Zinc-Induced Collapse of Augmented Inhibition by GABA in a Temporal Lobe Epilepsy Model

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In the kindling model of temporal lobe epilepsy, several physiological indicators of inhibition by γ-aminobutyric acid (GABA) in the hippocampal dentate gyrus are consistent with an augmented, rather than a diminished, inhibition. In brain slices obtained from epileptic (kindled) rats, the excitatory drive onto inhibitory interneurons was increased and was paralleled by a reduction in the presynaptic autoinhibition of GABA release. This augmented inhibition was sensitive to zinc most likely after a molecular reorganization of $GABA_A$ receptor subunits. Consequently, during seizures, inhibition by GABA may be diminished by the zinc released from aberrantly sprouted mossy fiber terminals of granule cells, which are found in many experimental models of epilepsy and in human temporal lobe epilepsy.

Synaptic inhibition in the mammalian forebrain is primarily mediated by GABA acting at its various receptors (*1*, *2*). It is not known by how much the balance between excitation and inhibition has to be offset for pathological changes to occur. A reduced synaptic inhibition will favor hyperexcitability, a condition long associated with epilepsy (*3*). Although in vitro studies of acute epilepsies have often relied on the experimental impairment of inhibition by GABA (*3*), the fate of inhibition by GABA in chronic epilepsy models and particularly in human epilepsies remains unclear (*3*–*6*). A distinctive sprouting of mossy fibers in the dentate gyrus is shared among human temporal lobe epilepsy (TLE) and several experimental epilepsy models (*7*–*10*). The aberrantly sprouted mossy fibers form recurrent excitatory synapses with other granule cells but may also contribute to the synaptic drive onto inhibitory interneurons (*11*). The kindling model of TLE, in which seizures are induced by initially subthreshold electrical stimuli delivered daily to limbic areas over several days or weeks, replicates many anatomical and pathological features, including the sprouting of mossy fibers as seen in human TLE (*12*). The model is characterized by an enhanced functional inhibition by GABA in the dentate gyrus (*6*, *13*, *14*), but there is no estimate of the excitatory drive onto the inhibitory interneurons.

We measured the degree of glutamatergic excitatory drive onto specific interneurons (*15*, *16*) responsible for generating spontaneous inhibitory postsynaptic currents (sIPSCs) in dentate gyrus granule cells after kindling by recording inhibitory currents in the whole-cell configuration (*17*) at the reversal potential (0 to +5 mV) of excitatory synaptic events (*18*). At this membrane potential, sIPSCs could be detected selectively (Fig. 1A), whereas the excitatory drive onto the interneurons remained intact and could be assessed by the

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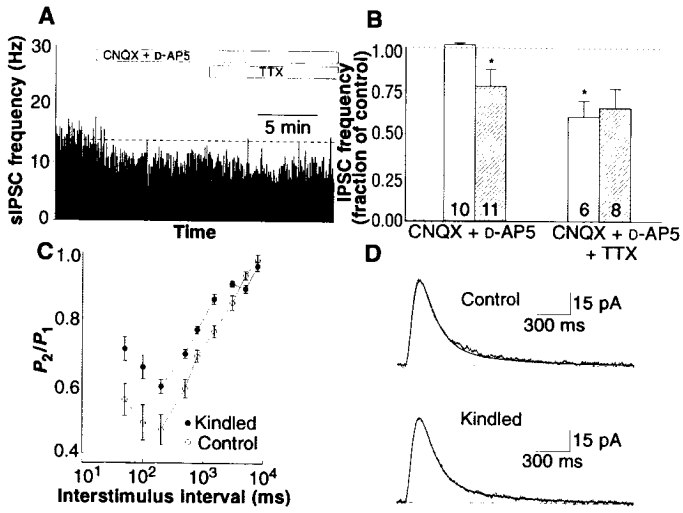

Fig. 1. Enhanced inhibition of kindled granule cells through increased excitatory drive onto interneurons and through a diminished autoinhibition of GABA release (**A**) for each cell, the average frequency over 1 to 2–min periods (dotted line) was measured at three different time points: (i) in control ACSF, (ii) during CNQX–D-AP5 perfusion, and (iii) during perfusion of CNQX–D-AP5 plus TTX. In this kindled granule cell, sIPSC frequency is plotted versus time and the horizontal bars indicate the perfusion of the various antagonists. (**B**) The ratios of the frequencies measured in CNQX–D-AP5 and in CNQX–D-AP5 plus TTX were calculated relative to the frequency in control ACSF (considered to be 1.0). The changes in relative frequency are plotted for control (open bars) and kindled (shaded bars) cells. The number of cells is indicated at base of the bars. Asterisks denote significant difference from the previous perfusate, $P < 0.01$, two-tailed t test. (**C**) The magnitude and time course of presynaptic autoinhibition was assessed in paired-pulse experiments on monosynaptically evoked, isolated $GABA_B$ currents (*25*). The population data are plotted; P_2/P_1 (the amplitude of the subtracted test pulse divided by the amplitude of the conditioning response) is displayed as a function of varying interstimulus intervals (50 to 8000 ms). The maximal inhibition is significantly reduced (by 25 to 45%, $P < 0.01$, two-tailed t test, control $n = 6$, kindled $n = 9$) after kindling at each interstimulus interval except at the two longest intervals (5000 and 8000 ms). (**D**) Isolated monosynaptically evoked $GABA_B$ currents were similar in a control (top) and a kindled (bottom) granule cell recorded in the presence of 10 μM CNQX, 40 μM D-AP5, and 75 μM picrotoxin (*17*). Each trace is the average of three to five successive responses.

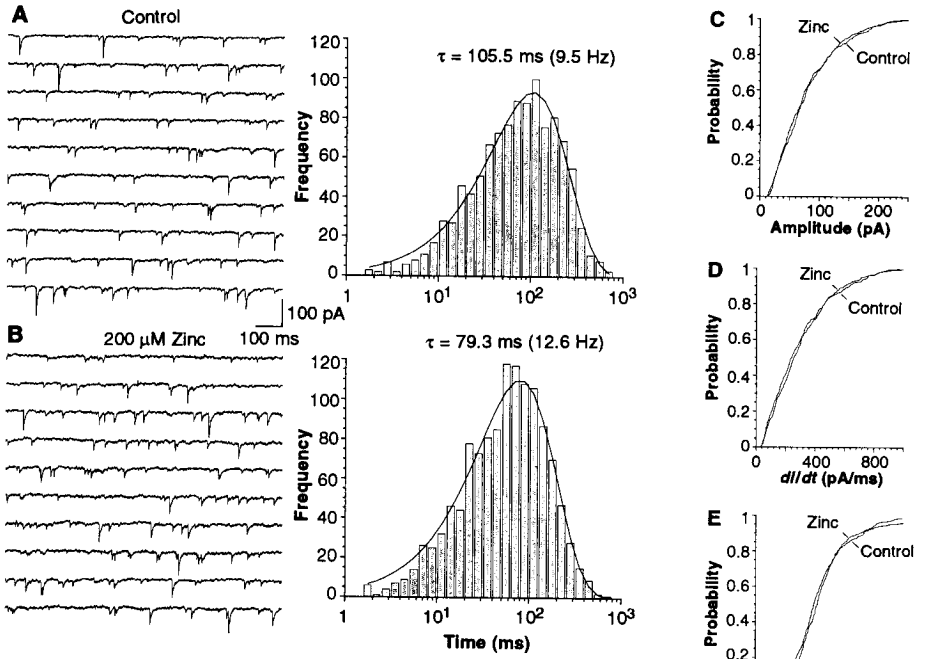

Fig. 2. Effect of bath-applied Zn^{2+} on mIPSC parameters in granule cells of control animals. (**A**) The left panel shows a 10-s recording of mIPSCs in a granule cell with the interevent-interval histogram of the same recording period to the right. Because of the chloride loading of the cells, IPSCs appear as inward currents. Mean mIPSC frequency during the control period was 9.5 Hz. (**B**) After bath application of 200 μM $ZnCl_2$ dissolved in ACSF, the frequency of mIPSCs increased to 12.6 Hz. (**C** through **E**) Cumulative probability plots of granule cell mIPSC parameters during control conditions and after bath application of 200 μM Zn^{2+}. In control granule cells ($n = 10$), none of the measured parameters, such as amplitude (C), rate of rise (D), and decay-time constants (E) was significantly altered (comparison of median values, two tailed t test, $P > 0.01$).

decrease in event frequency caused by perfusing the ionotropic glutamate receptor antagonists 6-cyano-7-nitro-quinoxaline-2,3-dione (CNQX, 10 μM) and D-2-amino-5-phosphonovalerate (D-AP5, 40 μM). Such treatment causes a reduction of less than 10% in the frequency of sIPSCs (*18, 19*). Consistent with a weak basal spontaneous excitatory drive onto interneurons in control slices (Fig. 1B), the average ratio of sIPSC frequencies observed after and before the CNQX + D-AP5 perfusion was 1.02 ± 0.01 ($n = 10$ slices). In contrast, after kindling, perfusion of CNQX + D-AP5 caused a significantly more-pronounced reduction (0.78 ± 0.1; $n = 11$ slices; $P < 0.01$, Student's t test) in the frequency of inhibitory events (Fig. 1, A and B). This finding can best be explained by an enhanced excitatory drive onto the interneurons responsible for generating spontaneous events in kindled granule cells. The anatomical substrate for this enhanced excitatory drive may be the sprouted mossy fibers that invade infra- and supragranular regions known to be abundant in interneuron processes (*20*).

Going a step beyond the excitatory drive onto the interneurons in the epileptic dentate gyrus, we also wanted to know what fraction of the spontaneous GABA release was related to invasion of the inhibitory terminals by action potentials (*2, 18, 19, 21*). In control cells, the frequency of miniature IPSCs (mIPSCs) recorded after perfusion of the Na^+ channel blocker tetrodotoxin [TTX, 1 μM (*18, 19, 21*)] was 60 ± 9% ($n = 6$ slices) of that recorded in the presence of CNQX + D-AP5 alone. However, in kindled preparations, once the excitatory amino acid receptor antagonists lowered sIPSC frequency, it could not be reduced further by perfusing TTX onto the slices (Fig. 1, A and B). Hence, in the absence of an excitatory drive onto the interneurons, most of the sIPSCs in kindled granule cells seem to result from a release of GABA that is independent of action potentials.

The release of GABA from inhibitory terminals is also under the control of presynaptic $GABA_B$ autoreceptors (*22*). These receptors can inhibit GABA release by as much as 40 to 60%, producing an activity-dependent disinhibition (*23*). An augmented presynaptic autoinhibition of GABA release, especially upon sustained firing of interneurons, could reduce dramatically the efficacy of inhibition during seizures (*23*). In kindled dentate gyri, we observed the opposite: The paired-pulse inhibition of monosynaptically evoked IPSCs, a paradigm used to measure activation of $GABA_B$ autoreceptors (*24, 25*), was significantly reduced (Fig. 1C). Reduced autoinhibition in epilepsy may guarantee a steady release of

GABA with repeated presynaptic activity, particularly during the sustained activity accompanying seizures of GABA-transmitting terminals. Contrasted with the diminished activation of presynaptic $GABA_B$ receptors after kindling, stimulus-evoked IPSCs mediated by $GABA_B$ receptors (*25*) were comparable in control and kindled granule cells (Fig. 1D).

These physiological findings appear to be consistent with enhanced inhibition mediated by $GABA_A$ receptors in the epileptic—that is, presumably hyperexcitable—dentate gyrus. With an augmented inhibition, how can the balance of excitability ultimately tip in favor of excitation and produce epilepsy? Excitatory neurotransmission mediated by the *N*-methyl-D-aspartate type of glutamate receptors is clearly enhanced after kindling (*26*), but other key factors may undermine the dampening effect of an augmented inhibitory activity.

We tackled this issue by examining another possible pathophysiological role of the massive and aberrant mossy-fiber sprouting characteristic of the epileptic dentate gyrus. Mossy fibers are loaded with Zn^{2+} that can be released on stimulation and that is estimated to reach local concentrations of a few hundred micromoles per liter (*27*). Sprouted to supragranular sites, mossy fiber boutons still contain Zn^{2+} (*7*, *10*) that could be released onto perisomatic and proximal dendritic regions of granule cells where little if any Zn^{2+} is being released normally under nonepileptic conditions. These regions are precisely where spontaneous inhibitory events originate on granule cells (*16*). Because Zn^{2+} inhibits certain types of $GABA_A$ receptors (*28*), mainly during early development (*29*), we considered whether it may also inhibit $GABA_A$ receptors of granule cells. First we examined the effect of Zn^{2+} on mIPSCs in control neurons. Perfusion of 200 μM $ZnCl_2$ had no effect on the amplitude, decay-time constant, or rates of rise of mIPSCs (Fig. 2, $n = 10$). The frequency of mIPSCs was moderately increased by Zn^{2+} (12 ± 5%; $n = 10$; $P = 0.038$, two-tailed t test), an effect that lasted beyond the wash, persisting in some cells for as long as 40 min after the return to the control perfusate. These findings reflect known presynaptic effects of Zn^{2+} (*30*) and are consistent with the presence of Zn^{2+}-insensitive $GABA_A$ receptors on control granule cells.

If $GABA_A$ receptors were similar in control and kindled granule cells, then contrary to our original hypothesis, Zn^{2+} release from sprouted mossy fibers could not cause a diminished inhibition in the epileptic dentate gyrus. The hypothesis could still be valid if marked differences were present in the sensitivity of control and "epileptic" $GABA_A$ receptors to Zn^{2+}. Trying to unravel possible differences in the Zn^{2+} sensitivity of control and epileptic $GABA_A$ receptors, we examined the effect of Zn^{2+} on mIPSCs recorded in slices obtained from kindled animals. In contrast to its effect in controls, Zn^{2+} produced a striking effect on mIPSCs recorded in kindled granule cells. Perfusion of $ZnCl_2$ (200 μM) blocked mIPSCs in kindled neurons, resulting in a significant decrease in mIPSC frequency (56 ± 8%; $n = 18$; Fig. 3, A and B). The presynaptic effect of Zn^{2+}, commonly seen as a lasting increase in the frequency of mIPSCs in control neurons, was masked in kindled cells and could only be observed after the wash of $ZnCl_2$ (Fig. 3C). Thus, the reduction of mIPSC frequency by perfusion of $ZnCl_2$ must have resulted solely from a reversible antagonism of epileptic granule-cell $GABA_A$ receptors by Zn^{2+} (Fig. 3, D through H). This antagonism was characterized by significantly ($P \ll 0.01$, paired two-tailed t test) reduced median amplitudes (by 33 ± 4%, from 57.9 ± 3.3 to 38.3 ± 3.3 pA; $n = 18$; Fig. 3, D and G), rates of rise (by 35 ± 5%, from 261 ± 16 to 168 ± 15 pA/ms; $n = 18$; Fig. 3, E and H, and faster decay-time constants (by 20 ± 5%, from 3.48 ± 0.08 to 2.82 ± 0.22 ms; $n = 18$; Fig. 3, F and H) of the mIPSCs recorded in the presence of Zn^{2+} in kindled neurons. We also simulated the effects of Zn^{2+} on mIPSCs (*31*). These simulations reflected the possibility that Zn^{2+} may block the epileptic $GABA_A$ receptor channels (*32*) through a noncompetitive mechanism (*33*).

The most obvious explanation for the Zn^{2+} sensitivity of kindled synaptic $GABA_A$ receptors would be the possible kindling-induced loss of γ subunits. However, because of the increased benzodiazepine binding after kindling (*34*), this pos-

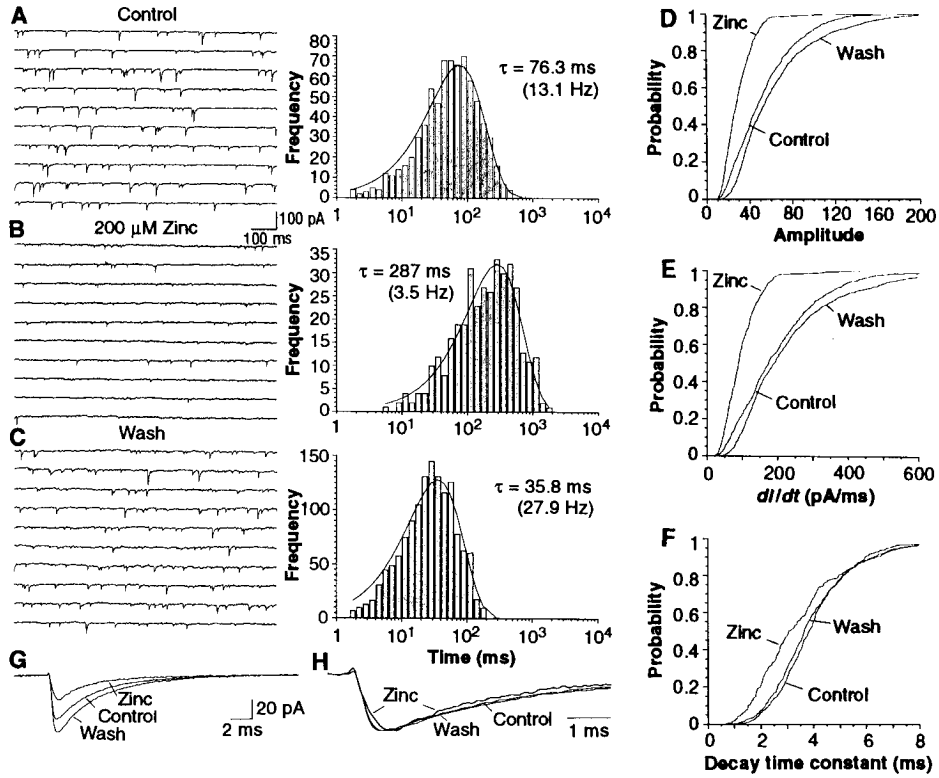

Fig. 3. Block of mIPSCs by bath-applied Zn^{2+} in a kindled granule cell. (**A**) The mean frequency of mIPSCs in control ACSF was 13.1 Hz. (**B**) Perfusion of 200 μM $ZnCl_2$ resulted in a dramatic reduction (to 27% of control, or 3.5 Hz) of mIPSC frequency. (**C**) The effect of Zn^{2+} was reversible. Moreover, indicating a long-term presynaptic effect comparable to that observed in controls, the frequency of mIPSCs more than doubled after washout and remained elevated for the remainder of the experiment (>30 min). The leftmost panels in (A) through (C) show 10-s consecutive recordings of mIPSCs in the presence of ionotropic glutamate antagonists. The corresponding panels to the right show the respective interevent-interval histograms fitted with exponential distributions. Cumulative probability plots of mIPSC parameters taken from the same cell illustrated in (A) through (C) indicated that Zn^{2+} resulted in significant reduction of mIPSC amplitudes (**D**), rates of rise (**E**), and decay-time constants (**F**), indicating a postsynaptic action on $GABA_A$ receptors in the granule cell. The effects on mIPSC parameters were readily reversible after superfusion with control ACSF. (**G**) After events had been sorted according to their rates of rise, those (±15%) scattered around the median were averaged and superimposed. Note the Zn^{2+}-induced reversible reduction of the average mIPSC. (**H**) The superimposition of normalized averages on a different time scale shows that Zn^{2+} perfusion reversibly reduced the mIPSC rate of rise and increased the rate of decay, consistent with an action of Zn^{2+} at synaptic $GABA_A$ channels.

sibility seems unlikely. Nevertheless, we tested the sensitivity of mIPSCs recorded in kindled preparations to the benzodiazepine agonist zolpidem. As in controls (*18*, *31*), perfusion of zolpidem (10 μM) produced a significant lengthening (327 ± 43%) of mIPSC decay-time constants in kindled granule cells (n = 6), consistent with the presence of functional benzodiazepine receptors after kindling. The absence of γ subunits, which are critical for benzodiazepine sensitivity (*28*), is commonly associated with the blocking effect exerted by Zn^{2+} on $GABA_A$ receptors during early ontogeny (*29*). Therefore, the preserved benzodiazepine sensitivity after kindling must reflect the continued presence of γ subunits in epileptic $GABA_A$ receptor channels (*35*); the Zn^{2+} sensitivity of these receptors must have arisen despite the functional γ subunits. Other subunits may also regulate the Zn^{2+} sensitivity of $GABA_A$ receptors: Some benzodiazepine-sensitive receptors are inhibited by Zn^{2+} (*33*), possibly through certain α subunits or the δ subunit (*36*).

In summary, two additional components of inhibition seem to compensate for hyperexcitability in the epileptic dentate gyrus: (i) an increased excitatory drive onto inhibitory interneurons, and (ii) a decreased autoinhibition of GABA release. Yet this enhanced inhibition by GABA ultimately collapses in the kindled hippocampus, and Zn^{2+} may be pivotal in this breakdown. During massive neuronal activity, Zn^{2+} released from sprouted mossy fibers around granule cell bodies and proximal dendrites (*37*) could cause a significant impairment in the function of epileptic $GABA_A$ receptors that are sensitive to Zn^{2+}. This Zn^{2+}-induced reduction of inhibition by GABA may promote the spread of epileptic activity. Furthermore, if Zn^{2+}-sensitive $GABA_A$ receptors were present in the normal adult brain, the activity-dependent Zn^{2+} release from neighboring excitatory boutons (*27*) may explain the intense predisposition of certain brain structures to epilepsy (*3*).

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17. The procedure for surgery and daily kindling of male Wistar rats through the hippocampal commissure was described previously in detail [G. Köhr, Y. De Koninck, I. Mody, *J. Neurosci.* **13**, 3612 (1993)]. Only cells that satisfied our criteria throughout the experiment for a constant low access resistance recording were included in the analysis. A total of 26 control cells from 2 age-matched and 11 sham-implanted controls, and 44 granule cells from 21 kindled animals with at least 15 stage five motor seizures were included. Coronal or horizontal 400-μm-thick brain slices were prepared from control and kindled rates (24 to 48 hours after the last seizure) as described previously (*20*). The artificial cerebrospinal fluid (ACSF) contained (in mM) 126 NaCl, 5 KCl, 2 $CaCl_2$, 2 $MgCl_2$, 26 $NaHCO_3$, 10 D-glucose, 0.01 CNQX (Tocris Cookson, Bristol, UK), 0.01 D-AP5 (Tocris Cookson, Bristol, UK), and 0.001 TTX (Calbiochem). The ACSF and the recording chamber were continuously bubbled with a mixture of 95% O_2 and 5% CO_2. Miniature IPSCs were recorded from dentate gyrus granule cells with tight-seal, whole-cell patch clamp pipettes containing 140 mM CsCl, 2 mM $MgCl_2$, 10 mM Hepes, and occasionally 0.2 mM EGTA or bis(aminophenoxy) ethanetetraacetic acid (BAPTA); CsOH was used to adjust the pH to 7.2. For experiments measuring isolated synaptic $GABA_B$ responses, the pipettes contained (in mM) 135 K-gluconate, 10 Hepes, 2 $MgCl_2$, and 0.2 tris-guanosine triphosphate (GTP); osmolarity of the filling solutions was between 265 and 275 mosm. All recordings were obtained at 35 ± 0.5°C and holding potentials of −70 ± 3 mV with either an Axopatch 1D, an Axopatch 200A, or a List EPC-7 amplifier. Access resistances (compensated 60 to 80%) were frequently monitored and were constant during the perfusion of $ZnCl_2$ and comparable between recordings in control and kindled granule cells. The signals were transmitted through a low-pass filter at 3 kHz (−3 dB, eight-pole Bessel filter, Frequency Devices 9002) and digitized at 20 kHz (DT 2821 A/D interface). Individual events were detected and analyzed off-line [CDR software; J. Dempster, University of Strathclyde, Glasgow, UK; see also (*16*) and (*18*)]. All experimental values are given as means ± SEMs. Significance was set at the 0.01 level.
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$$I(t) = A[1 - \exp(-t/\tau_m)]^4$$

$$[Aw_{h_1}\exp(-t/\tau_{h_1}) + Aw_{h_2}\exp(-t/\tau_{h_2})]$$

where $I(t)$ is current as a function of time, w_{h_1} and w_{h_2} are weighting factors (where $A = Aw_{h_1} + Aw_{h_2}$), τ_m is the time constant of the activation parameter, and τ_{h_1} and τ_{h_2} are the time constants of the inactivation parameters. The average values (± SEM) for τ_m, τ_{h1}, τ_{h2}, w_{h1}, and w_{h2} of the $GABA_B$ responses were comparable in milliseconds except w_{h1} and w_{h2}: 41.8 ± 2.7, 152.7 ± 10.8, 981 ± 198, 0.87 ± 0.02, and 0.11 ± 0.02 (control, n = 6), and 45.6 ± 1.2, 143.4 ± 7.6, 722 ± 86.9, 0.83 ± 0.06, and 0.17 ± 0.06 (kindled, n = 9).
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32. According to the model, to produce the observed effects of Zn^{2+} on kindled mIPSC kinetics without a concomitant reduction in single-channel conductance (*33*), the probability of opening (p_{open}) during bursts had to be decreased by 25 to 30%, an effect also observed in steady state single-channel recordings (*33*). Additionally, the unblocking rate had to be lowered by 71% (from 6666 to 1961 s^{-1}) and the closing rate of the channels had to be enhanced by 80% (from 303 to 543 s^{-1}). The ON rate, a parameter contributing to the rate of rise of mIPSCs (*38*), had to be reduced by 61% (from 7662 to 3000 s^{-1}).
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region of kindled slices in the presence of blockers of excitatory amino acid receptors with the aim to release endogenous Zn^{2+} onto the granule cells. In one of three experiments, we successfully reproduced the effects of perfused Zn^{2+} on sIPSCs, presumably through the release of Zn^{2+} from sprouted mossy fibers. Repetitive stimuli delivered to the same location in control slices had no effect on sIPSCs (n = 6). In slices, Zn^{2+} release experiments are difficult to control because even low-frequency stimuli used to test evoked responses can inadvertently release the bulk of Zn^{2+} from the mossy fibers. In the absence of any exogenous Zn^{2+} added to the ACSF, the lost Zn^{2+} cannot be replenished (C. J. Frederickson, personal communication).

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