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Systemic administration of the dietary constituent resveratrol inhibits the nociceptive jaw-opening reflex in rats *via* the endogenous opioid system

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ABSTRACT

The aim of the present study was to investigate whether, under *in vivo* conditions, systemic administration of resveratrol could attenuate the rat nociceptive jaw-opening reflex (JOR) *via* the endogenous opioid system. The JOR evoked by electrical stimulation of the tongue was recorded as digastric muscle electromyograms (dEMG) in pentobarbital-anesthetized rats. The amplitude of the dEMG increased significantly in proportion to the intensity of electrical stimulation (from 1× to 5 × threshold for the JOR). dEMG amplitude in response to 3× threshold electrical stimulation of the tongue was dose-dependently inhibited by intravenous administration of resveratrol (0.5–2 mg/kg). Maximum inhibition of dEMG amplitude was seen within approximately 10 min. These inhibitory effects were reversible, with dEMG responses returning to control levels after approximately 20 min. Pretreatment of rats with naloxone resulted in significant, dose-dependent attenuation of the inhibitory effects of resveratrol on dEMG amplitude compared with control. These findings suggest that resveratrol inhibits the nociceptive JOR *via* the endogenous opioid system. Further, the findings of the present study strongly support the idea that resveratrol, which is not known to have any toxic side effects, combined with an opioid could be a potential therapeutic agent for the prevention of acute trigeminal nociception.

1. Introduction

Resveratrol is plant polyphenol found in red wine and various food products (Fremont, 2000; Pervaiz, 2003). Resveratrol has been reported to have several beneficial biological actions, including anti-oxidative, anti-inflammatory, neuroprotective, anticancer, and cardioprotective effects (Leiro et al., 2005; Bermudez-Ocasna et al., 2006; Perez-Severiano et al., 2008; Pervaiz, 2003). Recent reports have described the use of complementary and alternative medicines (CAM), such as herbal medicines and acupuncture, for the treatment of persistent clinical 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). Because resveratrol has no known toxic side effects (Russo, 2007), it could be a candidate CAM for the therapeutic treatment of pain (Takeda et al., 2016). Takehana et al. (2016) reported that acute intravenous administration of resveratrol suppresses nociceptive trigeminal spinal nucleus caudalis (SpVc) wide-dynamic range (WDR) neurons *via* both peripheral and central mechanisms. Although local injection of resveratrol into the peripheral receptive field suppresses the excitability of SpVc neurons, possibly *via* inhibition of Na^+ channels in the nociceptive nerve terminals of trigeminal ganglion neurons (Shimazu et al., 2016), the central mechanisms involved in the inhibitory effects of resveratrol on nociceptive transmission remain to be determined.

Because the jaw-opening reflex (JOR) induced by electrical stimulation of the orofacial tissues, such as, tooth pulp (TP) is a valid index of reflex responses to noxious stimuli (trigeminal nociceptive reflex; Mahan and Anderson, 1970; Mason et al., 1985; Takeda et al., 1998), the JOR threshold is used as an indicator of the intensity of stimulus applied to the TP. It has been demonstrated previously that vagal afferent stimulation attenuates the activity of the trigeminal spinal nucleus oralis (SpVo) and the related JOR *via* the endogenous pain control system (Takeda et al., 1998). Ellrich (2004) reported

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electrical stimulation of tongue- evoked JOR was valid model for nociception. In addition, it has been reported that systemic administration of resveratrol results in dose-dependent antinociceptive effects *via* an opioidergic mechanism (Gupta et al., 2004). In that study, pretreatment of rats with naloxone blocked the analgesic effects of resveratrol. Together, these findings led us to speculate that intravenous injection of resveratrol would inhibit the nociceptive JOR *via* the endogenous pain control system. Thus, the aim of the present study was to investigate whether, under *in vivo* conditions, intravenous administration of resveratrol attenuated the rat nociceptive JOR *via* the endogenous opioid system.

2. Materials 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. Animal preparation

Electrophysiological recordings were made in 22 adult male Wistar rats weighing 230–280 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 administered through a cannula into the jugular vein, as required. The level of anesthesia was confirmed by the absence of the corneal reflex and a lack of response to paw pinching. Rectal temperature was maintained at 37.0 ± 0.5 °C with a homeothermic blanket during recording.

2.2. Recording of digastric muscle electromyograms in response to electrical stimulation of the tongue

Bipolar stimulating electrodes made from stainless steel wire (150 μm diameter, enamel insulated except for 0.5–1.0 mm at the tip) were inserted longitudinally into the tongue, as described previously (Ellrich, 2004). Electrical stimulation with constant current single pulses (0.1–3.8 mA, 0.1 ms, 1 Hz) was delivered through the bipolar stimulating electrodes using PowerLab and Chart 5 software (AD Instruments, Oxford, UK). Using stainless steel electrodes (interpolar distance 2 mm, insulated except for the tip), digastric electromyograms (dEMG) were recorded from the ipsilateral anterior belly of the digastric muscle as an indicator of the JOR. Electromyogram activity was recorded using PowerLab and Chart 5 software (ADInstruments). Recordings of dEMG activity in response to electrical stimulation of the tongue and data analyses were performed as reported previously (Takeda et al., 1998, 2002). To determine the threshold for the JOR from the dEMG, electrical stimulation was applied with a pulse duration of 0.1 ms at a stimulation frequency of 1 Hz, and the pulse intensity was increased until three responses to TP stimulation were obtained from five consecutive trials. The peak-to-peak amplitudes from five stimulus trials were averaged.

2.3. Experimental protocol

The effects of resveratrol (0.5, 1, and 2 mg/kg, i.v.; equivalent to 1, 5, and 10 mM, respectively), injected through a cannula into the jugular vein, were evaluated 5, 10, 20, and 30 min after administration because peak effect and recovery were thought to occur during this period. Resveratrol was dissolved in dimethyl sulfoxide (DMSO). The stock solution was stored at −20 °C in small aliquots until use and diluted in saline to the desired concentrations. Because the nociceptive JOR is considered a valid model of pain if it is evoked by adequate nociceptive stimulation (*e.g.* using a stimulus that is between 3× and 5× the threshold [3T and 5T, respectively] of the JOR, a threshold very close to the sensory threshold in human volunteers; Mason et al., 1985; Sotigiu and Bellinzona, 1991; Takeda et al., 1998, 2002), the mean dEMG amplitude, averaged across five stimulus trials, in response to electrical stimulation (3T) of the tongue was evaluated before and after intravenous administration of resveratrol. In addition, the effects of the opioid antagonist naloxone (0.5–1 mg/kg, i.v.) were evaluated using procedures reported in previous studies, which found that the peak effect of naloxone was observed approximately 10 min after its administration (Gupta et al., 2004; Takeda et al., 1998).

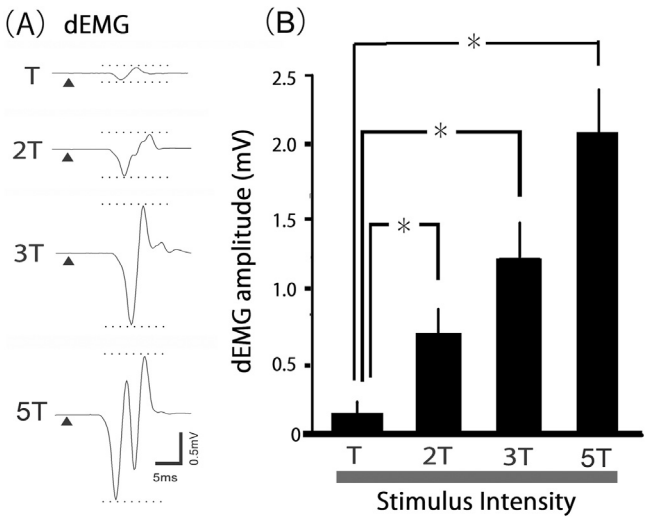

Fig. 1. Changes in digastric muscle electromyograms (dEMG) in response to electrical stimulation of tongue. (A) Changes on the dEMG in response to electrical stimulation of the tongue (1×–5× the threshold for the jaw-opening reflex [JOR]). (B) Summary of changes in the dEMG amplitude following electrical stimulation of the tongue at stimulus intensities between 1× and 5× the JOR threshold. The mean dEMG amplitude increased with increasing stimulus intensity. *$P < 0.05$, n = 9. Filled triangles indicate electrical stimulation of the tongue. T (threshold) *vs.* 2T, 3T and 5T.

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. Changes in dEMG activity in response to electrical stimulation of the tongue

Electrical stimulation of the tongue induced reflex responses of the digastric muscle with a latency of 5.4 ± 0.2 ms (n = 22). The mean threshold intensity was 0.2 ± 0.1 mA (n = 22). As shown in Fig. 1A, the peak-to-peak amplitude on the dEMG increased in proportion with increasing stimulation intensity (from 1T to 5T). These results are summarized in Fig. 1B. There was a significant increase in the mean dEMG amplitude with increasing stimulation intensity (from 1T to 5T; n = 5, F = 39.0, $P < 0.05$).

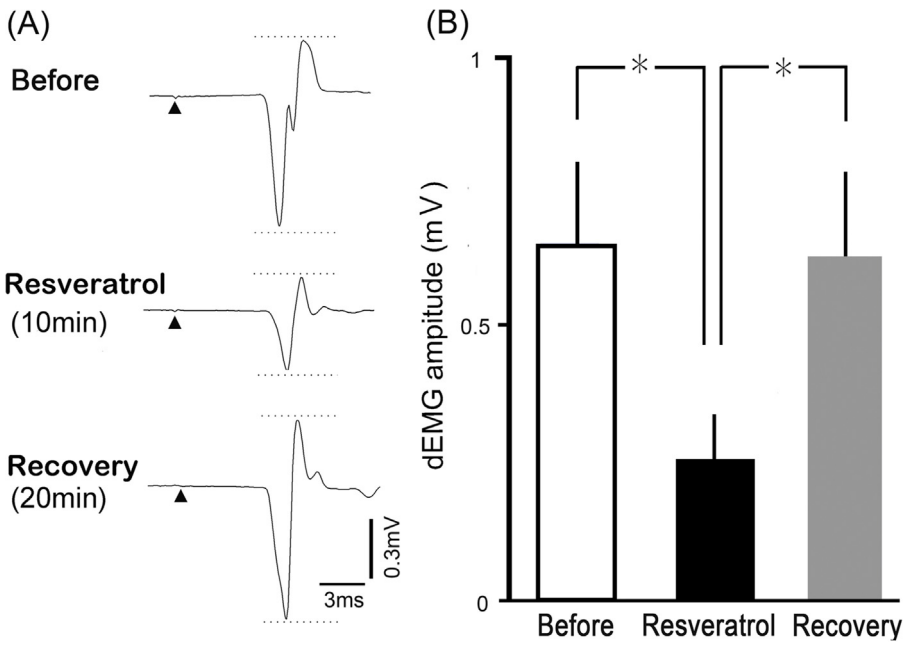

Fig. 2. Effects of intravenous resveratrol on the amplitude of dEMG following electrical stimulation of the tongue. (A) Typical example of dEMG responses to electrical stimulation of the tongue at a stimulus intensity of 3× the threshold for JOR and the effects of intravenous resveratrol (2 mg/kg, i.v.). (B) Summary of the effects of resveratrol on the mean dEMG amplitude following electrical stimulation of the tongue at a stimulus intensity of 3× the threshold for JOR. *$P < 0.05$, n = 5.

3.2. *Effects of i.v. resveratrol on dEMG responses to electrical stimulation of the tongue*

Fig. 2A shows a typical example of the effect of i.v. administration of resveratrol on the dEMG amplitude in response to electrical stimulation of the tongue at a stimulus intensity of 5T. Ten minutes after i.v. injection of resveratrol (2 mg/kg), inhibition of the peak-to-peak amplitude on the dEMG was maximal; thereafter, amplitude returned to control levels within approximately 20 min (Fig. 2A). As shown in Fig. 2B, the mean dEMG amplitude evoked by electrical stimulation of the tongue at a stimulus intensity of 3T was significantly reduced 10 min after resveratrol administration (amplitude 0.7 ± 0.1 *vs.* 0.3 ± 0.1 mV before *vs.* after resveratrol, respectively; $F = 9.4$, $P < 0.05$; n = 5). Again, the inhibitory effects of resveratrol were no longer evident after about 20 min. Resveratrol had a dose-dependent inhibitory effect on dEMG amplitude in response to electrical stimulation of tongue, with significantly greater effects seen with increasing doses of resveratrol ($18.1 \pm 7.2\%$, $28.1 \pm 5.2\%$ and $53.5 \pm 8.2\%$ reductions in dEMG amplitude with 0.5, 1 and 2 mg/kg resveratrol, respectively; n = 5; $F = 48.0$, $P < 0.05$; Fig. 3). Intravenous administration of vehicle had no significant effect on the dEMG amplitude in response to electrical stimulation of the tongue (n = 3; data not shown)

3.3. *Effects of naloxone pretreatment on resveratrol-induced suppression of dEMG activity*

The effects of the opioid antagonist naloxone on resveratrol-induced suppression of dEMG amplitude in response to electrical stimulation of the tongue at a stimulus intensity of 3T was evaluated in five rats. As shown in Fig. 4A, the inhibitory effect of 2 mg/kg, i.v., resveratrol on dEMG amplitude following electrical stimulation of the tongue was significantly attenuated in rats pretreated with 1 mg/kg, i.v., naloxone. The effects of naloxone (0.5 and 1 mg/kg) on resveratrol-induced suppression of dEMG amplitude are summarized in Fig. 4B. Naloxone (0.5 and 1 mg/kg, i.p.) pretreatment significantly attenuated the inhibitory effects of 2 mg/kg, i.v., resveratrol on dEMG amplitude in response to electrical stimulation of the tongue ($53.3 \pm 5.0\%$ reduction in dEMG amplitude with resveratrol alone *vs.* $30.1 \pm 2.5\%$ and $10.1 \pm 2.1\%$ reductions in amplitude after pretreatment with 0.5 and 1 mg/kg naloxone, respectively; n = 5; $F = 64.0$, $P < 0.05$).

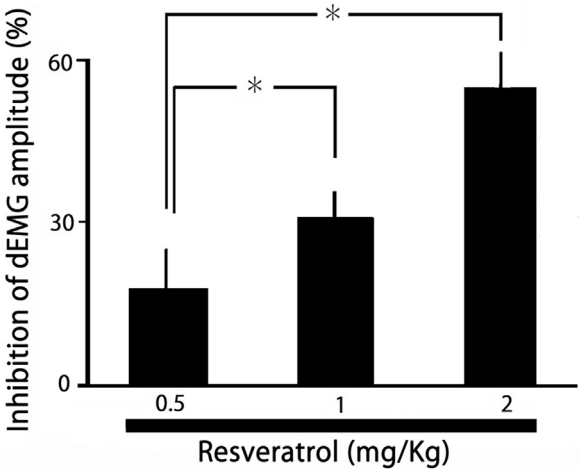

Fig. 3. Dose-dependent suppression by resveratrol (0.5–2 mg/kg) of the mean dEMG amplitude following electrical stimulation of the tongue *$P < 0.05$ for 0.5 *vs.* 1 and 2 mg/kg resveratrol, n = 5.

4. Discussion

The present study provides evidence that acute intravenous administration of resveratrol attenuates the nociceptive JOR *via* the endogenous opioid system. Therefore, the findings of the present study suggest that resveratrol, which is not known to have any toxic side effects, could be used as a CAM in combination with an opioid as a potential therapeutic agent for the prevention of acute trigeminal nociception.

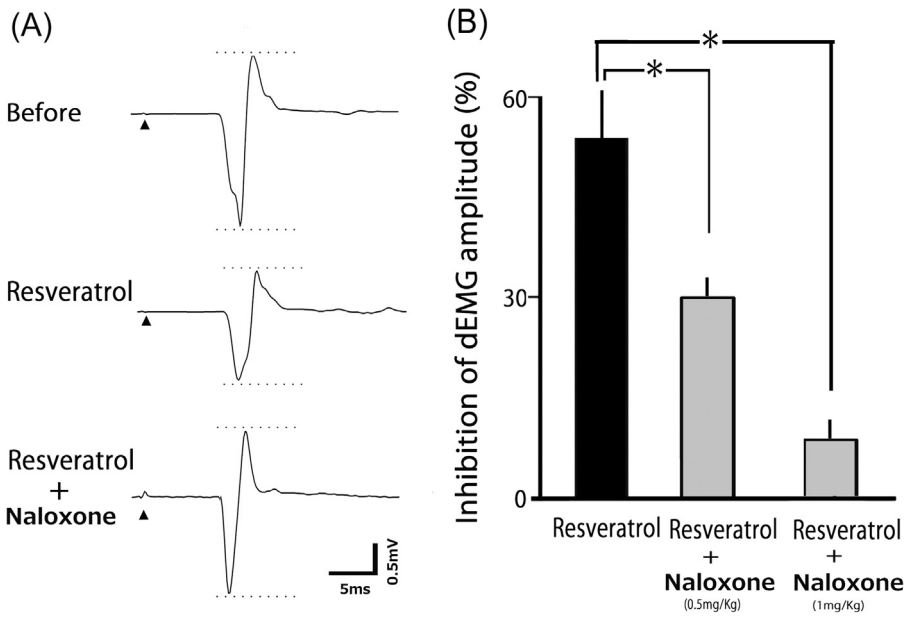

Fig. 4. Pretreatment with the opioid antagonist naloxone attenuated resveratrol-induced inhibition of the dEMG amplitude following electrical stimulation of the tongue. (A) Typical example of the effects of naloxone (1 mg/kg, i.v.) pretreatment on intravenous resveratrol-induced suppression of the dEMG amplitude following electrical stimulation of the tongue (3× the threshold for JOR). (B) Summary of the effects of naloxone on resveratrol-induced inhibition of the dEMG amplitude following electrical stimulation of the tongue. $^*P < 0.05$, resveratrol *vs*. naloxone (0.5 and 1 mg/kg), n = 5.

4.1. Validity of using the nociceptive JOR to assess the effects of resveratrol on the opioid system

Although the JOR is a masticatory reflex (Kidokoro et al., 1991), tooth-pulp evoked JOR is a nociceptive reflex that is suppressed by antinociceptive pathways mediated by the periaqueductal gray (PAG) matter, the nucleus raphe magnus or both, as well as by analgesic drugs (Curtis and Marwar, 1986; Oliveras et al., 1974; Sumino, 1971). The nociceptive JOR has been considered a valid model of pain if it is evoked by adequate nociceptive stimulation (*e.g.* using 3T–5T, a threshold very close to the sensory threshold in human volunteers; Mason et al., 1985; Sotigiu and Bellinzona, 1991; Takeda et al., 1998, 2002). The majority of sensory neurons in the JOR are located in the SpVo, which projects to the trigeminal motor nucleus (Mizuno et al., 1975; Sugimoto and Takemura, 1993). The spinal trigeminal nucleus is an important relay station in the transmission of orofacial sensory information, and this nucleus is functionally subdivided into three nuclei (from rostral to caudal): oralis, interpolaris and caudalis (Sessle, 2000). In addition, a recent study indicated that, in the absence of inflammatory or neuropathic pain, acute intravenous administration of resveratrol suppresses nociceptive SpVc neurons *via* both peripheral and central mechanisms (Takehana et al., 2016). Thus, the nociceptive JOR rat model is a valid model in which to test the effects of resveratrol on trigeminal nociceptive transmission *via* the endogenous opioid system.

4.2. Suppression of nociceptive JOR by resveratrol via *the endogenous opioid system*

The main findings of the present study are as follows: (i) dEMG responses to electrical stimulation of the tongue at a stimulus intensity of 3T were dose-dependently inhibited by systemic administration of resveratrol (0.5–2 mg/kg, i.v.); and (ii) maximum inhibition of dEMG amplitude was seen within approximately 10 min of resveratrol injection, with the inhibitory effects disappearing after approximately 20 min. The time course of inhibition of the nociceptive JOR reflex by resveratrol in the present study is in agreement with the findings of our previous study on the effects of resveratrol on SpVc WDR neuronal activity (Takehana et al., 2016). Therefore, it can be speculated that resveratrol inhibits the glutaminergic excitatory synaptic transmission in the SpVo by inhibiting post-synaptic glutamate receptors and presynaptic Ca^{2+} channels, as well as that of peripheral terminals of the trigeminal nerve by modulating both the generator potential and the initiation of action potentials (Meng et al., 2015; Kim et al., 2005; Shimazu et al., 2016).

Using the hot plate test, Gupta et al. (2004) reported that intraperitoneal administration of resveratrol results in dose-dependent antinociceptive effects *via* an opioidergic mechanism. In the present study, we demonstrated that pretreatment of rats with the opioid antagonist naloxone (0.5–1 mg/kg, i.p.) dose-dependently attenuated the suppressive effect of resveratrol on dEMG amplitude, suggesting that systemic administration of resveratrol attenuates the nociceptive JOR *via* the endogenous opioid system.

As summarized in Fig. 5, we hypothesized a possible mechanism of systemic administration of resveratrol could inhibits nociceptive transmission *via* endogenous opioid system as follows: (i) in slice preparations, enkephalin has been shown to inhibit nociceptive transmission from neurons in the superficial layer of the spinal dorsal horn by inhibiting glutamate release (Hori et al., 1992). Previously, Takeda et al. (2004) reported that activation of μ-opioid receptors inhibits the excitability of rat nociceptive small-diameter trigeminal ganglion neurons projecting to the superficial layer of the upper cervical dorsal horn and that this inhibition is mediated by potentiation of voltage-gated K^+ currents (Takeda et al., 2004). They have also found that reverse transcription-polymerase chain reaction analysis demonstrated expression of mRNA for μ-opioid receptor in the trigeminal ganglia and the μ-opioid receptor immunoreactivity was expressed in the small-diameter (nociceptive) trigeminal ganglion neuron (Takeda et al., 2004). Together, these results suggest that resveratrol activates μ-opioid receptors in presynaptic terminals of nociceptive trigeminal primary afferent

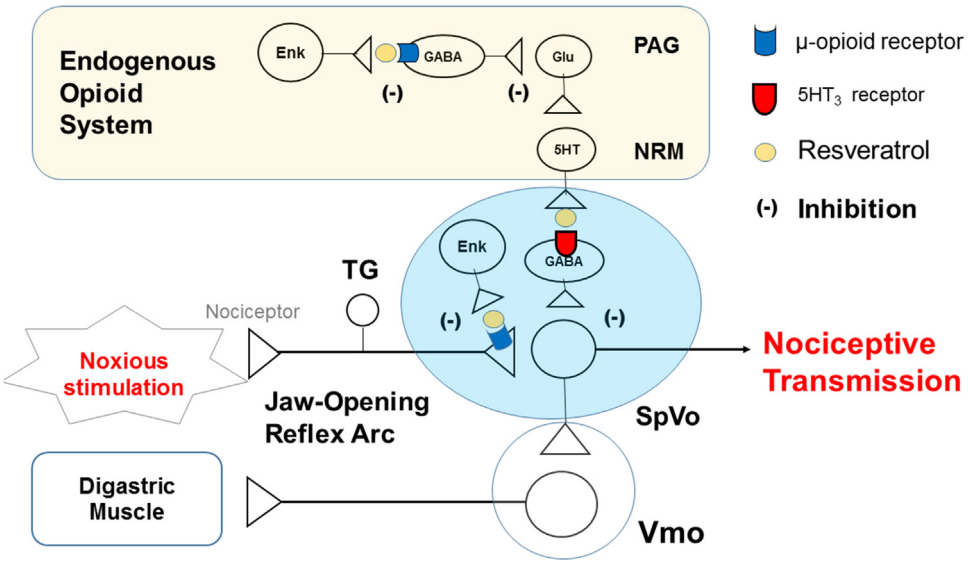

Fig. 5. Hypothesis for a possible mechanism of systemic administration of resveratrol inhibits nociceptive transmission *via* endogenous opioid system. Systemic administration of resveratrol could inhibit nociceptive transmission *via* endogenous opioid system as follows: (i) resveratrol activates μ-opioid receptors in presynaptic terminals of nociceptive trigeminal primary afferent neurons and potentiates voltage-gated K^+ currents. These actions inhibit the excitability of neuronal transmission in the SpVo by depressing presynaptic glutamate release. (ii) Activation of μ-opioid receptors by resveratrol increases neuronal activity in the PAG *via* GABAergic disinhibition, which can then activate serotonergic neurons in the nucleus raphe magnus (endogenous opioid system). These actions inhibit synaptic transmission of SpVo. (iii) resveratrol suppresses excitatory synaptic transmission of the SpVc *via* activation of 5-HT_3 receptor-mediated GABAergic inhibition. TG, trigeminal ganglion; SpVo, trigeminal spinal nucleus oralis; Vmo, trigeminal motor nucleus; PAG, periaqueductal grey; NRM, nucleus raphe magnus; Glu, Glutamate; Enk, Enkepahalin; GABA, ganma-aminobutyric acid; 5HT, 5-hydroxytrypytamine.

neurons and potentiates voltage-gated K^+ currents. These actions could inhibit the excitability of neuronal transmission in the SpVo by depressing presynaptic glutamate release (Fig. 5). (ii) it has been reported that opiates acting *via* μ-opioid receptors partially inhibit evoked inhibitory GABAergic pre- and postsynaptic potentials in the PAG (Chieng and Christie, 1994). Since previous studies indicate that activation of μ-opioid receptors increases neuronal activity in the PAG *via* GABAergic disinhibition, which can then activate serotonergic (5-hydroxytrypytamine [5-HT]) neurons in the nucleus raphe magnus (the PAG–nucleus raphe magnus–trigeminal pathway; Gebahrts and Randich, 1990; Takeda et al., 2002), it can be assumed that activation of μ-opioid receptors by resveratrol increases neuronal activity in the PAG *via* GABAergic disinhibition, which can then activate serotonergic neurons in the nucleus raphe magnus (endogenous opioid system). These actions inhibit the synaptic transmission of The SpVo (Fig. 5). (iii) it has also been known that resveratrol facilitates 5-HT_3 receptor-mediated ion currents (Lee et al., 2011). Previous studies have reported that nociceptive stimulation-evoked SpVo/C1 neuron activity is suppressed by conditioning peripheral nerve stimulation *via* 5-HT_3 receptor-mediated GABAergic inhibition (Tanimoto et al., 2004; Oshima et al., 2005). Collectively, these observations suggest that resveratrol suppresses excitatory synaptic transmission of the SpVo *via* activation of 5-HT_3 receptor-mediated GABAergic inhibition (Fig. 5). However, further studies are needed to confirm this possibility.

4.3. *Functional significance of resveratrol suppression of nociceptive JOR* via *the endogenous opioid system*

CAM, including herbal medicines and acupuncture, have been used to treat persistent clinical chronic pain (Rao et al., 1999; Konvicka et al., 2008; Rosenberg et al., 2008). Recent studies have reported on the potential effects of diet and dietary supplementation on conditions associated with pain (Shir et al., 2001; Ernest, 2003; Tall and Raja, 2004). Interestingly, resveratrol has no known toxic side effects (Russo, 2007), so it is possible that resveratrol may be a candidate CAM, specifically a therapeutic analgesic agent (Takeda et al., 2016). Recent studies provide evidence that: (i) local resveratrol injection into the peripheral receptive field suppresses SpVc neuron excitability, possibly by inhibiting generator and action potentials in the nociceptive nerve terminals of trigeminal ganglion neurons (Shimazu et al., 2016); (ii) trigeminal sensory transmission, including nociception, is suppressed by acute intravenous resveratrol (Takehana et al., 2016); and (iii) chronic administration of resveratrol attenuates inflammation-induced mechanical hyperalgesia, and this effect is due primarily to the suppression of hyperexcitability of SpVc WDR neurons *via* inhibition of peripheral and central cyclo-oxygenase cascade signaling pathways (Sekiguchi et al., 2016). These findings support the idea that resveratrol may be a potential therapeutic CAM for the alleviation of nociceptive pain and prevention of trigeminal inflammatory hyperalgesia.

Since surgical incisors cause acute pain, and surgery is a potential cause of chronic pain (Perkins and Kehlet, 2000; Kehlet et al., 2006), it is possible that resveratrol could effectively reduce clinical pain, including postoperative pain (Locher-Claus et al., 2005; Tillu et al., 2012). It has been recently reported that resveratrol induced apotosis in numerous human cancer cells (Zulueta et al., 2015; Feng et al., 2016; Bai et al., 2016). For example, Bai et al. (2016) demonstrated that resveratrol may be considered a novel agent for the treatment of bladder cancer *via* the inhibition of migration and invasion. Clinically, morphine has been well known used for alleviation for cancerous pain. However, the major side effects of morphine are respiratory depression, sedation and the development of addiction. In the present study, pretreatment of rats with the opioid antagonist naloxone attenuated the nociceptive JOR *via* the endogenous opioid system. Together, the findings of the present study support the idea that the combination of resveratrol, which has no known toxic side effects, with an opioid could be a potential therapeutic strategy for the alle *via*tion of palliative trigeminal nociception, including clinical pain (*e.g.* postoperative and/or cancerous pain).

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