Dual effects of eugenol on the neuronal excitability: an *in vitro* study

Jafar Vatanparast^{a*}, Samira Khalili^a, Maryam Naseh^{a,b}

^a Department of Biology, College of Science, Shiraz University, Shiraz, Iran

^b Department of Physiology, Shiraz University of Medical Sciences, Shiraz, Iran

Abstract

Besides its well-known actions on sensory afferents, eugenol also affects general excitability of the nervous system, but the mechanisms involved in the recent effect, especially through modulation of ion channels, have received much less attention. In this study, we studied the effects of eugenol on the excitability of central neurons of land snail *Caucasotachea atrolabiata* and tried to elucidate the underlying ionic mechanisms. The lower concentration of eugenol (0.5 mM) reversibly reduced the frequency of spontaneous action potentials that was associated with elevation of threshold, reduction of maximum slope of rising phase and prolongation of action potentials. These effects were mimicked by riluzole, suggesting that they might be mediated by inhibition of Na⁺ channels. Eugenol also prolonged the single-spike afterhyperpolarization and post stimulus inhibitory period, but these effects seemed to be consequent to action potential prolongation that indirectly augment Ca²⁺ inward currents and Ca²⁺-activated K⁺ currents. This concentration of eugenol was also able to prevent or abolish pentylenetetrazole-induced epileptiform activity. On the other hand, a higher concentration of eugenol (2 mM) reversibly increased the frequency of action potentials and then induced epileptiform activity in majority of treated neurons. Several criteria suggest that the inhibition of K⁺ channels by higher concentration of eugenol and indirect augmentation of Ca²⁺ currents are central to the hyperexcitability and epileptiform activity induced by eugenol. Our findings indicate that while low concentration of eugenol could have antiepileptic properties, at higher concentration it induces epileptiform activity. It seems that dose dependent inhibition of the ionic currents underlying rising and falling phases of action potential are relevant to the eugenol suppressant and excitatory actions, respectively.

Key words: Eugenol; Neuronal excitability; Epileptiform activity; Riluzole; Sodium channels; Potassium channels

1. Introduction

Eugenol, the major component of clove essential oil, is a phenylpropanoid with analgesic and anti-inflammatory effects and widely used as a toothache remedy. The analgesic action of eugenol has been mainly attributed to its modulatory actions on different ion channels that mediate nociceptive signaling in sensory neurons. Eugenol can primarily elicit irritation by activating TRPV1 channels, but it induces a long-lasting desensitization of these channels after chronic exposure that provides a basis for its analgesic action (Ohkubo and Shibata, 1997). In different neurons including dental primary afferents, it has been shown that eugenol inhibits action potentials and Na^+ and Ca^{2+} currents in a TRPV1-independent manner (Cho et al., 2008; Huang et al., 2012). Although most of the researches have focused on the mechanisms that underlie the effects of eugenol on the sensory transmission in the peripheral nervous system, a few works have sought its actions on the central nervous system. Eugenol easily penetrates into the central nervous system to alleviate neuropathic pain (Lionnet et al., 2010). It inhibits excitatory synaptic transmission in neocortical and hippocampal neurons (Ardjmand et al., 2006; Muller et al., 2006). Eugenol has shown antiepileptic action that has been mainly attributed to its inhibitory action on Na^+ current, although its actions on synaptic transmission has also been suggested as a contributor to its antiepileptic activity (Dallmeier and Carlini, 1981; Huang et al., 2012; Muller et al., 2006). Huang and colleagues (2012) reported that eugenol suppresses neuronal excitability and reduces the severity of pilocarpine-induced seizures. They found that eugenol inhibits both inactivating and non-inactivating Na^+ currents and suggested this effect as responsible for the suppressive and antiepileptic action of eugenol. On the other hand, clove oil overdose has been shown to be associated with development of generalized seizure (Hartnoll et al., 1993).

The modulatory action of eugenol on different ion channels of the neuronal membrane can affect different functions of the brain and its general excitability. The wide range application of eugenol containing products and its availability over-the-counter emphasize the necessity of more studies to clarify the effects of eugenol on neuronal excitability and the underlying mechanisms. In addition to the technical advantages offered by snail neurons for

electrophysiological studies, they have a rich repertoire of ion channels in their soma membranes that make them suitable model system to study neuronal excitability. They have the essential substrates to develop and sustain epileptiform activity that in many aspects resemble those that happen in the vertebrate neurons (Altrup et al., 2003). The structure and the function of ion channels show essential conservation and this is the basis for the assumption that the principal mechanisms underlying the epileptiform activity are constant in whatever nervous systems it appears (Altrup et al., 2003; Plummer and Meisler, 1999). In the current work we took advantage of central neurons of the land snail *Caucasotachea atrolabiata* (Krynicky, 1833) to study the effects of eugenol on the neuronal excitability and the underlying mechanisms.

2. Materials and methods

2.1. Animals and preparations

Experiments were performed on neurons in the subesophageal ganglia of land snail *Caucasotachea atrolabiata*. Animals were treated in accordance with the European Community guidelines and the experimental protocols were reviewed and approved by the animal care committee of the Shiraz University. The ganglionic mass with its main peripheral nerves and aorta was rapidly dissected out. The subesophageal ganglia were pinned by the nerve and edges of the connective tissue into a small (~ 1 ml) sylgard-grounded recording chamber containing normal snail Ringer and the overlying connective tissue was gently stripped off using fine forcipes.

2.2. Intracellular recording

SEC-10LX amplifier (npi electronic, Tamm, Germany) and Patchmaster 2x73.1 software (HEKA, Lambrecht, Germany) were used to record the membrane potentials and to inject current under current clamp conditions using low resistance (1– 5 M Ω) sharp microelectrodes. Data

were digitized using a LIH 8+8 data acquisition interface and the parameters describing action potentials waveform were analyzed using the Fitmaster software (HEKA, Lambrecht, Germany). The resting membrane potential (RMP), input resistance and action potential parameters were measured as described in our previous paper (Vatanparast and Andalib-Lari, 2015).

2.3. Solutions and drugs

The normal snail Ringer solution contained (in mM): NaCl 80, KCl 4, CaCl₂ 10, MgCl₂ 5, glucose 10, HEPES 5 and pH was adjusted to 7.4 with TRISMA-base. The Na⁺-free Ringer was prepared by substituting NaCl with equimolar quantities of Tris-HCl. Eugenol (99%), riluzole, dimethyl sulfoxide (DMSO) and nifedipine were purchased from Sigma-Aldrich (Germany). Other chemicals obtained from Merck (Darmstadt, Germany). Eugenol was prepared as 2 M stock in DMSO, stored under nitrogen in fridge and diluted (0.5 and 2 mM) daily in snail Ringer. Nifedipine was prepared in a stock solution of 100 mM in 95% ethanol. The final concentrations of DMSO and ethanol in bathing solutions were always less than 0.1% and 0.05%, respectively, which had no discernible effect on firing frequency and electrical properties of snail neurons.

2.4. Statistical analysis

Data were presented as means $\pm$ S.E.M. with n being the number of neurons on which the measurements were done. The statistical differences were analyzed with either paired t-tests, or repeated measures ANOVA followed by Bonferroni post-hoc tests. A value of $P < 0.05$ was considered as statistically significant.

3. Results

In normal snail Ringer, the studied neurons had mean RMP of -43.27 ± 1.9 mV and showed regular spontaneous action potential with mean frequency of 2.08 ± 0.2 Hz, amplitude of 73.54

1 ± 2.83 mV, duration of 12.38 ± 1.54 ms, threshold of -33.4 ± 1.67 , integral of 271 ± 42 and
2 maximum rising and falling slopes of 18.7 ± 2.3 V/s and -11.25 ± 1.9 V/s, respectively. Single
3 action potentials were followed by AHP with a mean amplitude of -8.36 ± 0.84 mV and
4 duration of 111 ± 27 ms (n=15). Depolarizing current injections (1–5 nA, 500 ms) greatly
5 increased the firing rate of action potentials that were followed by a prominent slow decaying
6 AHP, appearing as a post stimulus inhibitory period (PSIP).
7 Preliminary experiments showed that 0.5 mM was the minimum concentration of eugenol that
8 evidently and consistently suppressed the neuronal firing and modulated the waveform of
9 action potentials. On the other hand, exposure to 2 mM eugenol increased the firing frequency
10 and induced epileptiform activity in snail neurons, but the effects of 1 mM eugenol on neuronal
11 activity was not consistent. In most of neurons that were exposed to 1 mM eugenol, the firing
12 frequency was suppressed, in some neurons the neuronal activity was not notably affected and
13 even a few neurons showed periods of hyperexcitability. Here, we present the effects of 0.5
14 mM and 2 mM eugenol on the neuronal excitability and refer to them as lower and higher
15 concentrations of eugenol, respectively.

17 *3.1. The effects of lower concentration of eugenol on the spontaneous and evoked activity,* 18 *action potential characteristics and post stimulus inhibitory period*

19
20 Extracellular application of lower concentration of eugenol (0.5 mM) reduced the frequency of
21 action potentials in a time dependent manner. This effect was partially reversible after 3–5 min
22 washing at a rate of approximately 3ml/min with normal snail Ringer (Fig. 1). The effect of 0.5
23 mM eugenol on neuronal firing was associated with modulatory actions on action potential
24 waveform, but the resting membrane potential (RMP) and input resistance were not
25 significantly affected. As shown in Figure 2, the suppressive action of 0.5 mM eugenol on the
26 neuronal firing was associated with significant increase in action potential threshold and
27 reduction of action potential amplitude and maximum rising slope. The AHP duration was also
28 significantly prolonged after eugenol application (Fig. 1, Fig. 2 and Fig. 3C). Pearson's correlation
29 test showed that firing frequency is negatively correlated with action potential threshold ($r=-$

0.84, $P < 0.001$, $n = 8$) and AHP duration ($r = -0.78$, $P < 0.001$, $n = 8$). The AHP duration was itself positively correlated with the area under action potential curve ($r = 0.73$, $P < 0.001$, $n = 8$). Eugenol (0.5 mM) also reduced the mean frequency of action potentials in response to 2 nA depolarizing current steps from a control value of 11.5 ± 0.6 Hz to 9.7 ± 0.8 Hz ($n = 7$, $P < 0.05$), but the relevant PSIP was significantly increased from 1.27 ± 0.14 s to 3.1 ± 0.39 s ($n = 7$, $P < 0.001$, Fig. 3A and B).

3.2. Lower concentrations of eugenol suppressed pentylenetetrazole-induced epileptiform activity

A concentration of 20 mM pentylenetetrazole (PTZ) elicited epileptiform activity in the majority (14 out of 19) of studied neurons. Exposure to PTZ rapidly increased the frequency of action potentials and within 3-6 min, varying patterns of burst activity and paroxysmal depolarization shifts (PDS) were developed in responsive neurons. The PTZ-induced epileptiform activity was inhibited within 2-5 min of eugenol (0.5 mM) application in the presence of PTZ and the regular single spike activity was resumed, although at a lower frequency than the control condition. While exposure to PTZ did not significantly change the action potential amplitude and maximum slope of depolarization in proconvulsive period, single action potentials that resumed in the presence of eugenol (0.5 mM) showed reduced amplitude and maximum slope of depolarization phase of action potential and elevated threshold compared to control (Fig. 4G). Furthermore, bath application of PTZ was not able to induce epileptiform activity in any of the seven neurons that were pretreated with 0.5 mM eugenol (data not shown), which also supports the anti-epileptic action of lower concentrations of eugenol.

3.3. Riluzole mimicked the suppressive and antiepileptic action of lower concentration of eugenol

The finding that eugenol reduces the maximum slope of rising phase and amplitude of action potential along with an increase in the action potential threshold suggests that the suppressive

and anti-convulsive action of eugenol could be mediated through Na^+ channels blockade. To test this hypothesis we studied the effect of riluzole, a blocker of Na^+ channels, to find if Na^+ channel blockade may induce the same effects. Bath application of riluzole ($150\ \mu\text{M}$) to normal bathing solution resulted in decreased firing rate of spontaneous activity within 5 min of exposure (Fig. 5A and C) and two out of eight treated neurons were completely silenced (not shown). The suppressive action of riluzole on spontaneous activity was associated with an increase in the threshold and duration of action potential, and prolongation of AHP duration. Meanwhile, the action potential amplitude and slope of rising phase were concomitantly decreased, while the RMP was hyperpolarized from -42.75 ± 2.4 to -45.51 ± 1.7 mV ($P < 0.01$) and PSIP following to 2 nA current pulse was prolonged from 1.39 ± 0.21 s to 2.8 ± 0.33 s (Fig. 6A; $P < 0.01$, $n=6$). Application of PTZ (20 mM) to the neurons pretreated with riluzole did not induce epileptiform activity (not shown). Riluzole also abolished the PTZ-induced epileptiform activity and restored the single spike firing activity (Fig. 6; $n=7$).

3.4. Higher concentrations of eugenol induced hyperexcitability and epileptiform activity

Bath application of higher concentration of eugenol (2 mM) rapidly (in less than 2 min of exposure) increased the frequency of action potentials. Furthermore, the epileptiform activity developed later (within 3-6 min of exposure) in 7 out of 10 neurons that exposed to 2 mM eugenol. The epileptiform activity induced by eugenol was reversible and regular single spike activity was resumed after, 4-7 min washing with normal Ringer at a rate of approximately 3 ml/min (Fig. 7). The pattern of epileptiform activity showed considerable variation in different neurons and even changed by time in each neuron. During longer periods of exposure to eugenol (9-15 min), the epileptiform activity was gradually diminished (not shown). Action potentials in proconvulsive phase had lower amplitude, longer duration with lower steepness of both raising phase and falling phase, and were followed by suppressed AHPs (Fig. 7G). The RMP was significantly depolarized from -42.3 ± 2.1 mV to -37.6 ± 1.5 mV within 2 min of exposure to 2 mM eugenol ($n=8$). Resting membrane depolarization modulates the neuronal excitability by affecting different voltage gated ion channels and even may underlie burst firing.

To find if the eugenol-induced hyperexcitability and epileptiform activity are consequent to membrane depolarization, in some neurons the membrane potential was maintained close to the control level by injecting negative DC current in proconvulsive period. When RMP was held close to control level the mean frequency and threshold of action potentials were not significantly affected by eugenol, but the effects of eugenol on action potentials amplitude, AHP, slope of rising and falling phases and action potential duration were not dependent on membrane depolarization (Fig. 7G). Resetting the membrane potential to a value close to control value did not block the eugenol-induced epileptiform activity (not shown).

3.5. Eugenol-induced epileptiform activity is dependent on both Na^+ and Ca^{2+} currents

In some experiments the normal snail Ringer was exchanged with Na^+ -free Ringer in which NaCl had been replaced with Tris-HCl on an equimolar basis. All studied neurons showed spontaneous activity at a lower frequency after exchanging the bathing medium with Na^+ -free Ringer. Eugenol (2 mM) increased the frequency of spontaneous action potentials from 0.87 ± 0.17 Hz to 1.09 ± 0.12 Hz within 2 min of exposure ($P < 0.05$, $n=6$), but did not induce epileptiform activity (Fig. 8A). Meanwhile eugenol altered the pattern of voltage response during depolarizing current injection (2 nA, 500 ms) and reduced the PSIP from 5.6 ± 0.8 s to 3.5 ± 0.4 s in Na^+ -free Ringer (Fig. 8B and C, $P < 0.01$).

The eugenol-induced burst firing and associated PDS were suppressed and changed to single spike firing when non-selective T-type Ca^{2+} channel blocker, NiCl_2 (4 mM, $n=7$) or L-type Ca^{2+} channel blocker, nifedipine (50 μM , $n=6$) were bath applied (Fig. 8D and E). Following longer exposure to NiCl_2 (7-10 min), the amplitude of action potentials was strongly reduced and 6 out of 7 neurons were completely silenced (not shown).

4. Discussion

The present study indicated the inhibitory action of low concentration of eugenol on the neuronal activity and its ability to suppress epileptiform activity, and also showed for the first time that the higher concentration of eugenol can induce epileptiform activity in snail neurons. Considering the modulatory action of different concentrations of eugenol on the electrical properties of neurons and action potential characteristics, the possible interaction of eugenol with different ion channels and their relevance to neuronal excitability is discussed. Eugenol (0.5 mM) elevated action potential threshold and reduced both maximum rising slope and amplitude of action potentials, which are correlated with availability of the ion channels that underlie action potential upstroke. Park and colleagues showed that eugenol (1mM) suppresses action potentials and Na⁺ currents in dental afferents and suggested that this action contributes to its analgesic action (Park et al., 2006). Cho et al. also reported the inhibitory action of eugenol (0.3 mM) on Na⁺ currents in rat dorsal root ganglion neurons of rat (Cho et al., 2008). The neuroprotective/anticonvulsant agent riluzole employs several mechanisms of actions, but it primarily targets persistent Na⁺ current, which essentially contribute to the neuronal excitability and epileptogenesis (Diao et al., 2013; Ren et al., 2014). Riluzole showed almost comparable effect to that of low concentration eugenol in reducing the frequency, amplitude and slope of rising phase of action potentials and increasing the threshold of action potentials initiation. These findings support the assumption that the inhibitory action of low concentration of eugenol on Na⁺ channels most likely contributes to its suppressive action on the firing of snail neurons. It has been shown that eugenol (0.2 mM) diminishes the neuronal activity and gabazine-induced epileptiform activity in mouse cortical neurons (Pezzoli et al., 2014). Sub-millimolar concentrations of eugenol also inhibit compound action potentials and elevate the threshold for stimulation of phrenic nerve but do not affect RMP (Brodin and Roed, 1984). Szabadics and Erdélyi (2000) reported the effects of eugenol (0.25-1 mM) on *Helix pomatia* neurons and found that it reversibly inhibits synaptically evoked action potential, decreases the Ca²⁺ currents and reduces postsynaptic responses to acetylcholine and GABA. They also showed that the 1 mM eugenol reversibly depolarizes the RMP of *H. pomatia* neuron but did not report this effect for lower concentrations.

1 The finding that eugenol (0.5 mM) reduces the slope of falling phase of action potentials,
2 suggesting an inhibitory action on K^+ channels that underlie the repolarization phase, is in
3 accord with studies that have shown the inhibitory action of eugenol on K^+ channels (Huang et
4 al., 2012; Li et al., 2007). On the other hand, we found that the duration of AHP and post
5 stimulus inhibitory period, which are also underlain by K^+ currents, were prolonged by eugenol.
6 This contradiction could be explained by indirect augmentation of Ca^{2+} -activated K^+ currents. A
7 major component of AHP following single or trains of high frequency driven action potentials is
8 underlain by these currents in *C. atrolabiata* neurons (Vatanparast and Janahmadi, 2009). In
9 snail neurons, the Ca^{2+} inward current is activated mainly during falling phase of action
10 potential and could be strengthened by action potential prolongation (Chung et al., 2008; van
11 Soest and Kits, 1998). As eugenol (0.5 mM) reduced the slope of rising and falling phases and
12 increased the duration and integral of action potentials, an indirect increase of the Ca^{2+} inward
13 current and more activation of Ca^{2+} -activated K^+ currents is expected that may underlie the
14 prolongation of AHP and PSIP. In *C. atrolabiata* neurons, AHP duration is negatively correlated
15 with neuronal firing rate (Vatanparast et al., 2006; Vatanparast and Andalib-Lari, 2015;
16 Zeraatpisheh and Vatanparast, 2015), and the increase of AHP may contribute to the reduced
17 firing frequency after exposure to low concentration of eugenol.

18 Earlier *in vivo* studies have shown the anticonvulsant activity of eugenol and eugenol containing
19 essential oils. Dallmeier and Carlini (1981) showed that intraperitoneal injection of eugenol (1.2
20 mM/kg) significantly reduces the convulsions induced by transcorneal electroshock in mice.
21 Here we provided further support for the antiepileptic activity of eugenol by showing that
22 direct application of eugenol (0.5 mM) can prevent or inhibit the PTZ-induced epileptiform
23 activity under *in vitro* condition. Inhibition of Na^+ current is an essential mechanism of many
24 antiepileptic drugs (Diao et al., 2013; Ren et al., 2014). Here, the regular action potentials that
25 were recorded after application of eugenol (0.5 mM) on PTZ exposed neurons showed elevated
26 threshold and reduced amplitude and maximum depolarization slope. This finding together
27 with the almost similar antiepileptic activity of riluzole supports the assumption that the
28 inhibition of Na^+ currents most likely contribute to the antiepileptic action of low concentration
29 eugenol, although further experiments are required to confirm this possibility. The Ca^{2+} -

1 activated K^+ current is strengthened during periods of high frequency activity and act as a
2 feedback regulator of excitability and also an inhibitor of transition to burst firing in many
3 neurons (Rouchet et al., 2008; Stocker, 2004). Our finding that eugenol (0.5 mM) prolonged the
4 PSIP following to train of action potentials driven by depolarizing current step also supports the
5 indirect augmentation of Ca^{2+} inward current, which can potentiate Ca^{2+} -activated outward
6 current and contribute to the suppressive action of low concentration eugenol on PTZ induced
7 epileptiform activity.

8 Although eugenol is best known for its inhibitory effects on the neural activity, there are some
9 reports that describe the stimulatory action of eugenol on the neural activity (Lee et al., 2015).
10 Hartnoll et al. (1993) reported a case of ingestion of clove oil, usually contains 80-90% eugenol,
11 which was followed by a generalized seizure along with other symptoms. Earlier studies have
12 showed some mechanisms that may contribute to the excitatory action of higher concentration
13 of eugenol. It has been shown that eugenol (5 mM) activates TRPA1 channels in rat spinal
14 substantia gelatinosa neurons and enhances the spontaneous release of L-glutamate from
15 glutamate nerve terminals (Inoue et al., 2012). Eugenol (1 mM) inhibits GABA-induced current
16 in trigeminal neurons in a reversible, dose-dependent, and non-competitive manner (Lee et al.,
17 2015). The antagonism of GABAA receptors by eugenol might be mechanistically involved to the
18 induction of epileptiform activity in snail neurons, but further studies are required to confirm
19 this hypothesis. Several lines of evidences in the current study indicate that the inhibition of K^+
20 channels is involved in the epileptogenic action of 2 mM eugenol. The AHP duration and
21 amplitude and the absolute magnitude of falling phase of action potentials, which both are
22 underlined by K^+ currents (Cloues and Sather, 2003; Gola et al., 1986), were reduced by eugenol
23 during proconvulsive period. The K^+ outward currents not only affect the waveform of action
24 potentials but also contribute to the control of neuronal excitability and pattern of firing
25 (Cloues and Sather, 2003). We have previously reported that bath application of K^+ channel
26 blockers, especially tetraethyl ammonium as a blocker of delayed rectifier K^+ channels and big-
27 conductance (BK) Ca^{2+} -activated K^+ channels, can induce epileptiform activity in *C. atrolabiata*
28 neurons (Vatanparast and Andalib-Lari, 2015; Zeraatpisheh and Vatanparast, 2015).

1 The inhibition of K^+ channels has been implicated in the induction of burst firing and PDS by
2 several compounds in different neurons including snail neurons (Feher et al., 1988; Vatanparast
3 and Andalib-Lari, 2015; Zeraatpisheh and Vatanparast, 2015). Our data suggest that the strong
4 inhibition of K^+ channels by higher concentration of eugenol dominates its inhibitory effect and
5 elicits epileptiform activity. Huang et al. (2012) reported the inhibitory action of eugenol on Na^+ ,
6 Ca^{2+} and K^+ currents in the neuronal membrane. They found that lower concentration of
7 eugenol (1-3 μM) effectively inhibited Na^+ current. At higher concentration (10 μM) it also
8 reduced the amplitude of current through L-type Ca^{2+} channels and delayed rectifier K^+
9 channels. They also reported a reduction of neuronal excitability only by lower concentration
10 of eugenol and explained it by its ability to inhibit Na^+ current without a significant effect on
11 Ca^{2+} and K^+ currents. Although the effective concentration of eugenol on *C. atrolabiata*
12 neurons was found to be much higher than those reported by Huang et al. but, we also found
13 that the dominance of excitatory action of eugenol on its inhibitory effect, which most likely
14 results from significant inhibition of K^+ channels, is observed only at a concentration about four
15 times higher than that showing suppressive action, apparently by inhibition of Na^+ channels.
16 The higher concentration of eugenol increased the frequency of spontaneous activity, but it
17 was not able to induce epileptiform activity in Na^+ free Ringer. This finding shows that even
18 after exposure to higher concentration of eugenol a significant proportion of Na^+ permeable
19 channels are still available for activation and seems to be required for the induction of
20 epileptiform activity by eugenol. The eugenol-induced epileptiform activity was also dependent
21 on inward currents through Ca^{2+} channels as their blockers abolished the epileptiform activity.
22 Several studies have reported the inhibitory effect of eugenol on both T-type and L-type Ca^{2+}
23 channels in different cells (Chung et al., 2008; Seo et al., 2013). On the other hand, Ohkubo and
24 Kitamura (1997) showed that eugenol (1 mM) activates a Ca^{2+} inward current through TRPV1
25 channel. The essential contribution of Ca^{2+} inward currents through T-type and L-type channels
26 in the epileptiform activity induced by high concentration of eugenol suggests that either
27 eugenol does not significantly inhibit Ca^{2+} currents in snail neurons or this inhibitory action is
28 compensated by more intense inhibition of K^+ currents that indirectly potentiate the Ca^{2+}

currents. Further studies are required to test possible contribution of TRPV1 channels to the augmentation of Ca^{2+} inward currents by eugenol in snail neurons.

In conclusion, this study showed that eugenol can induce inhibitory and excitatory effects on snail neurons in a concentration-dependent manner. The inhibition of Na^+ channels by low concentration of eugenol seems to be involved to its inhibitory action on spontaneous activity and suppressive action on the PTZ-induced epileptiform activity. On the other hand, the inhibitory action of higher concentration of eugenol on K^+ channels and the consequent augmentation of Ca^{2+} currents apparently contribute to its excitatory and epileptogenic action. Although more researches are required to extend our finding to human intoxication conditions, this work reconfirms that eugenol might be beneficial as an antiepileptic agent, but our findings also recommend more caution in prescribing eugenol at high doses.

Conflict of interest

There are none.

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Figure Captions:

Fig. 1. Eugenol (0.5 mM) reduced the frequency of spontaneous action potentials that was associated with a reduction of their amplitude and slope of rising and falling phases in a time dependent and partially reversible manner. Spontaneous action potentials recorded from a representative neuron under control condition (A), 2 min (B) and 5 min after exposure to 0.5 mM eugenol (C), and 6 min after washout with normal snail Ringer (D). The small horizontal bars right to each graph show 0 mV. E: Superimposed action potentials from a neuron under control condition, 5 min after application of eugenol and 4 min after washout. Adjustment of superimposition was based on the threshold of action potentials.

Fig. 2. The effects on action potentials frequency and characteristics within 5 min exposure to 0.5 mM eugenol (EUG) and after washout. The absolute value of falling slope and AHP amplitude were considered for analysis. * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ compared to control; † $P < 0.05$ and †† $P < 0.01$ compared to eugenol ($n=8$). Frq, frequency; Amp, amplitude; Max Rd, maximum depolarization slope; Max Rr, maximum repolarization slope; AHP dur, afterhyperpolarization duration; AHP amp, afterhyperpolarization amplitude; Thr, threshold; Int, integral.

Fig. 3. Eugenol (0.5 mM) reduced the frequency of spontaneous and driven activity, and prolonged post stimulus inhibitory period (PSIP). The pattern of spontaneous firing and delay in resumption of spontaneous firing after a high frequency evoked activity by a constant depolarizing current step (2nA, 500ms) under control condition and 5min after exposure to 0.5 mM eugenol (EUG) (A). Pooled data of 8 neurons show that eugenol increases PSIP after train of action potentials evoked by 2nA current step (B). *** $P < 0.001$ compared to control. The areas within dashed rectangles in “A” have been overlayed at higher voltage and time resolution in C, indicating prolongation of afterhyperpolarization that follows single action potential in the presence of eugenol (gray trace) compared to control condition (black trace).

Fig. 4. Eugenol (0.5 mM) suppressed pentylenetetrazole-induced epileptiform activity. Spontaneous activity recorded from a representative neuron under control condition (A), 2 min (B), 4 min (C) and 6 min after exposure to 20 mM pentylenetetrazole (D), and 2 min (E) and 4 min after addition of eugenol to the bathing solution (final concentration 0.5 mM, F). G indicates superimposed action potentials recorded from a representative neuron under control condition (black trace), 2 min after exposure to pentylenetetrazole (proconvulsive period, dark grey) and 4 min after bath application of eugenol (light grey trace). Inset histogram shows modulatory action of eugenol on mean amplitude (Amp), maximum depolarization slope (Max Rd) and threshold (Thr) of action potentials (n=7). *P<0.05, **P<0.01 and ***P<0.001 compared to control.

Fig. 5. Riluzole (150 μ M) lowered the frequency of spontaneous activity, heightened action potential threshold, reduced maximum rising phase and increased post stimulus inhibitory period. The pattern of spontaneous activity and delay in resumption of spontaneous firing after a high frequency evoked activity by a constant depolarizing current step (2nA, 500ms) under control condition and 5 min after exposure to 150 μ M riluzole (A). Superimposed action potentials showing the reduction of action potential rising phase 5 min after application of riluzole compared to control condition (B). The modulatory effects observed 5 min after exposure to riluzole on action potentials frequency and characteristics (C). The absolute value of falling slope and AHP amplitude were considered for analysis. *P<0.05, **P<0.01 and***P<0.001 compared to control (n=6). Frq, frequency; Amp, amplitude; Dur, duration; Max Rd, maximum depolarization slope; Max Rr, maximum repolarization slope; AHP amp, afterhyperpolarization amplitude; AHP dur, afterhyperpolarization duration; Thr, threshold.

Fig. 6. Riluzole (150 μ M) prevented pentylenetetrazole-induced epileptiform activity. As shown for a representative neuron, riluzole reduced the number of action potential per burst in a time dependent manner and restored the single spike firing pattern, although at much lower frequency and with essential differences from control in action potential waveform.

Fig. 7. High concentration eugenol (2 mM) induced reversible epileptiform activity in snail neurons that was associated with modulation of action potential characteristics in proconvulsive period. Spontaneous activity of a representative neuron in control condition (A), 2 min (B), 3 min (C) and 5 min (D) after application of eugenol (2 mM). The paroxysmal depolarization shift and the associated burst were reduced within 4 min of washout with normal snail Ringer (E), and the regular firing was resumed within 6 min of washout with normal snail Ringer. The small horizontal bars right to each graph show 0 mV. The effects of eugenol (2 mM) on the mean characteristics of action potential within two min of exposure and after direct current (DC) injection in the presence of eugenol to restore the membrane potential to control level (G, n=6).

Fig. 8. Both sodium and calcium inward currents are required for eugenol-induced epileptiform activity. Spontaneous firing was sustained when normal Ringer was replaced with a Na^+ -free Ringer but eugenol (2 mM) was not able to induce epileptiform activity in this bathing medium (A). Eugenol shortened post stimulus inhibitory in Na^+ -free Ringer (B). The pattern of voltage response during depolarizing current injection (2 nA, 500 ms) in Na^+ -free Ringer and 3 min after eugenol application has been shown at a higher resolution in C. Bath application of NiCl_2 (4 mM) inhibited the eugenol-induced epileptiform activity within 5 min (D). Nifedipine (50 μM , NIF) also abolished eugenol-induced epileptiform activity and restored the regular single spike activity (E).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Regular Firing (Control)

(0.5 mM)

(2 mM)

?

Na⁺

K⁺

Ca²⁺

Suppressed Activity

Epileptiform Activity