5 pg/10^6 cells ($n = 6$) in cultured neurons, but the medium conditioned for 1 day by the cells was below the detection limit, confirming that FGF-1 lacks a signal peptide (Hicks et al., 1996). The intracellular FGF-1 content was maximally reduced to 20% of the original value by 24 hr after the addition of 100 μM cycloheximide, a protein synthesis inhibitor, demonstrating that most of the intracellular FGF-1 turned over within 24 hr. In light of this finding and the lack of secretion, the amount of intracellular FGF-1 was therefore considered to reflect the rate of FGF-1 synthesis.

Regulation of Intracellular FGF-1 Content or mRNA Level in Cultured Neurons

Among the ligands for neurotransmitter receptor tested, histamine, glutamate, and carbachol caused over a fivefold increase in the FGF-1 content of cultured cortical neurons (Fig. 1A). The time-dependent change after the addition of glutamate is shown in Figure 1B. The increase was significant by 3 hr, maximal by 12 hr, and sustained until 24 hr, demonstrating that the response was rapid and long lasting. The involvement of Ca^{2+}-activated signaling was postulated, because histamine H2 receptor (Bossa et al., 1991; Koizumi and Ohkawara, 1999), glutamate receptor (Hartley et al., 1993), and muscarinic-acetylcholine receptor (Wang et al., 2002) were reported to mediate

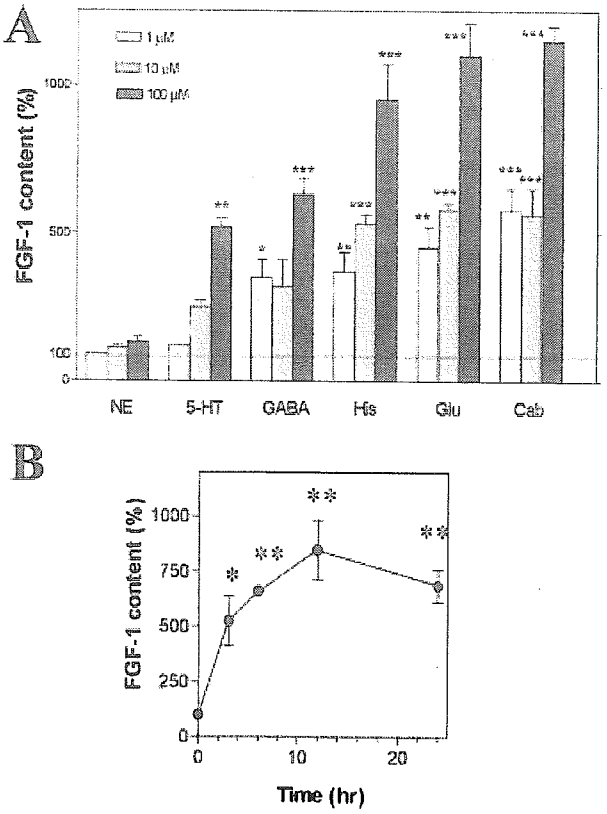

Fig. 1. Effects of various neurotransmitter receptor activation on FGF-1 content of cultured cortical neurons (**A**) and a time-dependent change in FGF-1 content after treatment with glutamate (**B**). Rat cortical neurons (10^5 cells/cm^2) were cultured in the serum-free defined medium containing insulin, transferrin, progesterone, and 5% BSA for 3 days. A: The cells were then treated for 24 hr with various ligands for neurotransmitter receptors prepared in the serum-free defined medium. The cell extracts were prepared and used for the EIA measurement of FGF-1. The FGF-1 content was 4.4 ± 0.5 pg/10^6 cells (n = 6) in control cultures. The values are the mean ± SE of the percent increase of six culture dishes. Significance vs. no-treatment group, $*P < 0.05$; $**P < 0.01$, $***P < 0.005$, Bonferroni's t-test for multiple pairwise comparisons. B: Confluent neurons were cultured in the serum-free defined medium containing 100 μM glutamate for the indicated times. FGF-1 content in the cell extracts was measured by EIA. Values are the mean ± SE of the percent control of six culture dishes. The SE is not shown when it is less than the width of the symbol. Significance vs. control, $*P < 0.01$; $**P < 0.005$, Bonferroni's t-test for multiple pairwise comparisons. NE, norepinephrine; 5-HT, 5-hydroxytryptamine/serotonin; His, histamine; Glu, glutamate; Cab, carbachol.

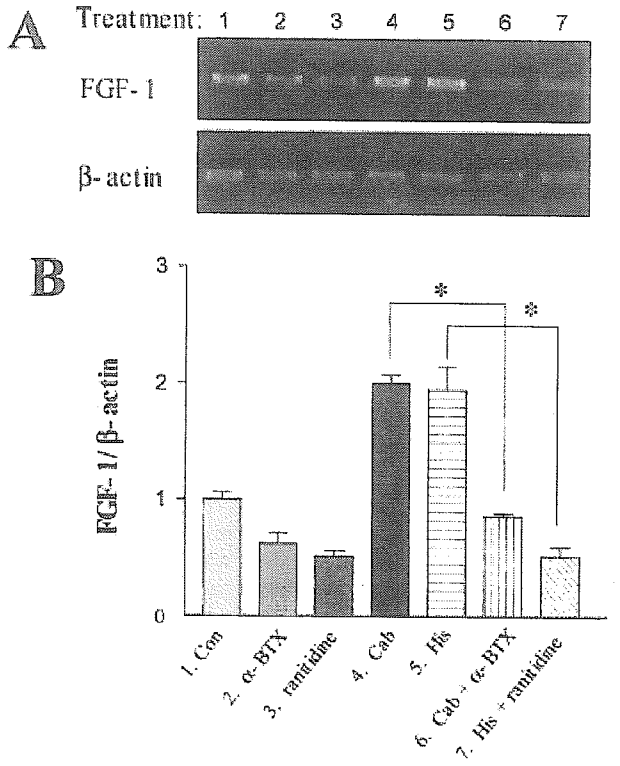

Fig. 2. Effects of antagonists against ACh or histamine receptors on FGF-1 mRNA expression of cultured cortical neurons. Rat cortical neurons were cultured for 3 days in the serum-free defined medium as described in the legend to Figure 1. **A:** 1, Control cells were maintained for 12 hr without any drugs. Cells were treated for 12 hr with: α-bungarotoxin (100 nM), an antagonist against nicotinic ACh receptors (2); ranitidine (50 μM), an antagonist against H2 receptors (3); carbachol (100 μM) alone (4); histamine (100 μM) alone (5); carbachol (100 μM) + α-bungarotoxin (100 nM) (6); or histamine (100 μM) + ranitidine (50 μM) (7). An aliquot of total RNA prepared from each culture was subjected to RT-PCR analysis using primers specific for FGF-1 or β-actin, and PCR products were electrophoresed in a 2% agarose gel. Gels were stained with ethidium bromide; photographs representing a typical result are shown. Structures of amplified DNA fragments were verified by sequencing analysis. The amounts of all PCR products were within range of linear increase at the indicated thermal cycles. **B:** The bands of PCR products corresponding to FGF-1 or β-actin mRNA-derived cDNA were densitometrically quantified, and the ratio of FGF-1/β-actin band intensity was calculated. Values are expressed as the means ± SE of the ratio of FGF-1/β-actin of five cultures. Significant differences were determined by Tukey's test. $*P < 0.005$. Con, control; α-BTX, α-bungarotoxin; Cab, carbachol; histamine, His.

Ca^{2+} influx or intracellular Ca^{2+} release. GABA moderately elevated the content at 1 μM and 100 μM, and 5-HT, at 100 μm. However, NE had no effect (Fig. 1).

The specificity of the ligands for neurotransmitter receptors such as carbachol and histamine was tested pharmacologically. First, the effect of carbachol on FGF-1 mRNA expression in cultured rat cortical neurons was examined in the presence or absence of α-bungarotoxin, an antagonist for nicotinic acetylcholine receptors (AChRs). FGF-1 mRNA expression was upregulated by the addition of carbachol in the absence of α-bungarotoxin, but unchanged in the presence of α-bungarotoxin (Fig. 2). Second, the effect of histamine on FGF-1 mRNA expression was evaluated in the pres-

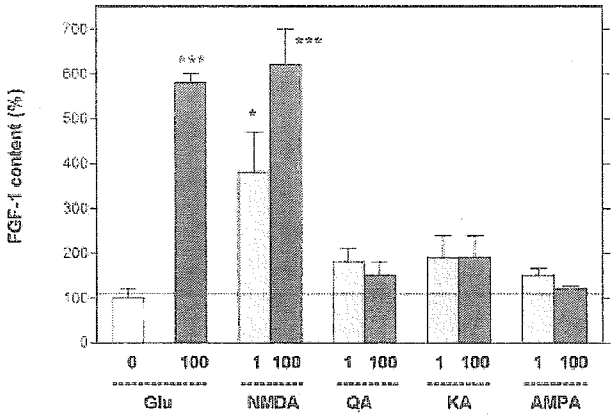

Fig. 3. Effects of the agonists for the glutamate receptors on FGF-1 content of cultured cortical neurons. Cortical neurons were treated with glutamate (Glu), or glutamate receptor agonists, NMDA, quisqualic acid (QA), kainic acid (KA) or AMPA for 24 hr at 1 or 100 μM. FGF-1 content in the cell extracts was measured by EIA. Intracellular FGF-1 content/10^6 cells is expressed as the percent of the value obtained in the absence of the reagent (control; Con). The values are the mean ± SE of six culture dishes. Significance vs. control, $*P < 0.05$; $***P < 0.001$, Bonferroni's t-test for multiple pairwise comparisons.

ence or absence of ranitidine, an antagonist for H2 receptors. FGF-1 mRNA expression was enhanced by the addition of histamine when ranitidine was absent, but unchanged when ranitidine was present (Fig. 2). These results demonstrated that FGF-1 mRNA expression by carbachol and histamine was specifically upregulated via α-bungarotoxin-sensitive AChRs and histamine H2 receptors, respectively. This implied that both drugs could enhance the synthesis of FGF-1 in a receptor-mediated specific manner.

To elucidate the mechanism of FGF-1 upregulation, we focused on the effects of glutamate. Glutamate receptors are activated selectively by agonists such as NMDA, quisqualic acid, kainic acid, or AMPA. Quisqualic acid is a full selective agonist for metabotropic glutamate receptors. The effect of glutamate was mimicked only by NMDA (Fig. 3), suggesting that activation of the NMDA glutamate receptor, resulting in Ca^{2+} influx, is involved in the mechanism to increase the neuronal FGF-1 content. To test this possibility, we examined the effect of glutamate or carbachol on neurons under Ca^{2+}-free conditions. The absence of Ca^{2+} completely abolished the effect (Fig. 4B), suggesting that FGF-1 synthesis is upregulated by the influx of Ca^{2+} and its subsequent signaling. Agents that stimulate Ca^{2+} influx via mechanisms different from the mechanism involving ligand binding-gated Ca^{2+} channels were then examined. Cellular depolarization by a high concentration of KCl (30 mM), which induces Ca^{2+} influx through voltage-dependent Ca^{2+} channels (Tabuchi et al., 2000), and by the Ca^{2+} ionophore A23187, which causes passive Ca^{2+} incorporation through the membrane (Reed and Lardy, 1972), also increased the FGF-1 content to a level comparable to that obtained with glutamate (Fig. 3A). These results demonstrate that intracellular Ca^{2+} influx is a key event to trigger FGF-1 gene expression in cultured cortical neurons.

Interaction Between cAMP/PKA and Ca^{2+}-Activated Pathways in the Regulation of Neuronal FGF-1 Synthesis

The cAMP/PKA pathway may be involved in the mechanisms that regulate the FGF-1 content in neurons (Iyengar, 1996). We tested the effects of glutamate or carbachol on neuronal FGF-1 content in the presence of 100 μM NE, and found that the increase in FGF-1 content was suppressed completely (Fig. 5A). Furthermore, co-administration of NE with 10 μM KT-5720, an inhibitor of PKA, restored the stimulatory activity of the neurotransmitters (Fig. 5A), suggesting that the adrenergic β-receptor-mediated cAMP/PKA signal pathway was involved in inhibition of the Ca^{2+}-induced FGF-1 synthesis. The interaction between glutamate and NE was also evaluated by examining FGF-1 mRNA expression. The ratio of FGF-1 mRNA to that of β-actin was significantly higher in glutamate-treated cells than in cells treated with NE alone and with NE and glutamate in combination (Fig. 5B). This was in good agreement with the change in FGF-1 content, demonstrating that NE antagonized the Ca^{2+}-activated signal pathway leading to FGF-1 gene expression.

We next monitored cAMP content and Ca^{2+} influx. Treatment with glutamate alone caused a slight but nonsignificant elevation of cAMP content during all experimental periods, whereas administration of NE alone or NE and glutamate in combination led to a significant increase in cAMP content (Table I). Combined treatment with NE and glutamate gave a much higher cAMP content than did NE alone at all times tested. These observations demonstrate that the high cAMP level was induced by the addition of NE irrespective of the coexistence of glutamate. The degree of Ca^{2+} influx elicited by glutamate was essentially unchanged in the presence of NE (Fig. 6), suggesting that there is indeed interplay between cAMP/PKA and Ca^{2+}-activated signals in neuronal FGF-1 synthesis.

Effects of Intracerebral Administration of Propranolol on Expression of FGF-1 Protein or mRNA in the Cerebral Cortex

To analyze the possible involvement of cAMP/PKA signal in the regulation of FGF-1 synthesis in the brain, we administered propranolol (100 μg/μl), a potent antagonist of adrenergic β-receptors, into the right side of the adult rat cerebral cortex, and compared the expression of FGF-1 mRNA with that of the left side, into which vehicle (PBS) had been injected (Fig. 7). FGF-1 mRNA was increased around the injection sites 6 and 12 hr after the injection, and returned to the original level by 24 hr (Fig. 8A). The increase of the expression occurred specifically and locally around the propranolol injection sites (Fig. 8B). The areas

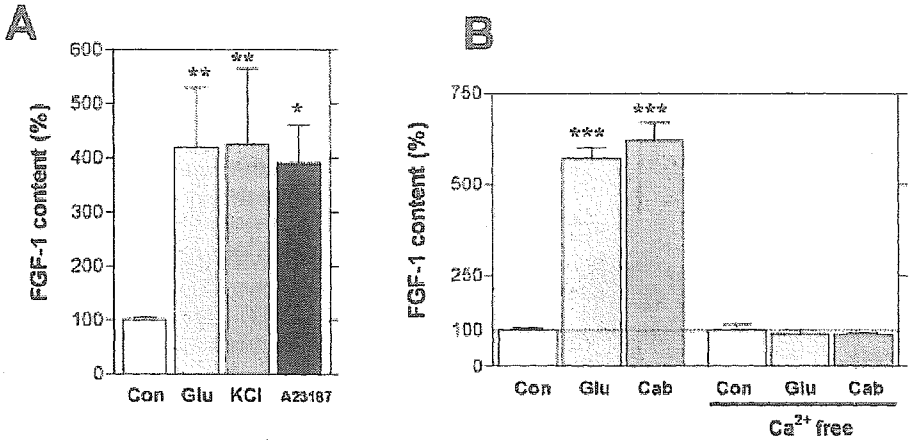

Fig. 4. Effect of agents that stimulate Ca^{2+}-influx (**A**) and the influence of Ca^{2+}-free medium (**B**) on FGF-1 content of cultured cortical neurons. A: Cortical neurons were treated with glutamate (100 μM, Glu), KCl (30 mM), or A23187 (0.6 μM) for 24 hr. B: Cortical neurons cultured in medium supplemented with Ca^{2+} or not were stimulated with glutamate (100 μM, Glu) or carbachol (100 μM, Cab) for 24 hr. Intracellular FGF-1 content/10^6 cells is expressed as the percent of the value obtained in the absence of the reagent (control; Con). Values are the mean ± SE of six culture dishes. Significance vs. control, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, Bonferroni's *t*-test for multiple pairwise comparisons.

5 mm distant from the propranolol injection site (i.e., area 2 shown in Fig. 7) did not show significant elevation. These results demonstrated that FGF-1 mRNA expression in the cerebral cortex was facilitated by propanolol, suggesting negative regulation of FGF-1 synthesis via adrenergic β-receptors. Immunohistochemical study revealed that administered propranolol markedly upregulated FGF-1 immunoreactivity, and that the elevated FGF-1 immunoreactivity colocalized with NeuN antigen, a 46/48-kDa nuclear protein antigen used widely to identify postmitotic neurons (Weyer and Schilling, 2003), but not with GFAP, demonstrating that FGF-1 synthesis was facilitated in neurons, but not in astrocytes (Fig. 9).

DISCUSSION

We showed that neuronal FGF-1 synthesis evoked through Ca^{2+} channels such as the NMDA receptor was abolished by cAMP/PKA signal through adrenergic β-receptors in cultured rat cortical neurons. Furthermore, blockade of adrenergic β-receptors by propranolol in the adult rat cerebral cortex markedly facilitated expression of FGF-1 mRNA and protein. These results suggest a close interplay between neurotransmitter receptor activation that evoke Ca^{2+}-mediated signals and those that generate cAMP/PKA signals in the regulation of FGF-1 synthesis in rat brain.

In accordance with the lack of a signal peptide, we could detect FGF-1 only in the cells, but not in the culture medium, irrespective of the addition of reagents used in the present study. In addition to the concentration of FGF-1 within the cells, rapid intracellular turnover of FGF-1 protein enabled us to evaluate de novo synthesis by measuring cellular FGF-1 protein.

Ca^{2+}-Activated Pathway to Upregulate FGF-1 Synthesis

Our present observations demonstrate that FGF-1 synthesis in cultured neurons is upregulated by Ca^{2+} influx and its subsequent signal. Ca^{2+} acts as a second messenger and triggers activity-dependent gene expression critical for adaptive changes in the nervous system (Finkbeiner and Greenberg, 1998), which includes gene expression of neurotrophic factors such as NGF, BDNF (Zafra et al., 1990; 1991; Rocamora et al., 1996; Shieh et al., 1998), neurotrophin-4/5 (NT-4/5) (Funakoshi et al., 1995), pleiotrophin (Amet et al., 2001), FGF-2 (Gomez-Pinilla et al., 1999), and neuregulin (Eilam et al., 1998). A leading paradigm of such regulation is activation of the nuclear transcription factor, Ca^{2+}/cAMP-responsive element binding protein (CREB) by Ca^{2+}/calmodulin (CaM) kinase IV (Sheng et al., 1990). Phosphorylated CREB drives the transcription of a many genes through interaction with its nuclear partner, CREB-binding protein (Chrivia et al., 1993). In the case of BDNF, one of the Ca^{2+}-responsive promoters is activated by CREB (Shieh et al., 1998). Instead, in the case of interleukin (IL)-6, another yet undefined factor may mediate the response to membrane depolarization (Sallmann et al., 2000). Amygdala stimulation of kindled animals greatly increased hippocampal and cortical BDNF mRNA levels; but the FGF-1 mRNA level was not altered, suggesting that BDNF and FGF-1 are recruited at different stages of kindling epileptogenesis (Simonato et al., 1998). These observations suggest that the expression of FGF-1 in neurons is regulated by a novel Ca^{2+}-responsive promoter element. The FGF-1 gene spans over 120 kb containing three protein-coding exons and at least four upstream untranslated exons (Liu et al.,

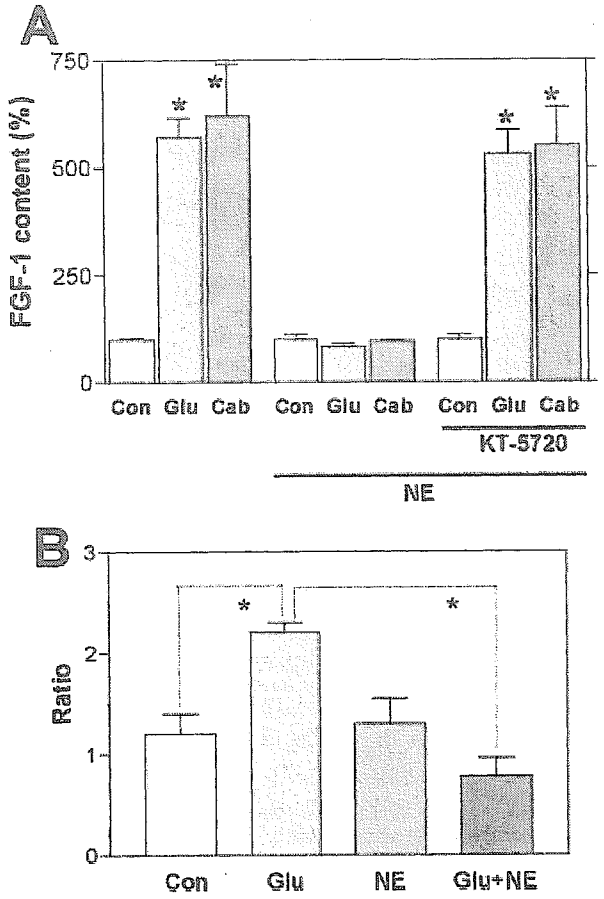

Fig. 5. Effect of coexistence of NE on glutamate- or carbachol-induced increase in FGF-1 content (**A**) or FGF-1 mRNA (**B**) of cultured cortical neurons. A: Cortical neurons were treated with glutamate (100 μM, Glu) or carbachol (100 μM, Cab) alone, or glutamate (100 μM) or carbachol (100 μM) with NE (100 μM) in combination for 24 hr. Co-administration of KT-5720 (10 μM) with NE was also carried out. Intracellular FGF-1 content/10^6 cells is expressed as the percent of the value obtained in the absence of the reagent (control: Con). The values are the mean ± SE of six culture dishes. Significance vs. control, $*P < 0.05$. Bonferroni's *t*-test for multiple pairwise comparisons. B: Cortical neurons were treated with glutamate (100 μM, Glu) or NE (100 μM) alone, or both in combination (Glu + NE) for 24 hr. Total RNAs were prepared, and an aliquot (500 ng) was reverse transcribed. Synthesized cDNAs of FGF-1 and β-actin were amplified by PCR, and the products were subjected to PAGE and visualized by ethidium bromide staining. The density of the corresponding bands was analyzed by image analysis software, and values are expressed as a ratio of the FGF-1:β-actin band density amplified from an identical RNA sample. Significance, $*P < 0.05$. Bonferroni's *t*-test for multiple pairwise comparisons.

TABLE I. Intracellular cAMP Level After Treatment With Glutamate, Norepinephrine, or Both*

	cAMP Level (fmol/dish)		
Treatment	10 min After treatment	3 hr After treatment	12 hr After treatment
No treatment	14.2 ± 2.6	12.8 ± 0.7	16.5 ± 4.4
Glutamate	18.1 ± 3.3	16.3 ± 2.0	19.8 ± 4.0
NE	21.8 ± 5.4[a]	21.4 ± 1.4[a]	25.7 ± 4.0[a]
Glutamate + NE	30.6 ± 2.4[c]	24.9 ± 1.2[b]	25.8 ± 3.5[a]

*Cortical neurons (10^5 cells/cm^2) were cultured for the indicated times in serum-free medium supplemented with 100 μm, glutamate, 100 μm norepinephrine (NE), or both. Cells were washed with phosphate-buffered saline, disrupted by sonication, and centrifuged at 100,000 × *g* for 10 min. Supernatants were used for cAMP determination.
Significance vs. no-treatment group, $^aP < 0.05$; $^bP < 0.01$; $^cP < 0.005$. Bonferroni's *t*-test for multiple pairwise comparisons.

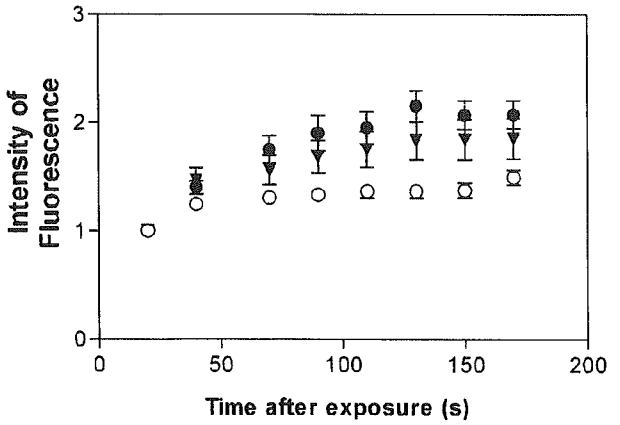

Fig. 6. Monitoring of Ca^{2+} influx after exposure to glutamate or NE alone or combined. Cultured cortical neurons were incubated with 1 μM fluo-3 AM for 30 min at room temperature. The fluorescence of each neuron was recorded at 488 nm with a fluorescence microscope after the addition of vehicle (open circles), 100 μM glutamate (filled circles) or glutamate and NE (downward triangles). Values are mean ± SE of 30–40 cells. Significance vs. control, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, Bonferroni's *t*-test for multiple pairwise comparisons.

1998). Splicing of each of these untranslated exons to the first protein coding exon generates four different mRNA transcripts. FGF-1.B transcript is expressed selectively in neural tissues, and is localized predominantly in the brainstem, ventral spinal cord, and cerebellum (Alam et al., 1996). A 23-base pair (bp) *cis*-element identified in the FGF-1.B promoter is thought to be linked to the enhanced functional activity of the promoter (Myers et al., 1995). Furthermore, a splice variant of the E2-2 gene product represses the transcriptional activities of the FGF-1.B promoter (Liu et al., 1998). Ca^{2+}-triggered regulatory mechanisms for FGF-1 gene expression, however, remain unknown.

Interplay Between Ca^{2+}-Activated and cAMP/PKA Signal Pathways

The cAMP/PKA pathway has been thought to regulate the activity of other signal transduction pathways (Iyengar, 1996). We found that the cAMP/PKA signal attenuated Ca^{2+}-induced FGF-1 synthesis. The degree of

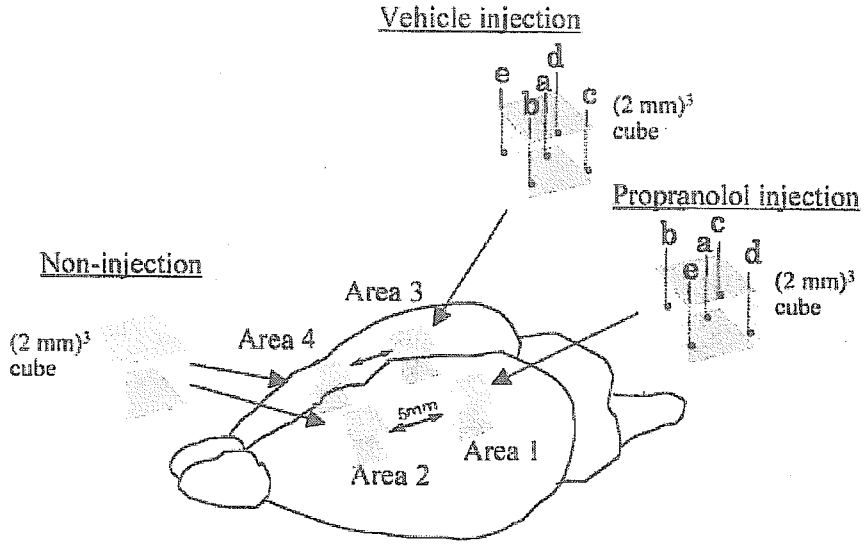

Fig. 7. Schematic illustration of the brain areas for vesicle or propranolol administration, and for RT-PCR or immunohistochemical analysis. One microliter each of vehicle (PBS) and propranolol (an antagonist of adrenergic β-receptors) in PBS (100 μg/μl) was injected into five sites from a–e within the brain areas 1 and 3, respectively. Area 1 or 3 was a $(2\ mm)^3$ cube surrounded by bregma −3.0 to −5.0 mm, lat. 2.0 to 4.0 mm, ht. 0.5 to 2.5 mm of the cerebral cortex of male Wistar rats (7 weeks old). Stereotaxic coordinates of the injection sites in area 1 or 3 were as follows: **a**, bregma −4.0 mm, lat. 3.0 mm and ht. 1.5 mm; **b**, −3.0, 2.0, 1.3; **c**, −5.0, 2.0, 1.2; **d**, −5.0, 4.0, 1.4; **e**, −3.0, 4.0, 1.8. Noninjection area 2 (or area 4) includes a $(2\ mm)^3$ cube area of bregma −1.0 to1.0 mm, lat. 2.0 to 4.0 mm, ht. 0.5 to 2.5 mm.

Ca^{2+}-influx by glutamate was essentially unchanged irrespective of the cellular cAMP content, suggesting that there is indeed interplay between cAMP/PKA and Ca^{2+}-activated signals in neurons. The regulatory mechanism(s) thus expected in vitro was confirmed in neurons of the adult rat cerebral cortex in vivo by analyzing effects of an adrenergic β-receptor antagonist: blockade of cAMP/PKA signal facilitated FGF-1 synthesis in cortical neurons. We could not observe such a change in astrocytes in vivo, however, which is probably because of a lack of FGF-1 synthesis in astrocytes of the mature rat brain (Wilcox and Unnerstall, 1991; Stock et al., 1992).

Elevation of intracellular cAMP activates PKA, which in turn phosphorylates some unknown protein "X" that might be involved in the major Ca^{2+}-dependent signaling pathway in FGF-1 gene expression. The phosphorylation inactivates X and blocks the FGF-1 gene expression, but dephosphorylation of X by phosphatase could reactivate the X. The resting levels of PKA and phosphatase activity in the cells are likely to reach a balance, leading to an equilibrium between active and inactive forms of X. As a candidate for X, Ca^{2+}/calmodulin (CaM) kinase kinase may be a plausible one. It is reported that CaM kinase cascades are blocked by cAMP/PKA-mediated inhibition of CaM kinase kinase by phosphorylation of Thr108, a mechanism to modulate the balance between cAMP- and Ca^{2+}-dependent signal transduction pathways (Matsushita and Nairn, 1999). Alternatively, in contrast to this consideration, there are reports showing alteration of Ca^{2+} influx by cAMP/PKA signals (Yang et al., 1996) through phosphorylation of Ca^{2+} channel proteins (Hell et al., 1995). It therefore cannot be excluded that cAMP/PKA signals alter the degree of Ca^{2+} channel-gated influx and modify the subsequent Ca^{2+}-dependent signal cascade directing FGF-1 gene expression. Plural *cis*-elements of the promoter and *trans*-acting factors that respond to Ca^{2+} may interplay in a complex fashion, depending on the Ca^{2+} concentration, and Ca^{2+}/calmodulin kinase cascades may be transduced in different ways.

Enhanced expression of FGF-1 after administration of propranolol, a nonselective β-adrenergic antagonist, suggests that FGF-1 synthesis is negatively regulated via adrenergic β-receptors in cortical neurons in vivo. The adrenergic nerve fibers with NE as a neurotransmitter are projected densely from the locus coeruleus into the cerebral cortex. NE secreted in a neuronal activity-dependent manner may suppress FGF-1 synthesis in cortical neurons, as suggested from the present in vitro experiment. It is likely that FGF-1 synthesis is highly sustained by neurotransmitter receptor activation that causes Ca^{2+} influx such as glutamate, acetylcholine, histamine, GABA, and serotonin in the absence of ligands of adrenergic β-receptors. These neurotransmitters can be substantially supplied from respective nerve terminals projected from areas other than the cerebral cortex.

FGF-1 synthesis regulated by neuronal activities in opposite ways is based on establishment and maintenance of particular neuronal circuits in the brain, which may be

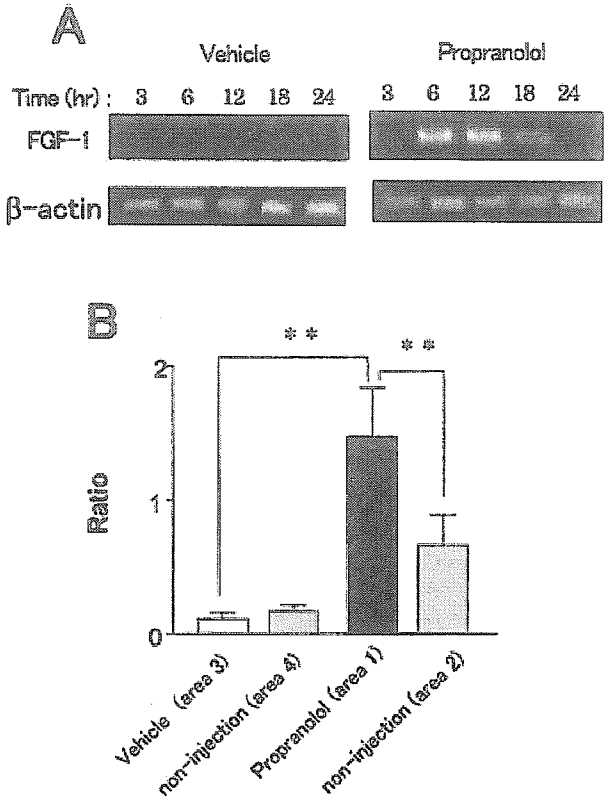

Fig. 8. Time-dependent (**A**) or site-specific (**B**) effects of propranolol injection on the expression of FGF-1 mRNA in the adult rat cerebral cortex. Vehicle (PBS) or propranolol, a blocker of adrenergic β-receptors, in PBS (5 μl, 100 μg/μl) was injected into the rat cerebral cortex as illustrated in Figure 7. A: Three animals were killed at the experimental times, and area 1 (propranolol) or 3 (vehicle) was dissected out and its total RNA was prepared. Each RNA was subjected to RT-PCR using primers specific for FGF-1 or β-actin, and PCR products were electrophoresed in a 2% agarose gel. Gels were stained with ethidium bromide, and photographs showing a representative result in each time were shown. B: Area 1, 2, 3, or 4 was dissected out 12 hr after the injection, and total RNA was prepared from these tissue samples. Each RNA was subjected to RT-PCR for FGF-1 or β-actin, and PCR products were analyzed in a 2% agarose gel. The bands corresponding to FGF-1 or β-actin mRNA-derived cDNA were densitometrically quantified, and the ratio of the FGF-1:β-actin band intensity was calculated. The values are expressed as the means ± SE of the five animals. Significant differences of the value of propranolol-injected tissue (area 1) from that of the vehicle-injected one (area 3), or that of the noninjected one of the ipsilateral side (area 2), were determined by Tukey's test. Significance, $*P < 0.05$ and $**P < 0.01$.

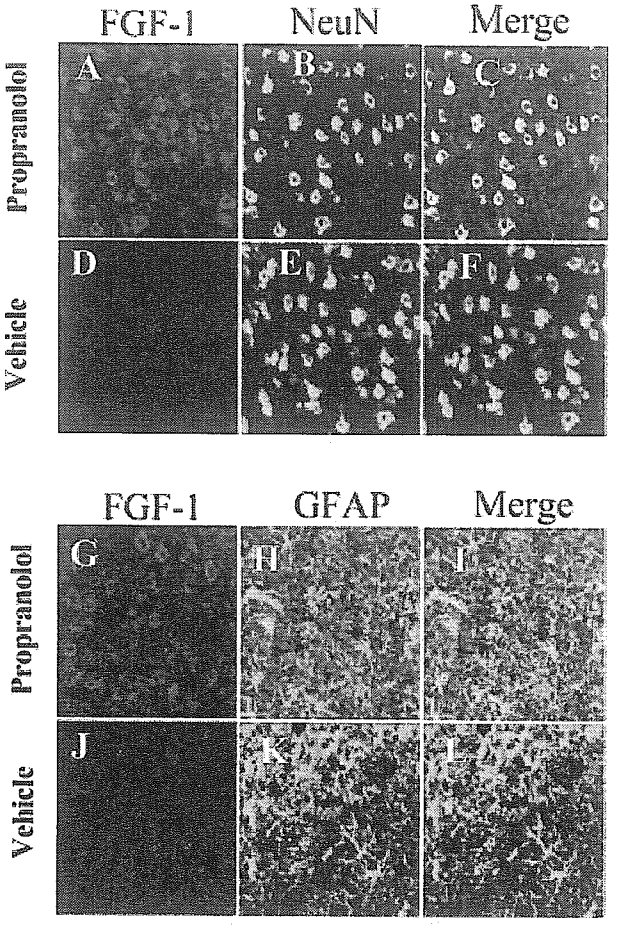

Fig. 9. Effects of propranolol injection on the expression of FGF-1 immunoreactivity in the adult rat cerebral cortex. Propranolol (a blocker of adrenergic β-receptors) or vehicle was injected into the rat cerebral cortex as indicated in Figure 7. Rats were anesthetized 24 hr after the injection, and cardio-perfused with 4% paraformaldehyde solution. The frozen brain tissues were cut into sections. The coronal sections including areas of 1 and 3 were reacted with antibody against FGF-1 (**A, D, G, J**). NeuN (**B, E**) or GFAP (**H, K**), and visualized as described in the text. The photographs of area 3 into which propranolol was administered (A–C, G–I) or area 1 into which vehicle was administered (D–F, J–L) are shown. The staining of FGF-1 (red color) and NeuN (green) are merged in C, F, I, and L. These experiments using three animals in each group were repeated three times. Scale bars = 20 μm.

one of the ways of neurotransmitters to regulate brain function. Further investigations are necessary to clarify interactions between both signal pathways in FGF-1 gene expression.

ACKNOWLEDGMENTS

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Hydrophobic Dipeptide Leu-Ile Protects Against Neuronal Death by Inducing Brain-Derived Neurotrophic Factor and Glial Cell Line-Derived Neurotrophic Factor Synthesis

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We investigated whether certain hydrophobic dipeptides, Leu-Ile, Leu-Pro, and Pro-Ile, which partially resemble the site on FK506 that binds to immunophilin, could stimulate glial cell line-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) synthesis in cultured neurons and found only Leu-Ile to be an active dipeptide. Leu-Ile protected against the death of mesencephalic neurons from wild-type mice but not from mice lacking the BDNF or GDNF gene. Next, we examined the effects of i.p. or i.c.v. administration of Leu-Ile on BDNF and GDNF contents. Both types of administration increased the contents of BDNF and GDNF in the striatum of mice. Also, peripheral administration of Leu-Ile inhibited dopaminergic (DA) denervation caused by unilateral injection of 6-hydroxydopamine (6-OHDA) into the striatum of mice. The number of rotations following a methamphetamine challenge was lower in the Leu-Ile-treated group than in the nontreated group. Next, we compared the calcineurin activity and immunosuppressant activity of Leu-Ile with those of FK506. Leu-Ile was not inhibitory toward calcineurin cellular activity in cultured neuronal cells. Furthermore, Leu-Ile did not suppress concanavalin A (ConA)-induced synthesis/secretion of interleukin-2 by cultured spleen cells, suggesting that the immunosuppressant activity of Leu-Ile may be negligible when used as a therapeutic tool for neurodegenerative diseases.

Key words: immunophilin; glial cell line-derived neurotrophic factor; brain-derived neurotrophic factor; FK506; Leu-Ile; dopamine; mice

The term *immunophilin* is used to designate receptors for immunosuppressant drugs, and FK506 is one of these drugs (Thomson, 1989; Srarzl et al., 1989). Immunosuppression is used therapeutically for a variety of purposes, one of the most important being the treatment of patients undergoing organ transplantation (Srarzl et al., 1989). Further additional action in the brain has been reported recently. FK506 can reduce ischemic brain damage in rats; for example, the drug can protect rats against quinolinate-induced excitotoxicity (Butcher et al., 1997). These findings suggest that the neuroprotective effects of FK506 may involve mechanisms distinct from N-methyl-D-aspatate (NMDA)-mediated signaling pathways (Butcher et al., 1997). FK506 administration diminished neural tissue damage following middle cerebral artery occlusion in rats (Phillips et al., 2002). Also, FK506 derivatives provided pronounced protection against neurotoxicity elicited by the β-amyloid peptide and serum derivation in cortical cultures (Lee et al., 1999). The ability of FK506 to

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block neurotoxicity in numerous models of important neurological diseases may have clinical relevance. FK 506 penetrates the blood–brain barrier reasonably well (Kochi et al., 1999). However, it would appear to be a poor therapeutic tool for neurodegenerative diseases because of its immunosuppressant activity. In this study, therefore, we looked for new neuroprotective immunophilin ligands without immunosuppressant activity and discovered the dipeptide Leu-Ile to be effective in neuroprotection. The neuroprotective mechanisms of this new ligand were shown to involve the induction of brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF). 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; Hallbook et al., 1991). BDNF affects the survival or differentiation of cultured motor neurons, mesencephalic dopaminergic neurons (Knusel et al., 1991), and septal cholinergic neurons. In adult rats, BDNF mRNA is more widely distributed in the whole brain than the mRNA of NGF or NT-3 and is regulated by glutamate or γ-aminobutyric acid neurotransmission (Phillips et al., 1990). Enhanced expression occurs following the establishment of long-term potentiation (Zafra et al., 1990; Rutherford et al., 1997). BDNF thus seems to participate in various activity-dependent events, including synapse plasticity. On the other hand, GDNF produced by a glial cell line (rat B49) is a factor that can affect dopaminergic neurons (Schubert et al., 1974). GDNF can promote the survival and function of dopamine neurons in vivo, in both the intact rat brain and after nigrostriatal lesioning (Hoffer et al., 1994; Bowenkampt et al., 1995; Jonhansson et al., 1995; Linder et al., 1995; Tomac et al., 1995; Collins et al., 1996; Granholm et al., 1997a,b). It was also shown that GDNF is secreted in the target (striatum) and transported retrogradely to the DA cell bodies in the mesencephalon (Tomac et al., 1996). These results suggest that GDNF may be effective against dopaminergic degeneration. Therefore, GDNF is expected to be useful as a therapeutic tool for dopaminergic neurological disorders.

BDNF and GDNF would seem to be very useful proteins for the treatment of various neurological disorders. However, there is an important obstacle to their therapeutic application; BDNF and GDNF are macromolecules that cannot pass through the blood–brain barrier, so it is difficult to deliver them from the periphery to the brain. Previously we reported on some stimulators for the synthesis of neurotrophic factors (Nitta et al., 1993, 1994, 1999a, 2002). Because the dipeptide Leu-Ile, designed from a part of FK506, provided neuroprotection attributable to induction of BDNF and GDNF, we also examined its immunosuppressant action and found it to have none. Therefore, this peptide appears to be a promising therapeutic tool for the treatment of various neurological disorders.

MATERIALS AND METHODS

Materials

FK506, GDNF, and BDNF were donated by Fujisawa Pharmaceutical Co., Ltd. (Tokyo, Japan), Amgen (Thousand Oaks, CA), and Sumitomo Pharmaceutical Co., Ltd. (Osaka, Japan), respectively. Dipeptides, including Leu-Ile, Leu-Pro, and Pro-Ile, which partially resemble the binding site of FK506, were purchased from Kokusan Chemical Co. Ltd. (Tokyo, Japan). All other materials used were of reagent grade. Rats and mice were purchased from Nippon SLC (Shizuoka, Japan). Mice lacking the BDNF gene were purchased from the Jackson Laboratory (Bar Harbor, ME) and mated. The generation of GDNF knockout mice was described elsewhere (Picher et al., 1996). All animals were treated according to the Guideline of Experimental Animal Care issued from the Office of the Prime Minister of Japan.

Cell Culture

Dopaminergic neurons were cultured from 13-day-old rat embryos as described previously (Nitta et al., 1999a,c). We selected the mesencephalic neuron population, which is rich in doapminergic neurons, because BDNF and GDNF have neuroprotective effects on and are synthesized by the dopaminergic neurons (Knusel et al., 1991; Hoffer et al., 1994; Bowenkampt et al., 1995; Jonhansson et al., 1995; Linder et al., 1995; Tomac et al., 1995; Collins et al., 1996; Granholm et al., 1997a,b). Antibody specific for tyrosine hydroxylase (TH; Chemicon, Temecula, CA) was used to identify dopaminergic neurons.

Enzyme Immunoassay

BDNF content was measured by an enzyme immunoassay (EIA) method (Nitta et al., 1999b,c). The EIA system for GDNF was based on the method originally developed for the EIA of NGF, BDNF, and NT-3 (Furukawa et al., 1983; Kaechi et al., 1993; Nitta et al., 1999b).

Antibodies against GDNF were produced by immunizing rabbits with purified human recombinant GDNF. GDNF protein (0.5 mg each) in phosphate-buffered saline (PBS; 5 ml) was emulsified with an equal volume of Freund's adjuvant and injected intradermally into rabbits four times at 2-week intervals. All blood was collected 1 week after the final injection. Antiserum (1 ml) was loaded onto a GDNF-linked column (1-ml bed volume; Affi-Gel 10; Bio-Rad, Hercules, CA). After extensive sequential washing with three types of loading buffer, i.e., 1) 0.1 M Tris-HCl (pH 7.4) containing 0.9% NaCl, 2) 0.05 M borate buffer (pH 8.0), and 3) 0.05 mM sodium acetate buffer (pH 5.0), the bound antibodies were eluted with 0.1 M glycine-HCl buffer (pH 2.0). A part of the purified anti-GDNF antibody preparation was eluted, biotinylated, and used as the secondary antibody.

For the determination of BDNF and GDNF contents in the conditioned medium and brain tissue, multiwell plates (Falcon 3910) were incubated with 5 μl of anti-BDNF or GDNF antibody in 0.1 M Tris-HCl buffer (pH 9.0, 10 μg/ml) per well overnight and washed with washing buffer [0.1 M Tris-HCl, pH 7.4, containing 1% (w/v) skim milk]. Sample or standard in washing buffer was then added to each antibody-coated well, and the plate was incubated for 12–18 hr at 4°. The well was

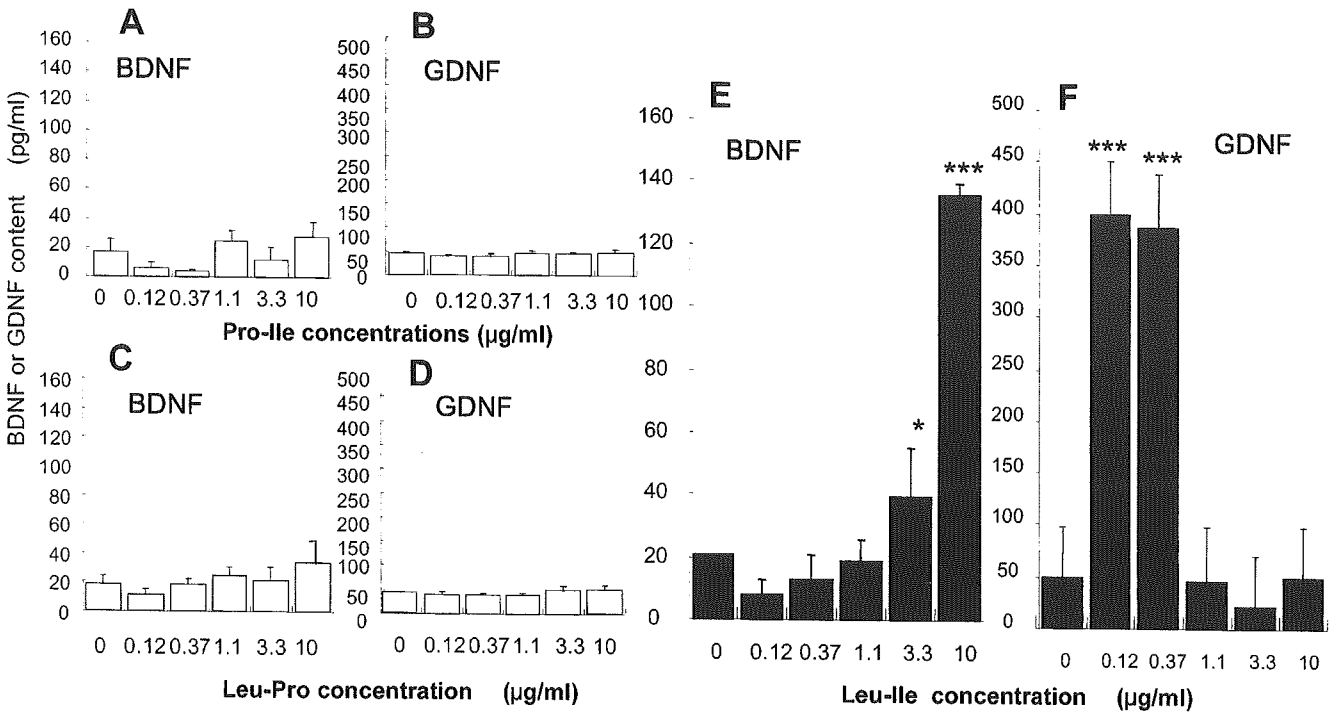

Fig. 1. Effects of dipeptides Pro-Ile, Leu-Pro, and Leu-Ile on BDNF and GDNF contents in medium from cultures of mesencephalic neurons. Neurons of the mesencephalon of 13-day-old rat embryos were cultured in the serum-free defined medium containing various concentrations of dipeptides. Conditioned media were taken 24 hr after the addition of each dipeptide, and their BDNF and GDNF contents were measured by EIAs. Values (n = 6) are expressed as mean ± SE. Three peptides, Pro-Ile (**A,B**), Leu-Pro (**C,D**), and Leu-Ile (**E,F**), were estimated for their inducing effects on the synthesis of BDNF (A,C,E) and GDNF (B,D,F). $*P < 0.05$, $***P < 0.005$ vs. nontreated neurons (Kruskal-Wallis test).

then washed with the washing buffer and incubated with the biotinylated secondary antibodies for 5 hr at 4°. After further washing with the washing buffer, avidin-conjugated β-galactosidase (Roche) was added to each well, and incubation was carried out for 1 hr. Then, after thorough washing with the washing buffer, the enzyme activity retained in each well was measured by incubation with a fluorogenic substrate, 4-methylumbelliferyl-β-D-galactoside (100 μM), dissolved in the washing buffer. The intensity of fluorescence was monitored with 360-nm excitation and 488-nm emission. The detection limit of the EIAs was as low as 1 pg/ml.

Preparation of Samples for EIA

The conditioned medium from cultured neurons was directly applied to the EIA system. Brain tissue from mice was added to sonication buffer (0.1 M Tris-HCl, pH 7.4, containing 1 M NaCl, 2% bovine serum albumin, 2 mM EDTA, 0.2% Na_3N) at the ratio of 1 g wet weight per 19 ml buffer, pulse-sonicated for 30 sec, and centrifuged at 100,000*g* for 30 min. The supernatant was then mixed vigorously with 100 μl chloroform and centrifuged at 20,000*g* for 15 min, after which the aqueous phase and cells were taken and kept in a deep freeze and used for the EIA measurements.

Unilateral 6-Hydroxydopamine-Induced Lesions

Twenty mice received 6-hydroxydopamine (6-OHDA; 20 μg/2 μl/mouse, calculated as free base; Sigma, St. Louis, MO) dissolved in ascorbate-saline (0.05%), and injected into the right substantia nigra. The injection rate was 0.4 μl/min, and the tip of the microsyringe (Hamilton 3020) was left in place for an additional 3 min before it was slowly retracted.

Behavioral Analysis

All rotational testing was in a glass cylinder (diameter was 20 cm). Control and lesioned mice were allowed to rest for 15 min to adapt to the testing environment and then were injected i.p. with 10 mg/kg methamphetamine sulfate (Dainippon Co. Pharmaceutical Ltd., Osaka, Japan) dissolved in PBS. Measurement of rotational activity began 10 min after the injection and lasted for 10 min. The number of rotations was recorded during the test period. Clockwise turns (ipsilateral to the lesion) were counted as turns. After the behavioral test, the brains of these mice were used for the immunostaining for TH.

Preparation of Splenic Lymphocytes

The preparation of splenic lymphocytes and measurement of interleukin-2 were carried out as described earlier (Sugiura et al. 2000). All procedures were conducted under aseptic conditions. Each group included four mice. Mice were sacrificed by cervical dislocation under ether anesthesia, and each single-cell suspension was prepared by pressing the spleen between two side glasses. The cell suspensions were passed through a 200-gauge stainless-steel sieve and then allowed to stand for 10 min

to remove tissue fragments. The cell suspensions were then centrifuged (600*g* for 10 min) and resuspended gently in fetal calf serum (FCS)-RPMI 1640 (Sigma) to a concentration of 4.0×10^6 viable cells/ml. The viability of the spleen cells, as determined by the trypan blue dye exclusion test, was greater than 95%.

Production of IL-2

One hundred microliters of spleen cell suspension at a concentration of 4.0×10^6 cells/ml were incubated with concanavalin A (ConA; 5 μg/ml) for 24 hr in an incubator at 37° with 5% CO_2 in humidified air. The IL-2 content in cultured supernatants was measured by using a commercial enzyme-linked immunosorbent assay (ELISA) kit for mouse IL-2 (Genzyme, Cambridge, MA).

Calcineurin Cellular Activity Assay

Calcineurin activity was measured by use of a calcineurin cellular activity assay kit (Calbiochem-Novabiochem Corporation, La Jolla, CA) as specified by the manufacturer. The cultured hippocampal neurons 5 days after the start were used for the measurement of calcineurin activity. Two hours after the addition of FK506 or Leu-Ile to the cultures, the cells were harvested and used as samples to be measured for calcineurin activity.

Statistical Analysis

Data are expressed as the mean ± SEM. ANOVA with Kruskal-Wallis test was used to establish statistical significance, set at $P < 0.05$.

RESULTS

Inducing Effects of Dipeptides on the Production of BDNF and GDNF in Cultured Hippocampal Neurons

In neuronal cultures (cell density 10^7 cells/mm^2), BDNF and GDNF contents were 16.8 ± 2.3 pg/ml and 45.2 ± 3.5 pg/ml, respectively, in medium conditioned for 1 day without any dipeptides. As shown in Figure 1A–D, neither Pro-Ile nor Leu-Pro affected BDNF and GDNF contents in the conditioned media from the cultured neurons 24 hr after the treatment, whereas Leu-Ile significantly increased both of them (Fig. 1E,F). The BDNF content was increased to 133.1 ± 23 pg/ml by Leu-Ile at the concentration of 10 μg/ml (Fig. 1E). As for GDNF, 0.12 and 0.37 μg/ml Leu-Ile increased its content to an amount about eight times that of the nontreated cells, whereas higher concentrations were ineffective (Fig. 1F).

Neuroprotective Effects of Leu-Ile Against Natural Neuronal Cell Death Among the Cultured Mesencepalic Cells

Neuronal cells seeded at a low density (3×10^4 cells/cm^2) were cultured for 3 days; during this period, they gradually died, the number of surviving cells decreasing to 50% of that at the beginning of the culture (Fig. 2). In the mesencephalic cells cultures, Leu-Ile protected against neuronal cell death in terms of both total cells and TH-positive cells (Fig. 2A). This result suggests that Leu-Ile can protect both dopaminergic and nondopaminergic neurons. Leu-Ile provided no neuroprotection

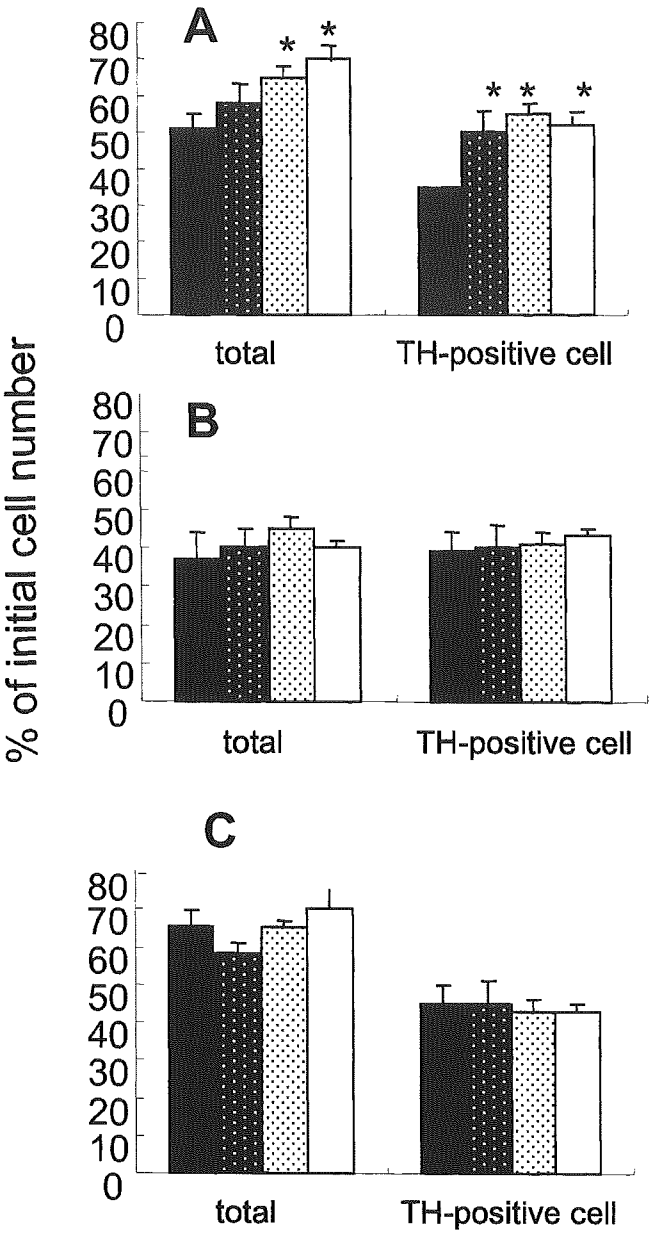

Fig. 2. Effects of Leu-Ile on the survival of mesencephalic neurons. Three-day cultures of mesencephalon from normal rats (**A**) or rats lacking the BDNF (**B**) or GDNF (**C**) gene were incubated with Leu-Ile for 24 hr. The number of surviving cells was then counted in eight arbitrarily selected fields (0.3 mm^2 each). The number in each field was averaged for each culture dish and was expressed as the mean ± SE ($n = 5$) percentage of the value of the initial cell numbers. Solid bars, Leu-Ile, 0 μM; hatched bars, Leu-Ile, 1 μM; stippled bars, Leu-Ile, 10 μM; open bars, Leu-Ile, 100 μM. *$P < 0.05$ vs. Leu-Ile 0 μM treated neurons (Kruskal-Wallis test).

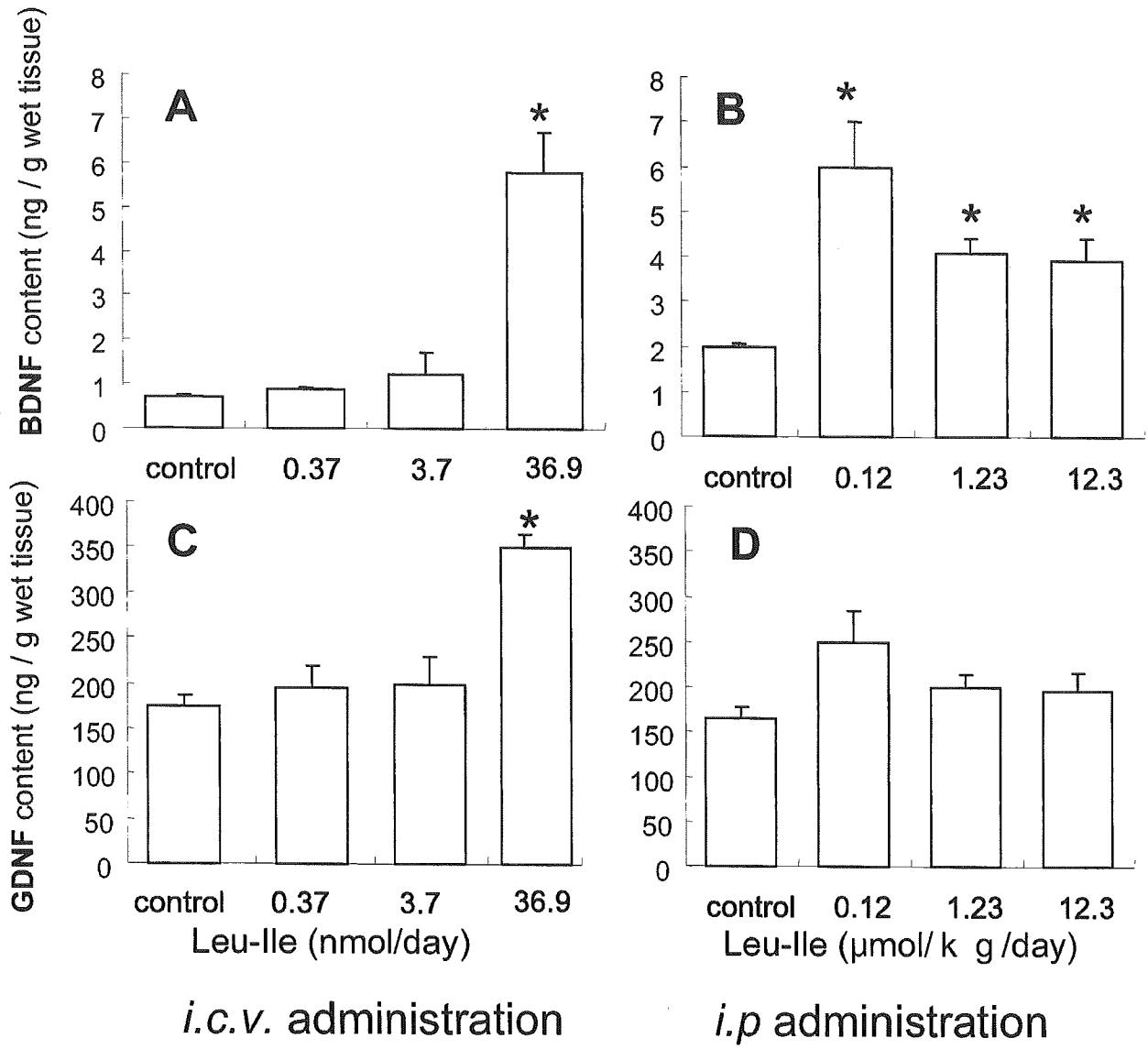

Fig. 3. Effects of Leu-Ile on the BDNF (**A,B**) and GDNF (**C,D**) contents in the rat striatum after i.c.v. (A,C) or i.p. (B,D) injections given once per day for 5 days. Each rat was decapitated 24 hr after the last injection, and the striatum was removed to prepare samples for measurements of BDNF and GDNF. $*P < 0.05$ vs. control (Kruskal-Wallis test).

of the mesencephalic neurons from mice lacking the BDNF or GDNF gene (Fig. 2B or C, respectively).

Effects of i.c.v. and i.p. Administration of Leu-Ile on the BDNF and GDNF Contents in the Mouse Striatum

Next, we investigated the inducing effect of Leu-Ile on BDNF and GDNF contents in the mouse striatum after i.p. or i.c.v. administration of the dipeptide once per day for 5 days (Fig. 3). Both routes of administration of Leu-Ile increased the BDNF and GDNF contents in the striatum. The i.p. administration required a much higher dose of Leu-Ile than the i.c.v. administration for the same effects. With i.c.v. administration, BDNF and GDNF contents in the striatum were increased dose dependently by Leu-Ile. However, by the i.p. route, 0.12 μmol/kg/day Leu-Ile increased the content of both, whereas higher concentrations were less stimulatory.

Effects of Leu-Ile on the Rotation Behavior Caused by 6-OHDA-Induced Dopaminergic Neuronal Degeneration

Mice with 6-OHDA-induced damaged to the dopaminergic neuronal system in their striatum were allowed to rest for 15 min to adapt to the testing environment and then were injected i.p. with 10 mg/kg methamphetamine

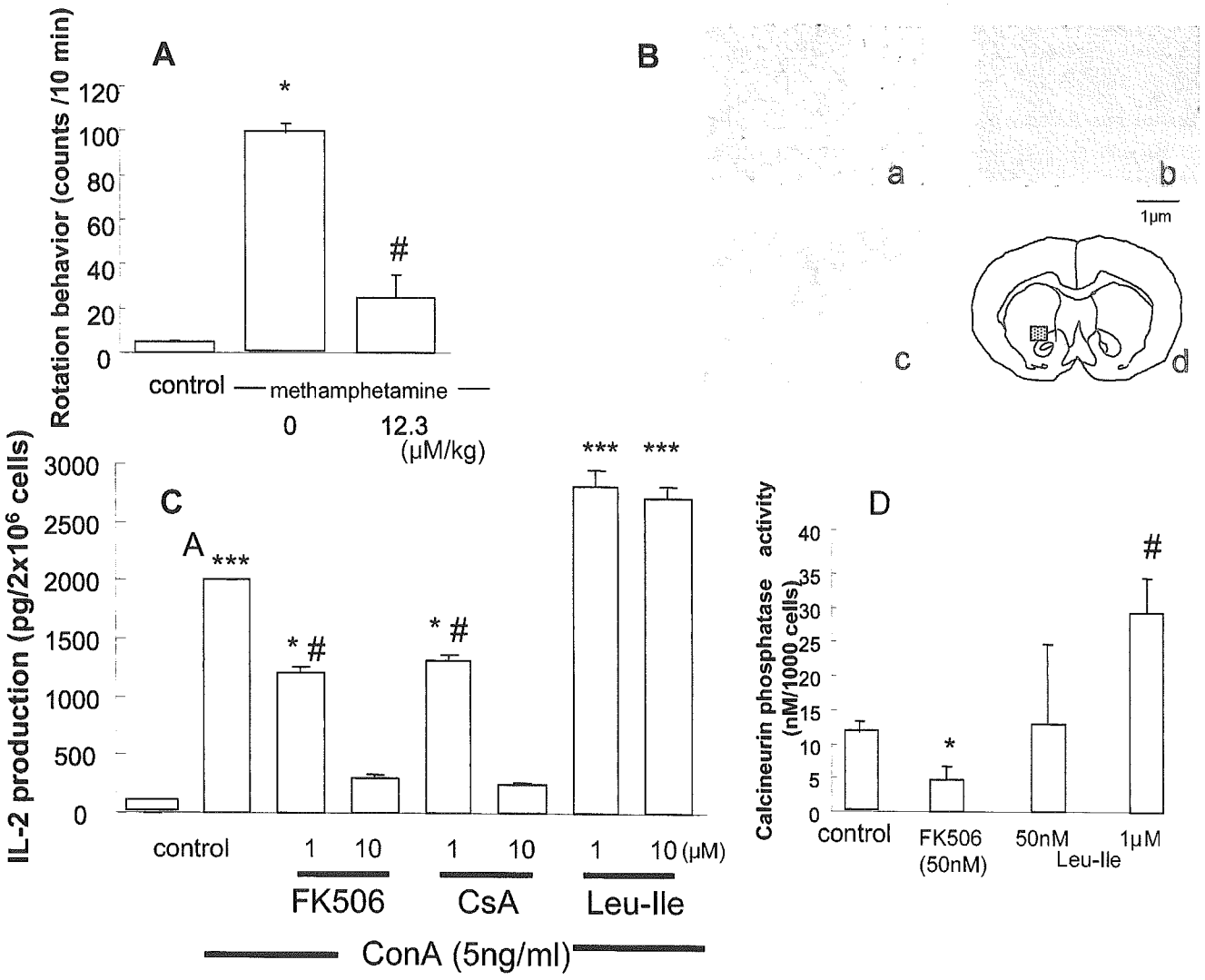

Fig. 4. **A:** Protective effects of Leu-Ile on mice lesioned with 6-OHDA. Each mouse received Leu-Ile i.p. for 10 days once per day after lesioning by 6-OHDA. Twenty-four hours after the last administration of Leu-Ile, the mice were challenged with methamphetamine (1.0 mg/kg). $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. 6-OHDA lesioned group (Kruskal-Wallis test). **B:** Effects of Leu-Ile on the dopaminergic denervation induced by 6-OHDA. The procedure was as described for A. a, Control; b, 6-OHDA injection and treatment of vehicle; c, 6-OHDA injection and treatment of Leu-Ile; d, indication of region for a–c. **C:** Effects of various immunophilin ligands on IL-2 produced by ConA-stimulated splenic lymphocytes. The procedure for preparation of the lymphocytes is described in Materials and Methods. The media were taken 24 hr after the stimulation with ConA. IL-2 concentration in the medium was measured by ELISA. $^{*}P < 0.05$ and $^{***}P < 0.005$ vs. control; $^{\#}P < 0.05$ vs. only ConA stimulation (Kruskal-Wallis test). **D:** Effects of Leu-Ile and FK506 on calcineurin activity in the cultured neurons. The cells were taken 30 min after the addition of FK506 or Leu-Ile. $^{*}P < 0.05$ vs. control, $^{\#}P < 0.05$ vs. FK506-treated group (Kruskal-Wallis test).

dissolved in PBS. Measurement of rotation activity began 10 min after the injection. Leu-Ile reduced the number of methamphetamine-induced turns experienced by the animals 10 days after the striatum and substantia nigra lesions (25 ± 3), compared with the number for control animals that received the vehicle only (97 ± 4, Fig. 4A). Immnostaining for TH in the striatum (Fig. 4Bd) was carried out using the brains after the behavioral test. TH-positive cells were found in the striatum in the control mice (Fig. 4Ba). 6-OHDA decreased these positive cells 10 days after the injection (Fig. 4Bb). Repeated i.p. administration of Leu-Ile protected the reduction of dopaminergic neurons (Fig. 4Bc).

IL-2 Induction

Cultured splenic lymphocytes produced IL-2 at a concentration of less than 1 pg/ml under normal culture conditions. The content of IL-2 in the medium was dramatically increased to 2,000 pg/ml by 24 hr after ConA stimulation. Both FK-506 and cyclosporine A

(CsA) tested at 1 and 10 μM inhibited the induction of IL-2 dose dependently, whereas Leu-Ile (1 and 10 μM) was not inhibitory.

Calcineurin Activity

FK506 at a concentration of 50 nM inhibited calcineurin activity in the cultured neurons. This concentration of FK506 was earlier reported to be the lowest effective concentration (Gaymes et al., 1997). Leu-Ile did not inhibit this activity at a concentration of either 50 nM or 1 μM, the latter of which was the effective concentration for the stimulation of GDNF production by the cultured neurons.

DISCUSSION

Immunophilins are used to define receptors for immnunosuppressant drugs such as CsA and FK506. In clinical fields, immunosuppression is used therapeutically for a variety of purposes. We have already reported that FK506 induces the synthesis of NGF, BDNF, and GDNF (Nitta et al., 2002). However, FK506 itself cannot be used for neuronal disorders, such as Parkinson's disease or Alzheimer's disease, because of its immunosuppressant actions. For this study, we selected three hydrophobic peptides, i.e., Leu-Ile, Leu-Pro, and Pro-Ile, because they are resemble the binding site of FK506 for immunophilin or FK-binding protein (Schreiber et al., 1991). As shown in Figure 1, only Leu-Ile increased the contents of BDNF and GDNF in the medium of the cultured neurons. Cameron et al. (1997) reported that FKBP12 binds inositol-1,4,5-trisphosphate receptors (IP3R) at Leu-Pro of FK506 and anchors calcineurin to this FK506-like domain. Immunosuppression appears to stem from the binding of the FK506-FKBP complex to the calcium-activated phosphatase (calcineurin), which inhibits its catalytic activity and results in the accumulation of phosphorylated calcinurin substrate. However, the present results show that Leu-Pro had no effects on the induction of BDNF or GDNF. The induction of BDNF and GDNF appears to be independent of the calcinurin activity, in that our results show that Leu-Ile did not inhibit the calcineurin activity and was not immunosuppressive. We have already found that Leu-Ile binds some transcription factors and regulates the apoptosis pathway (Nitta et al., unpublished data). Furthermore, Leu-Ile cannot bind to FKBP12 (Nitta et al., unpublished data). Immunophilin ligands regulate the immunosuppressive action via inhibition of calcineurin activity. Leu-Ile has inducible effects of calcineurin activity, and then immunological activity was increased. At the beginning of this study, Leu-Ile was designed to resemble the binding site of FK506 for FKBP; however, our results show that it is not an immunophilin ligand.

Next, we investigated the effects of Leu-Ile on the mouse brain after i.p. or i.c.v. injection. We had assumed that dipeptides such as Leu-Ile would not be stable in the blood stream and not be able to reach the brain tissue when they were peripherally administered. However, BDNF and GDNF contents in the striatum of mice were increased after repeated i.p. or i.c.v. injections. These results suggest that Leu-Ile can pass through the blood–brain barrier and initiate the synthesis of BDNF and GDNF in the brain. We attempted to obtain pharmacokinetic data by using Leu-Ile-labeled with a fluorescent marker at its N- or C-terminals; however, we were unable to detect it in the brain after i.p. injecton. The brain level of fluorescent Leu-Ile might have simply been too low to be detectable after the injection under physiological conditions.

The neuroprotective effects of Leu-Ile on the cultured neurons are at least partially the result of the induction of BDNF and GDNF, because the effect was not found when neurons from mice lacking the BDNF or GDNF gene were used. Some stimulators of neurotrophic factors have survival effects on cultured neurons (Nitta et al., 1997). For example interleukins-2, -4, and -5, all of which can induce NGF synthesis, also protect against neuronal death (Awatsuji et al., 1993ab). Furthermore, 4-methylcatechol (4MC), another stimulator of NGF and BDNF synthesis, also protects cultured neurons from dying, and 4MC enhances neuronal differentiation during brain development in rats (Fukumitsu et al., 1999; Nitta et al., 1999a; Sometani et al., 2002). The present data on mice lacking neurotrophic factor genes provide the first direct evidence that a stimulator of neurotrophic factors provides neuroprotection via such induction of neurotrophic factor synthesis.

The neurotrophic effects of the immunophilin ligands have been demonstrated in animal models of neurological disability. For example, FK506 stimulated regrowth of damaged sciatic nerves and functional recovery (Gold et al., 1994; Steiner et al., 1997a,b). We also demonstrated that FK506 had neuroprotective effects in mice with brain lesions induced by 6-OHDA (Nitta et al., 2002). Nonimmunosuppressive derivatives of FK506, e.g., GPI-1046, V-10,367 and L685,818, were also effective (Gold et al., 1997). Lesions made with 1-methyl-4-phenyl-1,2,3,6,-tetrahydropyridine (MPTP) or 6-OHDA elicit massive destruction of nigral doapminergic neurons (Schreiber, 1991; Gerlach et al., 1991; Gold et al., 1997; Rose et al., 2001). These are the same neurons that degenerate in patients with Parkinson's disease, making MPTP and 6-OHDA treatments experimental paradigms for the study of this disorder. Treatment with GPI-1046 before, during, or after the MPTP lesions substantially restored the damaged dopaminergic neurons, as demonstrated by increased striatal TH staining and reversal of haloperidol-induced catalepsy and akinesia (Gerlach et al., 1991). In rats lesioned by the unilateral intranigral administration of 6-OHDA, GPI-1046 elicited both morphological and functional recovery, with increased striatal catecholamine levels and a reduction in amphetamine-induced rotations (Gerlach et al., 1991). These pharmacological actions of GPI-1046 are attributed to the blocking of the calcineurin activity without the induction of neurotrophic factors. In present study, Leu-Ile also protected the dopaminergic denervations induced by 6-OHDA. Both GPI-1046 and Leu-Ile are compounds

related to FK506. However, their neuroprotective actions result from the different pathways, insofar as Leu-Ile has no effect on calcineurin activity (Fig. 4D). Thus, Leu-Ile should not be considered an immunophilin ligand but should be defined as a novel neurotrophic ligand.

The major 28-amino-acid neuropeptide known as vasoactive intestinal peptide (VIP) provides neuroprotection against the neuronal cell death induced by β-amyloid proteins (Gozes et al., 1999). The results of structure–function analysis of VIP indicated that, for most activities, the entire sequence of the peptide is required for full biological function. An active site comprising four amino acids has been discovered, and it mimics the neuroprotective activity of the 28-amino-acid peptides. The sequence of this four-amino-acid site is Ser-Ile-Leu-Asn, suggesting that the pair of Leu and Ile might be necessary (Gozes et al., 1999). Taken together, these suggestions and our present results suggest that Leu and/or Ile may be essential, key amino acids for the neuroprotective effects of neuropeptides. Leu-Pro and Pro-Ile did not provide the neuroprotection against natural death of cultured neurons. However, these peptides might have a neuroprotective effects on damaged neurons.

In conclusion, Leu-Ile, resembling part of the binding site on FK506 for FKBP12, had neuroprotective effects both in vivo and in vitro attributable to the induction of BDNF and GDNF synthesis. This dipeptide may thus have therapeutic potential in a wide range of neurological diseases. Conceivable targets include diabetic neuropathy, spinal cord injury, amyotrophic lateral sclerosis, and stroke. BDNF and GDNF have been used for therapy of these neurological diseases. However, delivering their potency to the central nervous system is a quite different matter. Leu-Ile should prove to be very useful clinically.

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