Effects of $R(-)$-BPAP on the Expressions of Neurotrophins and Their Receptors in Mesencephalic Slices

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Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family that plays an important role in regulating survival, differentiation, and functional integrity of central neurons. BDNF interacts with a specific trkB receptor kinase and with a low-affinity receptor p75NTR.1–3 Deficiencies of neurotrophins and their receptors are regarded as an important aspect of the pathogenesis of neurodegenerative disorders.4,5 Dopaminergic neuronal death in Parkinson’s disease has been associated with the depletion of neurotrophins, such as BDNF and nerve growth factor (NGF).5 Indeed, the levels of neurotrophins, especially BDNF, were markedly reduced in the substantia nigra of Parkinson’s disease patients, in whose selective degeneration of dopaminergic neurons is evident.5 Therefore, pharmacological stimulation of endogenous neurotrophin synthesis is expected to become a useful therapeutic approach for Parkinson’s disease.

$R(-)$-1-(Benzo furyl-2-yl)-2-propylaminopentane [$R(-)$-BPAP], a potent “catecholaminergic and serotonergic activity enhancer”, enhances the electric field stimulation-induced release of catecholamine from isolated brain stem and ameliorates motor deficits in rats. We evaluated the effects of $R(-)$-BPAP on the expression of brain-derived neurotrophic factor (BDNF) and neurotrophin-3 (NT-3), and their receptors, trkB and p75NTR, in rat mesencephalic slice cultures. Levels of mRNA and protein were measured at 48 h after $R(-)$-BPAP treatment by reverse transcription-polymerase chain reaction (RT-PCR) and enzyme-linked immunosorbent assay (ELISA), respectively. $R(-)$-BPAP significantly increased the mRNA and protein levels of BDNF, without affecting the level of NT-3 mRNA. In addition, $R(-)$-BPAP significantly increased the mRNA level of trkB, but not that of p75NTR. These effects of $R(-)$-BPAP may result in enhanced BDNF/trkB signaling, and could thus underlie the potential neurotrophic and antidepressant actions of this drug.

Key words $R(-)$-1-(benzo furyl-2-yl)-2-propylaminopentane; mesencephalon; brain-derived neurotrophic factor; trkB; slice culture; Parkinson’s disease

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family that plays an important role in regulating survival, differentiation, and functional integrity of central neurons. BDNF interacts with a specific trkB receptor kinase and with a low-affinity receptor p75NTR.1–3 Deficiencies of neurotrophins and their receptors are regarded as an important aspect of the pathogenesis of neurodegenerative disorders.4,5 Dopaminergic neuronal death in Parkinson’s disease has been associated with the depletion of neurotrophins, such as BDNF and nerve growth factor (NGF).5 Indeed, the levels of neurotrophins, especially BDNF, were markedly reduced in the substantia nigra of Parkinson’s disease patients, in whose selective degeneration of dopaminergic neurons is evident.5 Therefore, pharmacological stimulation of endogenous neurotrophin synthesis is expected to become a useful therapeutic approach for Parkinson’s disease.

$R(-)$-1-(Benzo furyl-2-yl)-2-propylaminopentane [$R(-)$-BPAP], a potent “catecholaminergic and serotonergic activity enhancer”, enhances the electric field stimulation-induced release of catecholamine and serotonin from isolated rat brain stem.6–9 Because of its “catecholaminergic and serotonergic activity enhancer” effects, $R(-)$-BPAP ameliorates motor deficits in reserpine-treated rats and improves active avoidance behavior in rats under tetraabenazine-induced depression.7,9 Therefore, this drug is a promising candidate as a treatment for symptoms of depression as well as for Parkinson’s disease.

Recently, $R(-)$-BPAP was shown to increase the expression of BDNF, NGF, and glial cell line-derived neurotrophic factor (GDNF) in cultured mouse astrocytes.10,11 Moreover, $R(-)$-BPAP protects several neuronal cell types such as cortical and hippocampal neurons and human dopaminergic neuroblastoma SH-SY5Y cells from various sorts of insults including serum deprivation, β-amyloid and endogenous neurotoxin N-methylD-aspartic acid.12,13 The protective action of $R(-)$-BPAP in SH-SY5Y cells is postulated to involve upregulation of neurotrophin synthesis. In the present study, we evaluated the effects of $R(-)$-BPAP on the expression of BDNF, neurotrophin-3 (NT-3), and their receptors in cultures of rat mesencephalic slices to determine whether the drug is effective in regulating neurotrophin signaling in preparations that retain the cellular architecture of intact brain tissue.

MATERIALS AND METHODS

Culture Preparation Experiments were performed in accordance with the guidelines of Fujimoto Pharmaceutical Corporation for the care and use of laboratory animals, which are based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Slice cultures were prepared according to a method reported previously.14 Postnatal day 3 Wistar rats (Nihon SLC, Shizuoka, Japan) were anesthetized by hypothermia, and the brain removed from the skull. Mesencephalic slices (350 µm thick) were cut with a tissue chopper (Narishige, Tokyo, Japan) and transferred onto a Millicell-CM insert membrane (Millipore, Bedford, MA, U.S.A.) in 6-well plates. Culture medium, consisting of 50% Earle’s minimal essential medium/HEPES, 25% Hanks’ balanced salt solution and 25% heat-inactivated horse serum (GIBCO BRL, Rockville, MD, U.S.A.) supplemented with 6.5 mg/ml glucose, 2 mM l-glutamine and 100 units/ml penicillin G sodium/100 µg/ml streptomycin sulfate was supplied at 700 µl/well. Slices were maintained in a 5% CO2 humidified atmosphere at 34 °C. The culture medium was exchanged for fresh medium every other day. After 10 d of cultivation, cultured slices were exposed to various concentrations of $R(-)$-BPAP (Fujimoto Pharmaceutical Corp., Osaka, Japan) for 48 h.

RNA Extraction and Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total RNA was extracted by the acid guanidinium isothiocyanate–phenol–chloroform extraction method. RT-PCR was performed using a GeneAmp® RNA Core Kit (Applied Biosystems, Carlsbad, CA, U.S.A.). Briefly, the first-strand cDNA of the isolated
mRNA was synthesized using MuLV reverse transcriptase. The synthesized cDNAs were then amplified by PCR using the following oligonucleotide primers: BDNF, sense 5'-ATG ACC ATC CTT TTC ACT ATG GT-3' and anti-sense 5'-TCT CCC TTT AAT GGT CAG TGT AC-3' (747 bp); NT-3, sense 5'-ATG CAG AGC ATA AGA GTC AC-3' and anti-sense 5'-AGC CTA CGA GTT TGT TGT TT-3' (294 bp); trkB, sense 5'-CTA CCT GCC ATC CCA ACA CT-3' and anti-sense 5'-TGG CCA ATG TCT GAA GGA GT-3' (440 bp); and p75NTR, sense 5'-TGG GTG AAG AGT GCC CAG AG-3' and anti-sense 5'-TGG GTG TGT CCA CAG AGA TG-3' (504 bp). PCR conditions were as follows: 95°C for 105 s, followed by 30 cycles of denaturing at 95°C for 15 s, annealing at 56°C (for BDNF and NT-3) or 60°C (for trkB and p75NTR) for 30 s, and extension at 72°C for 30 s. PCR products were separated on 1.5% agarose gel and stained with ethidium bromide. Signal densities of BDNF, NT-3, trkB and p75NTR PCR products amplified by a Model GS-700 Imaging Densitometer (Bio-Rad, Hercules, CA, U.S.A.) and expressed as the ratio to that of β-actin PCR products amplified from an identical RNA sample.

**Measurement of BDNF Levels** The protein levels of BDNF in slice cultures were determined by an enzyme-linked immunosorbent assay (ELISA: BNDF Emax® ImmunoAssay System, Promega, Madison, WI, U.S.A.). Each slice culture was homogenized in lysis buffer (20 mM Tris–HCl, pH 8.0, containing 137 mM NaCl, 10% glycerol, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml aprotinin, 1 μg/ml leupeptin, 1% nonylphenoxypolyethoxyethanol, and 0.5 mM sodium vanadate). The homogenates were then acid-treated, neutralized, and centrifuged at 14000×g for 15 min, and the supernatants were used as slice extract samples. Ninety-six-well plates for ELISA were coated by incubating with anti-BDNF antibody (BNDF Emax® ImmunoAssay System) in carbonate buffer (pH 9.7) at 4°C overnight, washed with washing buffer (20 mM Tris–HCl, pH 7.6, containing 150 mM NaCl and 0.05% Tween 20), and then blocked with ‘block and sample buffer’. A slice extract sample or BDNF standard solution was added to each antibody-coated well, and incubation was carried out for 2 h at room temperature. After 5 washes with washing buffer, anti-BDNF polyclonal antibody (BNDF Emax® ImmunoAssay System) was added to each well, and the plates were incubated for 2 h at room temperature. After another washing, the remaining horseradish peroxidase-conjugated anti-IgY solution was added, and the plates were then incubated for 1 h at room temperature. After a final washing, the remaining horseradish peroxidase activity was assessed by a colorimetric method following incubation with 3,3',5,5'-tetramethylbenzidine. Absorbance was measured at 450 nm. Protein concentrations were measured using Lowry’s method.15

**Statistical Analyses** Values represent as averages±S.E.M. Statistical analyses were performed with Least Significant Difference (LSD) and Dunnett’s tests (SAS preclinical package ver. 5.0, SAS Institute, Cary, NC, U.S.A.), and differences were considered significant at p<0.05.

**RESULTS**

**Effects of R-(−)-BPAP on the Expression of BDNF and NT-3** In the first set of experiments, we evaluated the effects of R-(−)-BPAP on the expression of BDNF and NT-3 in cultures of mesencephalic slices. When various concentrations (10−12—10−5 M) of R-(−)-BPAP were applied for 48 h, the drug at 10−9—10−7 M caused a marked and significant increase in the expression of BDNF mRNA with the maximal effective concentration being 10−7 M (Fig. 1A). R-(−)-BPAP caused an increase in the level of BDNF mRNA within 24 h, and the level remained elevated for up to 72 h (data not shown). In contrast, R-(−)-BPAP did not affect the mRNA levels of NT-3 at any concentrations tested (Fig. 1B). As R-(−)-BPAP at concentrations of 10−3—10−1 M induced a significant elevation of BDNF mRNA, the effect of the drug on BDNF protein level was examined by ELISA. In agreement with the results of mRNA expression, the protein levels of BDNF were significantly elevated 48 h after treatment with R-(−)-BPAP at 10−5 M. BDNF contents of R-(−)-BPAP-treated group (0.40±0.10 ng/mg protein, n=12) were higher than those of sham (0.09±0.03 ng/mg protein, n=12) (p<0.01).

**Effects of R-(−)-BPAP on the Expression of BDNF Receptors** Next, we examined whether R-(−)-BPAP or increased BDNF affected the expression of BDNF receptors trkB and p75NTR. R-(−)-BPAP at 10−9—10−7 M, which had effects on BDNF expression, also caused a significant increase in the mRNA level of trkB (Fig. 2A). On the other hand, the mRNA level of p75NTR was not influenced by R-(−)-BPAP at same concentrations (Fig. 2B).

**DISCUSSION**

The present study in cultures of mesencephalic slices demonstrated that R-(−)-BPAP significantly increased the
expression of BDNF mRNA, but not that of NT-3. \( R(-)\)-BPAP caused significant increases in the mRNA and protein levels of BDNF. As Ohta et al. suggested,\(^7\) we also consider that the increasing effects of \( R(-)\)-BPAP on BDNF protein are due to activating \textit{de novo} RNA synthesis. In a previous study using mouse astrocytes, the effective concentrations of \( R(-)\)-BPAP (\(5\times10^{-5} - 1\times10^{-3} \text{ M}\)) on BDNF expression were much higher than those in the present study using cultures of mesencephalic slices, which contain neuronal and glial cells preserved in an \textit{in vivo} neuronal network, and cell-to-cell interactions.\(^{10,11}\) Differences in the effective concentrations of \( R(-)\)-BPAP on BDNF expression in astrocytes and slice cultures may be altered by several factors including cell organizations and days of cultivation. \( R(-)\)-BPAP may be able to stimulate the BDNF synthesis of neurons, as well as astrocytes.

The effective concentrations of \( R(-)\)-BPAP on BDNF expression in the present study are in agreement with those of previous reports showing that \( R(-)\)-BPAP inhibits cell death of cortical neurons and SH-SY5Y cells.\(^{2,12,13}\) Notably, \( R(-)\)-BPAP was reported to induce the expression of anti-apoptotic protein Bcl-2 and its mRNA in SH-SY5Y cells at the concentration range of \(10^{-11} - 10^{-8} \text{ M}\).\(^{7,12,13}\) The neuroprotective effects of \( R(-)\)-BPAP may partly involve the enhancement of BDNF levels.

We demonstrated here for the first time that \( R(-)\)-BPAP significantly increased the expression of trkB mRNA without affecting the level of p75NTR mRNA. It is important to note that \( R(-)\)-BPAP (\(10^{-9} - 10^{-7} \text{ M}\)) increased the expression of trkB mRNA as well as BDNF, because BDNF exerts its neuroprotective actions through trkB.\(^{16}\) Furthermore, BDNF and some antidepressants that can upregulate BDNF expression were reported to increase the level of trkB mRNA in the hippocampus and the cerebral cortex.\(^{17,18}\) Recently, the induction of BDNF/trkB signal was implicated as an important mechanism of action of antidepressants.\(^{17,18}\) For example, the infusion of BDNF into the midbrain produces antidepressant-like effects in an animal model of depression.\(^{19}\) Interestingly, \( R(-)\)-BPAP is reported to produce antidepressant-like activity in rats with tetrabenazine-induced depression.\(^{7}\) Therefore, the \( R(-)\)-BPAP-induced increases in BDNF and trkB levels in mesencephalic slices may be related to its antidepressant properties. Moreover, the induction of BDNF by \( R(-)\)-BPAP in the mesencephalon may also contribute to the “catecholaminergic and serotonergic activity enhancer” effect advocated by Knoll \textit{et al.},\(^7\) because BDNF stimulates the basal and depolarization-induced release of dopamine \textit{via} activation of trkB and p75NTR in mesencephalic neurons.\(^{20}\)

In conclusion, we demonstrated that \( R(-)\)-BPAP could up-regulate the expressions of BDNF and trkB, which contribute to neuron survival in cultures of mesencephalic slices. Our findings suggest that \( R(-)\)-BPAP, in addition to action mediated by its “catecholaminergic and serotonergic activity enhancer” effect, may serve as a neuroprotective agent for the treatment of progressive neurodegenerative disorders.

REFERENCES