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# Neurotrophic effects of FPF-1070 (Cerebrolysin®) on cultured neurons from chicken embryo dorsal root ganglia, ciliary ganglia, and sympathetic trunks

**T. Satou**[1], **T. Itoh**[1,2], **Y. Tamai**[3], **H. Ohde**[2], **A. J. Anderson**[4], and **S. Hashimoto**[1]

[1] 2nd Department of Pathology, Kinki University School of Medicine,
[2] Fujimoto Pharmaceutical Corp., Research Institute, and
[3] 3rd Department of Internal Medicine, Kinki University School of Medicine, Osaka, Japan
[4] Institute for Brain Aging and Dementia, University of California, Irvine, CA, U.S.A.

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**Summary.** We examined the effect of FPF-1070 (Cerebrolysin®) on neurite outgrowth in explant cultures of dorsal root ganglia (DRG), sympathetic trunks (ST), and ciliary ganglia (CG) from 10- to 11-day chicken embryos. FPF-1070 significantly promoted neurite outgrowth in DRG and ST neurons at all concentrations examined, in comparison with phosphate buffered saline-treated negative controls; however, this effect on neurite outgrowth was not as significant as that observed for nerve growth factor-treated positive controls on DRG and ST neurons. Additionally, FPF-1070 exhibited an inverted U relationship between concentration and effectiveness in DRG and ST neurons. In contrast, FPF-1070 did not affect neurite outgrowth in CG neurons although ciliary neurotrophic factor-treated positive controls showed striking neurite outgrowth. Our results demonstrate that FPF-1070 has different neurotrophic effects depending on the subpopulation of neurons. This study clarifies a role for neurotrophic activity in the mechanism of action of FPF-1070.

**Keywords:** Cerebrolysin®, neurotrophic factor, culture, chicken, neuron, FPF-1070.

## Introduction

FPF-1070 is a commercially available drug (distributed under the trade name Cerebrolysin) that is prepared by standardized enzymatic breakdown of lipid-free pig brain proteins and consists of 85% free amino acids and 15% biologically active small peptides (10 < Kd) (Gschanes et al., 1997). While FPF-1070 has been employed in the clinical treatment of cognitive deficits in dementia

and stroke sequelae for more than 40 years (Gschanes et al., 1997), the mechanisms of action of this nootropic drug remain to be elucidated. Positive effects of FPF-1070 on learning and memory have been reported in animal models (Francis-Turner and Valoušková, 1996; Hutter-Paier et al., 1996) and clinical trials (Kofler et al., 1990; Rüther et al., 1994). Several potential mechanisms of action for these effects of FPF-1070 on neuronal tissue have been suggested. In particular, it has been reported that FPF-1070 provides metabolic stabilization of neurons via protein synthesis modulation (Piswanger et al., 1990), prevention of lactose acidosis (Windisch and Piswanger, 1985), prevention of free radical formation (Sugita et al., 1993), and interaction with adenosine (Xiong et al., 1995) or GABAb receptors (Xiong et al., 1996).

The role and function of neurotrophic factors, including nerve growth factor (NGF), in the morphogenesis and functional maintenance of the nervous system has been defined and expanded in recent years (Vantini, 1992; Yuen et al., 1996). FPF-1070 has been reported to exhibit similar effects to NGF in vivo, in particular, FPF-1070 ameliorates septal neuron loss after fimbria-fornix transection (Akai et al., 1992). Additionally, we have previously reported that FPF-1070 has effects on nerve fiber elongation similar to those of NGF on cultured chick embryo dorsal root ganglia (DRG) neurons (Satou et al., 1994). In this study, we examined the effects of FPF-1070 on cultured neurons derived from sympathetic trunks (ST) and ciliary ganglia (CG), in comparison with DRG explants, in order to clarify the effects of FPF-1070 on various aspects of the peripheral nervous system.

## Methods

Cell culture was performed according to the procedures described by Barde et al. (1980). These are summarized as follows: Thirty chicken embryos aged 10 to 11 days old were sacrificed to obtain approximately 300 pieces of neural tissue from the dorsal root ganglia (DRG), 30 pieces from the sympathetic trunks (ST) and 50 pieces from the ciliary ganglia (CG). These different neuronal tissues were separately collected in dishes (Falcon) filled with 0.1 M phosphate buffered saline (PBS) (pH of 7.4). After being washed with PBS once, the samples were transferred into centrifuge tubes, spun at 1,500 rpm for 5 minutes, and the supernates completely removed. 30 ml of a 0.02% EDTE (Nakarai Co. Ltd.) plus 0.05% trypsin (GIBCO) solution was added to the tubes containing DRG cells and 12 ml of this solution was added to the tubes containing ST and CG cells. After gently pipetting, the samples were incubated in a 5%-$CO_2$ incubator at 37°C for 40 minutes. Following incubation, the tubes were centrifuged at 1,500 rpm for 5 minutes, and the supernates carefully removed. 24 ml of Eagle-MEM culture media (Nissui Co. Ltd.) supplemented with 10% fetal calf serum (FCS) (GIBCO) was added to the tubes containing DRG cells and 12 ml of this supplemented media was added to the tubes containing ST and CG cells. Neuronal tissues were then resuspended by gently pipetting several times, the suspensions filtered through nylon mesh and recollected in 6 cm-diameterdishes (Falcon) at a volume of 12 ml per dish, and the dishes incubated in a 5%-$CO_2$ incubator at 37°C for 130 minutes. After incubation, a fraction of the media containing floating cells was carefully collected in the tubes. Floating cells in the collected media were counted by a counting chamber and each media were diluted with Eagle-MEM/10% FCS up to $3 \times 10^3$ cells/ml to prepare cell suspensions. 1 ml per well of these cell suspensions was plated in each well of 24-well polystyrene culture plates (Falcon) coated with 1% poly-d-lysine (SIGMA). Immediately after plating, a series of test samples was prepared by adding 5, 10, 20, 40 or 80 µl of FPF-1070 (provided by *E*BEWE *A*RZNEIMITTEL Ges.m.b.H. Pharmaceutical Laborato-

ries) per culture well. Negative controls were prepared using identical volumes of PBS. For DRG and ST cultures, which are known to be NGF responsive, positive controls were prepared by adding 5 or 10 μl of NGF (TAKARA Co. Ltd.) solution which was prepared by dissolving 1 μg NGF in 1 ml PBS including 0.04% bovine serum albumin. As a result, final concentration of NGF was 5 or 10 ng/ml in the culture medium. For CG cultures, which are known to be CNTF responsive, positive controls were prepared by adding CNTF (MBL Co. Ltd.) to a final concentration of 2 ng/ml in the culture medium. Additionally, the effect of 5 or 10 ng/ml NGF on CG cultures was tested as described above for DRG and ST cultures.

FPF-1070-treated cultures, negative control cultures, and positive control cultures were maintained in a 5%-$CO_2$ incubator at 37°C for 4 days, followed by evaluation for neurite outgrowth. For DRG cultures, the observed groups were as follows: FPF-1070-treated, n = 40 wells for each drug concentration; PBS-treated negative controls, n = 12 wells; 5 μl NGF-treated positive controls, n = 12 wells; 10 μl NGF-treated positive controls, n = 12 wells. For ST cultures, the observed groups were as follows: FPF-1070-treated, n = 40 wells for each drug concentration; PBS-treated negative controls, n = 16 wells; 5 μl NGF-treated positive controls, n = 24 wells; 10 μl NGF-treated positive controls, n = 29 wells. For CG cultures, the observed groups were as follows: FPF-1070-treated, n = 4 wells for each drug concentration; PBS-treated negative controls, n = 9 wells; 5 μl NGF-treated positive controls, n = 4 wells; 10 μl NGF-treated positive controls, n = 4 wells; CNTF-treated positive controls, n = 4 wells. Nerve fiber outgrowth was evaluated by observing neurons that formed nerve fibers twice or more as long as their own cytoplasm under a phase-contrast microscope, and quantifying outgrowth according to a 3 point scale. Evaluation and quantification was as follows: score 1, when elongating nerve fibers from a single neuron were observed; score 2, when elongating nerve fibers from a pair of neurons were observed; score 3, when nerve fibers extended from a cluster comprising more than three neurons. Scores obtained from all of the neurons observed in each well were summed to derive a total nerve fiber outgrowth score for that well, and analyzed for statistical significance by one way ANOVA followed by post-hoc t-tests.

## Results

PBS-treated negative controls showed little or no nerve fiber outgrowth in DRG, ST, or CG cells (Figs. 1a, 2a and 3a). In contrast, NGF-treated positive controls for DRG and ST, and CNTF-treated positive controls for CG, showed obvious nerve fiber elongation (Figs. 1b, 2b and 3b). FPF-1070 induced nerve fiber outgrowth in DRG and ST cells (Figs. 1c and 2c), but did not induce nerve fiber outgrowth in CG cells (Fig. 3c). As expected, NGF did not induce nerve fiber outgrowth from CG neurons (Fig. 3d).

The nerve fiber elongation scores of the FPF-1070-treated groups for DRG and ST cultures, which were significantly higher than those of the PBS-treated negative controls (Figs. 4 and 5), increased in a dose-dependent manner at concentrations between 5 and 20 μl/ml of FPF-1070. However, FPF-1070-induced elongation scores in DRG and ST cultures declined at concentrations above 20 μl/ml of FPF-1070, and did not significantly facilitate nerve fiber outgrowth in ST at a concentration of 80 μl/ml in comparison with PBS-treated negative controls (Figs. 4 and 5). In addition, FPF-1070, even at the maximally effective concentration of 10–20 μl/ml, was not as effective as either concentration of NGF in promoting nerve fiber elongation in DRG and ST cultures (Figs. 4 and 5). Finally, as described qualitatively above, CNTF-treated positive CG culture controls exhibited a significant increase in quantified nerve fiber elongation scores in comparison with either FPF-1070-

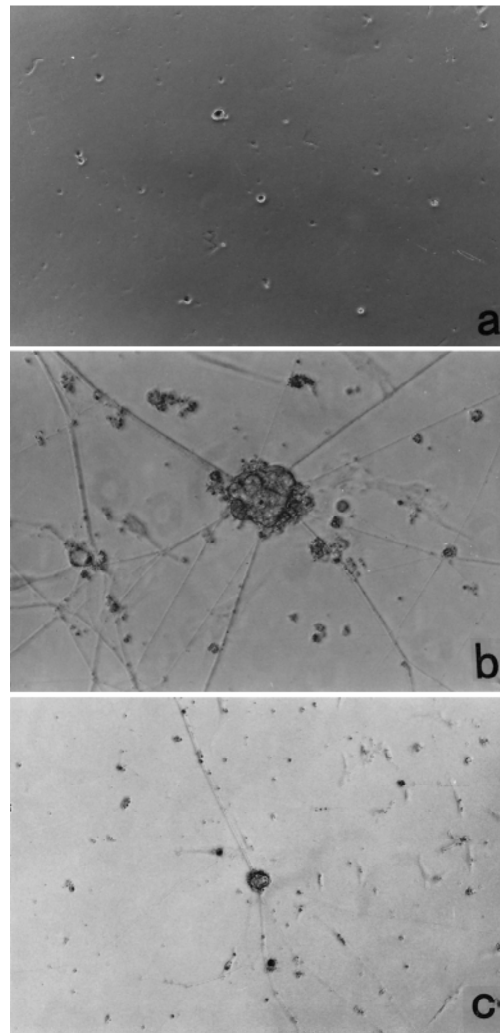


**Fig. 1.** Cultured neurons from dorsal root ganglia (DRG) under a contrast phase microscope. **a** DRG neurons do not exhibit neurite elongation in the PBS-treated negative control condition (3.3 × 10) **b** DRG neurons exhibit clump formation and multiple points of nerve fiber outgrowth and neurite elongation in the NGF-treated positive control condition (3.3 × 20) **c** Scattered DRG neurons exhibit neurite elongation in the FPF-1070 treated condition (3.3 × 20)

treated or PBS-treated negative control cultures, and no significant difference in CG neurite outgrowth was observed between FPF-1070-treated and PBS-treated negative control cultures (Fig. 6).

## Discussion

Research into the clinical applicability of neurotrophic factors for the treatment of various neurological disorders, such as neurodegenerative diseases (Hughes and O'Leary, 1996), dementia (Hefti et al., 1996), and peripheral

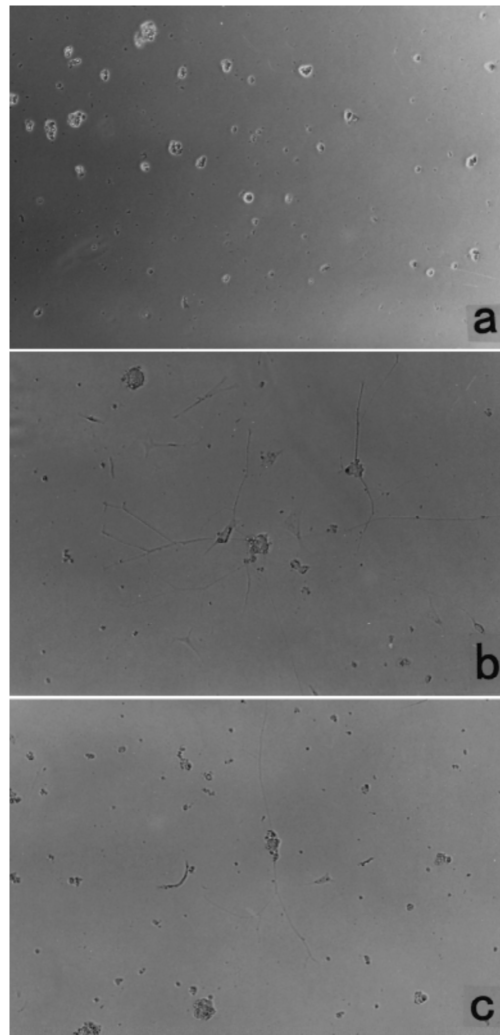


**Fig. 2.** Cultured neurons from sympathetic trunks (ST) under a contrast phase microscope. **a** ST neurons do not exhibit neurite elongation in the PBS-treated negative control condition ($3.3 \times 10$) **b** ST neurons exhibit neurite elongation and clump formation in the NGF-treated positive control condition ($3.3 \times 10$) **c** Scattered ST neurons exhibit neurite elongation in the FPF-1070-treated condition ($3.3 \times 10$)

neuropathy (Apfel and Kessler, 1996), has been an active area of investigation. However, several problems for the clinical use of neurotrophic factors remain to be addressed, including the route of administration, because these factors do not pass through the blood-brain barrier (Lindsay et al., 1993). The present study demonstrates that FPF-1070 has an effect analogous to that of neurotrophic factors such as NGF, as assessed by the induction of nerve fiber outgrowth on DRG and ST cells. Thus, FPF-1070, which can be administered intravenously, appears to be a useful clinical agent that may exert its nootropic (cognition enhancing) effects in part via neurotrophic activity.

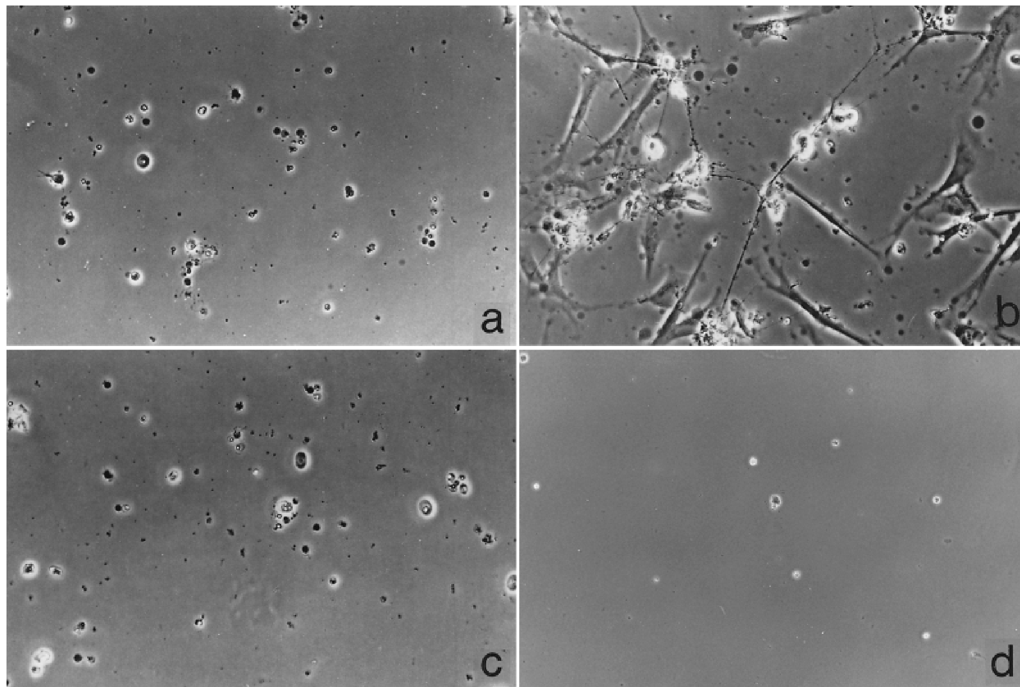


**Fig. 3.** Cultured neurons from ciliary ganglia (CG) under a contrast phase microscope. **a** CG neurons do not exhibit neurite elongation in the PBS-treated negative control condition ($3.3 \times 20$) **b** CG neurons exhibit neurite elongation in the CNTF-treated positive control condition ($3.3 \times 20$) **c** CG neurons do not exhibit neurite elongation in the FPF-1070-treated condition ($3.3 \times 20$) **d** CG neurons do not exhibit neurite elongation in the NGF-treated group ($3.3 \times 10$)

In contrast to its effect on DRG and ST cells, FPF-1070 did not induce nerve fiber outgrowth in CG neurons. This finding suggests that FPF-1070 exerts its neurotrophic actions in a neural cell subpopulation-dependent fashion. DRG and ST neuronal cultures are NGF-dependent, whereas CG neuronal cultures are not NGF-, but rather CNTF-dependent (Lindsay et al., 1994; Yuen et al., 1996). Thus, the clinical efficacy of FPF-1070 could vary depending upon the cell type predominantly affected in the presenting neurological disorder. Additionally, the differential effect of FPF-1070 on NGF- and CNTF-dependent neurons could suggest that this drug preparation might also mimic some of the negative potential effects of these neurotrophic factors. For example, NGF has been reported to have an adverse effect on myalgic syndrome, manifesting hyperalgesia (Lewin et al., 1993), degranulation of mast cells, and activation of nociceptors (Marshall et al., 1990), and CNTF has been reported to produce severe weight loss (Henderson et al., 1994). Consequently, investigation of the pharmacological effects of FPF-1070 on different neuronal subtypes may be important for the clinical application of FPF-1070. Nonetheless, clinical trials have demonstrated that FPF-1070 has therapeutic effects on diabetic neuropathy (Biesenbach et al., 1997), acute cerebrovascular diseases (Domżał and Zaleska, 1995), and Alzheimer's dis-

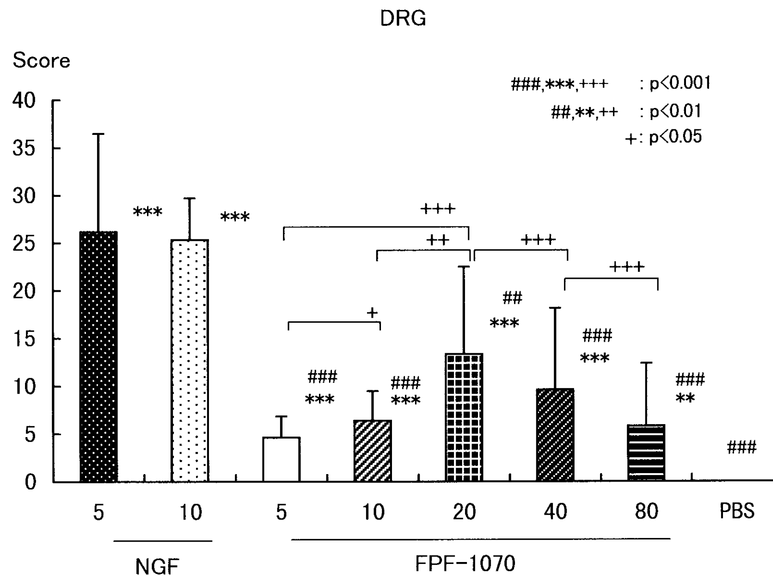


**Fig. 4.** Quantitative analysis of nerve fiber elongation in DRG neuron cultures. Nerve fiber elongation was assessed based on the growth and extension of neurites, scored on a scale from 1 to 3 as described under Methods. The number of observations for each condition was as follows: FPF-1070-treated cultures, n = 40 wells for each drug concentration; PBS-treated negative controls, n = 12 wells; 5 μl NGF-treated positive controls, n = 12 wells; 10 μl NGF-treated positive controls, n = 12 wells. Statistical analysis was via one way ANOVA followed by post-hoc t-test. FPF-1070 exhibited an inverted U curve for the relationship between concentration and nerve fiber outgrowth score. FPF-1070 exhibited its maximal neurotrophic effect at 20 μl FPF-1070 a well, although its effects were significantly weaker than NGF at all concentrations examined. ***($p < 0.001$), **($p < 0.01$); compared with PBS-treated negative control groups. ###($p < 0.01$), ##($p < 0.01$); compared with NGF-treated positive control groups

ease (AD) and cerebrovascular dementia (Rainer et al., 1997) without adverse side effects, contraindications, or measurable toxicity (Gschanes et al., 1997).

This study suggests that FPF-1070 has neurotrophic effects on NGF-dependent neurons in addition to the other mechanisms of action previously reported for this drug preparation, including metabolic stabilization of nerve cells (Piswanger et al., 1990), prevention of lactose acidosis (Windisch et al., 1985), and prevention of free-radical formation (Sugita et al., 1993). In this context, these findings suggest a potential mechanism for the clinical effectiveness of FPF-1070 in the AD brain, which exhibits a dramatic loss of NGF-dependent forebrain cholinergic neurons in association with memory loss and cognitive decline (Vantini, 1992). Interestingly, intracerebral administration of NGF has been suggested as a potential therapeutic to prevent brain atrophy in AD (Hefti et al., 1996).

The present study revealed that nerve fiber outgrowth in DRG and ST cells was suppressed by higher doses of FPF-1070. In accordance with this observation, Gschanes et al. reported that intraventricular administration of

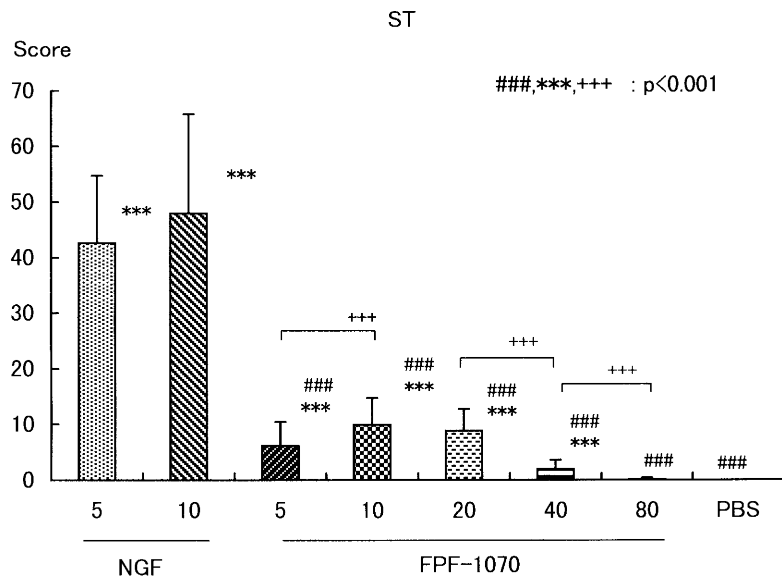


**Fig. 5.** Quantitative analysis of nerve fiber elongation in ST neuron cultures. Nerve fiber elongation was assessed based on the growth and extension of neurites, scored on a scale from 1 to 3 as described under Methods. The number of observations for each condition was as follows: FPF-1070-treated, n = 40 wells for each drug concentration; PBS-treated negative controls, n = 16 wells; 5 μl NGF-treated positive controls, n = 24 wells; 10 μl NGF-treated positive controls, n = 29 wells. Statistical analysis was via one way ANOVA followed by post-hoc t-test. FPF-1070 exhibited an inverted U curve for the relationship between concentration and nerve fiber outgrowth score. FPF-1070 exhibited its maximal neurotrophic effect at 10 to 20 μl FPF-1070 a well, although its effects were significantly weaker than NGF at all concentrations examined. ***($p < 0.001$); compared with PBS-treated negative control groups. ###($p < 0.001$); compared with NGF-treated positive control groups

high doses of FPF-1070 induced impairment of learning and memory in experimental rats (Gschanes et al., 1997). In contrast to intraventricular administration of high doses of FPF-1070 or direct exposure to FPF-1070 in culture cells, intravenous administration of FPF-1070 does not lead to a high concentration of FPF-1070 in the brain, because of the blood brain barrier. In this regard, it has been previously reported that only a small proportion of low molecular weight FPF-1070 peptides administered intravenously can pass through the blood-brain barrier to neuronal tissue, where they are biologically active (Gschanes et al., 1997). Thus, it appears that there is an optimal concentration of FPF-1070 which may be most effective in exerting neurotrophic effects on neural tissues. However, because the blood brain barrier may be severely compromised in some patients, further in vivo studies directed at examining this issue with FPF-1070 may be critical to optimizing the therapeutic applicability of this drug.

In conclusion, this study clarifies a role for neurotrophic activity in the mechanism of action of FPF-1070, and supports the clinical applicability of

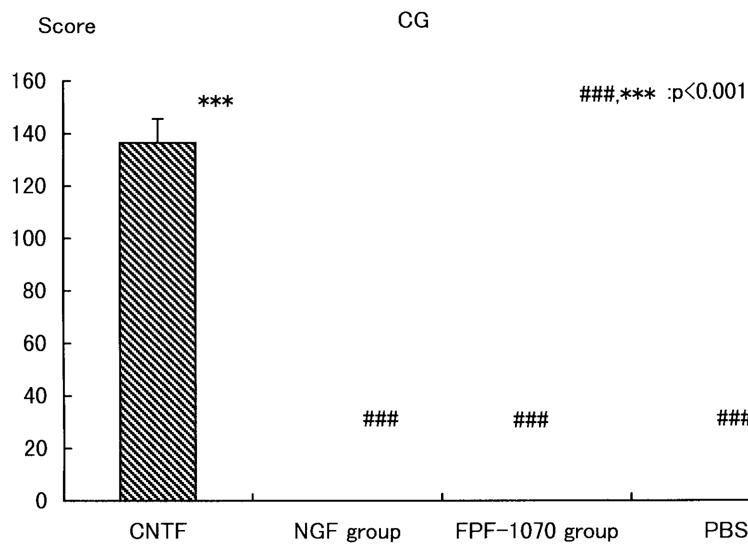


**Fig. 6.** Quantitative analysis of nerve fiber elongation in CG neuron cultures. Nerve fiber elongation was assessed based on the growth and extension of neurites, scored on a scale from 1 to 3 as described under Methods. The number of observations for each condition was as follows: FPF-1070-treated, n = 4 wells for each drug concentration; PBS-treated negative controls, N = 9 wells; 5 μl NGF-treated positive controls, n = 4 wells; 10 μl NGF-treated positive controls, n = 4 wells; CNTF-treated positive controls, n = 4 wells. Statistical analysis was via one way ANOVA followed by post-hoc t-test. In contrast to CNTF-treated positive controls, neither FPF-1070 nor NGF exhibited neurite-promoting effects on CG neurons. ***($p < 0.001$); compared with PBS-treated negative control groups. ###($p < 0.001$); compared with CNTF-treated positive control groups

FPF-1070 to the treatment of neurodegenerative diseases, dementia, and brain ischemia.

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Authors' address: Dr. T. Satou, 2nd Department of Pathology, Kinki University School of Medicine, 377-2 Ohnohigashi, Osakasayama, Osaka 589-8511, Japan