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FULL-LENGTH ORIGINAL RESEARCH

# Hippocampal zinc infusion delays the development of afterdischarges and seizures in a kindling model of epilepsy

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## Summary

**Purpose:** Zinc occurs in high concentration in synaptic vesicles of glutamatergic terminals including hippocampal mossy fibers. This vesicular zinc can be synaptically released during neuronal activity, including seizures. Zinc inhibits certain subtypes of N-methyl-D-aspartate (NMDA) and γ-aminobutyric acid $(GABA)_A$ receptors. By blocking NMDA excitation or GABA inhibition, an excess of zinc may alter the excitability of hippocampal circuits, which contribute to the development of seizures.

**Methods:** Twenty-one adult Wistar rats were implanted under anesthesia with Alzet pumps releasing vehicle, 10 μM $ZnCl_2$ or 1,000 μM $ZnCl_2$, at a rate of 0.25 μl/h continuously into the hippocampal hilus for 4 weeks. Kindling was performed by daily awake commissural stimulation at 60 Hz and afterdischarges were recorded from a dentate gyrus electrode. Development of behavioral Racine seizure stages was recorded by a blinded investigator.

**Results:** The development of behavioral Racine seizure stages was delayed only in rats infused with 1,000 μM $ZnCl_2$ ($p < 0.02$). With completion of kindling at stimulation number 20, all groups had reached the same maximum level of behavioral seizures. The expected increased progression of afterdischarge duration was inhibited by both 10 μM $ZnCl_2$ and 1,000 μM $ZnCl_2$ infusion compared to control animals ($p < 0.01$). At stimulation number 18, all groups had reached the same maximum duration of afterdischarges.

**Discussion:** We conclude that excess infused zinc delayed the development of behavioral seizures in a kindling model of epilepsy. These data support the hypothesis that zinc synaptically released during seizures may alter hippocampal excitability similar to zinc infused in our experiment.

**KEYWORDS:** Hippocampus, Hippocampal mossy fibers, NMDA receptors, $GABA_A$ receptor, Afterdischarge, Dentate gyrus.

## Introduction

### Zinc as a neuromodulator

Zinc ($Zn^{2+}$) is found in every mammalian cell, including neurons, as a constitutive element in hundreds of proteins and enzymes. In certain ''zinc-containing neurons'' (Frederickson, 1989; Christensen & Frederickson, 1998), about 10% of this zinc occurs in an unbound form, making it accessible to histochemical detection by Timm's stain (Sloviter, 1982; Danscher, 1996) or fluorescent indicators such as 6-methoxy-(8-p-toluenesulfonamido)quinoline (TSQ). This ''chelatable'' zinc is localized in synaptic vesicles of glutamate-releasing terminals (Frederickson, 1989; Danscher, 1996) and can be released upon neural activity (Assaf & Chung, 1984; Howell et al., 1984). Zinc uptake into synaptic vesicles requires the zinc transporter ZnT-3 (Wenzel et al., 1997; Cole et al., 1999).

Once released, zinc appears to remain in the form of a veneer layer of zinc in the extracellular space that maps onto the Timm's stained region of the hippocampus (Kay,

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2003) and could exert many effects on adjacent neurons. Zinc has been shown to modulate many voltage- and ligand-gated channels (Harrison & Gibbons, 1994; Smart et al., 1994).

### Zinc and epilepsy

Releasable, vesicular zinc is most abundant in brain regions that are prone to seizures, namely the limbic regions (Frederickson, 1989). Excessive release of zinc has been observed in an animal model of epilepsy (Takeda et al., 1999). Staining for vesicular zinc is most intense in the hippocampal mossy fibers (Slomianka, 1992), which project from granule cells in the dentate gyrus to the CA3 region of the hippocampus. In human temporal lobe epilepsy, as well as in several animal models of epilepsy, sprouting of mossy fibers (Cavazos et al., 1991) is observed by an increase in zinc-sensitive Timm's stain. Overall levels of zinc in the hippocampus appear to increase markedly as a result of kindling in rats (Mody & Miller, 1985; Slevin et al., 1986; Kasarskis et al., 1987).

Neuromodulatory effects of zinc released from sprouted mossy fibers could be proconvulsive or anticonvulsive. The only study suggesting proconvulsive effects of zinc showed that the duration of kindling-induced seizures and electrical afterdischarges was decreased by repeated intraperitoneal (i.p.) injections of the membrane-permeable zinc chelator diethyldithiocarbamate (DEDTC) before each stimulation (Foresti et al., 2008).

Most other studies of zinc suggest anticonvulsive effects of zinc. Mice lacking vesicular zinc because of the lack of the ZnT-3 zinc transporter (Cole et al., 2000) or a zinc-deficient diet (Takeda et al., 2003, 2006) are much more susceptible to kainic acid–induced seizures than mice with normal vesicular zinc levels, and epilepsy-prone rats have an increased zincergic innervation of their forebrain (Flynn et al., 2007), perhaps because of an upregulation of zinc to prevent the danger from seizures as the authors speculate. A delay of kindling-induced seizures was seen in cats fed a zinc-enhanced diet, and conversely an acceleration of kindling with a zinc-deficient diet (Sterman et al., 1986). However, this study did not determine if there was an actual change in hippocampal zinc levels or zinc release as a result of the dietary changes.

Most of the information on physiologic effects of zinc is based on studies in brain slices. The implications of slice studies for epilepsy are limited, since only short-term observations are possible and many neuronal connections have been transected. The present study was designed to evaluate potential physiologic effects of extracellular zinc in the intact brain. Because we considered dietary changes of synaptic zinc release as unreliable, and ZnT-3 knockout mice were not available, we chose the approach of infusing zinc via Alzet pumps in a rat kindling model of epilepsy to study the effects of extracellular hippocampal zinc on the development of epileptic seizures.

## Methods

### Hippocampal implants

Male Wistar rats of weights between 275 to 325 g at surgery were obtained from and housed in the UCLA vivarium. Care and use of the animals was approved by the UCLA Animal Research Committee and performed according to its policies and guidelines. Bipolar stainless steel electrodes were implanted under ketamine [40–50 mg/kg, intramuscular (i.m.)] and xylazine (5–6 mg/kg, i.m.) anesthesia into the midline hippocampal commissures (AP, −1.8; L, 0.0; V, 4.2) (Paxinos & Watson, 1986) for later stimulation.

### Zinc infusion

Alzet pumps (Durect Corp., Cupertino, CA, U.S.A.) delivering 0.25 $\mu$l/h of a 10 or 1,000 $\mu$M solution of $ZnCl_2$ in 0.9% saline or vehicle were connected to infusion cannulas implanted under pentobarbital sodium anesthesia (75 mg/kg, i.p.) aiming at the right hippocampal hilus (AP, −4.0; L, 2.0; V, 3.2) (Paxinos & Watson, 1986). The infusion cannulas (custom-made by Plastics One, Roanoke, VA, U.S.A.) were equipped with a bipolar recording electrode with wires to the right and left of the cannula. The electrode wire tips extended 0.5 mm below the cannula tip. Placement of cannula and electrodes was confirmed in histologic sections after sacrifice of the animals. An example of a cannula track and a drawing of the electrode–cannula are shown in Fig. 1A. Animals in which the hilus was not within a 45-degree wedge below the end of the cannula track were excluded from the study prior to unblinding of animal treatment status.

This strategy was based on the following observation. A rough estimate of the extent of zinc diffusion in the brain was obtained by examination of hippocampal sections of animals infused with the membrane-permeable metal chelator N,N,N′,N′-tetrakis (2-pyridylmethyl) ethylene-diamine (TPEN). Brain sections were stained using a modified Timm's sulfide/silver amplification technique (Sloviter, 1982). The diffusion track of TPEN was visible by the sharply delineated local loss of the vesicular zinc stain (Cuajungco & Lees, 1998) and labeled a wedge-shaped area with an angle of approximately 45 degrees below the cannula tip (Fig. 1B).

### Kindling procedure

After a postsurgical recovery period of 7 days, animals were stimulated daily through the implanted commissural electrode with a train of biphasic 150 mA pulses at 60 Hz for 1 s. The behavioral changes induced by the kindling process were scored according to the scale of Racine (Racine, 1972) by an investigator blinded to animal

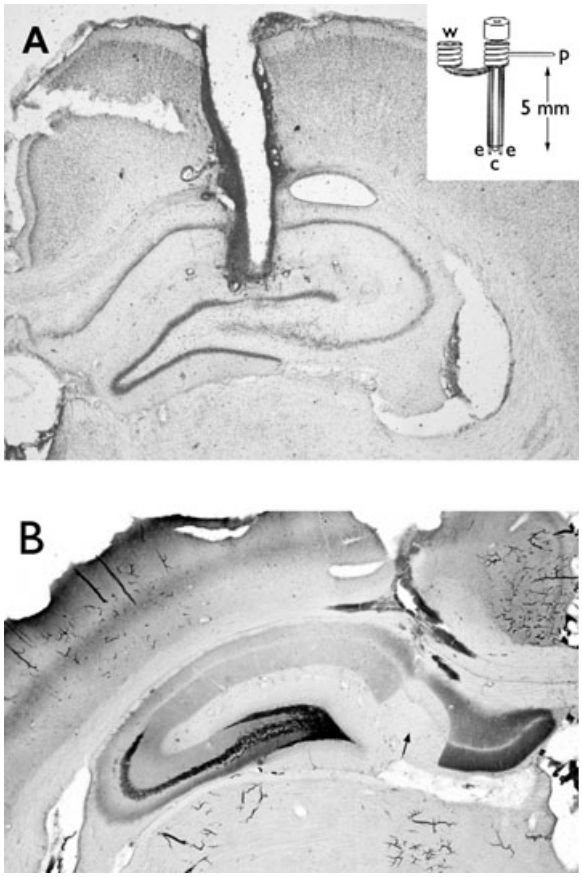


**Figure 1.**
(**A**) A Nissl-stained coronal section of a rat hippocampus is shown. The track left by the cannula delivering zinc solution is visible. The cannula tip was aimed at the hilus. The insert shows a drawing of the electrode-cannula used. The tips of the bipolar recording electrode (e,e) extended 0.5 mm below the opening of the cannula tip (c). The two wires were attached to the cannula laterally and medially. (w), contacts for the electrode wires. (p), connector to the pump delivering zinc solution. (**B**) A Timm's stained coronal section of a rat hippocampus is shown. The track of a cannula delivering the membrane-permeable zinc chelator TPEN is discernible in the upper right. Below the end of this track, a sharply delineated, wedge-shaped area is seen (arrow) where Timm's stain is not present, presumably due to chelation of zinc as a result of TPEN infusion. For the zinc infusion experiments, rats with a similar cannula location (hilus not within a 45° wedge below the end of the cannula track) were excluded from further analysis.


treatment status. Epileptic afterdischarges were recorded from the electrodes adjacent to the infusion cannula tip near the hippocampal hilus. The data were recorded via low noise, shielded cables and stored digitally with a sampling frequency of 2 kHz on a computer. The duration of the primary afterdischarge and interval and duration of the secondary afterdischarge were determined by an investigator blinded to animal treatment status.

### Histologic analysis

After the fully kindled state was reached at 16–20 stimulations, all animals were perfused under the i.p. anesthesia of a lethal dose of pentobarbital. Animals were perfused transcardially with normal saline for 1 min, then with 100 ml 0.1% sodium sulfide followed by 300 ml 4% paraformaldehyde solution in phosphate buffer. Brains were removed, postfixed in the same fixative overnight at 4°C, cryoprotected in a 20% sucrose solution overnight at 4°C, frozen, and then sectioned coronally on a freezing microtome at 60 $\mu$m spacing. Beginning from the rostral pole of the hippocampus where a complete, typical profile of the dentate gyrus is visible, every fourth coronal section of 60 $\mu$m thickness was stained with cresyl violet. This procedure yielded about 21 sections in 240 $\mu$m intervals across the entire rat hippocampal formation. Stained neurons in the hilar and CA3 regions were systematically examined by careful visual inspection as described in the results section.

### Exclusion of animals and data analysis

Of a total of 23 animals, 9 rats were excluded by an investigator blinded to animal treatment status and to any results of the study. One rat was excluded because of malfunction of the stimulating electrode, three rats were excluded because of poor quality recordings, and another five rats were excluded because of incorrect location of infusion cannula as determined by histologic analysis (e.g., a location similar to Fig. 1B). Of the animals remaining for data analysis, five rats were in the vehicle group, four rats were in the 10 $\mu$M zinc group, and five rats were in the 1,000 $\mu$M zinc group. Data analysis was performed by an investigator blinded to animal treatment status. After unblinding, Racine behavioral scores (Racine, 1972) (Fig. 2) and the duration of the primary afterdischarges (Figs. 3 and 4) were averaged for the animals in each treatment group and plotted over time as measured by kindling stimulation number.

### Experimental design and statistical analyses

The resultant design was a 3 (between subjects: 0, 10, or 1,000 $\mu$M $ZnCl_2$ solution) by 20 (within subject: 20 kindling stimulation trials), mixed factorial design with repeated measures on the second factor. There were two dependent variables: afterdischarge duration in seconds, a continuous variable, and Racine seizure stages, which may be considered ordinal. Three approaches were used to analyze the data; two of them overlapping. The first was the more common method of analysis of variance (ANOVA). Because of the noise in the recordings,

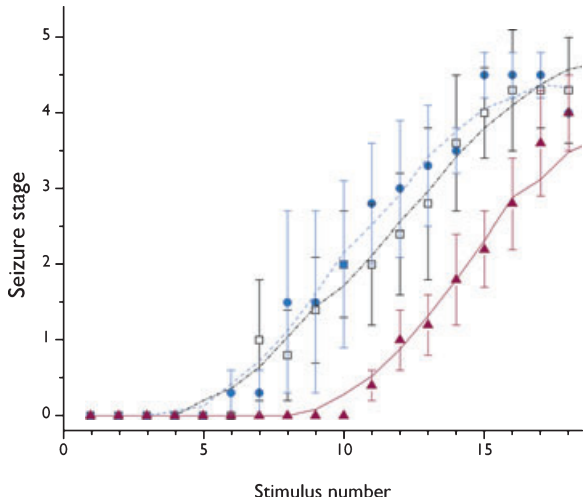


**Figure 2.**
Development of behavioral seizure stages over time during kindling. The normal saline group (NS; n = 5) is represented by black open squares with a dash-dot line, the 10 $\mu$M zinc-infused group (10 $\mu$M Zn; n = 4) by light blue filled circles with a dashed line, the 1000 $\mu$M zinc-infused group (1000 $\mu$M Zn; n = 5) by dark red filled triangles with a solid line. Data points are averages from all animals in a group, error bars are SEM. The lines are 5 point sliding averages of the averaged data points for each treatment group.


afterdischarge data for some animals were missing. So, ANOVA was used only for the Racine data, and then the results were considered only tentative given the ordinal nature of the data. SAS PROC GLM (SAS Institute, Cary, NC, U.S.A.) was used for this analysis.

The second approach used to analyze the data was the mixed model approach (Brown & Prescott, 2006). This method was developed to overcome certain problems that preclude the use of ANOVA and MANOVA, such as missing data in a repeated measures design (Singer & Willett, 2003). Both the afterdischarge data and the Racine data were analyzed using SAS PROC MIXED (SAS Institute).

The third approach to analyzing the data was non-parametric. The Kruskal-Wallace analysis of ranked data was used as an alternative, conservative method to determine if there existed differences between the groups.

## Results

### Development of behavioral seizures: Statistical analyses

The development of behavioral seizure stages over time during kindling is shown in Fig. 2. The 3 by 20 mixed ANOVA yielded a group by stimulation trial interaction, $F(38,209) = 2.0$, $p < 0.001$, reflecting a marginally significant group by stimulation trial quadratic component interaction, $F(2,11) = 3.3$, $p < 0.08$. Inspection of Fig. 2 aids in interpreting this interaction. No animal displayed any seizure through kindling trial 5, after which animals in both the vehicle and 10 $\mu$M groups began displaying seizures, but seizures in the 1,000 $\mu$M group were delayed until trial 11. Both the vehicle group and the 10 $\mu$M group reached Racine stage 5 by the 18th kindling stimulation trial, whereas the 1,000 $\mu$M group did not achieve Racine stage 5 until kindling stimulation trial 20 (data not shown). The Kruskal-Wallace tests confirmed these differences in achievement of Racine stages, with no statistically significant differences emerging until trial 10 ($p < 0.05$), after which trials 11 through 16 were all statistically significant ($p < 0.05$) or marginally significant ($p < 0.10$). These findings were also confirmed by the mixed model analysis, which also yielded a significant stimulation trial quadratic component by group interaction, $F(2,207) = 6.4$, $p < 0.002$.

To follow up the mixed model significant stimulation trial quadratic component by group interaction, the stimulation trial quadratic component was tested separately in each of the groups. For the 1,000 $\mu$M group, the stimulation trial quadratic trend was statistically significant, $F(1,209) = 24.7$, $p < 0.0001$. Neither of the other groups displayed a statistically significant stimulation trial quadratic trend (both $p > 0.50$). Based on both the ANOVA and mixed model approaches, it is clear that the onset of behavioral seizures during electrical stimulation was delayed in rats infused with 1,000 $\mu$M $ZnCl_2$.

### Epileptic afterdischarges: Qualitative description

Fig. 3 shows a typical afterdischarge with primary afterdischarge (pAD) and secondary afterdischarge (sAD).

The primary afterdischarge often consisted of two components: (1) Low-voltage fast activity seen in all animals, ranging in frequency typically from about 8–16 Hz. In the discharge shown in Fig. 3, this fast activity increases in amplitude for about 10 s before its termination (Fig. 3C). In other recordings, the termination of afterdischarge activity is characterized by replacement of the low-voltage fast activity by the background activity as seen before stimulation. (2) In many animals, the low-voltage fast activity was preceded by rhythmic high-amplitude spike and slow wave activity, with a frequency of about 3 Hz or lower. As shown in Fig. 3B, this activity then evolved into the low-voltage fast activity as described previously.

Many afterdischarges were terminated by an sAD of high-amplitude rhythmic sharp waves with a frequency of about 1 Hz (Fig. 3D). Motor seizures of a score of 3 or higher on the Racine scale (forelimb clonus; Racine, 1972) typically were associated with longer pADs and no sADs were seen within the time of recording.

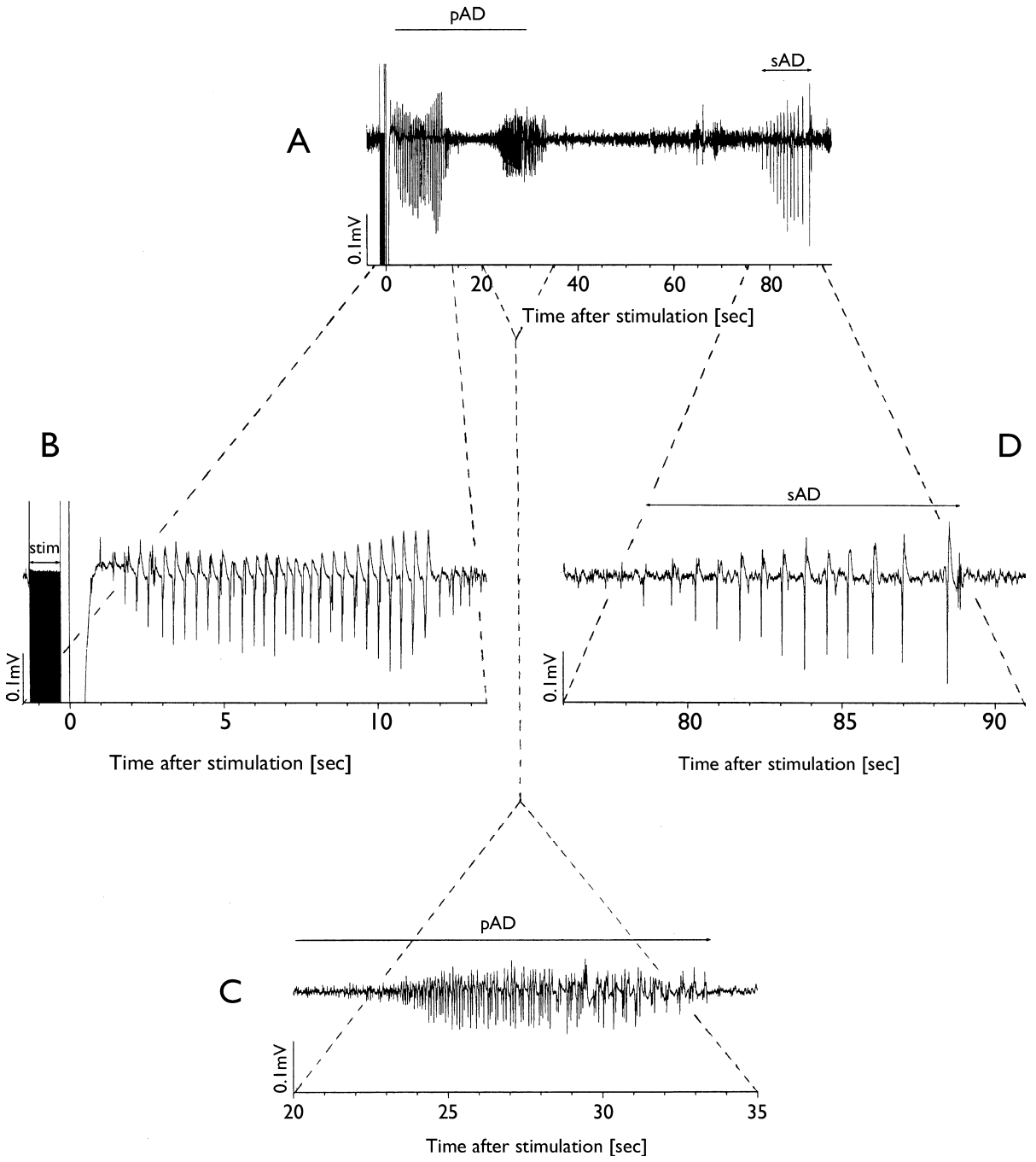


**Figure 3.**
A typical epileptic afterdischarge in a rat recorded by a commissural electrode. (**A**) entire duration of the afterdischarge; (**B**) close up of the initial portion of the afterdischarge; (**C**) close up of the end of the primary afterdischarge (pAD); (**D**) close up of the secondary afterdischarge (sAD).


To study the development of afterdischarges, the duration of the pADs was measured by an investigator blinded to animal treatment status. The duration of pAD was defined as the time in seconds from the end of electrical stimulation to the termination of low-voltage fast activity (Fig. 3A).

### Development of epileptic afterdischarges: Statistical analysis

The development of epileptic afterdischarges as defined previously is shown in Fig. 4. No ANOVA was possible because of missing data. The mixed model analysis similar to that described previously suggests that the development of afterdischarges was delayed in rats infused with either 10 or 1,000 $\mu$M $ZnCl_2$, as the stimulation trial by group interaction was not significant, but the effect for treatment groups was, $F(2,72.0) = 5.4$, $p < 0.01$. Inspection of Fig. 4 suggests that the delay was most prominent during the period of greatest change, at stimulation numbers 9–16, when afterdischarges of control animals appeared to be about 50% longer than afterdischarges in zinc-infused animals. There was no difference between the two zinc-infused groups. By the completion of kindling at stimulation number 18, all treatment groups had reached the same duration of afterdischarges.

### Nonparametric analysis

As a conservative alternative to the parametric approaches reported previously and thus to enhance our confidence in the findings, a Kruskal-Wallace analysis was performed for each stage, and for the sums of the stage data. The results of the K-W tests are included in Table 1.

As can be seen from the table, the means of the ranked data are remarkably consistent across all stages. The vehicle and the 10 $\mu$M groups were very similar or indistinguishable from one another, whereas the rank of the 1,000 $\mu$M group was approximately twice as great as the others. These data provide corroboration for the above-reported findings that the 1,000 $\mu$M zinc dosage level suppressed seizures relative to the administration of no zinc or a low level of zinc.

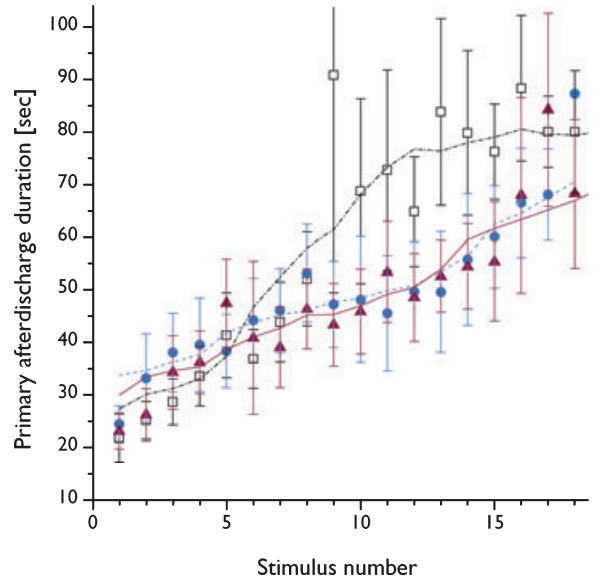


**Figure 4.**
Development of epileptic afterdischarges over time during kindling. The normal saline group (NS; n = 5) is represented by black open squares with a dash-dot line, the 10 $\mu$M zinc-infused group (10 $\mu$M Zn; n = 4) by light blue filled circles with a dashed line, the 1000 $\mu$M zinc-infused group (1000 $\mu$M Zn; n = 5) by dark red filled triangles with a solid line. Data points are averages from all animals in a group, error bars are SEM. The lines are 7-point sliding averages for the averaged data points for each treatment group.


### Histologic analysis: Procedure and qualitative description

Cresyl violet–stained neurons in the hilar and CA3 regions were systematically examined by careful visual inspection by an investigator blinded to animal treatment status. The hilar region was operationally defined as a 100 $\mu$m wide region deep to the granule cell layer, and the CA3 region was operationally defined as a 100 $\mu$m wide region enclosing the pyramidal cell layer, beginning at the lateral pole of the pyramidal cell layer and extending medially to the border of the hilar region. This was done in every fourth coronal section of 60 $\mu$m thickness, beginning from the rostral pole of the hippocampus, resulting in a total of 21 sections in 240 $\mu$m intervals across the entire rat hippocampal formation.

As visible in Fig. 5, some cell loss was noted in the hilar region of some of the animals. This was observed in all animal treatment groups with no apparent trend favoring any of the three groups after unblinding. Fig. 5 shows representative samples of the hilar region from each of the three treatment groups.

This analysis was done only to rule out that any observed physiologic changes might be caused by a major cell loss induced by zinc toxicity, which was not the case. Although the absence of major cell loss induced by zinc toxicity was an interesting finding, formal cell counting using stereology to determine potential minor differences in hippocampal cell numbers between the treatment groups was outside the scope of this investigation.

**Table 1. Average ranked number of trials to seizure by Racine stages and groups**

| Group | n | Stage 1 | Stage 2 | Stage 3 | Stage 4 | Stage 5 | All stages |
|---|---|---|---|---|---|---|---|
| Vehicle | 5 | 6.2 | 5.9 | 5.1 | 5.5 | 5.6 | 5.6 |
| 10 $\mu$M zinc | 4 | 4.9 | 4.9 | 5.9 | 5.8 | 5.4 | 5.3 |
| 1000 $\mu$M zinc | 5 | 10.9 | 11.2 | 11.2 | 10.9 | 11.1 | 11.2 |
| K–W statistic | | 5.5 | 6.4 | 6.3 | 5.2 | 5.8 | 6.1 |
| $\alpha$ | | 0.07 | 0.04 | 0.04 | 0.07 | 0.05 | 0.05 |

The K–W statistic is calculated as $\chi^2$ with 2 df.

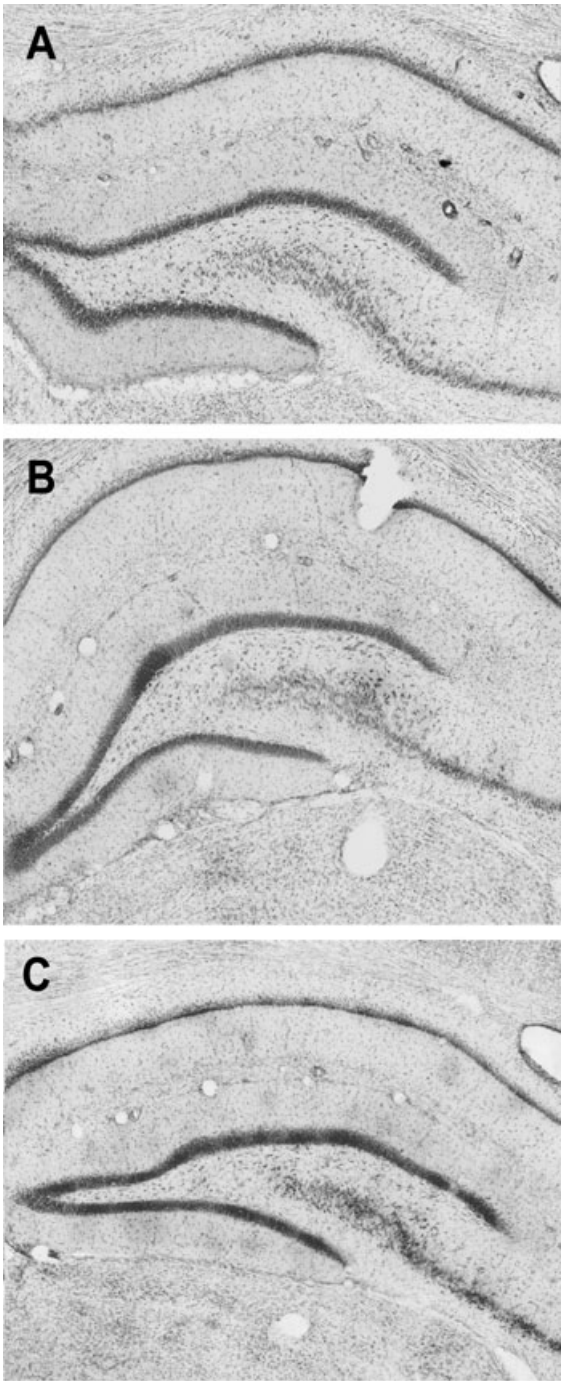


**Figure 5.**
Representative Nissl-stained coronal sections of a rat hippocampus from each of the treatment groups are shown. (**A**) normal saline infusion; (**B**) 10 $\mu$M zinc infusion; (**C**) 1000 $\mu$M zinc infusion. No apparent differences between the treatment groups in the loss of hilar neurons were seen after careful visual analysis as described in the text.


## Discussion

We found in the present kindling study that excess zinc delivered to the hippocampal hilus by continuous infusion delayed the development of primary afterdischarges at both low and high doses of zinc, whereas behavioral seizure development was only delayed at a high dose of zinc. Both changes appeared to be most prominent during the period of greatest change at stimulation numbers 9–16.

These findings need to be interpreted with caution. The present study of zinc effects in the intact brain has important advantages over slice recordings by allowing long-term observations of brain function with nearly intact neuronal connections. On the other hand, there are also clear limitations to this approach. Our strategy using chronic infusion leaves uncertain which levels of extracellular hilar zinc were actually achieved in the study.

The zinc concentrations achieved can be expected to have decreased rapidly with increasing distance from the delivering cannula. We used a mathematical model to estimate the time needed to achieve a steady state (i.e., the same zinc concentration as in the delivering pump, 10 $\mu$M or 1,000 $\mu$M). Delivering zinc at a rate of 0.25 $\mu$l/h, this steady state is reached in about 2 days at a distance of 1 mm from the cannula, whereas about 20 days are required to reach the same state at a distance of 2 mm. This model does not include the unknown effects of zinc uptake or metabolism.

Because the total length of the hippocampus extended over about 5 mm from rostral to caudal in our study, this suggests that only a small fraction of the total hippocampal tissue on one side was exposed to high zinc levels. The small size of the wedge of tissue below the cannula track lacking Timm's stain after TPEN infusion (Fig. 1B) similarly suggests coverage of only a small portion of the hippocampus on one side. These considerations were the reason we used unphysiologically high concentrations of zinc for this experiment—the aim was to achieve physiologically meaningful increases over a larger portion of the hippocampus while accepting unphysiologically high levels localized near the delivery site.

Use of this strategy included the risk of toxic effects of zinc where levels were highest, near the delivery site, including neuronal cell death (Yin & Weiss, 1995; Cuajungco & Lees, 1996, 1998). It is conceivable that zinc-induced neuronal death, rather than neuromodulatory effects of zinc, might have been responsible for the observed inhibitory effect of zinc on the development of seizures in our study. However, this appears unlikely, since careful visual analysis of all brains involved in the study found only occasional and very limited hilar cell loss, which was distributed equally in all treatment groups. In addition, a physiologic change due to neuronal death could be assumed to be permanent. In contrast, the

observed inhibitory effect of zinc on the development of seizures was only temporary. Although the absence of major cell loss induced by zinc toxicity was an interesting finding, formal cell counting using stereology to determine potential minor differences in hippocampal cell numbers between the treatment groups was outside the scope of this investigation.

Because cultured cortical neurons begin to die at zinc concentrations of 200–600 $\mu$M (Choi et al., 1988), the absence of major neuronal cell death in our experiment also lends additional support to the notion that actual zinc levels in most portions of the hippocampus were probably much lower than those in the delivery pump (10 $\mu$M or 1,000 $\mu$M).

It could be argued that continuous zinc infusion differs from normal physiology not only in zinc levels, but also lacks the localized and intermittent phasic peaks to be expected with synaptic release. However, a recent study using zinc fluorescence suggests the continuous presence of an extracellular layer of zinc, which mapped onto the Timm's stained region of the hippocampus, rather than intermittent phasic peaks with synaptic release (Kay, 2003). Although the implications of these data are limited, since they were obtained in slices, these findings suggest that our method of chronic zinc infusion may be adequate to the physiologic context of zinc as described above. In contrast, a physiologic study of the mossy fiber synapse (Vogt et al., 2000) estimates that concentrations of more than 10–20 $\mu$M zinc must be reached temporarily in the synaptic cleft during phasic release of zinc.

Our observations showed that a smaller amount of zinc was needed to achieve a delay in afterdischarge duration than was needed to affect the development of behavioral seizures. This suggests that afterdischarge, duration is more sensitive to subtle physiologic changes than externally observable seizures. This agrees with the common clinical observation of electrographic seizures in humans, which may only eventually become apparent as behavioral seizures after they have spread to involve a large enough area of cortex.

However, afterdischarge data needs to be interpreted with caution as there are sampling issues. Recording via a single bipolar electrode is limited and captures only a small fraction of the overall epileptiform activity of the entire rat brain during a seizure. In contrast, it is highly unlikely that the observed difference in behavioral seizures might result from inaccurate or biased observation. Observations were made by blinded investigators, and the results of the study contradicted our initial expectation that zinc might facilitate epileptic seizures.

Further studies are needed to determine the mechanism by which zinc caused inhibition of seizures in our experiment. In slice studies, zinc can induce the collapse of the enhanced $\gamma$-aminobutyric acid $(GABA)_A$ inhibitory drive in the dentate gyrus in epilepsy (Buhl et al., 1996; Cohen et al., 2003) and may also facilitate granule cell epileptiform activity (Timofeeva & Nadler, 2006). Both actions may contribute to a chronic hyperexcitable state. On the other hand, zinc may also block excitation and the development of seizures by blocking N-methyl-D-aspartate (NMDA) receptors (Molnar & Nadler, 2001). Native NMDA channels in cultured neurons are blocked by zinc (Christine & Choi, 1990; Legendre & Westbrook, 1990). A high-affinity binding site for zinc has been identified (Paoletti et al., 1997), which is subunit dependent (Chen et al., 1997). We can only speculate that the known inhibitory effects of zinc on NMDA receptors (Vogt et al., 2000; Molnar & Nadler, 2001) may have been more prominent under the conditions of our study than potential zinc effects on $GABA_A$ receptors.

This would make sense, since $GABA_A$ receptors appear to be sensitive to zinc only early in development (Draguhn et al., 1990; Smart & Constanti, 1990; Smart et al., 1991) or in the fully kindled state (Buhl et al., 1996), whereas NMDA receptors are zinc-sensitive in adult unkindled rats. It is possible that in our experiment zinc lost its inhibitory action on seizures in the late stages of kindling (inhibition of seizures was only temporary, see Figs. 2 and 4), because $GABA_A$ receptors were then also becoming zinc sensitive. This speculation would need to be confirmed by slice recordings from partially kindled rats.

Other observations in our laboratory showed a decrease in dentate granule cells and an increase in interneurons of $\delta$ subunit containing $GABA_A$ receptors, which are highly zinc-sensitive, after pilocarpine-induced status epilepticus in mice (Peng et al., 2004). This reorganization altered both tonic and phasic GABAergic inhibition in dentate granule cells (Zhang et al., 2007). It remains to be determined if a similar redistribution of the zinc-sensitive delta subunits of $GABA_A$ receptors occurred also in this kindling study and how it may have affected zinc sensitivity of $GABA_A$ receptors.

The findings of other investigators that mice lacking vesicular zinc due to lack of the ZnT-3 zinc transporter (Cole et al., 2000) or due to a zinc-deficient diet (Takeda et al., 2003) were more susceptible to kainic acid induced seizures support the notion that zinc blockage of kainate receptors may also have played a role in the anticonvulsant action of zinc seen in this kindling study. Zinc may also have blocked the release of glutamate from mossy fibers (Bancila et al., 2004; Quinta-Ferreira & Matias, 2004, 2005; Takeda et al., 2004).

Even intracellular modulatory effects of zinc are possible, since extracellular chelation of zinc does not affect hippocampal excitability (Lavoie et al., 2007). Zinc can enter neurons through $Ca^{2+}$-permeable AMPA type glutamate receptors (Yin & Weiss, 1995; Yin et al., 1998), voltage-gated $Ca^{2+}$channels (Freund & Reddig, 1994; Atar et al., 1995; Sensi et al., 1997) or NMDA channels (Sensi et al., 1997). Inside the cell, zinc can activate gene expression (Atar et al., 1995), inhibit NOS (Persechini et al., 1995),

activate PKC (Csermely et al., 1988), or may replace $Ca^{2+}$ in intracellular signaling pathways (Csermely et al., 1989).

The results of our study agree with the majority of studies examining the effect of zinc on seizures, which mostly indicate anticonvulsant effects of zinc. Decreased zinc due to dietary deficiency (Sterman et al., 1986; Takeda et al., 2003, 2006) or due to knockout of the synaptic vesicle zinc transporter ZnT-3 (Cole et al., 2000) resulted in increased susceptibility to kainate-induced seizures in mice and to kindling-induced seizures in cats (Sterman et al., 1986). While the kindling study has similar results to our own, potential changes in hippocampal zinc levels or zinc release due to the dietary changes were not investigated in this study.

There are only a few studies suggesting opposite (proconvulsant) effects of zinc. Zinc chelation in slices obtained from rats after pilocarpine-induced status epilepticus slowed the rate of development of NMDA receptor activated epileptiform activity in granule cells (Timofeeva & Nadler, 2006). In contradiction to the findings in our study, a decrease of kindling-induced seizure and afterdischarge duration was seen, without change in the progression of kindling stages, with repeated i.p. injections of the membrane-permeable zinc chelator DEDTC just before stimulations (Foresti et al., 2008). It cannot be excluded that these findings were due to toxic effects of the membrane-permeable chelator DEDTC, which may have interfered not only with several types of intracellular ions, but also with other aspects of intracellular metabolism and membrane stability. In our own preliminary studies using the membrane-permeable chelator TPEN we observed prominent toxic effects, which was the reason these studies were not taken further. A major difference is that the DEDTC study used rapid kindling over 2–3 days (10 stimuli per day), in contrast to the slow, daily kindling extended over several weeks used in our study. Therefore, it is also conceivable that during very rapid amygdalar kindling, as done in this study, $GABA_A$ receptors were converted to become zinc-responsive much faster than during the slow hippocampal kindling used in our study. This might have resulted in predominantly $GABA_A$ receptor-mediated proconvulsive effects of zinc, in contrast to the speculated predominantly NMDA receptor-mediated anticonvulsive effect of zinc in our own study of slow hippocampal kindling.

In any case, the results of our study should not be extrapolated too easily to the very different situation of spontaneous seizures in epileptic patients. Continuous chronic zinc infusion is substantially different from physiologic intermittent synaptic release of zinc. Whether the net effect of the highly complex neuromodulatory actions of zinc becomes inhibitory or excitatory is likely to depend on the circumstances. While our study investigated infused zinc, it does support the hypothesis that zinc which is synaptically released during seizures may alter excitability in hippocampal circuits in a similar fashion. A better understanding of the neuromodulatory actions of zinc could lead to therapeutic strategies aimed at preventing the development of seizures similar to kindling after traumatic or other types of brain injury by altering the release or the actions of zinc.

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We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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