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Original Article

Effects of Tokishakuyakusan on Regeneration of Murine Olfactory Neurons *In Vivo* and *In Vitro*

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Abstract

Post-upper respiratory tract infection related olfactory dysfunction typically occurs due to neural damage after an upper respiratory tract infection associated with a common cold or influenza. At present, Tokishakuyakusan, a Japanese traditional Kampo medicine, has been found to be effective for post-viral olfactory dysfunction. However, the pharmacodynamics of Tokishakuyakusan in the treatment of post-viral olfactory dysfunction remains unresolved. We investigated the effects of Tokishakuyakusan on the regeneration of olfactory neurons and expression of nerve growth factor (NGF) in neural systems, using *in vivo* murine studies and *in vitro* cell culture studies. Eight-week-old BALB/C female mice were fed a pellet diet with or without Tokishakuyakusan. Degeneration of cells in olfactory epithelium was induced by intraperitoneal methimazole injection. Regeneration of olfactory neurons was observed by histological and immunohistochemical procedures. NGF expression in the olfactory bulb was measured by enzyme-linked immunosorbent assay. NGF gene and protein expression were measured using rat primary cultured astrocytes by real-time polymerase chain reaction and enzyme-linked immunosorbent assay. We found that olfactory marker protein, Ki-67, and NGF were more highly expressed in the olfactory epithelium during the regeneration period in mice receiving Tokishakuyakusan. In cultured astrocytes, Tokishakuyakusan as well as its individual components, Atractylodes lancea rhizome and Japanese angelica root, increased NGF expression. Screening assays revealed that NGF production was increased by atractylodin and levistolide A, which are ingredients in Atractylodes lancea rhizome and Japanese angelica root, respectively. These results suggest that Tokishakuyakusan promotes regeneration of olfactory neurons by increasing NGF expression in the olfactory bulb.

Key words: Kampo, nerve growth factor, olfactory epithelium, post-upper respiratory tract infection related olfactory dysfunction

Introduction

Olfactory receptor neurons in the mammalian nervous system have 2 unique characteristics, including their ability to regenerate throughout life (Graziadei and Graziadei 1979a; Suzuki and Takeda 1993). Olfactory neuron progenitor cells are located near the basement membrane of the olfactory epithelium (OE). Olfactory neurons

can regenerate after olfactory axotomy or olfactory bulbectomy (Graziadei and Graziadei 1979b; Graziadei and Okano 1979; Harding and Write 1979; Wright and Harding 1982; Costanzo and Graziadei 1983). This regeneration is controlled by different types of growth factors and neurotrophins, including nerve growth factor (NGF; Miwa et al. 2002). Continuous infusion of NGF into the cranial cavity after olfactory bulbectomy increases the regeneration of olfactory neurons (Uramoto et al. 1998), and administration of NGF into the nasal cavity increases olfactory marker protein expression in the OE of axotomized rats (Yasuno et al. 2000).

NGF is mainly expressed in the hippocampus, cerebral cortex, and olfactory bulb in the central nervous system. The NGF produced in the olfactory bulb is transported to the horizontal limb of the diagonal band in the forebrain and is involved in the survival of cholinergic neurons, as well as in the survival and regeneration of olfactory neurons in the OE.

The other unique property of olfactory neurons is that they are a direct link between the outside and intracranial environments. Dendrites of olfactory neurons protrude from the surface of the OE where they can bind olfactory molecules present in the atmosphere. On the other hand, the axons of olfactory cells extend through the lamina propria of the mucosa to the olfactory bulb. Therefore, olfactory neurons are easily damaged by viruses or mechanical injury. On the other hand, olfactory neurons provide a useful drug delivery route into the central nervous system (Shiga et al. 2014).

Post-upper respiratory tract infection (post-URTI) related olfactory dysfunction results from neural damage after an URTI associated with a common cold or influenza. Post-URTI dysfunction is classified as a sensorineural olfactory dysfunction, and some patients recover spontaneously (Mott and Leopold 1991; Duncan and Seiden 1995; Hummel 2000; Reden et al. 2006; Rombaoux et al. 2010; Cavazzana et al. 2018), but recovery of olfactory function requires regeneration of the olfactory neurons. Although there has been no validated therapy for post-URTI dysfunction until recent years, efficacy of olfactory training has a been reported mainly in European countries based on randomized controlled trials (Hummel 2009; Konstantinidis et al. 2013; Damm et al. 2014; Altundag 2015; Konstantinidis et al. 2016).

Tokishakuyakusan (TSS), a traditional Japanese Kampo medicine, has been widely used in the treatment of patients with gynecological disorders, including climacteric disturbance, menstrual irregularity, dysmenorrhea, and infertility; it has also been approved by the Japanese Ministry of Health, Labor and Welfare.

In recent years, TSS has also been prescribed in Japan for patients with post-URTI dysfunction and has shown efficacy. Miwa et al. (2010) reported that the treatment of post-URTI dysfunction with TSS resulted in a greater improvement in olfactory function than that seen with intranasal steroid treatment. Uchida et al. (2009) treated patients with post-URTI dysfunction, who had not responded to intranasal steroids, with TSS or ninjin'yoeito, and the improvement rate was 43% and 36%, respectively. Ogawa et al. (2010) also reported that the improvement rate of patients with post-URTI dysfunction, who received treatment with intranasal steroid treatment alone, TSS oral administration alone, or a combination of steroids and TSS, for 3 months, was 29%, 55%, and 60%, respectively. However, the pharmacodynamics of TSS in the treatment of post-URTI dysfunction remains unknown. In this study, we investigated the effects of TSS on the regeneration of olfactory neurons and the expression of NGF in neural systems, using *in vivo* murine studies and *in vitro* cell culture studies.

Materials and methods

In vivo studies

Mice

Sixty-five 8-week-old BALB/C female mice purchased from Japan SLC, Inc. were divided 2 groups; 35 were assigned to the histological and immunohistochemical study, and 30 were assigned to the enzyme-linked immunosorbent assay study. The mice were housed in a temperature-controlled environment under a 12-h light–dark cycle, with access to food and water ad libitum. All animal experiments were conducted in accordance with institutional guidelines and with the approval of the animal care and use committee of Kanazawa Medical University (No. 2017-69).

Methimazole administration

To degenerate the OE, 30 mice were intraperitoneally injected on day 0 with methimazole (75 mg/kg; FUJIFILM Wako Pure Chemical Corporation) dissolved in 0.9% saline. It has been reported that methimazole induces apoptotic cell death in rodent olfactory neurons (Sakamoto et al. 2007). An additional 5 mice were intraperitoneally injected with 0.9% saline, without methimazole, as a normal control.

TSS administration

TSS was prepared by spray-drying a hot-water extract of the following 6 component drugs: Peony root (PR; root of *Paeonia lactiflora* Pallas, 4.0 g), Atractylodes lancea rhizome (ALR; rhizome of *Atractylodes lancea* De Candolle, 4.0 g), Oriental water plantain rhizome (OWR; rhizome of *Alisma orientale* Juzepczuk, 4.0 g), Poria sclerotium (PS; sclerotium of *Poria cocos* Wolf, 4.0 g), Cnidium rhizome (CR; rhizome of *Cnidium officinale* Makino, 3.0 g), and Japanese angelica root (JAR; root of *Angelica acutiloba* Kitagawa, 3.0 g). TSS was supplied by Tsumura & Co. as a lyophilized powder. The pharmaceutical quality of TSS is controlled according to the *Japanese Pharmacopoeia 17th edition* that regulates concentrations of (E)-ferulic acid, paeoniflorin, and atractylodi. TSS was included in the animal feed (CE-2; Crea Japan, Inc.) at 0.5% (w/w) of TSS. Fifteen animals in the TSS-treated group were given free access to animal feed containing TSS from 3 days after methimazole injection to the day of sacrifice, whereas 15 mice in the non-TSS control group were given free access to TSS-free food.

Dissection of nasal tissue and brain

For the histological observation of nasal tissue, mice were perfused via the left ventricle with 4% paraformaldehyde fixative at 14, 28, or 42 days following methimazole administration and after being deeply anesthetized with pentobarbital. Five mice were used for the TSS group and for the control group for each of the 3 recovering days. After decapitation and removal of the skin tissue, the mouse heads were fixed in 4% paraformaldehyde phosphate buffer for 24 h and then immersed in 10% sucrose solution, followed by 20% sucrose solution for 24 h, respectively. After decalcification with K-CX solution (Falma), the heads were dehydrated in a series of graded ethanol solutions, and then embedded in paraffin. Nasal tissues of the normal control mice ($N = 5$) were fixed and paraffin embedded according to the same protocol used for the methimazole-injected mice.

To measure NGF concentration in the olfactory bulb using the enzyme-linked immunosorbent assay, olfactory bulbs were dissected from 30 mice without perfusion fixation, following the induction of deep anesthesia and euthanasia by cervical dislocation at 14, 28, or 42 days after methimazole administration. Five mice were used

in the TSS group and non-TSS control group, respectively, for each time period.

Histological and immunohistochemical examination

Whole-head samples were cut in coronal sections at the level of 1 mm anterior to the section containing the anterior tip of the olfactory bulb. Six-micrometer-thick paraffin sections were deparaffinized in xylene and rehydrated in alcohol. One section was stained with hematoxylin and eosin and used for thickness measurements and another section was stained for immunohistochemistry (e.g., olfactory marker protein [OMP]). Data measurements were obtained from 3 randomly sampled sites along the septum from either the right or left side of the nasal cavity.

Immunohistochemistry was performed using anti-OMP (goat polyclonal, 1:100 dilution; FUJIFILM Wako Pure Chemical Corporation) and anti-Ki-67 (mouse monoclonal, 1:100; BD Biosciences, Franklin Lakes, CA, USA). OMP is an OMP exclusively expressed in mature olfactory receptor neurons (Buiakova et al. 1996). Ki-67 protein is a cellular marker of proliferation (Starborg et al. 1996), and Ki-67-positive cells are typically detected throughout the depth of the OE, mainly in the basal layer. Deparaffinized sections were boiled in citrate buffer for 10 min at 95 °C to activate each of the antigens. Sections were then reacted with each primary antibody, overnight. After washing sections with phosphate buffer, tissues were incubated with a biotin-conjugated secondary antibody for 30 min, followed by peroxidase detection. Cell counts and epithelial thickness measurements were made using a coronal section passing through the nasal cavity at a location 1 mm anterior to the section containing the anterior tip of the olfactory bulb. Because of the variability in epithelial thickness along the curved surfaces of the turbinates, we limited our measurements to the medial wall of the septum and sampled three 100-µm strips of epithelium beginning at the dorsal recess and moving ventrally down the epithelium. Counting was performed by a single examiner who was blinded to information about the sections.

Enzyme-linked immunosorbent assay of NGF in the olfactory bulb

Olfactory bulb tissues were homogenized by ultrasonication in extraction buffer and then centrifuged for 30 min at 15 000 × *g* and 4 °C. The supernatants were harvested and stored at –80 °C until use, after measuring total protein concentration. NGF in the olfactory bulb was measured using a Mouse Multi-Neurotrophin Rapid Screening ELISA Kit (Biosensis Pty Ltd), following the manufacturer's instructions. The results of the NGF quantification were reported as NGF content (ng) per milligram total protein.

In vitro studies: NGF activation in cultured astrocytes by TSS

Test drugs

TSS and the component drugs were supplied by Tsumura & Co. as a lyophilized powder. As compounds present in TSS, atractylodin and levistolide A, with sufficient purity for biological tests, were obtained from the Botanical Raw Materials Research Laboratories, Tsumura & Co.

Preparation and culture of primary cultured astrocytes

Astrocytes were cultured using a previously described procedure (Kawakami et al. 2009). In brief, forebrain cortices were dissected from 0- to 1-day-old Sprague–Dawley rats (Charles River Laboratories Japan Inc.) and the meninges were carefully removed. The neopallia were mechanically disrupted by pipetting in a 1:1 solution of Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich) and Ca- and Mg-free phosphate-buffered saline. After filtering through 100-µm mesh (BD Falcon) and lens-cleaning paper (Fujifilm), the cells (15×10^6) were seeded into a 75-cm^2 Primaria culture flask (Corning) and cultured in DMEM/F12 (Thermo-Fisher Scientific) containing 10% fetal bovine serum (MP Biomedicals). Culture medium was exchanged after 2 days, and every other day thereafter, and the cells were maintained in a humidified, 5% CO_2 incubator at 37 °C. After the primary culture reached confluence (12–14 days), the culture flasks were shaken at 260 rpm overnight to remove small process-bearing cells on the protoplasmic cell layer. The monolayer cells were trypsinized, seeded at approximately 20 000 cells/cm^2 into Primaria culture plates, and cultured in DMEM supplemented with 10% horse serum (Thermo-Fisher Scientific). After the culture reached confluence, at approximately 7 days, culture medium was replaced by DMEM supplemented with 0.5% horse serum, for 3 days. Astrocytes were identified by immunostaining using purified rabbit polyclonal antibodies to glial fibrillary acidic protein (GFAP), purchased from Chemicon; approximately 95% cells stained positive for GFAP. For the examination of test drugs, astrocytes were cultured for an additional 3, 6, 24, or 48 h in the presence of the test drug after the preculture described earlier. The culture fluids and cell lysates were harvested, and stored at –30 °C until used for the measurement of NGF, as described later.

TSS and the 6 component drugs were suspended in culture medium at a final concentration of 100, 150, 300, or 600 µg/mL, passed through a 0.22-µm filter after sonicating for 15 min, and then exposed to the astrocytes. Atractylodin and levistolide A were dissolved in Dimethyl sulfoxide (DMSO) at 100 mmol/L and diluted at final concentrations of 10, 30, and 100 µmol/L. Medium containing only DMSO was used as the vehicle control. To determine the signal pathway involved in NGF production by TSS, protein kinase (PKA) inhibitor H-89 (Sigma–Aldrich), inhibitor of MEK1 and MEK2, U0126 (Sigma–Aldrich), or estrogen receptor antagonist fulvestrant (Sigma–Aldrich) was dissolved in DMSO, and then diluted into the astrocyte culture 3 h before adding TSS. The final concentration of DMSO was 0.12% (v/v) in all wells, and each inhibitor was added at a concentration of 2 µmol/L, respectively, according to previous reports (Zhu et al. 2013; Cao et al. 2018; Yang et al. 2018).

Measurement of NGF and its receptor in primary cultured astrocytes

NGF expression was determined by real-time reverse transcription polymerase chain reaction (RT-PCR). In brief, total RNA was extracted from astrocytes using a RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The concentration of extracted total RNA was determined spectrophotometrically at 260 nm. Reverse transcription was performed using a High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) according to the manufacturer's instructions on a Gene Amp PCR System 9700 (Thermo Fisher Scientific). Next, real-time PCR was performed using TaqMan Fast Advanced Master Mix (Thermo Fisher Scientific). PCR was performed on an ABI QuantStudio 7 with a 384-well format (Thermo Fisher Scientific). The following TaqMan gene expression assay probes were used: NGF (Rn01533872_m1), TrkA (Rn00572130_m1), and Rps29 (Rn00820645_g1) (Thermo-Fisher Scientific). Real-time PCR was performed in duplicate for each sample. Differences in amplification were determined using the

$2^{-\Delta\Delta CT}$ method. Rps29 (ribosomal protein S29) was used as an endogenous control to normalize expression levels between samples. NGF contents in the culture fluids were measured using the rat beta-NGF ELISA kit (Thermo Fisher Scientific), according to the instructions provided by the manufacturer.

Statistical analysis

Data are presented as the mean ± standard error (SE). For in vivo studies using mice, Student's *t*-test was used to assess differences between groups. The statistical difference between groups in the cell culture experiments was assessed by one-way analysis of variance, followed by Dunnett's multiple comparisons test, or Student's *t*-test. The significance level in each statistical analysis was $P < 0.05$.

Results

In vivo studies

Thickness of OE

The thickness of the OE was reduced by 14 days after methimazole intraperitoneal injection in both the TSS group and the non-TSS control group and had increased gradually by 28 days and 42 days (Figure 1a). However, the OE thickness did not return to the level seen in normal control mice, even by 42 days after

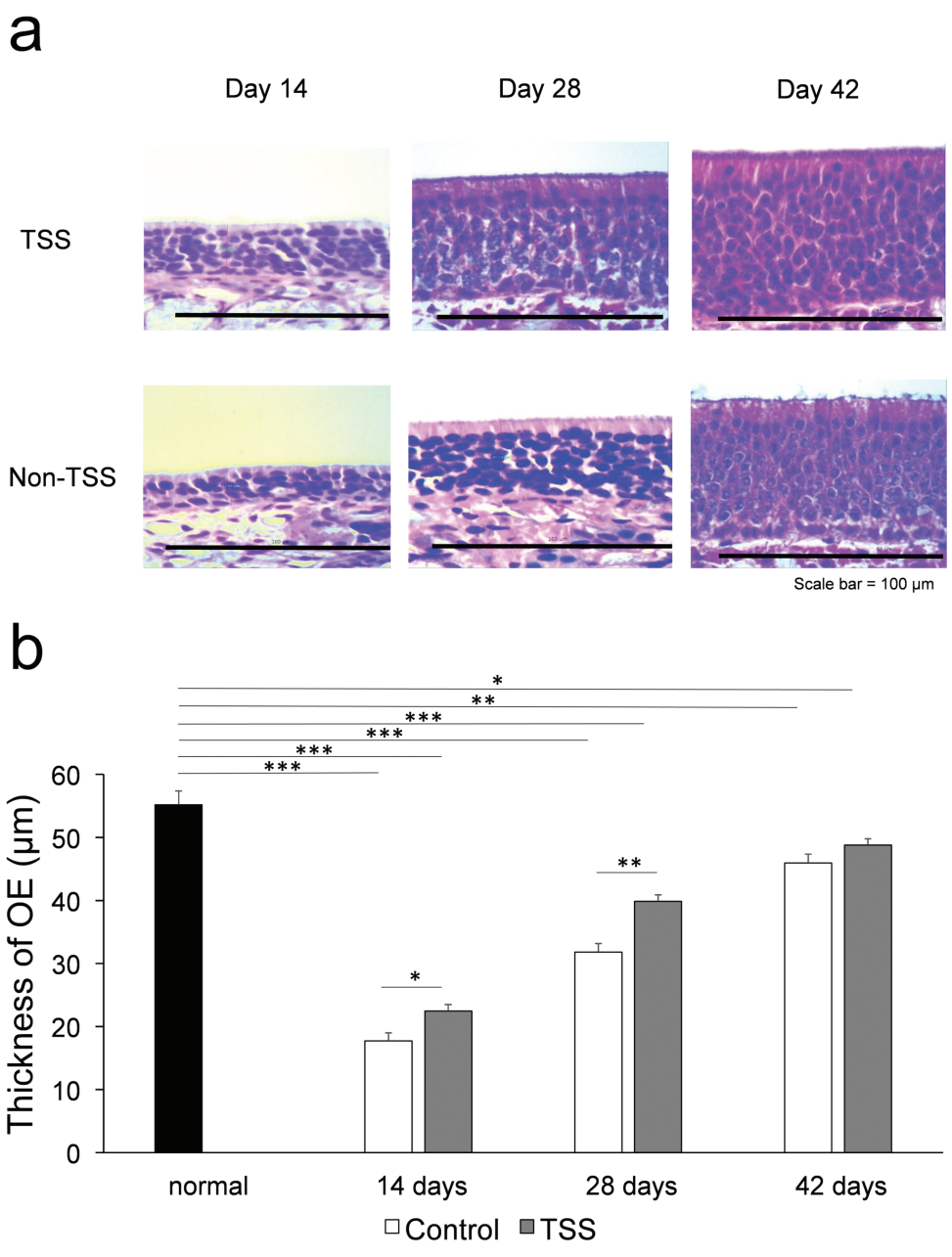

Figure 1. Olfactory epithelium thickness. Hematoxylin and eosin staining of the OE of TSS and non-TSS groups at 14, 28, and 42 days after methimazole injection. The OE thickness first decreased after methimazole injection, and then gradually increased. Scale bar = 100 µm. (**a**) The mean value of OE thickness in each group. (**b**) The OE thickness did not return to normal control levels even by 42 days. In the TSS feed group, the OE was thicker than that in the non-TSS control group at 14 and 28 days. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$: Student's *t*-test. TSS: Tokishakuyakusan.

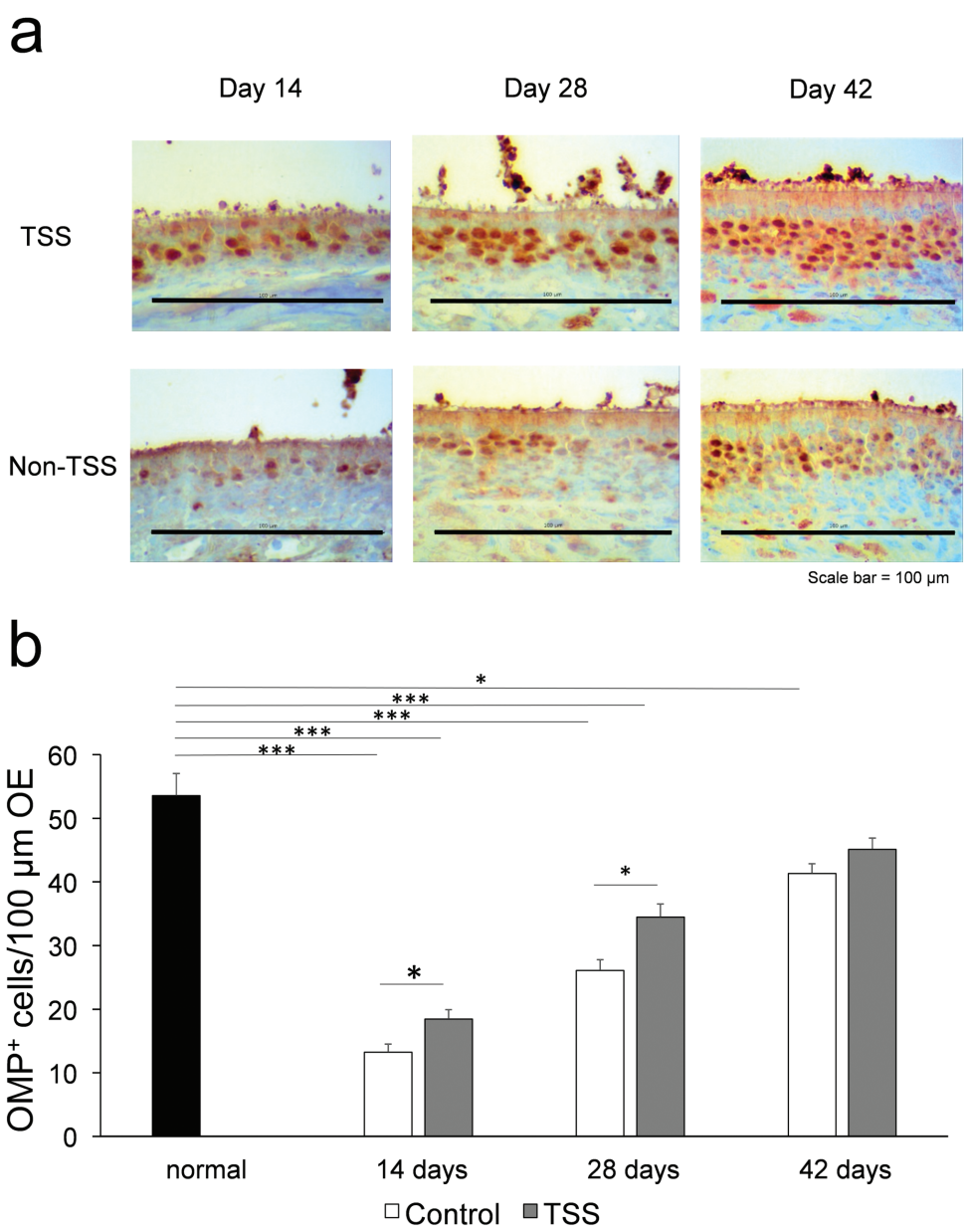

Figure 2. Immunohistochemical staining of OMP in the olfactory epithelium (OE). OMP-immunopositive cells were located in the upper half of OE. Scale bar = 100 µm. (**a**) The number of OMP-immunopositive cells was reduced at 14 days after methimazole injection in both groups, and increased gradually by 28 days and 42 days in both groups. The number of OMP-immunopositive neurons was significantly greater in the TSS group than in the non-TSS control group at 14 days and 28 days after injection. (**b**) $*P < 0.05$, $***P < 0.001$: Student's *t*-test. TSS: Tokishakuyakusan.

injection. Nevertheless, the OEs were significantly thicker in the TSS group mice than those in the non-TSS control group at 14 days and 28 days. There were no significant differences between the OEs in the TSS group and non-TSS control group by 42 days after methimazole injection (Figure 1b).

OMP and Ki-67 immunohistochemistry

Similar to the OE thickness, OMP-immunopositive olfactory neural cells were reduced by 14 days after methimazole injection in both groups and had increased gradually by 28 days and 42 days in both groups (Figure 2a). Although the OMP-immunopositive cells had not recovered to normal levels as the normal control mice in the non-TSS control group at 42 days, they had recovered to normal levels in the TSS group at this time point. There were significantly more OMP-immunopositive neurons in the TSS group than in the non-TSS control group at 14 days and 28 days after injection ($P < 0.05$). There were no statistically significant differences in the number of OMP-immunopositive neurons between the TSS group and non-TSS control group at 42 days after injection (Figure 2b).

The number of Ki-67-immunopositive cells in the OE was higher in the TSS group than in the normal control mice throughout the observation period; in contrast, Ki-67-immunopositive cells in the non-TSS control group did not differ from that in normal control mice. In addition, the number of Ki-67-immunopositive cells was greater in mice in the TSS group than in the non-TSS control group from 14 days to 42 days post-injection (Figure 3a and b).

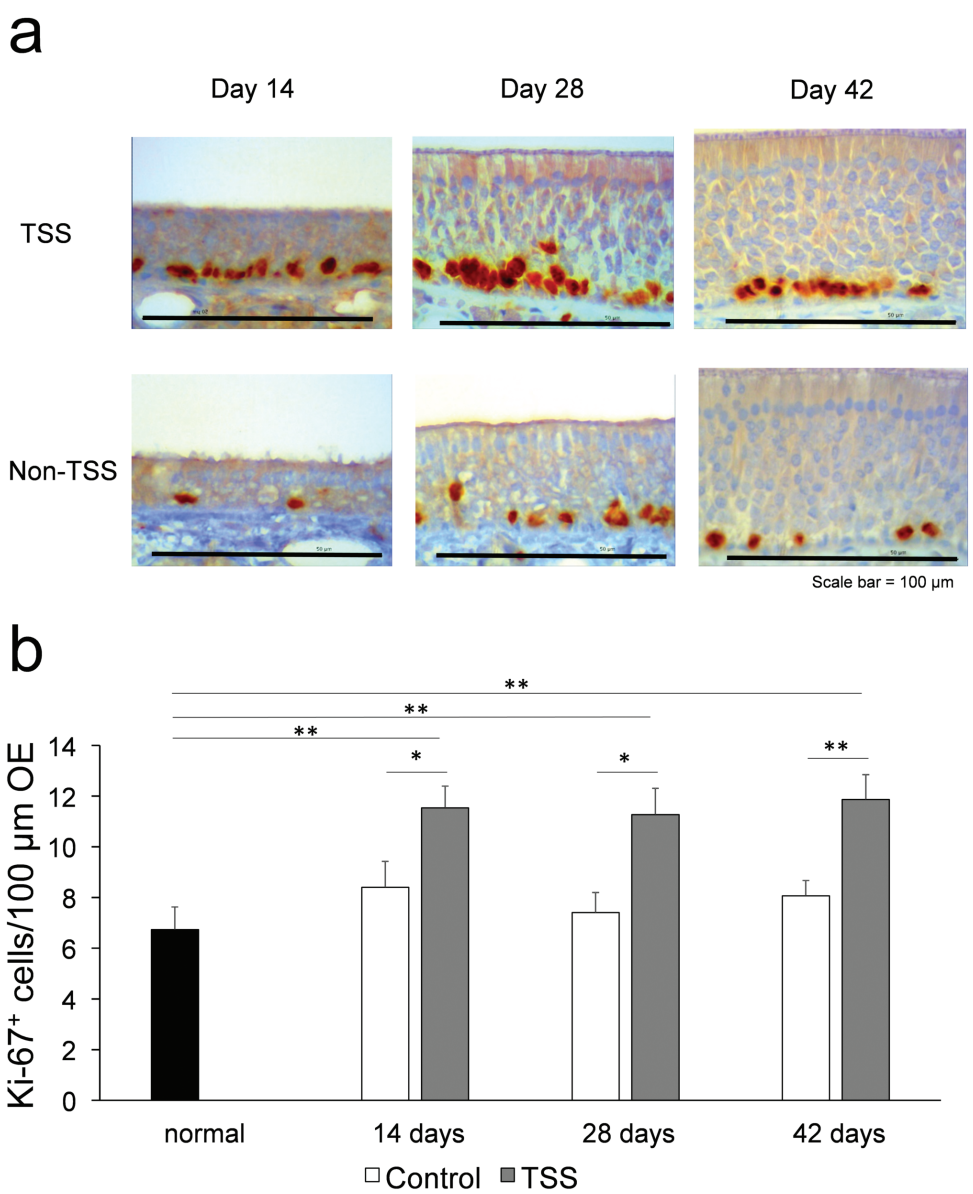

Figure 3. Immunohistochemical staining of Ki-67 in the OE. Ki-67 immunopositive cells were located just above the basement membrane in OE. Scale bar = 100 µm. (**a**) The number of Ki-67-immunopositive cells was greater in the TSS group than in normal control mice throughout the 42 days; in contrast, the number of Ki-67-immunopositive cells in the non-TSS group did not differ from that in normal control mice. The number of Ki-67-immunopositive cells were greater in the TSS group than in the non-TSS control group from 14 days to 42 days post-injection. (**b**) $*P < 0.05$, $**P < 0.01$: Student's *t*-test. TSS: Tokishakuyakusan.

NGF expression in olfactory bulb

NGF expression in the olfactory bulb had increased significantly more in the TSS-fed mice than in the mice in the non-TSS group by 28 days after methimazole injection, based on ELISA studies ($P < 0.05$). On the other hand, there was no significant difference between the TSS-fed group and non-TSS-fed group at 14 and 42 days post-injection, whereas the TSS group showed a tendency for increased expression at 14 and 42 days ($P < 0.10$; Figure 4).

In vitro studies

Effect of TSS on NGF expression in primary cultured astrocytes

NGF expression and/or signaling in the olfactory bulb is key to the recovery of damaged nerve tissue. The therapeutic effect of TSS in our animal model may have been due to a direct effect of TSS on NGF-expressing cells. Therefore, we next examined the effect of TSS on gene expression of NGF in primary cultured astrocytes. TSS, at a concentration of 300 µg/mL, significantly increased *NGF* messenger RNA (mRNA) levels at 24 and 48 h, by 10.5 and 9.7 times, respectively, compared with the control before starting the culture, whereas there was no change at 3 and 6 h (Figure 5a). TSS, at a concentration of 300 µg/mL, also significantly increased *NGF* mRNA at 48 h, to a level of 7.9 times more than that in the vehicle control (Figure 5b). We also examined the expression of TrkA, the NGF-specific receptor, in primary cultured astrocytes; we found no TrkA expression in cells with or without TSS treatment (data not shown).

Next, we examined NGF protein levels using an NGF-specific ELISA kit. TSS (300 µg/mL) significantly increased NGF protein concentration at 24 and 48 h to 182% and 214%, respectively, of

levels in the control before starting culture (Figure 6a). TSS significantly enhanced NGF concentration in the culture fluids for 48 h, in a concentration-dependent manner in the range of 300–600 μg/mL (Figure 6b). To investigate the signaling pathway involved in NGF upregulation by TSS treatment, we examined the influence of different pathway inhibitors on the TSS-induced NGF enhancement in astrocytes. U0126 inhibited the TSS-induced increase of NGF production, whereas H-89 and fulvestrant did not show any effect (Figure 6c). No morphological changes were observed in the cultures with any of the inhibitors.

Screening for NGF-enhancing active ingredients

Next, we investigated the component drugs of TSS: PR, ALR, OWR, PS, CR, and JAR. These test drugs were individually added to astrocyte cultures at a concentration of 100 μg/mL for 48 h, and NGF was measured by NGF-specific ELISA. ALR and JAR extracts significantly enhanced NGF concentration compared with the control, whereas the other drugs were inactive (Figure 7).

In addition, to identify NGF enhancers in TSS, 30 compounds present in TSS were examined at a single concentration (100 μmol/L); the results are shown in Supplementary Figure S1. Atractylodin, which is present in ALR (Chen et al. 2012), and levistolide A, which is present in CR (Zuo et al. 2011) and JAR (Gui and Zheng 2019), significantly enhanced NGF production in astrocytes. These active ingredients further showed concentration-dependent actions (Figure 8).

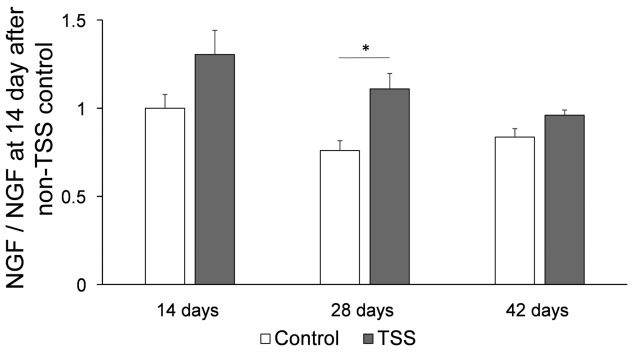

Figure 4. NGF expression in the olfactory bulb (ELISA study). NGF expression in the olfactory bulb increased significantly in the TSS group compared with the non-TSS control group at 28 days after methimazole injection. The TSS-fed group showed a tendency to increase compared with non-TSS control group at 14 and 42 days but was not significant. $^{*}P < 0.05$: Student's *t*-test. NGF: Nerve growth factor; TSS: Tokishakuyakusan.

Discussion

In this study, it was shown that TSS increased the regeneration of olfactory neurons after methimazole induced degeneration by activating NGF in the olfactory bulb and TSS and its components, ALR and JAR, enhanced NGF production in culture cells.

Kampo medicines have been widely used in Japan. Kampo drugs are used not only for indicated diseases or symptoms but also based on the individual's constitution or sex. Thus, different drugs could be prescribed for patients with the same disease, but with different constitutions. TSS has been widely used in the treatment of patients with gynecological disorders but has also been used for the treatment of cognitive impairment in recent years, based on its neural-activatory and neural-protective effects in the central nervous system (Itoh et al. 1996; Song et al. 2001; Hatip-Al-Khatib et al. 2007; Kitabayashi et al. 2007; Matsuoka et al. 2012). In the Japanese literature, it has been reported that TSS showed efficacy in patients with post-URTI dysfunction. Miwa et al. (2010) used TSS for the treatment of the patients with post-URTI dysfunction and reported a greater improvement in olfactory function with TSS than that seen with intranasal steroid treatment, with an improvement ratio in each group of 54% and 24%, respectively. Uchida et al. (2009) treated patients with post-URTI dysfunction, who had not responded to intranasal steroids, with TSS or another

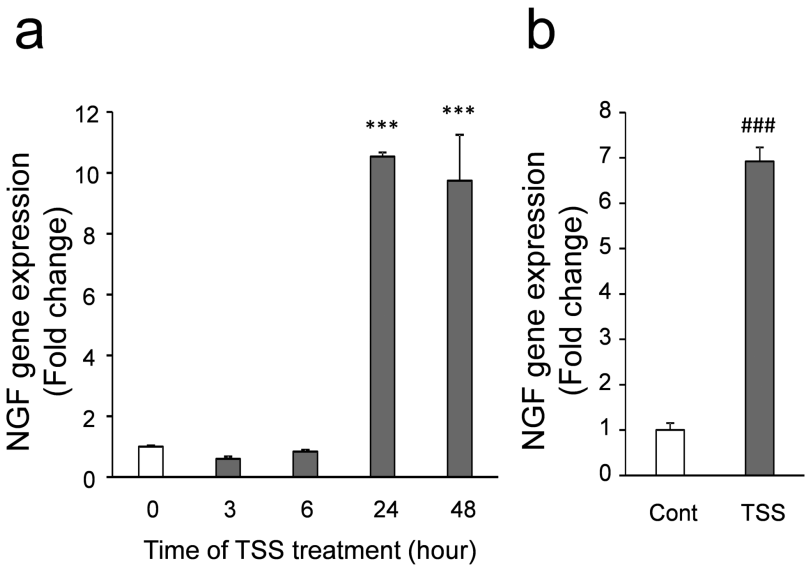

Figure 5. Effects of TSS on expression of *NGF* mRNA in the primary cultured astrocytes. The astrocytes were treated with 300 μg/mL TSS for 0–48 h (**a**) or 48 h (**b**), and *NGF* mRNA levels were quantified by real-time reverse transcription polymerase chain reaction (RT-PCR). Expression levels of *NGF* mRNA were normalized to that of *Rps29* mRNA and are shown as fold-change relative to the non-TSS treated control (a) or the vehicle control (b). The ΔCTs of *NGF* and *Rsp29* in the no-incubation control were 26.0 and 18.8, respectively. Each value represents the mean ± SE of 3 separate samples. $^{***}P < 0.001$ versus no incubation control, $^{###}P < 0.001$ versus vehicle control: Student's *t*-test. TSS: Tokishakuyakusan; NGF: Nerve growth factor.

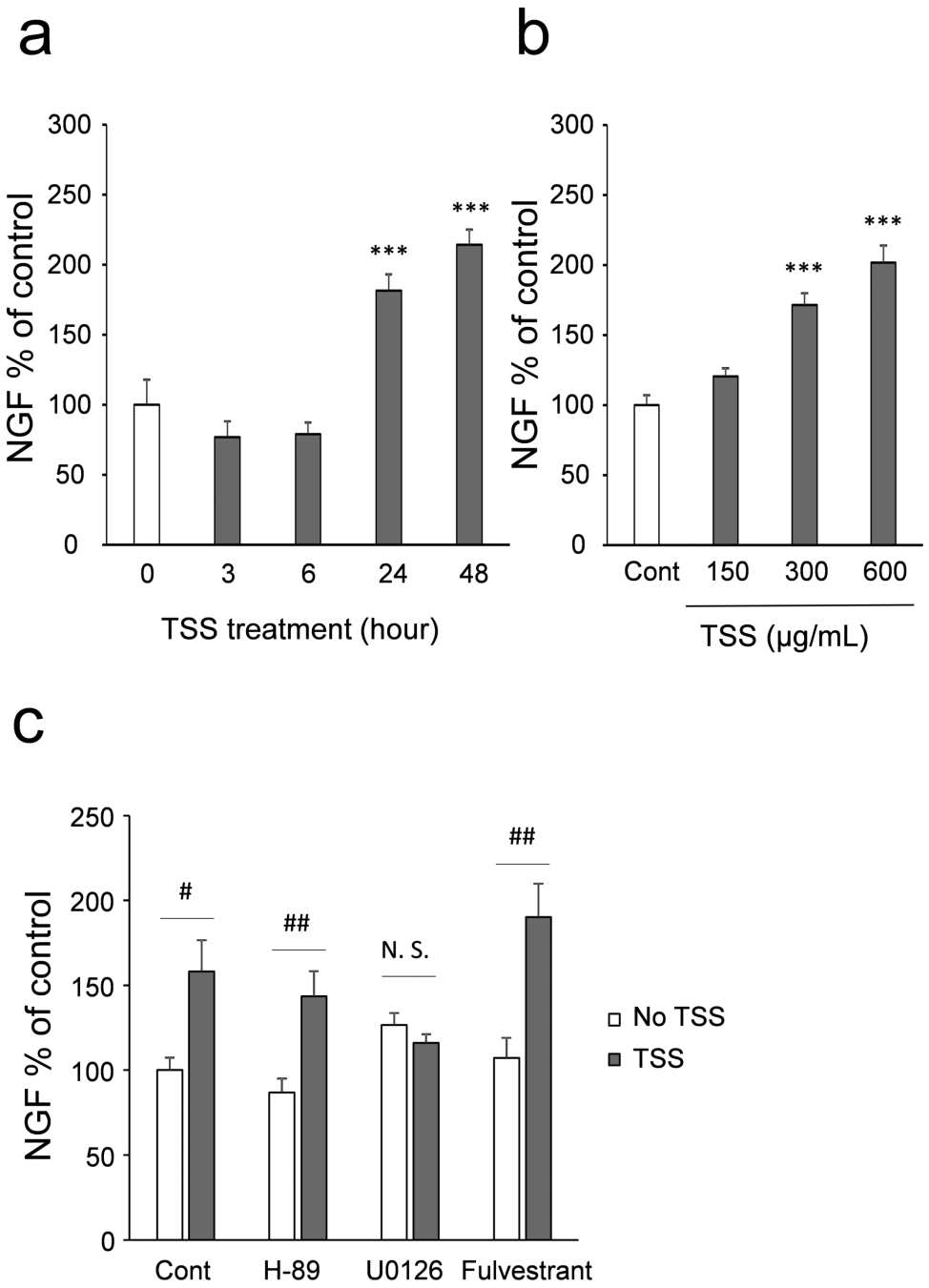

Figure 6. Effects of TSS on NGF production in primary cultured astrocytes. The astrocytes were treated with 300 μg/mL TSS for 0–48 h (**a**) or 150–600 μg/mL TSS for 48 h (**b**). The astrocytes were pre-treated with H-89 (2 μmol/L; protein kinase A inhibitor), U0126 (2 μmol/L; MEK1/2 inhibitor), or fulvestrant (2 μmol/L; estrogen receptor inhibitor) for 3 h, and then treated with 300 μg/mL TSS for 48 h (**c**). The concentration of NGF was measured using an NGF-specific ELISA kit. Each value represents the percentage of change against the no-incubation control (a), vehicle control (b), or vehicle control without inhibitor (c) in mean ± SE of 6 separate samples. $^{***}P < 0.001$ versus each control: one-way analysis of variance + Dunnett's test, $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus no TSS: Student's *t*-test, N. S.: not significant. TSS: Tokishakuyakusan; NGF: Nerve growth factor.

Kampo medicine, ninjin'yoeito, and found an improvement rate of 43% and 36%, respectively. Ogawa et al. (2010) reported that the improvement ratio of post-URTI dysfunction patients treated with only intranasal steroid treatment, only TSS oral administration, or combined steroid and TSS, for 3 months, was 29%, 55%, and 60%, respectively. In these articles, TSS appeared to be useful for the treatment of patients with post-URTI dysfunction; however, its mechanism has not been fully determined.

Regeneration of the OE occurs after axotomy or bulbectomy in mammals (Costanzo et al. 1983; Costanzo 1985). Several behavioral studies have also demonstrated recovery of olfactory function (Harding and Wright 1979; Yee and Costanzo 1995; Yee and Costanzo 1998). The OE is composed of olfactory receptor neurons, basal cells, and supporting cells. It has been proposed that the stem cells or progenitor cells for olfactory neurons are basal cells, located near the basal lamina of the OE (Graziadei and Graziadei 1979a).

Song et al. (2001) reported that oral administration of TSS increased NGF levels in the olfactory bulbs of mice with olfactory bulb lesions. NGF is expressed in various regions of the central nervous system, including the olfactory bulb. In our previous studies using mice, we reported that NGF in the olfactory bulb played an important role not only in the survival of cholinergic neurons in the horizontal limb of the diagonal band but also in the development, maintenance, and regeneration of olfactory neurons. In brief, ^{125}I-labeled NGF injected into the olfactory bulb was transported retrogradely into the OE (Miwa, Uramoto et al. 1998). Immunoreactivity for TrkA, a high-affinity NGF receptor, was high throughout regeneration of olfactory neurons after olfactory nerve transection (Miwa, Horikawa et al. 1998). Moreover, continuous administration of anti-NGF antibody into the olfactory bulb caused degeneration of olfactory neurons and olfactory dysfunction. In contrast, continuous infusion of NGF into the cranial cavity after olfactory bulbectomy increased regeneration of olfactory neurons (Uramoto 1998). On the basis of these findings, NGF in the olfactory bulb appears to play an important role in the maintenance and regeneration of olfactory neurons.

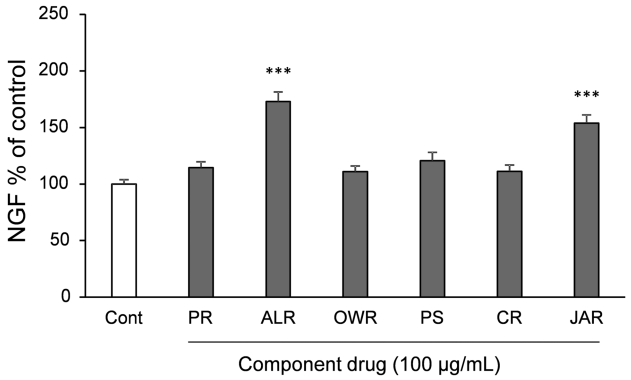

Figure 7. Random screening of active components of TSS on NGF production. The component drugs of TSS: PR, ALR, OWR, PS, CR, and JAR were applied to cultured astrocytes at a concentration of 100 μg/mL. Culture fluids were collected at 48 h, and the concentrations of NGF were measured using an NGF-specific ELISA kit. Each value is expressed as the percent of change against the vehicle control, as the mean ± SE of 6 separate samples. $^{***}P < 0.001$ versus vehicle control: one-way analysis of variance + Dunnett's test. TSS: Tokishakuyakusan; NGF: Nerve growth factor.

Suzukawa et al. (2011) reported that the OE was damaged 1 day after methimazole administration and returned to normal morphology by 32 days after administration. In our in vivo study, OMP-positive neurons and the thickness of the OE were reduced at 14 days after methimazole injection, but both increased gradually over time. At 14 days and 28 days, reflecting the regeneration period, both the OE thickness and number of OMP-positive cells had increased more in the TSS-treated mice than in non-treated animals. At 42 days, after completion of regeneration, both of these parameters showed no significant differences between control mice, TSS-fed mice, and mice receiving non-TSS food. On the other hands, Ki-67 immunoreactivity in TSS-fed mice was significantly increased over that of non-TSS-fed mice throughout the observation period. The number of Ki-67 cells was not different between the normal control and the non-TSS post-methimazole mice in our experiment. In past reports that observed the dynamics of Ki-67 during the chronological regeneration in mice olfactory neurons, the number of Ki-67 positive cells was increased on day 7 but returned to normal levels by day 14 (Ogawa 2014, Ueha 2016). However, we did not observe an increase in Ki-67 positive cells at days 14, 28, and 32 in this study. NGF expression in the olfactory bulb of TSS-fed mice was increased at 28 days; thus, NGF expression in the olfactory bulb was increased by TSS treatment and promoted regeneration of olfactory neurons in the OE. A major limitation of this study is that our histological findings are limited to a single region of the OE, the dorsal area of the septum. Although this

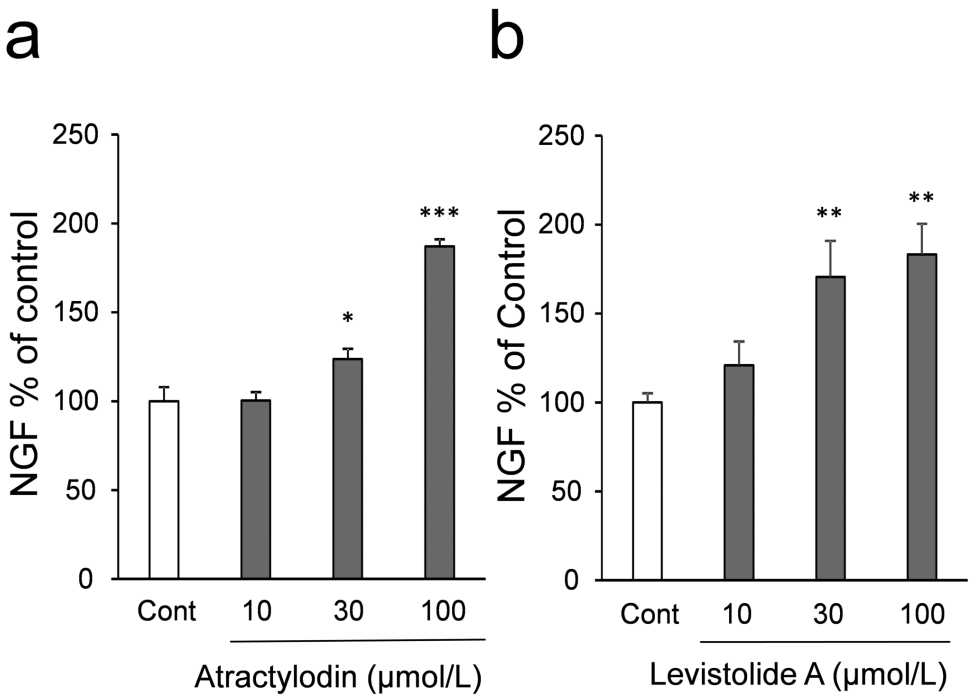

Figure 8. Enhancement of NGF production by atractylodin and levistolide A. Atractylodin and levistolide A were applied to cultured astrocytes at a concentration of 10, 30, and 100 μmol/L. Culture fluids were collected at 48 h, and the concentrations of NGF were measured. Each value is expressed as the percent of change against the vehicle control, as the mean ± SE of 6 separate samples. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus control: one-way analysis of variance + Dunnett's test.

area was selected because variations in epithelial thickness are less than in other regions of the nasal cavity our data may not accurately represent changes in other regions. Thus, our results represent only an estimate of the overall effects of TSS treatments.

This study, using an astrocyte culture system, clearly demonstrates that TSS increased NGF production, supporting the results in our *in vivo* study. Our results are consistent with those of a previous report, in which NGF was increased due to TSS administration in a mouse dementia model that was induced by intranasal irrigation with zinc sulfate (Song et al. 2001). The enhancing effect of TSS on NGF production is therefore plausible. Several reviews have demonstrated that phytochemicals modulate neurotrophic signaling pathways and upregulate NGF production (Moosavi et al. 2016; Venkatesan et al. 2015); we therefore examined the potential involvement of PKA, MEK1/2, and estrogen receptor signaling pathways in this context. On the basis of our astrocyte assay, NGF production was suppressed by addition of the MEK1/2 inhibitor U0126, but not the PKA inhibitor H-89 or the estrogen receptor antagonist fulvestrant, showing that the increase in NGF expression by TSS may be exerted through an ERK1/2-dependent pathway.

We identified 2 active ingredients that enhance NGF production in astrocytes. Atractylodin, an ingredient in ALR (Chen et al. 2012), is reported to have various biological actions, including augmentation of the ghrelin signal (Fujitsuka et al. 2011). A plasma pharmacokinetic study has shown that oral atractylodin was absorbed into the systemic circulation and into the brain when another traditional Kampo medicine that includes ALR, rikkunshito, was given orally to rats (Xiao et al. 2016; Nahata et al. 2018). Obara et al. (2002) reported that β-eudesmol, a major ingredient of ALR, had a direct effect on pheochromocytoma PC12 cells, which have some characteristics of nerve cells, resulting in morphological changes including neurite extension. Moreover, β-eudesmol increased the expression of neurotrophic factors, including NGF, IL-6, and glial cell-derived neurotrophic factor, when used at a concentration of 100–150 μmol/L. These lines imply that ALR potentially contains bioactive compounds affecting growth and/or differentiation of nerve-related cells. However, we examined β-eudesmol at 100 μmol/L and found out no activity, as shown in Supplementary Figure S1. Levistolide A, the other NGF-increasing active ingredient identified in this study, is found in CR (Zuo et al. 2011) and JAR (Gui and Zheng 2019). However, CR was unable to increase NGF in our study, as the concentration in the CR extract might have been low. A prompt absorption into the blood after oral administration of levistolide A has been reported (Zuo et al. 2011; He et al. 2015). These findings suggest that TSS leads to repair of chemically induced olfactory bulb lesions through upregulation of local NGF production, induced by absorbed active ingredients, such as atractylodin and levistolide A. We found no TrkA expression in cells with TSS treatment in this study. It has been proven that Trk A is transiently expressed in the OE during olfactory nerve regeneration (Miwa, Uramoto et al. 1998). In this study, astrocytes played a role in producing the ligand, NGF, and not the receptor; therefore, it did not express TrkA in itself.

Because post-URTI dysfunction is a sensorineural olfactory dysfunction, its recovery should require the regeneration of the olfactory neurons. Several drugs, such as zinc sulfate (Henkin 1976; Aiba et al. 1998), α-lipoic acid (Hummel et al. 2002; Welge-Lussen and Wolfensberger 2006), vitamin A (Reden et al. 2012), minocycline (Reden et al. 2012), theophylline (Henkin et al. 2009), pentoxifylline (Gudziol and Hummel 2009), and oral and intranasal steroids (Heilmann et al. 2004) have been used for the clinical treatment of post-URTI dysfunction. Some improvement has been noted in retrospective studies; however, none of these studies demonstrated statistically significant therapeutic effects based on double-blind, randomized, placebo-controlled trials. On the other hand, olfactory training using odorants has been reported to be effective in improving olfactory function in post-URTI dysfunction, based on randomized controlled trials (Hummel et al. 2009; Konstantinidis et al. 2013; Damm et al. 2014; Altundag 2015; Konstantinidis et al. 2016). The findings of this study suggest that TSS may be effective and useful for post-URTI dysfunction to activate NGF in the olfactory bulb. Further studies, including prospective, randomized, controlled trials, are required to confirm the clinical efficacy of TSS against post-URTI dysfunction. In addition, in mammals, 3 types of neurons have regenerative ability: olfactory neurons, granule cells in the olfactory bulb, and hippocampal neurons. These neurons are all present, from the periphery to the center, in the olfactory pathway. Intranasal administration reflects a drug-delivery route to the central nervous system, via the olfactory or trigeminal neurons. Recent studies have revealed that TSS increased extracellular acetylcholine levels in the dorsal hippocampus and improved the impairment of spatial memory induced by ovariectomy combined with beta-amyloid peptide (Egashira et al. 2018). Thus, TSS or its components, administered orally or nasally, may show some effects not only on sensorineural olfactory disorders but also neurodegenerative diseases, such as Alzheimer's disease, by activating NGF.

Conclusion

This study identified the active ingredients and at least a partial mechanism of action by which TSS exerts beneficial effects in post-URTI dysfunction, shedding some light on the pharmacodynamics of TSS in the treatment of this condition.

Supplementary material

Supplementary material can be found at http://www.chemse.oxfordjournals.org/.

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Conflicts of interest

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