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Granulatoside A, a Starfish Steroid Glycoside, Enhances PC12 Cell Neuritogenesis Induced by Nerve Growth Factor through an Activation of MAP Kinase

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Nerve growth factor (NGF) is the first and best characterized member of the neurotrophin family and an important factor for growth, differentiation, survival, and function maintenance of neurons.[1] Although NGF is expected to have therapeutic potential, it is also unstable and cannot readily pass through the blood-brain barrier. Thus, attempts have been made to identify the exogenous substances that mimic and/or enhance its physiological action.[2] Since the PC12 cell line derived from rat pheochromocytoma cells expresses neuronal differentiation in response to NGF, this is a useful model not only for screening studies but also for investigating the signal transduction pathways of PC12 cell neuritogenesis.[3] Our previous search for compounds that mimic the neuritogenic activity of NGF using the PC12 cell line system resulted in the isolation of a series of steroid glycosides named linckosides (for example, linckoside B (**4**)) from the Okinawan blue starfish *Linckia laevigata*.[4] A further examination of the related compounds led to identification of a known metabolite, granulatoside A (**1**), which was originally isolated from the starfish *Choriaster granulatus*.[5] Although granulatoside A (**1**) did not induce any neurite outgrowth in PC12 cells, it potently enhanced the neuritogenic activity of NGF. Here we report the novel NGF-enhancing activity of granulatoside A (**1**), a brief description of the structure–activity relationship, and the cellular mechanism involving mitogen-activated protein (MAP) kinase.

Granulatoside A (**1**) was obtained in 0.002% yield (based on the wet weight of the starfish *L. laevigata*) using the HPLC process that previously yielded linckoside F.[4c] This metabolite was originally isolated from a different starfish and identified by comparing its spectral properties with the reported data.[5]

Granulatoside A (**1**) did not induce the NGF-like neuronal differentiation in PC12 cells up to a concentration of 40 μM (Figure 1, 2a), despite its structural similarity to some active linckosides.[4] However, in the presence of a trace amount of NGF (1.5 ng mL^{-1}) that scarcely induced neurite outgrowth (Figure 1, 2b), granulatoside A (**1**) significantly induced neurite outgrowth in a dose-dependent manner (Figure 1). In the presence of **1** at 40 μM, the neuritogenesis reached the maximum of 95%, which was comparable to the activity of 40 ng mL^{-1} of NGF (Figure 1, 2c). The cells cultured without the agents did not exhibit any neurite outgrowth (Figure 2d). At a concentration higher than 80 μM, the compound showed a little cytotoxicity. The IC_{50} value of **1** against PC12 cells was estimated to be higher than 200 μM by MTT assay (data not shown), suggesting that **1** is a low-toxicity agent with interesting biological activity.

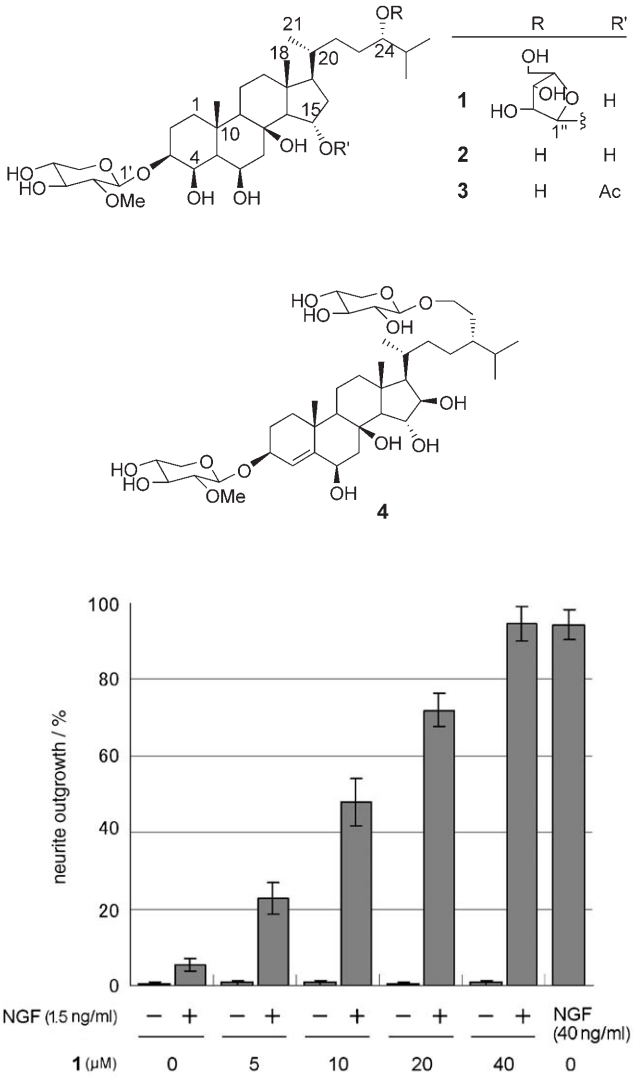

Figure 1. Dose-dependent enhancement of NGF-induced neuritogenesis in PC12 cells by granulatoside A (**1**) 3 days after treatment.

The synergistic effect exerted on the PC12 cell neuritogenesis by granulatoside A (**1**) and NGF was examined by varying their concentrations (Figure 3). The effect significantly increased as the concentration of **1** became higher and the concentration of NGF became lower, indicating that **1** potently enhanced NGF but not vice versa. For example, the peak activity was achieved using the combination of 1 ng mL^{-1} of NGF and 40 μM of **1**, and was comparable to the activity by 40 ng mL^{-1} of NGF alone, meaning an approximately 40-fold enhancement of the activity of NGF.

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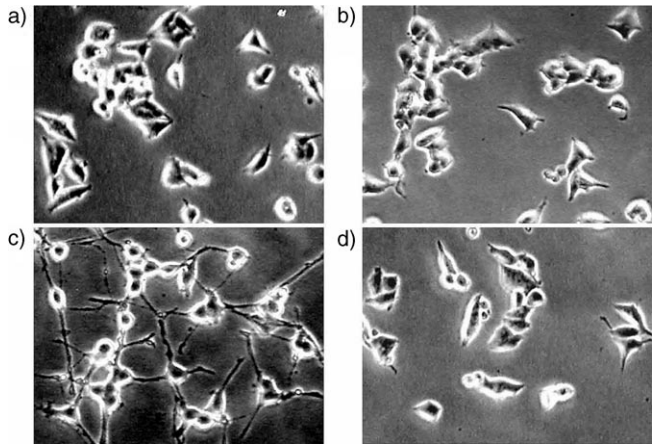

Figure 2. Phase-contrast photomicrographs of PC12 cells treated with granulatoside A (**1**) and/or NGF on day 3: a) 40 μM of **1**; b) 1.5 ng mL^{-1} of NGF; c) 1.5 ng mL^{-1} of NGF in the presence of 40 μM of **1**; d) control (1% DMSO).

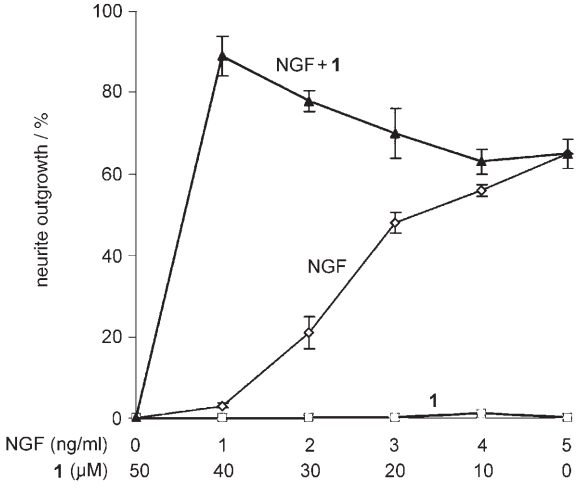

Figure 3. A synergistic effect of granulatoside A (**1**) and NGF on PC12 cells 3 days after treatment. Granulatoside A (**1**) at a higher concentration significantly enhanced the neuritogenic activity of NGF at a lower concentration.

The structural factors of **1** affecting the enhancement of NGF-induced neuritogenesis were then studied. Granulatoside A (**1**) was subjected to mild hydrolysis with aqueous acetic acid to give dearabinosyl granulatoside A (**2**) and monoacetate **3** in a ratio of approximately 1:1. The structure of **2** was identified as a known compound by comparing its NMR data with the previously reported data.[5] The full assignment of the ^{1}H NMR data was carried out in this study. The structure of **3** was determined by spectroscopic methods as follows. The molecular formula $C_{35}H_{60}O_{11}$ was determined by high-resolution ESI-TOFMS. The ^{1}H NMR signals due to L-arabinose at the side chain of **1** disappeared in **2**, and a methyl signal was observed at δ_H 2.00 ppm. Taking the molecular formula into consideration, monoacetylation seemed to occur during the hydrolysis followed by the concentration procedure. The acetylation at position C15 was finally determined from a lower-field shift of H15 (δ_H 4.29 to 5.15) in ^{1}H NMR, and the full signal assignment was performed by ^{1}H–^{1}H COSY and HOHAHA spectra.

The two derivatives **2** and **3** exhibited a relatively narrow range of effective concentrations for the enhancement of NGF-induced neuritogenesis. No neuritogenesis was observed for **2** at 10 μM or lower, and the low activity of **2** at 40 μM was due to cytotoxicity (Table 1). The derivative **3** showed a similar tendency but a higher cytotoxicity than **2**. The hydrophilic nature of the molecule, especially around the side chain, could play an important role in the enhancement of the NGF activity, possibly by suppressing cytotoxicity.

Table 1. Enhancement of NGF-induced neuritogenesis by granulatoside A (**1**) and its derivatives **2** and **3**.[a]

Conc. [μM]	**1**	**2**	**3**
10	48 ± 5	0	0
20	74 ± 4	70 ± 3	72 ± 2
40	95 ± 4	60 ± 5	toxic

[a] PC12 cells were treated with NGF (1.5 ng mL^{-1}) in the presence of **1**, **2**, or **3** at the indicated concentrations. Values are the percentages of cells with neurite outgrowth.

The cellular mechanism of the NGF-induced neuronal differentiation has been well investigated. NGF first binds to the specific transmembrane receptor TrkA to induce phosphorylation of the specific tyrosine residues located at the intracellular domain. This signaling event then leads to recruitment and activation of a number of signaling molecules including kinases. Among them, the MAP kinase extracellular signal-regulated kinase (ERK) is well recognized as a key enzyme during the NGF-induced neuritogenesis.[6] To investigate the cellular events during the enhancement of NGF-induced neuritogenesis by granulatoside A (**1**), phosphorylation of ERK in PC12 cells was first examined in the presence of **1** and NGF. NGF alone at a low concentration (1.5 ng mL^{-1}) induced a transitory phosphorylation of ERK1/2 for 1 h (Figure 4a), resulting in a little neurite extension in PC12 cells. Granulatoside A (**1**) alone at a concentration of 40 μM, which induced no neurite extension in PC12 cells (Figure 1 and 2), did not show phosphorylation of ERK1/2 over 24 h. However, sustained and enhanced ERK phosphorylation was observed over 4 h when the PC12 cells were treated with NGF (1.5 ng mL^{-1}) and **1** (20 μM) at the same time (Figure 4a). Next, phosphorylation of the NGF receptor TrkA was examined in a similar way. Granulatoside A (**1**) at 40 μM did not activate TrkA at all, and NGF at 1.5 ng mL^{-1} induced only slight phosphorylation. Unlike the case of ERK, phosphorylation of TrkA by NGF was not enhanced by the addition of granulatoside A (**1**) (Figure 4b). These results suggest that the enhancement of the NGF-induced neuritogenesis by granulatoside A (**1**) is dependent on the sustained activation of ERK and that a low concentration of NGF inducing only a transitory phosphorylation of ERK is inadequate for complete neuronal differentiation of PC12 cells. This is consistent with the previous reports that the persistent ERK phosphorylation is critical for neuronal differentiation of PC12 cells.[7]

In summary, a known marine steroid glycoside, granulatoside A (**1**), was newly isolated from a different marine source

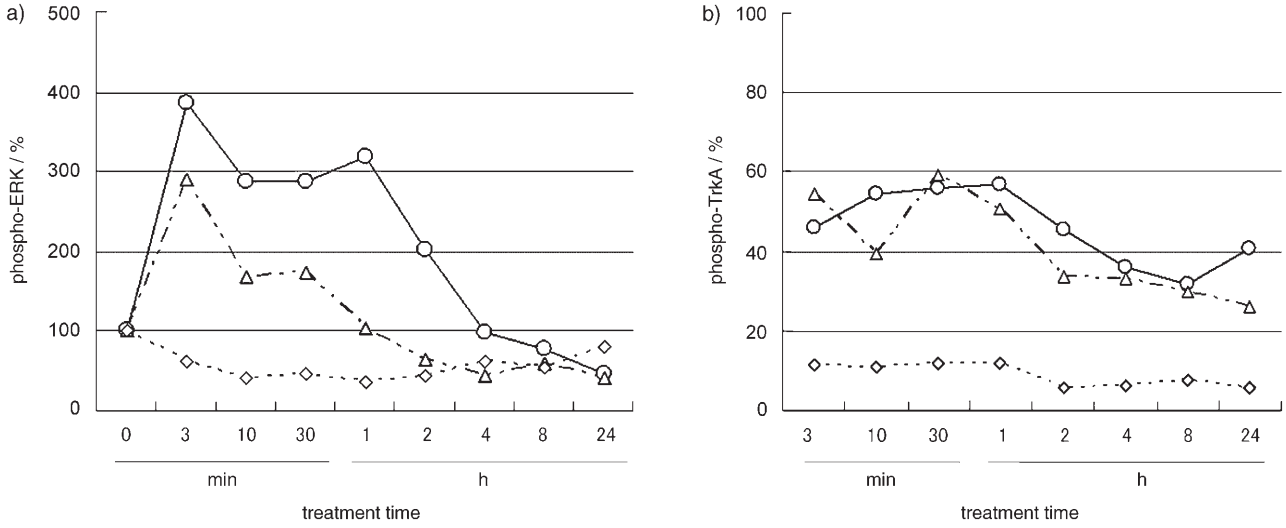

Figure 4. Phosphorylation of a) ERK and b) TrkA in the presence of granulatoside A (**1**) and/or NGF: ○ = NGF (1.5 ng mL^{-1}) + granulatoside A (**1**, 20 μM), △ = NGF (1.5 ng mL^{-1}) alone, ◇ = **1** (40 μM) alone. The phosphorylation levels (%) are measured by a luminescent image analyzer after Western blotting and normalized to the background value (100%) for phospho-ERK or to the value of 10 ng mL^{-1} NGF at 10 min (100%) for phospho-TrkA.

and found to potently enhance NGF-induced neuritogenesis. Although granulatoside A (**1**) itself did not induce neurite outgrowth in PC12 cells, it induced incredible neurite outgrowth in the presence of a trace amount of NGF which, by itself, scarcely affected PC12 cells. The compound was found to be innocuous to PC12 cells over a wide range of concentrations. A glycoside at the side chain might play an important role in the observed biological activities. The enhancement of NGF-induced neuritogenesis by granulatoside A (**1**) is attributable to both enhancement and maintenance of the phosphorylation of MAP kinase ERK1/2 in PC12 cells, although the upstream pathways are unclear. This marine metabolite has never been investigated as a biologically active substance, and its potential for improving NGF deficiency can be further explored.

Experimental Section

General. Optical rotations were measured on a DIP-370 digital polarimeter (Jasco, Tokyo, Japan). HR ESI-TOFMS spectra were recorded on a Mariner Biospectrometry Workstation (Applied Biosystems, CA, USA). NMR spectra were recorded on an AMX2–600 spectrometer (Bruker, Rheinstetten, Germany), and chemical shifts δ in ppm were referenced to the solvent peaks of δ_C 49.0 and δ_H 3.30 for CD_3OD.

Extraction and isolation. The extraction and purification procedures were described in detail in a previous report.[4c] The reversed-phase HPLC that yielded linckoside F gave several other fractions. Among them, the fraction eluted at the retention time of 74.3–81.6 min contained pure granulatoside A (**1**) (150 mg): colorless powder, $[\alpha]_D^{24} = -21$ ($c = 0.37$, MeOH) [reported data: $[\alpha]_D = -16$ ($c = 1.0$, MeOH)].[5]

Partial hydrolysis of granulatoside A (1) to give 2 and 3. A solution of **1** (3 mg) in 50% aqueous acetic acid (1 mL) was stirred at 60 °C for 48 h. The reaction mixture was concentrated and the residue was adsorbed on an ODS cartridge (Toyopak ODS-M, Tosoh, Tokyo, Japan), which was then eluted with 100% MeOH (3 mL). The eluate was concentrated and subjected to TLC [$CHCl_3$/MeOH (6:1), developed twice] to give **2** (1.0 mg $R_f = 0.44$) and **3** (1.2 mg $R_f = 0.66$).

Dearabinosyl granulatoside A (***2***): colorless powder, ^{1}H NMR (600 MHz, CD_3OD): $\delta = 4.46$ (d, $J = 7.6$ Hz, H-1′), 4.27 (m, 1H, H-15), 4.26 (m, 2H, H-4, H-6), 3.82 (dd, $J = 11.4$, 5.2 Hz, 1H, H-5′a), 3.64 (m, 1H, H-3), 3.63 (s, 3H, OMe), 3.48 (m, 1H, H-4′), 3.34 (t, $J = 9.0$ Hz, 1H, H-3′), 3.18 (m, 1H, H-24), 3.16 (dd, $J = 11.4$, 10.2 Hz, 1H, H-5′b), 2.91 (dd, $J = 9.0$, 7.6 Hz, 1H, H-2′), 2.41 (dd, $J = 14.8$, 2.4 Hz, 1H, H-7a), 1.73 (m, 1H, H-1a), 1.71 (m, 1H, H-2b), 1.96 (m, 2H, H-2a, 12a), 1.91 (m, 1H, H-16a), 1.80 (m, 1H, H-11a), 1.70 (m, 1H, H-16b), 1.61 (m, 1H, H-25), 1.59 (m, 2H, H-7b, 22a), 1.57 (m, 1H, H-23a), 1.48 (m, 1H, H-11b), 1.44 (s, 3H, H-19), 1.36 (m, 1H, H-20), 1.32 (m, 1H, H-17), 1.23 (m, 2H, H-5, 12b), 1.22 (m, 1H, H-23b), 1.17 (d, $J = 9.6$ Hz, 1H, H-14), 1.02, (m, 1H, H-1b), 0.99 (m, 1H, H-22b), 0.98 (m, 1H, H-9), 0.96 (s, 3H, H-18), 0.91 (d, $J = 6.8$ Hz, 1H, H-21), 0.90 (d, $J = 6.6$ Hz, 3H, H-26), 0.89 ppm (d, $J = 6.6$ Hz, 3H, H-27); HRMS (ESI-TOF) *m/z*: calcd for $C_{33}H_{58}O_{10}Na$: 637.3922 $[M+Na]^+$, found: 637.3964.

15-O-acetyl dearabinosyl granulatoside A (***3***): colorless powder, $[\alpha]_D^{25} = +6.3$ (*c* 0.03, MeOH); ^{1}H NMR (600 MHz, CD_3OD): $\delta = 5.15$ (ddd, $J = 9.9$, 9.9, 3.0 Hz, 1H, H-15), 4.45 (d, $J = 7.6$ Hz, H-1′), 4.25 (m, 1H, H-4, 6), 3.82 (dd, $J = 11.5$, 5.6 Hz, 1H, H-5′a), 3.64 (m, 1H, H-3), 3.62 (s, 3H, OMe), 3.18 (m, 1H, H-24), 3.47 (m, 1H, H-4′), 3.35 (t, $J = 8.8$ Hz, 1H, H-3′), 3.16 (dd, $J = 11.5$, 10.8 Hz, 1H, H-5′b), 2.90 (dd, $J = 8.8$, 7.6 Hz, 1H, H-2′), 2.04 (m, 1H, H-16a), 2.00 (s, 3H, Ac), 2.00 (m, 1H, H-12a), 1.97 (m, 1H, H-7a), 1.93 (m, 1H, H-2a), 1.83 (m, 1H, H-11a), 1.72 (m, 1H, H-1a), 1.70 (m, 1H, H-2b), 1.63 (m, 1H, H-16b), 1.61 (m, 1H, H-7b), 1.60 (m, 1H, H-25), 1.59 (m, 1H, H-22a), 1.53 (m, 1H, H-23a), 1.49 (m, 1H, H-11b), 1.47 (d, $J = 9.9$ Hz, 1H, H-14), 1.43 (s, 3H, H-19), 1.37 (m, 1H, H-20), 1.31 (m, 1H, H-17), 1.25 (m, 1H, H-12b), 1.24 (m, 1H, H-5), 1.19 (m, 1H, H-23b), 1.02 (m, 1H, H-1b), 1.01 (m, 1H, H-9), 0.99 (s, 3H, H-18), 0.95 (m, 1H, H-22b), 0.92 (d, $J = 6.4$ Hz, 1H, H-21), 0.89 (d, $J = 7.0$ Hz, 3H, H-26), 0.88 ppm (d, $J = 7.0$ Hz, 3H, H-27); HRMS (ESI-TOF) *m/z*: calcd for $C_{35}H_{60}O_{11}Na$: 679.4028 $[M+Na]^+$, found: 679.4017.

Bioassay for neurite outgrowth of PC12 cells. The activity was evaluated according to the methods described in our previous paper.[4] Briefly, twenty-thousand PC12 cells in MEME medium (1 mL) were placed in each well of a 24-well microplate and precultured in a CO_2 incubator at 37 °C. Twenty four hours later, the medium was replaced by 1 mL of serum-free MEME medium containing 1% DMSO and a test sample. For evaluation of enhancement of the NGF-mediated neurite extension, the medium was replaced with 1 mL of serum-free MEME medium containing 1.5 ng of NGF (Recombinant Human β-NGF, R&D Systems Inc., BC, Canada) and a test sample. The morphological changes of the cells were monitored under a phase-contrast microscope every 24 h for 6 days. The numbers of the cells with a longer process than the cell diameter were counted in three randomly chosen areas that included approximately a hundred cells.

Cell viability. Cell viability was quantified by the MTT assay. Briefly, PC12 cells incubated with **1** for 24 h were treated with 10 μL of 0.5% (*w/v*) MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in phosphate-buffered saline (PBS) for 4 h. The cells were then lysed with 0.04 N HCl in isopropyl alcohol, and the absorbance was read at 570 nm by a microplate reader.

Western blot analysis. PC12 cells (4×10^5 cells) in 1 mL of serum-containing MEME medium (10% horse serum and 5% fatal bovine serum) were placed in a 3.5 cm dish (Sumilon Petri dish for cell/tissue culture, Sumitomo Bakelite Co., Ltd., Tokyo, Japan) and incubated at 37 °C for 24 h. The medium was replaced with serum-free medium, and the cells were incubated for 16 h. The medium was replaced with serum-free medium containing a compound and 1% DMSO. The cells were incubated for the times indicated, rinsed twice with 0.5 mL of ice-cold PBS (137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, 1.47 mM K_2HPO_4), and then stored at −80 °C until use. The treated cells were lysed with an ice-cold mixture of 0.1 mL of lysis buffer (PBS containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 2 mM EDTA) and 1 μL of phosphatase inhibitor cocktail 1/2 (Sigma) on ice for 5 min. The cell lysate was centrifuged in a 1.5 mL tube at 10000 rpm at 4 °C for 5 min. A small portion of the supernatant was subjected to protein quantification with a protein assay reagent (Bio-Rad Lab., Inc., CA, USA). The rest was heated at 100 °C for 5 min with a half volume of SDS sample buffer (3×: 150 mM Tris-HCl, pH 6.8, 12% mercaptoethanol, 6% SDS, 30% glycerol). An aliquot (17.6 μg protein for TrkA or 10.6 μg protein for ERK) was run on a 6% (for TrkA) or 10% (for ERK) SDS-PAGE slab gel at 24 mA under the Laemmli conditions. The gel was transblotted onto a PVDF membrane (Immobilon-P Transfer Membrane, Millipore, MA, USA) with a Trans-Blot SD Cell blotter (Bio-Rad Lab.) at 2 mA cm^{-2}. The gel was stained with Quick CBB (Wako) to visualize proteins. The membrane was rinsed with TBS-T (20 mM Tris-HCl, pH 7.5, 140 mM NaCl, 0.1% Tween 20), incubated with 0.3% skim milk in TBS-T at room temperature for 1 h for blocking, washed three times with TBS-T, and agitated overnight at 4 °C with 5 mL of antiphospho-TrkA (Tyr490) antibody or anti-phospho-p44/42 MAP Kinase antibody (Cell Signaling Technology, Inc., MA, USA), which were diluted 1000 times with 5% BSA in TBS-T. The membrane was rinsed three times with TBS-T and treated for 1 h at room temperature with antirabbit IgG HRP-linked antibody (Cell Signaling Technology), which was diluted 2000 times with TBS-T. The membrane was rinsed three times with TBS-T and treated with ECL Western Blotting Detection Reagents (GE Healthcare Bio-Science Co., Ltd., NJ, USA). Bands were visualized by a Luminescent Image Analyzer LAS-1000 CH (Fuji Photo Film Co., Ltd., Tokyo, Japan) and analyzed by Science Lab 2005 Multi Gauge version 3.0 software.

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