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$GABA_A$ Receptor- and Non-NMDA Glutamate Receptor-Mediated Actions of Korean Red Ginseng Extract on the Gonadotropin Releasing Hormone Neurons

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Korean red ginseng (KRG) has been used worldwide as a traditional medicine for the treatment of various reproductive diseases. Gonadotropin releasing hormone (GnRH) neurons are the fundamental regulators of pulsatile release of gonadotropin required for fertility. In this study, an extract of KRG (KRGE) was applied to GnRH neurons to identify the receptors activated by KRGE. The brain slice patch clamp technique in whole cell and perforated patch was used to clarify the effect of KRGE on the membrane currents and membrane potentials of GnRH neurons. Application of KRGE (3 μg/μL) under whole cell patch induced remarkable inward currents (56.17±7.45 pA, *n*=25) and depolarization (12.91±3.80 mV, *n*=4) in GnRH neurons under high Cl^- pipette solution condition. These inward currents were not only reproducible, but also concentration dependent. In addition, inward currents and depolarization induced by KRGE persisted in the presence of the voltage gated Na^+ channel blocker tetrodotoxin (TTX), suggesting that the responses by KRGE were postsynaptic events. Application of KRGE under the gramicidin perforated patch induced depolarization in the presence of TTX suggesting its physiological significance on GnRH response. Further, the KRGE-induced inward currents were partially blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX; non-NMDA glutamate receptor antagonist, 10 μM) or picrotoxin (PIC; $GABA_A$ receptor antagonist, 50 μM), and almost blocked by PIC and CNQX mixture. Taken together, these results suggest that KRGE contains ingredients with possible GABA and non-NMDA glutamate receptor mimetic activity, and may play an important role in the endocrine function of reproductive physiology, via activation of $GABA_A$ and non-NMDA glutamate receptors in GnRH neurons.

Keywords: *Panax ginseng*, Korean red ginseng extract (KRGE), Gonadotropin-releasing hormone neurons, Patch-clamp techniques, $GABA_A$ receptor, Non-NMDA glutamate receptor

INTRODUCTION

The reproduction is the basis of improving and sustaining life. The gonadotropin releasing hormone (GnRH) neurons are central to sexual and behavioral maturation and the control of GnRH secretion is fundamental to reproductive maturation [1]. The GnRH neurons are the fundamental regulator of the pulsatile release of GnRH required for puberty and fertility [2].

The root of ginseng (*Panax ginseng* Meyer) has been known as folk medicine in East Asian countries since time immemorial, and is now one of the most largely

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used herbal drug in the world [3,4]. Korean red ginseng (KRG) is herb cultivated and aged for 4-6 years or more, and goes through extensive cleaning, steaming and drying processes before use [5]. Extract of KRG (KRGE) has been taken orally to improve both mental and physical health, and several studies have shown that KRGE helps preventing diabetes mellitus [6], atherosclerosis [7], erectile dysfunction [8], immune dysfunction [9], carcinogenesis [10], and physicochemical stress [11]. Additionally, administration of KRG water extract protects testicular function [12] and improves sperm survival rate and quality in guinea pigs exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin [13]. Furthermore, KRG also acts as antioxidant [14], potentiation of the erectile response [8], anti-tumor [15], enhancing of memory activities [16], anti-hypertensive [17], anti-diabetic [18], antistress [19], and aphrodisiac [20]. Although several studies have suggested the biological and pharmacological effects of KRGE on the reproductive physiology, the direct effects of KRGE on the neuronal membrane and the related ion channels are not completely understood. In this study, we used whole-cell patch clamp recording to examine the direct effects of KRGE on the membrane and related ion channels on the GnRH neurons.

MATERIALS AND METHODS

Animals

All experiments were approved by Chonbuk National University Animal Welfare and Ethics Committee. GnRH-green fluorescent protein tagged mice [21] were housed under 12-h light/12-h dark cycle (lights on at 07:00 h) with *ad libitum* access to food and water.

Brain slice preparation and electrophysiology

Brain slices were prepared as described by Bhattarai *et al*. [22]. Briefly, immature male and female mice (5-20 day old) were decapitated and their brains were removed rapidly and placed in ice-cold bicarbonate-buffered artificial cerebrospinal fluid (ACSF), with the following composition (in mM): 126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 11 D-glucose, 1.4 NaH_2PO_4 and 25 $NaHCO_3$ (pH 7.3-7.4, when bubbled with 95% O_2 and 5% CO_2). Coronal slices containing the preoptic hypothalamic area were cut using a vitratome (Microme, Walldrof, Germany). Patch pipettes were pulled from thin-wall borosilicate glass-capillary tubing (PG52151-4; WPI, Sarasota, FL, USA) on a Flaming/Brown puller (P-97; Sutter Instruments Co., Novato, CA, USA). The pipette solution was passed through a disposable 0.22 μm filter and contained the following (in mM): 140 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, 4 MgATP, 10 EGTA (pH 7.3 with KOH). The whole-cell patch clamp recordings were performed under voltage clamp and current clamp mode, using the Axopatch 200B system (Axon Instruments, Union City, CA, USA). The cells were voltage clamped at -60 mV after nullifying the junction potential between the patch pipette and bath solution. For perforated patch clamp recording, gramicidin (Sigma, St. Louis, MO, USA) was first dissolved in dimethylsulfoxide (Sigma) to a concentration of 2.5-5 mg/mL and then diluted in the pipette solution just before use to a final concentration of 2.5-5 μg/mL and sonicated for 10 min. In initial experiments, access resistance was monitored and experiments begun when resistance stabilized at 50-90 MΩ. This typically took 15 to 20 min after giga-seal formation and always corresponded to the resting membrane potential of the cell reaching a stable level below -45 mV. Spontaneous rupture of the membrane was evident by a sudden overshooting of action potentials above 0 mV. The changes in membrane current were sampled online using the Digidata 1322A interface (Axon Instruments, Union City, CA, USA) connected to an IBM PC. Acquisition and subsequent analysis of the acquired data were performed using Clampex9 software (Axon Instruments). The traces were plotted using Origin7 software (MicroCal Software, Northampton, MA, USA). All recordings were made at room temperature.

Chemicals and statistical analysis

KRGE was kindly provided by Korea Ginseng Corporation (Daejeon, Korea). Six-year-old fresh ginseng roots (*P. ginseng* Meyer) were harvested in field, Korea, in September 2009. KRGE was made by steaming fresh ginseng at 90°C to 100°C for 3 h and drying it at 50°C to 80°C. KRGE contains major ginsenosides Rb_1 (4.06%), Rb_2 (1.57%), Rc (1.31%), Rd (0.90%), Re (0.70%), Rf (0.50%), Rg_1 (0.48%), Rg_2 (0.69%) and Rg_3 (1.94%), as well as minor ginsenosides (Korea Ginseng Corporation). KRGE was prepared from water extract, which was extracted at 87°C for 8 h using six volumes of hot water circulating six times. The pooled extract was then concentrated to 36% water contents. The test compounds were dissolved in ACSF solution and tested by adding perfusing ACSF at known concentrations. Picrotoxin (PIC), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and chemicals for ACSF were purchased from Sigma. Tetrodotoxin (TTX), a Na^+ channel blocker, was obtained from Tocris bioscience (Bristol, UK). All values are expressed as the mean±SEM. Either paired *t*-test or

one sample *t*-test was used to assess the difference in the percentage of responding cells between the two groups and a *p*-value <0.05 was considered as statistically significant.

RESULTS AND DISCUSSION

Whole-cell patch clamp recordings were obtained from 40 GnRH neurons. Localization of the EGFP tagged GnRH neurons in the different preoptic hypothalamic areas was determined visually under the optic microscope with ×40 magnification. We performed voltage clamping under whole-cell patch with a holding potential of -60 mV in GnRH neurons of immature male and female mice.

Korean red ginseng extract-induced inward currents are concentration dependent

KRGE was applied to the GnRH neurons to clarify the hypothesis that KRGE can affect in the reproductive physiology and acts on the hypothalamic-pituitary-gonadal axis by regulating GnRH neurons and to determine the receptors activated by KRGE on the GnRH neurons. Both membrane currents and potential changes were recorded in whole-cell voltage and current clamp modes from the GnRH neurons in the preoptic region of coronal hypothalamic slices in the mouse brain. Thus, we measured the concentration-dependent effect of KRGE on GnRH neurons at different doses. At lower doses (<0.1 mg/mL) of KRGE, no responses were found on GnRH neurons. We used bath application of different doses of KRGE, ranging from 0.3 to 10 mg/mL, as shown in Fig 1A. Under high Cl^- pipette solution condition, KRGE produced inward currents with increasing doses in all neurons tested (n=6), with concentration-dependent increase in the KRGE-induced inward currents (0.3 mg/mL: -8.32±1.01 pA, 1 mg/mL: -17.23±2.84 pA, 3 mg/mL: -61.9±8.01 pA and 10 mg/mL: -225.83±33.41 pA) (n=6; p<0.05 in all) (Fig. 1A, B).

To check whether the KRGE-induced inward currents were repeatable without desensitization, 3 mg/mL KRGE was successively applied. The GnRH neurons showed short-lived and repeatable inward currents by successive KRGE applications (1st: -39.06±4.97 pA; 2nd: -37.80±4.68 pA; paired *t*-test p>0.05). The relative inward current by the 2nd application of KRGE was 0.97±0.2 (n=13) of that of the first application, indicating non desensitizing effect after repeated application of KRGE to GnRH neurons.

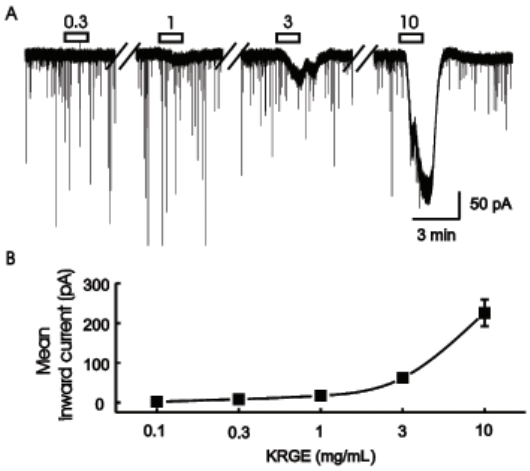

Fig. 1. The Korean red ginseng extract (KRGE) induced concentration-dependent inward currents in gonadotropin releasing hormone (GnRH) neurons. (A) Representative current trace of an immature GnRH neuron showing concentration-dependent increase in KRGE-induced inward currents by 0.3, 1, 3, and 10 mg/mL concentrations of KRGE. (B) Cumulative graph shows the mean inward currents by KRGE (0.1, 0.3, 1, 3, and 10 mg/mL).

Korean red ginseng extract acts on gonadotropin releasing hormone neurons directly

Further, to clarify whether KRGE acts directly on the postsynaptic GnRH neurons or via any action potential mediated mechanism, the effects of KRGE were tested in both current clamp and voltage clamp modes, in the presence of TTX, 0.5 μM, a voltage-gated Na^+ channel blocker, which blocks action potential-dependent transmission. The KRGE-induced inward currents were persistent in the presence of TTX in voltage clamp mode (Fig. 2B, n=5). The induction of inward currents in the presence of TTX clearly illustrated that KRGE acts directly on the GnRH neuronal membrane or dendrites. The bar graph besides each trace shows the relative current and the voltage change, respectively. In the current clamp mode, KRGE induced membrane depolarization with increased action potential firing, under high Cl^- pipette solution condition, and further application of TTX blocked the action potential, but failed to block KRGE-induced depolarization, suggesting that the KRGE-induced effects are not elicited by action potential-mediated transmission. Further to know the functional and physiological significance of KRGE on GnRH neurons, KRG was applied in gramicidin perforated patch clamp mode which allows preserving the intra cellular chloride concentration [23]. KRG in perforated mode also induced the membrane depolarization (8.0±1.9 mV, n=5) which remained persisted in the presence of TTX (6.24±1.17 mV, n=3) suggesting that KRGE induces excitatory effects on GnRH neurons.

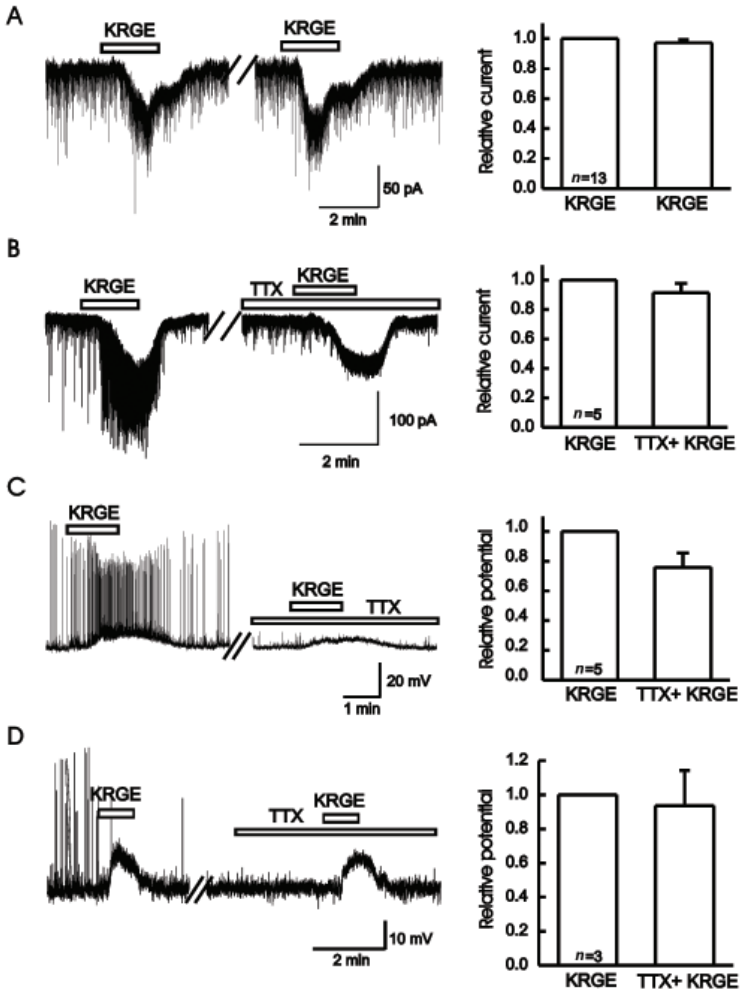

Fig. 2. The Korean red ginseng extract (KRGE) induces non-desensitizing inward currents on the post synaptic gonadotropin releasing hormone (GnRH) neurons. (A) Current trace showing the reproducible and non-desensitizing effects of KRGE (3 mg/mL) in an immature GnRH neuron. (B) The KRGE-induced inward currents persisted in the presence of tetrodotoxin (tetrodotoxin [TTX], 0.5 μM), a voltage-gated Na^+ channel blocker. (C) Representative voltage trace of a immature GnRH neuron showing membrane depolarization after application of KRGE alone and in the presence of TTX, (note: TTX quickly blocked the action potential induced by KRGE, but failed to block depolarization induced by KRGE). (D) Representative voltage trace of gramicidin perforated patch showing membrane depolarization by KRGE in intact and in the presence of TTX. Cumulative bar graphs shown on the right hand side of each trace represent the mean relative inward currents and voltage by KRGE, respectively (3 mg/mL) (p>0.05). KRG, Korean red ginseng.

Gramicidin perforated patch clamp recording and relative voltage data bar graph is illustrated in Fig. 2D.

Earlier studies have demonstrated that ginsenosides, the main components of ginseng, act on either glutamate or $GABA_A$ receptors in *Xenopus* oocytes and hippocampus neurons [24-26]. Further, our recent study in substantia gelatinosa neurons of the trigeminal subnucleus caudalis demonstrated that KRGE acts on non NMDA glutamate and GABA receptors [27]. Therefore, we investigated if KRGE-mediated inward currents are mediated by non-NMDA receptors in preoptic GnRH neurons. KRGE was applied in the presence of CNQX, a non-NMDA receptor antagonist. As shown in Fig. 3A, KRGE-induced inward currents were partially blocked by CNQX (20 μM) (control: -24.48±4.44 pA; CNQX: -7.58±1.15 pA). The percentage of inward currents induced by KRGE in the presence of CNQX was 31.8±1.9% (n=5) of that of KRGE alone (Fig. 3B, paired t-test, p<0.05), suggesting that KRGE contains a non-NMDA analogue which may directly act on the non-NMDA glutamate receptors in GnRH neurons. Furthermore, to investigate if KRGE-mediated currents are mediated by $GABA_A$ receptors in GnRH neurons, KRGE was also applied in the presence of PIC, a $GABA_A$ receptor antagonist. As shown in Fig. 4A, KRGE-induced currents were partially blocked by PIC (control: -57.5±21.5

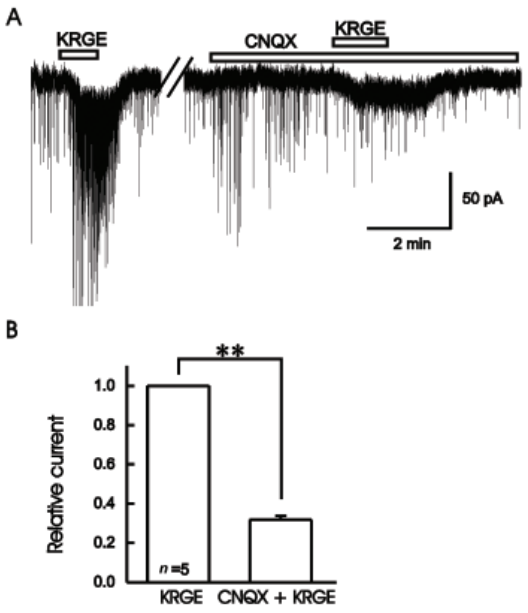

Fig. 3. The Korean red ginseng extract (KRGE)-induced inward currents were partly mediated by non-NMDA glutamate receptors. (A) A current trace of a immature gonadotropin releasing hormone neuron held at -60 mV, showing that the current induced by KRGE (3 mg/mL) was partly blocked by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM), a non-NMDA receptor antagonist. (B) Cumulative bar graph shows the mean relative current changes by KRGE (3 mg/mL) alone and KRGE (3 mg/mL) in the presence of CNQX (**p<0.01). KRG, Korean red ginseng.

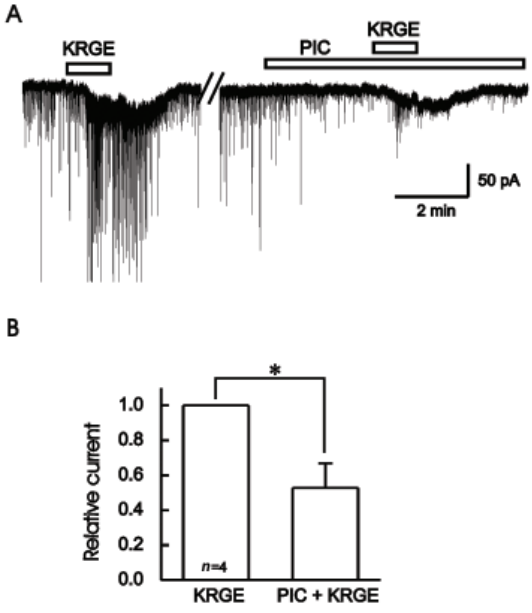

Fig. 4. The Korean red ginseng extract (KRGE)-induced inward currents were also partly mediated by $GABA_A$ receptors. (A) A current trace of a immature gonadotropin releasing hormone neuron held at -60 mV, showing that the current induced by KRGE was partly blocked by picrotoxin (PIC, 50 μM), a $GABA_A$ receptor antagonist. (B) Cumulative bar graph shows the mean relative current changes by KRGE (3 mg/mL) alone and KRGE (3 mg/mL) in the presence of PIC (*p<0.05).

pA; PIC: -24.9±6.15 pA) (50 μM, n=5). The percentage of inward currents induced by KRGE in the presence of PIC was 52.81±13.9% (n=4) of that of KRGE alone (Fig. 4B, paired t-test, p<0.05), suggesting that KRGE may directly target the $GABA_A$ receptors on GnRH neurons. Furthermore, KRGE was applied in the presence of both CNQX and PIC. KRGE-induced inward currents were almost blocked by CNQX combined with PIC (Fig. 5A) (control: -52.7±7.2 pA; CNQX+PIC: -7.28±3.37 pA). Fig. 5B shows the relative inward currents induced by KRGE in the presence of CNQX and PIC (n=5) (Fig. 5B). These results indicate that KRGE mainly acts on non-NMDA glutamate receptors and $GABA_A$ receptors on GnRH neuronal membranes and may influence the reproductive physiology by influencing GnRH neuronal activities. The KRGE-induced reproducible and short lasting inward currents on GnRH neurons were concentration-dependent. These repeatable non-desensitizing inward currents persisted in the presence of TTX, a Na^+ channel blocker, suggesting that the action sites for KRGE are directly on the recorded GnRH neuronal membrane or its dendrites, rather than via any action potential-mediated transmission mechanisms.

A variety of plants have been used in traditional herbal medicine to cure diseases and sustain longevity. Ginseng has been used in Asian countries from time immemorial. Currently, three types of common species of KRG have been used in research: *P. ginseng* (Asian ginseng), *P. quinquefolius* (American ginseng), and *P. japonicus* (Japanese ginseng) [28]. Most pharmacological actions of ginseng are attributed to ginsenosides [28], and more than 30 ginsenosides have been isolated to date [29].

P. quinquefolius extracts have been reported to reduce the discharge rate of the nucleus tractus solitarius neurons via regulation of GABAergic neurotransmission [30]. Choi *et al*. [24] demonstrated that the ginsenoside Rc affects the human recombinant $GABA_A$ receptor ($\alpha_1\beta_1\gamma_2S$) channel activity expressed in *Xenopus* oocytes. Further, ginsenoside Rc has been found to induce inward membrane currents in certain batches of oocytes expressing the $GABA_A$ receptor, which were blocked by PIC, suggesting that ginsenosides regulate the $GABA_A$ receptors expressed in *Xenopus* oocytes. Our recent study [27] also showed that KRG extract affected $GABA_A$ and non-NMDA glutamate receptors in SG neurons of trigeminal subnucleus caudalis, which are related to orofacial pain processing. Taken together, it is clear that KRGE activates both the $GABA_A$ receptors and non NMDA glu-

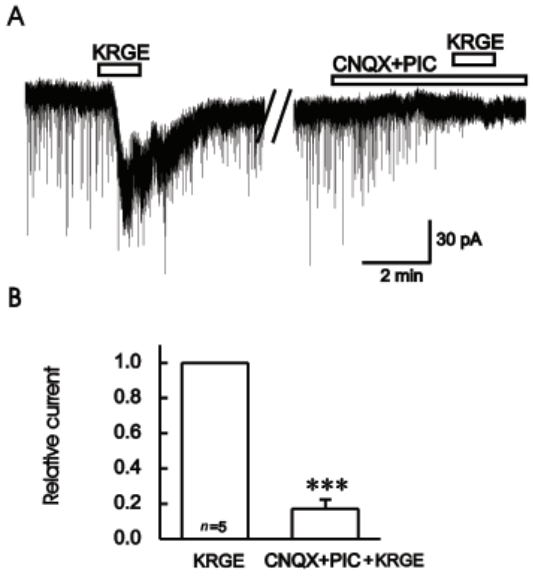

Fig. 5. The Korean red ginseng extract (KRGE)-induced inward currents were almost entirely blocked by combined non-NMDA glutamate receptor and $GABA_A$ receptor antagonists. (A) A current trace of a immature gonadotropin releasing hormone neuron held at -60 mV, showing the blockage of KRGE-induced inward currents by 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 μM) and picrotoxin (PIC, 50 μM), a non NMDA glutamate receptor antagonist and $GABA_A$ receptor antagonist. (B) Cumulative bar graph shows the mean relative current changes by KRGE alone and KRGE in the presence of blockers (**p<0.01).

tamate receptors and results of our present study are in good agreement with the above cited works.

Furthermore, GABAergic transmission regulates the activity of GnRH neurons in the hypothalamic preoptic area, which controls and governs puberty, as well as the behavioral changes that occur at puberty [31]. There is now substantial evidence that GABAergic neurons exert a potent and direct regulatory influence upon the electrical activity of GnRH neurons through the $GABA_A$ receptor [32-35]. In addition, earlier studies have demonstrated that GABAergic transmission on young GnRH neurons is excitatory resulting from high chloride concentration [36,37]. This suggests that, in our study the effect of KRGE on GnRH neurons is excitatory. Further, the present data demonstrated that the response induced through GABA receptor activation by KRGE was almost 50%, and we found that the inward currents induced by KRGE were suppressed by PIC, a $GABA_A$ receptor antagonist, suggesting that KRGE has GABA-mimetic action through the $GABA_A$ receptors in GnRH neurons. A recent study in GnRH neurons demonstrated that the hypothalamic neurons governing reproductive physiology can extend highly branched dendritic arborizations beyond the blood brain barrier [38]. This recent finding gives us more insight that both the drugs and neurotransmitters flowing directly in the blood may influence the GnRH neuronal activities. Therefore, it is conceivable that the circulating KRGE components that do not enter the blood brain barrier may also affect the GnRH neuronal activities. Furthermore, there are many studies showing that KRGE affects the reproductive physiology, but none of them examined the direct effect of KRGE on the membrane of GnRH neurons.

Taken together, this study demonstrated that KRGE directly acts on GnRH neuronal soma or dendrites by activating GABA and non-NMDA glutamate receptors, and thereby affects the reproductive physiology. These results suggest that KRGE may be a potential target for regulating the reproductive physiology via action on GnRH neurons. However, further studies are required to isolate and identify the compounds with KRGE-mediated action mechanisms on hypothalamic GnRH neurons.

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