Induction of Nerve Growth Factor by Butanol Fraction of *Liriope platyphylla* in C6 and Primary Astrocyte Cells

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Liriope platyphylla **(LP) has been used as a tonic, antitussive, and expectorant in Korea for many years. In this study, we found that the buthanol fraction of *Liriope platyphylla* (BLP)-conditioned media of C6 and primary astrocyte induced the neurite outgrowth of PC12 cells, and that the effect was reversed by addition of nerve growth factor (NGF)-antibody and GF109203X, an inhibitor of protein kinase (PKC). Furthermore, we demonstrated that BLP increased the expression and secretion of NGF. GF109203X also decreased NGF expression in C6 cells. Taken together, our results suggest that astroglial NGF enhanced by BLP in a PKC-dependent pathway contributed to the induction of neurite outgrowth of PC12 cells.**

Key words nerve growth factor; *Liriope platyphylla*; astrocyte; PC12; neurite outgrowth

Astrocytes play an essential role in maintaining the physiological function of neurons. In particular, neurotrophic factors (NFs) produced and released by astrocytes contribute to the differentiation and survival of neuronal cells.[1] Among the NFs, nerve growth factor (NGF), first identified to be a member of the NF family,[2] has been reported to have therapeutic potency in the treatment of neurodegenerative diseases such as Alzheimer's disease and cerebrovascular dementia.[3] However, since its large molecular weight and susceptibility to endogenous peptidase render NGF unable to penetrate the blood–brain-barrier, it can only be used for medical application when directly injected into the brain.[4] Therefore, much attention has been paid to the agents that either enhance the action of NGF or increase the NGF synthesis. Although the mechanism of NGF expression is not fully understood, it has been reported that the expression of NGF is regulated through a multiple signaling pathway. Phorbol ester increased NGF expression in astrocytes, suggesting the involvement of protein kinase C (PKC).[5]

Liriope platyphylla (LP) is a medicinal herb that has been used for treating cough and sputum in Korea. With regards to the pharmacological effect of LP, antibacterial and anticancer effects have been reported.[6,7] However, the neuroprotective effect of LP remains to be examined. In this study, we investigated whether the butanol fraction of LP (BLP)-conditioned media of astrocytes triggered neurite outgrowth in PC 12 cells that was differentiated into neuron-like cells.[8] In addition, the effects of BLP on astroglial NGF secretion and synthesis were also examined.

MATERIALS AND METHODS

Materials Dulbecco's modified Eagle medium (DMEM), RPMI 1640, fetal bovine serum (FBS), horse serum (HS), penicillin–streptomycin (PS), and NGF were purchased from Gibco-BRL (Gaithersburg, MD, U.S.A.). Antibody against mouse NGF was obtained from Chemicon (Temecula, CA, U.S.A.).

Preparation of Butanol Fraction of *Liriope platyphylla* LP was obtained from Milyang province of South Korea and identified by Prof. H. Kim of the Herbal Pharmacology Department of Kyung Hee University, where a voucher specimen (HP-SY-01) is deposited at the herbarium of the Graduate School of East-West Medical Science. The dried tubers of LP (6 kg) were extracted with 85% MeOH three times under an ultrasonic apparatus. The total extract concentrated with a rotary evaporator (572 g) was suspended in distilled water and partitioned with EtOAc (30 g) and BuOH (60 g), successively. No detailed chemical investigation appears to have been conducted on this plant. However, recently, the steroidal saponin, Spicatoside A, was isolated and characterized. BLP was standardized by determining the contents of spicatoside A, a major saponin.[9] The content of spicatoside A is 0.4% (data not shown).

Cell Culture C6, a rat astrocyte cell line, and PC 12 cells were obtained from the American Type Culture Collection (Rockville, MD. U.S.A.). C6 glioma cells were cultured in DMEM supplemented with 10% (v/v) heat-inactivated FBS and 1% (v/v) PS. Primary astrocytes were isolated from whole brain of 1-d-old neonatal Sprague–Dawley rats. Dissociated cells were plated in 75 mm tissue flasks and cultured in DMEM supplemented with 10% FBS and 1% PS. The incubated cells were detached by tapping and plated in 100 mm dishes to obtain a pure astrocyte cells culture. PC12 cells were cultured in RPMI 1640 supplemented with 5% FBS, 10% HS and 1% PS. All cells were maintained in a humidified incubator at 37 °C and 5% CO_2.

Preparation of Conditioned Media of Glial Cells C6 and primary astrocyte cells (3×10^5 cells/well) were seeded onto 100 mm dishes. After 24 h, the astrocyte cells were treated with BLP. The media were collected after 24 h of incubation, and stored at -20 °C until the treatment of PC 12 cells.

Neutralization with NGF-Antibody We neutralized NGF using an antibody against it. C6 cells were cultured with BLP (10 μg/ml) for 24 h and then the medium was transferred to a new tube. For the neutralization of NGF, we added the neutralizing antibody (0.5 μg/ml) into the medium followed by overnight incubation at 4 °C.[10] After the PC12 cells were cultured with this media, we observed the changes in morphology in the cells.

Neurite Outgrowth Assay For the neurite outgrowth

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assay, PC12 cells (3×10^4 cells/well) were seeded onto 6-well plates, and the media was exchanged with RPMI 1640 containing 2% HS and 1% FBS. After 24 h, the cells were treated with the BLP-conditioned media from the astrocytes and incubated for 6 d. Neurite length per cell was quantified after each day of incubation and photographs at 48 h obtained. Neurite length was quantified using an Olympus optical inverted phase-contrast microscope (model CK-2; ×100 magnification and Optimas 6.5 program (Media cybernetics, U.S.A.).

ELISA for NGF Determination The level of secreted NGF in the cell supernatant was determined using ELISA. The C6 and primary astrocyte cells were cultured in serum-free DMEM for 24 h, and then the medium was replaced with that including BLP (1, 5, 10 μg/ml). After incubation for the indicated time, the supernatant was harvested. ELISA was conducted according to the manufacturer's direction (Boehringer-Mannheim). The amount of NGFβ (Roche, Germany) in the samples was calculated from the standard curve. To investigate the mechanism of BLP action in the astrocytes, we cultivated C6 cells with BLP (10 μg/ml) prior to adding GF109203X, a PKC inhibitor (Wako Pure Chemical, Japan).

Reverse Transcription Polymerase Chain Reaction (RT-PCR) After treating the C6 and primary astrocyte cells with BLP (10 μg/ml) for 2 h, total RNA was extracted and first cDNA was synthesized using TRIzol reagent and oligo-dT (Gibco BRL). cDNA was amplified in 25 μl of PCR cocktail containing each specific primer, dNTP mixture and Taq DNA polymerase (TaKaRa, Shiga, Japan). The sets of primers were as follows: NGF, forward, 5′-TGGCCAGTG-GTCGTGCAGTC-3′, reverse, 5′-AAGTCAGCCTCTTG-CAGC-3′. Thirty cycles of PCR were performed (for NGF: denaturation at 95 °C for 30 s; annealing at 60 °C; extension at 72 °C for 160 s; for GAPDH: denaturation at 95 °C for 30 s; annealing at 56 °C; extension at 72 °C for 160 s). The thirty cycle reaction was followed by a final extension step at 72 °C for 3 min.

Statistics All data are expressed as the mean±S.E.M. from three independent experiments. Statistical significance was evaluated by the Student's *t*-test. Differences with a p value less than 0.05 were considered statistically significant.

RESULTS

Induction of PC12 Cell Neurite Outgrowth by BLP-Conditioned Media of C6 and Primary Astrocytes In this study, we examined the effect of BLP-conditioned media of astrocytes on the morphological changes in PC12 cells. C6 and primary astrocyte cells were incubated with BLP (1, 5, 10 μg/ml) for 24 h, and then the media were harvested and added to the PC12 cells. Induction of PC12 neurite outgrowth was observed in a dose-dependent manner (data not shown). The length of neurite-bearing cells was maximized at a concentration of 10 μg/ml of BLP-conditioned media (Fig. 1). Astroglial media treated with the EtOAc fraction of LP could not contribute to the induction of PC12 neurite outgrowth (data not shown).

Reverse of Conditioned Media-Induced PC12 Cell Neurite Outgrowth by NGF-Antibody We cultured PC12 cells with BLP-conditioned media in the presence and absence of NGF antibody. The differentiation of PC12 cells was reversed in BLP-conditioned media containing NGF antibody but not in BLP-conditioned media alone (Figs. 2A—G), indicating that NGF antibody blocked the differentiation of PC12 cells and BLP stimulated NGF secretion in astrocytes.

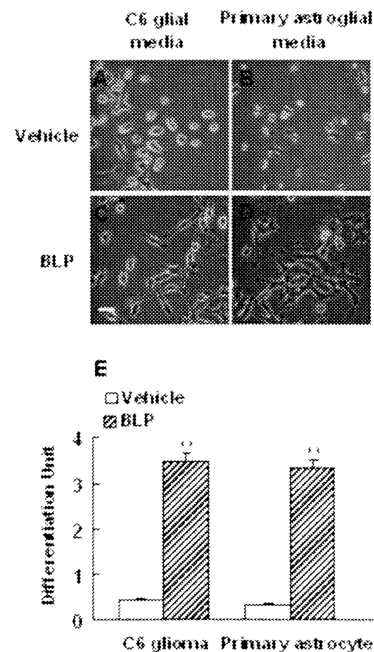

Fig. 1. Effect of BLP-Conditioned Glial Media on the Neurite Outgrowth from PC12 Cells

C6 and primary astrocyte cells were incubated for 24 h in DMEM containing 2% FBS with 10 μg/ml BLP, and then PC12 cells were cultivated in the BLP-conditioned media. After 48 h, randomly selected fields were taken using a camera attached to a microscope. (A) C6 glial media conditioned by vehicle (DMSO, 0.01%). (B) Primary astroglial media conditioned by vehicle (DMSO, 0.01%). (C) C6 glial media conditioned by BLP (10 μg/ml). (D) Primary astroglial media conditioned by BLP (10 μg/ml). (E) Assessment of differentiation unit of neurite outgrowth in each sample-treated PC12 cells. The differentiation of PC12 cells was scored as follows: cells without neurite outgrowth; 0, cells bearing neurite as long as one cell diameter; 1, cells bearing neurite two times longer length than their diameter; 2, and cells which had a synapse-like neurite; 4. Cells were photographed using a phase-contrast light microscope (×100). Scale bar is 50 μm. The results are expressed as the mean±S.E.M. Three independent experiments were carried out. The asterisks indicate a significant difference from the treatment with media conditioned by vehicle (** $p<0.01$).

Increase in NGF Synthesis by BLP To investigate the effects of BLP on NGF secretion in C6 cells and primary astrocyte cells, we determined the level of NGF protein in culture medium using ELISA. After 6 h of incubation with BLP (1, 5, 10 μg/ml), the NGF content in the culture medium of C6 and primary astrocyte cells increased in a dose-dependent manner (Fig. 3A). Furthermore, the RT-PCR results showed that BLP increased the level of NGF mRNA expression (Figs. 3B—D). Taken together, these results suggest that NGF derived from BLP-treated astrocytes caused the induction of PC12 cell neurite outgrowth.

Association of PKC-Dependent Pathway with Enhancement of NGF in BLP-Treated Astrocytes We also investigated whether the elevation of astroglial NGF by BLP is dependent on the PKC pathway. BLP-conditioned C6 glial media in the presence of GF109203X, an inhibitor of PKC, blocked the PC12 neurite outgrowth (Figs. 4A—E). In addition, as shown Fig. 3F, treatment of the C6 cells with GF109203X blocked the NGF triggered by BLP in the media, suggesting that PKC was associated with NGF syn-

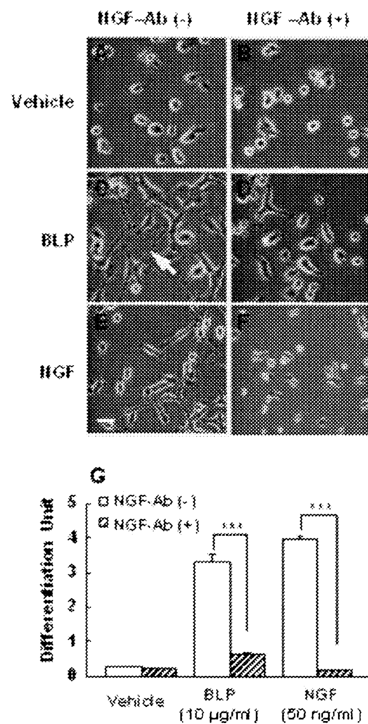

Fig. 2. Effect of NGF Antibody on Neurite Outgrowth in PC12 Cells

After C6 cells were incubated with BLP (10 μg/ml) for 24 h, PC12 cells were incubated in the conditioned media in the presence or absence of NGF antibody (0.5 μg/ml) for 48 h. (A) media conditioned by vehicle (DMSO, 0.01%). (B) Media conditioned by vehicle (DMSO, 0.01%) plus NGF antibody (0.5 μg/ml). (C) Media conditioned by BLP (10 μg/ml). (D) Media conditioned by BLP (10 μg/ml) plus NGF antibody (0.5 μg/ml). (E) Media conditioned by NGF (50 ng/ml). (F) Media conditioned by NGF (50 ng/ml) plus NGF antibody (0.5 μg/ml). (G) Assessment of differentiation unit of neurite outgrowth in sample-treated PC12 cells. Cells were photographed using a phase-contrast light microscope (×100). The differentiation of PC12 cells was scored according to the method described in Fig. 1. White arrow indicates the cell with a score of 4. Scale bar is 50 μm. The results are expressed as the mean±S.E.M. Three independent experiments were carried out. The asterisks indicate a significant difference from the treatment with conditioned media plus NGF antibody (*** $p<0.001$).

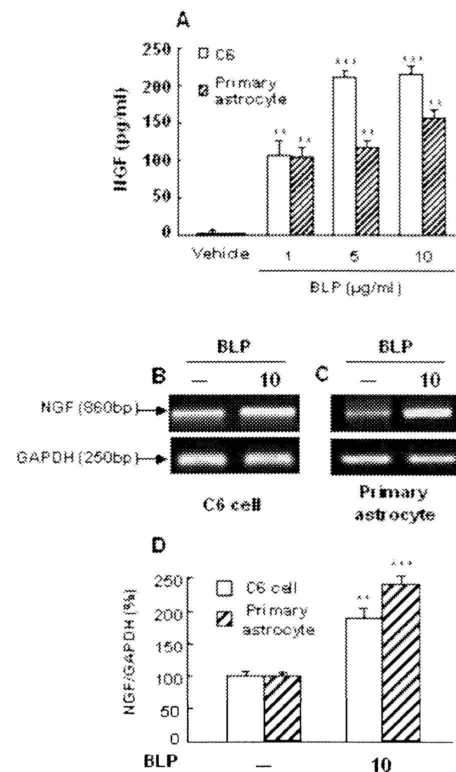

Fig. 3. Effect of BLP on Astrocyte NGF Synthesis

After incubation with BLP (1, 5, 10 μg/ml) for 6 h, NGF secretion from C6 cells and primary astrocytes was measured using an ELISA kit (A). RT-PCR was carried out to investigate whether BLP promoted the level of NGF mRNA expression. C6 (B) and primary astrocyte cells (C) were incubated with BLP (10 μg/ml) for 2 h. Total RNA was extracted and RT-PCR performed. NGF mRNA expression intensity was normalized through comparison with that of GAPDH mRNA expression (D). The results are expressed as the mean±S.E.M. Three independent experiments were carried out. The asterisks indicate a significant difference from the treatment with vehicle (** $p<0.01$, *** $p<0.001$).

thesis.

DISCUSSION

In previous studies, we reported that quinic acid from *Aster scaber* and phospholipids from *Bombycis corpus* have neurotrophic effects in PC12 cells.[11,12]

In the present study, we found that BLP-conditioned media of C6 and primary astrocyte cells caused the induction of PC 12 neurite outgrowth. Since NGF is known to be an endogenous molecule that contributes to the differentiation of PC12 cells,[13] we hypothesized that BLP might enhance NGF secretion and synthesis in astrocyte cells. To confirm this hypothesis, we added the NGF-antibody to BLP-conditioned media to neutralize the NGF. Treatment with NGF-antibody reversed the PC12 neurite outgrowth induced by BLP-conditioned media alone. This suggests that BLP stimulated the enhancement of NGF in astrocyte cells, which was responsible for the induction of PC12 neurite outgrowth. In addition, ELISA experiments clearly demonstrated that BLP elevated NGF secretion by astrocyte cells. RT-PCR was carried out in order to confirm that the increase in NGF in the media was due to up-regulated NGF mRNA synthesis. BLP (10 μg/ml) increased the level of NGF mRNA expression. it was reported that role of PKC is important in NGF synthesis mechanisms.[14,15] The experimental results that a PKC inhibitor reversed the neurite outgrowth and decreased the amount of NGF in the media revealed that the induction of glial NGF by BLP was at least in part dependent on a PKC pathway. However, something else should be considered. Although NGF plays an important role in the induction of PC12 neurite outgrowth, it is known that other NFs such as neurotrophic factor-3 (NT-3) and interleukin-6 (IL-6) also trigger the differentiation of PC12 cells.[16,17] Therefore, it is possible that in this study BLP might have stimulated the secretion and synthesis of other NFs as well as NGF. Nonetheless, neutralization of NGF, even if incompletely, reduced the induction of PC12 neurite outgrowth by BLP-conditioned media to a level similar to that of vehicle-treated cells (Fig. 2), suggesting that NGF was a major mediator of neurite outgrowth development. We also investigated the direct effect of BLP in PC 12 cells. When BLP was added to the PC 12 cells directly, neurite outgrowth was detected but NGF protein was not detected in PC 12 cells. Therefore, we hypothesized that BLP may act directly on PC 12 cells. These results suggest that BLP may activate TrkA-related signaling intermediates and mimic the neurotrophic actions of NGF in PC 12 cells. In Alzheimer's disease (AD), it has been reported that extensive degeneration and loss of cholinergic neurons in the basal forebrain cause severe memory impairment.[18] Although a therapeutic strategy for AD has not been developed, NGF is

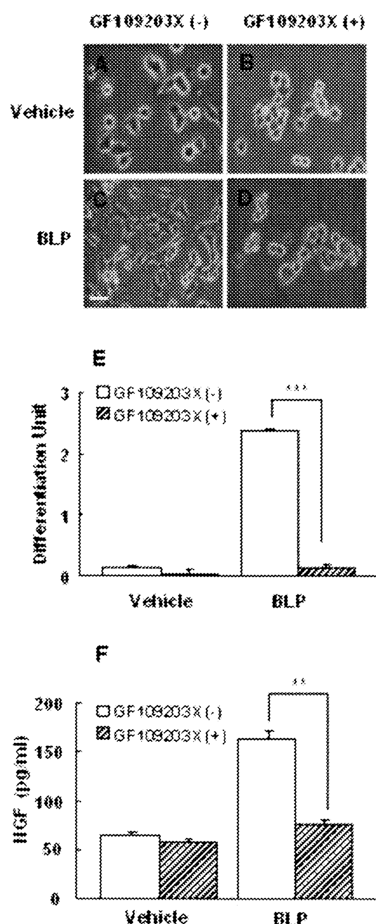

Fig. 4. Effect of GF109203X (10 μM) on BLP-Induced Neurite Outgrowth in PC12 Cells and NGF Secretion in C6 Cells

After C6 cells were incubated with BLP (10 μg/ml) in the presence or absence of GF109203X (5 μM) for 24 h, PC12 cells were incubated with the conditioned media for 48 h. (A) Media conditioned by vehicle (DMSO, 0.01%). (B) Media conditioned by vehicle (DMSO, 0.01%) plus GF109203X (5 μM). (C) Media conditioned by BLP (10 μg/ml). (D) Media conditioned by BLP (10 μg/ml) plus GF109203X (5 μM). (E) Assessment of differentiation unit of neurite outgrowth in sample-treated PC12 cells. The differentiation of PC12 cells was scored according to the method described in Fig. 1. (F) Effect of GF109203X on NGF secretion in C6 cells. Cells were incubated with the indicated concentration of BLP (10 μg/ml) with GF109203X (5 μM) for 6 h in C6 cells and the NGF released from C6 cells was measured by ELISA. GF109203X significantly reduced NGF protein in C6 cells compared with the treatment with BLP alone. Cells were photographed using a phase-contrast light microscope ($\times$100). Scale bar is 50 μm. The results are expressed as the mean $\pm$ S.E.M. Three independent experiments were carried out. The asterisks indicate a significant difference from the treatment with conditioned media plus GF109203X (** $p<0.01$, *** $p<0.001$).

expected to be useful in the therapy of AD.[18] Since NGF is produced physiologically in the brain, particularly by glial cells in the hippocampus and cerebral cortex, agents that can induce NGF synthesis/secretion in the brain should offer a useful and crucial alternative.[19] Compounds that exert effect on neurotrophin expression or secretion have been reported. Ginsenoside Rb1, a saponin from ginseng, up-regulated both NGF and TrkA, an NGF receptor.[20] In addition, kynurenine, a tryptophan metabolite, and *R*-(−)-1-(benzofuran-2-yl)-2-propylaminopentane enhanced the level of NGF mRNA expression in mouse astrocyte.[21,22] In the current study, despite the ability of BLP to enhance NGF synthesis/secretion, we were unable to identify which compound(s) is (are) responsible for these effects. However, since saponin was reported to induce NGF synthesis,[23] spicatoside A, major saponin present in LP, is thought to be a major NGF-inducing material. However, this remains to be verified.

NGF synthesis in astrocytes is regulated by various mechanisms. It has been reported that β-adrenergic agonists or cyclic AMP analogs as well as a PKC stimulator elicited NGF synthesis.[24] Thus, we could not exclude the possibility that other signal transduction pathways mediated the NGF synthesis in BLP-treated astrocyte cells.

In the light of the induction of astroglial NGF secretion and synthesis by BLP, BLP may possess therapeutic potential for treating neurodegenerative conditions. Further studies should focus on the precise correlation with a particular active constituent of BLP and the neurotrophic mechanism in this system.

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REFERENCES

1) Barres B. A., Barde Y., *Curr. Opin. Neurobiol.*, **10**, 64—68 (2000).
2) Levi-Montalcini R., *EMBO J.*, **6**, 1145—1154 (1987).
3) Pesavento E., Capsoni S., Domenici L., Cattaneo A., *Eur. J. Neurosci.*, **15**, 1030—1036 (2002).
4) Brinton R. D., Yamazaki R. S., *Pharm. Res.*, **15**, 386—398 (1998).
5) Jehan F., Neveu I., Phillippe N., Naveihan P., Wion D., Brachest P., *Brain Res.*, **672**, 128—136 (1995).
6) Baek N., Cho S. J., *Agri. Chem. Biotec.*, **41**, 390—394 (1998).
7) Kim S. W., Chang I. M., Oh K. B., *Biosci. Biotechnol. Biochem.*, **66**, 2751—2754 (2002).
8) Greene L. A., Tischler A. S., *Proc. Natl. Acad. Sci. U.S.A.*, **73**, 2424—2428 (1976).
9) Lee D. Y., Son K. H., Do J. C., Kang S. S., *Arch. Pharm. Res.*, **12**, 295—299 (1989).
10) Obara Y., Nakahata N., Kita T., Takaya Y., Kobayashi H., Hosoi S., Kiuchi F., Ohta T., Oshima Y., Ohizumi Y., *Eur. J. Pharmacol.*, **370**, 79—84 (1999).
11) Hur J. Y., Soh Y., Kim B. H., Suk K., Sohn N. W., Kim H. C., Kwon H. C., Lee K. R., Kim S. Y., *Biol. Pharm. Bull.*, **24**, 921—924 (2001).
12) Kwon H. C., Jung I. Y., Cho S. Y., Cho O. R., Yang M. C., Lee S. O., Hur J. Y., Kim S.Y., Yang J. B., Lee K. R., *Arch. Pharm. Res.*, **26**, 471—477 (2003).
13) Levi A., Biocca S., Cattaneo A., Calissano P., *Mol. Neurobiol.*, **2**, 201—226 (1988).
14) Yutaro O., Norimichi N., *Drug News Perspect.*, **15**, 290—298 (2002).
15) Pshenichkin S. P., Szekely A. M., Wise B. C., *J. Neurochem.*, **63**, 419—428 (1994).
16) Belliveau D. J., Krivko I., Kohn J., Lachance C., Pozniak C., Rusakov D., Kaplan D., Miller F. D., *J. Cell Biol.*, **136**, 375—388 (1997).
17) Satoh T., Nakamura S., Taga T., Hirano T., Kishimoto T., Aziro Y., *Mol. Cell. Biol.*, **8**, 1145—1150 (1988).
18) Whitehouse P., Price D., Struble R., Clarke A., Coyle J., Delong M., *Science*, **215**, 1237—1239 (1982).
19) Olson L., *Exp. Neurol.*, **124**, 5—15 (1993).
20) Saporito M. S., Carswell S., *J. Neurosci.*, **15** (3 Pt 2), 2280—2286 (1995).
21) Salim K. N., McEwen B. S., Chao H. M., *Brain Res. Mol. Brain Res.*, **47**, 177—182 (1997).
22) Lee D. R., Sawada M., Nakano K., *Neurosci. Lett.*, **244**, 17—20 (1998).
23) Ohta K., Ohta M., Mizuta I., Fujinami A., Shimazu S., Sato N., Yoneda F., Hayashi K., Kuno S., *Neurosci. Lett.*, **328**, 205—208 (2002).
24) Fukumoto H., Kakihana M., Suno M., *Glia*, **12**, 151—160 (1994).