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Modulation of inhibitory and excitatory neurotransmissions by Zn^{2+} on the substantia gelatinosa neurons of the trigeminal subnucleus caudalis in mice

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Abstract. The substantia gelatinosa of the trigeminal subnucleus caudalis has been considered to be an essential location for the transference of orofacial sensory signals. The co-localization of inhibitory and excitatory neurotransmitters in the same substantia gelatinosa (SG) neurons has demonstrated their essential part in the modification of nociceptive transmission. Zn^{2+} is particularly numerous in the mammalian central nervous system. There are proofs demonstrating the role of Zn^{2+} in the modulation of voltage- and ligand-gated ion channels. However, little is known about what roles Zn^{2+} may play in the modulation of signal transmission in the SG neurons of the trigeminal subnucleus caudalis (Vc). Therefore, in this study, we used the whole-cell patch clamp technique to find out the effect of Zn^{2+} on the responses of three main neurotransmitters (glycine, GABA, and glutamate) on SG neurons of the Vc in mice. We have proved that Zn^{2+} induces a big potentiation of glycine receptor-mediated response but attenuates GABA- and glutamate-induced responses at micromolar concentrations, however, enhances glutamate-induced response at nanomolar concentration. Taken together, these data demonstrated that Zn^{2+} can modulate glycine, GABA and glutamate-mediated actions on the SG neurons of the Vc and support an important mechanism in spinal sensory information signaling.

Key words: Substantia gelatinosa — Patch clamp techniques — Zn^{2+}

Introduction

The substantia gelatinosa (SG, lamina II) of the trigeminal subnucleus caudalis (Vc, also called the medullary dorsal horn) has been considered to be essential location for the transference of orofacial sensory signals, because it receives the nociceptive events from primary afferents, including thin myelinated Aδ- and unmyelinated C- fibers (Light and Perl 1979; Todd 2002; Santos et al. 2007). Glycine and γ-aminobutyric acid (GABA) are major inhibitory neurotransmitters, whereas glutamate is mainly an excitatory neurotransmitter. The co-localization of inhibitory and excitatory neurotransmitters in the same SG neurons has demonstrated their essential part in the modification of nociceptive transmission (Todd et al. 1996; Kohno et al. 1999; Price et al. 2005). For this reason, if any compound alters the functional properties of neurotransmitters in the SG neurons, it may modify significantly the pain-signaling messages proceeding from orofacial region to the brain.

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Zn^{2+}, known to be a necessary nutrient, is the second most plentiful trace element in the human body and has a fundamental effect on cellular growth, division, and differentiation (Vallee and Falchuk 1981; Coleman 1992). Among all transition metals, Zn^{2+} is also particularly numerous in the mammalian central nervous system (CNS) and is localized with a high concentration in the neuronal parenchyma (Frederickson et al. 1987; Frederickson 1989). This divalent element is also a required factor necessary for the normal operation of the nervous system (Hurley and Shrader 1972). However, paradoxically, at higher concentrations, it may serve as a neurotoxin that leads to some pathological brain diseases (Choi et al. 1988; Duncan et al. 1992; Gower-Winter and Levenson 2012).

There is much evidence demonstrating the role of Zn^{2+} in the modulation of voltage- and ligand-gated ion channels. For example, in the third-order neurons isolated from the crucian carp retina, Zn^{2+} was detected to modulate both glycine receptors and GABA receptors (Li and Yang 1999). In addition, Zn^{2+} also acts an inhibitory neuromodulator for the release of glutamate receptors in the rat hippocampus (Takeda et al. 2003). However, little is known about the roles that Zn^{2+} may play in the modulation of signal transmission in the SG neurons of the Vc. Therefore, in this study, we used the whole-cell patch clamp technique to find out the effect of Zn^{2+} on the responses of three main neurotransmitters (glycine, GABA, and glutamate) on SG neurons.

Materials and Methods

Animal and brain slice preparation

All experiments on living animals were ratified by the Experimental Animal Care and Ethics Committee of Chonbuk National University. Immature male and female Institude of Cancer Research (ICR) mice (7–20 postnatal days) (Damul Science, Suwon, Korea) tested in this study were housed under a stable environment including the 12-hour light/dark cycles (lights on at 06:00) with access to water and food *ad libitum*.

We used the same method to prepare brain slices as in our previous study (Nguyen et al. 2015). Firstly, ICR mice were beheaded; the brains were removed quickly and placed in ice-cold bicarbonate-buffered artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5 KCl, 2.4 $CaCl_2$, 1.2 $MgCl_2$, 11 D-glucose, 1.4 NaH_2PO_4, 25 $NaHCO_3$ and 0.5 sodium ascorbate (pH 7.3~7.4, bubbled with 95% O_2 and 5% CO_2). The brains were cut into coronal slices (180–200 μm in thickness) containing the Vc by a vibratome (VT1200S, Leica Biosystem, Nussluch, Eisfeld, Germany) in ice-cold ACSF and kept in oxygenated ACSF at room temperature for at least one hour before electrophysiological recording.

Electrophysiology and data analysis

Each individual brain slice was moved into the recording chamber. There, it was continuously submerged and perfused with oxygenated ACSF at a flow speed of 4–5 ml/min. To observe the slices, we used an upright microscope (BX51WI, Olympus, Tokyo, Japan) consisting of some Nomarski differential interference contrast optics. The SG (lamina II) of the medullary dorsal horn was identified as a translucent band that was medial to the spinal trigeminal tract and went along the lateral sides of the slice.

The patch pipettes were pulled in a thin-wall borosilicate glass-capillary tubing (PG52151-4, WPI, Sarasota, FL, USA) of the 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). After the glass-capillary electrode was loaded with the pipette solution, the resistance of the recording pipettes was measured at around 4–6 MΩ. To patch the cell, firstly, a gigaohm seal was formed with SG neuron, then the cell membrane patch was ruptured by negative pressure, and electrical measurement was done using a whole-cell patch-clamp recording mode with an Axopatch 200B (Molecular Devices, CA, USA). The currents of the cell membranes were sampled online using a Digidata 1322A (Molecular Devices, CA, USA) interface linked to a desktop computer. The electrophysiological signals were filtered (2 kHz, Bessel filter of Axopatch 200B) before being digitized at a rate of 1 kHz. The cell holding potential was maintained at –60 mV throughout the recordings. The acquisition and analysis of the data were done by using Clampex 10.6 software (Molecular Devices, CA, USA). All our recordings were done at room temperature.

Chemicals

Zinc sulfate heptahydrate, glycine, GABA, glutamate, and the chemicals to make ACSF were purchased from Sigma (USA). Stocks of all drugs were prepared according to their solubility in distilled water. We diluted the stock solutions to the desired final concentrations in ACSF just before use and were applied to the neurons *via* bath application.

Statistics

Software named Origin 7 (OriginLab Corp., Northampton, MA, USA) was used to plot the traces. All values were described in the form of the mean ± SEM. To compare the average amplitudes of inward currents between two groups, we used a paired *t*-test. A p-value < 0.05 was recognized as the statistically significant standard.

Results

To investigate whether there were any changes in the response induced by inhibitory or excitatory neurotransmitters in SG neurons, we compared the responses elicited by glycine (30 μM), GABA (30 μM), and glutamate (30 μM) alone and in the presence of Zn^{2+}. The cell-voltage clamp recordings were obtained from 43 SG neurons that belonged to 28 ICR mice of 7–20 postnatal days.

Zn^{2+} and glycine

First, we checked the effect of Zn^{2+} on glycine, an inhibitory neurotransmitter. When glycine was successively applied, the inward currents were induced. After that, Zn^{2+} at a low concentration (3 μM) was pretreated alone around five minutes; the Zn^{2+} did not elicit any detectable membrane currents. However, a glycine-induced inward current (I_{Gly}) was strongly potentiated when applied simultaneously with Zn^{2+} (Fig. 1A). As we observed in the bar graph, the mean amplitudes of I_{Gly} alone and in the presence of Zn^{2+} were 59.6 ± 13.5 pA and 149 ± 35.6 pA, respectively ($n = 8$, $p < 0.01$, Fig. 1B). Zn^{2+} potentiated these glycine currents when co-applied extracellularly at a concentration of 3 μM.

Besides, it is reported that the physiological extracellular Zn^{2+} concentration is rather in the nanomolar range (Thompson et al. 2000; Kay 2003; Frederickson et al. 2006). Therefore, the effect between Zn^{2+} and glycine was checked in a dose-response manner at different concentration of Zn^{2+} ranging from 10–3,000 nM (Fig. 1C). There was an increase of the I_{Gly} flowing, the rise of Zn^{2+} concentration with an EC_{50} of 4,093 nM.

Zn^{2+} and GABA

We continued to analyze the effect of Zn^{2+} on another inhibitory neurotransmitter, GABA. As shown in Fig. 2, successive application of GABA 30 μM created a detectable change in membrane current. When Zn^{2+} (3 μM) was treated together with GABA, the GABA-induced inward current (I_{GABA}) was decreased partially (Fig. 2A). The bar graph shows that the mean inward current induced by GABA (85.1 ± 15.7 pA) was reduced to 59.6 ± 19.7 pA in the presence of Zn^{2+} ($n = 6$, $p < 0.01$, Fig. 2B). These results indicate that Zn^{2+} at 3 μM concentration inhibits I_{GABA} on SG neurons. Besides, we also evaluated the effect Zn^{2+} in nanomolar concentrations to GABA 30 μM. However, 300 nM Zn^{2+} did not change GABA-mediated responses. There is no significant effect between the mean inward currents induced by GABA alone and in the presence of Zn^{2+} (87.3 ± 15.9 pA and 90.1 ± 14.7 pA, respectively) ($n = 9$, $p > 0.05$, Fig. 2C).

Zn^{2+} and glutamate

In the next stage of the experiment, we examined how Zn^{2+} affected the excitatory neurotransmitter of SG neurons, the

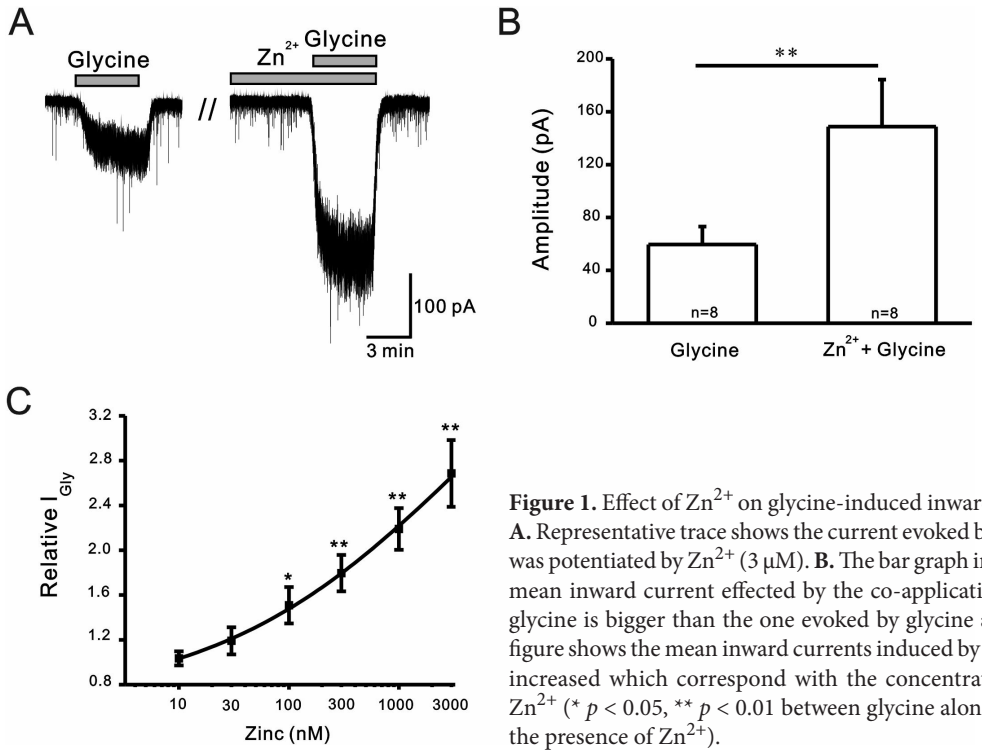

Figure 1. Effect of Zn^{2+} on glycine-induced inward current (I_{Gly}). **A.** Representative trace shows the current evoked by glycine 30 μM was potentiated by Zn^{2+} (3 μM). **B.** The bar graph indicates that the mean inward current effected by the co-application of Zn^{2+} and glycine is bigger than the one evoked by glycine alone. **C.** Curve figure shows the mean inward currents induced by glycine (30 μM) increased which correspond with the concentration changes of Zn^{2+} (* $p < 0.05$, ** $p < 0.01$ between glycine alone and glycine in the presence of Zn^{2+}).

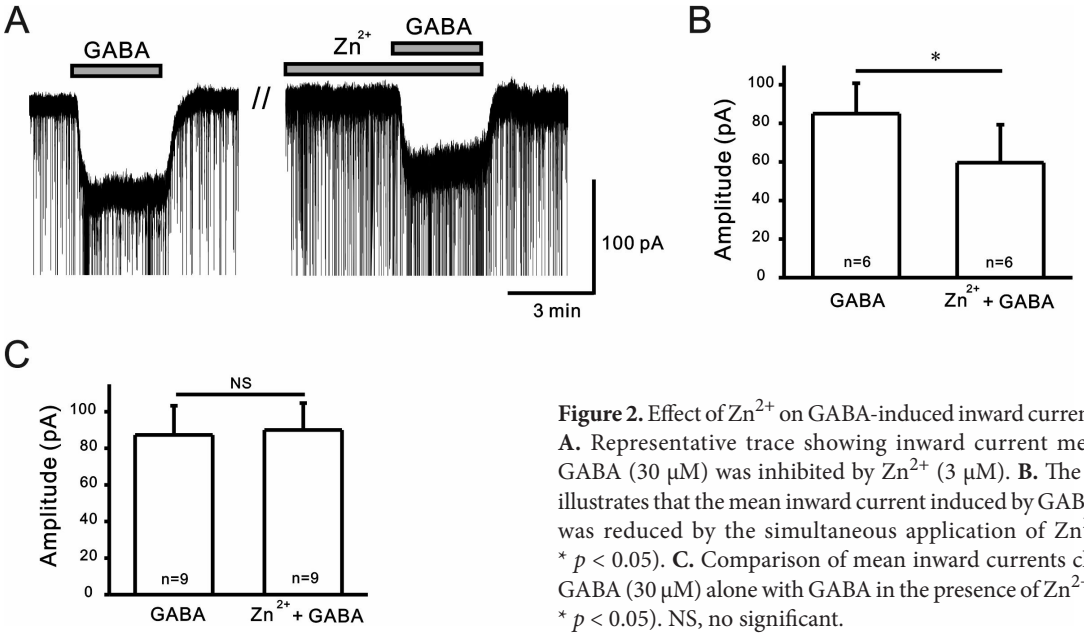

Figure 2. Effect of Zn^{2+} on GABA-induced inward current (I_{GABA}). **A.** Representative trace showing inward current mediated by GABA (30 μM) was inhibited by Zn^{2+} (3 μM). **B.** The bar graph illustrates that the mean inward current induced by GABA (30 μM) was reduced by the simultaneous application of Zn^{2+} (3 μM; * $p < 0.05$). **C.** Comparison of mean inward currents changed by GABA (30 μM) alone with GABA in the presence of Zn^{2+} (300 nM; * $p < 0.05$). NS, no significant.

glutamate receptors. First, Zn^{2+} was also applied at 3 μM, as in previous experiments. However, in this level of Zn^{2+} concentration, Zn^{2+} did not show any change on glutamate (30 μM)-induced inward current (I_{Glu}) (data not shown). As the Zn^{2+} concentration was increased to 10 μM, the glutamate-activated current was strongly decreased by the simultaneous application with Zn^{2+} (Fig. 3A). The mean amplitude of I_{Glu} alone (41.8 ± 8.5 pA) was decreased in the presence of Zn^{2+} 10 μM (20.7 ± 4.5 pA) ($n = 7$, $p < 0.01$, Fig. 3B). Again, these results provide evidence that Zn^{2+} inhibits the glutamate-mediated response.

Interestingly, at nanomolar concentration of Zn^{2+}, we found that Zn^{2+} (300 nM) increased the I_{Glu} (Fig. 3C). The mean inward current evoked by glutamate 30 μM in the absence and presence of Zn^{2+} 300 nM were –41.1 ± 10.9 pA and –53.8 ± 14.1 pA, respectively ($n = 6$, $p < 0.05$, Fig. 3D). To summarize all the data between Zn^{2+} and glutamate, these results provide evidence that Zn^{2+} has biphasic effects to glutamate: at the nanomolar concentration (300 nM), Zn^{2+} increases I_{Glu} but at the micromolar concentration (10 μM), Zn^{2+} inhibits I_{Glu}.

Discussion

Zn^{2+} has been known to play many physiological roles in the CNS, including synaptic messenger transmission (Christine and Choi 1990; Xie and Smart 1993), intracellular second messenger pathways (Forbes et al. 1991; Weinberger and Rostas 1991), and functional modulation of ion channels (Winegar and Lansman 1990; Li and Yang 1999). In this study, we used an exogenous Zn^{2+} application in order to examine the physiological role of synaptic Zn^{2+} on amino-acid neurotransmissions. By the electrophysiological approach, we have demonstrated that Zn^{2+} has different effects on different inhibitory and excitatory neurotransmitters in SG neurons of Vc. At a micromolar concentration (3 μM), Zn^{2+} induces a big potentiation of glycine receptor-mediated response but attenuates GABAergic inputs. With glutamate, Zn^{2+} has opposite effects depending on the concentration, Zn^{2+} with micromolar concentration (10 μM), decreases glutamate-induced inward currents but increases them with nanomolar concentration (300 nM)

Growing evidence suggests that released Zn^{2+} can perform as an extracellular modulator of inhibitory and/or excitatory synaptic events (Choi and Koh 1998). Besides, Zn^{2+} can enter postsynaptic neurons through the Ca^{2+}-permeable channels and thus exert intracellular effects on physiological signaling functions of ion channels (Weiss et al. 1993; Freund and Reddig 1994; Yin and Weiss 1995). As a signaling substance, an alteration in extracellular Zn^{2+} may change the operation of several membrane channels and neurotransmitters by modifying the transmitter releaser and/or the sensitivity of the postsynaptic cells to transmitter molecules (Harrison and Gibbons 1994; Smart et al. 1994).

Glycine is major fast inhibitory neurotransmitters in the spinal cord that is accumulated in small synaptic vesicles (Burger et al. 1991; Christensen and Fonnum 1991). Glycine receptors are composed of a combination of five distinct transmembrane protein subunits (Pfeiffer et al. 1982). Each receptor subunit includes a large extracellular N-terminal domain and four transmembrane spanning domains (term

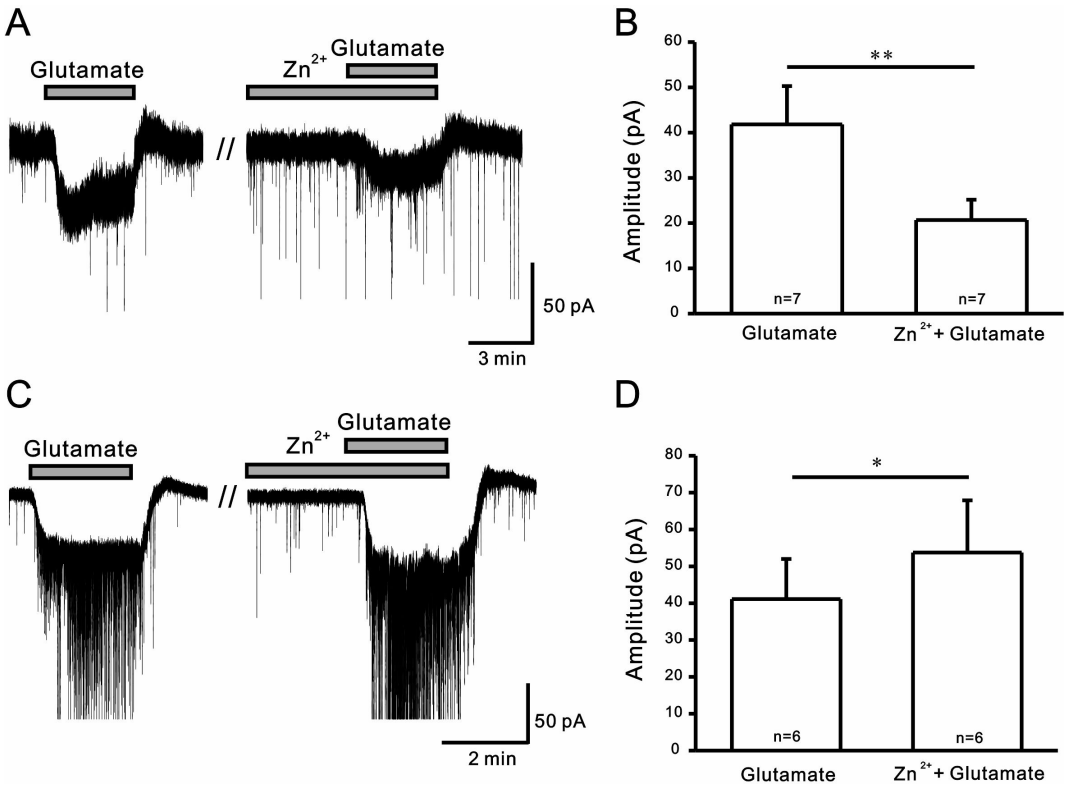

Figure 3. Effect of Zn^2 on glutamate-induced inward current (I_{Glu}). **A.** The representative trace showing current evoked by glutamate 30 μM was reduced by Zn^{2+} 10 μM. **B.** The bar graph compares the mean inward current changed by glutamate alone with glutamate in the presence of Zn^{2+} (10 μM). **C.** Glutamate (30 μM)-induced inward current was increased by the simultaneous application of Zn^{2+} (300 nM). **D.** There is a significant difference between the means values created by glutamate alone and glutamate in the presence of Zn^{2+} (300 nM) (* $p < 0.05$, ** $p < 0.01$).

M1-M4), in which the second segment (M2) composes the channel pore-lining α-helix (Karlin and Akabas 1995). Glycine-induced currents have been demonstrated to be potentiated by Zn^{2+} at a concentration between 0.1 and 10 μM in third-order neurons isolated from the crucian carp retina, in *Xenopus* oocytes and human embryonic kidney cells (Laube et al. 1995; Li and Yang 1999; Miller et al. 2005). At low concentrations, this ion metal modulates glycine-mediated currents by increasing the apparent agonist affinity without altering the maximal inducible current (Bloomenthal et al. 1994; Laube et al. 1995). With the results from molecular experiments, it has been concluded that this Zn^{2+} potentiation of glycine-gated currents was specifically mediated by the allosteric signal-transduction processing between ligand binding and channel activation, which involved the key control elements, the residues in the M1-M2 loop and the M2-M3 loop (Lynch et al. 1997, 1998; Miller et al. 2005). Conversely, a higher concentration level of Zn^{2+} (50 μM) significantly inhibited the glycine responses in the cultured rat spinal-cord neurons (Bloomenthal et al. 1994; Laube 2002). Zn^{2+} is a powerful modulator that can increase or decrease the open probability of a glycine channel in a way consistent with a strengthened or impaired affinity of the glycine receptor (Laube et al. 2000).

Another major inhibitory neurotransmitter in the CNS is GABA. These receptors contain some allosteric binding locations for several classes of chemicals that can modulate receptor function (Sivilotti and Nistri 1991; Bowery and Smart 2006). Many studies have demonstrated that Zn^{2+} inhibits $GABA_A$ responses on hippocampal neurons of rats, such as kindled adult hippocampal granule cells (Buhl et al. 1996) and cultured hippocampal neurons (Barberis et al. 2000), as well as on guinea-pig hippocampal neurons (Ruiz et al. 2004). The reduction of $GABA_A$ response by Zn^{2+} mainly appears to result from decreasing the opening frequency of $GABA_A$ single channels (Legendre and Westbrook 1991; Smart 1992). Each $GABA_A$ receptor has been shown to be formed by many different protein subunits (α, β, γ, and δ) and abundant subtypes by using the cDNA cloning techniques (Verdoorn et al. 1990). The inhibition of $GABA_A$ responses by Zn^{2+} obviously depends on the subunit components. $GABA_A$ receptor possessing αβ subunits

is more sensitive to Zn^{2+} inhibition than are the receptors consisted of γ subunits (Draguhn et al. 1990; Smart et al. 1991). In contrast, Zn^{2+} was reported to potentiate $GABA_A$ receptor activity in the retinal Müller glial cells in some receptor subunits (Qian et al. 1996). In addition, in the rat hippocampus, the extracellular Zn^{2+} also affected $GABA_B$ receptors in a biphasic manner by modulating $GABA_B$ binding biphasically (Xie and Smart 1991). As can be seen from those studies, Zn^{2+} has many effects on GABA.

Beyond effects on inhibitory neurotransmitters, glycine receptors, and $GABA_A$ receptors, Zn^{2+} also has a powerful modulation effect on glutamate-mediated responses. Glutamate or excitatory amino-acid receptors are considered to be the main neurotransmitter receptors that modulate the fast synaptic excitation in the CNS of the mammal (Gasic and Hollmann 1992). It has long been known that a large amount of Zn^{2+} is concentrated inside vesicles of the glutamatergic terminals in the CNS (Frederickson 1989; Choi and Koh 1998). This relation points toward the logical role of Zn^{2+} in the modulation of glutamate response. Depending on the pharmacological functions and the interaction of characteristic agonists, the glutamate receptors are classified into many subtypes (Gasic and Hollmann 1992). Many different effects of Zn^{2+} on different glutamate subtypes have been demonstrated. The presynaptic glutamate concentration released in the rat hippocampal CA1 and CA3, as well as the entorhinal cortex region, were attenuated by the perfusion with Zn^{2+} (Takeda et al. 2003, 2004). At the single-channel level, Zn^{2+} was proved to powerfully inhibit *N*-methyl-*D*-aspartate (NMDA) channel currents in murine neocortical neurons. Some main mechanisms explained for Zn^{2+} inhibition of NMDA receptors includes the decrease in channel open frequency and the voltage-dependent amplitude reduction, which suggested a fast channel block (Christine and Choi 1990). Besides, some lines also show that Zn^{2+} increased the excitation mediated by α-amino-3-hydroxy-5-methyl-4-isoxazoleproprionoc acid (AMPA) receptors and NMDA receptors in mouse cultured cortical and rat cultured hippocampal CA1 neurons, respectively (Peters et al. 1987; Kim et al. 2002). To supplement the above abundant effects of Zn^{2+}, our study has proved that Zn^{2+} also has biphasic effects on the glutamate-induced inward current in the SG neurons of Vc. The hypothesis for the opposite modulations of glutamatergic transmission by Zn^{2+} is the different actions on different types of glutamate receptors which co-localized at the glutamatergic postsynapses (Rassendren et al. 1990). These mechanisms happen in a concentration dependence of Zn^{2+} which corresponds with the difference in the apparent affinity values (300 nM for the potentiation and 10 μM for the inhibition). This specific characterization of Zn^{2+} on glutamate receptors was also reported in the *Xenopus* oocytes that at a low concentration, Zn^{2+} inhibited NMDA responses and increased non-NMDA response, but at higher concentration, Zn^{2+} inhibited non-NMDA currents (Rassendren et al. 1990). Further investigation needs to be done to find out which types of glutamate receptor involving in the potentiation and inhibition phenomena between Zn^{2+} and glutamate.

In conclusion, the above clear evidence reveals to some extent the diverse effects of Zn^{2+} on different neurotransmitters in the SG neurons. The opposite influences of this metal ion may originate from its different processes that interact with various binding sites on different receptors and with distinct affinities. Some growing studies have elucidated that Zn^{2+} also plays an important role in the modulation of pain transmission (Larson and Kitto 1997; Velazquez et al. 1999). Taken together, the regulatory action of Zn^{2+} to the neurotransmitters in the SG neurons implies an important mechanism in pain information processing in the CNS which has a part in the plasticity of neuronal circuits. Further research needs to be done to discover the concrete mechanism by which Zn^{2+} not only excites neurotransmitters but also inhibits receptors in the SG neurons of the Vc.

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Conflict of interest. The authors declare that they have no conflicts of interest.

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