Chronic Zinc Deficiency Alters Neuronal Function of Hippocampal Mossy Fibers

Abstract. *Low-frequency stimulation of hippocampal mossy fiber axons in zinc-deficient adult rats produced synaptic responses that declined in amplitude with successive stimuli. This response decrement is abnormal and suggests that the heavy deposits of zinc in mossy fiber boutons are important for synaptic transmission.*

One of the peculiar features of the mossy fiber system of the hippocampus is a high concentration of trace metals confined primarily to the terminal field of the axons. Several methods of histochemical analysis indicate that the predominant metal is zinc, which occurs primarily in association with vesicles within the mossy fiber giant synaptic boutons (*1*, *2*). This accumulation of zinc is generally presumed to be involved in some unspecified aspect of mossy fiber synaptic function (*3*).

Zinc in the mossy fiber system seems to be relatively labile (*4*); it is not implausible that chronic dietary deficiency of zinc might result in depletion of the metal from the mossy fibers and cause alteration of mossy fiber neuronal function. This possibility may explain some aspects of the striking behavioral symptoms that accompany severe zinc deficiency (*5*). The electrophysiological experiments reported here provide support for this notion by showing that neuronal transmission through the mossy fiber pathway is abnormal in adult rats that are severely deficient in zinc. Low-frequency stimulation of the mossy fiber axons in such rats produced synaptic field potentials that declined in amplitude with successive stimuli—unlike the response potentiation observed in control animals. These results support the possibility that zinc is involved in mossy fiber synaptic function and appear to be the first indication of a specific alteration in neuronal function associated with deprivation of an essential nutrient.

Male Sprague-Dawley rats were maintained on freely accessible zinc-deficient food beginning at 32 days of age. (*6*). Standard housing and handling precautions were observed to avoid significant zinc contamination. By the time of the electrophysiological experiments, the rats had experienced zinc deprivation for periods ranging from 48 to 63 days and exhibited a variety of deficiency symptoms, including anorexia, slow wound healing, alopecia, dermatitis, and behavioral abnormalities (*7*). Some control animals maintained under similar conditions were pair-fed zinc-supplemented food, whereas others were allowed free access to food.

The electrophysiological experiments were performed on the rats after urethane anesthetization (1.2 g/kg) (*8*). To evaluate mossy fiber electrophysiological properties, bipolar stimulating electrodes were placed in the dentate gyrus (the origin of the mossy fiber axons) and a micropipette electrode (NaCl-filled, 2 to 10 megohms) was placed in the CA3 region (the mossy fiber terminal field) of the hippocampus to record the extracellular, monosynaptically evoked field potentials (*9*). Additional stimulating electrodes were placed in the contralateral CA3 region to stimulate commissural axons that terminate on the same ipsilateral CA3 pyramidal cells as do the mossy fiber axons. These commissural synapses do not appear to contain a significant quantity of zinc, and thus the response to stimulation of the commissural axons provides an indication of whether zinc deficiency alters the properties of the mossy fiber pathway specifically or produces generalized changes in neuronal function (*10*).

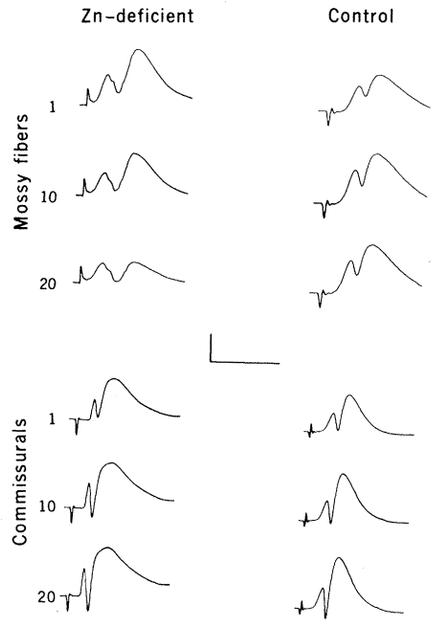

Fig. 1. Examples of field potentials evoked by stimulating mossy fiber and CA3 commissural axons in zinc-deficient and normal rats. Mossy fiber axons were activated with electrodes in the ipsilateral dentate hilus and commissural axons with electrodes in the contralateral CA3 region. Recording electrodes were located in the CA3 pyramidal layer. The top, middle, and bottom waveforms of each set are the potentials evoked by the first, tenth, and twentieth stimulus pulses, respectively, in a 4-Hz, 20-pulse train. Note that only the mossy fiber response of the zinc-deficient rats shows a response decrement under these conditions. Time and voltage calibrations are 10 msec and 1 mV (positive up).

Stimulation consisted of trains of 20 pulses delivered at frequencies between 0.33 and 4.0 Hz, with intertrain intervals of from 1 to 3 minutes (*11*). For each animal, the evoked responses to six to eight trains at each stimulation frequency were recorded on FM tape and then digitized at a sample rate of 8 kHz and analyzed by computer. The synaptic response amplitude was quantified by calculating the initial slope of the response in volts per second prior to the onset of any population spike discharge (*12*).

In the control animals, trains of low-frequency stimulation produced response potentiation. That is, the evoked synaptic response increased in amplitude during the course of a stimulus train (Figs. 1 and 2, A and B). The magnitude of this effect was frequency-dependent, with greater potentiation occurring at higher frequencies. Although the degree of potentiation varied among animals, the effect was clearly evident in all control animals and the magnitude was consistent throughout the course of an experiment with any particular animal. At the highest stimulation frequency used (4 Hz), potentiation of 24 to 38 percent was observed in eight control animals, whereas at 1 Hz a range of 7 to 18 percent was observed. At the lowest frequency (0.33 Hz), the response remained stable with no significant potentiation; declining response was not observed. Essentially the same results have been reported by others (*13*).

Stimulation of the mossy fibers in zinc-deficient animals, however, did not produce potentiation. Rather, the evoked synaptic response during a stimulus train declined in amplitude in each case (Figs. 1 and 2A). The effect was also dependent on the stimulation frequency. The response decrement was greatest at 4 Hz, where depressions of 22 to 43 percent were observed in seven experimental animals. Little or no depression was noted at 0.33 Hz, the lowest stimulation frequency. In none of the seven zinc-deficient animals did mossy fiber stimulation produce response potentiation. Since the response decrement was approximately exponential, best-fit exponential decay constants were calculated for each animal for each stimulation frequency. The combined data are plotted in Fig. 3 to show the dependency of the response decrement on frequency.

Although mossy fiber stimulation in the zinc-deficient animals produced a re-

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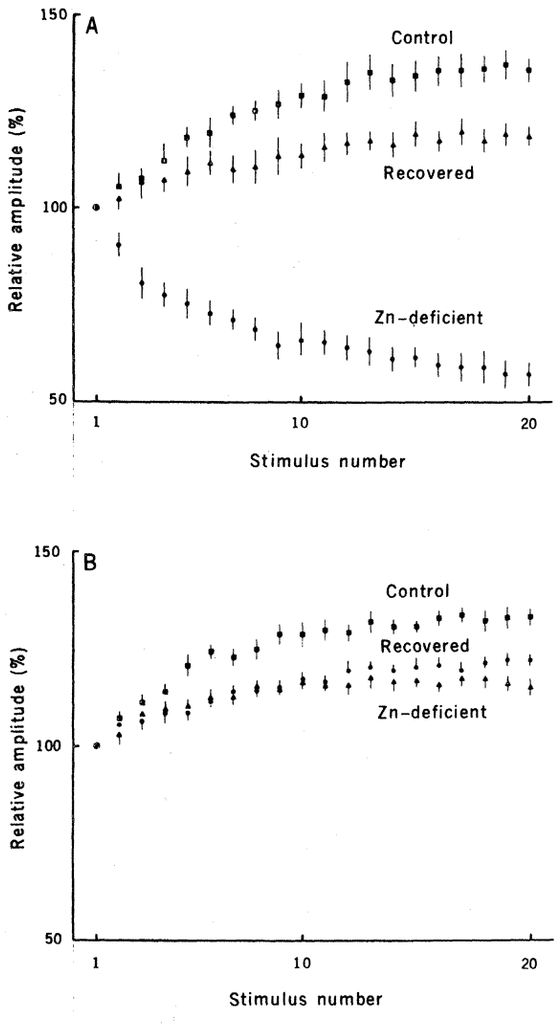

Fig. 2. (A) Relative amplitude of the initial slope of successive CA3 responses evoked by mossy fiber stimulation at 4 Hz from a normal rat, a zinc-deprived rat, and a rat whose zinc deprivation was reversed with supplements. Vertical bars indicate standard error. Recording electrodes in all cases were located in the CA3 pyramidal layer. (B) Similar to (A), but showing responses to stimulation of the commissural projections to the CA3 region. All three responses are within the normal range.

sponse decrement, this was not observed when the commissural axons were stimulated in the same animals (Figs. 1 and 2B). Low-frequency stimulation of the commissural axons in the experimental animals had the same effect as in the control animals; response potentiation was observed in every case, and the magnitude was comparable to that observed in the control animals.

Three observations seem important in interpreting these results. First, the severity of the mossy fiber depression appears to be related to the severity of zinc deficiency. In preliminary experiments with animals less severely deprived of zinc and showing minimal symptoms of zinc deficiency, the mossy fiber response decrement was less than one third of the maximum depression observed in the more severely deprived animals. Second, the effect appears to be reversible with dietary zinc supplements. One experimental animal, after 63 days of zinc deprivation, was given zinc-supplemented food for 48 hours prior to an experiment (*14*). The mossy fiber synaptic response of this rat potentiated (Fig. 2, A and B) (*15*). This rapid recovery from the effects of sustained zinc deprivation is consistent with both experimental and clinical experience (*5*, *14*, *16*). Finally, there were decided differences between the dithizone staining properties of the normal and the deprived animals (*17*). In normal rats, dithizone stains the hippocampal mossy fiber layer a conspicuous pink. But of six zinc-deficient animals examined, only one stained with moderate intensity; the rest either stained faintly or not at all (*18*). The mossy fibers of all six control animals examined stained with at least moderate intensity. Significantly, the mossy fibers of the rat whose zinc deficiency had been reversed were deeply stained by dithizone.

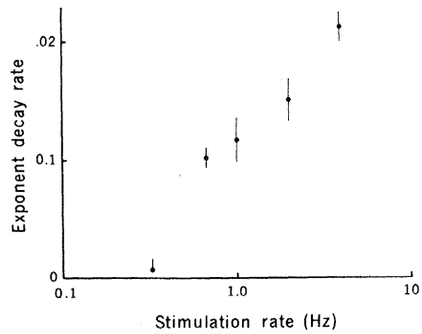

Fig. 3. The effect of stimulation rate on the rate of decrement of the mossy fiber response for the zinc-deficient rats. At each stimulation rate, the response amplitudes were fit to an exponential $y = K \exp(-an)$, where n is the stimulus number within a 20-pulse train. The exponential slope parameter a is plotted here. Vertical bars indicate standard error.

The major finding of these experiments was an abnormal decrement in the response to low-frequency stimulation of the hippocampal mossy fiber pathway in zinc-deficient rats. Clearly, this response decrement is related to the deficiency of zinc in the rats, since it was not observed in control animals and has not been reported by others working with normal rats (*13*). Although the mechanism involved in this response decrement is still unclear, it seems to have a presynaptic location. This is suggested by the normal responses obtained with stimulation of commissural axons in the same zinc-deficient animals that show decreasing responses to mossy fiber stimulation (*10*). The mechanism may be a progressive reduction in neurotransmitter release caused by deprivation-induced depletion of mossy fiber zinc, with consequent reduction of the activity of some zinc-dependent enzyme necessary to maintain transmitter availability at the synapses. Although the evidence to fully evaluate this hypothesis remains insufficient, it is consistent with (i) the known effects of zinc deficiency on the activity of some zinc metalloenzymes (*19*), (ii) the presumed or demonstrated mechanism of synaptic response decrement in a variety of preparations (*20*), and (iii) the rapid and specific effects of zinc chelating agents on mossy fiber neuronal transmission (*21-23*).

It is unlikely that hippocampal mossy fiber synapses are the only ones containing significant quantities of heavy metals such as zinc. Evidence is accumulating that heavy metals are widely distributed in neuropil—primarily within presynaptic structures, as is mossy fiber zinc (*3*, *24*). If such metals as zinc are important to neurotransmission in the synapses in which they are located, then a variety of neurobehavioral disorders may be related to abnormalities in trace metal metabolism (*16*, *25*).

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9. Electrodes were placed by using a combination of stereotaxic coordinates and electrophysiological control. Electrode positions were confirmed following an experiment in cresyl violet-stained sections with recording pipette tip positions marked with fast green dye [R. C. Thomas and V. J. Wilson, *Nature (London)* **206**, 211 (1965)]. In some cases, electrode tips were clipped off and left in place while the brain was fixed.
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11. Stimulation was with biphasic square waves of 100-μsec duration. Intensity was usually adjusted to give approximately one half of the maximal response (about 200 to 400 μA). Although similar results were obtained with higher and lower stimulation intensities, only moderate intensities were investigated systematically, since they present fewer problems of interpretation [O. Steward, W. F. White, C. W. Cotman, *Brain Res.* **134**, 551 (1977)].
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14. Food consumption during this period approximately doubled over that of the previous 48 hours, a strong indication of recovery from zinc deficiency. The dietary supplement provided 24 mg of zinc during the 48 hours. Consumption of a comparable amount of zinc results in increased food consumption by zinc-deficient rats within a few hours [C. F. Mills *et al.* in (*7*); J. K. Chesters and J. Quarterman, *Br. J. Nutr.* **24**, 1061 (1970)].
15. Absence of a mossy fiber response decrement was also observed during preliminary experiments within about 36 hours of inadvertently supplementing deprived animals; no data on zinc intake are available.
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17. Dithizone (100 mg/kg) was administered by slow (20 minutes) intraperitoneal injection [K. Fleischhauer and E. Horstmann, *Z. Zellforsch.* **46**, 598 (1957)]. Fifteen minutes after the injection, the animal was killed with an overdose of pentobarbital and the brain was quickly removed without fixation. Dithizone was used in preference to a Timm stain because of its relative simplicity and because it is a somewhat more specific indicator for zinc.
18. These results should be viewed with caution, since two of the rats died after receiving about two thirds of the full dose of dithizone, and a third rat (the only moderately stained animal) died shortly after receiving the full dose. None of the control animals died prematurely. This differential effect of dithizone on viability raises the possibility that some confounding factor may have reduced the stainability of the deprived animals.
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22. It seems doubtful that the mossy fiber response decrement could be due to nonspecific effects of zinc deprivation on mossy fiber oxidative metabolism. The major oxidative enzyme in mossy fibers (glycerophosphate dehydrogenase; *23*) probably contains iron, not zinc [Y. Hatafi and D. L. Steggall, in *The Enzymes*, P. D. Boyer, Ed. (Academic Press, New York, 1976), vol. 13, p. 175]. In addition, other hippocampal projections showing high zinc metalloenzyme activity (*23*) do not have a zinc content comparable to that of mossy fibers (*1-3*). Finally, the prominently staining mossy fiber zinc is not, for the most part, associated with mitochondria (*2, 24*). It seems unlikely that these results could be attributed to developmental failure or to degeneration of the mossy fibers in the deprived animals, since (i) the mossy fiber system is morphologically [S. A. Bayer and J. Altman, *J. Comp. Neurol.* **158**, 55 (1974)] and chemoarchitectonically [I. L. Crawford and J. D. Connor, (*2*); S. I. Mellgren, *Z. Zellforsch.* **141**, 347 (1973)] mature before the zinc-deficient diet was instituted and since (ii) most mossy fiber zinc is unlikely to have a structural role (*4*).
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Genetic Effects of Impure and Pure Saccharin in Yeast

Abstract. *Yeast cells were grown in media containing impure or purified saccharin preparations. Dose-dependent increases in frequencies of cells possessing aberrant cell morphologies were revealed by light microscopy. At each test dose, cells grown in impure saccharin exhibited up to sevenfold higher frequencies of mitotic crossing-over or gene conversion in three of four assays for genetic recombination than cells grown in purified saccharin from the same lot. With one exception, the sweetener produced by the Maumee process caused larger increases in recombination and gene reversion than the sweetener produced by the Remsen-Fahlberg process. The several test markers did not respond equally to any test saccharin. Cells grown in liquid media containing no saccharin or two of three test concentrations of saccharin produced cell titers that were approximately equivalent.*

The saccharin (lot S1022, Sherwin-Williams) that induced bladder cancer in two generations of rats (*1*) was produced by the Maumee process and contained impurities that, when highly concentrated, were weakly mutagenic in some *Salmonella* test strains (*2, 3*). Both commercial S1022 saccharin and saccharin purified from the same lot did not cause mutagenic or other genetic alterations in several short-term tests (*4, 5*), including a mitotic recombination test in yeast D3 (*4*) and widely used *Salmonella* assays (*2, 4-6*). In contrast, the same impure [organic solvent-soluble impurities, 10 to 15 parts per million (ppm)] and partially purified saccharin (organic solvent-soluble impurities, 1 to 5 ppm) increased frequencies of sister chromatid exchanges in both Chinese hamster ovary cells and human lymphocytes (*4, 7*), caused urines of saccharin-fed mice to be weakly mutagenic to *Salmonella* TA100 (*6*), caused weak mutagenic responses at the TK+/TH− locus in mouse lymphoma L5178Y cells (*4*), and exhibited cocarcinogenic activity in C3H/10T1/2 mouse embryo cells in culture (*8*). There is also evidence that the same highly purified saccharin from lot S1022 induces a variety of chromosome aberrations in Chinese hamster cells in culture (*4*). The National Academy of Sciences' Panel I for the Study of Saccharin and Its Impurities re-

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