ZINC MODULATES PRIMARY AFFERENT FIBER-EVOKED RESPONSES OF VENTRAL ROOTS IN NEONATAL RAT SPINAL CORD *IN VITRO*

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Abbreviations: ACSF, artificial cerebrospinal fluid; AP-5, DL-2-amino-5-phosphonovaleric acid; bicuculline, (S),9(R)-(-)-bicuculline methobromide; CNS, central nervous system; DRP, dorsal root potential; IPSCs, inhibitory postsynaptic currents; MSR, monosynaptic reflex; NMDA, *N*-methyl-D-aspartate; PPADS, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid; fPSR, fast polysynaptic reflex; sVRP, slow ventral root potential; TNP-ATP, 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate

Abstract—Zinc ions (Zn^{2+}) are known to modulate the functions of a variety of channels, receptors and transporters. We examined the effects of Zn^{2+} on the reflex potentials evoked by electrical stimulation and responses to depolarizing agents in the isolated spinal cord of the neonatal rat *in vitro*. Zn^{2+} at low concentrations (0.5-2 μM) inhibited, but at high concentrations (5 and 10 μM) augmented, a slow depolarizing component (slow ventral root potential; sVRP). Zn^{2+} had no effect on fast components (monosynaptic reflex potential; MSR, fast polysynaptic reflex potential; fPSR). Unlike Zn^{2+}, strychnine (5 μM), a glycine receptor antagonist, and bicuculline (10 μM), a $GABA_A$ receptor antagonist, potentiated both fPSR and sVRP. Zn^{2+} (5μM) did not affect depolarizing responses to glutamate and *N*-methyl-D-aspartate (NMDA). Zn^{2+} enhanced the substance P-evoked depolarization in the absence of tetrodotoxin (0.3 μM) but not in its presence. The dorsal root potential (DRP) was inhibited by bicuculline (10 μM) but not by Zn^{2+} (5 μM). The Zn^{2+}-potentiated sVRP was inhibited by the NMDA receptor antagonists, ketamine (10 μM) and DL-2-amino-5-phosphaonovaleric acid (AP-5, 50 μM) but not by P2X receptor antagonists, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid (PPADS, 30 μM) and

2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate (TNP-ATP, 10 μM). Ketamine (10 μM) and AP-5 (50 μM) almost abolished spontaneous activities increased by Zn^{2+}. It is concluded that Zn^{2+} potentiated sVRP induced by primary afferent stimulation, which was mediated by the activation of NMDA receptors but not by activation of P2X receptors or blockade of glycinergic and GABAergic inhibition. Zn^{2+} does not seem to directly affect NMDA receptors. The release of glutamate from interneurons may play an important role in Zn^{2+}-induced potentiation of sVRP in the spinal cord of the neonatal rat.

Key words: NMDA receptors, ketamine, slow ventral root potential, synaptic reflex potential

Zinc ions (Zn^{2+}) are abundant in the central nervous system (CNS), including the hippocampus, olfactory bulb, amygdala and cortex (Takeda, 2001). The concentration of Zn^{2+} in human cerebrospinal fluid is 0.1-0.2 μM (Palm et al., 1983). In addition, Zn^{2+} is stored in presynaptic terminals with some neurotransmitters (Beaulieu et al., 1992; Palmiter et al., 1996; Wang et al., 2002) and released by electrical activity (Assaf and Chung, 1984; Howell et al., 1984). Therefore, the activation of these nerve fibers is reported to result in increases in the Zn^{2+} concentration to around 300 μM at the synaptic cleft of the rat hippocampus (Assaf and Chung, 1984). As Zn^{2+} modulates long-term potentiation in the rat hippocampus, it is suggested that Zn^{2+} is involved in learning and memory mechanisms (Xie and Smart, 1994; Lu et al., 2000). The roles of Zn^{2+} appear to be intricate in the CNS because of its excitatory and inhibitory modulation of various channels, receptors and transporters such as P2X, glycine, $GABA_A$, glutamate NMDA, non-NMDA receptors (Harrison and Gibbons, 1994; Smart, et al., 1994; 2004) and glutamate transporters (Vandenberg et al., 1998; Mitrovic et al., 2001) in neurons and glia.

As Zn^{2+} exists in the dorsal and ventral horns in the mouse and rat spinal cord,

Zn^{2+} is considered to be involved in sensory and motor functions (Danscher et al., 2001; Wang et al., 2001b). Zn^{2+} is found to co-localize with glutamate or GABA in the mouse spinal cord (Danscher et al., 2001; Wang et al., 2001a). In cultured rat spinal neurons, low concentrations of Zn^{2+} (<10 μM) potentiate inhibitory postsynaptic currents (IPSCs) induced by glycine, whereas high concentrations of Zn^{2+} (>50 μM) suppress them (Laube et al., 1995). The biphasic effect of Zn^{2+} on glycinergic transmission is mediated via both presynaptic P2X receptors and postsynaptic glycine receptors (Laube, 2002). The activity of some P2X receptor subtypes has been shown to be potentiated by Zn^{2+} (Acuña-Castillo et al., 2000; Coddou et al., 2003; Ohta et al., 2005). In cultured chick spinal neurons, Zn^{2+} has also been shown to inhibit GABA receptors (Celentano et al., 1991). Although Zn^{2+} can affect several receptors and channels in the isolated spinal neurons in culture, it is still unclear whether Zn^{2+} affect neuronal activities such as reflex potential changes of the spinal cord.

In the isolated spinal cord of the neonatal rat, stimulation of the dorsal root evokes reflex potentials at the ipsilateral ventral root and the adjacent dorsal root (Akagi and Yanagisawa, 1987; Nussbaumer et al., 1989; Woodley and Kendig, 1991). The early

part of the ventral root potentials is monosynaptic reflex potential (MSR) mainly mediated by glutamate non-NMDA receptors. The MSR is followed by a slow ventral root potential (sVRP) which is mediated in large part by glutamate NMDA receptors and in small part by various metabotropic receptors including NK_1 receptors. The dorsal root potential (DRP) is a $GABA_A$ receptor-mediated response (Seno and Saito, 1985). This preparation provides a useful model to examine the effect of Zn^{2+} on spinal transmission. The purpose of the present study was to examine the effects of Zn^{2+} on spinal reflex potential responses and the responses to several receptor agonists, and to evaluate the site of action of Zn^{2+} in the spinal cord.

EXPERIMENTAL PROCEDURES

Preparations and electrophysiology

All experiments conformed to the guidelines set by NIH and were approved by the Animal Research Committee of the Graduate School of Veterinary Medicine, Hokkaido University. All efforts were made to minimize animal suffering and to reduce the number of animals used. Both male and female neonatal rats (Wistar, 1-3 days old) were

used in this experiment.

Neonatal rats were anesthetized with diethyl ether and decapitated. The isolated spinal cord preparation was prepared as described previously (Otsuguro et al., 2005) with some modifications. The spinal cord was removed together with lumbar dorsal and ventral roots (L3-L5) from rats. The spinal cord was hemisected and placed in a chamber, and was superfused with artificial cerebrospinal fluid (ACSF) at a flow rate of about 3.5 ml/min. The composition of ACSF was as follows (mM): NaCl 138, $NaHCO_3$ 21, NaH_2PO_4 0.6, $CaCl_2$ 1.25, KCl 3.5, $MgCl_2$ 1.2, glucose 10. In the experiments on effects of NMDA and P2X receptor antagonists, the concentration of $MgCl_2$ was increased to 2 mM to depress spontaneous activities. The ACSF was equilibrated with a gas mixture of 95% O_2 and 5% CO_2 at 28±1ºC. A suction stimulating electrode was placed on the dorsal root. A suction recording electrode was placed on the ipsilateral ventral root to record the monosynaptic (MSR), fast polysynaptic reflex potential (fPSR) and slow ventral root potential (sVRP) or on the adjacent dorsal root to record the dorsal root potential (DRP). The depolarizing responses to glutamate, NMDA and substance P were also recorded from the ventral root. The magnitude of the MSR,

fPSR, DRP and the depolarizing responses to agents were expressed as amplitude (mV), and sVRP was expressed as an integral of depolarization (mV s). The spontaneous activity was also expressed as an integral (mV s) above the resting level of the ventral root potential. In the most experiments, the dorsal root was stimulated every 2 min throughout the experiments by a single square wave pulse of 500-700 μs duration and 30-40 V amplitude, which is the supramaximal intensity producing sVRP. In some experiments, three different stimulus intensities were used, including the high-stimulus intensity described above, an intermediate-stimulus intensity (80-300 μs, 30 V) evoking about half the magnitude of sVRP induced by the high-stimulus intensity and a low-stimulus intensity (30-150 μs, 30 V) evoking small but constant and detectable MSR. DRP was evoked by a single square wave pulse of 30-70 μs duration and 30 V amplitude. The effects of drugs and Zn^{2+} on spinal reflex potentials were evaluated by the mean of 4 responses about 20 or 30 min after their application, and expressed as a percentage of the mean of 4 responses immediately before application. The preparation was allowed to equilibrate for about 1 h before recordings. Drugs were applied to the preparation in known concentrations by adding them to the superfusate. Electrical

responses were detected with high gain amplifiers (MEZ-8300 and 8301, Nihon Kohden, Japan). MSR and fPSR were recorded by a thermal arraycorder (WR7900, Graftec, Japan) with a sampling time of 80 μs. The sVRP, DRP and the depolarizing responses to drugs were digitized by an analog/digital converter (PowerLab, AD Instruments, Australia) with a sampling time of 10 ms. Data were stored in a personal computer and analyzed thereafter with software (Chart 5, AD Instruments, Australia).

Drugs

DL-2-amino-5-phosphonovaleric acid lithium salt (AP-5), (S),9(R)-(-)-bicuculline methobromide (bicuculline), (±)-ketamine hydrochloride, L-703,606 oxalate salt, pyridoxal-phosphate-6-azophenyl-2',4'-disulphonic acid tetrasodium salt (PPADS), *N*-methyl-D-aspartic acid (NMDA) and 2',3'-O-(2,4,6-trinitrophenyl)adenosine 5'-triphosphate monolithium trisodium salt (TNP-ATP) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). L-Glutamic acid hydrochloride was from Tokyo Kasei Kogyo Co. (Tokyo, Japan). Strychnine sulfate and tetrodotoxin were from Wako Pure Chemical Ind. (Osaka, Japan). Substance P was from Peptide Institute (Minoh,

Japan).

Statistical Analysis

Results are expressed as mean±S.E.M. Statistical comparisons between two groups were performed by Student's *t*-test. A *P* value of less than 0.05 was considered significant.

RESULTS

Effects of Zn^{2+} on spinal reflex responses

We first examined the effect of Zn^{2+} (5 μM) on electrical activities of ventral roots in the isolated spinal cord preparation of neonatal rats. The dorsal root was stimulated every 2 min and Zn^{2+} was applied. The bath application of Zn^{2+} (5 μM) caused a small depolarization (0.25±0.06 mV, n= 6) of baseline ventral root potential (Fig. 1A). In some preparations, Zn^{2+} greatly increased spontaneous activities. Electrical stimulation of the dorsal root elicited monosynaptic and fast polysynaptic reflex potentials (MSR and fPSR) followed by a slow ventral root potential (sVRP) lasting about 20 s in the

ipsilateral ventral root of the same segment (Fig. 1B). In addition to the baseline change, Zn^{2+} (5 μM) markedly potentiated sVRP but not MSR and fPSR. Since Zn^{2+} markedly increased the area of depolarization of sVRP but not its peak amplitude, the integral of sVRP above the baseline was used to estimate the effect of Zn^{2+}. As the spontaneous activities increased by Zn^{2+} interfered with an accurate estimate of the sVRP integral, the effects of Zn^{2+} on the spinal reflex responses were summarized without these data (about 15% of preparations treated with 5 μM Zn^{2+}). Zn^{2+} (5 μM) slightly decreased sVRP during first 6 min and then increased sVRP, which reached a maximum at around 10 min after its application (Fig. 1C). The effect of Zn^{2+} almost disappeared 20 min after washing with normal ACSF.

Next, the effects of various concentrations of Zn^{2+} on spinal reflex responses were examined. Whereas Zn^{2+} (10 μM) potentiated sVRP, low concentrations of Zn^{2+} (0.5 and 2 μM) inhibited sVRP, the maximum suppression of which was attained around 4 min after application (Fig. 2A). In 1 of 6 and 2 of 8 preparations, Zn^{2+} at 2 and 5 μM, respectively, caused a marked but transient decrease in sVRP, which returned to near the control level. The mean of 4 responses between 14 and 20 min after application of Zn^{2+}

was expressed as a percentage of the mean of 4 responses before the application, and Zn^{2+} concentration-response relationships were summarized as shown in Fig. 2B. Although MSR and fPSR were not affected by Zn^{2+} at any concentration examined, Zn^{2+} inhibited sVRP at low concentrations but potentiated it at high concentrations. Reversal concentrations of Zn^{2+} were between 2 and 5 μM. Concentrations of Zn^{2+} higher than 10 μM could not be examined because of marked increases in spontaneous activities. Cu^{2+} (0.5-30 μM), another important trace metal in the CNS, inhibited MSR, fPSR and sVRP to the same extent in a concentration-dependent manner (Fig. 2C). Unlike Zn^{2+}, Cu^{2+} had no excitatory effect on the spinal reflex responses.

It has been reported that stimulation with different intensities causes activation of different types of neuronal pathways (Akagi et al., 1985; Nussbaumer et al., 1989). Therefore, we also examined the effects of Zn^{2+} on spinal reflex potentials evoked by stimulation with different intensities (Fig. 3A and Table 1). The responses to stimulation with low- and intermediate-intensities were compared to those with high-stimulus intensity. Low- and intermediate-stimulus intensities evoked 42.5±11.0 and 99.4±1.2% (n=6) of MSR evoked by high-stimulus intensity, 68.0±6.8 and

100.8±9.0% (n=6) of fPSR, and 8.5±2.1 and 48.4±4.8% (n=6) of sVRP, respectively. Zn^{2+} (5 μM) significantly potentiated sVRP evoked by intermediate- and high- but not by low-stimulus intensities. On the other hand, Zn^{2+} (5 μM) did not affect MSR and fPSR evoked by any stimulus intensity.

Effects of strychnine and bicuculline on spinal reflex responses

It is possible that the excitatory effect of Zn^{2+} is due to the suppression of inhibitory mechanisms in the spinal cord. Therefore, we compared the effects of Zn^{2+} with those of strychnine and bicuculline, glycine and $GABA_A$ receptor antagonists, respectively, on the spinal reflex responses. Similar to 5 μM Zn^{2+}, strychnine (5 μM) increased sVRP to 167.9±14.4 % (n=4) but not MSR (114.2±2.7%, n=4). However, dissimilar to Zn^{2+}, strychnine enhanced fPSR to 267.2±20.1 % (n=4). Bicuculline (10 μM) also potentiated sVRP (201.6±32.5%, n=3) and fPSR (149.2±4.8%, n=3) but not MSR (99.9±2.5%, n=3). The effects of strychnine and bicuculline seemed to be somewhat different from that of Zn^{2+}. Therefore, we examined the effects of strychnine and bicuculline on sVRP evoked by low-stimulus intensity (Fig 3B and C). As described above (Fig. 3A and Table 1),

Zn^{2+} (5 μM) failed to increase sVRP evoked by this stimulus intensity. However, strychnine (5 μM) and bicuculline (10 μM) markedly increased sVRP to 1040.0±203.4% (n=4) and 3187.8±1198.7% (n=5), and fPSR to 305.5±45.4% (n=4) and 157.1±20.2% (n=6) but not MSR (75.2±27.1%, n=4 and 113.6±13.9%, n=5), respectively.

Electrical stimulation of the dorsal root elicited a dorsal root potential (DRP) in the adjacent dorsal root. As shown in Fig. 4, the DRP was markedly and reversibly suppressed by 10 μM bicuculline to 48.5±2.8% (n=4), indicating that this reflex potential was mediated by $GABA_A$ receptors. However, Zn^{2+} (5 μM) did not affect the DRP (96.1±2.4%, n=4).

Effects of Zn^{2+} on depolarizing responses to glutamate, NMDA and substance P

The bath application of glutamate (30-300 μM), NMDA (20-100 μM) or substance P (0.03-1 μM) to the isolated spinal cord for 1 min produced a concentration-dependent depolarization of the ventral root (Fig. 5). Zn^{2+} (5 μM) did not affect depolarizing responses to glutamate and NMDA (Fig. 5A and B). On the other hand, substance P (10

nM)-evoked depolarization was slightly but significantly enhanced by Zn^{2+} (Fig. 5C). As reported previously (Otsuka and Yanagisawa, 1980), tetrodotoxin (0.3 μM) inhibited the depolarizing response to substance P and shifted the concentration-response curve for substance P to the right. In the presence of tetrodotoxin, Zn^{2+} failed to enhance the depolarizing responses to substance P.

Substance P depolarizes spinal neurons through NK1 receptors. sVRP are reported to be inhibited by NK1 receptor antagonists (Otsuka et al., 1995). We therefore examined the effect of Zn^{2+} (5 μM) on sVRP in the presence of a NK1 receptor antagonist, L-703-606. The bath application of L-703,606 (1 μM) for 50 min decreased sVRP to 78.4±2.6% (n=4). Under this condition, Zn^{2+} (5 μM) showed only a small potentiation of sVRP (123.3±4.1% of control in the presence of L-703,606, n=4).

The effects of antagonists for NMDA receptors and P2X receptors on sVRP increased by Zn^{2+}

NMDA receptors and P2X receptors are reported to play important roles in spinal transmission (Burnstock and Wood, 1996; Dickenson et al., 1997). In particular, it is

known that sVRP is mainly mediated by NMDA receptors and the activity of some P2X receptors is up-regulated by Zn^{2+}. We therefore examined the effects of NMDA receptor antagonists, ketamine and AP-5, and P2X receptor antagonists, PPADS and TNP-ATP, on sVRP increased by Zn^{2+} (Fig. 6). The application of Zn^{2+} (5 μM) for 20 min increased sVRP (150 -200%). Subsequently, each antagonist was applied in the presence of Zn^{2+}. The sVRP increased by Zn^{2+} (5 μM) was significantly suppressed by ketamine (10 μM) or AP-5 (50 μM) but not by PPADS (30 μM) or TNP-ATP (10 μM).

To further examine the effect of ketamine on sVRP increased by Zn^{2+}, ketamine (3-30 μM) was cumulatively applied in the presence of Zn^{2+}. Ketamine inhibited sVRP increased by Zn^{2+} in a concentration-dependent manner (Fig. 7A). Next, the effect of ketamine on sVRP increased by Zn^{2+} was compared with that on sVRP without Zn^{2+} (Fig. 7B). In the absence of Zn^{2+}, ketamine also inhibited sVRP in a concentration-dependent manner. However, ketamine at 3 μM selectively suppressed the sVRP increased by Zn^{2+} to 70.8±8.3% (n=7) without any effect on sVRP in the absence of Zn^{2+} (103.3±7.8%, n=8). Ketamine (10 and 30 μM) inhibited sVRP to the same extent as that increased by Zn^{2+}.

The effects of NMDA receptor antagonists on the spontaneous activity increased by Zn^{2+}

As mentioned above, spontaneous activity was observed in some preparations, in which Zn^{2+} (5 μM) further increased the activity. Therefore we investigated the effects of the NMDA receptor antagonists on the spontaneous activity (Fig. 8). Ketamine (10 μM) and AP-5 (50 μM) almost abolished the spontaneous activity in the presence of Zn^{2+}.

DISCUSSION

In the present study, we found that low concentrations of Zn^{2+} (0.5-2 μM) inhibited sVRP, whereas higher concentrations of Zn^{2+} (≥3 μM) potentiated sVRP without affecting MSR, fPSR and DRP in the isolated spinal cord of the neonatal rat. Characterization of the excitatory effect of Zn^{2+} indicated that Zn^{2+} potentiated sVRP through the activation of NMDA receptors but not the activation of P2X receptors or the blockade of glycine or $GABA_A$ receptors. The release of glutamate from interneurons may play an important role in Zn^{2+}-induced potentiation of sVRP.

The inhibitory effects of Zn^{2+} on responses to glycine and GABA are well documented (as reviewed in Harrison and Gibbons, 1994; Smart, et al., 1994; 2004). As blockade of glycine or $GABA_A$ receptors enhances spinal reflex potentials, glycinergic and GABAergic neurons play a crucial role as the inhibitory interneurons in the spinal cord (Akagi and Yanagisawa, 1987; Deshpande and Warnick, 1988). In cultured chick and rat spinal neurons, Zn^{2+} inhibits GABA- (Celentano et al., 1991) and glycine-induced currents (Laube, 2002). In the present experiments, however, the effects of strychnine and bicuculline, glycine and $GABA_A$ receptor antagonists, respectively, on the spinal reflex responses were incompatible with the effect of Zn^{2+}. Both strychnine and bicuculline, but not Zn^{2+}, potentiated fPSR and the low-stimulus intensity-evoked sVRP. Furthermore, DRP was inhibited by bicuculline but not by Zn^{2+}. These results indicate that the excitatory effect of Zn^{2+} is not due to relief from the endogenous glycinergic and GABAergic inhibition in the neonatal rat spinal cord.

It is reported that Zn^{2+} and Cu^{2+} increase activities of some P2X receptor subtypes in the spinal cord (Acuña-Castillo et al., 2000; Coddou et al., 2003). In fact, in the cultured spinal neurons, Zn^{2+} (5 μM) is known to enhance glycinergic transmission,

which is inhibited by PPADS, a P2X receptor antagonist (Laube, 2002). ATP is also capable of releasing an excitatory transmitter, glutamate, from dorsal horn neurons of the rat spinal cord via P2X receptors (Gu and MacDermott, 1997; Nakatsuka and Gu, 2001). In the present study, however, neither PPADS nor TNP-ATP, P2X receptor antagonists, inhibited sVRP increased by Zn^{2+}. Moreover, Cu^{2+} failed to potentiate sVRP at any concentration examined. It is, therefore, concluded that the activation of P2X receptors is not involved in the excitatory effect of Zn^{2+} in the isolated spinal cord of the neonatal rat.

In the present experiments, low concentrations of Zn^{2+} inhibited sVRP but high concentrations potentiated it. sVRP is inhibited selectively by some analgesics such as opiates and α_2-adrenoceptor agonists (Otsuguro et al., 2005) and thus reflects nociceptive responses in the neonatal rat spinal cord. It has been reported that intrathecal injection of Zn^{2+} causes antinociceptive effects in the mouse writhing assay (Larson and Kitto, 1997). It seems likely that low concentrations of Zn^{2+} inhibit sVRP and thus nociceptive transmission in the spinal cord. On the other hand, high concentrations of Zn^{2+} potentiated sVRP without affecting MSR. In the neonatal rat

spinal cord, a large part of sVRP is reported to be associated with NMDA receptors and MSR with non-NMDA receptors (Woodley and Kendig, 1991; Brockmeyer and Kendig, 1995). It has, however, been reported that Zn^{2+} at 50 μM or more inhibits NMDA receptor-mediated responses but potentiates non-NMDA receptor-mediated responses in cultured mouse hippocampal neurons (Mayer, et al., 1989) and in *Xenopus* oocytes expressing rat brain NMDA and non-NMDA receptors (Rassendren et al., 1990). Higher concentrations of Zn^{2+} might also potentiate MSR in the neonatal rat spinal cord.

NMDA receptors are composed of heterometric compositions of NR1 and NR2 subunits (Ozawa et al., 1998; Cull-Candy et al., 2001). NR2 subunits further divided into NR2A, B, C and D. Zn^{2+} inhibits NR2A more potent than other subtypes (Williams, 1996; Paoletti et al., 1997). It has been reported that NR2A do not play a dominant role at early postnatal development in CNS including rat spinal cord (Portera-Cailliau et al., 1996; Stegenga and Kalb, 2001). It might be reason why Zn^{2+} (≥3 μM) did not show inhibitory effect on NMDA receptor-mediated responses in neonatal rat spinal cord. In present study, Zn^{2+} did not affect depolarizing responses to glutamate and NMDA, although it significantly potentiated sVRP, which was suppressed by NMDA receptor

antagonists, ketamine and AP-5. These results suggest that Zn^{2+} does not directly affect NMDA responses, but that the activation of NMDA receptors is necessary for the excitatory effect of Zn^{2+}.

Zn^{2+} enhanced substance P-evoked depolarization in our study. It seems unlikely that Zn^{2+} directly potentiates responses to substance P because the enhancement was abolished by tetrodotoxin. It is reported that NK_1 receptors are expressed in dorsal horn neurons receiving inputs from C-fibers (Labrakakis and MacDermott, 2003) and that substance P causes the release of glutamate from the spinal cord of the neonatal rat (Maehara et al., 1995). Zn^{2+} also increases the extracellular concentrations of excitatory amino acids such as aspartate and glutamate in the rat hippocampus (Takeda et al., 2004) and the substantia nigra (Dopico et al., 2004). Therefore, it is reasonable to suggest that Zn^{2+} affects glutamate-containing interneurons expressing NK_1 receptors and thus enhances sVRP through the release of glutamate in the neonatal rat spinal cord. This hypothesis is supported by the fact that only a small potentiation of sVRP occurs with Zn^{2+} in the presence of NK1 receptor antagonist. One possible explanation for the increase in extracellular glutamate is that Zn^{2+} influences

the function of glial cells that modulate synaptic transmission by maintaining the glutamate/glutamine cycle or releasing neuronal modulators (Schousboe, 2003; Watkins and Maier, 2003). Zn^{2+} has been reported to inhibit the activity of glutamate transporters (Vandenberg et al., 1998; Mitrovic et al., 2001) that lower the extracellular glutamate level in the synaptic cleft. Another possibility is that Zn^{2+} inhibits potassium channels in neurons and thus potentiates sVRP, because Zn^{2+} is shown to increase neuronal excitability by inhibiting calcium-dependent potassium channels in the rat hippocampus (Sim and Cherubini, 1990) and a transient A-like potassium channel in dopaminergic neurons of the rat substantia nigra (Chung et al., 2000). In the present study, ketamine (3 μM) inhibited sVRP potentiated by Zn^{2+} but not control sVRP. It seems likely that Zn^{2+} potentiates some glutamate-containing interneurons which are sensitive to the NMDA receptor antagonist.

In the spinal cord, NMDA receptors have been reported to play a crucial role in long-term potentiation and hyperalgesia (Dickenson, et al., 1997; Sandkühler et al., 2000). The long-term potentiation evoked by tetanic electrical stimulation is prevented by NMDA receptor antagonists in the rat spinal cord (Randi•, et al, 1993; Liu and

Sandkühler, 1995). Ketamine is clinically used as an anesthetic agent and alleviates hyperalgesia and allodynia in the rat (Qian et al., 1996; Chaplan et al., 1997) and human (Felsby et al., 1995; Ilkjaer, et al., 1996). The fact that ketamine inhibits the excitatory effect of Zn^{2+} suggests that Zn^{2+} enhances nociceptive transmission in the spinal cord of the neonatal rat.

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Figure Legends

Fig. 1. Effects of Zn^{2+} on ventral root potentials in the neonatal rat spinal cord. (A) The slow ventral root potential (sVRP) was evoked by a single electrical stimulation every 2 min (arrowheads). Zn^{2+} (5 μM) was added to ACSF. In some preparations as shown in the lower panel, Zn^{2+} caused frequent spontaneous activities. (B) Representative traces of fast reflex responses (monosynaptic reflex: MSR and fast polysynaptic reflex: fPSR) and sVRP in the same preparation are shown in the upper and lower panels, respectively. The left, middle and right traces show the responses before (Control), after the application of 5 μM Zn^{2+} for 20 min, and after control (Wash), respectively. (C) The time course of the effects of Zn^{2+} on the peak amplitude (MSR and fPSR) and the area under the curve (sVRP) of depolarization, the magnitude of each response is expressed as a percentage of the response immediately before the application of Zn^{2+}. Each symbol and error bar represent mean±S.E.M. (n=6-8)

Fig. 2. The time course of effect of Zn^{2+} on sVRP and concentration-dependent effects of Zn^{2+} and Cu^{2+} on spinal reflex responses. (A) The time course of changes in sVRP in

the presence of Zn^{2+} (0.5-10 μM), which is expressed as a percentage of the response immediately before the application of Zn^{2+}. Electrical stimulation was applied to the dorsal root every 2 min. The data on 5 μM Zn^{2+} are transferred from Fig. 1C. Each symbol and error bar represent mean±S.E.M. (n=6-8). (B, C) Concentration-dependent effects of Zn^{2+} (0.1-10 μM, B) and Cu^{2+} (0.5-30 μM, C) on sVRP, MSR and fPSR. The mean of the last 4 responses in the presence of metals at each concentration is shown. Each symbol and error bar represent mean±S.E.M. (n=4-6 for Zn^{2+}, n=4 for Cu^{2+}).

Fig. 3. The effects of Zn^{2+} on the spinal reflex responses to electrical stimulation with different intensities. (A) Representative traces of fast reflex responses (MSR and fPSR) and sVRP in the same preparation. The spinal reflex potentials were evoked by three different stimulus intensities: low (30-150 μs, 30 V,●), intermediate (80-300 μs, 30 V, ○) and high (500-700 μs, 30-40 V, ▲). Twenty min after the application of 5 μM Zn^{2+}, the responses were again evoked by the three different stimuli. (B, C) The effects of strychnine and bicuculline on the spinal reflex responses to stimulation with low intensity. The representative traces of fast reflex responses (MSR and fPSR) and sVRP

evoked by low-stimulus intensity in the same preparation. Twenty min after the addition of 5 μM strychnine (B) and 10 μM bicuculline (C), the reflex responses were again evoked.

Fig. 4. The effects of Zn^{2+} on dorsal root potentials in the neonatal rat spinal cord. (A, B) Representative traces of the dorsal root potential (DRP) before, during the application of 5 μM Zn^{2+} (A) and 10 μM bicuculline (B), and after their washout.

Fig. 5. The effects of Zn^{2+} on glutamate-, NMDA- and substance P-evoked depolarization. (A, B, C) The concentration-response curves for glutamate (n= 6, A), NMDA (n=6, B) and substance P (n=6-7, C) in the presence (closed circle) or absence (open circle) of Zn^{2+}. These agents were added to ACSF and applied for 1 min at intervals of 10-15 min. Subsequently, Zn^{2+} (5 μM) was applied for at least 20 min and the agents were applied in the presence of Zn^{2+}. Then the peak amplitude of stimulant-evoked depolarization was measured from the basal potential in the presence of Zn^{2+}. Tetrodotoxin (TTX, 0.3 μM) was also added to ACSF for at least 20 min in the

presence (closed triangle) or absence (open triangle) of Zn^{2+}. Each symbol and error bar represent mean±S.E.M. *P< 0.05 vs. control (paired-Student's t-test).

Fig. 6. The effects of the antagonists for NMDA and P2X receptors on sVRP. (A, B) Representative traces of the effects of ketamine (A) and PPADS (B) on sVRP in the presence of Zn^{2+}. After Zn^{2+} (5 μM) was applied to the spinal cord for at least 20 min, ketamine (10 μM) or PPADS (30 μM) was applied. (C) The effects of 10 μM ketamine (n=5), 50 μM AP-5 (n=4), 30 μM PPADS (n=4) and 10 μM TNP-ATP (n=3) on sVRP increased by 5 μM Zn^{2+} are summarized. These antagonists were applied to the spinal cord for 20 min (ketamine, AP-5 and TNP-ATP) or 30 min (PPADS) in the presence of Zn^{2+}. Each column represents the mean of the area under the curve of the sVRP before (open columns) and after (solid columns) the application of the antagonists in the presence of Zn^{2+}. Error bars represent S.E.M. **P< 0.01 vs. the response before the application of the antagonists in the presence of Zn^{2+} (paired-Student's t-test).

Fig. 7. The effect of ketamine on sVRP. increased by Zn^{2+} (A) Representative traces of

the sVRP suppressed by ketamine in the presence of Zn^{2+}. At least 20 min after the application of 5 μM Zn^{2+}, ketamine was cumulatively applied for 20 min at each concentration (3, 10 and 30 μM). (B) The effects of ketamine on sVRP in the presence (solid columns) or absence (open columns) of 5 μM Zn^{2+}. The data represent the area under the curve of the sVRP as percentages of responses before the application of ketamine without Zn^{2+}. Each symbol and error bar represent mean±S.E.M. (n=7-8).

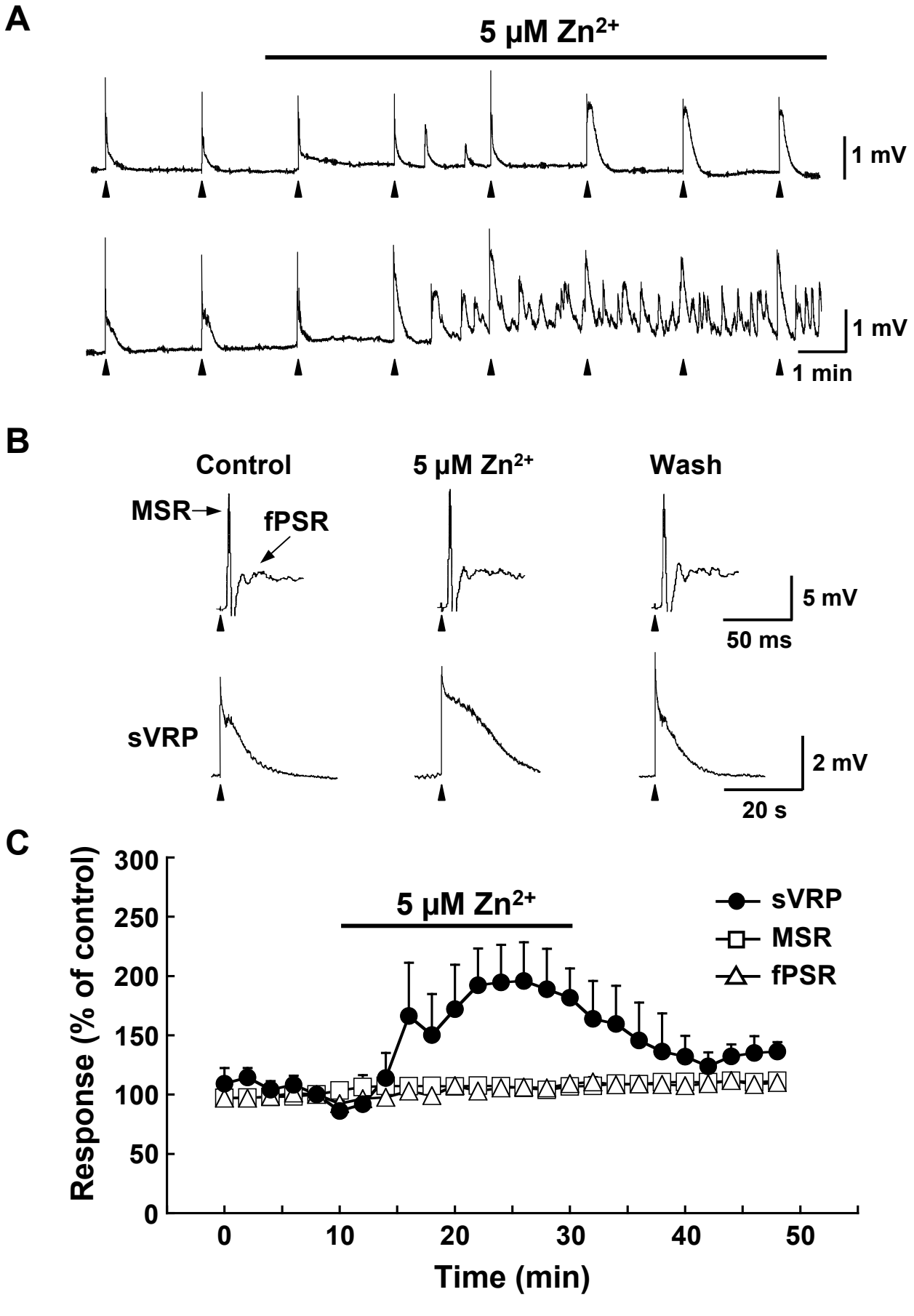
Fig. 8. The effects of NMDA receptor antagonists on the spontaneous activity increased by Zn^{2+}. (A) Representative traces of the spontaneous activity in the same preparation. Dotted lines show the resting level of the ventral root potential. At least 20 min after the application of 5 μM Zn^{2+}, 10 μM ketamine was applied for 20 min in the presence of Zn^{2+}. (B) The effects of ketamine (10 μM, n=4) and AP-5 (50 μM, n=6) are expressed as the percentage of the spontaneous activity before the application of Zn^{2+}. Each column represents the mean of the area under the curve of the spontaneous activity above the resting level for 10 min before (open columns) and after (solid columns) the application of the antagonists in the presence of Zn^{2+}. Error bars represent S.E.M.

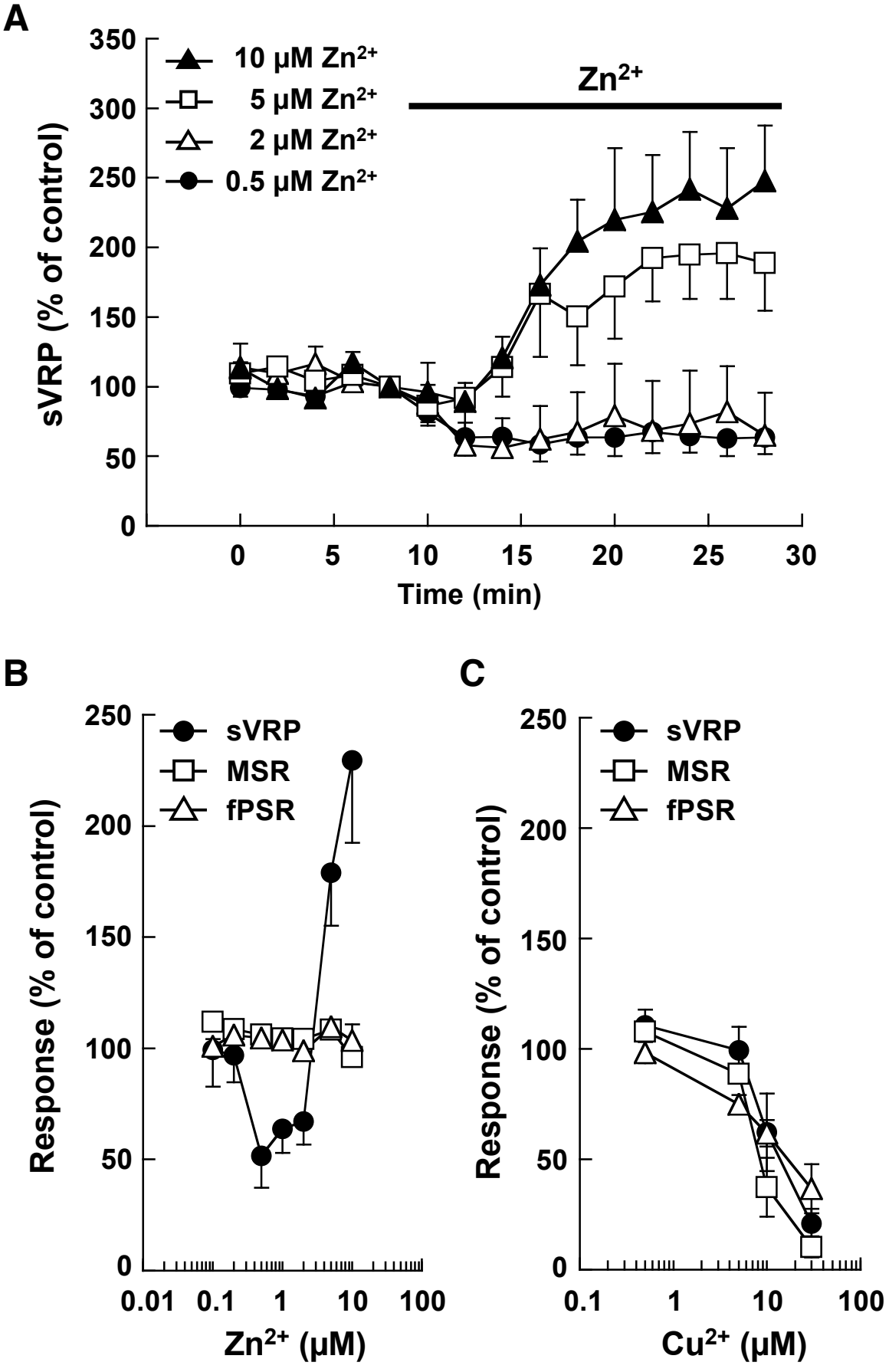
*P<0.05, **P< 0.01 vs. the response before the application of the antagonists in the presence of Zn^{2+} (paired-Student's *t*-test).

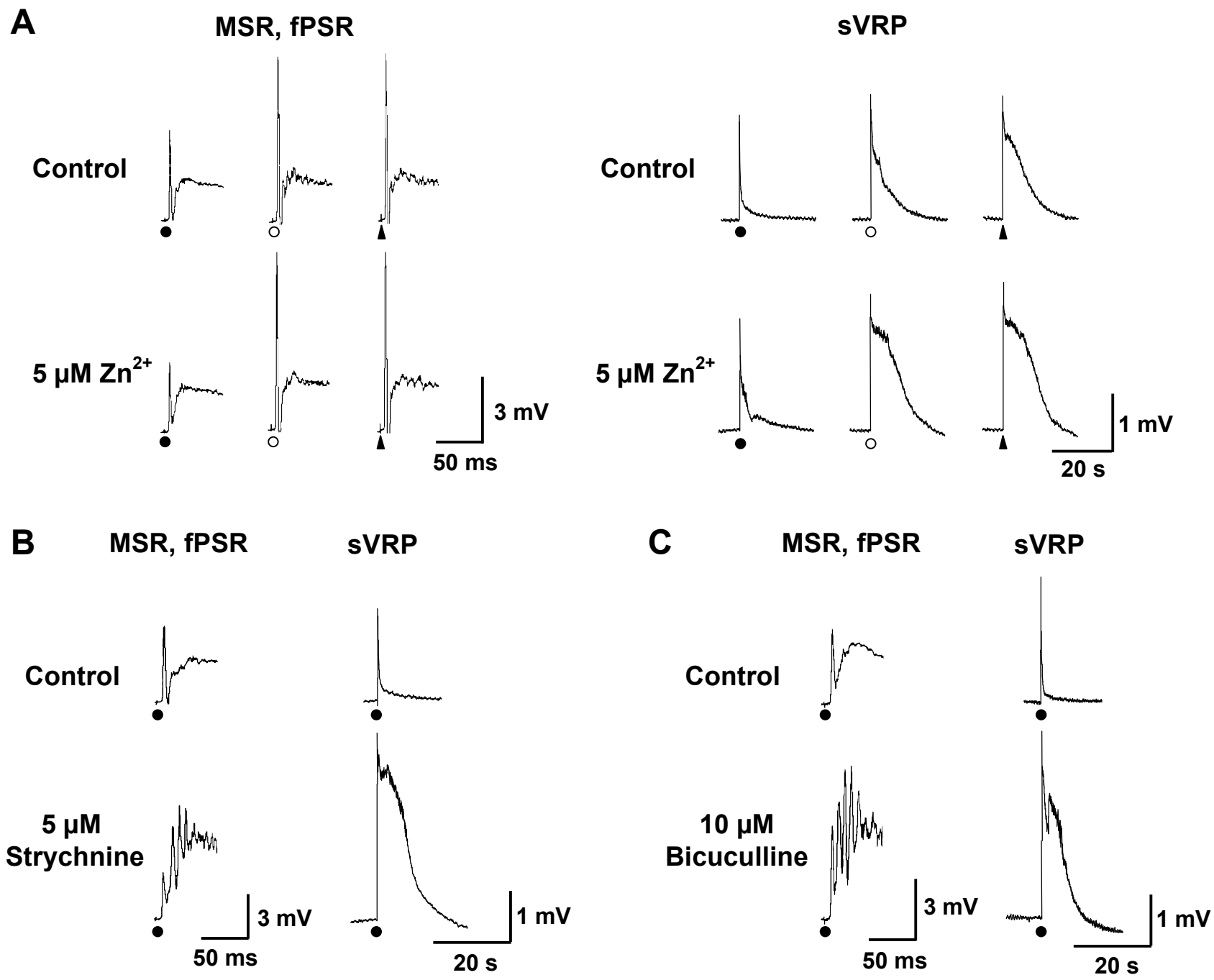
Table 1 Effects of Zn^{2+} on MSR, fPSR and sVRP evoked by different stimulus intensities

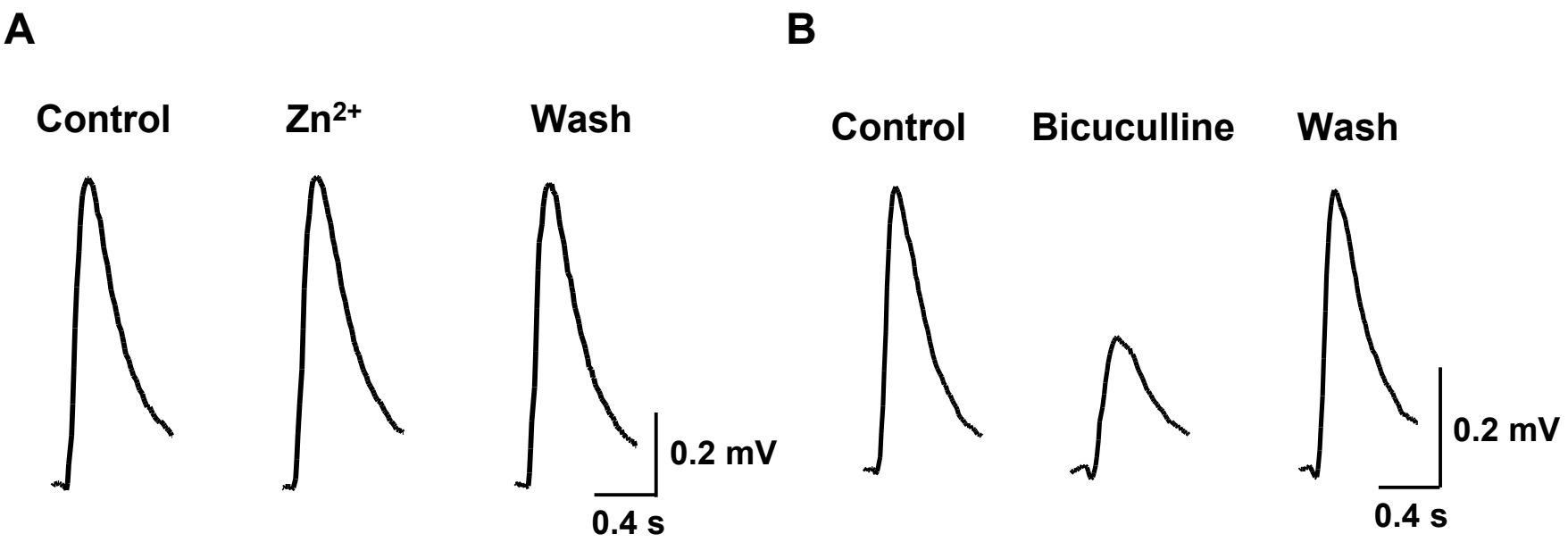
Stimulus intensity	Time-matched control (%)			5 μM Zn^{2+} (%)		
	MSR	fPSR	sVRP	MSR	fPSR	sVRP
low	78.1±9.0	103.1±11.1	160.9±25.9	81.2±3.4	102.8±6.9	189.5±57.0
intermediate	103.6±4.8	105.1±9.3	130.3±17.6	102.4±2.5	95.8±7.7	278.7±42.4**
high	104.3±4.8	111.2±10.1	111.4±12.9	88.0±15.8	94.9±5.2	176.5±18.9*

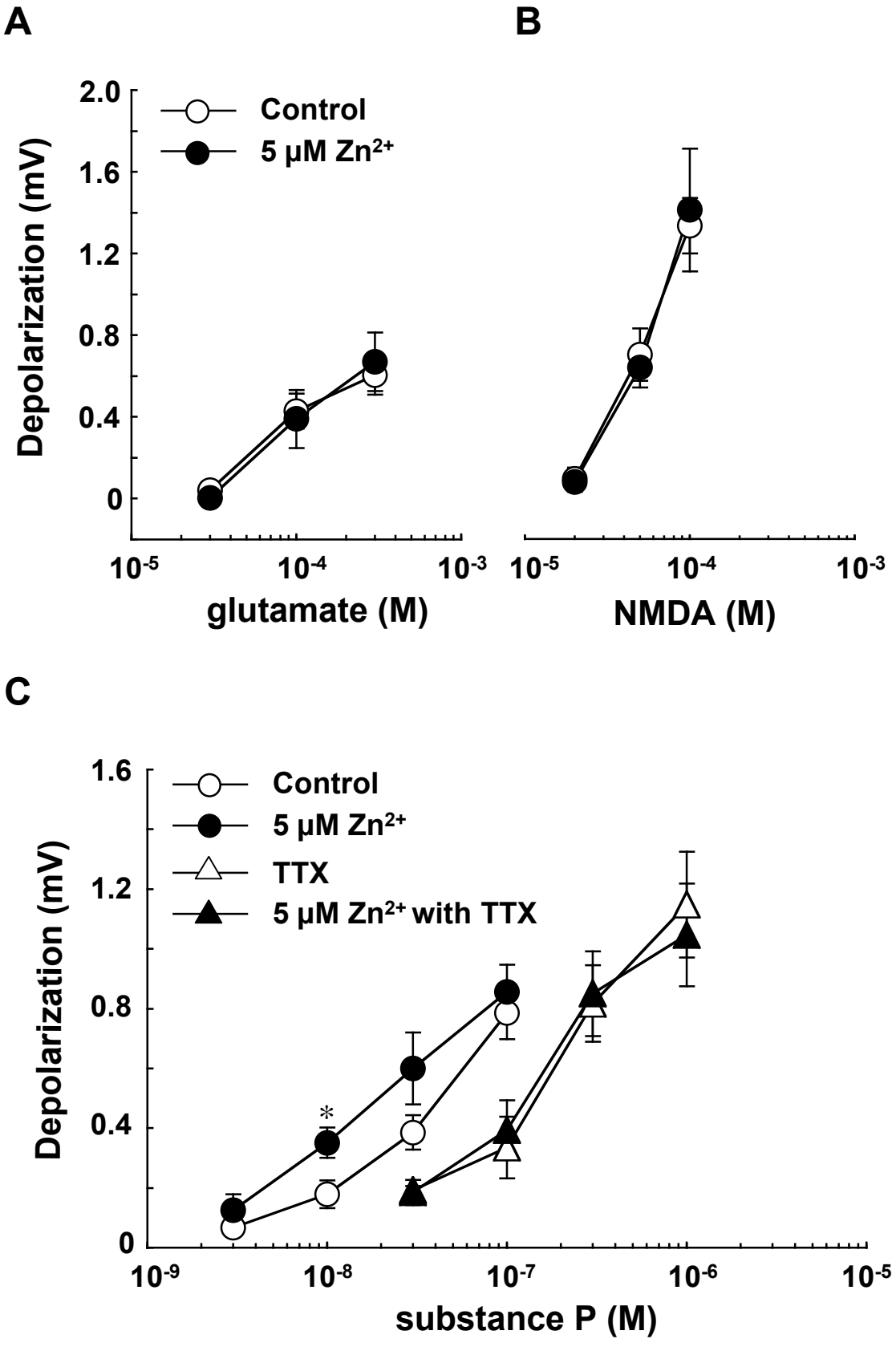
The spinal reflex potentials (MSR, fPSR and sVRP) were evoked by different stimulus intensities (low, intermediate and high). After application of 5 μM Zn^{2+} for 20 min, the reflex potentials were again evoked. In the time-matched control, the preparation was perfused for 20 min in the absence of Zn^{2+}. Each value is expressed as a percentage of the magnitude of the spinal reflex potential evoked by the second stimulation compared with that evoked by the first stimulation. Each value represents mean ±S.E.M. (n=6). *P<0.05, **P<0.01 vs. time-matched control (unpaired Student's t-test).

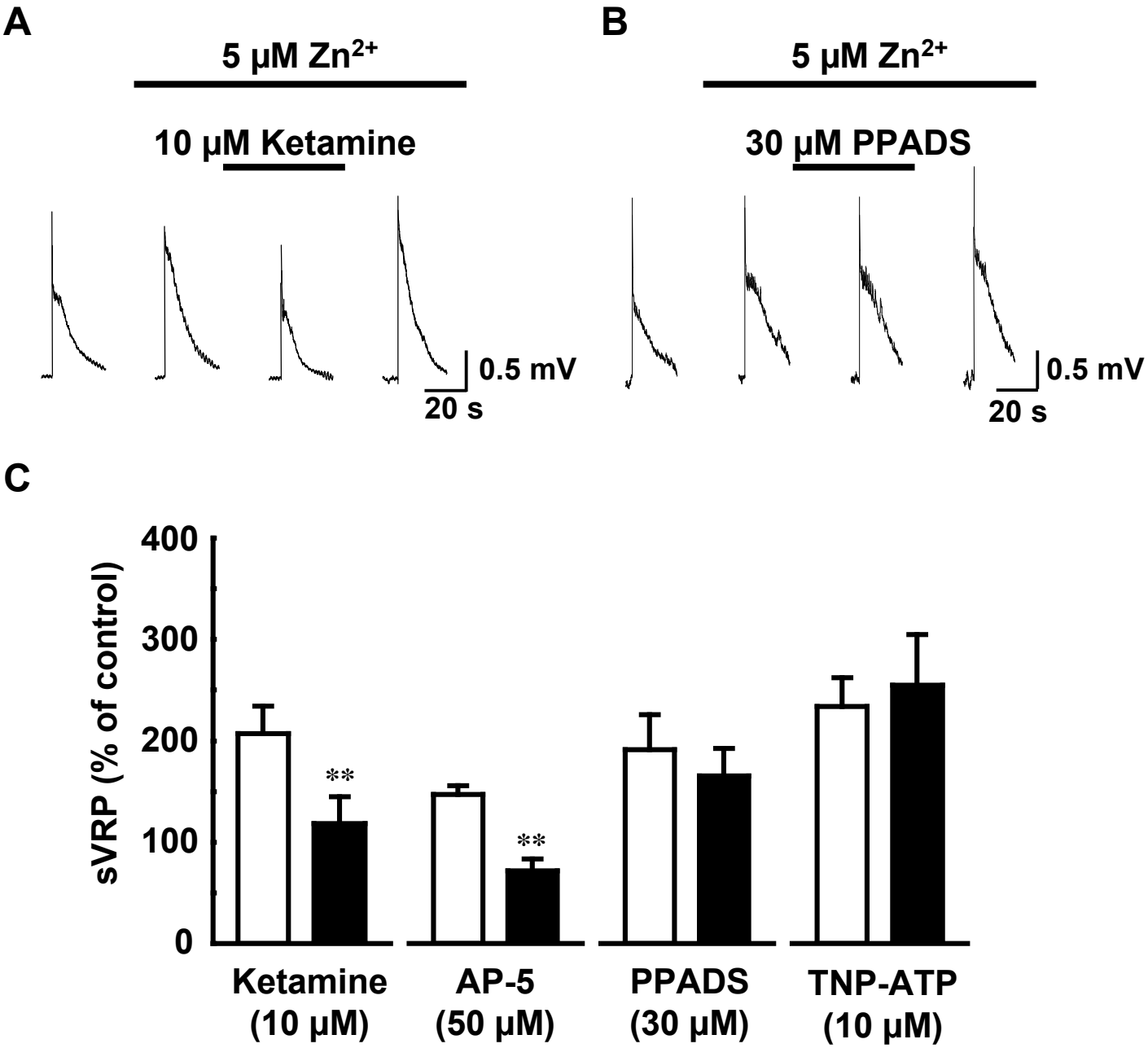

A
5 μM Zn2+
1 mV
1 mV
1 min
B
Control
5 μM Zn2+
Wash
MSR
fPSR
5 mV
50 ms
sVRP
2 mV
20 s
C
5 μM Zn2+
sVRP
MSR
fPSR
Response (% of control)
300
250
200
150
100
50
0
0
10
20
30
40
50
Time (min)

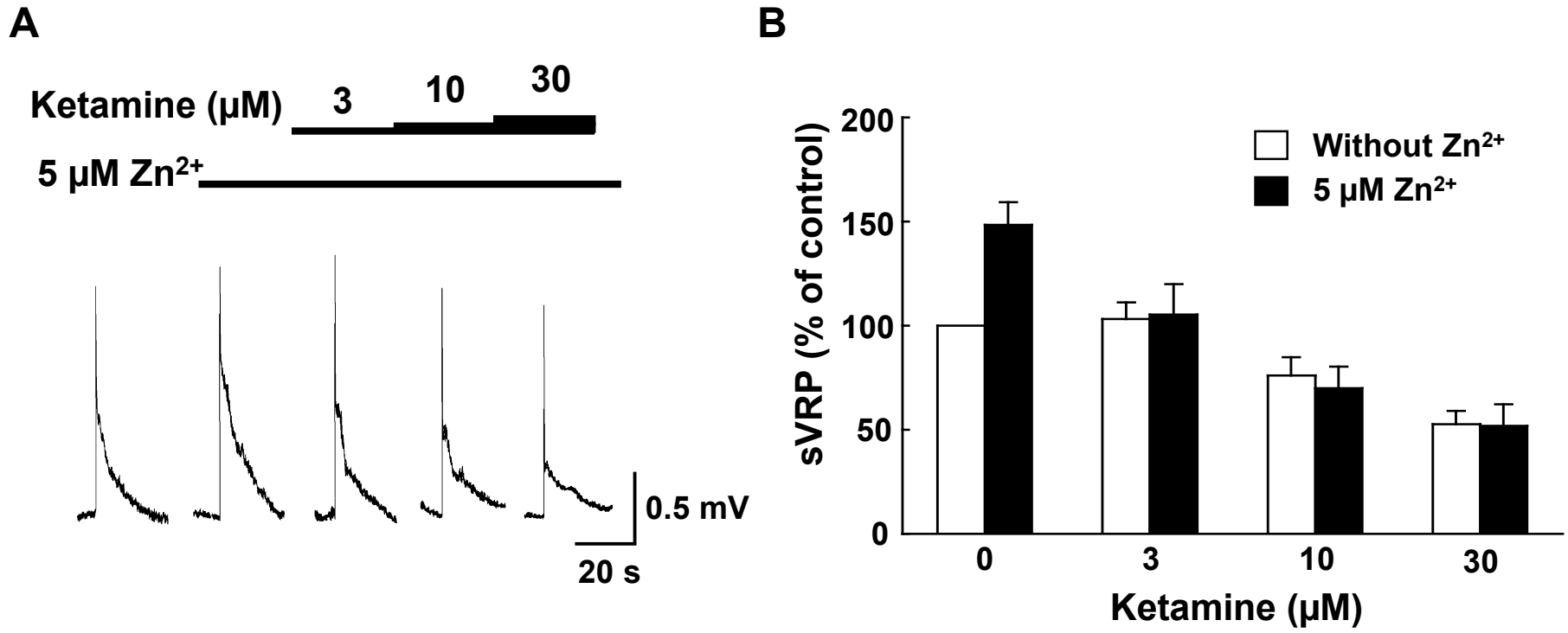

A
10 μM Zn2+
5 μM Zn2+
2 μM Zn2+
0.5 μM Zn2+
Zn2+
sVRP (% of control)
Time (min)
B
sVRP
MSR
fPSR
Response (% of control)
Zn2+ (μM)
C
sVRP
MSR
fPSR
Response (% of control)
Cu2+ (μM)

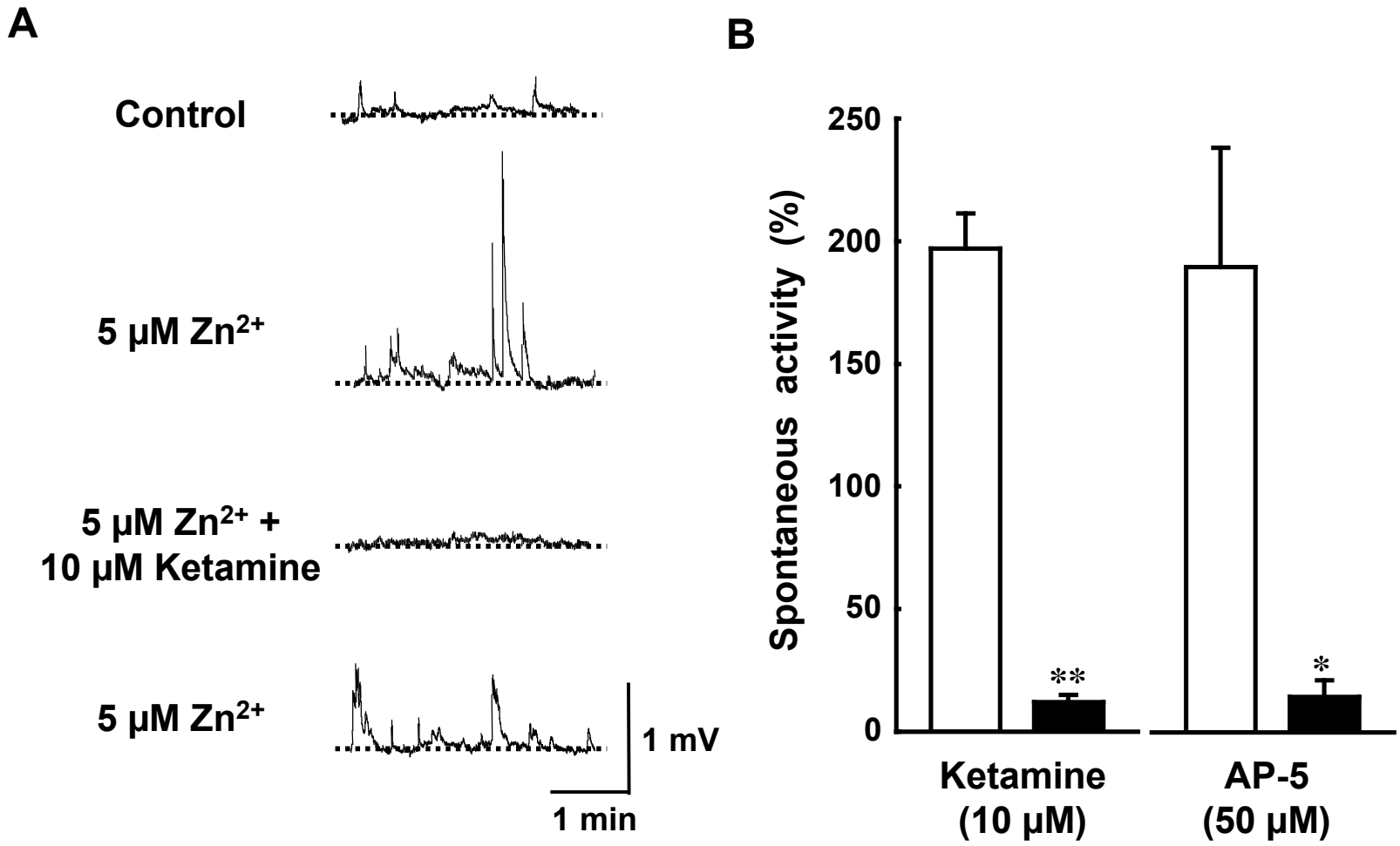

A
MSR, fPSR
sVRP
Control
5 μM Zn2+
3 mV
50 ms
1 mV
20 s
B
MSR, fPSR
sVRP
Control
5 μM
Strychnine
3 mV
50 ms
1 mV
20 s
C
MSR, fPSR
sVRP
Control
10 μM
Bicuculline
3 mV
50 ms
1 mV
20 s


A
Control
Zn2+
Wash
0.2 mV
0.4 s
B
Control
Bicuculline
Wash
0.2 mV
0.4 s


A
B
C
Control
5 μM Zn2+
TTX
5 μM Zn2+ with TTX
Depolarization (mV)
glutamate (M)
NMDA (M)
substance P (M)
*


A
5 μM Zn2+
10 μM Ketamine
0.5 mV
20 s
B
5 μM Zn2+
30 μM PPADS
0.5 mV
20 s
C
sVRP (% of control)
400
300
200
100
0
**
**
Ketamine (10 μM)
AP-5 (50 μM)
PPADS (30 μM)
TNP-ATP (10 μM)


A
Ketamine (μM)
3
10
30
5 μM Zn2+
0.5 mV
20 s
B
sVRP (% of control)
200
150
100
50
0
Without Zn2+
5 μM Zn2+
0
3
10
30
Ketamine (μM)


A
Control
5 μM Zn2+
5 μM Zn2+ +
10 μM Ketamine
5 μM Zn2+
1 mV
1 min
B
Spontaneous activity (%)
250
200
150
100
50
0
**
*
Ketamine
(10 μM)
AP-5
(50 μM)