General Physiology and Biophysics
Revised manuscript #1

Title: Modulation of inhibitory and excitatory neurotransmissions by Zn2+ on the substantia gelatinosa neurons of the trigeminal subnucleus caudalis in mice
Running title: Modulation of neurotransmitters by Zn2+ on the SG neurons
Create date: 2019-07-05

Name	*Affiliations*
Mrs Hoang Thi Thanh Nguyen	1. Department of Oral Physiology, School of Dentistry and Institute of Oral Bioscience, Chonbuk National University, Jeonju, South Korea
Mrs Seon Hui Jang	1. Department of Oral Physiology, School of Dentistry and Institute of Oral Bioscience, Chonbuk National University, Jeonju, South Korea
Mrs Soo Joung Park	1. Department of Oral Physiology, School of Dentistry and Institute of Oral Bioscience, Chonbuk National University, Jeonju, South Korea
Mr Dong Hyu Cho	1. Department of Obstetrics and Gynecology, Chonbuk National University Hospital and School of Medicine, Jeonju, South Korea
Mr Seong Kyu Han	1. Department of Oral Physiology, School of Dentistry and Institute of Oral Bioscience, Chonbuk National University, Jeonju, South Korea

Corresponding author: Mr Dong Hyu Cho <obgyn2001@jbnu.ac.kr>

Corresponding author: Mr Seong Kyu Han <skhan@jbnu.ac.kr>

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 SG neurons has demonstrated their essential part in the modification of nociceptive transmission. Zn2+ is particularly numerous in the mammalian central nervous system. There are proofs demonstrating the role of Zn2+ in the modulation of voltage- and ligand-gated ion channels. However, little is known about what roles Zn2+ 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 Zn2+ on the responses of three main neurotransmitters (glycine, GABA, and glutamate) on SG neurons of the VC in mice. We have proved that Zn2+ 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 Zn2+ 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.

Keywords: patch clamp techniques; substantia gelatinosa; Zn2+

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

Hoang Thi Thanh Nguyen [1], Seon Hui Jang [1], Soo Joung Park [1], Dong Hyu Cho [2,*], Seong Kyu Han [1,*]

[1] Department of Oral Physiology, School of Dentistry and Institute of Oral Bioscience, Chonbuk National University, Jeonju, Republic of Korea, 54896

[2] Department of Obstetrics and Gynecology, Chonbuk National University Hospital and School of Medicine, Jeonju, Republic of Korea, 54896

First author: Hoang Thi Thanh Nguyen. Email: thanhhoangrhm@gmail.com

Running title: Modulation of neurotransmitters by Zn^{2+} on the SG neurons

Correspondence to: Seong Kyu Han, Department of Oral Physiology, School of Dentistry and Institute of Oral Bioscience, Chonbuk National University, Jeonju, Republic of Korea. Tel: (+82) 63-270-4028. E-mail: skhan@jbnu.ac.kr

Dong Hyu Cho, Department of Obstetrics and Gynecology, Chonbuk National University Hospital and School of Medicine, Jeonju, Republic of Korea. Tel: (+82)10-6424-4121. E-mail: obgyn2001@jbnu.ac.kr

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 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 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 V_C 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.

Keywords 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.

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

1. 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 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 (*p*H 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 V_C 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.

2. 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 (*p*H 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.

3. 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

107 solubility in distilled water. We diluted the stock solutions to the desired final concentrations
108 in ACSF just before use and were applied to the neurons via bath application.

109 *4. Statistics*

110 A software named Origin 7 (OriginLab Corp., Northampton, MA, USA) was used to plot
111 the traces. All values were described in the form of the mean ± S.E.M. To compare the
112 average amplitudes of inward currents between two groups, we used a paired *t*-test. A *p*-value
113 < 0.05 was recognized as the statistically significant standard.

114 **Results**

115 To investigate whether there were any changes in the response induced by inhibitory or
116 excitatory neurotransmitters in SG neurons, we compared the responses elicited by glycine
117 (30 μM), GABA (30 μM), and glutamate (30 μM) alone and in the presence of Zn^{2+}. The cell-
118 voltage clamp recordings were obtained from 43 SG neurons that belonged to 28 ICR mice of
119 7-20 postnatal days.

120 **1. Zn^{2+} and Glycine**

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

129 Besides, it is reported that the physiological extracellular Zn^{2+} concentration is rather in
130 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.

2. 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).

3. Zn^{2+} and glutamate

In the next stage of the experiment, we examined how Zn^{2+} affected the excitatory neurotransmitter of SG neurons, the 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 3.B). 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

179 1994; Yin and Weiss 1995). As a signaling substance, an alteration in extracellular Zn^{2+} may
180 change the operation of several membrane channels and neurotransmitters by modifying the
181 transmitter releaser and/or the sensitivity of the postsynaptic cells to transmitter molecules
182 (Harrison and Gibbons 1994; Smart et al. 1994).

183 Glycine is a major fast inhibitory neurotransmitters in the spinal cord that is accumulated
184 in small synaptic vesicles (Burger et al. 1991; Christensen and Fonnum 1991). Glycine
185 receptors are composed of a combination of five distinct transmembrane protein subunits
186 (Pfeiffer et al. 1982). Each receptor subunit includes a large extracellular N-terminal domain
187 and four transmembrane spanning domains (term M1-M4), in which the second segment (M2)
188 composes the channel pore-lining α-helix (Karlin and Akabas 1995). Glycine-induced
189 currents have been demonstrated to be potentiated by Zn^{2+} at a concentration between 0.1 and
190 10 μM in third-order neurons isolated from the crucian carp retina, in *Xenopus* oocytes and
191 human embryonic kidney cells. (Laube et al. 1995; Li and Yang 1999; Miller et al. 2005). At
192 low concentrations, this ion metal modulates glycine-mediated currents by increasing the
193 apparent agonist affinity without altering the maximal inducible current (Bloomenthal et al.
194 1994; Laube, Kuhse et al. 1995). With the results from molecular experiments, it has been
195 concluded that this Zn^{2+} potentiation of glycine-gated currents was specifically mediated by
196 the allosteric signal-transduction processing between ligand binding and channel activation,
197 which involved the key control elements, the residues in the M1-M2 loop and the M2-M3
198 loop (Lynch et al. 1997; Lynch et al. 1998; Miller, Da Silva et al. 2005). Conversely, a higher
199 concentration level of Zn^{2+} (50 μM) significantly inhibited the glycine responses in the
200 cultured rat spinal-cord neurons (Bloomenthal, Goldwater et al. 1994; Laube 2002). Zn^{2+} is a
201 powerful modulator that can increase or decrease the open probability of a glycine channel in
202 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. A $GABA_A$ 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, Minami et al. 2003; Takeda et al. 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 V_C. 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, Lory 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 to inhibits receptors in the SG neurons of the V_C.

Acknowledgements

This research was supported by Basic Research Program through the National Research Foundation of Korea (NRF) funded by Ministry of Education (2016R1D1A3B03932241) and Ministry of Science, ICT & Future Planning (2015R1C1A1A02036793).

Conflict of interest

The authors declare that they have no conflicts of interest.

References

Barberis A, Cherubini E, Mozrzymas JW (2000): Zinc inhibits miniature GABAergic currents

by allosteric modulation of GABAA receptor gating. J. Neurosci. **20**, 8618-8627

Bloomenthal AB, Goldwater E, Pritchett DB, Harrison NL (1994): Biphasic modulation of the strychnine-sensitive glycine receptor by Zn2+. Mol. Pharmacol. **46**, 1156-1159

Bowery NG, Smart TG (2006): GABA and glycine as neurotransmitters: a brief history. Br. J. Pharmacol. **147**, S109-119

Buhl EH, Otis TS, Mody I (1996): Zinc-induced collapse of augmented inhibition by GABA in a temporal lobe epilepsy model. Science **271**, 369-373

Burger PM, Hell J, Mehl E, Krasel C, Lottspeich F, Jahn R (1991): GABA and glycine in synaptic vesicles: storage and transport characteristics. Neuron **7**, 287-293

Choi DW, Koh JY (1998): Zinc and brain injury. Annu. Rev. Neurosci. **21**, 347-375

Choi DW, Yokoyama M, Koh J (1988): Zinc neurotoxicity in cortical cell culture. Neuroscience **24**, 67-79

Christensen H, Fonnum F (1991): Uptake of glycine, GABA and glutamate by synaptic vesicles isolated from different regions of rat CNS. Neurosci. Lett. **129**, 217-220

Christine CW, Choi DW (1990): Effect of zinc on NMDA receptor-mediated channel currents in cortical neurons. J. Neurosci. **10**, 108-116

Coleman JE (1992): Zinc proteins: enzymes, storage proteins, transcription factors, and replication proteins. Annu. Rev. Biochem. **61**, 897-946

Draguhn A, Verdorn TA, Ewert M, Seeburg PH, Sakmann B (1990): Functional and molecular distinction between recombinant rat GABAA receptor subtypes by Zn2+. Neuron **5**, 781-788

Duncan MW, Marini AM, Watters R, Kopin IJ, Markey SP (1992): Zinc, a neurotoxin to cultured neurons, contaminates cycad flour prepared by traditional guamanian methods. J. Neurosci. **12**, 1523-1537

298 Forbes IJ, Zalewski PD, Giannakis C (1991): Role for zinc in a cellular response mediated by
299 protein kinase C in human B lymphocytes. Exp. Cell. Res. **195**, 224-229
300 Frederickson CJ (1989): Neurobiology of zinc and zinc-containing neurons. Int. Rev.
301 Neurobiol. **31**, 145-238
302 Frederickson CJ, Giblin LJ, Krezel A, McAdoo DJ, Mueller RN, Zeng Y, Balaji RV, Masalha
303 R, Thompson RB, Fierke CA, Sarvey JM, de Valdenebro M, Prough DS, Zornow MH
304 (2006): Concentrations of extracellular free zinc (pZn)e in the central nervous system
305 during simple anesthetization, ischemia and reperfusion. Exp Neurol. **198**, 285-293
306 Frederickson CJ, Kasarskis EJ, Ringo D, Frederickson RE (1987): A quinoline fluorescence
307 method for visualizing and assaying the histochemically reactive zinc (bouton zinc) in
308 the brain. J. Neurosci. Methods. **20**, 91-103
309 Freund WD, Reddig S (1994): AMPA/Zn(2+)-induced neurotoxicity in rat primary cortical
310 cultures: involvement of L-type calcium channels. Brain Res. **654**, 257-264
311 Gasic GP, Hollmann M (1992): Molecular neurobiology of glutamate receptors. Annu. Rev.
312 Physiol. **54**, 507-536
313 Gower-Winter SD, Levenson CW (2012): Zinc in the central nervous system: From molecules
314 to behavior. Biofactors **38**, 186-193
315 Harrison NL, Gibbons SJ (1994): Zn2+: an endogenous modulator of ligand- and voltage-
316 gated ion channels. Neuropharmacology **33**, 935-952
317 Hurley LS, Shrader RE (1972): Congenital malformations of the nervous system in zinc-
318 deficient rats. In: Neurobiology of the Trace Metals Zinc and Copper (Ed. CC Pfeiffer),
319 pp. 51-60, Academic Press, New York
320 Karlin A, Akabas MH (1995): Toward a structural basis for the function of nicotinic
321 acetylcholine receptors and their cousins. Neuron **15**, 1231-1244

322 Kay AR (2003): Evidence for chelatable zinc in the extracellular space of the hippocampus,
323 but little evidence for synaptic release of Zn. J. Neurosci. **23**, 6847-6855
324 Kim TY, Hwang JJ, Yun SH, Jung MW, Koh JY (2002): Augmentation by zinc of NMDA
325 receptor-mediated synaptic responses in CA1 of rat hippocampal slices: mediation by
326 Src family tyrosine kinases. Synapse **46**, 49-56
327 Kohno T, Kumamoto E, Higashi H, Shimoji K, Yoshimura M (1999): Actions of opioids on
328 excitatory and inhibitory transmission in substantia gelatinosa of adult rat spinal cord.
329 J. Physiol. **518**, 803-813
330 Larson AA, Kitto KF (1997): Manipulations of zinc in the spinal cord, by intrathecal injection
331 of zinc chloride, disodium-calcium-EDTA, or dipicolinic acid, alter nociceptive
332 activity in mice. J. Pharmacol. Exp. Ther. **282**, 1319-1325
333 Laube B (2002): Potentiation of inhibitory glycinergic neurotransmission by Zn2+: a
334 synergistic interplay between presynaptic P2X2 and postsynaptic glycine receptors.
335 Eur. J. Neurosci. **16**, 1025-1036
336 Laube B, Kuhse J, Betz H (2000): Kinetic and mutational analysis of Zn2+ modulation of
337 recombinant human inhibitory glycine receptors. J. Physiol. **522**, 215-230
338 Laube B, Kuhse J, Rundstrom N, Kirsch J, Schmieden V, Betz H (1995): Modulation by zinc
339 ions of native rat and recombinant human inhibitory glycine receptors. J. Physiol. **483**,
340 613-619
341 Legendre P, Westbrook GL (1991): Noncompetitive inhibition of gamma-aminobutyric acidA
342 channels by Zn. Mol. Pharmacol. **39**, 267-274
343 Li P, Yang XL (1999): Zn2+ differentially modulates glycine receptors versus GABA
344 receptors in isolated carp retinal third-order neurons. Neurosci. Lett. **269**, 75-78
345 Light AR, Perl ER (1979): Spinal termination of functionally identified primary afferent

346 neurons with slowly conducting myelinated fibers. J. Comp. Neurol. **186**, 133-150
347 Lynch JW, Jacques P, Pierce KD, Schofield PR (1998): Zinc potentiation of the glycine
348 receptor chloride channel is mediated by allosteric pathways. J. Neurochem. **71**, 2159-
349 2168
350 Lynch JW, Rajendra S, Pierce KD, Handford CA, Barry PH, Schofield PR (1997):
351 Identification of intracellular and extracellular domains mediating signal transduction
352 in the inhibitory glycine receptor chloride channel. EMBO J. **16**, 110-120
353 Miller PS, Da Silva HM, Smart TG (2005): Molecular basis for zinc potentiation at
354 strychnine-sensitive glycine receptors. J. Biol. Chem. **280**, 37877-37884
355 Nguyen HT, Bhattarai JP, Park SJ, Lee JC, Cho DH, Han SK (2015): Enhanced GABA action
356 on the substantia gelatinosa neurons of the medullary dorsal horn in the offspring of
357 streptozotocin-injected mice. J. Diabetes Complications **29**, 629-636
358 Peters S, Koh J, Choi DW (1987): Zinc selectively blocks the action of N-methyl-D-aspartate
359 on cortical neurons. Science **236**, 589-593
360 Pfeiffer F, Graham D, Betz H (1982): Purification by affinity chromatography of the glycine
361 receptor of rat spinal cord. J. Biol. Chem. **257**, 9389-9393
362 Price TJ, Cervero F, de Koninck Y (2005): Role of cation-chloride-cotransporters (CCC) in
363 pain and hyperalgesia. Curr. Top. Med. Chem. **5**, 547-555
364 Qian H, Malchow RP, Chappell RL, Ripps H (1996): Zinc enhances ionic currents induced in
365 skate Muller (glial) cells by the inhibitory neurotransmitter GABA. Proc. Biol. Sci.
366 **263**, 791-796
367 Rassendren FA, Lory P, Pin JP, Nargeot J (1990): Zinc has opposite effects on NMDA and
368 non-NMDA receptors expressed in Xenopus oocytes. Neuron **4**, 733-740
369 Ruiz A, Walker MC, Fabian-Fine R, Kullmann DM (2004): Endogenous zinc inhibits

370 GABA(A) receptors in a hippocampal pathway. J. Neurophysiol. **91**, 1091-1096
371 Santos SF, Rebelo S, Derkach VA, Safronov BV (2007): Excitatory interneurons dominate
372 sensory processing in the spinal substantia gelatinosa of rat. J. Physiol. **581**, 241-254
373 Sivilotti L, Nistri A (1991): GABA receptor mechanisms in the central nervous system. Prog.
374 Neurobiol. **36**, 35-92
375 Smart TG (1992): A novel modulatory binding site for zinc on the GABAA receptor complex
376 in cultured rat neurones. J. Physiol. **447**, 587-625
377 Smart TG, Moss SJ, Xie X, Huganir RL (1991): GABAA receptors are differentially sensitive
378 to zinc: dependence on subunit composition. Br. J. Pharmacol. **103**, 1837-1839
379 Smart TG, Xie X, Krishek BJ (1994): Modulation of inhibitory and excitatory amino acid
380 receptor ion channels by zinc. Prog. Neurobiol. **42**, 393-441
381 Takeda A, Minami A, Seki Y, Oku N (2003): Inhibitory function of zinc against excitation of
382 hippocampal glutamatergic neurons. Epilepsy. Res. **57**, 169-174
383 Takeda A, Minami A, Seki Y, Oku N (2004): Differential effects of zinc on glutamatergic and
384 GABAergic neurotransmitter systems in the hippocampus. J. Neurosci. Res. **75**, 225-
385 229
386 Thompson RB, Whetsell WO Jr, Maliwal BP, Fierke CA, Frederickson CJ (2000):
387 Fluorescence microscopy of stimulated Zn(II) release from organotypic cultures of
388 mammalian hippocampus using a carbonic anhydrase-based biosensor system. J.
389 Neurosci. Methods **96**, 35-45
390 Todd AJ (2002): Anatomy of primary afferents and projection neurones in the rat spinal dorsal
391 horn with particular emphasis on substance P and the neurokinin 1 receptor. Exp.
392 Physiol. **87**, 245-249
393 Todd AJ, Watt C, Spike RC, Sieghart W (1996): Colocalization of GABA, glycine, and their

receptors at synapses in the rat spinal cord. J. Neurosci. **16**, 974-982

Vallee BL, Falchuk KH (1981): Zinc and gene expression. Philos. Trans. R. Soc. Lond. B. Biol. Sci. **294**, 185-197

Velazquez RA, Cai Y, Shi Q, Larson AA (1999): The distribution of zinc selenite and expression of metallothionein-III mRNA in the spinal cord and dorsal root ganglia of the rat suggest a role for zinc in sensory transmission. J. Neurosci. **19**, 2288-2300

Verdoorn TA, Draguhn A, Ymer S, Seeburg PH, Sakmann B (1990): Functional properties of recombinant rat GABAA receptors depend upon subunit composition. Neuron **4**, 919-928

Weinberger RP, Rostas JA (1991): Effect of zinc on calmodulin-stimulated protein kinase II and protein phosphorylation in rat cerebral cortex. J. Neurochem. **57**, 605-614

Weiss JH, Hartley DM, Koh JY, Choi DW (1993): AMPA receptor activation potentiates zinc neurotoxicity. Neuron **10**, 43-49

Winegar BD, Lansman JB (1990): Voltage-dependent block by zinc of single calcium channels in mouse myotubes. J. Physiol. **425**, 563-578

Xie X, Smart TG (1993): Giant GABAB-mediated synaptic potentials induced by zinc in the rat hippocampus: paradoxical effects of zinc on the GABAB receptor. Eur. J. Neurosci **5**, 430-436

Xie XM, Smart TG (1991): A physiological role for endogenous zinc in rat hippocampal synaptic neurotransmission. Nature **349**, 521-524

Yin HZ, Weiss JH (1995): Zn(2+) permeates Ca(2+) permeable AMPA/kainate channels and triggers selective neural injury. Neuroreport **6**, 2553-2556

Figure legends

Figure 1: Effect of Zn^{2+} on I_{Gly}. (A) The 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 Gly alone. (C) Curve figure shows the mean inward currents induced by glycine 30 µM increased which correspond with the concentration changes of Zn^{2+} (* $p < 0.05$, ** $p < 0.01$).

Figure 2: Effect of Zn^{2+} on 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).

Figure 3: Effect of Zn^{2} on 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 (* $p < 0.05$, ** $p < 0.0.1$).

Fig. 1 Download full resolution image

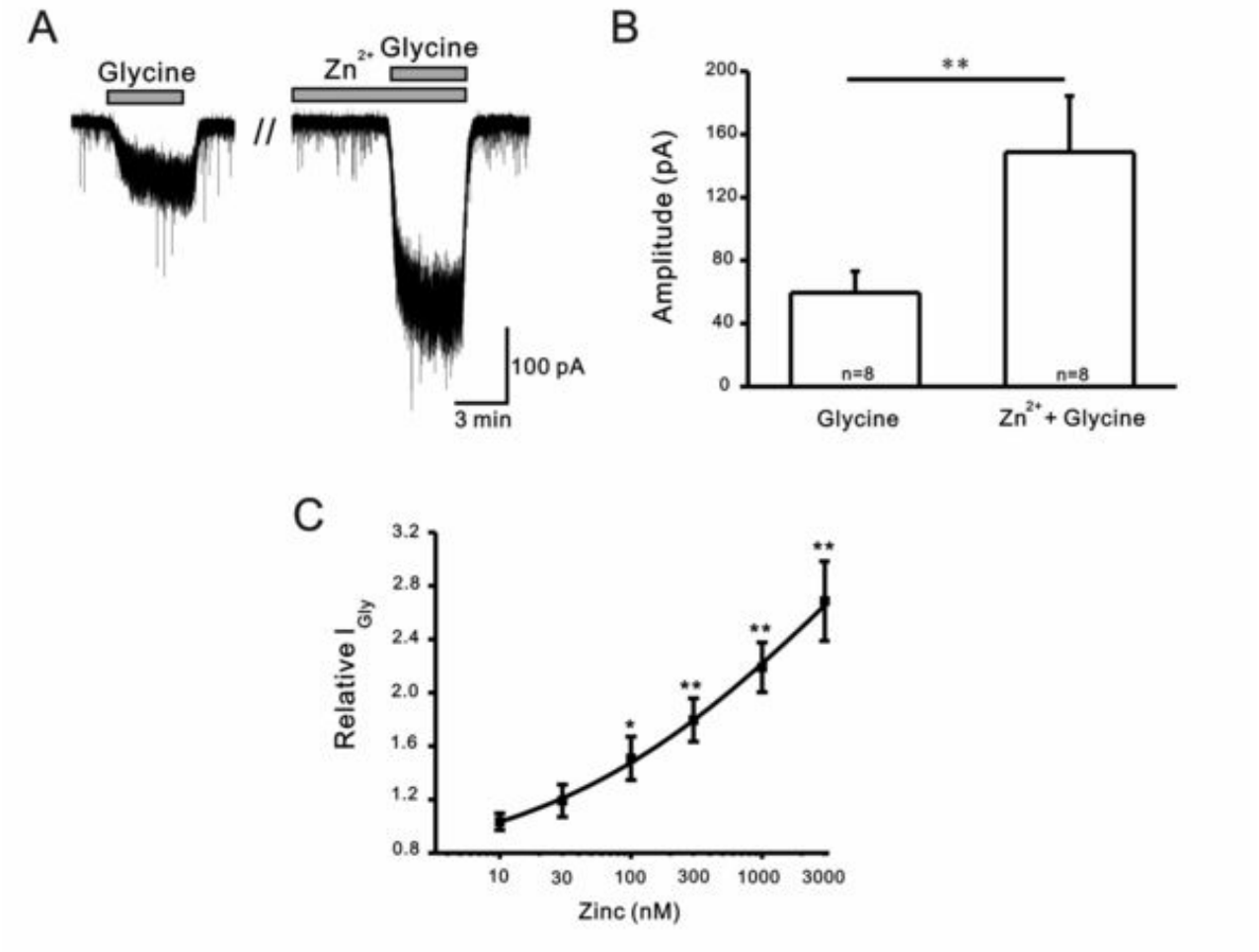

Fig. 2 Download full resolution image

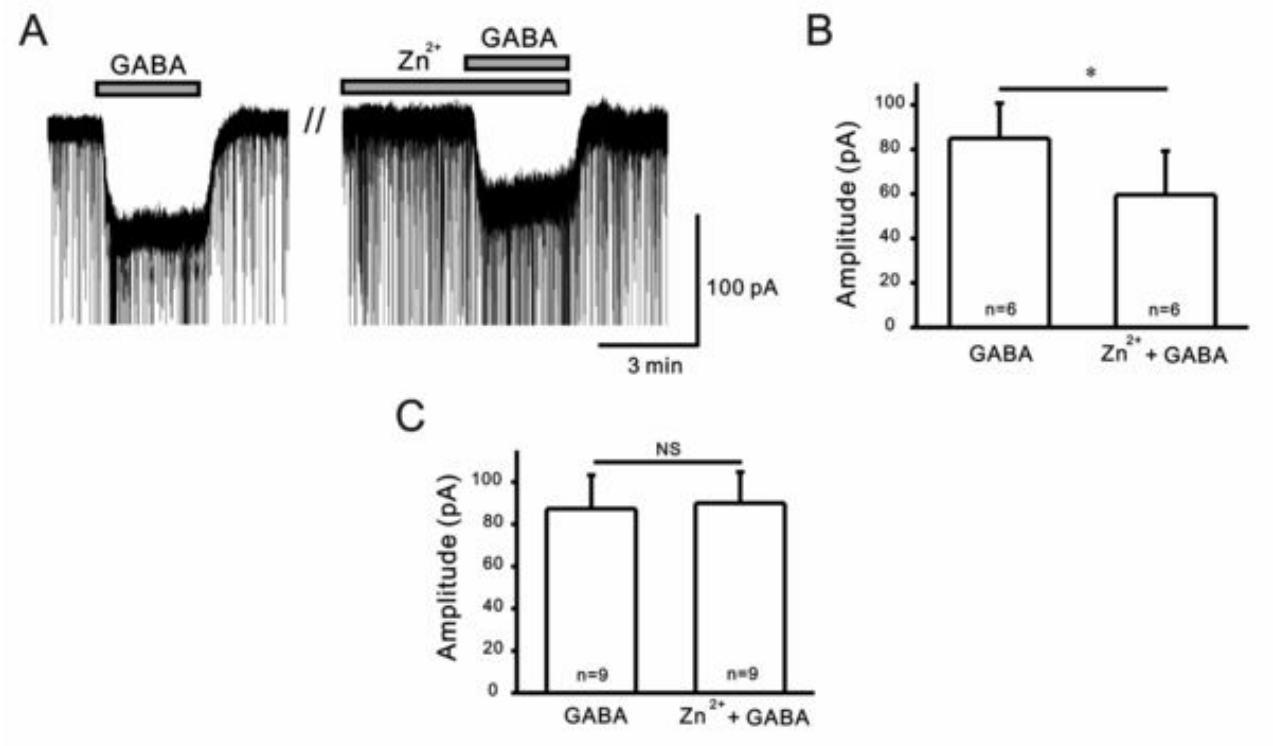

Fig. 3 Download full resolution image