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# Metabolite 1 of Protopanaxadiol-Type Saponins, an Axonal Regenerative Factor, Stimulates Teneurin-2 Linked by PI3-Kinase Cascade

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We previously showed that 20-*O*-$\beta$-D-glucopyranosyl-20(*S*)-protopanaxadiol (M1), a metabolite of protopanaxadiol-type ginseng saponins by intestinal bacteria had axonal extension activity in degenerated neurons, and improved memory disorder and synaptic loss induced by an active fragment of amyloid $\beta$, A$\beta$(25–35). It is unknown how M1 shows these effects in neurons. To clarify the signal transduction mechanism of M1-induced axonal extension, phosphorylated proteins by M1 stimulation were identified because most cellular signal pathways are regulated by phosphorylation/dephosphorylation. The combination of immunoprecipitation and MALDI-TOF-MS revealed that teneurin-2 and mPar3 were specifically phosphorylated by M1 stimulation. Because mPar3 is known as an axonal specifying molecule and to be regulated by phosphatidylinositol 3-kinase (PI3-kinase), the involvement of teneurin-2 and PI3-kinase in the M1 signal was studied. In teneurin-2-deficient cortical neurons, M1-induced axonal extension and PI3-kinase activation were significantly inhibited. In addition, treatment with PI3-kinase inhibitor also reduced M1-induced axonal extension. These results suggest that M1 induces axonal outgrowth through the teneurin-2—PI3-kinase cascade.
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## INTRODUCTION

Ginseng, the root of *Panax ginseng*, is widely used as a tonic medicine throughout the world, and has also been found to be efficacious in the treatment of amnesia. We previously studied the neurite outgrowth activity of methanol extracts from seven ginseng drugs and 19 constituents isolated from Ye-Sanchi (rhizome of *Panax vietnamensis* HA et GRUSHV. var. *fuscidiscus* K.KOMATSU, S.ZHU & S.Q.CAI) and Kouzichi (rhizome of *P. japonicus* C.A. MEYER var. *major* C.Y.WU et FENG) in human neuroblastoma SK-N-SH cells, and found that protopanaxadiol-type (ppd-type) saponins (ginsenoside Rb$_1$, ginsenoside Rb$_3$, notoginsenoside R$_4$ and notoginsenoside Fa) were the active compounds (Tohda *et al*, 2002). Ppd-type saponins are known to be completely metabolized into 20-*O*-$\beta$-D-glucopyranosyl-20(*S*)-proto-

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panaxadiol (M1) by intestinal bacteria when taken orally (Odani *et al*, 1983; Tawab *et al*, 2003). Our previous report showed that M1 had axonal extension activity in degenerated neurons, and ameliorated memory disorder and synaptic loss in an Alzheimer's mouse model induced by A$\beta$(25–35) (Tohda *et al*, 2004).

M1 was also reported to have anticancer effect (Wakabayashi *et al*, 1998; Lee *et al*, 1999) in addition to axonal regeneration and antidementia effects. Binding molecules and the signal transduction mechanism of M1, however, is still unknown. Therefore, we aimed to identify the target molecules of M1, and designed an approach to detect phosphorylated proteins by M1 stimulation using immunoprecipitation and the matrix-associated laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS). As a result, two interesting candidates were revealed. Teneurin-2 is a neuronal cell surface protein with one transmembrane domain. Putative functions of teneurin-2 may be recognizing and forming synapses, because teneurin-2 overexpression leads to the enlargement of filopodia and growth cones (Rubin *et al*, 1999), and teneurin-2 is expressed when axons find their targets (Rubin *et al*, 2002). Another candidate is mPar3, a key molecule for polarizing and axonal specifying signals in

neurons, which is regulated by phosphatidylinositol 3-kinase (PI3-kinase) (Shi et al, 2003). Therefore, it was investigated whether the M1 signal was related to teneurin-2 and PI3-kinase.

## METHODS

### Materials

M1 was enzymatically prepared from ginsenoside Rd, and 800 mg was dissolved in 60 ml of 0.2 M sodium dihydrogenphosphate buffer (pH 4.0), to which was gradually added 12 ml ethanol, 2 g of naringinase (Sigma-Aldrich, St Louis, MO), and 8 ml toluene. After incubation at 40°C for 7 days, the mixture was extracted three times with 100 ml ether and dehydrated by filtration over magnesium sulfate. After concentration under reduced pressure, the product was purified by column chromatography over silica gel to give 190.9 mg of M1. The chemical structure of M1 was identified as follows: a white amorphous powder, $^1$H-NMR (pyridine-$d5$): signals for the aglycone moiety at $\delta$ 0.78–2.61, 3.41 (1H, m, H-3), and 5.24 (1H, t-like, H-24). Signals for a glycosyl moiety at $\delta$ 3.84–4.45 (6H, m, including a proton signal for H-12 of the aglycone) and 5.15 (1H, d, $J = 7.5$ Hz, H-1′). LY294002 and A$\beta$(25-35) were purchased from Biomol (Plymouth Meeting) and Sigma-Aldrich, respectively.

### Procedures

*Primary culture.* Embryos were removed from pregnant Sprague–Dawley rats (Japan SLC, Shizuoka, Japan) at 17 or 18 days of gestation. The animals were handled in accordance with the Guide for Animal Experiments, Toyama Medical and Pharmaceutical University. The cortices were dissected, and the dura mater was removed. The tissue was chopped, the cells were dissociated, and plated onto eight-well chamber slides (BD Falcon, Bedford, MA) at a density of $1.0 \times 10^5$ cells/cm$^2$ (Figures 1, 3–6), $7.35 \times 10^4$ cell/cm$^2$ (Figure 7), or onto a 100-mm diameter dish at a density of $8.8 \times 10^4$ cells/cm$^2$ (Figure 2) coated with poly-D-lysine (5 μg/ml), and grown at 37°C in a humidified atmosphere with 10% CO$_2$.

*Analysis of neurite outgrowth.* Rat cortical neurons were cultured for several days as shown in each figure. The cells were fixed with 4% paraformaldehyde and immunostained with a monoclonal antibody to phosphorylated NF-H (dilution 1:1000; Sternberger Monoclonals Inc., Lutherville, MD) as an axonal marker, or with a monoclonal antibody to MAP2 (dilution 1:1000; Chemicon, Temecula, CA) as a dendritic marker as shown in our previous report (Tohda et al, 2004). Fluorescence images were captured using a fluorescence microscope (AX-80, Olympus, Tokyo, Japan) at $1709.4 \times 2273.5$ μm, and 4–6 images were captured per treatment. The lengths of neurites positive for phosphorylated NF-H or MAP2 were measured using an image analyzer (Scion Image, Scion, Frederick, MD) for each cell.

*Analysis of phosphorylated Akt.* The cells were fixed and double-immunostained with the combination of a polyclonal antibody to phosphorylated Akt (S473) (dilution
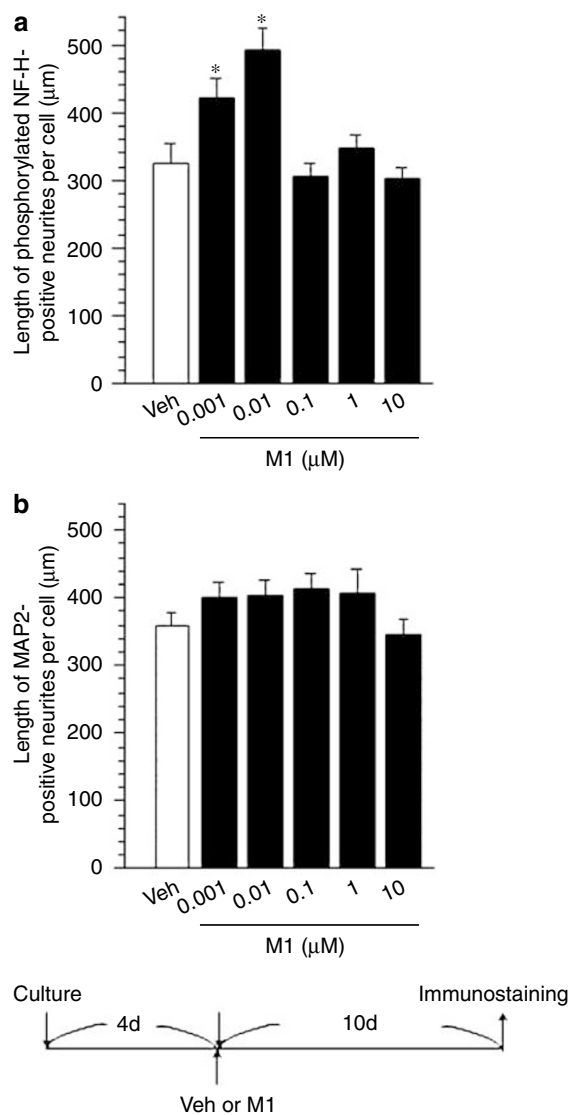
**Figure 1** Effect of M1 on the extension of axons and dendrites. M1 (0.001–10 μM) or a vehicle (Veh, 0.1% DMSO) was added to rat cortical neurons 4 days after the start of the culture period. After 10 days, cells were fixed and immunostained for phosphorylated NF-H (a) and MAP2 (b). The lengths of neurites positive for phosphorylated NF-H or MAP2 per cell were measured. Values represent the means and SEM of 24–37 neurons. *$P < 0.05$ when compared with Veh.

1:1000; R&D Systems, Inc., Minneapolis) and a monoclonal antibody to phosphorylated NF-H (Sternberger Monoclonals Inc.), or with a polyclonal antibody to phosphorylated Akt and a monoclonal antibody to MAP2 (Chemicon). Alexa Fluor 488-conjugated goat anti-mouse IgG and Alexa Fluor 546-conjugated goat anti-rabbit IgG (dilution 1:200; Molecular Probes) were used as secondary antibodies. Fluorescence images were captured using a fluorescence microscope (AX-80) at $437 \times 328$ μm, and four images were captured per treatment. The area of positive puncta to phosphorylated Akt on each axon or dendrite was measured using an image analyzer (ATTO densitograph, ATTO, Tokyo, Japan). The length of the axons and dendrites was measured with Scion Image software (Scion).
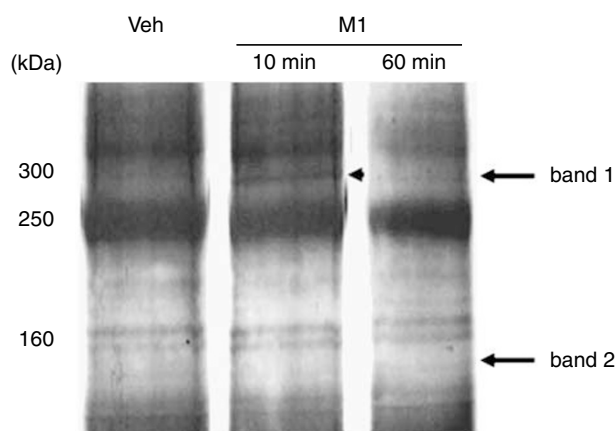
**Figure 2** Phosphorylated proteins by M1 stimulation. Rat cortical neurons were treated by 0.01 μM M1 or the vehicle (Veh, 0.1% DMSO), and after 10 and 60 min incubation, the reaction was stopped. Cell lysate was immunoprecipitated with antiphosphorylated tyrosine antibody. Precipitated samples were electrophoresed on SDS-PAGE, and silver stained. In a lane of M1-treated cells, a band of approximately 300 kDa (band 1) after 10-min stimulation, and a band of less than 160 kDa (band 2) after 60-min stimulation were seen, which were hardly detected in the control lane (60 min after stimulation).

*Immunoprecipitation.* Cells were treated with drugs for 10 or 60 min, and lysed with 1% Nonided-TNE buffer (1% Nonided P40, 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin) for 30 min on ice, and centrifuged at 10 000 r.p.m. for 5 min. Quantified proteins of the supernatant (1.5 mg) were suspended in 0.2% Nonided-TNE buffer, and were added to protein G solution. After shaking for 1 h at 4°C, samples were centrifuged at 5000 r.p.m. for 3 min. Antiphosphorylated tyrosine monoclonal antibody (dilution 1:1000; Chemicon) was added to the supernatants. After incubation for 15 h at 4°C, protein G solution was added, the solution further incubated for 1 h at 4°C, and centrifuged for 1 min at 5000 r.p.m. Precipitates were dissolved in 0.2% Nonided-TNE buffer, and centrifuged for 1 min at 5000 r.p.m. Final precipitates after three repeats of centrifugation were immunoprecipitated samples.

*SDS-PAGE and silver staining.* The immunoprecipitated sample and an equal volume of sample buffer (125 mM Tris-HCl (pH 6.8), 4% SDS, 10% sucrose, 10% 2-mercaptoethanol, 0.004% bromophenol blue) were mixed, and boiled for 3 min. The 10% gels were run at 250 V for 2 h until the dye front reached the bottom of the gel. Silver staining of gels was performed as described in the manufacturer's procedures (Amersham Pharmacia Biotech Inc., Piscataway, NJ).

*In gel digestion.* Protein bands were excised from the gel and washed for destaining with a 1:1 ratio of 30 mM potassium ferricyanide and 100 mM sodium thiosulfate for 10 min. After rinsing with water 3 times, the gels were incubated in 200 mM ammonium bicarbonate for 20 min, and the liquid was discarded. The gels were incubated in a solution of 50% acetonitrile in 100 mM ammonium bicarbonate for 15 min, and then the solution was displaced with 10 mM dithiothreitol in 100 mM ammonium bicarbonate. After incubation for 1 h at 50°C, an equal volume of

50 mM iodoacetamide in 100 mM ammonium bicarbonate was added to the gels, followed by incubation for 40 min at room temperature. The gels were rinsed with 50% acetonitrile in 100 mM ammonium bicarbonate for 10 min twice. After drying the gels, 0.01 mg/ml trypsin in 0.01% SDS/100 mM ammonium bicarbonate was absorbed onto gels, and incubated overnight at 37°C.

*MALDI-TOF-MS analysis.* Peptide extracts were dissolved in 30 μl of 50% acetonitrile solution with 0.5% trifluoroacetic acid (TFA) and desalted using C18 ZipTips (Millipore, Billerica). The peptide mixtures were eluted repeatedly in 5 μl of 70% acetonitrile/0.1% TFA. One microliter of peptide mixture was deposited onto a target disk, immediately after 1 μl of α-cyano-4-hydroxycinnamic acid matrix solution was added to the peptide samples. All the samples were analyzed using AutoFLEX (Bruker Daltonics, Billerica). External calibration was performed using a peptide calibration standard (Bruker Daltonics). Internal calibration was also performed using the auto-digested peaks of trypsin. Monoisotopic peptide masses were assigned and peptides in the mass range of 1000–3000 Da were recorded.

*Database search.* Proteins were identified using Mascot (http://www.matrixscience.com) and Ms-fit (http://www.abcc.ncifcrf.gov/ucsfhtml3.2/msfit.htm) software. Probability-based MOWSE scores were calculated by comparing the search results against an estimated random match population and were reported as $-10 \times \log_{10}(P)$, where $P$ is the absolute probability. Scores greater than 68 were considered significant ($P < 0.05$).

*siRNA transfection.* siRNA for teneurin-2 was designed by a Takara siRNA design support system (http://www2.takara-bio.co.jp), and was synthesized by Takara. Sense (5′-UGUGACCAGUAUCUUGGAGUU-3′) and antisense (5′-CUCCAAGAUACUGGUCACAUU-3′) RNAs were annealed and used. Transfection was carried out in serum-free medium following the manufacturer's procedures using *Trans*IT-TKO (Mirus, Madison). The optimal incubation period (2 days) until RNA extraction was determined previously.

*Reverse transcription-polymerase chain reaction.* DNase I-treated total RNAs extracted from the sample were used for reverse transcription (RT), and polymerase chain reaction (PCR) was performed using a primer set for teneurin-2 designed to amplify 2703–3114 bp of rat teneurin-2, sense; 5′-YTGCTGCCTCCAGTCMRCYTG-3′, antisense; 5′-GGCATAGAAGCTGTTCCACGGC-3′. As a positive control, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA was also detected using a primer set designed to amplify 547–1073 bp of rat GAPDH, sense; 5′-CCAAGGTCATCCATGACAAC-3′, antisense; 5′-TTACTCCTTGGAGGCCATGT-3′. The PCR program for teneurin-2 was carried out at 40 cycles of 30 s at 94°C, 1 min at 63.5°C, and 1 min at 72°C. The program for GAPDH was carried out at 25 cycles of 30 s at 94°C, 1 min at 58°C, and 1 min at 72°C. Optimal annealing temperatures and cycle numbers for quantitative comparison were previously determined.

*Statistical analysis.* Statistical comparisons were made by Student's $t$-test, or one-way analysis of variance, followed by Dunnett's *post hoc* test. Values of $P < 0.05$ were considered significant. The means of the data are presented together with SEM.

## RESULTS

### Effect of M1 on Neurite Extension in Cortical Neurons

We investigated the effects of M1 on neurite extension in cultured cortical neurons. Rat cortical neurons were treated by M1 at day 4. At day 14 (treated for 10 days), cells were fixed and immunostained with an antibody of phosphorylated NF-H (P-NF-H, axonal marker) or MAP2 (dendritic marker). Treatment with 0.001 and 0.01 μM M1 significantly enhanced the length of phosphorylated NF-H-positive neurites compared with vehicle-treated cells (Figure 1a). In contrast, the length of MAP2-positive neurites was not increased by treatment with any doses of M1 (0.001–10 μM) (Figure 1b).

### Phosphorylated Proteins by M1 Stimulation in Rat Cortical Neurons

To investigate the target molecule and signal transduction of M1, proteins phosphorylated by M1 stimulation were identified. Rat cortical neurons were treated with 0.01 μM M1, and after 10 and 60 min incubation, the reaction was stopped. Cell lysate was immunoprecipitated with antiphosphorylated tyrosine antibody. Precipitated samples were electrophoresed on SDS-PAGE and silver stained. Protein bands, which were increased by M1 stimulation compared with the control, were excised from the gel, and analyzed by MALDI-TOF-MS. In a lane of M1-treated cells, a band of approximately 300 kDa (band 1) after 10-min stimulation, and a band of less than 160 kDa (band 2) after 60-min stimulation were seen, which were hardly detected in the control lane (Figure 2). Mass data of the bands were researched on peptide mass databases, Mascot and MS-Fit. As a result, bands 1 and 2 were suggested to be teneurin-2 (MOWSE score: 21850) and mPar3 (MOWSE score: 371), respectively.

Teneurin-2 consists of 2765 amino acids (306.6 kDa), and was cloned in chicks, mice, rats, and humans (Minet and Chiquet-Ehrismann, 2000). The expression pattern of teneurin-2 is predominant in the brain (Otaki and Firestein, 1999; Rubin *et al*, 1999; Tucker *et al*, 2001), and is richer in the developmental stage than in the adult period (Otaki and Firestein, 1999; Rubin *et al*, 1999). Previously known functions of teneurin-2 are the formation of filopodia (Rubin *et al*, 1999), growth cone enlargement (Rubin *et al*, 1999), and promoting cell–cell adhesion (Rubin *et al*, 2002). The putative structure of teneurin-2 is a type II transmembrane protein. In the extracellular domain, there are eight EGF-like repeats (Otaki and Firestein, 1999). Although teneurin-2 is speculated to act as a homophilic adhesive protein, we supposed another scenario in which the membrane-spanning protein acts as a signaling molecule, like growth factor receptors.

mPar3 is a 150 kDa protein, making a complex with mPar6 and atypical protein kinase C (aPKC), and this complex is crucial for cell polarity. Especially in neurons, the polarized localization of the complex is important for axon specification (Shi *et al*, 2003). The localization of the mPar3 complex is regulated by PI3-kinase (Shi *et al*, 2003; Schwamborn and Püschel, 2004). Therefore, we hypothesized that PI3-kinase might be located upstream of mPar3 phosphorylation, and teneurin-2 might transduce signals through PI3-kinase.

### Effect of Decreased Teneurin-2 on PI3-Kinase Activity and Axonal Extension

Rat cortical neurons were treated with siRNA for teneurin-2 at 10 or 50 nM for 2 days. Total RNA was then extracted, and the expression level of teneurin-2 mRNA was measured by semiquantitative RT-PCR. Teneurin-2 mRNA was decreased by treatment with 10 nM siRNA compared with control and mock transfectants (Figure 3). Although 50 nM siRNA also reduced teneurin-2 mRNA, this inhibition was not potent compared with 10 nM, and cell toxicity was weak.

To investigate whether M1 stimulation (treated for 1 h) results in PI3-kinase activation through teneurin-2, phosphorylated Akt (P-Akt) was immunostained in neurons treated by siRNA for teneurin-2. Double immunostaining for P-Akt and P-NF-H, or P-Akt and MAP2 was carried out to distinguish PI3-kinase activity on axons or dendrites. In mock transfectants, P-Akt was distributed mainly in cell bodies when treated by the vehicle (Figure 4). However, P-Akt-positive puncta were localized in many axons 1 h after M1 administration. On the other hand, the localized puncta of P-Akt decreased in teneurin-2 siRNA-treated and M1-stimulated neurons. These results were quantified in Figure 5a. Significantly increased P-Akt expression in axons was induced by M1 stimulation in mock transfectants; however, P-Akt expression was not enhanced in teneurin-2-
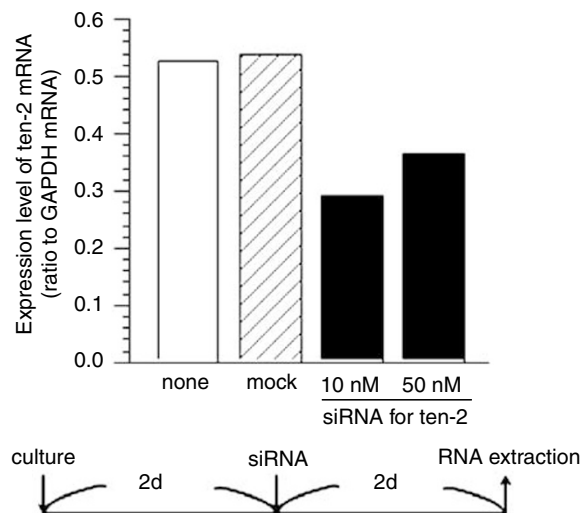


**Figure 3** Effect of siRNA transfection on teneurin-2 mRNA expression. siRNA for teneurin-2 (10, 50 nM) or water (mock) was transfected into rat cortical neurons at 2 days after culture. Untransfected cells (none) were also used as a control. After 2 days, total RNA was extracted, and the expression levels of teneurin-2 mRNA and GAPDH were measured by semiquantitative RT-PCR. Ratios of teneurin-2 mRNA to GAPDH mRNA were shown.
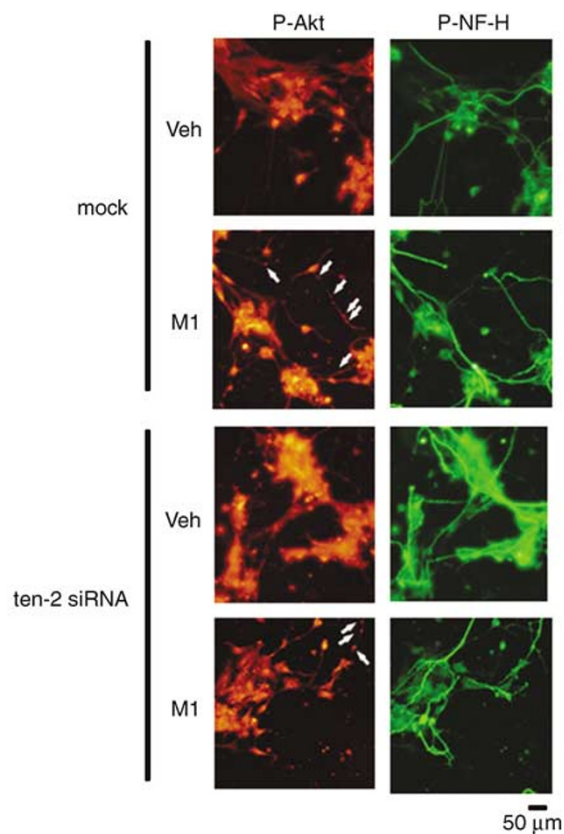
**Figure 4** Effect of teneurin-2 knockdown on M1-induced PI3-kinase activation in axons. siRNA for teneurin-2 (10 nM) or water (mock) was transfected into rat cortical neurons at 3 days after culture. After 2 days, 0.01 μM M1 or the vehicle (Veh, 0.1% DMSO) was administered to the cells. After 1 h later, cells were fixed and double-immunostained for phosphorylated Akt and phosphorylated NF-H. Arrows show phosphorylated Akt-positive puncta. Scale = 50 μm.

deficient neurons even after M1 treatment. The length of axons was measured in those neurons (Figure 5b). Significantly increased axon length was induced by M1 stimulation in mock transfectants; however, axons were not extended in teneurin-2-deficient neurons even after M1 treatment. Double staining for P-Akt and MAP2 showed that localized P-Akt puncta were seen in M1-treated neurons that were not dendrites (Figure 6).

To confirm the involvement of PI3-kinase in M1 signal transduction, neurons were treated with PI3-kinase inhibitor, LY294002 (Figure 7). P-NF-H-positive neurites, axons, were significantly extended by M1 treatment for 4 days. When LY294002 (0.1 and 1 μM) was simultaneously treated with M1, axonal extension was significantly inhibited.

## DISCUSSION

Treatment with M1, a metabolite of ginseng protopanax-adiol-type saponins, extended axons, but not dendrites, in rat cortical neurons (Figure 1). As described in our previous report, M1 can regenerate axons and synapses, and therefore recover memory impairment in amyloid $\beta$(25–35)-injected mouse brain (Tohda *et al*, 2004). In addition,
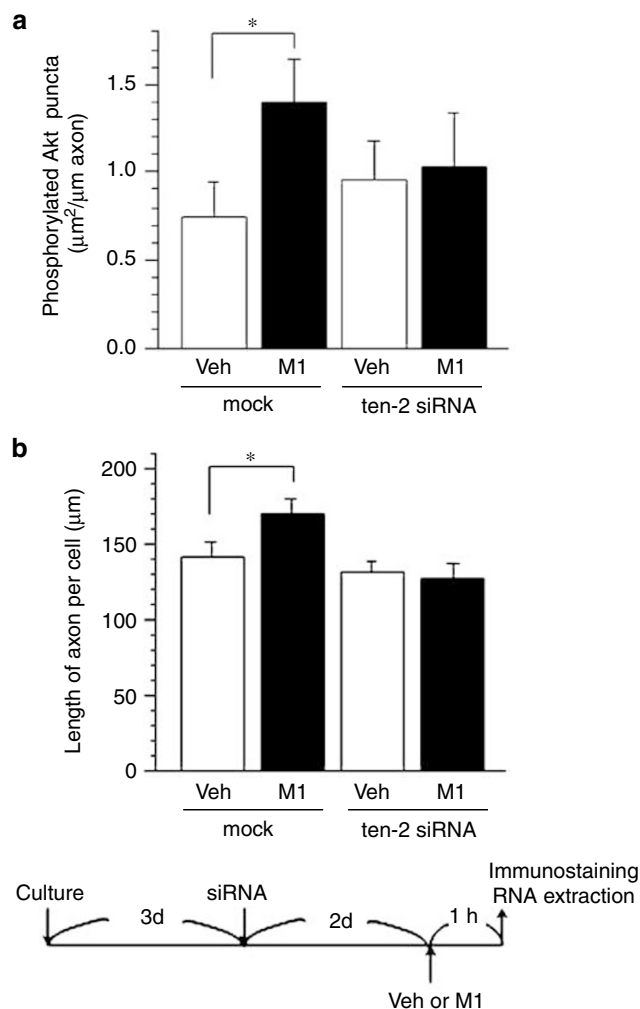


**Figure 5** Effects of teneurin-2 knockdown on M1-induced PI3-kinase activation in axons, and axonal extension. (a) Quantified data of Figure 4. Areas of phosphorylated Akt-positive puncta per 1 μm of axon were measured. (b) Axonal lengths per neuron. The values represent the means and SEM of 71–95 axons. *$P < 0.05$.
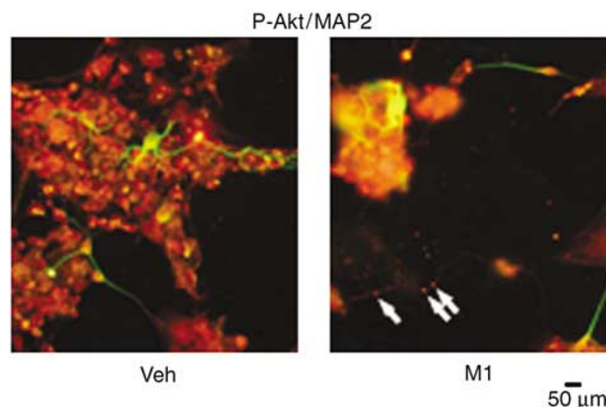


**Figure 6** Effect of teneurin-2 knockdown on M1-induced PI3-kinase activation in dendrites. siRNA for teneurin-2 (10 nM) or water (mock) was transfected into rat cortical neurons at 3 days after culture. After 2 days, 0.01 μM M1 or the vehicle (Veh, 0.1% DMSO) was administered to the cells. After 1 h, cells were fixed and double-immunostained for phosphorylated Akt (red color) and MAP2 (green color). Arrows show phosphorylated Akt-positive puncta. Scale = 50 μm.
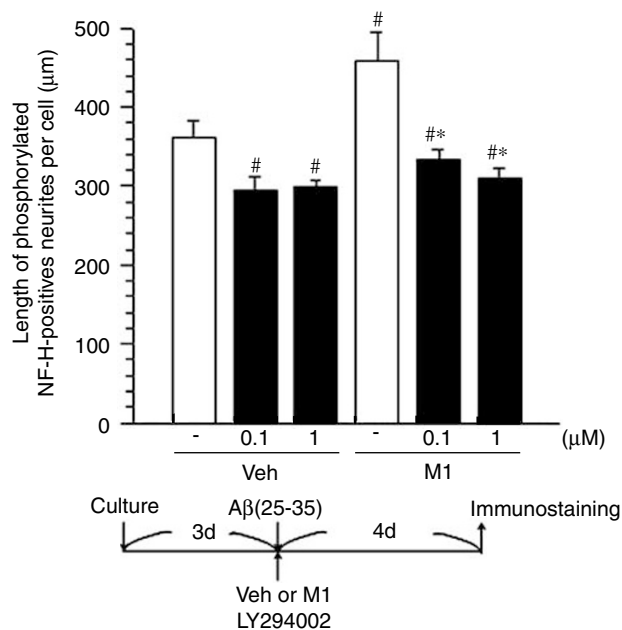
**Figure 7** Effect of PI3-kinase inhibitor on M1-induced axonal extension. M1 (0.01 μM) or the vehicle (Veh, 0.1% DMSO) were added with 10 μM Aβ(25–35) to rat cortical neurons 3 days after the start of the culture period. PI3-kinase inhibitor, LY294002 was simultaneously added at 0.1 or 1 μM. After 4 days, cells were fixed and immunostained for phosphorylated NF-H. The lengths of neurites positive for phosphorylated NF-H per cell were measured. Values represent the means and SEM of 23–30 neurons. *$P < 0.05$ when compared with Veh/LY294002 (−), [#]$P < 0.05$ when compared with M1/LY294002 (−).

treatment with M1 recovers synaptic loss in amyloid β(25–35)-treated cultured cortical neurons (unpublished data). Although M1 was also reported to have an anticancer effect (Wakabayashi *et al*, 1998; Lee *et al*, 1999), no target molecule of M1 has been clarified. Knowing the target and signal transduction of M1 may elucidate the mechanism of axonal formation.

Most intracellular signal transductions are through phosphorylation cascades. Therefore, we had the idea that target molecules of M1 might be identified by analyzing phosphorylated proteins after M1 treatment. After stimulation with M1 (0.01 μM), two bands were shown as specifically phosphorylated proteins by M1 in tyrosine residue (Figure 2): teneurin-2 and mPar3. By sequence motif analysis, one intracellular tyrosine kinase phosphorylation site exist in teneurin-2. *Drosophila* homolog of teneurin, odz was shown to be phosphorylated on tyrosine (Levine *et al*, 1994). In mPar3, three putative sites exist as tyrosine kinase phosphorylation sites. Therefore, it is reasonable that teneurin-2 and mPar3 were identified as tyrosine phosphorylated proteins.

Interestingly, mPar3 is known as a key molecule for axonal specification, and upstream PI3-kinase regulates mPar3 localization in axons (Shi *et al*, 2003). PI3-kinase has been shown to be involved in neurite outgrowth signaling (Rodgers and Theibert, 2002), especially in axonal formation (Edström and Ekström, 2003; Ménager *et al*, 2004). On the other hand, teneurin-2 is a type II transmembrane protein (Otaki and Firestein, 1999), and is involved in the formation of filopodia, and growth cone enlargement

(Rubin *et al*, 1999). Teneurin-2 is speculated to be not only a homophilic adhesive protein, but also a molecule necessary for neurite fasciculation and/or neuron target recognition (Rubin *et al*, 2002). As most growth factor receptors are transmembrane proteins, we hypothesized that teneurin-2 may function like an M1 receptor.

PI3-kinase activity can be indirectly evaluated by increased P-Akt. The puncta of P-Akt in axons were significantly increased by M1 (0.01 μM) stimulation for 1 h (Figures 4 and 5a). As shown in Figure 6, P-Akt is not distributed in dendrites. In addition, axons were extended by M1 stimulation although the enhanced length was not particularly long due to the short period of stimulation (Figure 5b). P-Akt is known to localize in axons like a dot when axons are extending (Shi *et al*, 2003; Schwamborn and Püschel, 2004). By siRNA treatment, teneurin-2 mRNA could be decreased in cortical neurons (Figure 3). The phosphorylation of Akt, that is, the activation of PI3-kinase, and axonal extension were not seen in teneurin-2 knock-down neurons (Figures 4 and 5), suggesting that PI3-kinase activation by M1 occurs in axons, leading to axonal extension, and that teneurin-2 is necessary for the M1 signal to activate PI3-kinase.

Many transmembrane-type receptors, such as trkA and trkB (Klesse and Parada, 1999), are coupled with PI3-kinase at the cytoplasmic domain. The localized property and function of the filopodia formation of teneurin-2 are inhibited without its cytoplasmic domain (Rubin *et al*, 2002), suggesting that the cytosolic domain must be critical for the signal transduction of teneurin-2. It is also shown that the intracellular domain of teneurin-2 may be released into the cytosol, and may regulate nuclear transcription factor zic-1 (Bagutti *et al*, 2003). Although the entire mechanism of teneurin-2 function remains unclear, teneurin-2 may have several action forms by different types of ligand stimulation. An associating site or adaptor molecule for the interaction of teneurin-2 and PI3-kinase are now under investigation. In addition, it is still unknown whether M1 binds to teneurin-2 directly, and if so, which domain of teneurin-2 is needed for M1 interaction. It is also important to study the existence of physiological ligands for teneurin-2 other than M1, and whether the M1 signal through teneurin-2 activates only mPar3 or also other molecules.

In conclusion, we have found for the first time in this study that M1 induces axonal extension through the teneurin-2–PI3-kinase cascade. Teneurin-2 is a possible novel target of axonal and synaptic regeneration.

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