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Research report

Acute intravenous administration of dietary constituent theanine suppresses noxious neuronal transmission of trigeminal spinal nucleus caudalis in rats

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ABSTRACT

Theanine is a non-dietary amino acid linked to the modulation of synaptic transmission in the central nervous system, although the acute effects of theanine in vivo, particularly on nociceptive transmission in the trigeminal system, remain to be determined. The present study investigated whether acute intravenous theanine administration to rats attenuates the excitability of wide dynamic range (WDR) spinal trigeminal nucleus caudalis (SpVc) neurons in response to nociceptive and non-nociceptive mechanical stimulation in vivo. Extracellular single unit recordings were made from 15 SpVc neurons in response to orofacial mechanical stimulation of pentobarbital-anesthetized rats, and responses to non-noxious and noxious mechanical stimuli were analyzed. The mean firing frequency of SpVc WDR neurons in response to all mechanical stimuli was dose-dependently inhibited by theanine (10, 50, and 100 mM, i.v.) with the maximum inhibition of discharge frequency reached within 5 min. These inhibitory effects were reversed after approximately 10 min. The relative magnitude of theanine's inhibition of SpVc WDR neuronal discharge frequency was significantly greater for noxious than non-noxious stimulation. Iontophoretic application of L-glutamate induced the mean firing frequency of SpVc WDR neuron responding to noxious mechanical stimulation was also inhibited by intravenous administration of 100 mM theanine. These results suggest that acute intravenous theanine administration suppresses glutaminergic noxious synaptic transmission in the SpVc, implicating theanine as a potential complementary and alternative therapeutic agent for the treatment of trigeminal nociceptive pain.

1. Introduction

The spinal trigeminal nucleus is an important relay station in transmitting orofacial sensory information and is functionally subdivided into three nuclei from rostral to caudal: oralis, interpolarlis, and caudalis (Sessle, 2000). In particular, the spinal trigeminal nucleus caudalis (SpVc) relays trigeminal nociceptive inputs from sites of inflammation and tissue injury (Sessle, 2000; Takeda et al., 2012). Chronic pathological conditions such as tissue inflammation can change the properties of somatic sensory pathways, leading to hyperalgesia and allodynia (Scholz and Woolf, 2002), while changes in the excitability of primary afferent neurons (peripheral sensitization) alter information processing in the spinal trigeminal nucleus or higher centers (Millan, 1999). Previous studies have demonstrated that wide dynamic range (WDR) neurons in the SpVc region are important in the mechanism underlying hyperalgesia/allodynia and/or referred pain associated with orofacial pain (Takeda et al., 2000, 2005, 2012). Complementary and alternative medicine (CAM), such as herbal remedies and acupuncture, has been used clinically to treat persistent chronic pain (Rao et al., 1999; Konvicka et al., 2008; Rosenberg et al., 2008), and considerable research has focused on the potential effects of diet and dietary supplementation on conditions associated with pain (Shir et al., 2001; Ernest, 2003; Tall and Raja, 2004). We recently showed that intravenous administration of dietary resveratrol suppresses nociceptive SpVc WDR neuronal activity via the glutaminergic exci-

Abbreviations: SpVc, trigeminal spinal nucleus caudalis; WDR, wide dynamic range; CAM, complementary and alternative medicine; DRG, dorsal root ganglion; ANOVA, analysis of variance; AMPA, α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid; NMDA, *N*-methyl-D-aspartate; GABA, γ-aminobutyric acid; SSS, superior sagittal sinus; C1, first cervical dorsal horn.

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tatory synaptic transmission of SpVc by inhibiting postsynaptic glutamate receptors (Takehana et al., 2016). Also, local injection of resveratrol into the peripheral receptive field suppresses the excitability of SpVc WDR neurons, possibly via inhibition of voltage-gated Na^+ channels in the nociceptive nerve terminal of trigeminal ganglion neurons (Shimazu et al., 2016). In addition, choronic administration of resveratrol has been shown to attenuate inflammation-induced nociceptive SpVc WDR neuronal excitability associated with hyperalgesia via inhibition of both peripheral and central cyclooxygenase-2 cascade signaling pathways (Sekiguchi et al., 2016). Together, these findings support the idea that dietary constituents such as resveratrol could be potential therapeutic CAM for the alleviation of nociceptive pain and prevention of trigeminal inflammatory hyperalgesia.

Theanine (γ-glutamylethylamide) is an amino acid derivate of glutamic acid found almost exclusively in certain teas. Theanine extracts from green tea have been suggested to prevent and cure cancer via intrinsic biological activity (Weiner et al., 2009). Theanine is structurally similar to glutamic acid, a primary excitatory neurotransmitter in the central nervous system (Nathan et al., 2006), and can bind to glutamate receptors, α-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA), kainite, and *N*-methyl-D-aspartate (NMDA) receptors (Kakuda et al., 2002; Maruyama and Takeda, 1994). Hence, theanine acts as a natural antagonist of glutamate in synaptic neurotransmission (Shinozaki and Ishida, 1978: Kakuda et al., 2002). Since it has been demonstrated that blockade of glutamatergic transmission via NMDA and non-NMDA receptors results in significant inhibition of excitatory neurotransmission of SpVc, glutamatergic transmission mechanisms in the SpVc have clinical importance for primary headache syndromes, such as migraine and cluster headache (Storer and Goadsby, 1999). Chan and MaassenVanDenBrink (2014) indicated that glutamate receptor antagonists play an important role in the management to migraine. Intraperitoneal injection of theanine into mice also elevated the intracerebral level of γ-aminobutyric acid (GABA) within 30 min, suggesting that theanine is transported into the brain through the blood-brain barrier and can alter intracerebral GABA levels (Kimura and Murata, 1971; Egashira et al., 2007). These findings suggest that theanine could modulate excitatory and inhibitory neuronal transmission in the central nervous system. Based on these observations, we hypothesized that theanine administration would attenuate noxious stimulation-induced excitability of SpVc neuronal activity through a central mechanism, as is the case for local anesthetic agents and/or analgesic drugs, it could be a candidate CAM for pain. However, the acute effects of theanine on trigeminal neuronal activity in vivo in response to nociceptive and non-nociceptive mechanical stimulation remain to be determined. Therefore, the present study investigated whether acute intravenous administration of theanine to rats could attenuate the excitability of nociceptive SpVc WDR neuronal activity in vivo in response to mechanical stimulation.

2. Material and methods

The experiments described herein were approved by the Animal Use and Care Committee of Azabu University and were performed in accordance with the guidelines of the International Association for the Study of Pain (Zimmermann, 1983). Every effort was made to minimize the number of animals used and their suffering.

2.1. Extracellular single unit recording of WDR neuronal activity in the SpVc

Electrophysiological recordings were made in 13 adult male Wistar rats weighing 230–290 g. Rats were anesthetized with pentobarbital sodium (45 mg/kg, i.p.) and anesthesia was maintained with additional doses of 2–3 mg/kg per h pentobarbital sodium through a cannula into the jugular vein, as required. The level of anesthesia was confirmed by the absence of corneal reflex and lack of response to paw pinching. Rectal temperature was maintained at $37.0 \pm 0.5\,^{\circ}C$ with a homeothermic blanket during recording. Rats were placed in a stereotaxic apparatus, and the activity of a single neuron from the SpV_C region was recorded extracellularly. Single neuron activity was recorded using a glass micropipette filled with 2% pontamine sky blue and 0.5 M sodium acetate according to the stereotaxic coordinates of Paxinos and Watson (1986). Neuronal activity was amplified (DAM 80; World Precision Instruments), filtered (0.3–10 kHz), monitored with an oscilloscope (SS-7672; Iwatsu, Tokyo, Japan), and then recorded on a polygraph (NEC-Sanei 8M14) for subsequent off-line analysis using PowerLab and Chart 5 software (ADInstruments, Oxford, UK).

In some experiments (n = 5), the single neuron activity was instead recorded using a three-barreled glass micropipette filled with 2% pontamine sky blue and 0.5 M sodium acetate as described in our previous studies (Takeda et al., 2000, 2005), with iontophoretic application as described in our previous reports (Takeda et al., 2000, 2005; Tanimoto et al., 2004). Of the two lateral barrels of the micropipette, one barrel containing 160 mM NaCl was used for balancing currents to prevent the occurrence of tip polarization artifacts. The remaining barrel contained L-glutamate (100 mM in 160 mM NaCl, pH 8.5; Nacalai Tesque, Kyoto, Japan), as described previously (Takeda et al., 2005, 2006). The currents for ejecting, retaining, and balancing were provided by a constant current unit (Dia Medical, DPI-25, Japan). The drugs were ejected with 10–90 nA cationic currents, and 10–25 nA retaining currents were used.

2.2. Animal experiment protocols

Extracellular recordings of SpVc WDR unit activity were made as follows. Mechanical stimulation was used to identify the receptive field quickly and to avoid sensitizing peripheral receptors. In this way, we identified single units that responded to stimulation on the left side of the orofacial facial skin (whisker pad) with a brush and a set of von Frey hairs (Semmes-Weinstein Monofilaments; North Coast Medical, Gilroy, CA, USA). Noxious pinch stimulation, which evoked a pain sensation when applied to a human subject, was applied to the orofacial area of rats using forceps. After identification of WDR SpVc neurons responding to stimulation of the whisker pad, we checked for spontaneous discharge. The threshold for mechanical stimulation was determined using non-noxious and noxious mechanical stimulation (5 s) with von Frey hairs (1, 2, 4, 6, 10, 15, 26, 60 g) at 5-s intervals. The mechanical receptive field of neurons was mapped by probing the facial skin with von Frey hairs, and then outlined on a life-sized drawing of a rat on tracing paper. The WDR neuronal discharges induced by mechanical stimulation were quantified by subtracting background activity from evoked activity, and spontaneous discharge frequencies were determined over 2–5 min. If no discharge was recorded, the cell was deemed a silent neuron. The mean firing rate of SpVc WDR neurons evoked by mechanical stimulation was compared before and after drug administration. Since previous studies have demonstrated the importance of WDR neurons in the SpVc region in the mechanism underlying hyperalgesia and referred pain associated with orofacial pain (Takeda et al., 2000, 2012), the present study focused on the effects of theanine on SpVc WDR neuronal activity, although we did not examine nociceptive-specific neurons (Ness and Randich, 2000). Post-stimulus histograms (bin = 100 msec) were generated in response to each stimulus.

The effects of theanine (10, 50, and 100 mM, i.v.), injected through a cannula into the jugular vein, were evaluated 5, 10, 20, and 30 min after administration when peak effect and recov-

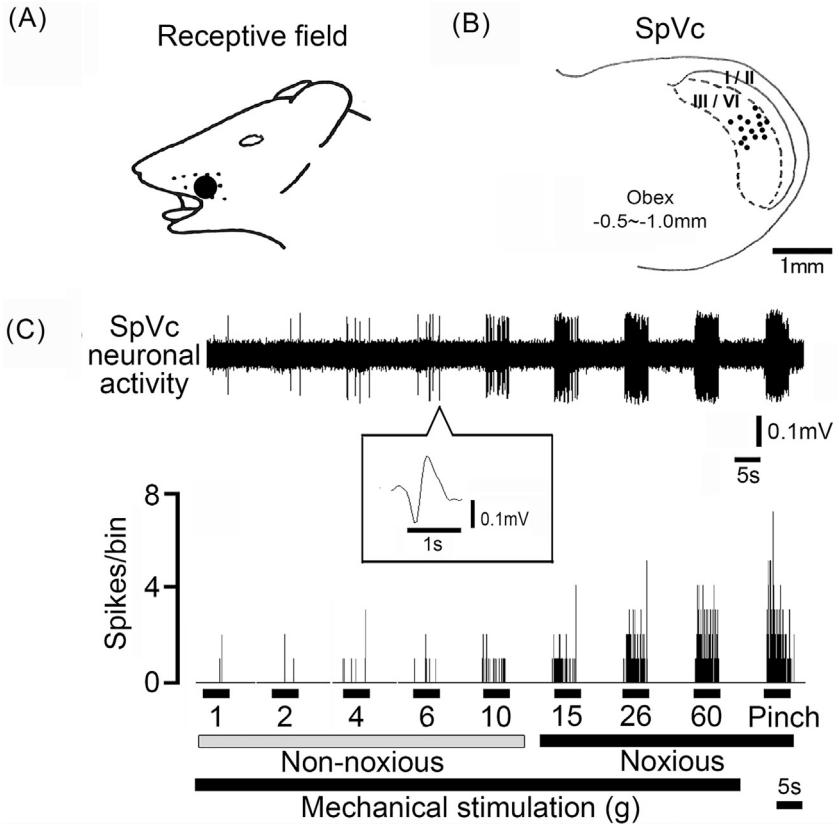

Fig. 1. General characteristics of spinal trigeminal nucleus caudalis (SpVc) wide dynamic range (WDR) neuron activity in response to mechanical stimulation. (A) Receptive field of the whisker pad in the facial skin. The shaded area indicates the location and size of the receptive field. (B) Distribution of SpVc WDR neurons responding to non-noxious and noxious mechanical stimulation of the facial skin (n = 15). (C) Typical example of SpVc WDR neuron activity evoked by non-noxious (2–10 g) and noxious mechanical stimulation (15, 26, and 60 g and noxious pinch) of the orofacial skin. Upper trace, SpVc WDR neuron activity; lower trace, post-stimulus histogram. Inset, example of the action potential waveform evoked by mechanical stimulation.

ery are thought to occur. Theanine was dissolved in saline, and the stock solution was stored at −20 °C in small aliquots until use. The mean spontaneous and mechanical stimulation-induced discharges rates, as well as the mechanical threshold before and after intravenous administration of theanine, were evaluated in the present study.

2.3. Identification of recording sites

Recording sites of SpVc WDR neuronal activity were identified as described previously (Takeda et al., 2000, 2012). Briefly, at the end of the recording sessions, rats were deeply anesthetized and anodal DC currents (30 μA, 5 min) were passed through a recording micropipette. The rats were then perfused transcardially with saline and 10% formalin. Frozen coronal sections (30 μm) were cut and stained with hematoxylin–eosin. Recording sites were identified from the blue spots, and the path of electrode tracks was constructed in combination with the micromanipulator readings.

2.4. Data analysis

Values are expressed as the mean ± SEM. Statistical analyses were performed using two-way repeated-measures analysis of variance (ANOVA) followed by Tukey–Kramer or Dunnett's post hoc tests for electrophysiological data. $P < 0.05$ was considered significant.

3. Results

3.1. General properties of SpVc WDR neurons innervating facial skin

Extracellular single unit activity recorded from 15 neurons in the SpVc, responding to non-noxious and noxious mechanical stimulation, exhibited a somatic receptive field in the orofacial area (mainly whisker pad; Fig. 1A), as described previously (Takehana et al., 2016; Sekiguchi et al., 2016; Takeda et al., 2000). Every neuron recorded belonged to the WDR category (Takehana et al., 2016; Sekiguchi et al., 2016; Takeda et al., 2012). Two of the 15 units exhibited spontaneous discharges. As shown in Fig. 1B, recording sites were found in layers I–III (n = 6; 40%) and IV–V (n = 9; 60%) of the SpVc (obex from −0.5 to −2 mm). Fig. 1C shows typical examples of SpVc WDR neuronal unit responses. Graded mechanical stimulation was applied to the most sensitive area of the receptive field, which exhibited increased a firing frequency of SpVc WDR neurons in proportion to the stimulus intensity. The mean mechanical stimulation-induced spike threshold was 2.8 ± 1.3 g (n = 15).

3.2. Effects of intravenous administration of theanine on excitability of SpVc WDR neurons in response to non-noxious stimuli

Fig. 2 represents a typical effect of theanine (100 mM, i.v.) on the excitability of SpVc WDR neurons in response to non-noxious mechanical stimulation. Five minutes after injection of theanine, non-noxious (1–10 g) mechanical stimulation-evoked SpVc WDR neuronal activity was inhibited, but this inhibition disappeared

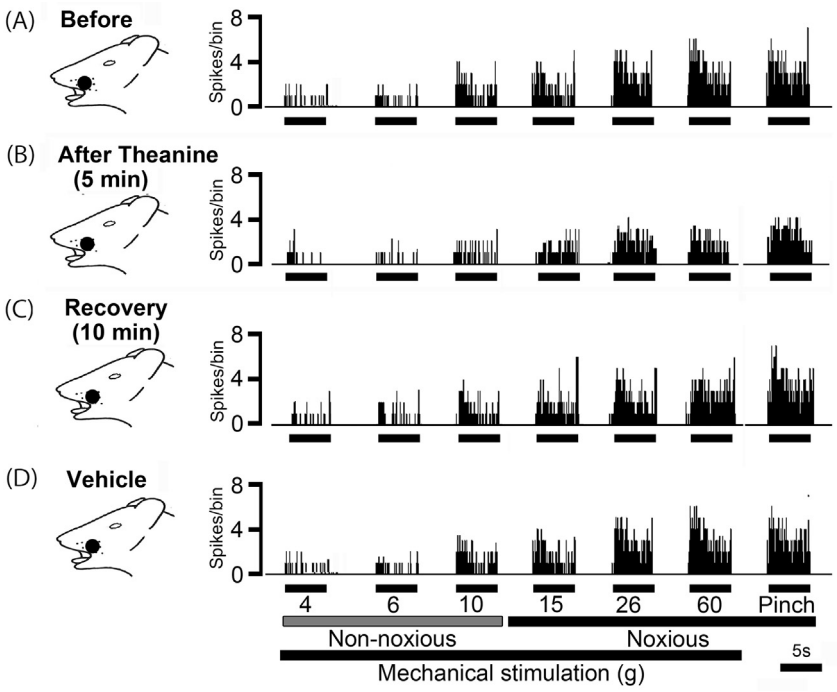

Fig. 2. Effect of acute intravenous injection of theanine on SpVc WDR neuronal activity evoked by non-noxious, noxious, and mechanical stimulation. Typical example of non-noxious (6 and 10 g), noxious (15 and 60 g) mechanical, and noxious pinch stimulation-evoked SpVc WDR neuron activity, before (A) and at 5 (B) and 10 min (C) after theanine administration (100 mM, i.v.) (D) Effects of intravenous administration of vehicle. Receptive field of whisker pad in the facial skin. Black area indicates the location and size of the receptive field.

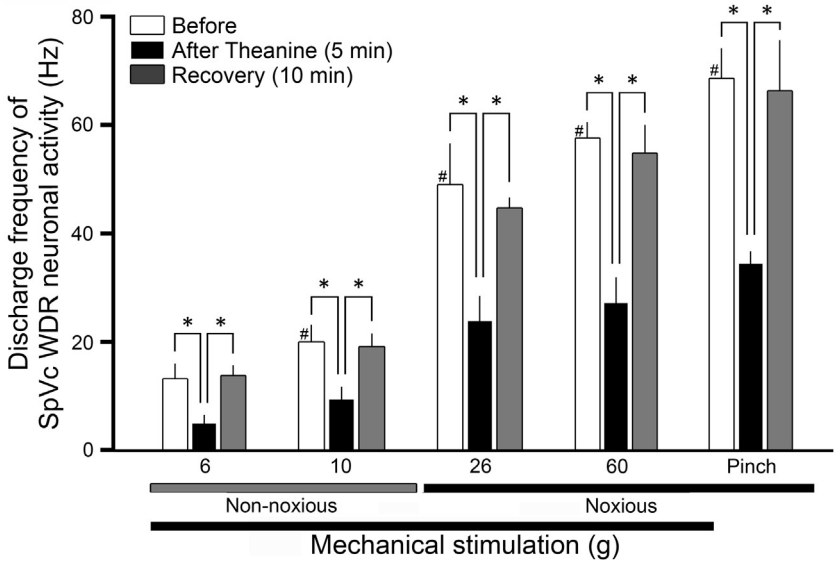

Fig. 3. Time course of intravenous theanine administration on the mean firing frequency of SpVc WDR neurons responding to non-noxious, noxious, and noxious pinch mechanical stimulation. # $P < 0.05$ compared with 6-g stimulus; *$P < 0.05$ compared with before theanine administration (100 mM), n = 5, # $P < 0.05$, 6 g vs. 10, 26, and 60 g, and pinch.

within approximately 10 min, with activity returning to control levels. There were no obvious changes in the size of the receptive field ($17.5 \pm 0.3\ mm^2$ vs. $18.1 \pm 0.1\ mm^2$, n = 15, NS) or in the mechanical threshold after theanine administration. SpVc WDR neuronal activity evoked by non-noxious mechanical stimulation is summarized in Fig. 3. After theanine injection, there was a significant decrease in the mean firing rate of non-noxious mechanical stimulation-evoked SpVc WDR neuronal activity, which eventually returned to control levels (before theanine injection). Mean firing rates before and after theanine were 13.5 ± 2.8 vs. 5.0 ± 1.5 Hz, respectively, in response to the 6-g stimulus and 20.1 ± 3.1 vs. 9.5 ± 2.3 Hz, respectively, in response to the 10-g stimulus (n = 5, $P < 0.05$ for both). As indicated in Fig. 4, The suppression of SpVc WDR neuronal firing in response to non-noxious stimulation was significantly greater following intravenous injection of 10 vs. 100 mM theanine ($10.5 \pm 8.1\%$ vs. $35.6 \pm 5.4\%$, respectively, for the 6-g stimulus; $14.4 \pm 6.0\%$ vs. $38.1 \pm 4.9\%$, respectively, for the 10-g stimulus (n = 5, $P < 0.05$ for all; Fig. 4).

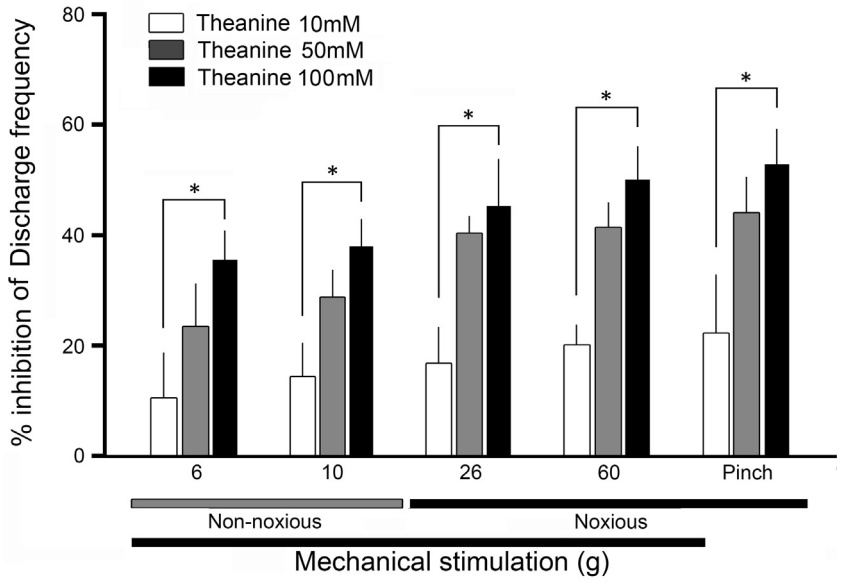

Fig. 4. Dose-dependent suppression by theanine of the mean firing frequency of SpVc WDR neurons responding to non-noxious, noxious, and noxious pinch mechanical stimulation. *$P < 0.05$, 10 mM vs. 100 mM i.v, theanine, n = 5.

3.3. Effects of intravenous administration of theanine on excitability of SpVc WDR neurons in response to noxious stimuli

Fig. 2 also shows the typical effects of theanine (100 mM, i.v.) on the excitability of SpVc WDR neurons in response to noxious mechanical and noxious pinch stimulation. Noxious (15–60 g) mechanical stimulation-evoked SpVc WDR neuronal activity was inhibited 5 min after the injection of theanine, and again this inhibition disappeared within approximately 10 min, with activity returning to control levels. Similarly, SpVc WDR neuronal activity in response to noxious pinch stimulation was inhibited 5 min after the theanine injection, and then returned to control levels (before theanine injection). within 10 min.

As indicated in Fig. 3, the mean firing rates of SpVc WDR neurons evoked by noxious mechanical and pinch stimulation decreased significantly after injection of theanine compared with (before theanine injection): 49.0 ± 7.5 vs. 23.9 ± 4.7 Hz, respectively, for the 26-g stimulus; 57.6 ± 2.9 vs. 27.3 ± 4.7 Hz, respectively, for the 60-g stimulus; and 68.7 ± 5.4 vs 34.4 ± 2.2 Hz, respectively, for the pinch stimulus (n = 5, $P < 0.05$ for all). The suppression of SpVc WDR neuronal firing in response to noxious mechanical and pinch stimulation was significantly greater following intravenous injection of 10 vs. 100 mM theanine (16.9 ± 6.4% vs. 45.4 ± 8.4, respectively, for the 26-g stimulus; 20.2 ± 3.6% vs. 50.2 ± 5.9%, respectively, for the 60-g stimulus; and 22.3 ± 10.6% vs. 52.9 ± 6.5%, respectively, for the pinch stimulus (n = 5, $P < 0.05$ for all; Fig. 3). No significant changes were observed in the mean receptive field size after theanine administration (before vs after 14.5 ± 0.1 mm^2 vs. 15.9 ± 0.3 mm^2, n = 5, NS). There were also no changes in the spontaneous firing rate after theanine administration. Intravenous administration of vehicle had no significant effect on either spontaneous or evoked (non-noxious, noxious mechanical, and pinch stimulation) activity of SpVc WDR neurons (n = 3; Fig. 2).

3.4. SpVc WDR neuronal activity in response to noxious vs. non-noxious stimuli after theanine

As shown in Fig. 5, we also compared the relative inhibitory effect of 100 mM i.v. theanine on responses to non-noxious and noxious stimuli. The mean magnitude of inhibition by theanine of

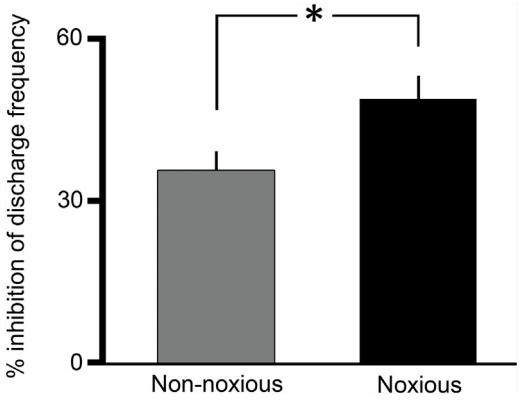

Fig. 5. Comparison of the mean magnitude of inhibition by theanine (100 mM) of SpVc WDR neuronal discharge frequency between non-noxious and noxious stimulation.

SpVc WDR neuronal discharge frequency was significantly greater for noxious than non-noxious stimuli (35.8 ± 3.4% vs. 49.0 ± 4.6%, respectively; n = 13, $P < 0.05$).

3.5. Iontophoretic application of L-glutamate-evoked neuronal discharge frequency of SpVc WDR neurons responding to noxious mechanical stimulation

In this study we found that five of the SpVc WDR neurons responding to noxious mechanical stimulation of the whisker pad were also activated by iontophoretic application of glutamate (Fig. 6). As shown in Fig. 6A, we observed that these neurons were also activated by iontophoretic application of glutamate, as previously described (Takeda et al., 2005, 2006), and that the glutamate-evoked discharge was reproducible across the 5-s intervals (Fig. 2B). The firing frequency of iontophoretic glutamate application-evoked spike frequency was current-dependently increased (30, 50, and 70 nA), as were the mean discharge frequencies of SpVc WDR neuronal activity evoked by iontophoretic glutamate application (Fig. 6B; n = 5). There were no significant differences in mean discharge frequency between noxious stim-

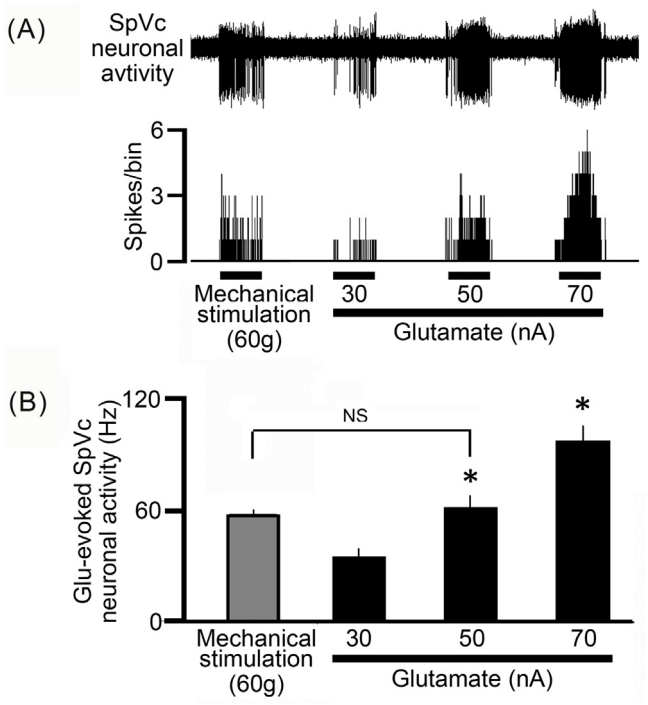

Fig. 6. Glutamate iontophoretic application-evoked SpVc WDR neuronal activity responding to noxious mechanical stimulation. (A) Typical examples of glutamate iontophoretic application (30, 50, and 70 nA, 5 s) on evoked neuronal discharges of SpVc WDR neurons responding to noxious mechanical stimulation (60 g). (B) Summary of glutamate iontophoretic application on evoked mean neuronal discharges of SpVc WDR neurons responding to mechanical stimulation of the orofacial area. *, $P < 0.05$, n = 5, 30 nA vs. 50 nA, 70 nA.

ulations (60 g) and iontophoretic application of glutamate (50 nA; Fig. 6B). We also observed that iontophoretic application of vehicle (160 mM, NaCl pH 4.5) had no significant effect on the SpVc WDR spinal neuronal activity, as previous described (n = 3, data not shown; Takeda et al., 2006).

3.6. Suppression of iontophoretic application of glutamate-evoked neuronal discharge frequency after intravenous theanine administration

Fig. 7 shows the effect of intravenously administered theanine on the iontophoretic application of glutamate-evoked SpVC WDR neuronal activity responding to noxious stimulation. Glutamate-evoked discharges of SpVc WDR were inhibited in a reversible manner (Fig. 7A), and the mean firing frequency of these neurons responding to iontophoretic application of glutamate was significantly inhibited by intravenous theanine (100 mM), with the maximal inhibition of discharge frequency revealed within 5 min (30 nA, 20.3 ± 1.8 vs. 31.4 ± 3.5 Hz, 50 nA, 35.0 ± 4.2 vs. 57.8 ± 5.4 Hz, 70 nA, 59.0 ± 2.9 vs. 97.8 ± 7.9 Hz, n = 5, $P < 0.05$, Fig. 7B). These inhibitory effects were reversed within approximately 10 min.

4. Discussion

This study provides the first evidence that acute intravenous theanine administration suppresses glutaminergic excitatory synaptic transmission in the SpVc. These findings suggest that dietary constituent, theanine, could have potential as a CAM therapeutic agent for trigeminal nociceptive pain.

4.1. Intravenous acute administration of theanine suppresses excitability of SpVc WDR neurons

Our main study findings are as follows: (i) mean SpVc WDR neuronal firing rate was dose-dependently inhibited by theanine (1–100 mM, i.v.) in response to both non-noxious and noxious mechanical stimuli; (ii) this discharge inhibition was reversible within approximately 10 min; (iii) intravenous administration of vehicle had no significant effect on either the spontaneous or evoked (non-noxious, noxious mechanical, and pinch stimulation) activity of SpVc WDR neurons. Theanine is known to pass through the blood-brain barrier via a leucine-preferring transport system (Yokogoshi et al., 1998). Following the systemic administration of 100 mM theanine in the present study, the drug was detected in the

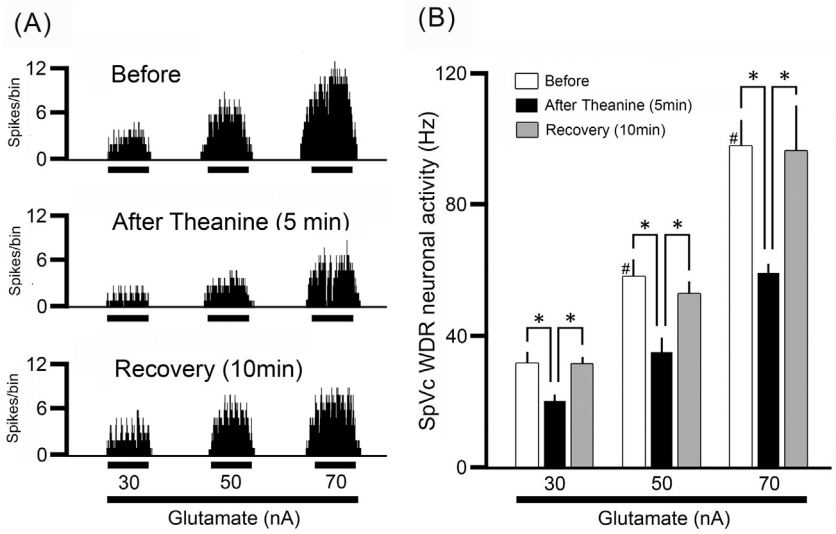

Fig. 7. Effect of intravenous administration of theanine on the glutamate iontophoretic application-evoked SpVc WDR neuronal discharge frequency. (A) Typical examples of glutamate iontophoretic application (30, 50 and 70 nA, 5 s) on evoked discharges of SpVc WDR neurons responding to mechanical stimulation of the orofacial area before and at 5 and 10 min after theanine intravenous administration of 100 mM theanine. (B) Summary of glutamate iontophoretic application on evoked discharges of SpVc WDR neurons responding to mechanical stimulation of the orofacial area before and at 5 and 10 min after theanine intravenous administration. *, $P < 0.05$, n = 5, before vs. after 100 mM, i.v. theanine administration. #, $P < 0.05$, n = 5, 30 nA vs 50 nA, 70 nA.

bloodstream to a calculated concentration of approximately 1 mM, and this concentration still had a significant effect on the nociceptive transmission of SpVc firing. In crayfish, 1 mM theanine also significantly inhibited glutaminergic excitation at the neuromuscular junction (Shinozaki and Ishida, 1978). Together, these findings suggest that under in vivo conditions, acute intravenous theanine administration suppresses trigeminal nociceptive transmission in the SpVc.

4.2. Mechanism underlying the suppression of nociceptive glutaminergic synaptic transmission by theanine

Several previous findings indicate that theanine can modulate excitatory and inhibitory neuronal transmission in the central nervous system (Kimura and Murata, 1971; Egashira et al., 2007; Kakuda et al., 2002; Maruyama and Takeda, 1994; Shinozaki and Ishida, 1978), including elevation of intracerebral GABA levels within 30 min of intraperitoneal theanine administration that suggested transport into the brain through the blood-brain barrier (Kimura and Murata, 1971; Egashira et al., 2007). In this study we found that theanine-induced inhibition of the discharge frequency of SpVc WDR neurons in response to both non-noxious and noxious mechanical stimuli was reversible and occurred within approximately 10 min. Considering the different time course of theanine effectiveness between previous studies and the experimental findings presented herein, it is reasonable to speculate that the inhibitory effect of nociceptive and non-nociceptive transmission evoked by acute theanine administration may not happen via a GABAergic mechanism. Our finding of no significant change in receptive field size before and after theanine administration supports such a speculation, particularly in light of a previous study indicating that a local GABAergic mechanism in the SpVc modulates the mechanical receptive field properties (Takeda et al., 2000). Clearly, further studies are needed to identify the precise inhibitory mechanism at play with nociceptive transmission in the presence of theanine.

Interestingly, theanine binds to glutamate receptors, AMPA, kainite, and NMDA receptors (Kakuda et al., 2002; Maruyama and Takeda, 1994), and a previous study demonstrated that iontophoretically applied glutamate induced the amplitude of excitatory junctional potentials was reversible inhibited by theanine, implicating theanine as a glutamate antagonist (Shinozaki and Ishida, 1978). Di et al. (2010) also reported that theanine attenuates L-glutamate-induced apoptosis via NMDA receptor-related signaling pathways. In the present study, we found that (i) the relative magnitude of inhibition by theanine of SpVc WDR neuronal discharge frequency was significantly greater for noxious than non-noxious stimulation; (ii) the mean firing frequency of SpVc WDR neurons responding to iontophoretic application of glutamate was inhibited by intravenous administration of theanine; (iii) there was no significant difference in mean discharge frequency between noxious mechanical stimulation (60 g) and iontophoretic application of glutamate (50 nA); and, (iv) the mean firing frequency of SpVc WDR neurons responding to iontophoretic application of 50-nA glutamate was significantly inhibited by intravenous administration of theanine, with the maximal inhibition of discharge frequency observed by 5 min and reversed within 10 min. Taken together, our findings suggest that acute intravenous theanine administration suppresses the glutaminergic excitatory post synaptic transmission, possibly via an NMDA receptor signaling pathways in the SpVc convergence inputs from noxious rather than non-nonxious sensory information. However, further studies are needed to elucidate the mechanistic basis of the speculations.

4.3. Functional significance of suppressing nociceptive glutaminergic synaptic transmission by theanine

The SpVc nucleus is an important relay station for trigeminal nociception, including for the orofacial region. Thus, because SpVc WDR neurons contribute to hyperalgesia and/or referred pain associated with dental pain (Takeda et al., 2000, 2005, 2012; Nishikawa et al., 2004), only the effect of theanine on SpVc WDR neuronal activity was evaluated in the present study and nociceptive-specific neurons were not tested. Following injury and inflammation of the neural tissue innervating the orofacial area, changes in the properties of these neurons lead to pathological pain, such as hyperalgesia and allodynia (Iwata et al., 1999; Takeda et al., 2012). In addition, we reported previously that temporomandibular joint inflammation-induced hyperexcitability of SpVc WDR neurons contributes to the ectopic mechanical allodynia innervating facial skin (Takeda et al., 2005, 2012). Previous our study showed that there is a convergence of sensory inputs from the superior sagittal sinus (SSS) and tooth pulp afferent on the first cervical dorsal horn (C1) neurons, and NMDA and non-NMDA receptors located in the C1 neurons contributes to the mechanism of trigeminal referred pain associated with migraine (Fujimi et al., 2006). Storer and Goadsby (1999) has been demonstrated that blockade of glutamatergic transmission via NMDA and non-NMDA receptors results in significant inhibition of excitatory neurotransmission of SpVc. Since trigeminovascular nociceptive fibers are activated by the functionally specific stimulation of the SSS, it is likely that glutamatergic transmission has trigeminal transmission in acute primary headache syndromes, such as migraine and cluster headache (Storer and Goadsby, 1999). Chan and MaassenVanDenBrink (2014) indicated that glutamate receptor antagonists play an important role in the management to migraine. Actually, it has been known that kynurenic acid is an endogenous glutamate receptor blocker, a potent NMDA receptor antagonist (Birch et al., 1988). Csáti et al. (2015) recently reported that administration of kynurenic acid resulted in the inhibition of inflammation-induced activation of neuronal signaling system in the trigeminal ganglia. Thus, our present study suggests that acute theanine administration could indeed attenuate the excitability of SpVc WDR neurons associated with the trigeminal area and tissue injury/inflammation, including migraine and cluster headache.

It is also known that patients frequently turn to CAM therapies such as herbal medicines and acupuncture for pain control when other medical treatments are ineffective (Konvicka et al., 2008; Rosenberg et al., 2008). Consequently, the potential influence of diet and dietary supplementation on conditions associated with pain have been the focus of considerable research (Shir et al., 2001; Rivat et al., 2008), and increasing CAM agents are being developed for the treatment of persistent chronic pain (Rosenberg et al., 2008; Kessler et al., 2001). The safety of theanine is important when considering its potential clinical applications. To this end, theanine administered in a dietary mixture to rats for 13 weeks produced no adverse effects even at a dose of 4000 mg/Kg/day (Borzelleca et al., 2006). In addition, Tarumizu et al. (2015) reported recently that a compound health supplement containing theanine provided some relief for patients with low back pain, suggesting that theanine could be a safe alternative or complement to non-steroidal anti-inflammatory drugs therapy for conditions such a lower back pain. Because incisions associated with surgery cause acute pain and surgery has been identified as a potential major cause of chronic pain (Perkins and Kehlet, 2000; Kehlet et al., 2006), it can be speculated that theanine may effectively reduce clinical pain, such as postoperative pain (Locher-Claus et al., 2005; Tillu et al., 2012). Taken together, the results of the preset study support the idea that dietary constituents theanine could be a part of an effective CAM

strategy for alleviating nociceptive pain and preventing trigeminal inflammatory hyperalgesia.

References

Birch, P.J., Grossman, C.J., Hayes, A.G., 1988. Kynurenic acid antagonises responses to NMDA via an action at the strychnine-sensitive glycine receptor. Eur. J. Pharmacol. 154, 85–87.

Borzelleca, J.F., Peters, D., Hall, W., 2006. A 13 week dietary toxicity and toxicokinetic study with Lĥeanine in rats. Food Chem. Toxicol. 44, 1158–1166.

Chan, K., MaassenVanDenBrink, A., 2014. Glutamate receptor antagonists in the management of migraine. Drugs 74, 1165–1176.

Csáti, A., Edvinsson, L., Vécsei, L., Toldi, J., Fülöp, F., Tajti, J., Warfvinge, K., 2015. Kynurenic acid modulates experimentally induced inflammation in the trigeminal ganglion. J. Head. Pain 16, 99.

Di, X., Yan, J., Zhao, Y., Zhang, J., Shi, Z., Chang, Y., Zhao, B., 2010. L-theanine protects the APP (Swedish mutation) transgenic SH-SY%Y cell against glutamate-induced Excitatotoxicity via inhibition of NMDA receptor pathway. Neuroscience 168, 778–786.

Egashira, N., Hayakawa, K., Osajima, M., Mishima, K., Iwasaki, K., Oishii, R., Fujiwara, M., 2007. Involvement of $GABA_A$ receptors in the neuroprotective effect of theanine on focal cerebral ischemia in mice. J. Pharmacol. Sci. 105, 2111–2214.

Ernest, E., 2003. Complementary medicine. Curr. Opin. Rheumatol. 15, 151–155.

Fujimi, Y., Takeda, M., Tanimoto, T., Matsumoto, S., 2006. N-methyl-aspartate (NMDA) and non-NMDA receptor antagonists suppress the superior sagittal sinus-evoked activity of C1 spinal neurons responding to tooth pulp electrical stimulation in rats. Odontology 94, 22–28.

Iwata, K., Tashiro, A., Tsuboi, Y., Imai, T., Sumino, R., Morimoto, T., Dubner, R., Ren, K., 1999. Medullary dorsal horn neuronal activity in rats with persistent temporomandibular joint and perioral inflammation. J. Neurophysiol. 82, 1244–1253.

Kakuda, T., Nozawa, A., Sugimoto, A., Nishino, H., 2002. Inhibition by theanine of binding of [3H]AMPA, [3H]Kainate and [3H]MDL105,519 to glutamate receptors. Biosci. Biotechnol. Biochem. 66, 2683–2686.

Kehlet, H., Jensen, T.S., Woolf, C.J., 2006. Persistent postoperative pain: risk factors and prevention. Lancet 367, 1618–12625.

Kessler, R.C., Davis, R.B., Foster, D.F., Van Rompay, M.I., Walters, E.E., Wilkey, S.A., Kaptchuk, T.J., Eisenberg, D.M., 2001. Long-term trends in the use of complementary and alternative medical therapies in the United States. Ann. Intern. Med. 135, 262–268.

Kimura, R., Murata, T., 1971. Influence of alkylamides of glutamic acid and related compounds on the central nervous system I. Central depressant effect of theanine. Chem. Pharm. Bull. 19, 1257–1261.

Konvicka, J.J., Meyer, T.A., McDavid, A.J., Roberson, C.R., 2008. Complementary/alternative medicine use among chronic pain clinic patients. J. Perianesth. Nurs. 23, 17–23.

Locher-Claus, M.T., Erickson, T.E., Law, A.S., Johnson, W.T., Gebaharts, G.F., 2005. Effect of pre-emptive morphine, ibuprofen of local anesthetic on fos-expression in the spinal trigeminal nucleus following tooth pulp exposure in rat. J. Endod. 31, 578–583.

Maruyama, M., Takeda, K., 1994. Electrophysiologically potent non-competitive glutamate antagonists at crayfish neuromuscular junctions are also potent inhibitors of [3H]MK801 binding to synaptic membranes from rat central nervous system. Comp. Biochem. Physiol. 107C, 105–110.

Millan, M.J., 1999. The induction of pain: an integrative review. Prog. Neurobiol. 57, 1–164.

Nathan, P.J., Lu, K., Gray, M., Oliver, C., 2006. The neuropharmacology of L-theanine (N-Ethyl-L-Glutamine): a possible neuroprotective and cognitive enhancing agents. J. Herb. Pharmacother. 6, 21–30.

Ness, T.J., Randich, A., 2000. Intravenous lidocaine inhibits nociceptive reflex and spinal neurons in the rat. Anesthesiology 92, 1685–1691.

Nishikawa, T., Takeda, M., Tanimoto, T., Matsumoto, S., 2004. Convergence of nociceptive information from temporomandibular joint and tooth-pulp afferents on C1 neurons in the rat. Life Sci. 75, 1465–1478.

Paxinos, G., Watson, C., 1986. The Rat Brain in Stereotaxic Coordinates, 2nd ed. Academic Press, New York.

Perkins, F.M., Kehlet, H., 2000. Chronic pain as an outcome of surgery. A review of predictive factors. Anesthesiology 93, 1123–1133.

Rao, J.K., Mihaliak, K., Kroenke, K., Bradley, J., Tierney, W.M., Weinberger, M., 1999. Use of complementary therapies for arthritis among patients of rheumatologists. Ann. Intern. Med. 131, 409–416.

Rivat, C., Richebé, P., Laboureyras, E., Laulin, J.P., Havouis, R., Noble, F., Moulinoux, J.P., Simonnet, G., 2008. Polyamine deficient diet to relieve pain hypersensitivity. Pain 137, 125–137.

Rosenberg, E.I., Genao, I., Chen, I., Mechaber, A.J., Wood, J.A., Faselius, C.J., 2008. Complementary and alternative medicine use by primary care patients with chronic pain. Pain Med. 9, 1065–1072.

Scholz, J., Woolf, C.J., 2002. Can we conquer pain? Nat. Neurosci. 5, 1062–1067.

Sekiguchi, K., Takehana, S., Shibuya, E., Matsuzawa, N., Hidaka, S., Kanai, Y., Inoue, M., Kubota, Y., Shimazu, Y., Takeda, M., 2016. Resveratrol attenuates inflammation-induced hyperexcitability of trigeminal spinal nucleus caudalis neurons associated with hyperalgesia in rats. Mol. Pain 12 (April (11)).

Sessle, B.J., 2000. Acute and chronic craniofacial pain: brainstem mechanisms of nociceptive transmission and neuroplasticity and their clinical correlates. Crit. Rev. Oral. Biol. Med. 11, 57–91.

Shimazu, Y., Shibuya, E., Takehana, S., Sekiguchi, K., Oshima, K., Kamata, H., Karibe, H., Takeda, M., 2016. Local administration of resveratrol inhibits excitability of nociceptive wide-dynamic range neurons in rat trigeminal spinal nucleus caudalis. Brain Res. Bull. 124, 262–268.

Shinozaki, H., Ishida, M., 1978. Theanine as a glutamate antagonist at a crayfish neuromuscular junction. Brain Res. 151, 215–219.

Shir, Y., Raja, S.N., Weissman, C.S., Campbell, J.N., Seltzer, Z., 2001. Consumption of soy diet before nerve injury preempts the development of neuropathic pain in rats. Anesthesiology 95, 1238–1244.

Storer, R.J., Goadsby, P.J., 1999. Trgeminovascular nociceptive transmission involves N-methyl-D-aspartate and non-N-methyl-D-aspartate glutamate receptors. Neuroscience 90, 1371–1376.

Takeda, M., Tanimoto, T., Matsumoto, S., 2000. Change in mechanical receptive field properties induced by $GABA_A$ receptor activation in the trigeminal spinal nucleus caudalis neurons in rats. Exp. Brain Res. 134, 409–416.

Takeda, M., Tanimoto, T., Ito, M., Nasu, M., Matsumoto, S., 2005. Role of capsaicin-sensitive afferent inputs from the masseter muscle in the C1 spinal neurons responding to tooth-pulp stimulation in rats. Exp. Brain Res. 16, 107–117.

Takeda, M., Tanimoto, T., Takahashi, M., Kadoi, J., Nasu, M., Matsumoto, S., 2006. Activation of α-adrenoreceptors suppresses the excitability of C1 spinal neurons having convergent inputs from tooth pulp and superior sagittal sinus in rats. Exp. Brain Res. 174, 210–220.

Takeda, M., Takahashi, M., Mastumoto, S., 2012. Suppression of neurokinin-1 receptor in trigeminal ganglia attenuates central sensitization following inflammation. J. Peripher. Nerv. Syst. 17, 169–181.

Takehana, S., Sekiguchi, K., Inoue, M., Ito, Y., Kubota, Y., Yui, K., Shimazu, Y., Takeda, M., 2016. Systemic administration of resveratrol suppress the nociceptive neuronal activity of trigeminal spinal nucleus caudalis in rats. Brain Res. Bull. 120, 117–122.

Tall, J.M., Raja, S.N., 2004. Dietary constituents as novel therapeutics for pain. Clin. J. Pain 20, 19–26.

Tanimoto, T., Takeda, M., Nishikawa, T., Matsumoto, S., 2004. The role of $5\text{-}HT_3$ receptors in the vagal afferent activation-induced of C1 spinal neurons projected from tooth-pulp in the rat. J. Pharmacol. Exp. Ther. 311, 803–810.

Tarumizu, C., Ohno, T., Handa, S., Matsuoka, S., Orimo, H., 2015. Effectiveness of a compound supplement containing piperines, theanine, creatine, α-lipoic acid, and proteoglycan for low back pain: a double-blind placebo-controlled parallel comparison study. J. Pain Relief 4, 4.

Tillu, D.V., Melemedjian, O.K., Asiedu, M.N., Qu, N., Feline, M.D., Dussor, G., Price, T.J., 2012. Resveratrol engages AMPK to attenuates ERK and mTOR signaling in sensory neurons and inhibits incision-induced acute and chronic pain. Mol. Pain 8, 5.

Weiner, L.M., Dhodapkar, M.V., Ferrone, S., 2009. Monoclonal antibodies for cancer immunotherapy. Lancet 21, 1033–1040.

Yokogoshi, H., Kobayashi, M., Mochizuki, M., Terashima, T., 1998. Effect of theanine, γ-glutamylethylamine, on brain monoamine and striatal dopamine release in conscious rats. Neurochem. Res. 23, 667–673.

Zimmermann, M., 1983. Ethical guidelines for investigations of experimental pain in conscious animals. Pain 16, 109–110.